Universidade Federal do Rio Grande
Instituto de Ciências Biológicas
Pós-graduação em Biologia de
Ambientes Aquáticos Continentais

Adaptação intraespecífica em peixes:

análises em ambientais naturais e antropizados

Giselle Xavier Perazzo

Orientadora: Profª Drª Adriana Gava

Rio Grande
2019

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Tese apresentada ao Programa de Pós-graduação em Biologia de Ambientes Aquáticos Continentais como requisito parcial para a obtenção do título de Doutora em Biologia de Ambientes Aquáticos Continentais.

Rio Grande
2019

"... EU GOSTO DO GOSTO DA CORAGEM,
A MELHOR VIAGEM É SEGUIR A TRILHA QUE EU ABRI..."
(Banda do Mar)

Dedico esta tese aos que diariamente me encorajam a seguir a trilha que eu abri

Vera, Gilnei, Giovanni
e Geordano

AGRADECIMENTOS

"A teoria da evolução é notadamente a mais importante das teorias biológicas, e sob todos os aspectos, é sempre um prazer fazer parte da classe dos que têm a sorte de estudá-la." (Ridley, 2006)

Muitas pessoas contribuíram para que eu pudesse, ao longo desses quatro anos de doutoramento, continuar fazendo parte do grupo daqueles que se dedicam ao estudo da Evolução. E a elas dedico meu profundo agradecimento, pois nada construímos sozinhos.

Agradeço a minha orientadora, Dra. Adriana Gava, pela acolhida no meu retorno à FURG, pela confiança no meu trabalho e pela conclusão de mais uma parceria, que se iniciou em 2007, ainda na minha graduação, seguiu-se durante meu Mestrado e chegou ao Doutorado.

Agradeço ao meu supervisor na University of Basel, Dr. Walter Salzburger, pela grande oportunidade a mim confiada, abrindo as portas de seu laboratório e permintindo que eu pudesse realizar o período de estágio sanduíche na UniBasel.

Em nome da Profa. Dra. Fabiana Schneck, coordenadora do Programa de Pós-Graduação em Biologia de Ambientes Aquáticos - PPGBAC, agradeço aos professores e técnicos do ICB que contribuíram com meu aprendizado e desenvolvimento do projeto de pesquisa. Em especial, agradeço ao apoio logístico para e nas coletas das técnicas Dra. Bruna F. Nornberg e da Dra. Daiane Carrasco. Também agradeço ao Dr. Fernando Marques Quintela pela ajuda em campo e nas contribuições do artigo defendido na qualificação.

O desenvolvimento deste trabalho envolveu a parceria com diversos pesquisadores, sem os quais eu não conseguiria desenvolver os artigos aqui apresentados. Primeiramente, agradeço ao Dr. Felipe Alonso e Prof. Pablo Calviño, que após meu contato prontamente se dispuseram a compartilhar suas amostras de *Jenynsia* de Punta del Este, e também realizar novas amostragens, além de apresentar valiosas discussões sobre os resultados. Agradeço também ao Dr. Gustavo E. Chiaramonte e a Prof. Ricardo Ferriz pelo acesso a coleção ictiológica do Museo Argentino de Ciencias Naturales Bernardino Rivadavia, onde estão depositadas as amostras da população marinha de *Jenynsia*. Também agradeço a parceria de trabalho com o Dr. Fabiano Corrêa, que me apresentou a ideia de trabalhar com os *Bryconamericus* do Chasqueiro. Por último, agradeço preciosa equipe do Grupo de Pesquisa em Biologia Computacional, do Centro de Ciências Computacionais da FURG, que me acolheu para as análises de bioinformática: Professores Dr. Adriano Velasque Werhli e Dra. Carina dos Santos Machado, obrigada pela acolhida e partilha de

seus equipamentos e conhecimentos; Dr. Bruno Rodrigues Oliveira obrigada pelas excelentes contribuições no desenvolvimento das atividades; I.C. Gabriel Moraes obrigada pelo empenho, atenção e paciência comigo e com meus *big data*.

Agredeço aos professores membros da banca avaliadora pelas contribuições ao trabalho apresentado. Em especial agradeço a Profa. Dra. Lizandra Robe também pela atenção e paciência ao discutir aspectos da RADSeq, e ao Prof. Rodrigo Fornel pelos ensinamentos sobre Morfometria Geométrica no seu curso, ministrado na UFRGS, em 2015.

Agradeço aos meus amigos pelo fundamental suporte nos momentos mais difíceis e também pelos momentos de alegria e risadas que compartilhamos. Um especial obrigada para a Daiana Garcez, Thaísa Bozetti, Emanuela Castro, Claúdio Rosano e Murilo Fernandes.

Agradeço ao amor e suporte da minha família, sempre presente e tão importante.

Agradeço ao meu querido marido, Geordano, companheiro e incentivador do dia-a-dia dessa conquista. Obrigada pelos conselhos, discussões, força nas coletas, por segurar a barra quando foi necessário. Te amo!

Finalizo agradecendo às instituições onde conduzi meu doutorado - Universidade Federal do Rio Grande e University of Basel – e as agências financiadoras - Coordenação de Aperfeiçoamento de Pessoal de N vel Superior (CAPES) e Swiss Government Excellence Scholarship for Foreign Students – pelas bolsas de estudo.

RESUMO

Compreender a diferenciação intraespecífica entre populações de peixes que habitam distintos ambientes permite contribuir com o entendimento de como as espécies evoluem, além de colaborar com a compreensão das interações entre os organismos aquáticos e o seu meio, cada vez mais sujeito às alterações antrópicas. Dessa forma, a presente Tese teve como objetivo principal estudar a adaptação intraespecífica de peixes expostos a ambientes heterogêneos, tanto de origem natural como antrópica. Para as análises em ambientes naturais, foram realizadas abordagens de morfometria geométrica e linear e de genômica populacional para avaliar a diferenciação intraespecífica em *Jenynsia lineata* de distintos habitats. As análises morfométricas revelaram um padrão de dimorfismo sexual dependente de habitat, além da forma e tamanho do corpo serem distintos na população marinha. Com relação às análises genômicas, observamos estruturação populacional com a formação de três clusters, com a população marinha apresentando maior estruturação em relação às demais. Além disso, ficou evidenciado que há diferenças em parâmetros de diversidade genética entre espécimes de ambientes lóticos e lênticos. Também foram identificados SNPs sob potencial seleção divergente entre os ambientes estudados, os quais podem estar localizados em genes com função de desenvolvimento corporal, assim como de osmorregulação, entre outros, corroborando a hipótese de que *J. lineata* é uma espécie localmente adaptada a variáveis condições ambientais. Já para avaliar como o processo de adaptação local ocorre em ambientes alterados via ação humana, foi analisada a variação na forma e tamanho do corpo de *Bryconamericus iheringii* expostos a ambientes naturais e alterados, ao longo da bacia do Arroio Chasqueiro, sul do Brasil. Evidenciou-se que *B. iheringii* apresenta dimorfismo sexual de forma (SShD), com ambos os sexos apresentando o corpo mais fusiforme em ambiente lótico, com as fêmeas apresentando variação na posição da boca entre esses ambientes. Trabalhos prévios com *B. iheringii* não revelaram diferenças entre córregos naturais (sem represamento), o que enfatiza a potencial força seletiva da alteração ambiental causada pela construção da barragem. Observa-se, por fim, que nosso conhecimento sobre adaptação local em peixes da região Neotropical ainda é incipiente, especialmente na região compreendida pelo estudo conduzido nesta Tese, e que os resultados aqui apresentados contribuem com dados iniciais para o conhecimento sobre adaptação local em peixes da região costeira do Rio Grande do Sul e do Uruguai.

Palavras-chave: Adaptação Local; Barragem; *Bryconamericus iheringii; Jenynsia lineata*; Planície Costeira.

ABSTRACT

The study of the intraspecific differentiation among fish populations that inhabit different environments can contribute to the understanding about the species evolution, contributing with knowledge about the interactions between aquatic organisms and their environment, increasingly subject to anthropic alterations. Thus, the main purpose of this Thesis was to study the intraspecific adaptive process of fishes exposed to heterogeneous environments, both natural and anthropogenic. For analyzes in natural environments, approaches using geometric and linear morphometry and population genomics analyzes were performed to evaluate the intraspecific variation in *Jenynsia lineata* from different habitats. Morphometric analyzes revealed a pattern of habitat-dependent sexual dimorphism, besides the body shape and size being distinct in the marine population. In relation to the genomic analyzes, we observed population structuring with the formation of three clusters, and the marine population showing a greater distinction form the all others. In addition, it was evidenced that there are differences in genetic parameters between lotic and lentic specimens. We also identified SNPs under potential divergent selection between the environments studied herein, which seem to be in genes with development function, as well as osmoregulation, among others, corroborating the hypothesis that *J. lineata* is a species locally adapted to variable environmental conditions. To evaluate how the local adaptation process occurs in altered environments via human action, the differences in the body shape and size of the *Bryconamericus iheringii* exposed to natural and altered environments along the Chasqueiro stream basin, southern Brazil, were analyzed. It was evidenced that *B. iheringii* shows shape sexual dimorphism (SShD), with both sexes presenting the body more fusiform in lotic environment, with females showing differentitation in the position of the mouth between these environments. Previous work with *B. iheringii* revealed no differences between natural streams (without damming), which emphasizes the potential selective force of the environmental change caused by the construction of the dam. Finally, it is observed that our knowledge about local adaptation in fish from the Neotropical region is still incipient, especially in the region of study, and the results presented here contribute with initial data for knowledge about local adaptation in fish of the coastal region of Rio Grande do Sul and Uruguay.

Keywords: Local Adaptation; Dam; *Bryconamericus iheringii; Jenynsia lineata*; Coastal Plain.

APRESENTAÇÃO

A presente Tese resulta do projeto de pesquisa que desenvolvi durante o curso de Doutorado em Biologia de Ambientes Aquáticos Continentais - PPGBAC, na Universidade Federal do Rio Grande - FURG. O tema central da minha tese foi evolução de peixes em ambientes aquáticos da região costeira do Rio Grande do Sul e do Uruguai. Busquei compreender como espécies com distribuição em diferentes tipos de habitats estão adaptadas a distintos ambientes. Assim, pude avaliar a variação tanto em ambientes naturalmente distintos, como naqueles alterados por ação humana.

Na Introdução Geral apresento uma descrição do meu tema de estudo, bem como as ferramentas utilizadas, além dos ambientes e das espécies estudadas. Os resultados, separados em três artigos, estão apresentados nos capítulos que compõem a Tese. Os dois primeiros se referem ao estudo da variação morfológica e genética, respectivamente, encontrada na espécie *Jenynsia lineata* de diferentes habitats. O primeiro artigo, que aborda a variação morfológica utilizando a morfometria como ferramenta, foi publicado da revista **Hydrobiologia** e está apresentado no Capítulo 1. O segundo artigo versa sobre a variação a nível genômico da estrutura populacional de *J. lineata*. Este artigo será submetido para o **Journal of Evolutionary Biology**, e está apresentado no capítulo 2. O terceiro artigo, que compõe o Capítulo 3, apresenta os potenciais efeitos da construção de uma barragem na variação morfológica de *Bryconamericus iheringii* de um riacho subtropical. Este artigo foi submetido para a revista **Reviews in Fish Biology and Fisheries**. Finalizo a Tese com uma conclusão abordando um apanhado geral dos achados deste projeto e apontando perspectivas para futuros estudos nesta linha de pesquisa. Cada capítulo está escrito de acordo com as normas das respectivas revistas para as quais os artigos foram/serão submetidos (contudo, mantendo o tamanho e tipo de letra ao longo da Tese para fins de facilitar a leitura); tais normas estão disponíveis nos links ao final desta sessão. A Introdução Geral e a Conclusão estão apresentadas seguindo as normas da ABNT.

Conduzi as atividades de pesquisa deste projeto contando com a colaboração de diversos pesquisadores e instituições. Além do Laboratório de Genética do Instituto de Ciências Biológicas da FURG, onde estive sediada durante meu doutorado, também conduzi análises em outros locais, dentro da própria instituição, como também fora dela. Realizei estágio no Salzburger Laboratory, da University of Basel, Suíça, por meio da concessão de bolsa de pesquisa do programa Swiss Government Excellence Scholarship for Foreign Students. Lá realizei as atividades de laboratório (RADSeq), bem como aprofundei meus conhecimentos em Morfometria Geométrica com o grupo de pesquisa do Prof. Salzburger. Já as análises de bioinformática foram conduzidas junto ao Grupo

de Pesquisa em Biologia Computacional – ComBi-L, do Centro de Ciências Computacionais - C3, da FURG.

Foram meus colaboradores durante este projeto de pesquisa, além da minha orientadora do PPGBAC, Prof. Dr. Adriana Gava, e do meu supervisor no estágio de doutorado sanduíche, Prof. Dr. Walter Salzburger (University of Basel), os seguintes pesquisadores:

- do ComBi-L/C3 – FURG: o Prof. Dr. Adriano Velasque Werhli, a Prof. Dr. Karina dos Santos Machado, o I.C. Gabriel Moraes e o Dr. Bruno Rodrigues Oliveira;
- do Instituto de Bio y Geociencias del NOA - IBIGEO/CONICET, Argentina: Dr. Felipe Alonso
- do Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" - MACN e do Grupo de Investigación y Conservación de Killis – GICK: Prof. Pablo Calviño
- da Universidade Federal do Acre: Dr. Fabiano Corrêa

As amostragens realizadas no Brasil foram feitas com autorização do Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio/MMA) sob o número 49103-4. A utilização dos animais para fins de pesquisa foi certificada pela Comissão de Ética em Uso Animal – CEUA, da FURG, sob o número P023/2016 (certificado em anexo). A aquisição de amostras de exemplares do Uruguai foi possibilitada pela parceria com pesquisadores do IBIGEO/CONICET e do MACN que possuem autorização uruguaia para amostragem em Punta del Este.

Links para normas utilizadas na elaboração de cada capítulo

Capítulo 1: *Hydrobiologia*

https://www.springer.com/life+sciences/ecology/journal/10750

Capítulo 2: *Journal of Evolutionary Biology*

https://onlinelibrary.wiley.com/page/journal/14209101/homepage/forauthors.html

Capítulo 3: *Reviews in Fish biology and Fisheries.*

https://www.springer.com/life+sciences/ecology/journal/11160

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INTRODUÇÃO GERAL

Espécies que habitam ambientes heterogêneos ao longo de sua distribuição estão sujeitas a diferentes pressões seletivas. Dependendo das distintas condições as quais as populações estão submetidas, a seleção natural pode propiciar a existência de variáveis das espécies em diferentes locais (TOWNSEND; BEGON; HARPER, 2006), mesmo na presença de fluxo gênico. Assim, diferentes pressões de seleção podem resultar na diferenciação fenotípica de características que conferem vantagem adaptativa local, a qual pode ocorrer em virtude da adaptação genética e/ou da plasticidade fenotípica (sem diferenciação genética entre as populações) (TORRES-DOWDAL et al., 2012). Este processo é conhecido como adaptação local, cujo conhecimento além de contribuir para o entendimento acerca da evolução das espécies, pois tem implicação direta no processo de especiação, também se torna relevante uma vez que pode explicar como as espécies se adaptam a distintas condições e, também, como respondem as mudanças ambientais.

O conhecimento sobre o processo de adaptação local vem trazendo luz a uma das mais desafiadoras questões da ecologia evolutiva: Como a adaptação pode ocorrer mesmo havendo fluxo gênico entre populações? O desafio reside no fato da coexistência de forças antagônicas; se por um lado, adaptação está baseada na divergência por meio da seleção de genótipos, o fluxo gênico tende a homogeneizar os genótipos entre as populações (FREELAND; KIRK; PETERSEN, 2011). A resposta a esta questão envolve um balanço entre a quantidade de fluxo gênico e a força da pressão seletiva atuando em determinada característica, pressão essa que pode ser variável de acordo com as diferenças ambientais que as populações de determinada espécie enfrentam. Ou seja, mesmo que haja fluxo gênico, a variabilidade genética entre as populações pode propiciar a ocorrência de variados fenótipos, os quais podem ser selecionados distintivamente entre as populações, em virtude das diferentes pressões seletivas de cada habitat. Dessa forma, adaptação local pode ocorrer tanto por meio de respostas fenotípicas plásticas (quando o mesmo genótipo apresenta amplo espectro de respostas fenotípicas, de acordo com variações ambientais) ou por meio de divergência genética adaptativa (quando ocorre diferenciação genotípica entre as populações associada à adaptação) (CRISPO, 2008).

Dessa forma, compreender as causas e as consequências da divergência fenotípica entre as populações é um dos objetos fundamentais da ecologia evolutiva (TORRES-DOWDAL et al., 2012). Os peixes têm se mostrado um grupo interessante para este tipo de investigação uma vez que há vários relatos de variação fenotípica intraespecífica, muitas delas associadas com recorrentes pressões seletivas. Como um agente de seleção para variação na forma do corpo de peixes, por exemplo, destaca-se, entre outros, o fluxo de água (CURETON; BROUGHTON, 2014;

GASTON; LAUER, 2015; HAAS; BLUM; HEINS, 2010; MISE et al., 2015). Alterações como as causadas pelo represamento e canalização dos corpos de água impõem mudanças fundamentais em paisagens naturais; ou seja, cria novos desafios ecológicos e evolutivos para organismos aquáticos que são capazes de responder à alteração de fluxo (HAAS; BLUM; HEINS, 2010). Assim, aquelas espécies hábeis a responder rapidamente a essas mudanças por meio da plasticidade fenotípica possuem a habilidade intrínseca necessária para sobreviver em ambientes onde mudanças ambientais dinâmicas estão ocorrendo, podendo ocasionar, inclusive, alteração da estrutura da assembleia de peixes destes locais (GASTON; LAUER, 2015).

Em geral, espécies cujas populações habitam ambientes lóticos e lênticos podem apresentar variações morfológicas em função do nível de atividade de nado, diferenciado de acordo com a velocidade de fluxo da água (LAUDER, 2015). Populações de ambientes de água mais calmas tendem a apresentar o corpo mais comprimido (maior eixo dorsoventral e menor eixo anteroposterior) em comparação com aquelas de águas lóticas, com a forma do corpo mais fusiforme (HAAS; BLUM; HEINS, 2010) e maior pedúnculo caudal (GASTON; LAUER, 2015). Os estudos sobre os efeitos da alteração antrópica do fluxo de água no processo evolutivo de ictiofauna submetida a estas alterações estão em expansão (ASSUMPÇÃO et al., 2012; CURETON; BROUGHTON, 2014b; FRANSSEN, 2011; FRANSSEN; STEWART; SCHAEFER, 2013; GASTON; LAUER, 2015; HAAS; BLUM; HEINS, 2010; JACQUEMIN; PYRON, 2016; SANTOS; ARAÚJO, 2015), mais ainda escassos especialmente nos subtrópicos da região Neotropical. Desta forma, estudos que visam entender como as populações respondem a mudanças ambientais amplas e rápidas é a primeira etapa para elucidar as consequências que habitats perturbados têm sobre a evolução dos organismos (FRANSSEN; STEWART; SCHAEFER, 2013).

Obviamente, a diferença no fluxo de água não têm sido o único fator abiótico associado à variação fenotípica intraespecífica em peixes. Por exemplo, diversas espécies têm apresentado diferenças adaptativas e/ou plásticas em resposta a gradientes de salinidade (ARAÚJO et al., 2014; BAKER et al., 2015; BERG et al., 2015; BERNER; GRANDCHAMP; HENDRY, 2009; DEFAVERI; MERILÄ, 2014; DENNENMOSER et al., 2017; FOSTER et al., 2015; JØRGENSEN et al., 2008; LANGERHANS et al., 2004; MARCHINKO; SCHLUTER, 2007; NORRIS; DEVRIES; WRIGHT, 2010; OLSEN; ANDERSON; MCDONALD, 2016; ROESTI et al., 2012). Além da necessidade de ajuste fisiológico para viver em ambientes com distintas concentrações salinas, as espécies cujas distribuições incluem tanto os ambientes dulcícolas como os estuarinos e marinhos podem apresentar variações morfológicas, como distintos tamanhos corporais, como observado, por exemplo, em *Gambusia affinis* (LANGERHANS et al., 2004),

Poecilia vivipara (ARAÚJO et al., 2014; GOMES; MONTEIRO, 2008), *Clupea harengus* (JØRGENSEN et al., 2008), *Jenynsia lineata* (FONTOURA et al., 1994; MAI; GARCIA; VIEIRA, 2005) e *Gasterosteus aculeatus* (BAKER et al., 2015; FOSTER et al., 2015). Mudanças no tamanho podem estar relacionadas com o próprio ajuste fisiológico uma vez que a salinidade influencia o crescimento de diversas espécies de peixes por afetar a taxa metabólica padrão, bem como a ingestão e conversão de alimentos, além da estimulação hormonal envolvida com a osmorregulação e o crescimento (BOEUF; PAYAN, 2001). Nesses casos, a variação de tamanho pode ser um subproduto de mecanismos fisiológicos, não apresentando valor adaptativo.

Entretanto, a variação ambiental entre ambientes dulcícolas e salinos causa alterações na estrutura do habitat, como, por exemplo, na própria assembleia de peixes, consequentemente variando a presença e abundância de predadores entre esses ambientes. A predação, por sua vez, é reconhecidamente uma pressão seletiva em ambientes aquáticos (LANGERHANS et al., 2004). Ela atua tanto por meio da mortalidade de presas induzindo rápidas mudanças evolutivas em conjuntos de características antipredadores na população, como também induzindo respostas plásticas em caracteres sob seleção direta dos predadores (TORRES-DOWDAL et al., 2012). Além disso, a diferenciação ecológica entre ambientes salinos e dulcícolas impõe às espécies que habitam esses lugares diferentes disponibilidade de alimento, de modo que suas populações podem apresentar adaptações fenotípicas específicas associadas às distintas dietas (VERA-DUARTE; BUSTOS; LANDAETA, 2017). De modo geral, a observação de padrões convergentes de variações na forma do corpo compartilhados entre espécies de gradientes ambientais similares tem permitido a elaboração de um paradigma ecomorfológico para a correlação entre a forma no corpo e a predação como pressão de seleção (LANGERHANS et al., 2004). Populações de espécies distantes filogenética e geograficamente têm apresentado variação na forma do corpo em ambientes com e sem (ou com menor) pressão de predação, apresentando fenótipos associados ou não com a habilidade de escape (nado explosivo) (LANGERHANS; DEWITT, 2004). Portanto, percebe-se que ambientes contrastantes são uma importante fonte para seleção natural divergente, de forma que a adaptação a estes habitats, sob determinadas circunstâncias, pode levar a especiação (NOSIL, 2012; SCHLUTER, 2009).

Desta forma, observa-se que a compreensão dos mecanismos de adaptação local envolve não somente o reconhecimento das variações do fenótipo frente a heterogeneidade ambiental, mas também a análise do comportamento genético dessas populações. Isso decorre principalmente pelo fato de que a divergência fenotípica pode ocorrer em função da adaptação genética local ou da plasticidade fenotípica da espécie em questão (KAWECKI; EBERT, 2004). Ambas situações englobam respostas adaptativas uma vez que no caso de adaptação genética local, os genótipos

residentes produzem fenótipos com maior valor adaptativo do que genótipos originados de outros habitats; alternativamente, a plasticidade fenotípica é adaptativa quando a capacidade de resposta a alguma variável ambiental pode mover os fenótipos da população para uma condição ótima localmente, mesmo sem diferenciação genética (TORRES-DOWDAL et al., 2012).

Ferramentas de estudo

Como visto, compreender o processo de adaptação local envolve compreender, por exemplo, de que forma uma mesma espécie é capaz de habitar distintos ambientes; ou seja, envolve a análise de variação intraespecífica. Esta variação pode se manifestar fenotípica e/ou geneticamente, de modo que a investigação tanto da variação de fenótipos como da variabilidade genotípica é fundamental para o estudo da adaptação local. Nesse sentido, nesta tese foram utilizadas duas ferramentas para o estudo da adaptação local: a morfometria para o estudo da variação de fenótipos, e a genômica populacional para o estudo da variabilidade genética.

Morfometria Geométrica e Linear

Historicamente, o estudo da variação da forma dos organismos tem se mostrado muito útil para entender os processos evolutivos, uma vez que a partir do conhecimento morfológico podemos ilustrar como estruturas podem se modificar e, portanto, sugerir mecanismos que podem estar associados às mudanças evolutivas (ANTHWAL; TUCKER, 2017). Os clássicos estudos de Charles Darwin muito se pautaram na compreensão e comparação de estruturas corpóreas entre animais, de forma que, para ele, a morfologia é "uma das partes mais interessantes da História Natural, e quase pode dizer-se que é sua verdadeira essência" (DARWIN, 1859). As análises morfológicas ganharam ao longo do tempo suporte estatístico e geométrico, seguindo a tendência de quantificação de fenômenos biológicos por meio de modelos matemáticos, culminando em uma nova área de pesquisa na fronteira entre a biologia, a estatística e a geometria: a morfometria geométrica (MONTEIRO; REIS, 1999).

O campo de estudo da morfometria busca quantificar de maneira precisa e prática as informações sobre forma e tamanho do organismo ou de parte dele (ELEWA, 2010) e de sua correlação com outras variáveis (BOOKSTEIN, 1991). Tradicionalmente, a morfometria esteve relacionada a aplicação de análises estatísticas multivariadas a partir de variáveis quantitativas lineares, como comprimento, largura e altura, o que é conhecido como ***morfometria linear***. Entretanto, no final dos anos 1980 e início dos anos 1990, uma revolução ocorreu no modo como

as estruturas morfológicas eram quantificadas e analisadas: a geometria dessas estruturas foi capturada e mantida ao longo das análises estatísticas, surgindo o que hoje conhecemos como ***morfometria geométrica*** (ADAMS; ROHLF; SLICE, 2004). Em geral, a abordagem geométrica fornece melhores informações sobre as relações funcionais subjacentes do que as medições tradicionais lineares (FABRE et al., 2014; SIDLAUSKAS; MOL; VARI, 2011). Entretanto, a combinação de medições lineares com morfometria geométrica deve ser usada em harmonia para produzir uma compreensão mais completa sobre a morfologia (GINTER et al., 2012).

A morfometria geométrica é baseada em coordenadas cartesianas de marcos anatômicos, permitindo a exploração e visualização de conjuntos de dados em larga escala (MITTEROECKER; GUNZ, 2009). Sendo assim, a escolha dos marcos anatômicos é o primeiro passo para a análise geométrica da forma de uma estrutura ou mesmo de um organismo. Os marcos anatômicos (*landmarks*, no inglês) são sítios anatômicos discretos que podem ser reconhecidos como o mesmo ponto em todos os espécimes em estudo, e a escolha de quais marcos anatômicos irão compor a análise em questão envolve, idealmente, os seguintes critérios (ZELDITCH; SWIDERSKI; SHEETS, 2012):

i. Homologia entre os marcos anatômicos
ii. Cobertura adequada da morfologia em estudo
iii. Marcos anatômicos devem ser encontrados repetida e confiavelmente em todos os exemplares estudados
iv. Não deve haver troca de posição entre os marcos anatômicos (o que pode ocorrer em alguns casos ao longo do desenvolvimento dos organismos)
v. Marcos anatômicos devem estar no mesmo plano (no caso da análise de dados em duas dimensões)

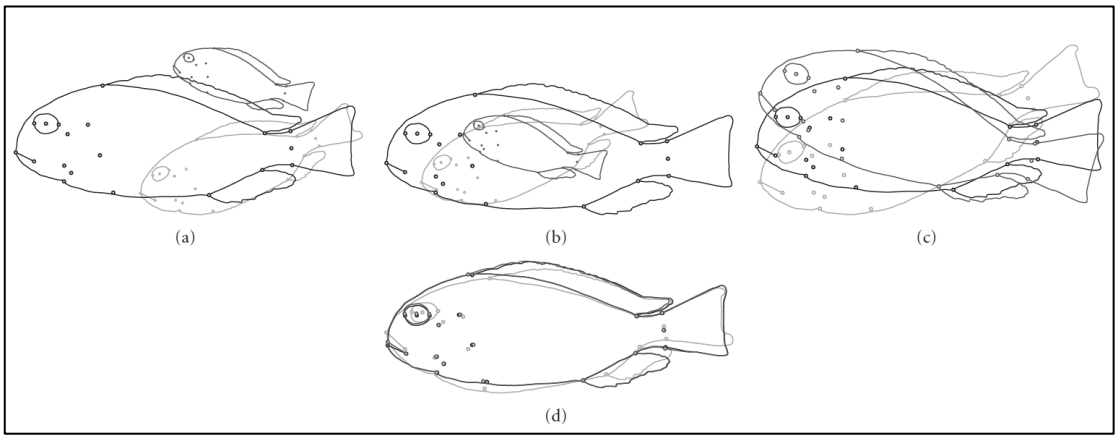
Por fim, a união dos marcos anatômicos irá compor a forma do organismo ou da estrutura em estudo. Forma (*shape*, no inglês) é "toda informação geométrica que permanece quando efeitos de posição, escala e rotação são retirados de um objeto" (KENDALL, 1977). Ou seja, para analisarmos comparativamente diversas formas (por exemplo, vários organismos/estruturas) devemos excluir as variáveis que não são de interesse para a explicação da variação geométrica observada. Como os dados de morfometria geométrica são capturados a partir de coordenadas dos marcos anatômicos, para que possamos avaliar a variação da forma entre indivíduos ou estruturas é preciso eliminar a variação relacionada a diferentes tamanhos, bem como a distintos posicionamentos no momento da aquisição da imagem, que, por conseguinte afetará a matriz de coordenadas.

Sendo assim, estes efeitos indesejáveis de posição, escala e rotação devem ser removidos para obtenção das coordenadas de forma e um dos métodos mais utilizados é o de sobreposição de Procrustes (ZELDITCH; SWIDERSKI; SHEETS, 2012). Em resumo, a proposta de Rohlf & Slice (1990) para utilização da sobreposição de Procrustes envolve (Figura 1):

i. a centralização de cada configuração de marcos anatômicos para a sua origem (subtraindo as coordenadas do centroide pelas coordenadas de cada marco anatômico, individualmente para cada configuração);
ii. o escalonamento da configuração dos marcos anatômicos para a unidade do tamanho do centroide (dividindo as coordenadas de cada marco anatômico pelo tamanho do centroide desta configuração);
iii. a escolha de uma configuração como referência, a partir da qual a segunda configuração será rotacionada de modo a minimizar o somatório das distâncias quadradas entre as formas, seguindo sucessivamente com as demais configurações disponíveis.

Como visto, a sobreposição de Procrustes remove toda a informação sobre tamanho. Contudo, podemos utilizar uma medida de tamanho da forma, conhecida como o ***tamanho do centroide***, o qual é obtido a partir da raiz quadrada do somatório das distâncias quadradas de cada marco anatômico ao ponto centroide. O tamanho do centroide é diferente das medidas lineares de tamanho pois é uma medida geometricamente independente da forma (BOOKSTEIN, 1991). Isso quer dizer através do tamanho do centroide captura-se a informação do tamanho de toda a forma. Ou seja, quando cada dimensão é aumentada ou diminuída isso ocorre na mesma proporção, o que torna o organismo maior ou menor como um todo, sem alterar sua forma (ZELDITCH; SWIDERSKI; SHEETS, 2012).

Figura 1 - Representação da sobreposição de Procrustes. Em (a) observam-se as configurações originais (coordenadas brutas). Primeiramente, o centroide de cada configuração é transformado para a origem (b). Logo em seguida as configurações são escalonadas para uma unidade comum (c) e rotacionadas para minimizar a diferença entre os marcos anatômicos (d).



Fonte: Kerschbaumer & Sturmbauer (2011).

O modo mais comum de acessar dados para morfometria geométrica é através de imagens digitalizadas (fotografias ou *scanners*), as quais são subsequentemente submetidas a *softwares* para digitalização dos marcos anatômicos (MONTEIRO; REIS, 1999). Através dessa metodologia trabalhamos com a morfometria geométrica em duas dimensões. Atualmente existem digitalizadores em três dimensões que possibilitam a captura de marcos anatômicos considerando-se a profundidade da forma em estudo. Dados tridimensionais também podem ser medidos através de varreduras de superfície ou de volume (por meio de tomografias e ressonâncias) (MITTEROECKER; GUNZ, 2009). Estes equipamentos são menos comuns, mais caros e não portáteis, o que, por vezes, inviabiliza a análise morfométrica em três dimensões. Além disso, os resultados obtidos de dados tridimensionais podem ser difíceis de visualizar devido à dificuldade de projetá-los dentro de duas dimensões, como nas páginas das publicações (ZELDITCH; SWIDERSKI; SHEETS, 2012).

Genômica Populacional

Obviamente, o estudo da adaptação local também envolve a análise da variação genotípica entre populações, uma vez que existe o potencial de um único genótipo se desenvolver em múltiplos fenótipos alternativos sob diferentes condições ambientais, por meio da plasticidade fenotípica (FREELAND; KIRK; PETERSEN, 2011). Nesse sentido, a genética de populações tem

sido uma importante ferramenta no campo da pesquisa evolutiva pois possibilita o estudo das forças que resultam em mudanças evolutivas nas espécies ao longo do tempo, desvendando o arcabouço genético dentro do qual a evolução ocorre. Tradicionalmente, um pequeno número de marcadores moleculares neutros têm sido utilizados no estudo de padrões de variação genética entre indivíduos e populações (EKBLOM; WOLF, 2014). Contudo, atualmente o uso de abordagens que incluem o sequenciamento do genoma são uma realidade e expandiram o campo de ação da genética de populações, para o que hoje chamamos de ***genômica populacional*** (HARTL; CLARK, 2010).

A transição entre os clássicos estudos de genética de populações para a era das "ômicas" (genômica, transcriptômica, proteômica) teve início a partir dos rápidos avanços nas tecnologias de sequenciamento e de bioinformática ocorridos nas últimas décadas (EKBLOM; WOLF, 2014). Uma das grandes inovações no campo da biologia molecular foi o desenvolvimento do sequenciamento de segunda geração (*next-generation sequencing* - NGS) o qual possibilita a geração de grande quantidade de dados genômicos (assim como transcriptômicos) de qualquer organismo (ELMER, 2016). A partir desses dados, é possível realizar escaneamento genômico para busca de variação genética potencialmente adaptativa, assim como estimar parâmetros demográficos (NARUM et al., 2013). Existem várias ferramentas moleculares para acessar as bases genômicas da adaptação local (SAVOLAINEN; LASCOUX; MERILÄ, 2013). Dentre elas a mais utilizada é o polimorfismo de nucleotídeo único – SNP, o qual se refere a uma única posição de par de base ao longo da sequência de DNA que varia entre indivíduos (FREELAND; KIRK; PETERSEN, 2011). Uma das abordagens genômicas amplamente utilizadas para identificação e genotipagem de SNPs em estudos evolutivos e ecológicos de organismos não-modelos é o sequenciamento de DNA associado a sítios de restrição (*restriction site-associated DNA sequencing* – RADSeq) (ANDREWS et al., 2016).

RADSeq são pequenos fragmentos de DNA adjacentes a cada instância do sítio de reconhecimento de uma determinada enzima de restrição (BAIRD et al., 2008). Originalmente, RADSeq foi o nome dado para descrever uma particular metodologia, mas tem sido subsequentemente adotado para se referir a uma variedade de técnicas relacionadas que dependem de enzimas de restrição para determinar o conjunto de loci a ser sequenciado. No Quadro 1 estão sumarizadas as metodologias de RADSeq mais comuns, descritas em ANDREWS et al. (2016).

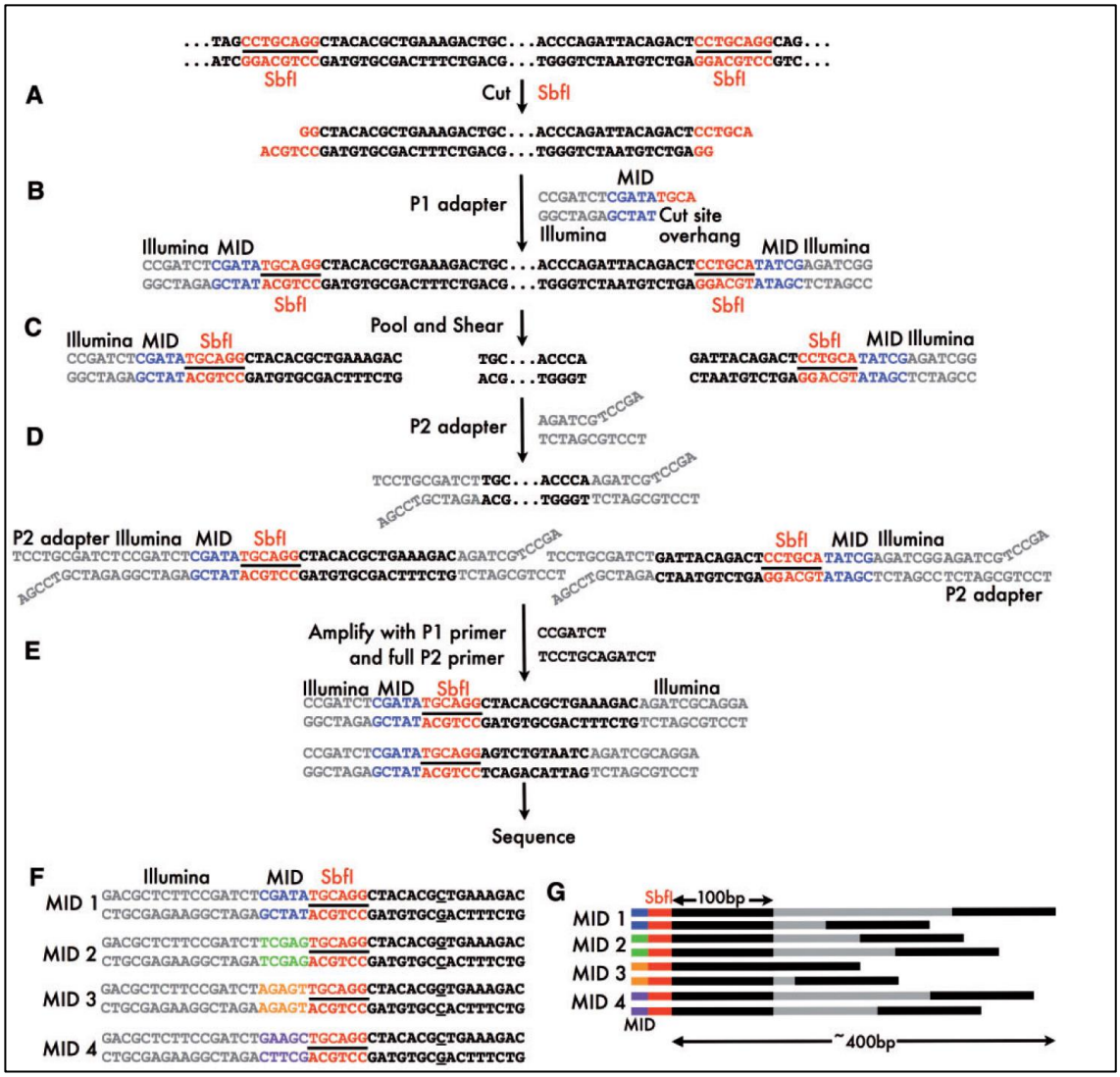
Quadro 1 – Métodos mais comuns de RADSeq (existem outras variações não descritas aqui).

Métodos que sequenciam fragmentos adjacentes a um único sítio de enzima de restrição
RADSeq original: digere DNA genômico com uma enzima de restrição, seguido por cisalhamento mecânico para reduzir fragmentos a um tamanho apropriado para o sequenciamento, criando variação no tamanho dos fragmentos em cada locus (BAIRD et al., 2008; MILLER et al., 2007).
RADSeq 2bRAD: utiliza enzimas de restrição do tipo IIB, a qual cliva o DNA *upstream* a *downstream* ao sítio de reconhecimento, resultando em fragmentos curtos e uniformes (33 – 36 pares de bases) (GUO et al., 2014; WANG et al., 2012).
Métodos que sequenciam fragmentos flanqueados por dois sítios de enzima de restrição
GBS: *Genotyping by sequencing* utiliza uma enzima de corte comum (cortes mais frequentes ao longo do DNA) e amplificação, via PCR, de fragmentos preferencialmente pequenos (ELSHIRE et al., 2011).
SBG: *Sequence-based genotyping* utiliza uma enzima cujos cortes ocorrem mais espaçadamente (*rare-cutter enzime*) e uma ou duas enzimas comuns, seguida de PCR com amplificação preferencial de fragmentos curtos (TRUONG et al., 2012).
CRoPS: *Complexity reduction of polymorphic sequence* utiliza duas enzimas e um kit de preparação de bibliotecas próprio (VAN ORSOUW et al., 2007).
RRLs: *Reduced representation libraries* utilizam uma enzima comum, seguida por uma etapa de seleção de tamanho e um kit de preparação de bibliotecas próprio do sequenciador Illumina (GREMINGER et al., 2014; VAN TASSELL et al., 2008).
MGS: *Multiplexed shotgun genotyping* utiliza uma enzima comum e uma etapa de seleção de tamanho (ANDOLFATTO et al., 2011).
ezRAD: utiliza uma ou mais enzimas de restrição e um kit próprio do sequenciador Illumina para preparação das bibliotecas (TOONEN et al., 2013).
ddRAD: *Double-digest RAD* utiliza duas enzimas de restrição, com adaptadores específicos para cada enzima e com seleção de tamanho via corte de gel automatizado (PETERSON et al., 2012).

Fonte: Adaptado de ANDREWS et al. (2016).

De maneira geral, a RADSeq apresenta algumas etapas básicas, resumidas na Figura 2 (retirada de DAVEY; BLAXTER, 2010). Primeiramente, o DNA genômico é cortado com a enzima de restrição escolhida. Logo em seguida, um adaptador (P1) é adicionado aos fragmentos. Este adaptador contém uma região para reconhecimento no sequenciador (Illumina), uma sequência de identificação única (MID – *molecular identifier*; utilizada para identificar cada amostra) e uma sequência para identificação do sítio de restrição. Cada amostra (espécime) deverá receber um adaptador com uma sequência única de MID, pois as amostras serão agrupadas para formar as bibliotecas que serão futuramente sequenciadas. As bibliotecas são então submetidas a quebra mecânica dos fragmentos, que ocorre de maneira aleatória. Após o processo de quebra, apenas um subconjunto dentro da biblioteca será constituído de fragmentos com o sítio de restrição e os adaptadores P1. Anterior à amplificação dos fragmentos, é adicionado ainda um segundo adaptador (P2), o qual apresenta uma das extremidades diferenciadas, com uma estrutura em forma de "Y". Esta estrutura permite que apenas fragmentos contendo o primeiro adaptador (P1) sejam amplificados. Sendo assim, a amplificação via PCR com *primes* P1 e P2 selecionará apenas os fragmentos contendo ambos adaptadores (consequentemente, contendo também a sequência para identificação, a sequência de reconhecimento do sequenciador e o sítio de restrição, além da RAD *tag* que será o alvo de análises a *posteriori*). Após o sequenciamento, as amostras agrupadas com diferentes MIDs são separadas e os SNPs são identificados, por meio de ferramentas de bioinformática (DAVEY; BLAXTER, 2010).

Figura 2 – O processo da RADSeq. (A) O DNA genômico é cortado com a enzima de restrição escolhida (no exemplo, *Sbf*I). (B) O Adaptador P1 é ligado aos fragmentos cortados por *Sbf*I. (C) As amostras de múltiplos indivíduos são agrupadas e todos os fragmentos são aleatoriamente cisalhados. (D) O adaptador P2 é ligado a todos os fragmentos. (E) Amplificação via PCR com primers P1 a P2. (F) Amostras agrupadas com diferentes MIDs são separadas computacionalmente e os SNPs são identificados (C/G, sublinhado no exemplo). (G) Como os fragmentos são cortados aleatoriamente, sequências finais emparelhadas de cada fragmento sequenciado irão cobrir uma região de 300 a 400 pb downstream ao sítio de restrição.



Fonte: DAVEY; BLAXTER (2010).

As sequências de RADSeq podem ser alinhadas a um genoma de referência e serem genotipadas com as ferramentas utilizadas para o sequenciamento de genomas inteiros, ou também podem ser utilizadas para gerar grandes conjuntos de marcadores quando não há genoma de referência, por meio da abordagem conhecida como *assembly de novo* (DAVEY et al., 2013).

Quando se trabalha com espécies sem o genoma sequenciado, as RAD tags podem ser montadas *de novo* (com os erros de sequenciamento devidamente tratados) a partir de elementos com sobreposição, de modo a se inferir o alelismo dessas sequências (DAVEY; BLAXTER, 2010). STACKS (CATCHEN et al., 2013) é um software suíte amplamente utilizado para as análises de *novo*, o qual utiliza um algoritmo que conservativamente monta alelos putativos, seguido por loci putativos. Inicialmente, o algoritmo *de novo* forma pilhas (stacks) de correspondências a partir das sequências brutas (Figura 3a). Em seguida, a sequência consenso de cada stack é quebrada em *kmers* e estocada em um dicionário (Figura 3b). Dois stacks que têm um certo número de *kmers* em comum são considerados potencialmente coincidentes e são alinhados juntos. Se o número de erros de nucleotídeos for menor que a distância permitida entre stacks, os stacks são fundidos dentro de um locus. Uma vez que um locus é formado, os fragmentos secundários serão trazidos de volta para a análise e alinhados contra o locus montado previamente, usando um valor de erro de nucleotídeo mais permissivo. Esse processo fornece mais profundidade que auxilia o modelo de chamada de SNPs para a detecção de polimorfismos (Figura 3c). Esse processo ocorre individualmente em cada amostra no conjunto de dados para construir loci. Uma vez que esta etapa está completa, os dados de cada indivíduo serão fundidos dentro um catálogo (Figura 4), no qual está contida todos os loci e alelos da população (STACKS, 2019).

A partir da formação dos haplótipos temos a identificação dos SNPs, que serão os marcadores moleculares utilizados para estimativas de diversidade genética entre populações (ou entre outros grupos de interesse, como habitats, por exemplo). Cada haplótipo representa um SNP, ou seja, um polimorfismo identificado naquela posição. Tendo os SNPs identificados, podemos proceder com diversas estimativas de diversidade genética para comparação interpopulacional. O método mais comum de quantificar a diferenciação genética entre populações está baseado nas estatísticas $\boldsymbol{F}$, desenvolvidas por Wright na década de 1950 (FREELAND; KIRK; PETERSEN, 2011). As estatísticas $\boldsymbol{F}$ utilizam coeficientes de endocruzamento para descrever o particionamento da variação genética dentro e entre populações, que pode ser calculada em três diferentes níveis: $\boldsymbol{F_{IS}}$ – coeficiente de endocruzamento; $\boldsymbol{F_{ST}}$ –índice de fixação (estima a diferenciação genética entre populações); $\boldsymbol{F_{IT}}$ – estima um coeficiente de endocruzamento para o indivíduo considerando tanto os cruzamentos não aleatórios dentro da população como a diferenciação entre as populações (FREELAND; KIRK; PETERSEN, 2011). As estatísticas $\boldsymbol{F}$ são estimadas a partir das seguintes fórmulas:

$$\boldsymbol{F_{IS}} = \frac{H_S - H_I}{H_S} \qquad \boldsymbol{F_{ST}} = \frac{H_T - H_S}{H_T} \qquad \boldsymbol{F_{IT}} = \frac{H_T - H_I}{H_T}$$

onde $\boldsymbol{H_I}$ é a heterozigosidade observada em uma subpopulação no momento da investigação (heterozigosidade individual), $\boldsymbol{H_S}$ é a heterozigosidade esperada na subpopulação, e $\boldsymbol{H_T}$ é a heterozigosidade esperada na população inteira (Figura 5).

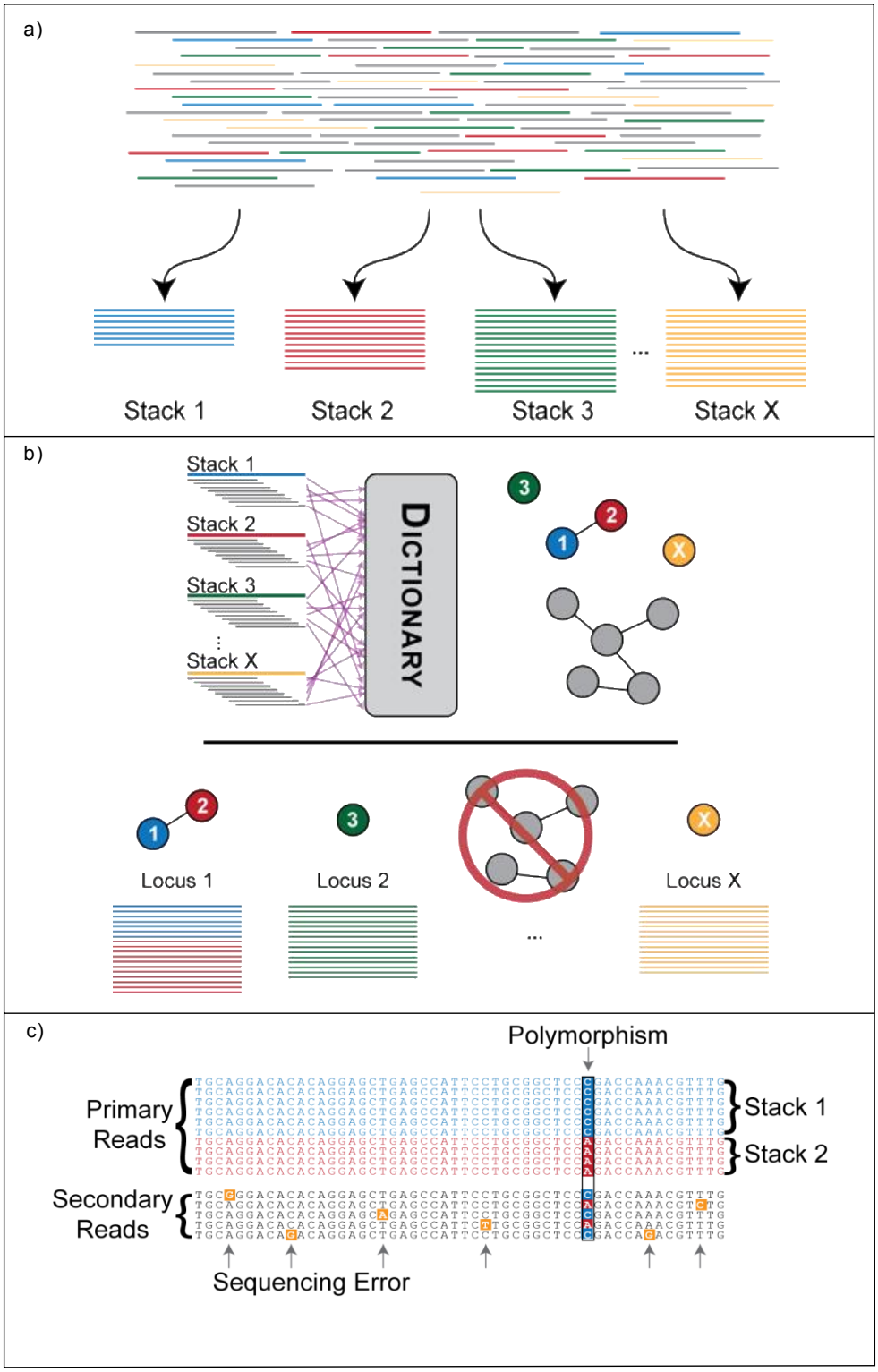
Como visto, os cálculos para as estatísticas ***F*** envolvem basicamente o conhecimento sobre a heterozigosidade, ou seja, o conhecimento de quão diverso um loco é dentro de uma população (FRANKHAM; BALLOU; BRICOE, 2004). Sendo assim, a heterozigosidade observada será obtida a partir da frequência alélica obtida para cada haplótipo. Para o cálculo das estatísticas ***F***, além da heterozigosidade observada também precisamos da heterozigosidade esperada, obtida por meio das equações derivadas do princípio do equilíbrio de Hardy-Weinberg. Segundo o modelo (e considerando suas premissas), a partir das frequências alélicas podemos estimar as frequências genotípicas, e vice-versa, uma vez que o modelo propõe a seguinte relação entre frequências alélicas e genotípicas:

$$\mathbf{AA}: p^2 \qquad \mathbf{Aa}: 2pq \qquad \mathbf{aa}: q^2$$

em que p^2, $2pq$, q^2 são as frequências dos genótipos **AA**, **Aa**, **aa** em qualquer geração, p e q são as frequências alélicas de **A** e **a** nos gametas da geração precedente e $p + q = 1$ (HARTL; CLARK, 2010).

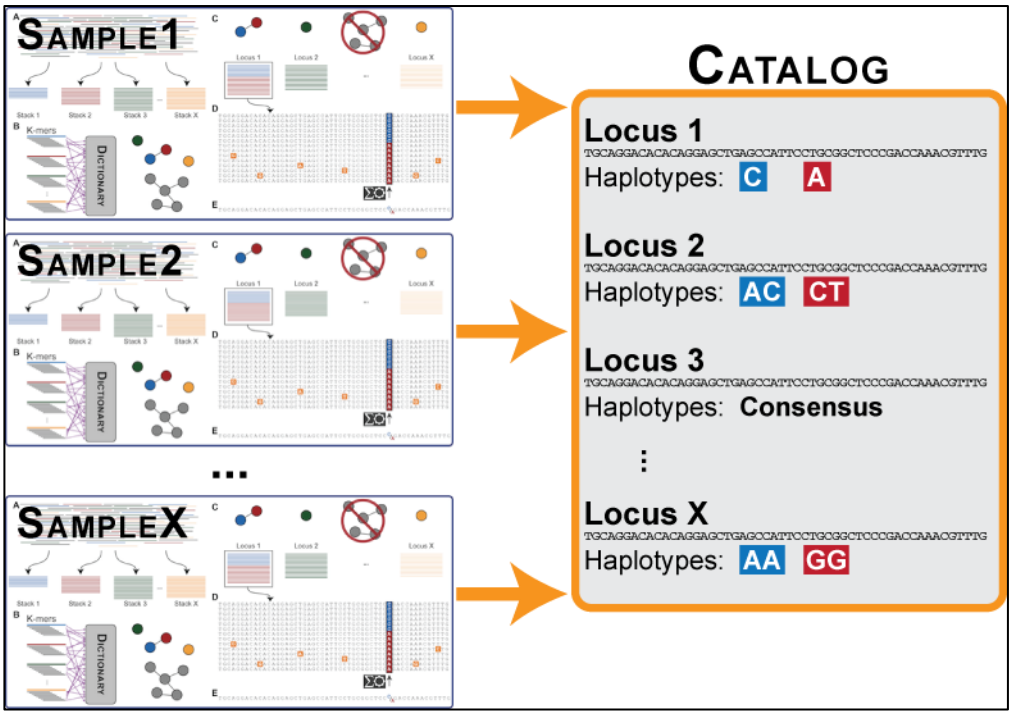
$\boldsymbol{F_{ST}}$ mede a extensão pela qual populações estão diferentes umas das outras, sendo por isso a principal medida utilizada para estudos de comparação populacional. Obviamente, outras estimativas genéticas devem ser utilizadas para comparação entre populações, como, por exemplo, a porcentagem de loci polimórficos e a diversidade nuc ca (π), além das ferramentas de análise que não consideram grupos (subpopulações) *a priori*. Tais análises buscam calcular a probabilidade de os dados serem divididos em um número de clusters, a fim de maximizar o equilíbrio de Hardy-Weinberg. Aqueles cluster mais prováveis tendem a refletir as estruturas (subpopulações) dentro da amostragem estudada (FREELAND; KIRK; PETERSEN, 2011).

Figura 3 – Montagem de genótipos *de novo* no STACKS. (a) Etapa inicial da montagem *de novo* formando *stacks* a partir de emparelhamentos exatos entre as sequências brutas. (b) A sequência consenso de cada *stack* é quebrada em kmers e estocada em um dicionário. (c) Representação de um STACKS locus com um polimorfismo, apresentando as sequências primárias e as secundárias.



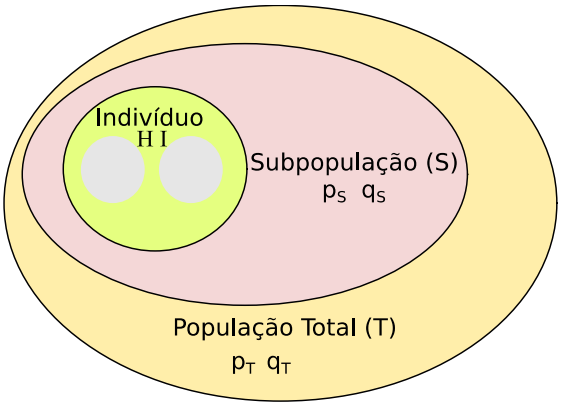
Fonte: http://catchenlab.life.illinois.edu/stacks/param_tut.php

Figura 4 – Representação da formação de catálogos formados pelo STACKS. Um catalogo corresponde uma sequência (*read*) com "x" locus identificados. Cada locus terá seu haplótipo, que corresponde a um determinado SNP identificado.



Fonte: http://catchenlab.life.illinois.edu/stacks/param_tut.php

Figura 5 – Diagrama apresentando a natureza hierárquica das estatísticas F. Os dois círculos centrais representam dois alelos de um indivíduo. A partir da comparação da heterozigosidade presente em cada indivíduo obtemos a heterozigosidade observada (H_I), a heterozigosidade esperada para a subpopulação ($H_S = 2p_Sq_S$) e a heterozigosidade esperada para a população como um todo ($H_T = 2p_Tq_T$).



Fonte: Adaptado de Graham Coop (disponível em https://cooplab.github.io/popgen-notes/)

Além das comparações entre populações, os dados gerados pela RADSeq também possibilitam a investigação de loci candidatos a seleção positiva ou divergente. O método comumente empregado em organismos não modelos é a análise de ***F_{ST} outliers***, o qual contrasta

F_{ST} de loci individuais com a distribuição de F_{ST} esperada em uma distribuição baseada em um modelo neutro. Assim, loci com níveis muito altos de diferenciação entre populações são considerados candidatos para seleção positiva ou divergente enquanto loci com níveis de F_{ST} excepcionalmente baixos são candidatos a seleção balanceadora (NOSIL; BUERKLE, 2010). Sendo assim, é possível propor possíveis genes envolvidos com adaptação a partir da identificação de loci consistentemente distintos entre populações, mesmo em organismos que não possuem o genoma sequenciado.

Caracterização das áreas de estudo

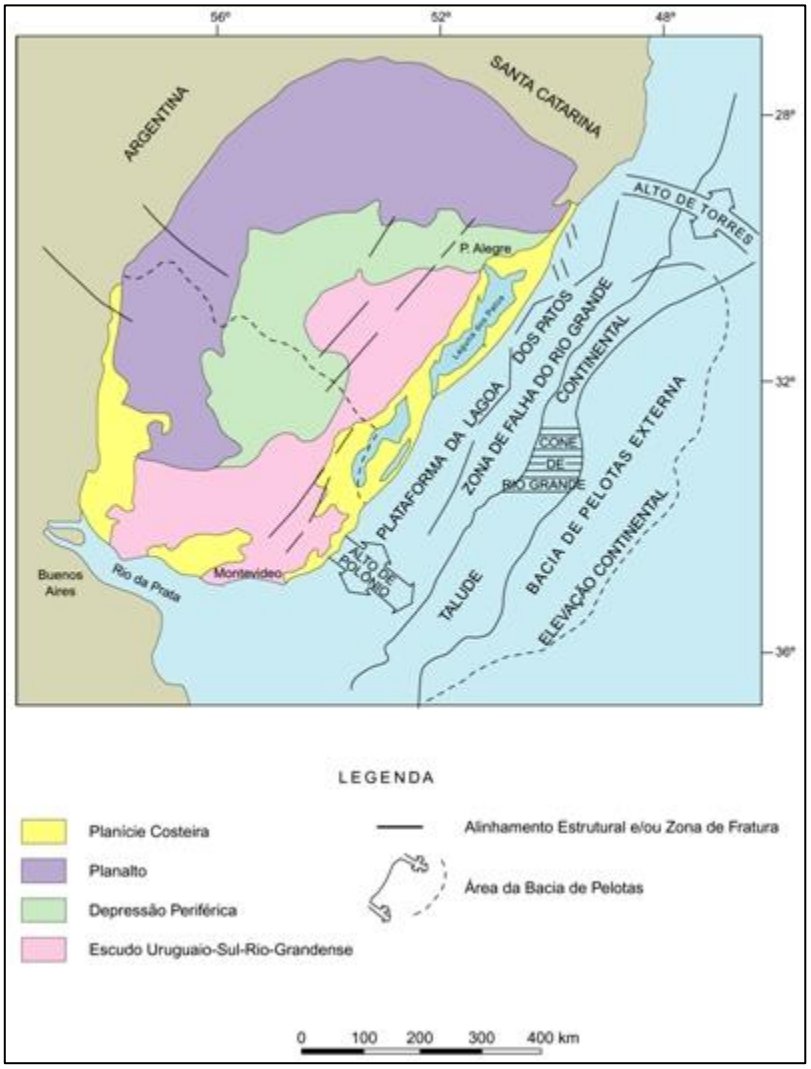
Na presente tese, busquei investigar os processos de adaptação local ocorridos em peixes que habitam distintos ambientes costeiros no sul do Rio Grande do Sul e na costa uruguaia. Parte das amostras foram coletadas na região da Planície Costeira (Figura 6), a qual é caracterizada por uma longa costa retilínea, com extensos campos de dunas e numerosas lagoas (incluindo o sistema Patos-Mirim), além de outros corpos de água que podem estar temporariamente conectados através de pequenos canais (CASTELAO; MOLLER-JR, 2006). Esta região possui uma fisiografia muito particular devido a sua origem a partir de eventos de variação do nível do mar em processos transgressivos nos períodos interglaciais e por processos regressivos nos períodos glaciais, durante o Quaternário (TOMAZELLI; VILLWOCK, 2005). Outros pontos de amostragens se localizam no Escudo Uruguaio-Sul-Rio-Grandense (Figura 6). Esta região apresenta associações de rochas ígneas, metamórficas e sedimentares com idades muito antigas, que variam desde o Arqueano até o Eopaleozóico (~3,85 a ~5,42 milhões de anos) (SPERANDIO; GOMES, 2016). A área de amostragem como um todo apresenta clima Temperado Subtropical Mesotérmico Úmido (segundo a classificação de Köpen), com grande variação sazonal (verões quentes e invernos rigorosos). As temperaturas médias variam entre 15 e 18 ˚C, com precipitação média entre 1000 e 1500 mm (GOVERNO DO ESTADO DO RIO GRANDE DO SUL, 2019; INUMET, 2019).

Para os estudos de adaptação local foram escolhidos os seguintes ambientes: **lagoa**, **estuário**, **riacho**, **sangradouro**, **piscinas rochosas marinhas** e **reservatório** (origem antrópica), os quais são detalhados a seguir (os mapas de cada ponto de amostragem, bem como a descrição de suas variáveis físicas e químicas, estão disponíveis nos capítulos que compõem a tese).

Lagoas

As lagoas são corpos de água lênticos e com rasa lâmina de água que permite a presença de macrófitas ao longo de sua extensão devido a entrada de radiação até o substrato (ESTEVES, 1998). Uma das lagoas amostradas está localizada no interior da Ilha dos Marinheiros (ao sul do estuário da Lagoa dos Patos, Rio Grande do Sul, Brasil) e é conhecida como Lagoa da Noiva (Figura 7a). É um ambiente de água doce, substrato arenoso e com vegetação típica de lagos rasos, podendo variar de tamanho de acordo com as condições climáticas, apresentando baixos níveis de água durante o verão (período de seca) e conexões temporárias com lagos pequenos e rasos durante o inverno (período chuvoso) (QUINTELA et al., 2009, 2018).

Figura 6 - Fisiografia do Rio Grande do Sul e do Uruguai. As regiões de estudo da presente tese englobam a Planície Costeira e o Escudo Uruguaio-Sul-Rio-Grandense.



Fonte: https://saopelotas.furg.br/pt/o-projeto

Localizado na região central da Planície Costeira está o Parque Nacional da Lagoa do Peixe, cuja lagoa que o nomeia também fez parte dos ambientes aqui estudados (Figura 7b). Assim como as demais lagoas da região, a Lagoa do Peixe apresenta águas rasas (média de 30 centímetros de profundidade, exceto nos canais) e extensão de 35 quilômetros com largura média de 1 quilômetro (LOEBMANN; VIEIRA, 2005). A salinidade pode variar de acordo com a época do ano e a região da lagoa, visto que há intrusão de água do mar devido a uma abertura artificial que ocorre periodicamente nos meses de inverno persistindo até o início do verão quando a desembocadura é bloqueada pela deposição de sedimentos marinhos.

A terceira lagoa amostrada localiza-se no Uruguai, no departamento de Maldonado, sendo conhecida como Laguna del Diario (Figura 7c). É classificada como uma lagoa semifechada, pois em condições naturais apresentava trocas com águas marinhas e salobras do Oceano Atlântico e Rio da Prata. Hoje, está lagoa é considerada como um reservatório de água doce desde que foi isolada do mar pela construção de uma rodovia costeira, em 1955 (BASSET; BAXTER; DABORN, 2014; PANARIO; GUTIÉRREZ, 2011). Apresenta um espelho de água de aproximadamente 200 hectares, fazendo parte de uma série de ecossistemas aquáticos que se estendem em direção ao departamento de Rocha até o sul do Brasil, tendo sua formação ocorrida durante o Quaternário pelo desenvolvimento de dunas litorâneas na última transgressão marinha (CALVIÑO, 2007).

Estuários

Os estuários são habitats expostos tanto a água doce como a salgada, dependendo das marés, da drenagem de terras, do vento, bem como da morfologia local, apresentando grande variação de salinidade (SCANES; FERGUSON; POTTS, 2017). No extremo sul do Brasil encontra-se o estuário da Laguna dos Patos. Esta que é a maior laguna do Brasil e segunda da América Latina (com mais de 265 km de comprimento e 10.227 km^2 de superfície). Caracteriza-se por apresentar enseadas rasas e numerosas ilhas, com típica vegetação de marismas (PALMA-SILVA et al., 2012). As amostras no estuário foram realizadas nas margens de duas ilhas do estuário da Laguna dos Patos: na Ilha dos Marinheiros (Figura 7d) e na ilha da Torotama (Figura 7e).

Ambas ilhas são de origem recente (não superior a 5.000 anos), estando em plena evolução geomorfológica (VIEIRA, 1983). Marinheiros é a maior das ilhas deste complexo lagunar, estando distante aproximadamente 1.6 km da boca do estuário. Nos períodos de chuvas intensas pode haver conexão entre os sistemas internos e as águas do estuário, devido a inundações das áreas periféricas da ilha (QUINTELA et al., 2018). Nossas amostragens ocorreram na face nordeste na ilha, na

localidade da Marambaia, ao longo de um pequeno canal de escoamento de águas internas, próximo à costa da ilha. O segundo ponto de amostragem no estuário se deu na costa da ilha da Torotama, em sua porção norte. Esta ilha apresenta pequena cota de emersão, com pobre cobertura vegetal, estando quase ligada ao continente (VIEIRA, 1983).

Piscinas rochosas marinhas

As piscinas rochosas são ambientes formados por covas e depressões na zona entre marés que retém água na maré baixa, agindo como refúgio para muitos organismos (WHITE; HOSE; BROWN, 2015). Constituem-se de micro-habitat extremamente produtivos com diversidade de plantas, invertebrados e peixes. Contudo, são ambientes isolados e irregularmente distribuídos ao longo da costa, sendo variáveis no tempo e no espaço (MARTINS; HAWKINS; THOMPSON, 2007). Dessa forma, estes ambientes são extremamente variáveis, com drásticas mudanças físicas entre as marés, o que propicia a ocorrência de adaptação fisiológica, morfológica e comportamental para os organismos que vivem nesse tipo de ambiente (ZANDER; NIEDER; MARTIN, 1999).

Nossas amostragens se concentraram nas piscinas rochosas localizadas próximo ao porto de Punta del Este, Uruguai (Figura 7f). São ambientes de águas marinhas rasas compreendendo cerca de 200 m de comprimento, sem contato com ambientes de água doce (o mais próximo está localizado a 5 km de distância) (CALVIÑO; ALONSO, 2016).

Sangradouros

A costa do extremo sul é formada por praias arenosas, dunas e áreas úmidas localizadas atrás da linha de dunas costeiras (PALMA-SILVA et al., 2012), com poucas descargas pluvio-lagunares ao longo dos 640 km de litoral do Rio Grande do Sul. Contudo, observam-se numerosos pequenos cursos de água, denominados sangradouros, que fazem parte da drenagem da planície costeira, dando escoamento às águas pluviais coletadas nas depressões e banhados (FIGUEIREDO; CALLIARI, 2005). Estes córregos apresentam dinâmica espacial e temporal específicas, podendo apresentar variação nos níveis de salinidade na região supra-litoral (próximo e entre as dunas), uma vez que esta zona está sob efeito do spray marinho podendo sofrer inundações pelas águas oceânicas durante eventos extremos (marés altas e tempestades) (GIANUCA, 1998). Contudo destaca-se a baixa variabilidade na salinidade ao longo do ano em sangradouros da praia do Cassino (BASTOS et al., 2014). O curso de água dos sangradouros é variável dependendo da

quantidade de chuvas de modo que a localização dos mesmos pode ser alterar entre estações (FIGUEIREDO; CALLIARI, 2006).

Foram amostrados sete sangradouros ao longo da costa dos municípios de Rio Grande e Santa Vitória do Palmar (Figura 7g). Todos ambientes apresentam substrato arenoso com macrófitas submersas e emergentes e água corrente em sentido ao mar.

Riachos (arroios)

Ambientes lóticos, como rios e riachos, são caracterizados por um fluxo de água forte e unidirecional, sendo um sistema aberto com características que se alteram ao longo de seu curso (UIEDA; CASTRO, 1999). Organismos que habitam ambientes lóticos refletem seu nicho localizado com adaptações específicas para os variados habitats que ocorrem desde a nascente até a foz (MCCABE, 2019). No sul do Brasil, por influência da fronteira com países de língua espanhola, os riachos recebem o nome de arroio (*arroyo*, em espanhol). Aqui adoto a expressão riacho (*stream*) como equivalente a arroio.

Localizado no município de Arroio Grande, sul do Rio Grande do Sul, a bacia do Arroio Chasqueiro, possui uma área de 500 m^2, afluindo para a margem oeste da Lagoa Mirim. A bacia é formada pela congruência de dois riachos (Arroio Chasqueiro e Arroio Chasqueirinho) (Figura 7h), os quais são represados formando a Barragem do Chasqueiro, seguindo o curso de água por um único riacho à jusante da barragem (Arroio Chasqueiro) (COSTA-FILHO, 2016). Os Arroios Chasqueiro e Chasqueirinho, a montante da barragem, apresentam margens formadas por pastagem e vegetação arbórea, respectivamente, com substrato formado predominantemente por areia. Nas regiões a jusante observa-se a presença de macrófitas com arbustos e árvores nas margens (CORRÊA et al., 2015).

Reservatório

A construção de reservatórios a partir do represamento de rios e riachos é uma prática usual, especialmente para o desenvolvimento da agricultura de larga escala, como a de arroz irrigado, cujo maior produtor do Brasil é o estado do Rio Grande do Sul (PALMA-SILVA et al., 2012). Além da alteração causada ao ambiente natural (de um ambiente lótico temos um ambiente lêntico), os reservatórios são diferentes dos lagos naturais, pois o regime de operação determinado de acordo com propósito pelo qual o reservatório foi criado pode alterar significativamente suas características físico-químicas e respostas biológicas (SCHMUTZ; MOOG, 2018).

O reservatório estudado na presente tese pertence à bacia hidrológica do Arroio Chasqueiro (Figura 7i). A construção da Barragem do Chasqueiro foi finalizada em 1983. Apresenta uma superfície máxima de 1800 ha e capacidade de acumulação de água de 117 milhões de m^3 (COSTA-FILHO, 2016). As margens do reservatório são predominantemente de campos de gramíneas utilizados para pastagem e o substrato é arenoso com ocorrência de pedregulhos (de atividade humana) (CORRÊA et al., 2015).

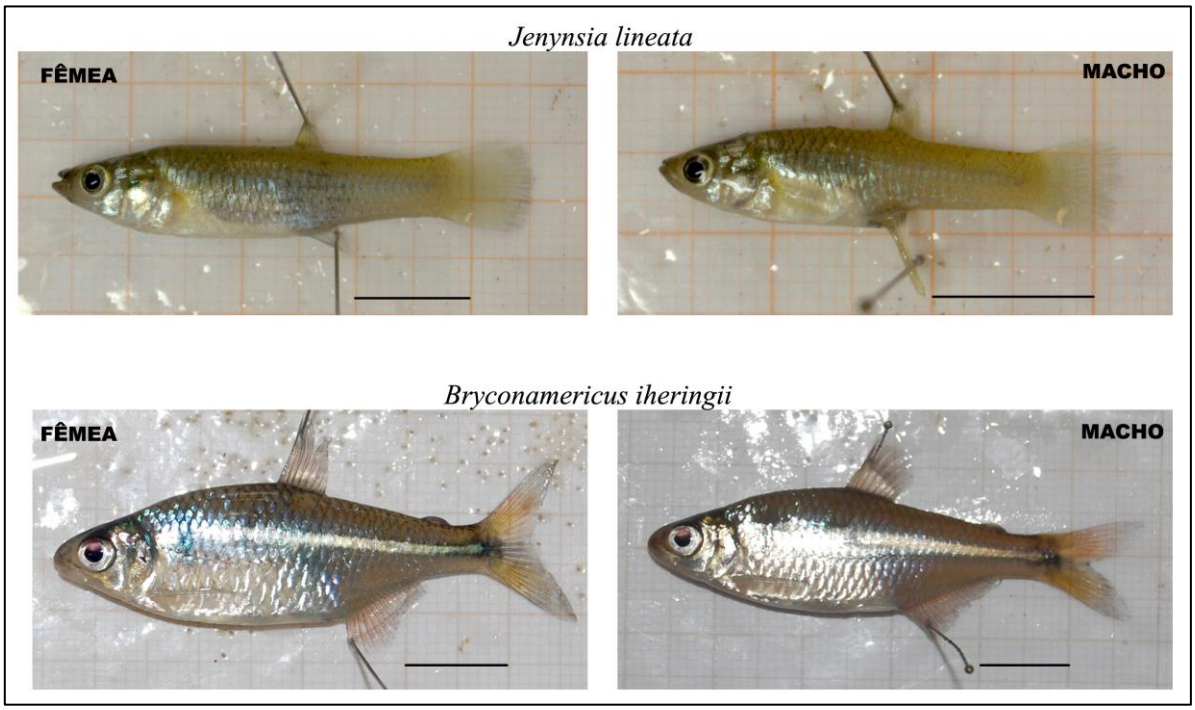
Figura 7 – Ambientes de amostragem. (a) Lagoa da Noiva, (b) Lagoa do Peixe, (c) Laguna del Diario, (d) Ilha dos Marinheiros, (e) Ilha da Torotama, (f) Piscinas Rochosas Marinhas, (g) Sangradouros, (h) Arroio Chasqueiro, (i) Reservatório do Arroio Chasqueiro. As setas indicam o local onde foram realizadas as coletas (nas imagens sem setas, as amostragens ocorreram ao longo do corpo de água representado pela fotografia).

Fonte: (b) http://parnalagoadopeixe.blogspot.com/; (c) http://www.visionmaritima.com.uy/; demais imagens: autoria própria.

As espécies estudadas

Na presente tese foram estudadas duas espécies de peixes: o anablepídeo *Jenynsia lineata* (Jenyns 1842) e o caracídeo *Bryconamericus iheringii* (Boulenger 1887) (Figura 8). Estas espécies foram escolhidas atendendo principalmente o requisito de frequência e abundância nos diferentes sistemas de estudo. Vários estudos prévios haviam apontado a ocorrência em abundância de *J. lineata* em diversos tipos de habitats (BASTOS; CONDINI; GARCIA, 2013; BASTOS et al., 2017; CALVIÑO; ALONSO, 2016; CORRÊA et al., 2015; GARCIA et al., 2004; GOYENOLA et al., 2011; MAI et al., 2007; RAMOS; VIEIRA, 2001; VOLCAN et al., 2012), tornando-a uma boa espécie candidata ao estudo de adaptação local em ambientes naturalmente distintos. Já a escolha de *B. iheringii* se deu pela abundância ao longo da bacia hidrológica do Chasqueiro relatada por CORRÊA et al. (2015), bem como seu hábito alimentar generalista (MELLO; GONZÁLEZ-BERGONZONI; LOUREIRO, 2011) tornando-a interessante para a análise da adaptação local em ambientes alterados.

Figura 8 – Exemplares das espécies estudas. A barra em cada imagem representa 1 cm.



Fonte: Autoria própria.

Jenynsia lineata

Recentemente, *J. lineata* foi reconhecida como sinônimo sênior de *J. multidentata* (AMORIM, 2018), sendo, portanto, a nomenclatura utilizada nesta tese. Pertencente a ordem Cyprinodontiformes e a família Anablepidae, *J. lineata* apresenta ampla distribuição pelo sul da América do Sul, do rio Colorado, na Argentina, até a porção baixa da bacia do rio Paraná e rios e riachos costeiros entre o Uruguai e o estado do Rio de Janeiro (GHEDOTTI, 2003). São animais de pequeno tamanho com corpo alongado, levemente comprimido lateralmente e coberto por escamas. Apresentam de 5 a 7 linhas longitudinais com manchas pretas em forma de traços curtos, estando ausente ou pouco evidente em fêmeas grandes (MALABARBA et al., 2013).

Assim como observado para outros Cyprinodontiformes, *J. lineata* apresenta dimorfismo sexual. Machos são facilmente distinguidos de fêmeas por seu tamanho menor (comprimento padrão médio de machos: 4,5 cm; comprimento padrão médio de fêmeas: 8,5 cm) e por apresentarem a nadadeira anal modificada em um gonopódio (órgão copulatório) (BETITO, 2006; LOPEZ-RODRIGUEZ; BARROS; PERRY, 2017). São animais ovovivíparos, com fertilização interna e sem cuidado parental (MELLO; GONZÁLEZ-BERGONZONI; LOUREIRO, 2011). Apresentam ciclo reprodutivo anual, com duas cortes (indivíduos que nascem de dezembro a março se reproduzem de setembro da novembro; indivíduos nascidos de setembro a novembro se reproduzem a partir de março) (GARCIA et al., 2004).

É uma espécie de hábito alimentar onívoro, ingerindo tanto plantas (macrófitas, algas marinhas) como animais (microcrustáceos, insetos, poliquetas), embora o consumo de vegetação possa ser acidental tendo em vista os indícios de que essa espécie não assimila este alimento (BASTOS et al., 2017). Ocorrem em uma variedade de habitats, tais como lagoas (FONTOURA et al., 1994), estuários (BASTOS et al., 2017; GARCIA et al., 2004; MAI et al., 2007; RAMOS; VIEIRA, 2001), piscinas rochosas marinhas (CALVIÑO; ALONSO, 2016), sangradouros (BASTOS; CONDINI; GARCIA, 2013) e riachos (CORRÊA et al., 2015; VOLCAN et al., 2012). Essa variabilidade de ambientes, associada com sua abundância fazem de *J. lineata* um interessante organismo para entender os processos de adaptação local a ambientes costeiros na região Neotropical.

De fato, algumas variações morfológicas foram observadas em *J. lineata* de distintos ambientes, especificamente com diferenças de salinidade. Primeiramente, FONTOURA et al. (1994) relataram diferenças no tamanho corporal de *J. lineata* de uma lagoa (água doce) em comparação aos dados obtidos anteriormente para os animais que habitam estuário (água salobra). Observou-se que, em média, os animais de águas com maior salinidade eram maiores do que os

animais de água doce. Posteriormente, MAI; GARCIA; VIEIRA (2005) comprovaram por meio de testes em laboratório que *J. lineata* cresce mais (comprimento total e peso) em ambientes salinos do que em ambientes dulcícolas. Dessa forma, sabemos que *J. lineata*, que apresenta ampla distribuição e ocorrência em uma variedade de habitats, com variação no tamanho entre populações de ambientes com diferentes concentrações salinas. Estes são indicativos de que esta espécie apresenta potencial para análises sobre adaptação local, uma vez que o estudo de espécies com similares características de variação trouxeram importantes conhecimentos sobre o tema (ARAÚJO; MONTEIRO, 2013; BAKER et al., 2015; BERNER; GRANDCHAMP; HENDRY, 2009; FOSTER et al., 2015; GOMES; MONTEIRO, 2008; LANGERHANS et al., 2004, entre outros).

Bryconamericus iheringii

Pertencente a grande família Characidae, *B. iheringii* é uma espécie de pequeno porte podendo alcançar 11,4 cm de comprimento padrão (ZANIBONI-FILHO et al., 2004). É comumente encontrado em variados ambientes como riachos, rios e lagoas, distribuindo-se ao longo das bacias da Laguna dos Patos, do rio Uruguai, do rio da Prata e do rio Paraguai (KOKUBUN et al., 2018; MELLO; GONZÁLEZ-BERGONZONI; LOUREIRO, 2011; TATSUMI, 2006). Não apresenta dimorfismo sexual evidente, exceto pela presença de diminutos ganchos nas nadadeiras anal e pélvica em machos maduros, e seu período reprodutivo ocorre nos meses de verão e primavera do hemisfério sul (LAMPERT; AZEVEDO; FIALHO, 2004).

São generalistas podendo apresentar comportamento oportunista caso haja restrição de itens alimentares no ambiente, o que é conhecido como adaptabilidade trófica (GERKING, 1994). Essa capacidade de mudança de dieta conforme as condições ambientais (disponibilidade de alimento e interação com outras espécies) evidenciam o oportunismo da espécie e a capacidade de adaptação frente a alterações de paisagens. KOKUBUN et al. (2018) relataram que *B. iheringii* apresentam variação intrapopulacional na forma do corpo, o que pode estar associado ao hábito alimentar generalista da espécie. Contudo os autores não consideraram a variação inerente aos sexos, tendo em vista que a espécie apresenta crescimento alométrico, além de machos, em geral, apresentarem tamanho ligeiramente menor que as fêmeas (FERRIZ et al., 2010). Sendo assim, é possível que haja variação na forma do corpo entre *B. iheringii* que habitam distintos ambientes, tendo em vista que a espécie apresenta amplo espectro alimentar.

De acordo com o levantamento feito por CORRÊA et al. (2015) na bacia hidrológica do Arroio Chasqueiro, *B. iheringii* foi uma das duas espécies mais abundantes ao longo do sistema,

com ocorrência tanto no reservatório como nas porções à jusante e à montante do mesmo. Considerando as características da espécie, que indicam adaptação trófica de acordo com as condições do ambiente e o registro de abundância em ambientes alterados, *B. iheringii* apresentou-se como uma boa opção para análise de adaptação local em ambientes com impacto antrópico decorrente da construção de barragens.

Justificativa e objetivos

Compreender a variação intraespecífica entre populações de peixes que habitam distintos ambientes torna-se relevante sob a perspectiva do estudo da evolução desses organismos, pois permite contribuir com o entendimento de como as espécies evoluem, incorporando a capacidade de variação de origem genética ou plástica e de sua importância para a seleção natural. Além disso, contribui para a compreensão das interações entre os organismos aquáticos e o seu meio, cada vez mais sujeito às alterações antrópicas (FLORENTINO; SÚAREZ, 2014).

Dessa forma, a presente tese de doutorado teve como objetivo principal estudar o processo adaptativo intraespecífico de peixes expostos a ambientes heterogêneos, tanto naturais como antropizados. Para as análises em ambientes naturais, utilizei a morfometria geométrica e linear e a genômica populacional para avaliar a variação intraespecífica a nível morfológico e genético em *J. lineata* de distintos habitats. Neste caso os objetivos específicos foram:

- investigar se existe variação fenotípica em *J. lineata* de distintos habitats e como ela se manifesta;
- relacionar a variação morfométrica com fatores ambientais, a fim de inferir quais variáveis ambientais podem influenciar a variação na forma e tamanho do corpo;
- avaliar se machos e fêmeas apresentam as mesmas respostas fenotípicas frente a variação ambiental;
- analisar a estrutura genômica populacional de *J. lineata* de distintos habitats a partir de SNPs obtidos através de RADSeq;
- identificar SNPs potencialmente sob seleção divergente e associados com variáveis ambientais, indicando quais genes podem estar relacionados com adaptação em *J. lineata*.

Para avaliar como o processo de adaptação local ocorre em ambientes alterados via ação humana, utilizei a morfometria geométrica e linear para avaliar a variação intraespecífica em *B.*

iheringii expostos a ambientes naturais e alterados, ao longo da bacia do Arroio Chasqueiro. Especificamente, os objetivos foram:

- investigar se há variação fenotípica entre populações de *B. iheringii* expostas a distintas condições ambientais (ambiente lótico *versus* ambiente lêntico), avaliando as alterações na forma e tamanho do corpo que podem ocorrer em espécies submetidas a uma relativamente recente alteração no curso de um riacho;
- analisar se no caso de espécies sem dimorfismo sexual evidente, machos e fêmeas respondem de forma semelhante à alteração ambiental.

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CAPÍTULO 1

SHAPE AND SIZE VARIATION OF *JENYNSIA LINEATA* (JENYNS 1842) (CYPRINODONTIFORMES: ANABLEPIDAE) FROM DIFFERENT COASTAL ENVIRONMENTS

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Este artigo está publicado na revista **Hydrobiologia** (2019) 828: 21

https://doi.org/10.1007/s10750-018-3794-6

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Abstract

A key question in ecological speciation is to understand the causes and consequences of phenotypic divergence among populations. In this work, we analyzed the body shape and size variation in *Jenynsia lineata* across different coastal habitats along the Atlantic coast of South America. We hypothesized that *J. lineata* presents morphological variations to inhabit contrasting environments and that these adaptations are sex-specific. We analyzed 13 populations from five coastal habitats, using linear and geometric morphometry, and tested the correlation of body shape variation with environmental variables to understand which environmental factors may influence body shape and size variation. *Jenynsia lineata* showed differences in body shape and size among populations, and these differences are specific to each sex. While females showed a variation in the caudal peduncle correlated with water current, we did not find such trait variation and correlation in males. Alternatively, individuals from marine rocky-pools have a convex body curvature along the dorsal profile and larger body sizes, in both sexes. With these results we describe the shape and size morphological variation of *J. lineata* and discuss this uncommon habitat-dependent sexual dimorphism in a Neotropical livebearer fish.

Keywords

Geometric and linear morphometrics; *Jenynsia multidentata*; local adaptation; phenotypic plasticity; sexual dimorphism

Introduction

Morphological variation in populations of fish inhabiting divergent environments provides important examples of the processes related to evolution (Langerhans & DeWitt, 2004; Haas et al., 2010; Torres-Dowdal et al., 2012; Theis et al., 2014; Rowiński et al., 2015). Different environments across a species' distribution range may generate an array of distinct selection regimes, which could promote adaptive variation among populations (Townsend et al., 2008), driving plastic responses and/or genetic adaptive divergence (Crispo, 2008). In the absence of other evolutionary mechanisms and constraints, these individuals with local trait variation would have, on average, a higher relative fitness in their local environment than individuals from other habitats (Kawecki & Ebert, 2004).

A key question in the research about ecological speciation is to understand the causes and consequences of phenotypic divergence among populations. In fishes, some environmental conditions have been recognized as important causes of morphological variation and are often related to locomotion (Haas et al., 2010; Theis et al., 2014; Gaston & Lauer, 2015; Lauder, 2015) or feeding (Langerhans et al., 2004; Gomes & Monteiro, 2008; Araújo et al., 2014; Zanella et al., 2015; Ingley et al., 2016). Another common observation is that fishes show convergence in body shape when populations occur along similar environmental gradients, allowing the elaboration of an eco-morphological paradigm for the correlation between body shape and environmental selective pressure (Langerhans et al., 2004). For example, different fish species tend to have a more compressed body shape (longer dorsal-ventral axis and shorter anterior-posterior axis) in populations of lentic water environments compared to those of lotic environments, where body shape is typically more fusiform (Haas et al., 2010; Gaston & Lauer, 2015). Such an eco-morphological paradigm has also been established for different predation pressures. In this case, populations of phylogenetically or geographically distinct species feature a pattern of variation in the body shape, according to the presence or absence of predators, that is related to the ability of predator escape (burst-swim) (Langerhans et al., 2004).

The one-sided livebearer *Jenynsia lineata* (Jenyns, 1842) is one of the most abundant brackish and freshwater fish in the subtropical regions of South America (Garcia et al., 2004; Goyenola et al., 2011) and has recently been recognized as a senior synonym of *J. multidentata* (Jenyns, 1942) (see Amorim, 2018). This species is omnivorous (Bastos et al., 2017), with viviparous reproduction, and shows a remarkable sexual dimorphism whereby females are larger than males, while males feature a copulatory organ (gonopodium) formed by a modification of the anal fin (Betito, 2006; Lopez-Rodriguez et al., 2017). Popularly known as '*barrigudinho'* (Brazil),

'*overito'*, or '*madrecita'* (Argentina and Uruguay), *J. lineata* is widely distributed and occurs in a variety of habitats, such as estuaries (Ramos & Vieira, 2001; Garcia et al., 2004; Mai et al., 2007; Bastos et al., 2017), coastal washouts (Bastos et al., 2013), lagoons (Fontoura et al., 1994), freshwater streams (Volcan et al., 2012; Corrêa et al., 2015), and marine rocky-pools (Calviño & Alonso, 2016). This variability of environments, associated with its abundance, makes *J. lineata* an interesting organism to understand local adaptation to Neotropical coastal habitats.

Heterogeneous aquatic environments, such as coastal habitats, have long been in the focus of research related to adaptive divergence in fish, and body variation are often correlated with a salinity gradient characteristic for such habitats (Norris et al., 2010; Olsen et al., 2016; Dennenmoser et al., 2017). In *J. lineata*, for example, specimens are larger in brackish populations than in those from freshwater environments (Fontoura et al., 1994; Mai et al., 2005). Other Cyprinodontiformes, such as *Poecilia vivipara* (Bloch & Schneider, 1801) (Gomes & Monteiro, 2008; Araújo et al., 2014) and *Gambusia affinis* (Baird & Girard, 1853) (Langerhans et al., 2004), or even fishes from other orders, such as *Clupea harengus* (Clupeiformes) (Jørgensen et al., 2008) or *Gasterosteus aculeatus* (Linnaeus, 1758) (Gasterosteiformes) (Marchinko & Schluter, 2007; Berner et al., 2008, 2009; Baker et al., 2015; Foster et al., 2015), show a similar variation in body size and shape along salinity gradients. Different environments are an important source of divergent natural selection, and adaptation to those habitats may, under some circumstances, lead to speciation (see e.g. Schluter, 2009; Nosil, 2012). To understand the dynamics of adaptation to different habitats, it is important to characterize morphological variation at the level of individuals and populations as well as at ecological scales (Shukla & Bhat, 2017).

An important issue when one is interested in understanding the contribution of habitat environments to body shape variation is to consider the patterns of sex differences in body shape (Mokodongan et al., 2018). Since body shapes could be specific to each sex, species with remarkable sexual dimorphism might show males and females with distinct habitat-related body shape variation. In such cases, trade-offs between natural and sexual selection often underlie the diversification of sexes (Heinen-Kay et al., 2015), especially because evolutionary forces that have shaped the breeding success of males are fundamentally different from those acting on females (Bronson, 1985). Species with marked sexual dimorphism are the primer evidence that sexual selection is a strong force in the evolution of this intraspecific divergence (Kocher, 2004). However, habitat choice can also contribute to adaptative correlations between phenotype and environment (Porter & Akcali, 2018). In this context, *J. lineata* seems to be an adequate species to investigate inter- and intrapopulation body variation and the phenotype *versus* habitat

correlations in Neotropical coastal environments, since this organism shows a remarkable sexual dimorphism and inhabits distinct ecosystems across its distributional range.

In this study, we investigated the body shape and size variation in *J. lineata* across different coastal habitats along the Atlantic coast of South America. For this purpose, we analyzed, through linear and geometric morphometrics approaches, 13 populations of *J. lineata* from five different coastal ecosystems. Geometric morphometrics is based on landmark coordinates, permitting the exploration and visualization of large high-dimensional data sets (Mitteroecker & Gunz, 2009). Linear measurements, or traditional morphometrics, involve summarizing morphology in terms of length measurements, ratios, or angles (Webster & Sheets, 2010). In general, the geometric morphometrics approach provides better insights into the underlying functional relationships than linear traditional measurements (Sidlauskas et al., 2011; Fabre et al., 2014). However, the combination of linear measurements and geometric morphometrics should be used in harmony to yield the most complete understanding of morphology (Ginter et al., 2012). Here, we used geometric morphometrics to evaluate the variation in body shape and size (through centroid size, a composite size measure based on all landmarks (Mitteroecker et al. 2013)), with the aim to investigate the phenotypic variation in *J. lineata* among distinct habitats. We used linear measurements to improve the size analyses and to obtain information regarding the size of specific structures of the body (such as fins and body lengths), not available from geometric morphometrics data. We then related body shape with environmental variables to understand which environmental factors could influence body shape variation. Beyond, we investigated how each sex responds morphologically to the habitat variation. With this study, we aimed to determine how a fish species with remarkable sexual dimorphism could be phenotypically adapted to distinct environments. Specifically, we wanted to understand whether *J. lineata* presents phenotypic variation between environments, what varies in body size and shape, which environmental factors could be associated with such variation, and if males and females present the same responses. Our hypothesis was that *J. lineata* should present morphological adaptation to inhabit contrasting environments, such as marine versus freshwater or lotic versus lentic habitats, and that adaptation is sex-specific: due to the morphological size and shape distinction between sexes, males and females would show distinct morphological adaptation, even considering the same habitat.

Materials and Methods

Study area

We analyzed shape and body variation of *J. lineata* from five different environments: coastal washout, estuary, freshwater stream, lagoon, and marine rocky-pool (Fig. 1, Table 1). The coastal washouts, estuaries, and lagoon populations were in the coastal plain of Rio Grande do Sul, Brazil. This large plain (~ 620 km long) is characterized by a rectilinear coastline, extensive dune fields, and numerous lagoons (including the Patos-Mirim system) and others water bodies, which may temporarily be connected through small channels (Castelao & Moller-Jr, 2006).

The coastal washouts, locally known as '*sangradouros'*, are freshwater streams (creeks) that cross the dune belt towards the beach. They play an important role in the drainage of pluvial water from swamps located behind the frontal dune systems (Figueiredo & Calliari, 2006). These washouts have specific spatial and temporal dynamics with varying levels of salinity (Gandara-Martins et al., 2014). In this study, we sampled fish from seven coastal washouts in the supra-littoral region (near/between dunes), a zone under the effect of sea spray that can be inundated by ocean water during extreme events (high tides or storms) (Gianuca, 1998). The distance between the sampled washouts varied from approximately 2 km (between the coastal washouts 1, 2, 3, and 4) to more than 48 km (between the coastal washouts 5, 6, and 7) (the distance between washouts 4 and 5 was approximately 20 km) (see Fig. 1a). Note that these distances are not strict, as the coastal washouts change their course depending on the season (Figueiredo & Calliari, 2006). The substrate in the washouts was dominated by fine sand and submerse and emergent macrophytes were observed in these water bodies.

Located at coastal margins, estuarine habitats are exposed to both fresh- and salt water, depending on the tides, land drainage, wind, and local morphology, thereby showing a great variation in salinity (Scanes et al., 2017). In this study, we sampled two estuarine populations along the coast of the Torotama and Marinheiros Islands, located in the Patos Lagoon. On Torotama Island, sampling was performed along the island coast, an open beach region without vegetation and with sandy substrate. On the Marinheiros Island, we sampled along a small channel (approximately 100 m long) near to the island coast, a region characterized by muddy substrate with vegetation (including trees) along the channel borders.

On Marinheiros Island, we also sampled a second population of *J. lineata* from a shallow lagoon, known as Noiva Lagoon. This lagoon is in the middle of the island, behind sand dunes, and is constituted by freshwater, sandy to muddy substrate, and the typical vegetation found in

shallow lakes. This environment can vary in size according to climatic conditions, with low water levels during the dry season (summer) and temporal connections with adjacent small and shallow lakes during the rainy season (winter) (Quintela et al., 2009, 2018). We also sampled in a second lagoon, called Peixes Lagoon, with shallow water depths (average of 30 cm, except on the channels) (Loebmann & Vieira, 2005); in this lagoon, the northern part (limnetic region) was sampled.

A freshwater stream population was sampled in the Sul-Riograndense shield, South Brazil. The sampled stream is called Chasqueiro and belongs to the Mirim Lagoon hydrographic system. It is in the city Arroio Grande, where it is used as freshwater reservoir. Our sampling was performed upstream of the reservoir, a region that has an average width of about 8 m and a depth of about 30 cm, with a strong water current (lotic environment) (Corrêa et al., 2015).

Additionally, a marine rocky-pool population was sampled in Uruguay, near the port of Punta del Este (Maldonado, Uruguay). This population was described as the first record of *J. lineata* in a truly marine environment (Calviño & Alonso, 2016). At that location, *J. lineata* inhabits shallow rocky-pools that have no connection to freshwater.

Sampling and data acquisition

Thirteen populations of *J. lineata* (N = 554, females = 357, males = 197) were sampled between January 2016 and February 2017, using a beam trawl and hand nets (see Table 1 for details). In the laboratory, the individuals were anesthetized by immersion in clove oil solution and digital images were taken in a standardized way. Specifically, photographs of the lateral left side of each living organism were taken using a digital camera (Nikon® D90 or P600) mounted at approximately 50 cm. The specimens were positioned on a graph paper, their fins were extended, and the dorsal and anal fins were pinned.

Digital images were converted into .tps files, using the tpsUtil 1.64 software (Rohlf, 2013). For each specimen, 12 landmarks and 12 linear measurements (Fig. 1b) were recorded using the software tpsDIG2 2.30 (Rohlf, 2015). Linear measurements were recorded between some landmarks (LM1–LM4: estimation to head size; LM7–LM8: dorsal fin width; LM11–LM12: dorsal fin width; LM10–LM11: ventral width of caudal peduncle; LM9–LM10: caudal peduncle depth) and between landmarks and the extremities of the anal, caudal, and dorsal fins (LM3, LM2 and LM1, respectively). To avoid bias related to the acquisition of landmarks and linear measurements, the same person performed all processes (GXP). We used only adult specimens

(standard length > 19 mm, according to Garcia et al., 2004) and avoided pregnant females (identified visually).

Environmental parameters were recorded at each sampling location. Dissolved oxygen, pH, salinity, and water temperature were measured using a multi-parameter water quality checker (Horiba®, model U50). Surface water current and microhabitat current were determined according to Theis et al. (2014), with the following modifications: surface water current was estimated by measuring the time a 500 ml plastic bottle filled with 250 ml of water traveled 10 m. Microhabitat current was estimated considering the level of the water current. To this end, we used lollipops (Florestal® Flopito Baby, strawberry flavor, individually wrapped) to measure the relative rate of dissolution. Prior to the measurement, each lollipop was precisely weighted. For the measurements, four lollipops were mounted underwater for 6 minutes (using a fixed line) while one was exposed, for the same period, to water taken from the same location, but left in a beaker filled with 500 ml. This control treatment was performed to determine the baseline dissolution rate for each site, without water current. After recovery, the lollipops were dried at ambient temperature for at least 24 h and then weighted to calculate the mass lost relative to the baseline for each site. The water column depth of each environment was determined using a measuring tape. Substrate type was classified as sandy (type 1), muddy (type 2), or rocky (type 3).

All parameters were determined five times at each sampling site, except for microhabitat current, which was measured four times. The different measurements were performed at slightly different sites, about 10 m away from the previous one. For habitat characterization, the mean across all measurements was used. These environmental variables were measured during the spring season of 2017 for 12 populations (note that it was not possible to obtain environmental information from the coastal washout 7, Fig. 1). Although habitat parameters might seasonably vary to some extent, the relative differences between sampling habitats are likely to be consistent.

Data analysis

Geometric Morphometry: Due to a pronounced sexual dimorphism in this species, females and males were analyzed separately. Geometric morphometric analyses were performed with shape coordinates obtained from Generalized Procrustes Analyses (GPA) (Rohlf & Slice, 1990), which minimizes the differences of translation, scaling, and rotation between landmarks (Zelditch et al., 2012). We also obtained the centroid size values from GPA, characterized as the square root of the sum of the squares of the distance of each landmark from the centroid (mean of all coordinates) of the configuration (Bookstein, 1991). Normality distribution and homogeneity of

variances of centroid size values were checked by Shapiro-Wilk and Levene's tests, respectively, and the size variation among populations was analyzed through Analysis of Variance (ANOVA), followed by Tukey´s *pos-hoc* test with Bonferroni correction. A Welch F test was performed in the cases that homogeneity of variances was rejected. Possible allometric effects, caused by different ontogenetic stages among specimens, were removed by regressing Procrustes coordinates (shape variable) into centroid size (size variable). Multivariate analyses were performed with the covariance matrix calculated from the resulting regression residuals (Stange et al., 2016). Principal Components Analysis (PCA) was used to identify the axes of maximal shape variance among all specimens and the patterns associated with this variance as well as to identify grouping of variance among the specimens. Multivariate Analysis of Variance (MANOVA), followed by pairwise comparisons, was performed to analyze shape statistical differences using the scores of informative principal components (based on a Broken-Stick distribution) as dependent variable and habitat as independent variable. The MANOVA was followed by Wilks' λ test to identify the proportion of the variance that is explained by the independent variable (population). We used the Canonical Variates Analysis (CVA) to describe the differences among groups (habitats) and to form mathematical functions, which were used to assign specimens to groups through jackknife cross-validation analyses (Zelditch et al., 2012).

Linear Morphometry: Standardized relative measures (called RM) were used for linear morphometric analyses, which were obtained by dividing each measure by the individual standard length, according to Shukla & Bhat (2017). A total of 525 specimens (335 females and 190 males) were used for the linear morphometry analyses because it was not possible to obtain measures for all sampled specimens (some specimens presented damaged fins, making its measurements impossible). Normality distribution and homogeneity of variances for relative distances were checked by Shapiro-Wilk and Levene's tests, respectively. The variation among populations for each RM was analyzed through Analysis of Variance (ANOVA), followed by Tukey´s *pos-hoc* test with Bonferroni correction. A Welch F test was performed in the cases that homogeneity of variances was rejected. Factorial analyses were performed to describe the interdependent relationship between the linear measures. To this end, Bartlett's test of sphericity and the Kaiser-Meyer-Olkin (KMO) criterion were used to check the assumptions of non-correlation among variables and the adequacy of the data matrix, respectively, with factors with eigenvalues > 1 being selected.

General Linear Models: To understand the relationships of shape variation and environmental variables, we developed predictive models using the scores of informative principal components as response variables and selected environmental variables as predictive variables.

First, we checked the correlation of these variables individually with the informative principal components scores, separately for each sex. Variables with Pearson´s correlation coefficient > 0.2 were selected to develop the models. When predictive variables correlated with each other (r > 0.7), we selected the one with higher correlation with the response variable. Linear regression analyses were performed independently for each informative PC (as response variable), using the selected environmental variables as predictors. The models were developed based on a stepwise regression modeling method, where the environmental variables that best explained the response variables (shape variation) were selected as the best model for each shape variable, using the adjusted R^2, p-value, and the AIC (Akaike information criterion) values as criteria for the selection of predictive models (Shukla & Bhat, 2017). The assumptions of linearity, normality, and homogeneity of variances were checked through plots of residuals versus fitted values, normal Q-Q, and scale-location (squared root of standardized residuals versus fitted values), respectively. When some of these assumptions were not achieved, logarithmical transformations were performed with the predictive variables to fit assumptions, and subsequently, the predictive models were developed. Extreme values were cut off based on Cook's D plot.

Analyses were performed in R environment (R Core Team, 2013), using the Geormorph (Adams & Otárola-Castillo, 2013; Adams et al., 2017) and MASS (Venables & Ripley, 2002) packages for geometric morphometric analyses, the Hmisc (Harrell, 2014) and Psych (Revelle, 2017) packages for predictive models, and nFactors (Raiche, 2010) and REdaS (Hatzinger et al., 2014) for factorial analyses. Graphs were edited using the software Inkscape v0.92. Differences were considered significant at $p < 0.01$.

Results

Geometric morphometric analyses

The results of the MANOVA indicated that mean body shape was distinct among the habitats, both in females ($F_{4, 352} = 26.498$, Wilks' $\lambda = 0.45805$, $p < 0.001$) and in males ($F_{4, 192} = 9.232$, Wilks' $\lambda = 0.49896$, $p < 0.001$). The difference was significant among all habitats ($p < 0.001$), except for males from stream and coastal washouts habitats ($p = 0.1603$). The jackknife cross-validation analyses indicated an overall classification accuracy of 82.1% for females and 72.1% for males, with correct classification among habitats varying from 42 to 96% (Table 2).

For females, the Broken-Stick model indicated PC1, PC2, and PC3 as the informative principal components, which together accounted for 58.7% of the total variation. These PC axes

were mainly related to the caudal peduncle length, curvature of the body, as well as the head size (Table 3). Specimens from lagoon environments were distributed mainly in the negative end of the PC1 (which explained 28.3% of the total variation), having shorter and wider caudal peduncles when compared with specimens from the stream and coastal washouts, which have longer and narrower caudal peduncles. The PC2 (explaining 17.8% of the total variation) distinguished two groups based on body curvature. Specimens from marine rocky-pools showed a convex body curvature along the dorsal profile, with a more ventral position of the mouth and the caudal peduncle, as compared to specimens from all other populations (Fig. 2).

For males, we identified the first four PC axes as informative components, accounting for 65.9% of the total variation (Table 3). In contrast to females, it was not possible to clearly distinguish groups along the males' PC axes. However, males from marine rocky-pools also occupied a distinct position in the morphospace indicating the same body curvature as females from the same environment, as observed in both PC1 (22.2% of total variation) and PC2 (19.4% of total variation) (Fig. 2).

The CVA, for both females and males, evidenced the distinction among the specimens from marine rocky-pools and the others along CV1, which accounted for 56.7 and 47.4% of the total variation, respectively (Fig. 3). This distinction was also clear when the Procrustes distance was observed (Table 4). This difference regarding the marine rocky-pools was also observed for body size, where an ANOVA indicated that centroid size was different among the populations, irrespective of sex (Table 5). Mean centroid size of marine rocky-pools specimens was larger than the mean centroid size from specimens from other populations (Fig. 4). However, regarding centroid size, pairwise comparisons showed that most populations were statistically different ($p < 0.001$), i.e., mean centroid size was significantly different within habitats.

Linear measurement analyses

Total length (RM5) was different among the populations for both sexes (Fig. 5). For females, body depth (RM6), caudal peduncle length (RM9), and depth (RM10) also differed among populations, whereas males differed with respect only to caudal fin length (RM2) (Table 5). For males, the specimens from marine rocky-pools were larger in terms of total length.

In females, factorial analysis indicated four factors with eigenvalues > 1, accounting for 59% of the total variation. The higher loadings for the factor 1 were those associated with the caudal peduncle (RM9 and RM10) and with the body depth (RM6), whereas the total length (RM5)

and caudal fin length (RM2) loadings were higher for factor 2. The remaining factors were mainly related to eye diameter (RM12) and dorsal fin length (RM1). In males, we identified five factors with eigenvalues > 1, with 67% of the total variation. The first factor, which accounted for 21% of the total variation, was mainly related to body length (RM5) and caudal fin length (RM2). Factors 2 and 3 (12% of total variation each) were associated with gonopodium size (RM3) and body depth (RM6). The remaining factors were associated with caudal peduncle (RM9 and RM10) and eye diameter (RM12) (Table 6, Fig. 6).

Predictive models

There was a strong correlation between microhabitat current and superficial water current ($r > 0.7$). For this reason, only microhabitat water current was used to develop the models. In females, multivariate linear regression with stepwise selection indicated microhabitat water current and substrate as the main predictors for the PC1 scores ($F_{2, 309} = 40.99$, $p < 0.001$, $R^2 = 0.205$), but only microhabitat water current was statistically significant at $p < 0.01$. Substrate and pH fit the model with the PC2 scores ($F_{2, 309} = 59.61$, $p < 0.001$, $R^2 = 0.274$). For males, the model indicated a correlation between the PC1 scores and salinity and water temperature ($F_{2, 179} = 15.08$, $p < 0.001$, $R^2 = 0.135$), with salinity being the main predictor. Substrate and water temperature best explained PC2 (lower AIC value and higher R^2 adjusted). The remaining informative PC scores did not show any significant correlation with the available environmental variables (Table 7).

Discussion

Fishes often exhibit phenotypic divergence in response to habitat differences (Bruckerhoff & Magoulick, 2017). Variation in the water current has been identified as one of the most important factors associated with morphological adaptation in fishes (see e.g. Gomes & Monteiro, 2008; Berner et al., 2009; Haas et al., 2010; Foster et al., 2015). However, only relatively few studies have looked at differences in body shape related to water current between males and females. Here, we examined phenotypic divergence in body shape in *J. lineata* inhabiting different coastal habitats. We found that females and males showed distinct shape variations with respect to the analyzed environmental variables. In females, shape variation primarily involved the caudal peduncle, whereas males did not show any variation in this trait. Females from environments with lower microhabitat current showed wider and shorter caudal peduncles compared with specimens

living in habitats with higher microhabitat current, which is concordant to what has been observed in other species (Haas et al., 2010; Theis et al., 2014; Gaston & Lauer, 2015; Lauder, 2015). Surprisingly, water current was not correlated with shape variation in males of *J. lineata*.

This habitat-related sexual dimorphism means that the environmental conditions might exert different selection pressures in variation of body shape and size among sexes. This pattern of sex differences in body shape and size is critical for our understanding of the role of natural selection (e.g., resource availability, presence of predators, water current) in sexual dimorphism evolution, especially in species with remarkable morphological sex differences, such as *J. lineata.* Regarding to sexual dimorphism, two main hypotheses have been proposed as drivers of the evolution of this characteristic: sexual selection and intraspecific niche divergence with adaptation of each sex to different ecological niches within the same environments (Shine, 1986). Both hypotheses are not mutually exclusive. Hence, it is possible that females and males of *J. lineata* occupy distinct niches, where females are more exposed to water current than males, occupying different places in the microhabitat. Other *Jenynsia* species (*J. alternimaculata* Fowler (1940) and *J. maculate* Regan (1906)) have been observed using different microhabitats in northwestern Argentina when recorded with underwater videos and visualizations; larger individuals used the central portion of the creek, while smaller individuals and males occupied the margins (Felipe Alonso, pers. obs. unpublished data). Indeed, it is well established that sexual dimorphism in size is extremely important in driving sexual segregation, at least in ungulates (see Ruckstuhl, 2007). This sexual segregation occurs when females and males of a species have a differential use of space (Peterson & Weckerly, 2017). This behavioral phenomenon is widespread in the animal kingdom, but poorly documented in aquatic environments (Wearmouth & Sims, 2008).

On the other hand, mating preferences could play a role in the sexual dimorphism observed in *J. lineata*, since organisms exhibiting genitalia that cannot be retracted are particularly susceptible to premating sexual selection and natural selection (Langerhans, 2010). Analyzing the effects of male genital size on attracting mates in different predation regimes, Langerhans et al. (2005) found that females from other livebearer fish species exhibited mating preference for the large-gonopodium males. However, relatively large gonopodia seem to incur in a cost of reduced burst-swimming speed because of increased hydrodynamic drag (however, see Booksmythe et al., 2016). In this case, gonopodium size seems to reflect an evolutionary trade-off between premating sexual selection, favoring a larger gonopodium, and natural selection pressures related to predation avoidance, favoring a smaller gonopodium (Langerhans et al., 2005). It is possible that this kind of trade-off could be also occurring in *J. lineata*. Besides, this species features coercive mating, whereby males approach females from behind and try to thrust their copulatory organ, the role of

females is not a passive one; when observed together with males, females showed avoidance and aggression, which leads us to infer that struggling may represent a way by which the female assesses the skill and endurance of males (Bisazza et al., 2000). The trade-off between sexual selection and selection of swimming performance could promote a specific adaptive body shape in males of *J. lineata*, for which occupying micro-habitats with less water current than females (as observed for others *Jenynsia* species) should be better due to the drag caused by the prominent copulatory organ. Another explanation for such caudal peduncle differentiation between sexes could be related to a different swimming performance. However, with morphological data alone it is not possible to answer the question why females and males show distinct phenotype responses associated with the environmental variables studied herein.

We also observed a second pattern of body shape variation in *J. lineata* of both sexes from marine rocky-pools. Their bodies were more curved, and both mouth and the caudal fin had a more ventral position. This characteristic might be related to the specific environmental conditions in their habitat: permanent water current in variable directions due to the action of waves, getting water in and out of the pools. Also, variations associated with the tides could play a role as drastic alterations in environmental factors can occur in these habitats (i.e., temperature, salinity, dissolved oxygen, pH). As a result, some fish species living in this kind of environment have developed a range of adaptations (including morphological ones) that allow them to tolerate this variable environment (Laming et al., 1982; Gibson, 1986). Adults of *Kelloggella disalvoi* (Randall, 2009) (a Gobiidae from the Easter Island, Pacific Ocean) show a similar pattern of body curvature as those found in *J. lineata* from Punta del Este rocky-pools (Vera-Duarte et al., 2017). In *K. disalvoi*, the specific adaptation has been associated with diet: specimens with an inferior mouth feed more on bivalves, whereas specimens with an anterior mouth primarily prey upon copepods (Vera-Duarte et al., 2017). There is no data about the diet of *J. lineata* from rocky-pools. However, this species has been found to live on an omnivorous diet, with diet shifts between environments (Bastos et al., 2017). Possibly, the morphological variations in *J. lineata* from rocky-pool populations might also be associated with foraging habits. However, it is also highly possible that numerous complex evolutionary processes act together to shape the local morphological differences among the studied populations.

An additional interesting characteristic of *J. lineata* from rocky-pools is its body size. Specimens from Punta del Este showed the largest centroid size in both sexes, and males of that population were the largest ones of all tested. Previous works in *J. lineata* reveled variations in size according to salinity (Fontoura et al., 1994; Mai et al., 2005). This relation between size and salinity has been described for numerous fish species (Langerhans & DeWitt, 2004; Gomes &

Monteiro, 2008; Jørgensen et al., 2008; Araújo et al., 2014; Baker et al., 2015; Foster et al., 2015). One explanation for this variation could be the different predation pressure regime present in marine habitats with higher salinity. Salinity changes the environmental conditions, causing alterations in the habitat structure and influencing the entire ecological community. Generally, brackish water environments contain fewer piscivorous fishes than freshwater ones, where prey needs to spend more energy to escape from predators, leading to shorter bodies (Gomes & Monteiro, 2008). We did not find any piscivorous fishes in the marine rocky-pools from Punta del Este (Calviño & Alonso, 2016), and it is therefore possible that the size difference observed in *J. lineata* could be related to the predation pressure (however, it is also possible that other fishes inhabit these pools and were not sampled). However, size changes could be a byproduct of physiological mechanisms under other constrains with no adaptive positive value. Salinity influences growth in numerous fish species by affecting standard metabolic rate, food intake, food conversion, and/or hormonal stimulation. In fact, numerous hormones are involved in osmoregulation and growth regulation (Boeuf & Payan, 2001). Different environments may present divergent environmental conditions that may act together and interact. Therefore, experiments manipulating those variables under controlled conditions are necessary to assess the extent and contribution of those factors to morphological changes and to evaluate if those are due to genetic adaptive responses, phenotypic plasticity, consequences of other restrictions, or random effects such as genetic drift and founder effects.

In conclusion, in different habitats, *J. lineata* shows variations in relation to body shape and size, and these variations are not the same for males and females. Water current seems to be an important environmental factor correlated to body shape variation, while the salinity degree is strongly correlated with body size. We highlight both locomotor and foraging habits as the main functions that might be related with the body shape and size variation observed in *J. lineata*. Apart from some issues that could not be resolved in this study, our findings present the morphological adaptation of *J. lineata* inhabiting contrasting environments, and this adaptation is sex-specific. To test whether the observed phenotypic variation is due to phenotypic plasticity or to genetic polymorphism (allelic variation at coding loci), common garden experiments are needed. In this sense, genomic investigations could also be interesting to identify polymorphisms that could be related to the observed phenotypic variation.

Acknowledgments

The authors are grateful to Dr. Bruna F. Nornberg, Dr. Daiane Carrasco, Dr. Fernando Quintela and MSc. Daiana K. Garcez for the help in the field work; Dr. Madlen Stange and Dr. Rodrigo Fornel for great guidance about geometric morphometric analyses; and Dr. Gustavo E. Chiaramonte and Prof. Ricardo Ferriz for the ichthyologic collection access at Museo Argentino de Ciencias Naturales Bernardino Rivadavia. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de N vel Superior - Brasil (CAPES) - Finance Code 001 and by the Swiss Government Excellence Scholarship for Foreign Students - Switzerland.

Tables

Table 1. Environmental parameters (mean ± SD) and number of females and males analyzed for each population. Environmental variables for CW-7 population was not available for analyses, and also depth for CW-6. OD = oxygen dissolved; Sal = salinity; Temp = water temperature; Depth = depth of water column; Micro = microhabitat water current; Super = Superficial water current.

Environment	Population	Females	Males	pH	OD (mg/ml)	Sal (ppm)	Temp (°C)	Depth (cm)	Micro (mg/s)	Super (m/s)	Substrate
Coastal washouts (CW)	CW-1	55	12	8.0±0.1	8.9±1.9	0.3±0.0	22.9±0.8	100.0±3.8	0.9±0.2	0.1±0.0	Sandy
	CW-2	25	10	8.0±0.0	8.4±1.2	0.3±0.0	23.4±0.1	8.9±5.5	6.2±0.8	0.3±0.0	Sandy
	CW-3	15	11	8.1±0.0	9.6±1.7	0.2±0.0	22.2±0.0	21.0±7.0	5.6±0.1	0.4±0.0	Sandy
	CW-4	24	7	8.0±0.1	10.1±2.6	0.2±0.0	22.8±0.1	29.0±9.2	5.8±1.3	0.2±0.0	Sandy
	CW-5	21	14	7.3±0.1	10.6±2.6	0.1±0.0	19.9±0.0	53.0±8.6	4.5±0.7	0.3±0.0	Sandy
	CW-6	11	10	7.4±0.1	11.2±3.1	0.1±0.0	18.7±0.7	---	2.6±0.5	~0	Sandy
	CW-7	39	16	---	---	---	---	---	---	---	---
Estuary	Torotama Island	22	19	7.0±0.1	11.0±0.4	0.0±0.0	16.3±0.1	36.0±3.8	3.5±0.9	0.2±0.0	Sandy
	Marinheiros Island	20	11	6.5±0.1	5.2±0.6	0.0±0.0	18.4±0.1	32.0±1.9	0.5±0.4	~0	Moody
Lagoon	Noiva Lagoon	46	17	7.9±0.3	10.0±0.3	0.0±0.0	18.9±0.7	84.0±7.2	1.2±0.9	0.1±0.0	Moody
	Peixe Lagoon	36	36	8.0±0.3	10.4±0.7	0.1±0.0	21.3±0.1	33.0±2.6	1.3±0.1	0.1±0.0	Moody
Stream	Chasqueiro Stream	17	14	6.1±0.3	10.4±0.9	0.0±0.0	18.6±0.2	120±23.5	1.4±0.6	0.1±0.0	Moody
Marine rocky-pools	Punta del Este	26	20	8.8±0.1	9.9±0.4	21.3±0.6	19.6±1.1	31.0±16.5	1.1±0.5	~0	Rocky

Table 2 Classification results (%) from CVA jackknife cross-validation.

Females						
Habitat	Estuary	Stream	Lagoon	CW	Marine	Correct classification
Estuary	47.6	0.0	16.7	35.7	0.0	47.6
Stream	5.9	52.9	0.0	41.2	0.0	52.9
Lagoon	6.1	0.0	79.3	12.2	2.4	79.3
CW	2.6	1.6	4.2	91.6	0.0	91.6
Marine	0.00	0.0	0.0	3.8	96.1	96.1
Males						
Habitat	Estuary	Stream	Lagoon	CW	Marine	Correct classification
Estuary	60.0	0.0	20.0	16.7	3.3	60.0
Stream	7.1	42.9	7.1	42.9	0.0	42.9
Lagoon	3.8	0.0	71.7	24.5	0.0	71.7
CW	3.7	2.5	12.5	78.7	2.5	78.7
Marine	5.0	0.0	0.0	10.0	85.0	85.0

Table 3 Explained variations of the informative principal components of the shape attributes related with the higher loadings landmarks.

Principal component	**Explained variation (%)**	**Landmarks with higher loadings**	**Fish shape attributes**
Females			
PC1	28.31	9, 10, 11, 12 (x-axis)	Caudal peduncle length
PC2	17.77	9 (y axis), 4 (x-axis), 12, 11 (y-axis)	Body curvature
PC3	12.59	6, 4, 1, 5 (x-axis)	Head size
Males			
PC1	22.18	12, 9, 6 (x-axis), 1 (y-axis)	Caudal peduncle and head length, and mouth position
PC2	19.39	4, 1, 5, 2 (x-axis)	Head size
PC3	13.91	10, 8, 7, 4 (x-axis)	Caudal peduncle length and head size
PC4	10.42	12, 7, 8 (x-axis), 10 (y-axis)	Caudal peduncle length

Table 4 Procrustes distances from least square means generated in CVA analyses.

Females					
	Estuary	Stream	Lagoon	CW	Marine
Estuary	0	0.01536845	0.01419506	0.01249562	0.03260140
Stream		0	0.02669847	0.01426437	0.03968148
Lagoon			0	0.02182658	0.03020874
CW				0	0.03403277
Marine					0
Males					
	Estuary	Stream	Lagoon	CW	Marine
Estuary	0	0.01963892	0.02252585	0.01919011	0.03234595
Stream		0	0.01853569	0.01195373	0.03219391
Lagoon			0	0.01461908	0.03052316
CW				0	0.02594600
Marine					0

Table 5 Centroid size and linear measurements differences among populations, through analysis of variance (ANOVA). It is presented just those measurement which were statistically important (i. e., with p-value < 0.01 and with the population sum of squares higher/equal than residuals sum of squares).

		Females			
	DF	Sum Sq	Mean Sq	F value	p-value
RM5 – total length					
population	12	0.13486	0.011239	53.13	<0.001
residual	322	0.06811	0.000212		
RM6 - body depth					
population	12	0.07129	0.005941	60.76	<0.001
residual	322	0.03148	0.000098		
RM9 - ventral width of caudal peduncle					
population	12	0.06780	0.005650	25.21	<0.001
residual	322	0.07215	0.000224		
RM10 - caudal peduncle depth					
population	12	0.02201	0.0018345	42.88	<0.001
residual	322	0.01378	0.0000428		
Centroid Size					
population	12	25.92	2.1597	48.85	<0.001
residual	322	14.23	0.044		
		Males			
	DF	Sum Sq	Mean Sq	F value	p-value
RM2 – caudal fin length					
population	12	0.06373	0.005311	24.3	<0.001
residual	177	0.03868	0.000219		
RM5 – total length					
population	12	0.06155	0.005129	24.32	<0.001
residual	177	0.03734	0.000211		
Centroid Size					
population	12	18.444	1.5370	51.65	<0.001
residual	177	5.267	0.0298		

Table 6 Loadings and proportion of total variation of factor analysis from linear relative measures (RM).

Females					***Males***					
Measure	Factor 1	Factor 2	Factor 3	Factor 4	Measure	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
RM6	0.84				RM2	0.95				
RM9	-0.63				RM5	0.98				
RM10	0.86				RM6		-0.59			
RM2		0.90			RM7		0.94			
RM5		0.96			RM8			0.97		
RM11			0.52		RM9				0.98	
RM12			0.89		RM10				-0.51	
RM1				0.68	RM12					0.84
RM3				0.47	RM1					
RM7				0.49	RM3					
RM8					RM11					
% variatio	20%	18%	12%	9%	% variation	21%	12%	12%	12%	10%

Table 7 Predictive models indicating the relationship between the shape variation (from informative principal components scores – PCs) and the environmental variables through linear models.

Sex	Response variables	Selected models	AIC value	F value	p-value	Adjusted R^2
Females	PC1 scores	microhabitat water current	-2617.7	78.39 $_{(1, 310)}$	<0.001	0.199
		substrate	-2682.8	5.298 $_{(1, 310)}$	0.022	0.014
		microhab.+ substrate	-2683.9	40.99 $_{(2, 309)}$	<0.001	0.205
	PC2 scores	substrate	-2731.4	96.26 $_{(1, 310)}$	<0.001	0.234
		pH	-2788.5	28.85 $_{(1, 310)}$	<0.001	0.082
		pH+ substrate	-2803.5	59.61 $_{(2, 309)}$	<0.001	0.274
Males	PC1 scores	log(salinity)	-1506.7	11.44 $_{(1, 180)}$	<0.001	0.054
		water temperature	-1512.2	5.647 $_{(1, 180)}$	0.018	0.025
		log(salinity) + temp.	-1527.4	15.08 $_{(2, 179)}$	<0.001	0.135
	PC2 scores	water temperature	-1556.8	15.77 $_{(1, 181)}$	<0.001	0.075
		substrate	-1544.9	28.98 $_{(1, 181)}$	<0.001	0.133
		temp. + substrate	-1576.6	27.63 $_{(2, 180)}$	<0.001	0.226

Figures

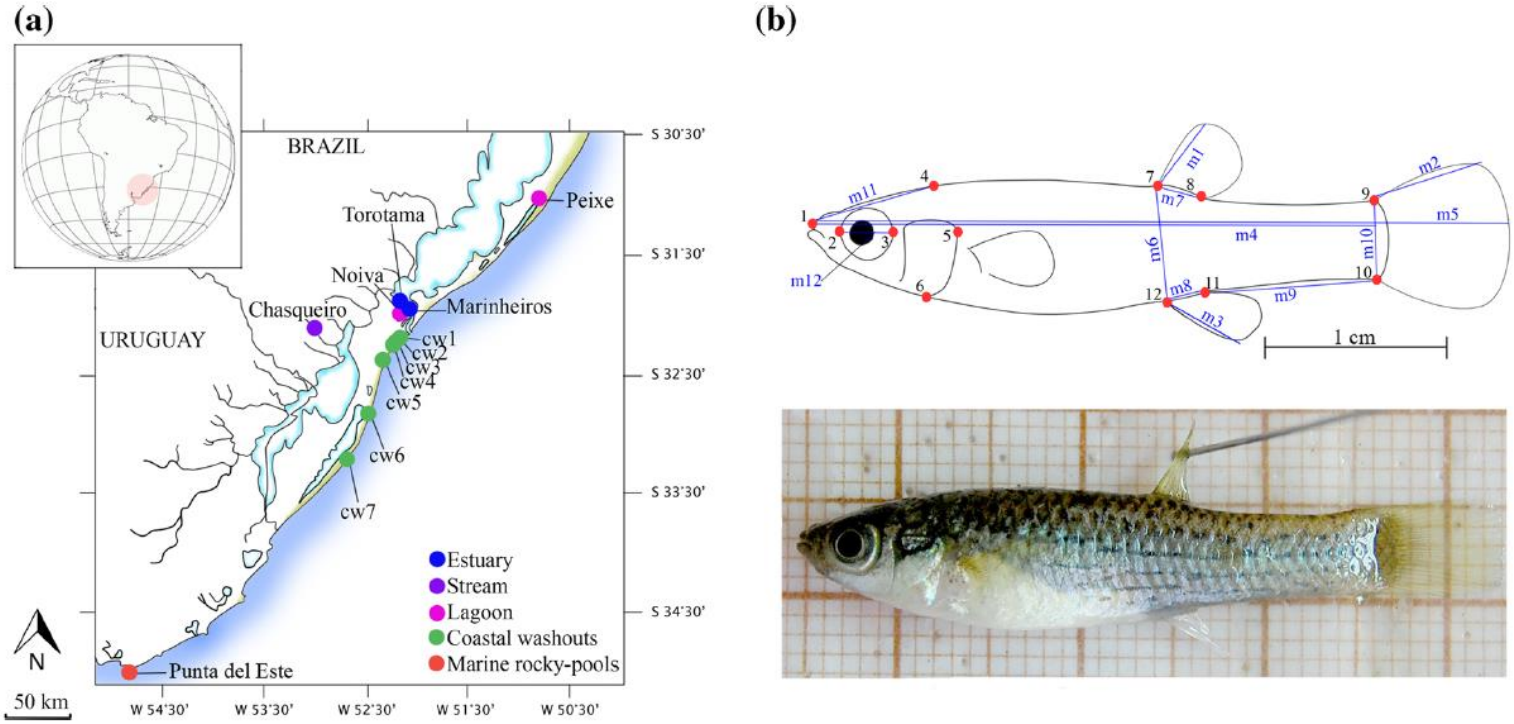

Fig. 1 Sampling sites (a) and landmark positions and linear measurements of *Jenynsia lineata* in left lateral view (b). Landmarks (red dots): 1 – snout anterior margin upper jaw; 2- eye anterior most margin; 3 – eye posterior most margin; 4 – supraoccipital process posterior margin; 5 – dorsal margin of gill opening; 6 – ventral margin of gill opening; 7 – dorsal fin origin; 8 – dorsal fin base posterior margin; 9 – caudal fin base dorsal margin; 10 – caudal fin base ventral margin; 11 – anal fin base posterior margin; 12 – anal fin origin. Linear measurements (blue lines): m1 – dorsal fin length; m2 – caudal fin length; m3 – anal fin length; m4 – standard length; m5 – total length; m6 – body depth; m7 – dorsal fin width; m8 – anal fin width; m9 – ventral width of caudal peduncle; m10 - caudal peduncle depth; m11 – head length; m12 – eye diameter.

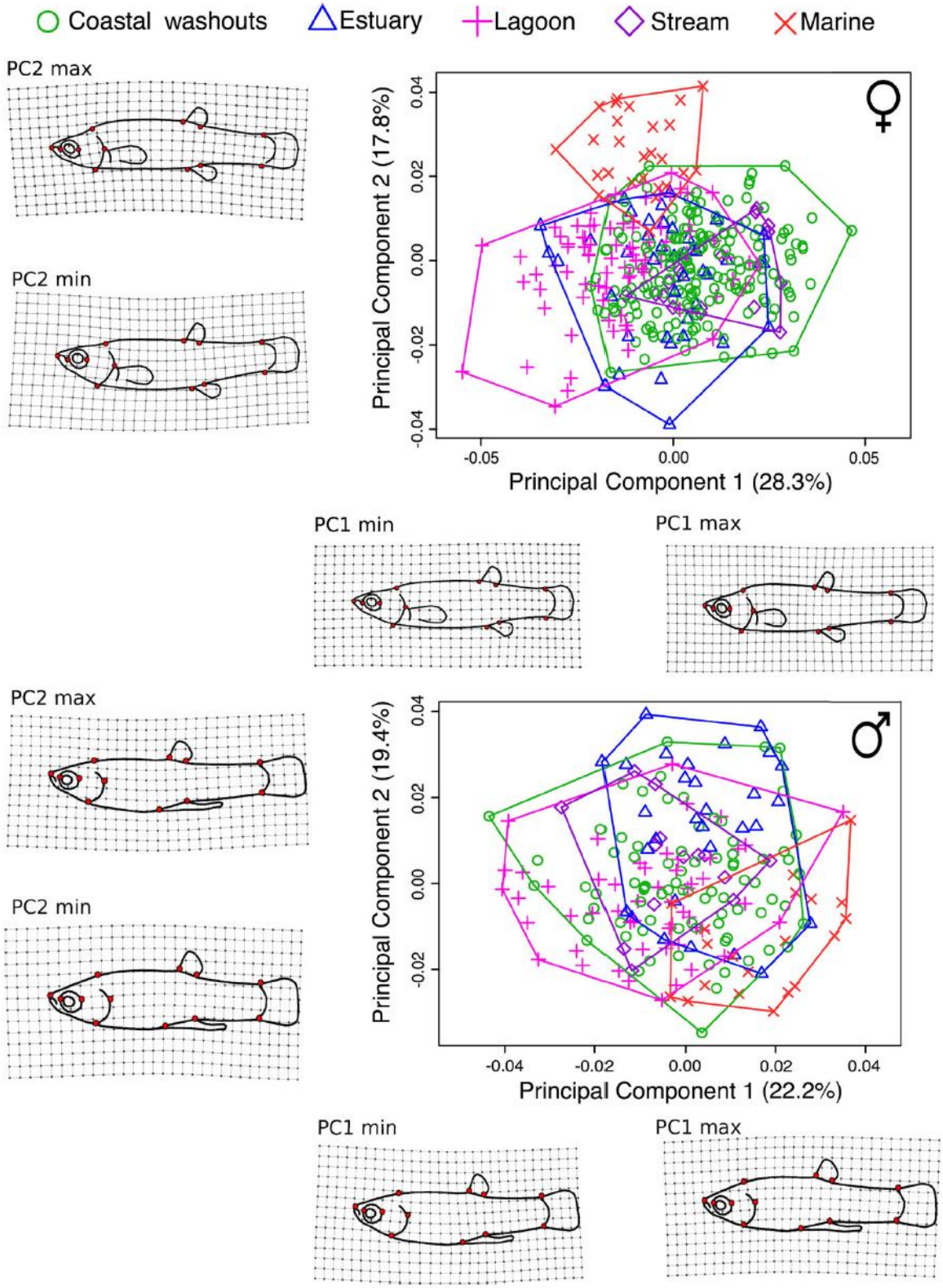

Fig. 2 Principal Components Analysis of body shape in females and males, showing PC1 versus PC2. For each PC, the shape alteration of extreme PC values in relation to the mean shape through fishes' warped drawings on grid of deformation is represented.

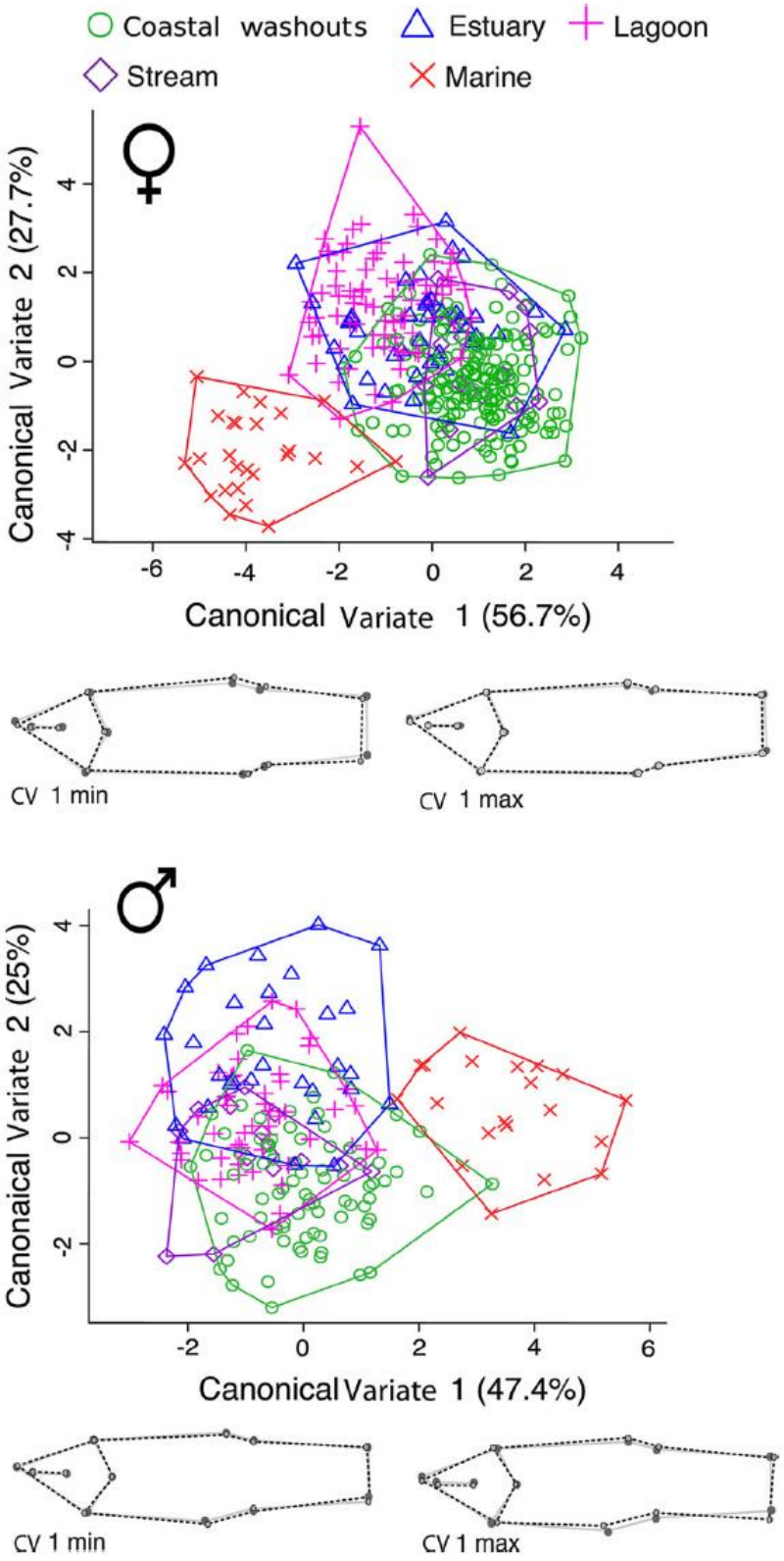

Fig. 3 Canonical Analysis of Variance among habitats for body shape in females and males, showing CV1 versus CV2. Maximum and minimum body shape alteration estimates are shown for CV1 from each sex through warped drawings.

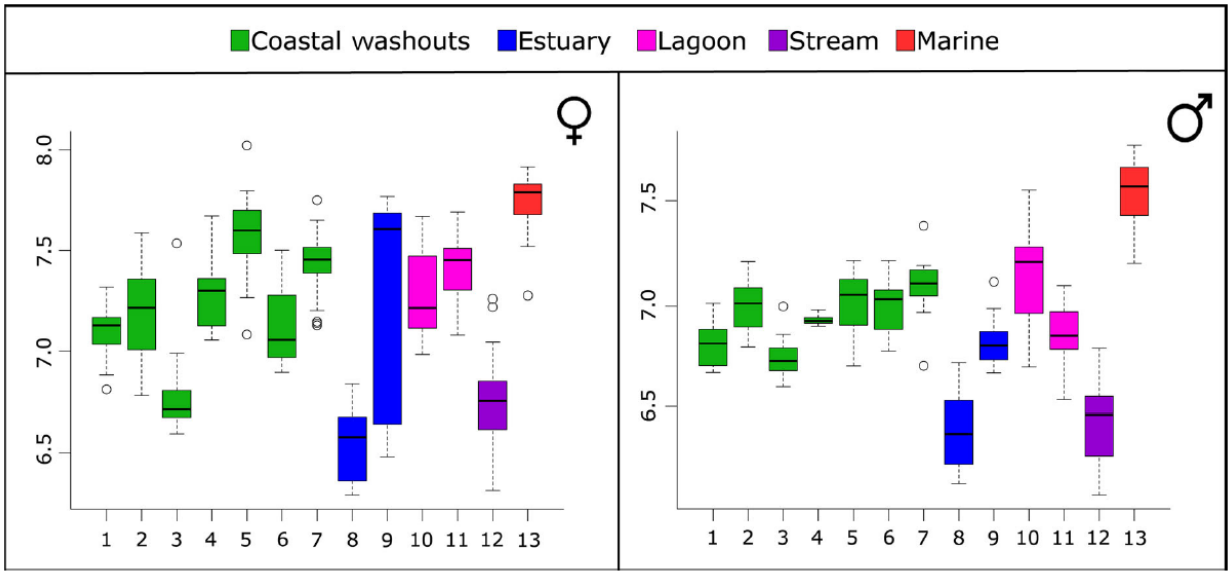

Fig. 4 Centroid size boxplot for females and males from each population (numbered from 1 to 13 in the same order as in Table 1).

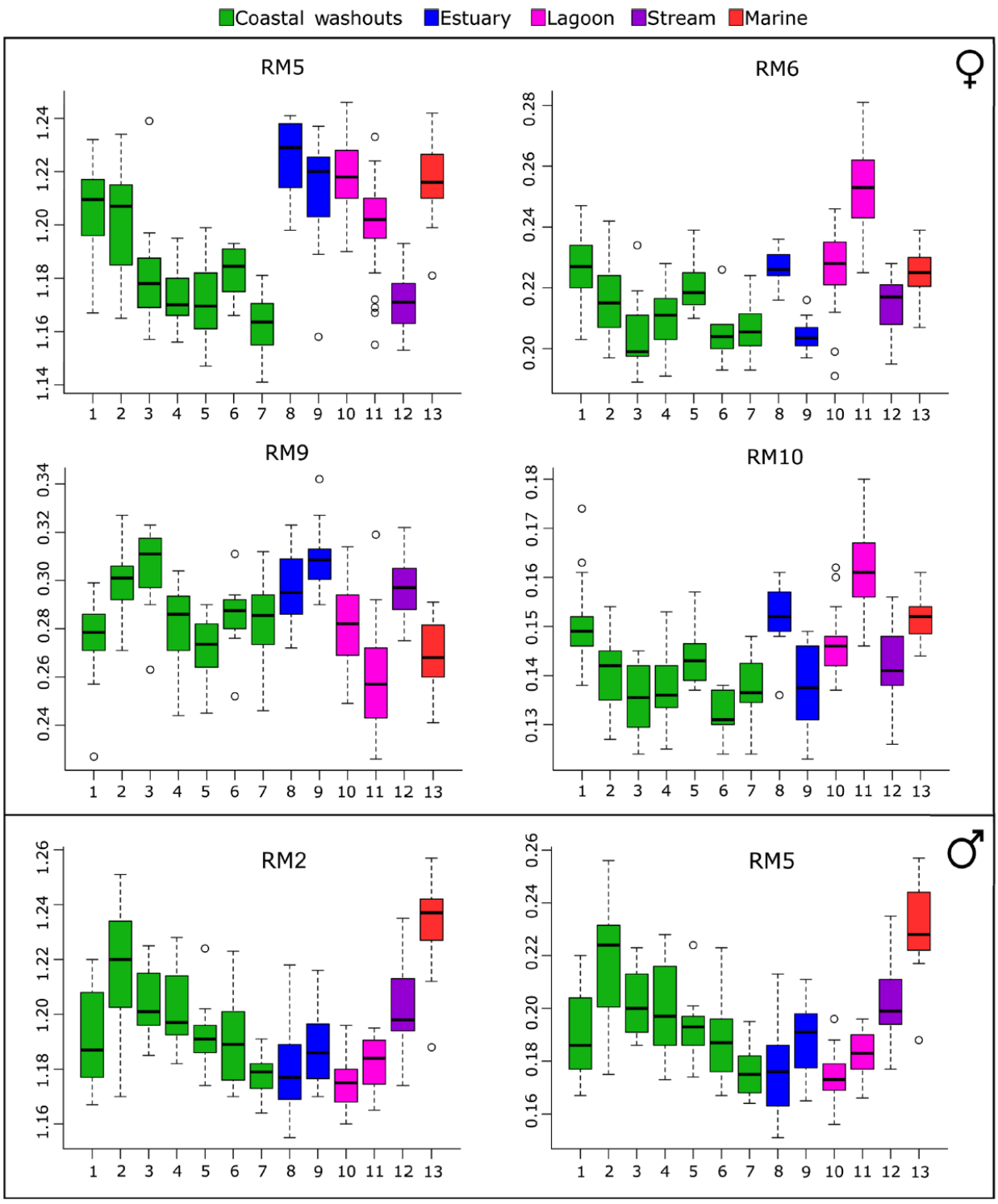

Fig. 5 Boxplots with linear measures statistically different among the 13 populations (numbered from 1 to 13 in the same order as in Table 1) for females and males.

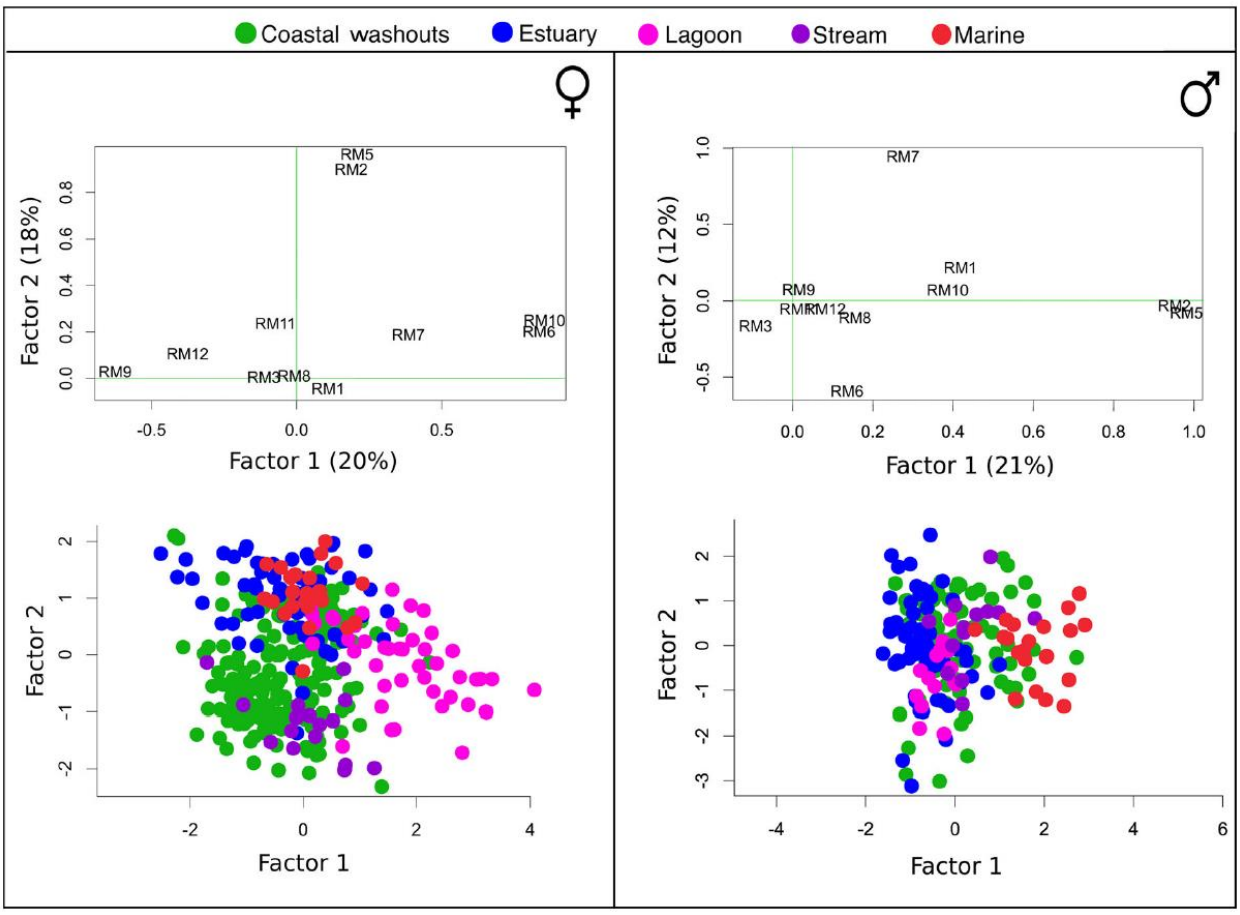

Fig. 6 Factorial analysis of linear measurements. For each sex, a plot with Factor 1 versus Factor 2 is shown, along with the contribution of the relative linear measures explaining each factor (superior plots) and the distribution of each specimen according its Factor 1 versus Factor 2 scores (inferior plots).

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CAPÍTULO 2

GENOMIC INSIGTHS OF LOCAL ADAPTATION PROCESS IN *JENYNSIA LINEATA* (JENYNS 1842) (CYPRINODONTIFORMES: ANABLEPIDAE) FROM DIFFERENT COASTAL ENVIRONMENTS

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Running Title

Genomic investigation of *Jenynsia lineata*

Este artigo será submetido para o periódico Journal of Evolutionary Biology.

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Abstract

With a distribution along a wide range of habitats and showing phenotypical variation among distinct coastal environments, *Jenynsia lineata* is one of the most abundant brackish and freshwater fish in the subtropical regions of South America, which highlights this species as an interesting organism to understand local adaptation to Neotropical coastal habitats. Using reduced-representation libraries of genomic DNA, we successfully identified 3934 SNPs in nine populations of *J. lineata* from five distinct habitats along the southern coast of Brazil and Uruguay. These populations showed pronounced genetic differentiation, with distinct patterns of genetic diversity between environments. Some SNPs were identified putatively under selection and were associated with developmental body structures, in agreement with the phenotypic variation observed among these populations. Marine rocky-pools population was genetically distinct from all other populations, with indications that it was formed by a small set of founder individuals, with privation of gene flow with other populations, as expected due the isolation condition of these habitats. In opposite, habitats subject to constant changes (coastal washouts and estuaries) showed specimens with high genetic diversity, but also with high coefficients of inbreeding, suggesting the occurrence of subpopulations at these habitats. This genomic investigation about population structure, genetic diversity and discovery of SNPs putatively under selection supported the morphological variation already observed for *J. lineata* in these environments, corroborating with the hypothesis that this species is locally adapted to variable habitat conditions.

Keywords

Jenynsia multidentata; Livebearer fish; Neotropical region; Population genomics; RADSeq; SNPs

Introduction

Species inhabiting heterogeneous environments are subject to distinct selection pressures (Townsend *et al.*, 2008), which may promote local adaptation. This process explains why adaptation may evolve in face of gene flow, since different phenotypes are expected to be selected for in different parts of a species' range, according to the selective pressures specific of each environment (Freeland *et al.*, 2011). Local adaptation may occur through plastic phenotypical responses and/or genetic adaptive divergence (Crispo, 2008), and these variation are often associated with the fitness to a specific environment (Kawecki & Ebert, 2004). If adaptation occurs through genetic adaptive divergence, it might create new species, through ecological speciation (Nosil, 2012).

New approaches in molecular biology, such as the high throughout 'next-generation' sequencing (NGS), have exciting implications for research on ecology and evolution of populations, due to the possibility of generating amounts of genomic or transcriptomic data on almost any organism (Elmer, 2016). Through this technology, it is possible to perform genome scans to search for potentially adaptive genetic variation in a population genomic context, as well as to estimate demographic parameters (Narum *et al.*, 2013). There are various approaches that can be used to dissect the genomic basis of local adaptation (Savolainen *et al.*, 2013), including the single nucleotide polymorphism (SNP) which refers to a single base pair position along DNA sequence that varies among individuals (Freeland *et al.*, 2011). The most widely used genome-wide scan for high-throughput SNP discovery and genotyping in ecological and evolutionary studies of non-model organisms is the restriction site-associated DNA sequencing (RADSeq) (Andrews *et al.*, 2016).

RADSeq are short fragments of DNA adjacent to each instance of a particular restriction enzyme recognition site (Baird *et al.*, 2008). The RADSeq reads can be aligned to a reference genome and genotyped using standard tools designed for whole genome sequencing data, and be also used to assembly *de novo*, generating large marker sets when no reference genome is available (Davey *et al.*, 2013). When working with a species for which there is no reference genome sequence, the RAD tags must be assembled *de novo* (with sequence error dealt with efficiently), identified from repetitive elements and allelism inferred (Davey & Blaxter, 2010). Such approach has fueled studies in ecological, evolutionary and conservation genomics by using NGS to uncover hundreds or thousands of polymorphic loci across the genome of non-model organisms (Andrews *et al.*, 2016).

Popularly known as '*barrigudinho'* (Brazil), '*overito'*, or '*madrecita'* (Argentina and Uruguay), *Jenynsia lineata* Jenyns, 1842 (senior synonym of *J. multidentata* Jenyns, 1942 (Amorim, 2018)) is a viviparous Anablepidae fish (Cyprinodontiformes) and one of the most abundant brackish and freshwater fish in the subtropical regions of South America (Garcia *et al.*, 2004; Goyenola *et al.*, 2011). *Jenynsia lineata* is widely distributed and occurs in a variety of habitats, such as estuaries (Ramos & Vieira, 2001; Garcia *et al.*, 2004; Mai *et al.*, 2007; Bastos *et al.*, 2017; Perazzo *et al.*, 2019), coastal washouts (Bastos *et al.*, 2013; Perazzo *et al.*, 2019), lagoons (Fontoura *et al.*, 1994; Perazzo *et al.*, 2019), freshwater streams (Volcan *et al.*, 2012; Corrêa *et al.*, 2015; Perazzo *et al.*, 2019), and marine rocky-pools (Calviño & Alonso, 2016; Perazzo *et al.*, 2019). Phenotypic variation has been identified in *J. lineata* both for body size and shape (Fontoura *et al.*, 1994; Mai *et al.*, 2005; Perazzo *et al.*, 2019). Individuals from higher salinity habitats show largest bodies and a convex body curvature along the dorsal profile. It was also demonstrated that females from higher water flow environments show a more fusiform body when compared with females from lentic habitats, revealing a habitat-dependent sexual dimorphism, since water flow has no correlation with body variation in males (Perazzo *et al.*, 2019).

This wide range of habitats associated with the phenotypic variation makes *J. lineata* an interesting organism to understand local adaptation to Neotropical coastal environments. In this context, the main aim of this work was to analyze the genomic population structure of *J. lineata* from five different ecotypes to evaluate wheter the observed phenotypic divergence is the result of genetic divergence, while also identifying putative genes involved that are target to divergent natural selection. Briefly, we identified 3934 quality-filtered RAD-SNPs, which were used to investigate divergence among ecotypes, the presence of outlier loci putatively under divergent selection, and SNP-environment association. Then, those loci containing SNPs putatively under selection were used for a BLAST query against the livebearer fish *Poecilia reticulata* genome database, to identify possible genes under selection in *J. lineata*.

Materials and Methods

Study area and sampling

We analyzed nine populations of *J. lineata* from five different environments: coastal washout, estuary, lagoon, marine rock-pools, and freshwater stream (see Fig. 1, Table 1). These populations are in the coastal plain of South Brazil and coast of Uruguay. The coastal washouts,

locally known as '*sangradouros'*, are freshwater streams (creeks) that cross the dunes belt towards the beach. These washouts undergo specific spatial and temporal dynamism with varying levels of salinities (Gandara-Martins *et al.*, 2014). In this study, we sampled fish from three coastal washouts (CW1, CW2, CW3) at the Cassino beach (city Rio Grande, Brazil), in the supra-littoral region (near/between dunes), a zone under the effect of sea spray that can be inundated by ocean water during extremes events (high tides or storms) (Gianuca, 1998). We sampled two populations along the Patos Lagoon Estuary (city Rio Grande, Brazil), at the coast of Marinheiros (EST1) and Torotama (EST2) Islands. As estuarine habitats, such populations are exposed to both fresh- and salt water, depending on the tides, land drainage, wind, local morphology, and, hence, showing a great variation in salinity (Scanes *et al.*, 2017). On Marinheiros Island, we also sampled a second population of *J. lineata* from a shallow lagoon, known as Noiva Lagoon (LAG1). This lagoon is in the middle of the island, behind sand dunes. It is constituted by freshwater, sandy to muddy substrate and the typical vegetation found in shallow lakes. This environment can vary in size according to climatic conditions, with low water levels during the dry season (summer) and temporal connections with adjacent small and shallow lakes during the rainy season (winter) (Quintela *et al.*, 2009, 2018a). Another lagoon population was sampled at Punta del Este, Uruguay, which is called Diario Lagoon (LAG2). This lagoon is classified as a semi-closed lagoon – originally, in natural conditions there were interchanges of water with the Atlantic Ocean. Today, this lagoon is considered a freshwater reservoir, since it was isolated from the sea due to the construction of a coastal highway, in 1955 (Panario & Gutiérrez, 2011). In Uruguay, at the same city, we sampled a population from marine rocky-pool environment (MAR). This population was described as the first record of *J. lineata* in a truly marine environment (Calviño & Alonso, 2016), without connection to freshwater. Lastly, we sampled a freshwater stream population called Chasqueiro (STR), in the Sul-Riograndense shield, South Brazil. This stream is in the city Arroio Grande where it is used as freshwater reservoir and our sampling was done upstream of the reservoir.

The fish samplings were performed between January 2016 and February 2017, using beam trawl and hand nets. In laboratory, the individuals were anesthetized by immersion in clove oil solution to take a clip of caudal fin, which was stored in 96% ethanol for subsequent genome extraction. Salinity was recorded at each sampling site using a multi-parameter water quality checker (Horiba®, model U50) (Table 1). For each place, salinity was determined five times; each measurement was performed at slightly different sites, about 10 m away from the previous one. For habitat characterization, the mean across all measurements was used. These environmental parameters were measured during the spring season of 2017 for all populations.

RADSeq library preparation and sequencing

Genomic DNA was extracted from clip fins of 132 specimens of *J. lineata* from nine populations (Fig. 1, Table 1) using E.Z.N.A.® tissue DNA kit, according to manufacture instructions. RADSeq libraries were prepared according to Roesti *et al.* (2012). Briefly, genome of each specimen was digested with the restriction enzyme *Sbf*I, followed by 5-nucleotide barcode adapter ligation to the restriction site of each sample. Next, samples were multiplexed in libraries, with 44 samples per library, and single-end-sequenced to 250bp reads in Illumina HiSeq2500 at the Genomics Facility Basel operated by ETH Zurich Department of Biosystems Science and Engineering and University of Basel. Illumina reads will be available from the Sequence Read Archive (SRA) at NCBI under the accession numbers SAMN10873280-SAMN10873411.

Bioinformatics and quality filtering

Raw sequence data quality was analyzed trough FASTQC software v.0.11.7 (www.bioinformatics.babraham.ac.uk/projects/). STACKS software v1.46 (Catchen *et al.*, 2013b) was used to demultiplex libraries, to identify loci and to call genotypes. Firstly, the libraries were de-multiplexed, according to the individual barcodes, and filtered using *process_radtag*. In this process, reads were trimmed to 150bp to remove low-quality bases at the 3'-ends reads due to increased sequencing error rate (Bernatchez *et al.*, 2016)**.** Then, the parameters for assembly were chosen based on running series of *de novo* analyses tests with a subset of samples, as suggested by Rochette & Catchen (2017). Based on the tests results (Fig. S1), we run *ustacks* module to align the short-read sequences into putative alleles, using the following parameters: M = 5 (maximum distance in nucleotide allowed between stacks), and m = 3 (minimum depth of coverage required to create a stacks). With the processed samples, we build catalogues through *cstacks* module, which create a set of consensus loci, merging alleles together, using the parameter n = 5 (number of mismatches allowed between sample loci when building the catalog). Using *sstacks* module, the putative loci constructed by the *ustacks* program were searched against a catalog produced by *cstacks*. Subsequently, we used the *rxstacks* unit to improve SNP genotype calls, to filter out unlikely haplotypic combinations (loci that were assembled differently in >10% of the samples) and to remove loci having high error rates (Rochette & Catchen, 2017). After, we run *cstacks* and *sstacks* units again with the filtered RAD loci from *rxstacks* unit. The *population* module was then used to filter and export the genotypes calls, beyond to calculate population genomic statistics. At

this module, we retained the genotypes that were available for at least in 80% of the samples within a population and shared by at least 70% of populations, beyond to present a minor allele frequency (MAF) > 0.05 and heterozygosity < 0.7. Samples that did not meet the quality standards were excluded from subsequent analyzes.

Population genomics analyses

Major allele frequency, percent of polymorphic loci, nucleotide diversity (π) and Wright's F statistics (F_{IS} and F_{ST}) were calculated for every SNP and population using *populations* from STACKS. To assess population differentiation, we used the filtered SNPs to calculate pairwise F_{ST} values for all population pairs, using GenoDive software. The significance of these differences was assessed using 10000 randomized permutation tests, with Bonferroni correction for multiple comparisons ($p < 0.001$). With GenoDive we also calculated the partition of the genetic variation across populations and habitats (marine, stream, lagoon, estuary, and coastal washout) through an Analysis of Molecular Variance – AMOVA. We run AMOVA using all SNPs and using SNPs putatively under selection (see below). We tested for isolation by distance performing a correlation between F_{ST} values and the geographical distance, through the Mantel test with 10,000 permutation, using the Genepop v4.2 software (Raymond & Rousset, 1995). To perform an ordination of specimens and populations on the genetic space, we performed an Analysis of Principal Components (PCA), using the Adegenet v2.1.1 package (Jombart & Ahmed, 2011) on R environment (R Core Team, 2013). Finally, we used two approaches to infer the populational structure. The Admixture software was used to estimate the putative genetic clusters (K) among the nine populations (Alexander *et al.*, 2009). To estimate which is the best value of K (the number of ancestral populations), we run the Admixture with default settings testing K values (K = 1 to K = 10) and accessing the best of K value though a cross-validation procedure. We also used the fineRADstruture software to infer co-ancestry matrix from RADSeq data, which represents a summary of nearest neighbor haplotype relationships in the data set (Malinsky *et al.*, 2018).

Discovery of SNPs putatively under selection

We used two methods to identify the presence of outlier loci putatively under divergent selection. Firstly, we performed a genome scan using the default setting of BAYESCAN 2.1 (Foll & Gaggiotti, 2008). Briefly, this approach is a Bayesian method that estimates the probability that each locus is subject to selection using differences in allele frequencies between populations. For

this, the analyses were based on 1:10 prior odds for the neutral model and included 20 pilot runs (with 5000 interactions each). After 50000 interactions, a locus with $\log_{10}$ (Bayes factor according to Jeffrey's scale) > 2 and with a false discovery rate (FDR) of 0.05 was considered under divergent selection.

The second method was a gene-environment association performed at BAYENV2.0, which tests for unusual allele frequency patterns correlated with environmental variables (Coop *et al.*, 2010; Günther & Coop, 2013). The environmental variable tested was salinity. After 100,000 interactions we get an estimation of Bayes factors and Spearman's Rho. Those loci resulting from the intersection between the tail of Bayes factor's distribution (top 5%) and the tail of Spearman's Rho distribution (top 5%) were considered to have allele frequency correlated with salinity, according to Günther & Coop (2013) and Bernatchez *et al.* (2016).

We used the nucleotide sequences (150 bp) containing the SNPs putatively under selection detected by BAYESCAN and by BAYENV2 for a BLASTN query (https://blast.ncbi.nlm.nih.gov/) against the genome database of the livebearer guppy *Poecilia reticulata* (Künstner *et al.*, 2016). We used the guppy genome database because it is the closest relative to *J. lineata* with an available genome, as Poeciliidae is the sister clade of Anablepidae (Amorim & Costa, 2018). We consider those hits with E-value < 1e-08 and identity > 70% to describe genes potentially under selection among ecotypes in *J. lineata*. The function of each gene was searched at UniProt database (https://www.uniprot.org/), using fish species as reference (specifically, *P. reticulata*, *Danio rerio*, and *Salmo salar*).

Results

SPNs discovery

The number of raw reads obtained was 621,942,397. After filtering process, the average number of reads per specimen was 3,314,819 ± 2,506,364 and a total of 123 individuals (37 males, 66 females and 20 of undetermined sex) were retained for genomic analysis. After running *rxstacks* unit, the mean number of loci per individual was 54,374.1 ± 16,644.65 (Fig. S2). A total of 3934 SNPs successfully passed quality filters and were retained for subsequent analyses.

Population genomics

The average major allele frequency per population (P) ranged from 0.8256 to 0.8572, and the observed heterozygosity ranged from 0.0620 to 0.2082 when considering all loci that were polymorphic in at least one population The highest observed heterozygosity was found at lagoon populations (LAG1 and LAG2), while the EST1 and EST2 populations showed the lowest values of heterozygosity. For all nucleotide positions (polymorphic and fixed) the average major allele frequency ranged from 0.9978 to 0.9982 (Fig. 2), whereas the observed heterozygosity ranged from 0.0008 to 0.0027. Considering the variant positions, the percentage of polymorphic loci indicates that populations of marine rocky-pools (MAR) and stream (STR) have a reduced genetic diversity when compared with the populations of the costal washouts (CW1, CW2, CW3), estuaries (EST1, EST2) and lagoons (LAG1, LAG2). The nucleotide diversity ($\boldsymbol{\pi}$) ranged from 0.2005 to 0.2555, with the lowest values for the marine rocky-pools specimens and the highest for coastal washouts specimens (Table 2).

Genetic divergence of *J. lineata* from different environment was assessed through the average F_{ST} for pairwise comparisons of all sampled populations in the present study (Table 3). Considering the permutation tests, we observed significant genetic differentiation between all pairs comparisons, except for the coastal washouts populations ($p > 0.001$). The Uruguayan populations (MAR and LAG2) showed the highest F_{ST} values among the pairwise comparisons ($0.117 \geq F_{ST} \geq 0.365$), including the comparison between themselves ($F_{ST} = 0.220$). Besides to be statistically different, stream population seems to be more genetically related with the coastal washouts populations than with any other. The lagoon populations (LAG1 and LAG2) were more genetically distant between each other ($F_{ST} = 0.213$), and closer each one to coastal washouts and estuaries populations (LAG1: $0.051 \geq F_{ST} \geq 0.066$; LAG2: $0.162 \geq F_{ST} \geq 0.185$). The Mantel test yielded a coefficient of correlation $r^2 = 0.5910$ ($p = 0.00019$) that evidences that the populations fit a model of Isolation by Distance.

Regarding population structure, the cluster analysis revealed that K = 3 model was the most likely number of genetically distinct populations, according to ancestry fractions generated by Admixture software (Fig. 3b). Individuals of streams and marine rocky-pools formed two of these clusters, without evidence of admixture. The third cluster was composed by individuals of the estuary (many of which presenting admixture with the stream cluster), lagoons (with LAG2 presenting signals of admixture with the marine rocky-pools clusters) and coastal washouts (also presenting signals of admixture with the stream cluster). Such results were corroborated by the co-ancestry matrix generated by fineRADstruture software (Fig. 3a). Such analysis also pointed out

the high coancestry among individuals from marine rocky-pools population, and among individuals from stream, and lower shared ancestry among individuals from coastal washouts, estuaries and lagoons. The PCA plot generated with the eigenvalues of the first two major axis shows that the PC1 defines the differences based on geographical distance (MAR and LAG2 are from Uruguay and the others from Brazil). Along the PC2 the main distinction was observed between STR and LAG1 populations. Coastal washout and estuary populations present a distribution coherent with the habitat classification. However, lagoons populations (LAG1 and LAG2) are clearly distant from each other along the PC1(Fig. 3c).

Results from the AMOVA using all loci showed that the majority (~25%) of the genetic variance that is not attributed to variation among individuals was partitioned across populations. In this case, the use of habitat to sort the populations was not supported (~6%; p = 0.042). However, using the SNPs putatively under selection, generated by BAYESCAN analysis, we obtained significant statistical variation associated to habitat (> 28%; p = 0.002). The SNPs selected to be putatively under selection associated with the environmental variable (salinity) do not support the sort of populations by habitat (p = 0.419) (Table 4).

In general, we obtained larger values for F_{IS}, which can be observed at the distribution of F_{IS} values across the polymorphic loci in each population (Table 2; Fig. 4). The estuary populations (EST1 and EST2) and the coastal washouts populations showed a large proportion of loci with F_{IS} between 0.5 and 1 (around 20 to 40%). Beyond, marine and stream populations also presented a considerable number of loci with $F_{IS} > 0.5$. On the other hand, the Lagoon populations (LAG1 and LAG2) showed the lowest F_{IS} values among the polymorphic loci (Table 2).

Discovery of SNPs putatively under selection

BAYESCAN suggested 44 loci candidates for diversifying selection, of which 11 loci presented Bayes factor > 2 (Fig. 5; Table S1). The BLAST searches of these 11 loci against the guppy *P. reticulata* genome database retrieved one loci that was associated with two scaffolds, related with chondrocyte and brain morphogenesis, synaptic regulation and swimming behavior (Table 5). SNP-environment association analysis identified 32 loci putatively under selection (Table S1). From these, two loci showed Bayes factor > 2. We used all 32 loci identified by BAYENV to BLASTN query against the guppy genome. It resulted in 12 hits that successfully passed by the filter of E-value < 1e-08 and identity > 70%. These sequence hits were associated to 67 scaffolds, which are related to many gene functions, such as blood coagulation, component

of eyes (crystalline), regulations of ion transmembrane channel activity, steroid metabolic process, neuronal, muscle, cartilage and egg development, cholesterol homeostasis, swimming behavior, among others. Of the two loci with higher Bayes factor (> 2), only the loci 60936_80 hits with one scaffold of the guppy genome, which is associated with growth plate cartilage chondrocyte morphogenesis (Table 5).

Discussion

A previous study has identified that *J. lineata* shows phenotypic divergence among distinct coastal environments, specifically associated with water flow and salinity variation (Perazzo *et al.*, 2019). In this study, we performed a genomic investigation of those populations with the main aim to identify polymorphisms that could be associated with the phenotypic variation observed among them. Using reduced-representation libraries of genomic DNA, we successfully identified 3,934 SNPs in nine populations of *J. lineata* from five distinct habitats along the southern coast of Brazil and Uruguay. With these SNPs we accessed the genetic variability among populations and habitats, and identified loci putatively under selection, detecting the genes potentially involved in such selection. This is the first genome data report for *J. lineata*.

Genomic population

In general, all populations exhibited a distribution of major allele frequency consistent with genetically diverse populations, with high percentage of polymorphic loci (between 60 – 82%). Moreover, we obtained tree ancestral cluster for *J. lineata* populations, mainly segregating the populations from marine, stream and lagoon habitats (specimens from coastal washouts and estuaries have shared ancestry with lagoon and stream). An exception was the LAG2 population, which shared ancestry mainly with marine habitat (LAG2 is located near the marine population; Fig. 1). These results reinforce the hypothesis of local adaptation of *J. lineata* to distinct coastal habitats, given the recent origin of these environments (~6,000 years BP) and the genomic diversity observed herein.

The marine rocky-pools population showed low values of the percentage of polymorphic loci and the highest average of major allele frequency. This population also shows the lowest level of nucleotide diversity and much more single-nucleotide variants (127) than the other populations. Marine environment is an uncommon habitat for *J. lineata*, although this species tolerates high salinity environments (Monteiro-Neto *et al.*, 2003; Garcia *et al.*, 2004; Mai *et al.*, 2005; Calviño

& Alonso, 2016; Quintela *et al.*, 2019). The population from Punta del Este rocky-pools represent a completely distinct environment condition for this species, specifically because of its composition by marine water, without connection with freshwater (Calviño & Alonso, 2016). Such unique environmental conditions associated with the observed pattern of low genetic diversity, high allele fixation and great number of private sites indicate that marine rocky-pools population may be younger (formed by a small set of founder individuals) and smaller than those from the other habitats, with privation of gene flow with other populations (high coancestry, Fig. 3a), as expected due to the isolation of these habitats (Catchen *et al.*, 2013a), potentially susceptible to founder events.

The opposite situation was observed for coastal washouts (CW1, CW2, CW3) and estuaries (EST1, EST2) populations. Specimens from these environments showed high percentage of polymorphic loci and nucleotide diversity, with very low number of private alleles (0 or 1). We also observed low values of observed heterozygosity and consequently higher values of Wright's inbreeding coefficient F_{IS}. Both environments, estuaries and coastal washouts, are habitats with constant changes of conditions (most of them related with the salinity variation). The coastal washouts are small channels with periodical inflow of water from swamps located behind the frontal dunes at the beach, with specific spatial and temporal dynamics (Figueiredo & Calliari, 2006; Gandara-Martins *et al.*, 2014). It can be inundated by ocean water during extremes events (high tides or storms), which are common at Cassino beach (Gianuca, 1998), and its flow velocity may change abruptly depending on rainfall regime. Similarly, estuaries are exposed to both fresh- and salt water, depending on the tides, land drainage, and local morphology, thereby showing great variation in salinity (Scanes *et al.*, 2017). It is probable that the inconstancy in such habitats creates the opportunity to population subdivision, with small populations inhabiting adequate environments (acting as refuges) under harsh conditions, surviving in alternative environments. In such case, individuals from a single sampled population could be from distinct subpopulations, which explains increases in the number of polymorphic loci and other values of genetic diversity well as the high values of inbreeding.

By the other hand, specimens from lagoon habitat (LAG1 and LAG2) showed the highest values of average observed heterozygosity (approximately twice as much), and consequently lowest F_{IS}, beyond of high values of polymorphic loci and nucleotide diversity, indicating high genetic diversity in these populations. In general, lagoons are habitats with relatively shallow, quiet water (Audrey *et al.*, 2017), representing a completely distinct scenario described for coastal washouts, stream, estuaries, and even for marine rocky-pools. Small- to medium-sized standing water bodies are generally short-lived and discontinuous in time and space. In such cases, species

tend to have critically higher mobility and colonization ability, which can lead to more dynamic and larger geographical ranges, faster colonization of new areas and greater occupancy of potential niches than lotic species/populations. On average, lentic species are predicted to maintain higher levels of gene flow between populations due the higher mobility, and also are expected to be older, with larger intraspecific genetic variability (Ribera, 2008). It is worth to highlight that LAG1 and LAG2 are considerably distant from each other and with large genetic differentiation (~ 417 km, F_{ST} = 0.254; LAG1 is in Brazil and LAG2 is in Uruguay; Fig1, Table 3). This means that these two populations do not share the same polymorphic loci, but share the same pattern of high heterozygosity, in agreement with the idea above mentioned about species inhabiting lentic environments.

According to the Habitat Templet concept proposed by Southwood (1977), habitat is the templet that drives the evolution of organisms, such that habitats constraints characteristics influence the ecological traits of the species living on it. A classic constraint characteristic in aquatic environments is the lotic *versus* lentic conditions. In our study, *J. lineata* from lotic environment (stream) showed considerable number of private variable loci, low values of polymorphic loci, heterozygosity and nucleotide diversity, beyond of high F_{IS}. These results are in opposition of those observed for populations of lentic habitats (LAG1 and LAG2). In contrast to standing water bodies, running waters bodies generally persist over longer geological periods and remain connected to other water bodies within the drainage basin. Considering dispersal as the ability of a species to establish a new population in a non-contiguous habitat patch (Bowler & Benton, 2005), species living in geologically long-lived habitats will be expected to have reduced dispersal ability, and to be more sensible to global change, as they will have more difficulty tracking rapid changes in a discontinuous habitat matrix (Ribera, 2008). Consequently, it is expecting a reduction in intrapopulation genetic variability with higher phylogeographical structure, as observed for *J. lineata* from stream habitat. Beyond to presenting reduced genetic diversity (Table 2), this population represents one cluster generated by ADMIXTURE program, which was partially shared with the coastal washouts populations (which might be considered lotic environments). “Stream” category was also distinguished from other populations by PCA and fineRADstruture analysis. Such contrast between lotic versus lentic habitat was the main factor associated with morphological variation in *J. lineata* from the same populations investigated in the present work (Perazzo *et al.*, 2019), which corroborates the hypothesis of local adaptation for *J. lineata* from distinct coastal environments.

Discovery of SNPs putatively under selection

We used two approaches to investigate SNPs that could be under divergent selection. BAYESCAN identifies candidate loci under selection using differences in allele frequencies between populations, through a method that separates neutral effects from adaptive effects (Foll & Gaggiotti, 2008). From this approach, we obtained 44 SNPs with diversifying selection, of which 11 loci showed a posterior probability of 0.99 to 1.0 (considered as decisive evidence of selection). These 11 loci have an increase in the proportion of variance explained by habitat (~28%) in comparison with the analysis using all SNPs (~6%). We obtained one hit against the genome of *P. reticulata* (Künstner *et al.*, 2016) with two predicted genes, the collagen alpha-1 type XXI (col21a1) and the SH3 and multiple ankyrin repeat domains 3 (shank3a). Collagen is an important extracellular matrix structural constituent, which is essential in cartilage and bone (Olsen *et al.*, 2000). Using QLT approach, SNPs found near the col21a1 gene were associated with body length in catfish (Geng *et al.*, 2017). *Jenynsia lineata* also present differences related to body length (Fontoura *et al.*, 1994; Mai *et al.*, 2005; Perazzo *et al.*, 2019). We found at the present work consistent evidence of putative selection at gene col21a1, which associated with the evidence of morphological variation among habitats (Perazzo *et al.*, 2019) corroborates with the hypothesis of local adaptation for *J. lineata* from distinct coastal environments.

The second predicted gene for this RAD-sequence (at the locus 15547_81) was the shank3a gene. This gene is a member of Shanks proteins family, which contains multiple sites for protein-protein interaction, and role connecting neurotransmitter receptors, ion channels and other membrane proteins to the actin cytoskeleton and G-protein-coupled signaling pathway (Sheng & Kim, 2000; Boeckers *et al.*, 2002). According the gene ontology annotations for zebrafish (*Danio rerio*), shank3a is related with neurological process, such as brain morphogenesis, long-term synaptic potentiation, postsynaptic density assembly, regulation of AMPA receptor activity, swimming behavior, among others (The UniProt Consortium, 2017). In experimental conditions, Kozol *et al.* (2015) identified disruption in motor behaviors that manifest as unproductive swim attempts, and spontaneous, seizure-like behavior in zebrafish with knockdown of shank3a gene, revealing the relationship of this gene with the swimming behavior. We found distinct morphological phenotypes for *J. lineata* which were mainly associated with locomotor habits (Perazzo *et al.*, 2019). It is possible that shank3a gene might be involved in local adaptation of *J. lineata*, favoring distinct swimming behavior among distinct coastal washout.

In addition, gene-environment association performed at BAYENV identified unusual allele frequency patterns correlated with salinity for 32 loci. The RAD-sequences of these loci putatively

under selection related with salinity had 12 hits with the guppy genome, which are related with many gene functions (see Table 6). Some biological processes are expected to be distinct between populations of species inhabiting marine and freshwater environments, such as the osmoregulation. Here, we identified a locus potentially under selection associated with the potassium voltage-gated channel gene. In sticklebacks, repeats flanking the chromosomal XI inversion contained alternative 3' exons for voltage-gated potassium channel gene KCNH4, which alternative inversion orientations was associated to generate marine- and freshwater-specific KCNH4 isoforms (Jones *et al.*, 2012). Indeed, freshwater sticklebacks have increased expression of KCNH4 when compared with marine sticklebacks (Taugbøl *et al.*, 2014). Other gene identified in responses to salinity environmental variation is the stress-related heat-shock protein, known to be affected by osmotic stress (Sørensen *et al.*, 2003). In our BAYENV analysis, we identified two loci putatively under selection that hit with the *P. reticulata* Hsp40 gene (member B5 and C6). Hps40 proteins works together with Hsp70 proteins (Song *et al.*, 2014), which had slightly higher expression in freshwater than in saltwater sticklebacks (Taugbøl *et al.*, 2014).

Although BAYESCAN and BAYENV do not overlap SNPs putatively under selection, some gene functions annotated from BLAST queries revealed repeated gene functions. One of the two loci with higher Bayes factor identified by BAYENV (loci 60936_80) is also associated with the collagen alpha-1, the same gene identified though BAYESCAN (loci 15547_81). *Jenynsia lineata* from marine rocky-pool environment showed distinct body morphology, specifically with a body dorsal profile curved and larger body length compared with populations from other coastal environments (Perazzo *et al.*, 2019). These differences might be related with distinct SNPs at genes involved in developmental body structures, such as the collagen alpha-1.

Conclusions

This is the first view of *J. lineata* genome population structure, which has revealed interesting answers, and some other questions, about local adaptation process in coastal environments in the subtropics of the Neotropical region. Previously, we demonstrate that *J. lineata* differ phenotypically in different environments (Perazzo *et al.*, 2019). At the present work, we show that this species also varies genetically among populations of these environments. As observed at morphological study, lentic versus lotic constraint seems to be an important selective strength among the coastal habitats studied herein. We observed distinct genetic diversity patterns between lentic and lotic environments, and some SNPs identified as putatively under selection

were associated with developmental body structures, in agreement with the phenotypic variation observed among these populations.

The marine rocky-pool population was genetically distinct from all other populations, including from a near freshwater population (LAG2), but shares some similarities with the nearby freshwater population LAG2. According to Calviño & Alonso (2016) and Amorim & Costa (2018), *Jenynsia* is a marine-derived lineage, with the origin of the genus associated with transition of the ancestor from marine to freshwater environments favored by Miocene marine transgressions, but constituted by a predominantly freshwater genus today. The east coast of South America experienced considerable relative sea level changes during the Holocene (García-Rodríguez *et al.*, 2006), until the formation of actual landscapes. We found evidences that marine population from Punta del Este rocky-pools should be younger (formed by a small set of founder individuals) and smaller than those from the other habitats. The origin of the marine rocky-pool population remains unclear. However, we know that this population show specific genomic characteristics related with the adaptation to live in an uncommon environment for this species, such as the SNPs potentially under selection related with osmoregulation and body development.

In conclusion, we found phenotypical (Perazzo *et al.*, 2019) and genotypical (present work) variation in *J. lineata* from distinct coastal environments, corroborating with the hypothesis that this species is locally adapted to variable habitat conditions. However, common garden experiments may improve the knowledge about local adaptation elucidating whether the phenotypical variation among environments is or not inheritable. Moreover, it is important to investigate what are the genomic bases of the previously described habitat-dependent sexual dimorphism of *J. lineata* to better understand the local adaptation process in this Neotropical livebearer fish.

Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de N vel Superior - Brasil (CAPES) - Finance Code 001, FAPERGS (Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul) FAPERGS/CNPq-PRONEX number 16/0485-4 and by the Swiss Government Excellence Scholarship for Foreign Students - Switzerland.

Tables

Table 1 Populations sampled with the description of habitat, geographical coordinates, salinity and number of individuals sampled.

Population	Habitat	Salinity	Geographical coordinates		Individuals sampled			
					Males	Females	Unidentified Sex	Total
CW1	Coastal Washout	0.3±0.0	32˚12'56"S	52˚11'10"W	4	10	0	14
CW2	Coastal Washout	0.3±0.0	35˚12'33"S	52˚10'44"W	2	13	0	15
CW3	Coastal Washout	0.2±0.0	32˚13'53"S	52˚12'18"W	8	4	0	14
EST1	Estuary	0.0±0.0	31˚59'11"S	52˚06'20"W	2	6	0	8
EST2	Estuary	0.0±0.0	31˚54'51"S	52˚09'06"W	8	11	0	19
LAG1	Lagoon	0.0±0.0	32˚00'48"S	52˚08'12"W	8	13	0	21
LAG2	Lagoon	0.1±0.0	34˚54'28"S	55˚00'21"W	0	0	10	10
MAR	Marine Rocky-pools	21.3±0.6	34˚58'26"S	54˚57'08"W	1	6	10	17
STR	Freshwater Stream	0.0±0.0	32˚07'39"S	53˚03'43"W	3	13	0	16
				Total	36	76	20	132

Table 2 Summary of genetic statistic for all populations. *Variant positions* are those nucleotide that are polymorphic in at least one population. *All positions* are all nucleotide positions across all RAD sites (regardless of wheter they are polymorphic or fixed). N= average number of indivuals genotyped at each locus; Private = number of variable sites unique to each population; Sites = number of polymorphic (variant positions) or total (all positions) nucletide sites; % Poly = percentage of polymorphic loci; P = the average frequency of the major allele; H_{obs} = average observed heterozigosity per locus; π = average nucleotide diversity; F_{IS} = average Wright's imbreeding coeficient.

	N	Private	Sites	% Poly	P	H_{obs}	π	F_{IS}
Variant positions								
CW1	9.98	1	57180000	77.63	0.8259	0.1345	0.2555	0.3320
CW2	13.59	1	61480000	82.37	0.8279	0.1595	0.2516	0.2715
CW3	10.37	0	41760000	76.92	0.8256	0.1014	0.2552	0.4321
LAG1	19.74	43	56860000	75.56	0.8288	0.2082	0.2435	0.0979
LAG2	9.86	20	48180000	63.85	0.8442	0.2043	0.2223	0.0456
STR	13.56	52	34600000	59.79	0.8475	0.0938	0.2102	0.3048
EST1	7.15	0	11520000	64.57	0.8399	0.0620	0.2349	0.4386
EST2	11.27	0	31350000	77.89	0.8265	0.0933	0.2524	0.4504
MAR	15.43	127	45300000	61.19	0.8572	0.1275	0.2005	0.2150
All positions (variant and fixed)								
CW1	10.03	1	5783290000	0.99	0.9978	0.0017	0.0033	0.0042
CW2	13.81	1	5868470000	1.05	0.9978	0.0020	0.0032	0.0035
CW3	10.40	0	4321670000	0.97	0.9978	0.0013	0.0032	0.0054
LAG1	19.88	43	5885510000	0.97	0.9978	0.0027	0.0031	0.0013
LAG2	9.92	20	5897510000	0.82	0.9980	0.0026	0.0028	0.0006
STR	13.60	52	4557340000	0.76	0.9981	0.0012	0.0027	0.0039
EST1	7.14	0	1395830000	0.83	0.9980	0.0008	0.0030	0.0056
EST2	11.28	0	3161190000	0.99	0.9978	0.0012	0.0032	0.0057
MAR	15.48	127	5775940000	0.78	0.9982	0.0016	0.0026	0.0028

Table 3 Pairwise comparisons of genetic and geographic distances among *Jenynsia lineata* populations. Above diagonal are the genetic distances represented by F_{ST} values. Bellow diagonal are the geographical distances in kilometers between populations. All comparisons showed significant statistical differences, except for the coastal washouts populations.

Populations	CW1	CW2	CW3	LAG2	LAG1	STR	EST1	EST2	MAR
CW1		0.002	-0.000	0.167	0.088	0.161	0.054	0.034	0.260
CW2	1.43		0.012	0.162	0.095	0.154	0.067	0.045	0.257
CW3	2.54	1.04		0.166	0.096	0.168	0.045	0.037	0.258
LAG2	397.7	396.9	395.5		0.213	0.282	0.185	0.177	0.220
LAG1	20.9	22.0	23.0	417.2		0.254	0.066	0.051	0.288
STR	82.4	81.7	81.0	361.8	84.9		0.231	0.207	0.365
EST1	24.6	25.8	33.1	421.4	3.5	87.3		-0.004	0.267
EST2	30.0	33.1	33.9	424.4	11.9	84.5	8.9		0.260
MAR	400.4	399.6	398.5	9.9	419.1	364.7	422.6	426.3	

Table 4 Results from AMOVA testing the partitioning of genetic variation across populations and distinct habitats (marine rocky-pools, stream, lagoon, estuary and costal washouts), using all SNPs, and using the SNPs under putative selection generated by BAYESCAN and BAYENV programs.

	Source of variation	Nested in	% Variance	F-stat	F-value	p-value
All SNPs						
	Within individuals	--	0.628	F_{IT}	0.372	--
	Among individuals	Population	0.246	F_{IS}	0.282	0.000
	Among populations	Habitat	0.065	F_{SC}	0.069	0.000
	Among habitat	--	0.061	F_{CT}	0.061	0.042
BAYESCAN SNPs						
	Within individuals	--	0.425	F_{IT}	0.575	--
	Among individuals	Population	0.209	F_{IS}	0.329	0.000
	Among populations	Habitat	0.084	F_{SC}	0.117	0.000
	Among habitat	--	0.282	F_{CT}	0.282	0.002
BAYENV SNPs (environmental variable: salinity)						
	Within individuals	--	0.549	F_{IT}	0.451	--
	Among individuals	Population	0.226	F_{IS}	0.291	0.000
	Among populations	Habitat	0.227	F_{SC}	0.263	0.000
	Among habitat	--	-0.052	F_{CT}	-0.052	0.419

Table 5 BLAST hits from sequences containing a SNP found to be putatively under selection by BAYESCAN and BAYENV programs. Locus ID is the SNP identification. Sequence ID and Gene ID are the identification the sequence and gene of *Poecilia reticulata*, respectively. E-value represents the statistical significance of the hits. Are indicated the percentage of query cover and identity with the target sequence, and the gene description with the principal known functions.

Locus ID	Sequence ID	E-value	Query cover (%)	Identity (%)	Gene ID	Gene description	Principal known functions
BAYESCAN							
15547_81	XM_008438486.1	6e-12	41	85	103482359	Collagen alpha-1 (XXI) chain-like	Growth plate cartilage chondrocyte morphogenesis
	XM_017302735	8e-10	48	80	103459384	SH3 and multiple ankyrin repeat domains 3	Brain morphogenesis; synaptic regulation; swimming behavior
BAYENV							
7479_16	XM_008408258.2	1e-41	100	85%	103464273	RNA binding motif protein 15B	RNA binding
16283_146	XM_008403110.2 XM_008403109.2	2e-20	63	81	103460804	Fibrinogen alpha chain (fga), transcript variant X1, X3	Blood coagulation, fibrin clot formation; platelet activation
19313_40	XM_008411811.2	3e-36	64	95	103466313	Gamma-crystallin M3-like	Dominant structural components of the vertebrate eye lens
	XM_008438049.1	8e-31	64	91	103482102	Gamma-crystallin M3- like, transcript variant X1	
	XM_008417951.1	3e-29	66	88	103469900	Gamma-crystallin M2-like	
	XM_008438003.1	1e-28	68	87	103482072	Gamma-crystallin M3-like	
	XM_008438013.1	1e-27	68	86	103482081	Gamma-crystallin M3-like	

	XM_008438033.1	6e-26	68	85	103482102	Gamma-crystallin M3-like
	XM_008437951.1	6e-26	68	85	103482036	Gamma-crystallin M3-like
	XM_008396830.2	7e-25	68	84	103456941	Gamma-crystallin M3-like, transcript variant X1
	XM_008438093.1	7e-25	68	84	103482134	Gamma-crystallin M1-like
	XM_008438073.1	7e-25	68	84	103482119	Gamma-crystallin M2-like
	XM_008437989.1	7e-25	67	84	103482069	Gamma-crystallin M3-like
	XM_008426436.1	2e-24	68	84	103475049	Gamma-crystallin M3-like
	XM_008437969.2	4e-22	68	82	103482054	Gamma-crystallin M2-like
	XM_008401615.1	4e-22	68	82	103459776	Gamma-crystallin M2-like
	XM_017302509.1	4e-21	68	82	103456941	Gamma-crystallin M3-like
	XM_017302515.1	2e-20	68	81	103456941	Gamma-crystallin M3-like
	XM_008438063.1	2e-20	68	81	103482114	Gamma-crystallin M2-like
	XM_008438023.1	2e-20	68	81	103456941	Gamma-crystallin M3-like
	XM_008437958.1	7e-19	64	81	103482045	Gamma-crystallin M3-like
	XM_008426406.1	1e-15	65	79	103475026	Gamma-crystallin M2-like

	XM_008436832.1	2e-13	47	83	103481427	Gamma-crystallin M2-like	
	XM_008426477.1	6e-13	65	77	103475076	Gamma-crystallin M2-like	
	XM_008426426.2	8e-12	62	77	103475040	Gamma-crystallin M3-like	
33572_132	XM_017304977.1 XM_008410595.2XM_008410596.2 XM_008410597.2	4e-15	36	93	103465608	SHQ1, H/ACA ribonucleoprotein assembly factor (shq1), transcript variant X1, X2, X3, X4	Processing of ribosomal RNAs
35928_28	XM_008436759.2 XM_008436760.2 XM_008436761.2	5e-14	49	86	103481356	Potassium voltage-gated channel subfamily H member 2-like, transcript variant X1, X2, X3	Regulation of ion transmembrane channel activity
37272_28	XM_008419858.2	2e-43	92	79	103471060	Ovochymase-2-like	Egg envelop conversion to a sperm-penetrable form
40399_45	XM_008413001.2	9e-16	68	79	103466995	TBC1 domain family member 25	Activation of GTPase activity; intracellular protein transport; regulation of autophagosome maturation
	XM_008426756.2	1e-14	69	78	103475265	Uncharacterized, transcript variant X1	
	XR_535000.1	2e-12	71	76	103473321	Uncharacterized	
	XM_008421222.1	7e-11	58	79	103471931	3-oxo-5-alpha-steroid 4-dehydrogenase 2-like	Steroid metabolic process

45207_112	XM_008402804.2	1e-34	66	92	103460570	Growth arrest-specific protein 7-like	Neuronal development
	XM_008437743.2	7e-19	66	81	103481921	Growth arrest specific 7 (gas7)	
48477_129	XM_008407772.2	9e-24	96	77	103463985	Protein FAM69A-like	Cystein-rich type II transmembrane protein
	XM_017304343.1	1e-22	73	81	103463143	Adenomatosis polyposis coli 2 (apc2)	Stabilizes microtubules; Regulate actin fiber dynamics
	XM_008396215.2	4e-22	93	76	103456575	Kelch like family member 40 (klhl40)	Negative regulation of protein ubiquitination; skeletal muscle fiber development; Swimming
	XM_008396217.2	4e-22	93	76	103456576	Zinc finger and BTB domain containing 47 (zbtb47)	Cellular response to DNA damage stimulus
	XM_008415211.2 XM_008415213.2 XM_017306308.1	2e-18	96	75	103468265	DnaJ heat shock protein family (Hsp40) member C6 (dnajc6), transcript variant X1, X2, X3	Clathrin-dependent endocytosis
	XM_008407907.2	3e-17	73	77	103464075	Von Willebrand factor A domain-containing protein 7-like	Transcription, DNA repair, ribosomal and membrane transport
	XM_008413892.2	5e-14	42	88	103467477	Chromosome LG7 open reading frame, human C20orf24 (clg7h20orf24), RAB5 interacting factor	
	XM_008418796.2 XM_008418795.2	8e-12	50	84	103470381	DnaJ heat shock protein family (Hsp40) member B5 (dnajb5), transcript variant X1, X2, X3	Chaperone cofactor-dependent protein refolding; Negative regulation of transcription by RNA polymerase II

	XM_008418797.2						
	XM_017309189.1	8e-12	38	86	103476948	WD repeat- and FYVE domain-containing protein 4, transcript variant X1, X2	Ubiquitin-protein transferase activity
	XM_008429715.2						
	XR_001777469.1	3e-11	50	80	103478937	Uncharacterized	
	XM_008412657.2						
	XM_017305323.1						
	XM_008412658.2	3e-11	34	88	103466800	PHD finger protein 21A (phf21a), transcript variant X1, X2, X3, X4, X5	Face development
	XM_008412660.2						
	XM_008412661.2						
	XM_008411500.2	9e-11	85	73	103466122	Uncharacterized	
	XM_008428641.2	9e-11	36	87	103476361	Angiomotin-like	Angiogenesis; cell migration; embryonic pattern specification; regulation of cell shape
54918_19	XM_017309794.1	4e-15	38	91	103478868	Eukaryotic translation initiation factor 2B subunit epsilon	Guanyl-nucleotide exchange factor activity
60350_21	XR_536031.2	2e-18	91.5	76	103480031	Uncharacterized	
60936_80	XM_017306123.1	1e-16	39	92	103468879	Collagen alpha-1 (XI) chain-like	Growth plate cartilage chondrocyte morphogenesis
64915_47	XM_017303436.1	1e-46	100	87	103460668	Apolipoprotein B (apob), mRNA	Cholesterol homeostasis; digestion; lipid transport;

Figures

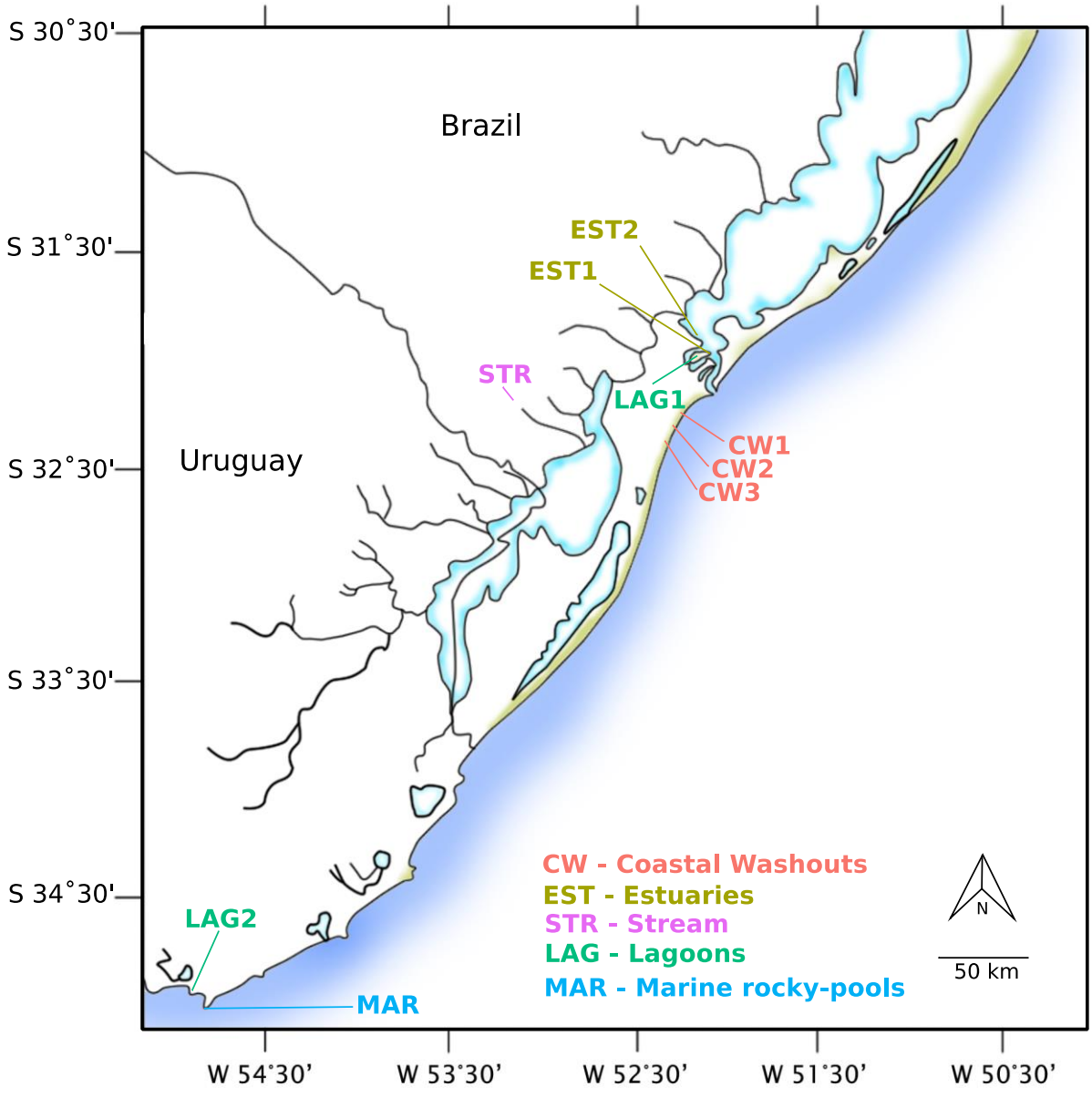

Fig. 1 Sampling sites of the nine populations (from five environments) of *Jenynsia lineata*.

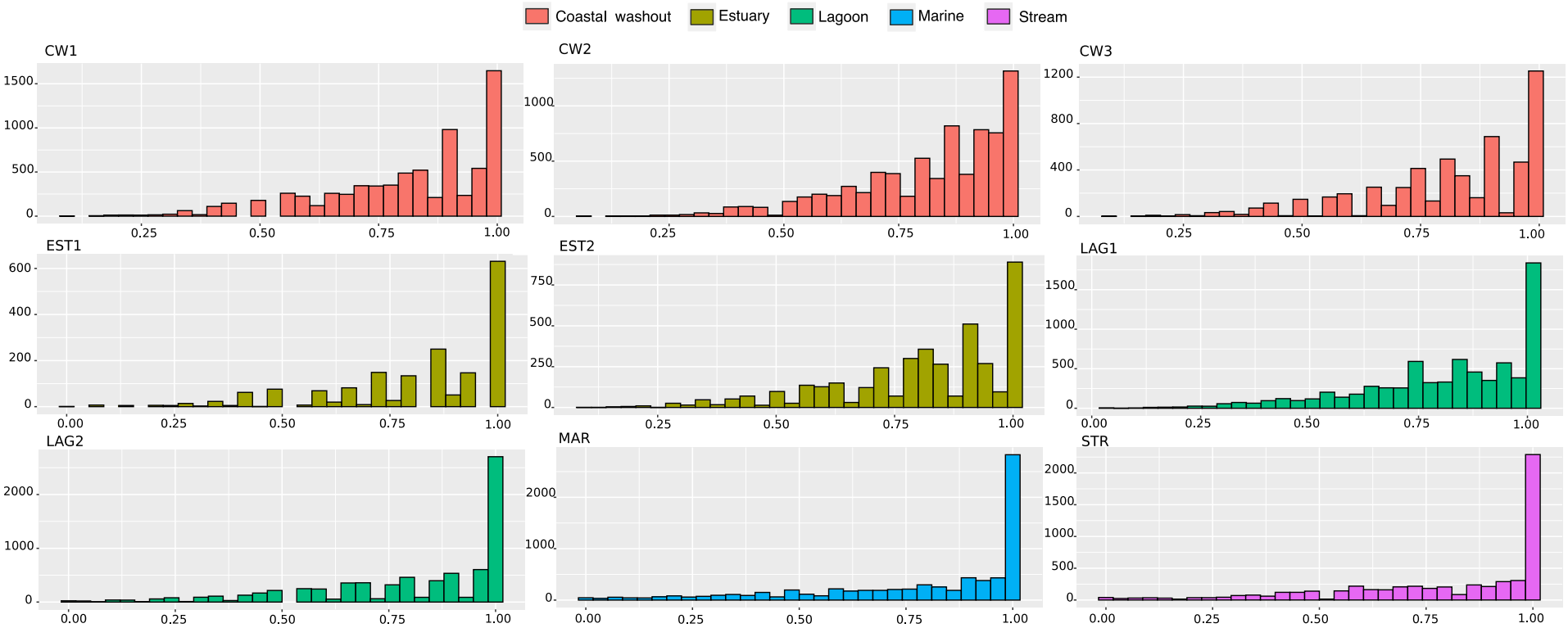

Fig. 2 Allele frequency distribution per loci in each population. X-axis represents the allele frequency and Y-axis represents the number of loci. Allele frequency was obtained from the major allele frequency.

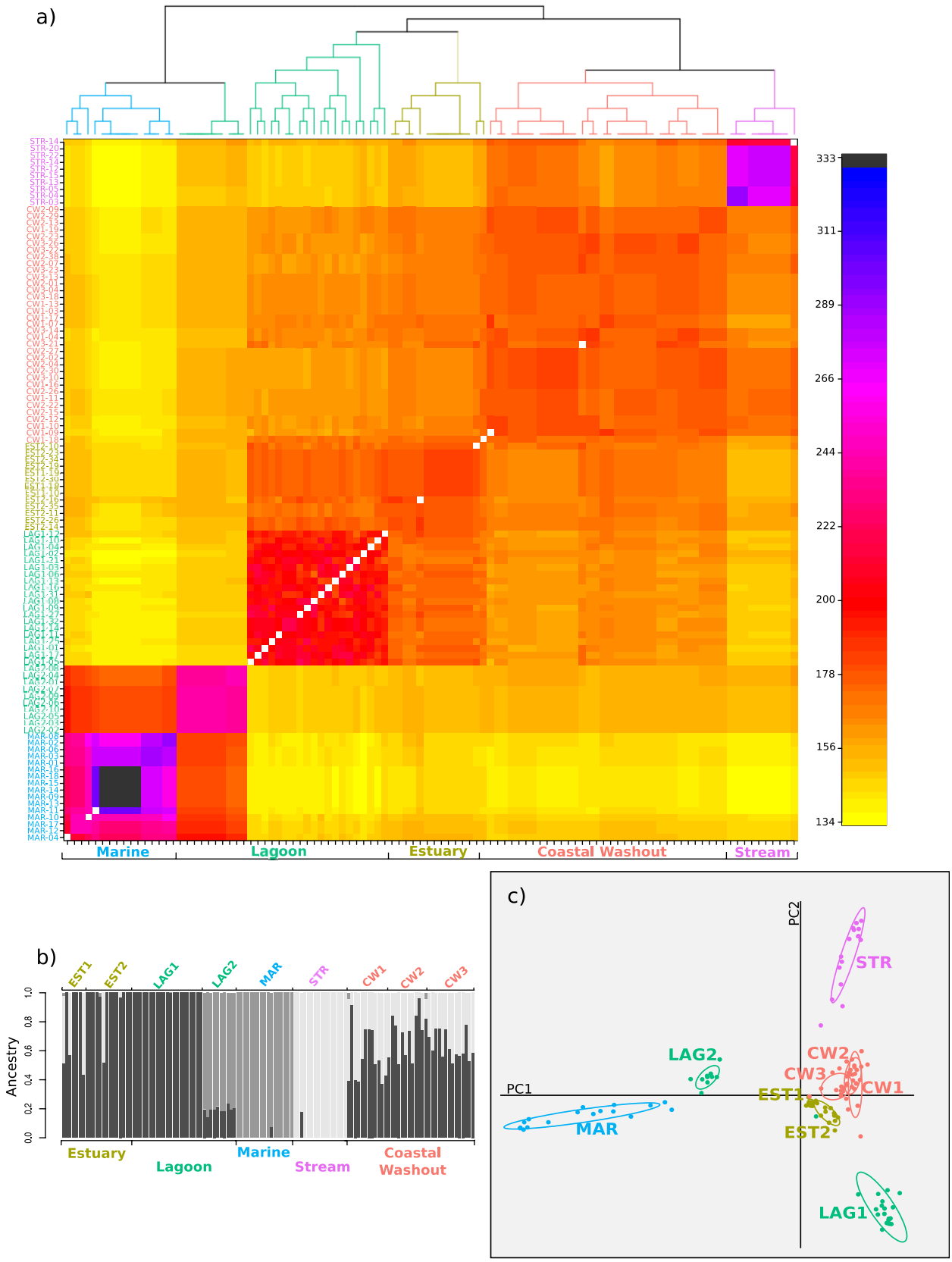

Fig. 3 Population structure analyses of *Jenynsia lineata* populations. a) The coancestry heat map generated by fineRADstruture. The highest levels of co-ancestry are noticeable among specimens from the Marine rocky-pools population (MAR), indicated by black, blue and

purple colors. The lowest levels of co-ancestry sharing are given between MAR and all the other populations, indicated by yellow coloration, exception made to LAG2 population, with which MAR population has geographic proximity, indicated by red color. The stream population also presents noticeable distinction from the others populations. b) Barplot of ancestry generated by ADMIXTURE with k = 3, with each bar representing a specimen and the color indicating a putative population cluster. c) Principal Component Analysis of allele frequencies among specimens from nine populations. Ellipses indicate the distribution of the individuals from different groups.

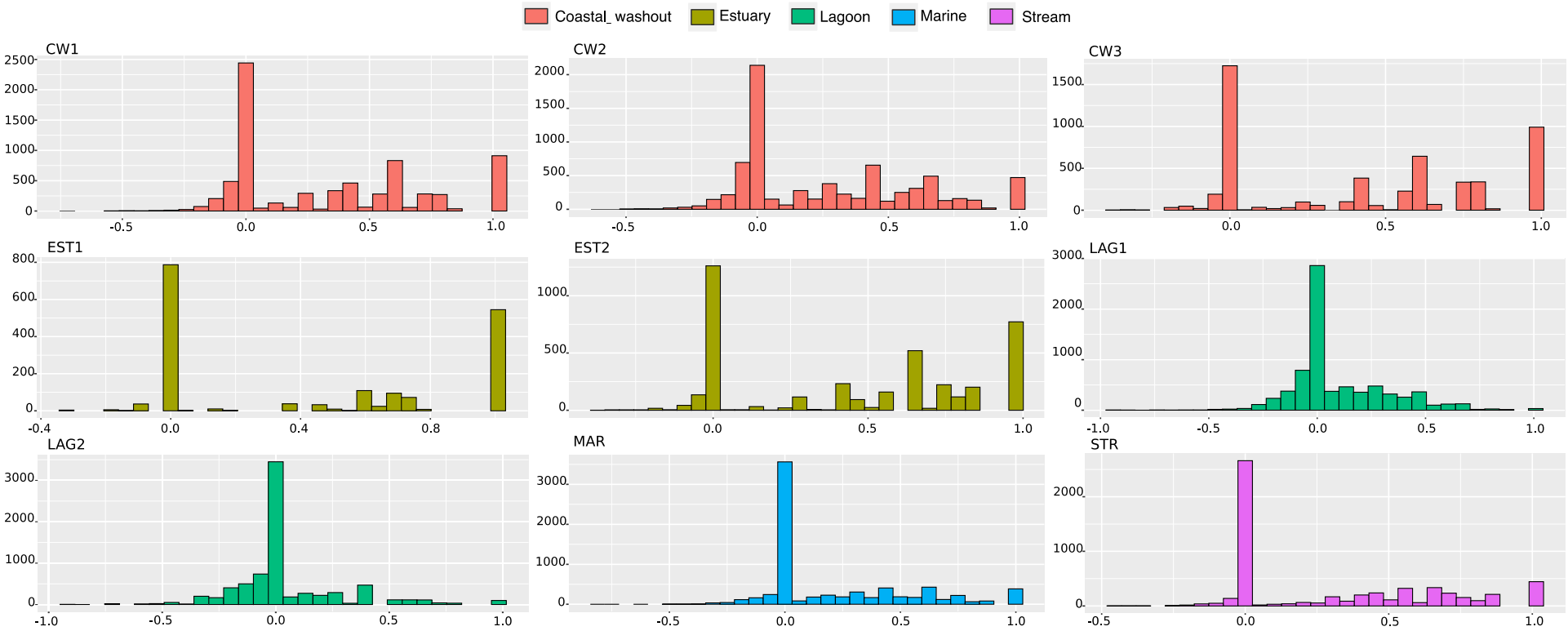

Fig. 4 F_{IS} values distribution per loci in each population of *Jenynsia lineata*. Different habitats are represented by different colors: light blue is the coastal washout, pink is the lagoon, yellow is the stream, green is the estuary, and dark blue is the marine.

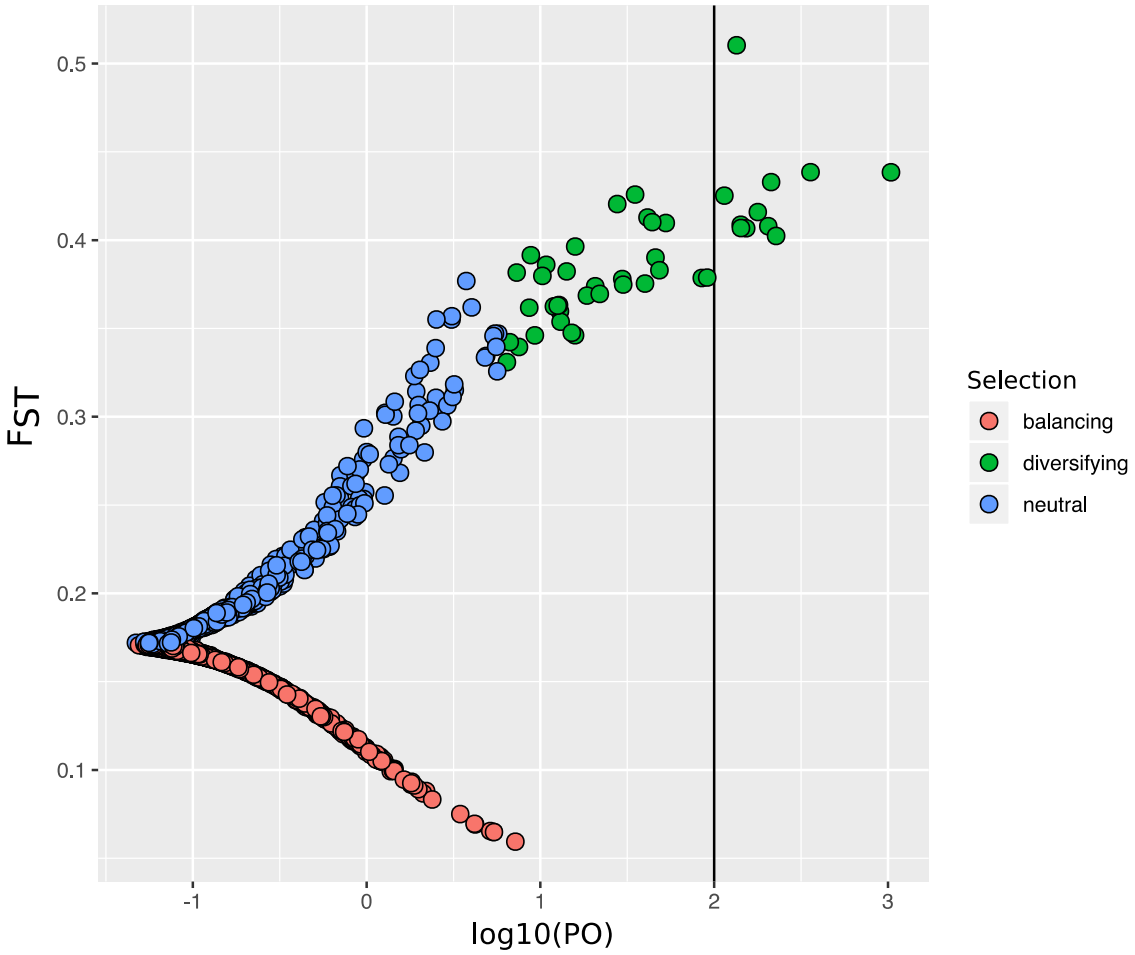

Fig. 5 Outlier loci identified for *Jenynsia lineata* by BAYESCAN. Plot of 3934 SNPs according to F_{ST} and log10(PO), with the indication of the threshold (=2) used to identify the decisive SNPs under selection. Colors demonstrate the proportion of SNPs under balancing, diversifying and neutral selection.

Supporting information

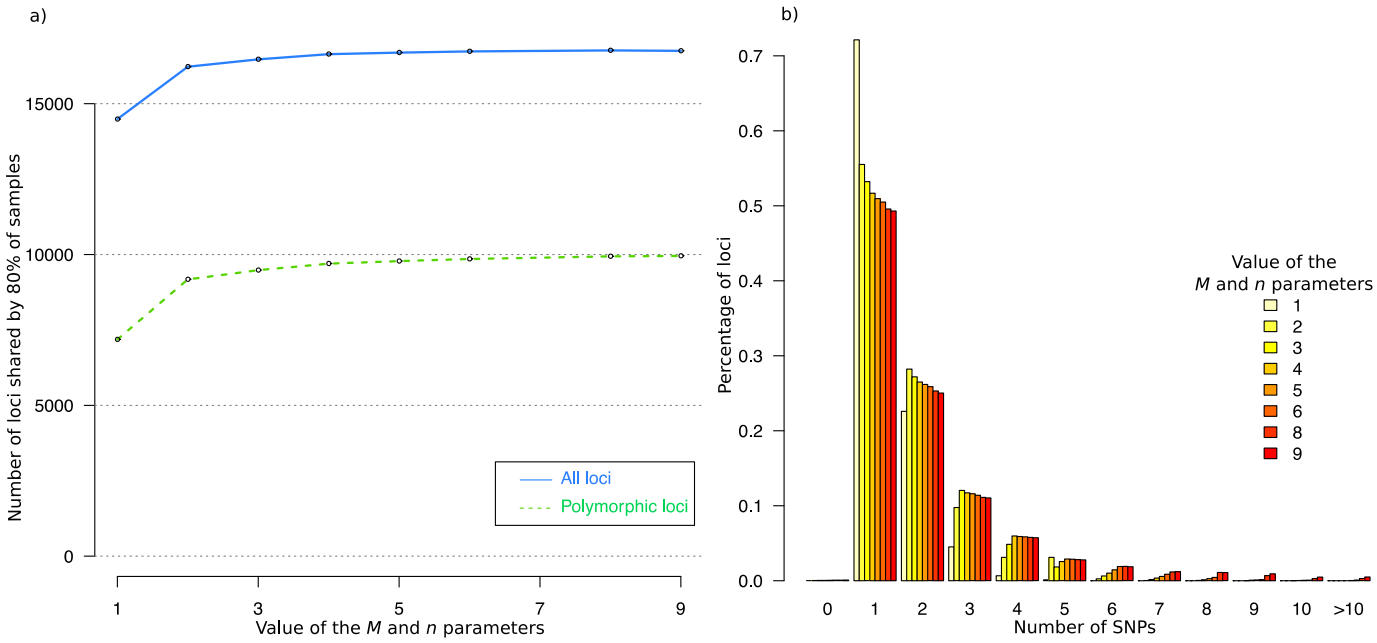

Fig. S 1 Selection of assembly parameters in *de novo* STACKS analysis of *Jenynsia lineata*. In (a) it is showed the effects of increasing the *M* and *n* parameters in the number of all loci (solid line) and polymorphic loci (dashed line) shared by 80% of samples or more, and in (b) it is showed the distribution of SNPs per locus. In both plots, the *M* and *N* parameters are kept equal, and the *m* parameter is fixed to 3. We selected *M* and *n* = 5 for assembly because at this value the number of loci plateaus and the distribution of SNPs is mostly unaffected

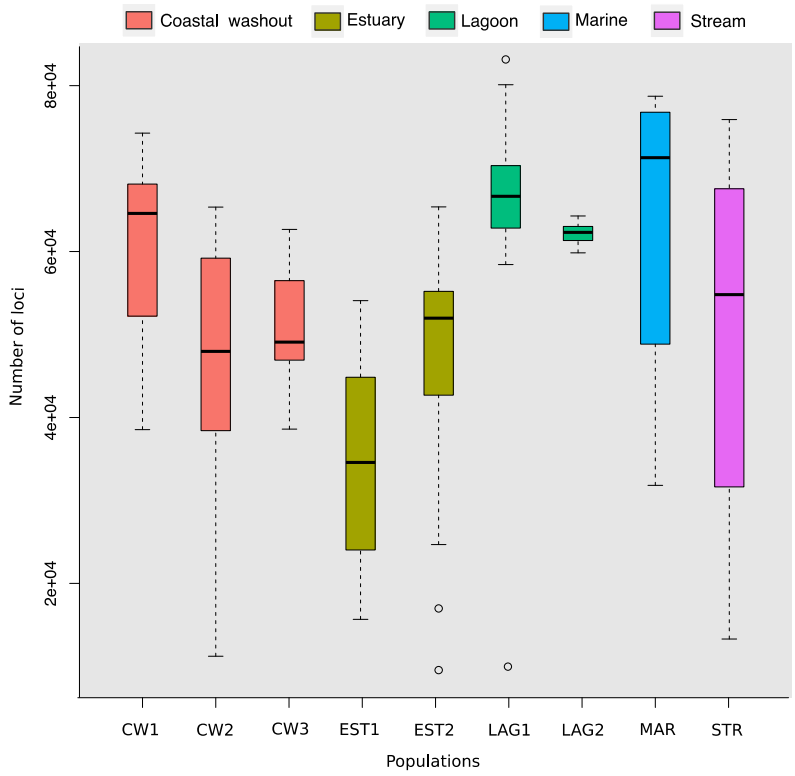

Fig. S 2 Average number of loci per individual for each population, after assembly *de novo* process.

Table S 1 Genome scans summary results from BAYESCAN and BAYENV, with the putatively loci under selection. For each program results are show the Bayes factor, major allele frequency and whether there is occurrence of hit at the BLAST query. For each SNP are also showed the Q value and the F_{ST} results from BAYESCAN results, and the Spearman and Pearson coefficients from BAYENV results.

BAYESCAN

SNP ID	Locus ID	log10(BF)	Q value	F_{ST}	Allele frequency	BLAST hit
6539	61130_72	3.01730	0.00096	0.43844	0.45	No
3258	31191_36	2.55470	0.00187	0.43847	0.64	No
4160	40704_122	2.35660	0.00271	0.40239	0.51	No
4625	44607_56	2.32770	0.00320	0.43290	0.67	No
1609	15547_81	2.31120	0.00353	0.40791	0.54	Yes
4648	44738_72	2.25090	0.00387	0.41592	0.69	No
1610	15547_95	2.18420	0.00425	0.40670	0.54	No
6613	61845_9	2.15430	0.00459	0.40687	0.55	No
792	6255_78	2.15310	0.00485	0.40874	0.40	No

904	7100_85	2.12870	0.00511	0.51037	0.67	No
6771	63489_74	2.05870	0.00543	0.42524	0.64	No

BAYENV

SNP ID	Locus ID	Bayes Factor	Spearman	Pearson	Allele frequency	BLAST hit
965	7479_16	0.23	0.27	0.35	0.57	Yes
973	7507_36	0.32	0.41	0.43	0.81	No
1189	9329_62	0.15	0.29	0.27	0.92	No
1479	13956_55	0.21	0.31	0.34	0.91	No
1514	14375_64	0.35	0.38	0.37	0.81	No
1702	16283_146	0.23	0.29	0.29	0.99	Yes
1945	19022_15	23.90	0.33	0.32	0.79	No
1947	19022_106	0.14	0.30	0.29	0.89	No
1966	19313_40	0.31	0.30	0.26	0.85	Yes
2094	20381_116	0.44	0.34	0.32	0.74	No
2326	22264_42	0.22	0.25	0.28	0.58	No
2932	28528_102	0.21	0.30	0.33	0.88	No
3421	32668_113	1.20	0.36	0.39	0.89	No
3532	33572_132	0.24	0.40	0.31	0.67	Yes
3535	33652_98	0.42	0.33	0.32	0.92	No
3793	35928_15	0.13	0.30	0.34	0.73	No
3885	37272_28	0.60	0.23	0.28	0.95	Yes
4117	40399_45	0.16	0.35	0.30	0.85	Yes
4703	45207_112	1.16	0.25	0.27	0.93	Yes
4781	46020_125	0.14	0.33	0.28	0.92	No
4782	46060_56	0.14	0.29	0.36	0.88	No
4957	48477_129	0.14	0.29	0.24	0.77	Yes
5089	50263_127	0.77	0.26	0.37	0.62	No
5267	51902_96	0.23	0.39	0.41	0.87	No
5655	54662_30	0.27	0.29	0.34	0.64	No
5679	54918_19	0.28	0.23	0.32	0.58	Yes
6426	60350_21	0.19	0.30	0.31	0.83	Yes
6506	60936_80	7.08	0.27	0.25	0.72	Yes
6749	63198_105	0.14	0.38	0.41	0.86	No
6776	63591_147	0.14	0.31	0.41	0.73	No
6984	64915_47	0.38	0.27	0.33	0.93	Yes

7538	98052_96	0.14	0.25	0.30	0.92	No

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CAPÍTULO 3

IMPACT OF A RECENT WATER RESERVOIR CONSTRUCTION ON FISH MORPHOLOGY IN A SUBTROPICAL STREAM

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ABSTRACT

Dam construction causes fundamental changes in the natural landscapes, creating new ecological and evolutionary challenges to aquatic organisms that can respond the water current alteration. Thereby, to understand how populations respond to wide and fast environmental changes is the first step to elucidate the consequences of disturbed habitats on species evolution. In this work we analyzed shape and size variation in *Bryconamericus iheringii* Boulenger 1887 from the Chasqueiro stream basin, south of Brazil, which was recently dammed. We used linear measurements and geometric morphometrics to identify morphological differences among specimens from reservoir (lentic habitat), upstream and downstream (lotic habitat) of the dam. We also checked for size and shape sexual dimorphism. *Bryconamericus iheringii* showed distinct shape and size between specimens from the reservoir and the stream. The size variation between environments was the same for both sexes, but the shape variation was distinct for males and females. Considering the linear measurements, lotic populations showed larger measurements (body length and width, dorsal and pectoral fins base length, and caudal peduncle length), which seem to facilitate sustained swimming. Regarding body shape we observed that both sexes have a more fusiform body in lotic habitat than in reservoir, but females, differently of males, also showed mouth position distinct between these environments. In previous analysis of body shape and size variation, *B. iheringii* did not show differences between populations from natural streams, which emphasizes the putative strong selective force of the environmental alteration caused by the impoundment acting on these populations.

KEYWORDS

Geometric morphometrics; Dam; Characidae; Neotropical Region; Local Adaptation; Phenotypic Plasticity

Introduction

Demand for fresh water has more than tripled since 1950, growing as well as the human interventions in the water resources. These are mainly related with the modification of the flow regime from many rivers and streams, with the drying up of water bodies and the creation of reservoirs (Goudie 2018). Reservoirs may look like natural lakes. However, the operating regime determined by the purpose for which the reservoirs were created may significantly change their physical and chemical characteristics and the biology of their organisms (Schmutz and Moog 2018). Specimens inhabiting such altered habitat are exposed to different environmental variables than those specimens from the original habitat, eventually showing different phenotypes. Intraspecific morphological divergence in fishes has been recorded in response to anthropogenic disturbances, such as the impoundments (Haas et al. 2010; Franssen 2011; Franssen et al. 2013; Grabowski et al. 2018). Impoundment causes interruption of the river continuity, affecting migration of fishes and transport of sediments and nutrients. It creates a completely new environment with homogenization of habitats and alteration of flow regime, which affects water quality and downstream flow. Thereby, to understand how populations respond to wide and fast environmental changes should be the first step to predict the consequences of habitat disturbance on species evolution (Franssen et al. 2013).

Alterations such as those caused by dams lead to fundamental changes in the natural landscapes, creating new ecological and evolutionary challenges to aquatic organisms (Haas et al. 2010). Some of the biological responses that organisms may present are those related to the water flow regime change. Therefore, analyzing the persistent species in these altered environments may serve as a model system to investigate population responses to rapid environmental disturbances (Franssen et al. 2013). In general, fish species that inhabit lotic and lentic environments may show morphological variation due the swimming performance, that is altered according to water current (Lauder 2015). Individuals from lentic environments or with very slow water current tend to have a deeper body (larger dorsal-ventral axis and smaller anterior-posterior axis) when compared with population from high water current, usually with a more fusiform body (Haas et al. 2010) and with larger caudal peduncle (Gaston and Lauer 2015). But this is not a rule (see Franssen 2011). The construction of reservoirs does not only alter the water current but also the entire ecological community, potentially creating different selective pressures, such as those related to feeding, predator evasion and maneuverability. The ability to escape from predators or even to feed on different food between altered and native environments may be different, creating a range of

morphological differences, which have been noted for some fish species (Langerhans and DeWitt 2004; Langerhans et al. 2004; Gomes and Monteiro 2008).

Reservoirs-induced morphological shifts are potentially due to phenotypic plasticity (Franssen et al. 2013), which is the ability of one genotype to produce more than one phenotype under different environmental conditions, not accounting for incremental change observed over several generations (Garland and Kelly 2006; Cureton and Broughton 2014). Although the phenotypic plasticity does not incorporate genetic variation, at first, the average plasticity of the population should evolve if the selective agent imposes more than instantaneous pressure (Garland and Kelly 2006). In the case of the alteration caused by the dam construction, directional selection can promote local adaptation since morphological divergence in reservoirs may confer greater fitness to reservoir-resident individuals (Franssen et al. 2013). This was observed in some investigations of phenotypic variation in fishes between lake-stream pairs despite of close proximities of populations (Berner et al. 2009; Moser et al. 2016; Rajkov et al. 2018). Although environmental disturbances, such as the water impoundments, have grown very rapidly around the world, few studies have evaluated their impact on the evolution of fish species inhabiting such disturbed sites (Haas et al. 2010; Franssen 2011; Assumpção et al. 2012; Franssen et al. 2013; Cureton and Broughton 2014; Gaston and Lauer 2015; Santos and Araújo 2015; Jacquemin and Pyron 2016).

Morphological changes in fishes due to anthropogenic habitat alteration have been described as a fast process, at least for those related to the alteration caused by the impounded streams (Haas et al. 2010; Franssen 2011). Phenotypic variation also is related to the characteristics of the reservoir, i.e. same or related species may respond differently to water impoundment (Haas et al. 2010). Moreover, males and females may show specific responses to environmental distinct conditions, which was described for species with remarkable sexual dimorphism (Perazzo et al. 2019). Considering these aspects and that distinct lotic environments will show specific features after the impoundment, it is relevant to evaluate how fishes are responding morphologically to the impacts caused by the impoundments in distinct ecosystems. In the present work, we analyzed the fish morphology variation in a species from a recently dammed subtropical stream from Neotropical region. Specifically, we described shape and size variation in *Bryconamericus iheringii* Boulenger 1887 from lentic and lotic habitat present in the Chasqueiro Stream basin, Southern Brazil. We are also interested in testing the presence and degree of sexual dimorphism of size (SSD) and shape (SShD) in individuals from both environments (lentic/lotic). We choose *B. iheringii* because this is an abundant species along this basin (Corrêa et al. 2015), besides being a generalist with opportunist dietary habits and with a high degree of trophic plasticity (Kokubun

et al. 2018). The species presents none sexual dimorphism, except by the presence of small fin-ray hooks in mature males (Lampert et al. 2004). This set of characteristics make *B. iheringii* a good candidate to investigate the hypothesis that the alteration of the water current may be an environmental factor affecting the fish morphology in a dammed subtropical stream, and that this variation is the same for both sexes, since *B. iheringii* does not present remarkable sexual dimorphism.

Materials and Methods

Study area

Sampling sites were at the hydrographic basin of the Chasqueiro Stream, which belongs to Mirim Lagoon system, city of Arroio Grande, south of Brazil (31˚6'51"S and 50˚5'17"W) (Fig. 1). This stream was chosen because of the recent construction of a water reservoir in the decade of 1980. This stream is lotic both upstream and downstream of the dam, with the reservoir typically lentic, enabling the comparison between these two kinds of environments (lentic versus lotic). In general, this region presents a subtropical climate with the average annual rainfall ranging from 1,200 to 1,450 mm and the average temperature varying from 11˚C to 25˚C. The basin is formed by two main streams (Chasqueiro and Chasqueirinho) with approximately 248 km^2, which were dammed to create a water reservoir with an area of 1,800 ha, used primarily for rice irrigation. Beyond the distinction related to water current, these lentic (reservoir) and lotic (downstream and upstream) environments also present differences in relation to geomorphological features, vegetation composition and aquatic fauna (Corrêa et al. 2015).

Sampling and Data acquisition

We sampled in the reservoir and in the upstream and downstream portions of the Chasqueiro stream. Ninety-four specimens (42 females and 52 males) were sampled between May and September 2016, using a beam trawl and hand nets (Table 1). In the laboratory, the individuals were anesthetized by immersion in clove oil solution and digital images were taken in a standardized way. Specifically, photographs of the lateral left side of each living organism were taken using a digital camera (Nikon® D90) mounted at approximately 50 cm. The specimens were positioned on a graph paper, their fins were extended, and the dorsal and anal fins were pinned.

Soon after the photographic records, the identification of the sexes was performed observing the gonads in an optical microscope. Digital images were converted into .tps files, using the tpsUtil 1.64 software (Rohlf 2013). For each specimen, 12 landmarks were recorded using the software tpsDIG2 2.30 (Rohlf 2015) (Fig 1). We used the same software to get the following linear measurements: total length (TL), standard length (SL), body width (BW), and caudal peduncle width (CPW). To avoid bias related to the acquisition of landmarks and linear measurements, the same person performed all processes (GXP).

Data analysis

Linear morphometry: We used standardized relative measurements for linear morphometry, to avoid issues related with different developmental stages. The standardization was obtained by dividing each linear measurement by the individual standard length. Normality distribution and homogeneity of variances for relative distances were checked by Shapiro-Wilk and Levene's tests, respectively. The variation between sites and sexes for each relative measurement was analyzed through two-way Analysis of Variance (ANOVA) followed by pairwise Tukey test, and visualized using boxplots.

Geometric morphometrics: Geometric morphometric analyses were performed with shape coordinates obtained from Generalized Procrustes Analyses (GPA) (Rohlf and Slice 1990), which minimizes the differences of translation, scaling, and rotation between landmarks (Zelditch et al. 2012). We also obtained the centroid size (CS) values from GPA, characterized as the square root of the sum of the squares of the distance of each landmark from the centroid (mean of all coordinates) of the configuration (Bookstein 1991). Normality distribution and homogeneity of variances of centroid size values were checked by Shapiro-Wilk and Levene's tests, respectively. We tested centroid size differences between sexes and habitats by two-way Analysis of Variance (ANOVA). Possible allometric effects, caused by different ontogenetic stages among specimens, were removed by regressing Procrustes coordinates (shape variable) into centroid size (size variable). Multivariate analyses were performed with the covariance matrix calculated from the resulting regression residuals. Principal Components Analysis (PCA) was used to identify the axes of maximal shape variance among all specimens and the patterns associated with this variance as well as to identify grouping of variance among the specimens. Multivariate Analysis of Variance (MANOVA), followed by pairwise comparisons, was performed to analyze shape statistical differences using the scores of informative principal components (based on a Broken-Stick

distribution) as dependent variable and habitat as independent variable. The MANOVA was followed by Wilks' λ test to identify the proportion of the variance that is explained by the independent variable (population). We used the Canonical Variates Analysis (CVA) to describe the differences among groups (habitats) and to form mathematical functions, which were used to assign specimens to groups through jackknife cross-validation analyses (Zelditch et al., 2012).

Sexual size dimorphism (SSD) and sexual shape dimorphism (SShD): We used the results of linear and geometric morphometrics analyses to check sexual size and shape dimorphism. Beyond, we tested SShD through Procrustes ANOVA, using Procrustes coordinates as response variable and sex and site as factors. An estimation SSD was obtained using an SSD index (Tamagnini et al., 2018), described as

$$SSD\ index = \frac{[female\ mean\ CS] - [males\ mean\ CS]}{[female\ mean\ CS]}$$

SSD index was calculated considering all specimens and separately for each site. For SSD, value of zero indicates none sexual dimorphism (Tamagnini et al., 2018).

Analyses were performed in R environment (Team 2018), using the Geomorph package (Adams and Otárola-Castillo 2013; Adams et al. 2017) for geometric morphometrics analyses. Graphs were edited using the software Inkscape v0.92. Differences were considered significant at $p < 0.01$.

Results

The average standard length was 4.26 ± 0.73 cm, without statistical differences between sexes ($p = 0.0249$) but differing between sites ($p < 0.0001$). Specimens from lentic environment were larger than those form the lotic habitats. We also analyzed linear measurements that could be related with the different habitats (lotic versus lentic environments). We found that total length, body width, dorsal fin base length, pectoral fin base length and caudal peduncle length were statistically significant distinct between lotic and lentic environments ($p < 0.01$). Only body width was different between sexes ($p = 0.0024$) (table 2). Despite of largest standard length of dam specimens, the standardized measurements reveled that lotic specimens show largest total length, body width, caudal peduncle length, dorsal and pectoral base lengths. We also observed that

specimens from upstream and downstream (lotic environments) were similar for all such measurements (Fig. 2).

Bryconamericus iheringii was statistically different regarding to centroid size in both site and sex comparisons ($p < 0.01$), with the site variation being more important than sex variation (Table 2, Fig. 3). These results are coherent with the relatively low SSD indices (Table 3). Moreover, the interaction between habitat and sex was not significant ($p = 0.8508$), which indicates that degree of SSD does not differ between lotic and lentic environments.

We found allometric effects on shape, which was different for males and females ($p = 0.001$) and between environments ($p = 0.006$). Because of this, shape analyses were performed with regression residuals of shape on centroid size. Broken-Stick model with PCA components revealed the first five PCs as the most important and its scores were used for MANOVA to compare shape variation between sex and environments. We found statistical differences between sexes ($F_{1, 88} = 2.4815$, Wilk's $\lambda = 0.58165$, $p = 0.0028$) and among environments ($F_{2, 88} = 2.8045$, Wilk's $\lambda = 0.30426$, $p = <0.0001$). The PC1 and PC2 explained 20.4 and 18.1% of total variation, respectively, with the landmarks 5, 9, 8, 10 and 12 as main loadings. Exploring the morphospace generated by PCA, the main body shape of females and males occupy distinct distribution (females: negative PC1 and positive PC2 axis; males: positive PC1 and negative PC2 axis) (Fig. 4). Such body distinction between sexes is then related with the fish shape attributes associated with the position of anal fin and body width and length, since these features are represented by the landmarks with higher loadings values. These finds were corroborated by the CVA (Fig. 6), with correct classification of 71.1% for females and 62% for male. With these results we demonstrate that *B. iheringii* shows sexual shape dimorphism, and the habitat-related shape alteration was then analyzed separately for males and females.

Females

Females showed different shape among environments ($F_{2, 47} = 3.4346$, Wilk's $\lambda = 0.5807$, $p = 0.0017$), specifically between upstream and dam ($p = 0.0011$) and between upstream and downstream ($p = 0.0005$). The shape size (centroid size) was also different between females from dam and downstream ($p = 0.0005$), but not for the remaining comparisons. Through the PCA, it is possible to observe a grouping related to different kind of environment along the PC1, which encompass 22.3% of total variation. The females from upstream occupy the positive end of the PC1, whereas females from dam occupy the opposite position, with females from downstream in a central position. The main loadings for PC1 were the landmarks 10, 5, 1 and 12, which are

primarily related with body width and mouth position. Therefore, females from lentic environment showed body shapes less slimmed than females from upstream. Beyond, we also observed variation in relation to mouth position, with females from dam having more ventral mouth position than females from upstream. This grouping was confirmed by CVA, which accounted approximately 65% of total variation in the first CV (Fig. 6). At the CV1 it is possible to visualize the distinction between females from upstream + downstream and females from dam, and the main variation was mouth position (Fig. 7). The overall classification accuracy was 88% (dam = 85.7%, downstream = 92%, upstream = 81.8%).

Males

Differently of females, males did not show shape variation between environments ($F_{2, 37}$ = 1.499, Wilk's λ = 0.6641, p = 0.1597), despite of significant centroid size difference between specimens from downstream and dam ($F_{2, 37}$ = 5.679, p = 0.005). The PCA reveled the first two PCs with 22.3 and 20.4% of total variance, respectively, with landmarks 10, 4, 5 and 7 as main loadings (Fig. 5). As observed in females, these landmarks are related with the body width and caudal peduncle shape. Although the MANOVA showed no statistical difference in shape between environments, the CVA was able to classify groups with an overall classification accuracy of 95%, which means that the variation between groups is higher than within groups. The first CV accounted almost 80% of total variation, with upstream and dam specimens occupying the negative and positive CV1 ends, respectively (Fig. 6). This variation was related with the displacement of landmark 8, indicating shorter caudal peduncle in males from dam when compared with males from upstream (Fig. 7).

Discussion

Bryconamericus iheringii from Chasqueiro basin shows morphological differences between stream and reservoir populations, corroborating with our first hypothesis. Such result has been observed for other persistent species in distinct altered aquatic ecosystems by the construction of impoundments (Haas et al. 2010; Franssen 2011; Assumpção et al. 2012; Franssen et al. 2013; Cureton and Broughton 2014; Gaston and Lauer 2015; Santos and Araújo 2015; Jacquemin and Pyron 2016). The environmental alteration caused by the dam construction seems to be a factor that is promoting phenotypical divergence along this stream basin. Specimens from upstream and downstream were similar for most of our comparisons, diverging from specimens from the reservoir. A previous analysis of body shape and size variation of *B. iheringii* did not show

differences between populations from 22 streams along the Campos Sulinos ecosystem (geographically near to our sampling area) (Kokubun et al. 2018), which emphasizes the putative strong selective force of the environmental alteration caused by the impoundment acting on these populations.

Linear measurements together with geometric morphometrics data analyses clearly indicate differences among environments, with the main distinction between specimens from upstream and the reservoir. The new lentic environment (reservoir) seems to select specimens with shorter body length and width, smaller dorsal and pectoral fins base, and shorter caudal peduncle length. Note that the linear measurements did not differ between sexes, which means that *B. iheringii* does not present sexual size dimorphism (SSD). However, sex is an important factor when we analyzed the body shape, indicating that *B. iheringii* has sexual shape dimorphism (SShD). Then, males and females did not respond equally the habitat alteration, not corroborating with our second hypothesis of same morphological variation between sexes for species without sexual dimorphism (or not so easily identified). This pattern was also identified in *Jenynsia lineata*, which shows remarkable sexual dimorphism and internal fertilization (Perazzo et al. 2019). Despite the differences regarding the sexual dimorphism between these two species, maybe males and females of *B. iheringii* occupy distinct ecological niches, and consequently are responding differently the selective pressure, as was proposed for *J. lineata*. This is a hypothesis that should be tested to better understand the process of sexual shape dimorphism and its influence on species local adaptation.

In general, intraspecific morphological variation in fishes, disregarding the sexual dimorphism, are related with two main factors: locomotion (Langerhans 2008; Haas et al. 2010; Franssen 2011; Franssen et al. 2013; Cureton and Broughton 2014; Lauder 2014; Theis et al. 2014; Gaston and Lauer 2015; Perazzo et al. 2019) and feeding (Langerhans et al. 2004; Gomes and Monteiro 2008; Heinen-Kay and Langerhans 2013; Araújo et al. 2014; Zanella et al. 2015; Ingley et al. 2016), which are features directly affected by contrasting environments. Regarding to locomotion, a general pattern has been observed with fishes from lotic habitats often having fusiform morphologies, to reduce drag and facilitate sustained swimming, and fishes from lentic habitats with shallower anterior region and increased caudal region, to facilitate faster burst and increasing maneuverability (Langerhans and DeWitt 2004; Langerhans 2008; Franssen et al. 2013). In relation to feeding, the main morphological variations are associated with the head, specifically the mouth position. Although these patterns are not universal (Franssen et al. 2013), we also observed in *B. iheringii* from lentic habitats a body less fusiform than in those from lotic habitats for both sexes, but prominently in females. The mouth position of females (the anterior

end of head) also was an important morphological feature distinguishing *B. iheringii* from reservoir and from stream. CVA revealed that females from the reservoir showed a more anterior position of the snout end when compared with females from stream. *Bryconamericus iheringii* is an generalist species, capable of preying switching based on the availability of resources (Kokubun et al. 2018). The environmental changes caused by the alteration of lotic to lentic habitat on the reservoir possibly include the alteration of food resources for *B. iheringii*, since this seems to be an important selective factor, at least for females. Actually, it is expected that the reservoir operation creates an completely altered environment, affecting abiotic and biotic factors of the ecosystem (Schmutz and Moog 2018). The morphological variation observed in *B. iheringii* seems to reflect water flow and food availability alterations due the construction of the dam.

In fact, the dam construction means the creation of a novel environment, and the species able to survive on this new environment need to exhibit plasticity in a range of traits. Plasticity is the ability of one genotype to produce more than one phenotype according to environmental conditions (Garland and Kelly 2006; Cureton and Broughton 2014). Although plasticity does not necessarily means genotypic variation, phenotypical plasticity may be adaptive since the average trait plasticity in a population should evolve in the case of directional selective pressure (Garland and Kelly 2006), which may be the alteration of lotic to lentic environments. Though these anthropogenic alterations apparently create new environments, promoting new evolutionary paths, it is necessary to keep in mind that some species can inhabit the new environments and other are not. In Chasqueiro stream basin was observed lower species diversity at the reservoir in comparison with the upstream and downstream of the dam (Corrêa et al. 2015). Which means that species, such as *B. iheringii*, exhibit phenotypic plasticity, which enables them to explore and to adapt to the new altered environment. However, the lower diversity in reservoir indicates that the plasticity seems to be not a rule for all species.

In conclusion, we observed that a fish species for which was not observed shape and size variation in natural environments (Kokubun et al. 2018), showed morphological variation probably related to the damming of a subtropical stream, 30 years ago. The size variation was the same for both sexes of *B. iheringii*, but the shape variation was distinct for males and females, being more prominent in females. Considering the linear measurements, specimens from lotic populations showed larger averaged measurements (body length and width, dorsal and pectoral fins base length, and caudal peduncle length), which seem to facilitate sustained swimming. Regarding body shape, we observed that both sexes have a more fusiform body in lotic habitat than in reservoir, but females differently than males, also showed a distinct mouth position between these environments. We could observe that the reservoir is an important factor influencing the

morphological variation in *B. iheringii*, a species with shape sexual dimorphism described in the present work. This shape sexual distinction should be considered in morphological studies with *B. iheringii*. Future studies should also consider the effects of dam construction on the fish community, specifically in relation to those species with co-occurrence along the stream and reservoir, to analyze if the variation observed in *B. iheringii* also is occurring in the other species. That would provide a test to the existence of a reservoir-induced modification rule. It is also interesting to evaluate if there is genetic variation among such populations, with the aim to understand what is the evolutionary path of species that are facing the alterations caused by reservoirs construction.

Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil (CAPES) - Finance Code 001, FAPERGS (Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul) FAPERGS/CNPq-PRONEX number 16/0485-4 and by the Swiss Government Excellence Scholarship for Foreign Students, Switzerland. We also are grateful to MSc. Daiana K. Garcez, Prof. Dr. Ricardo Berteaux Robaldo, and the Dr. Bruna F. Nornberg and Dr. Daiane Carrasco for the field support. Ethics Committee of Animal Use of the University of Rio Grande (CEUA/FURG, number P023/2016) has approved this research.

Tables

Table 1 Number of specimens per sex per site of sampling.

Sex	Upstream	Dam	Downstream	Total
Males	10	19	13	42
Females	13	14	25	52
Total	23	33	38	94

Table 2 ANOVA two-way results for size comparisons (centroid size + linear measurements) and Procrustes ANOVA results for shape comparisons (Procrustes coordinates), respectively testing for SSD and SShD between environments.

			Df	*SS*	*MS*	*F*	*p-value*
Size	Centroid Size	Sex	1	0.1579	0.1579	7.242	**0.0085**
		Site	2	0.5957	0.2978	13.659	**<0.0001**
		Sex:Habitat	2	0.0071	0.0035	0.162	0.8508
		Residuals	88	1.9188	0.0218		
	Standard length	Sex	1	2.05	2.051	5.211	0.0249
		Site	2	16.85	8.423	21.404	**<0.0001**
		Sex:Site	2	0.04	0.019	0.048	0.9532
		Residuals	88	34.63	0.394		
	Total length	Sex	1	0.0005	0.0005	0.955	0.3310
		Site	2	0.0111	0.0055	10.500	**<0.0001**
		Sex:Site	2	0.0019	0.0009	1.794	0.1720
		Residuals	88	0.0464	0.0005		
	Body width	Sex	1	0.0002	0.0002	11.061	**0.0013**
		Site	2	0.0003	0.0001	8.795	**0.0003**
		Sex:Site	2	<0.0001	<0.0001	0.232	0.7931
		Residuals	88	0.0015	<0.0001		
	Peduncle width	Sex	1	<0.0001	<0.0001	0.076	0.7838
		Site	2	<0.0001	<0.0001	3.108	0.0496
		Sex:Site	2	<0.0001	<0.0001	0.489	0.6151
		Residuals	88	<0.0001	<0.0001		
	Dorsal fin base length	Sex	1	<0.0001	<0.0001	0.059	0.8079
		Site	2	0.0016	0.0008	5.334	**0.0065**
		Sex:Site	2	0.0003	0.0001	1.083	0.3432
		Residuals	88	0.0129	0.0001		
	Pectoral fin base length	Sex	1	<0.0001	<0.0001	1.082	0.3012
		Site	2	0.0008	0.0004	9.811	**0.0001**
		Sex:Site	2	<0.0001	<0.0001	0.656	0.5216
		Residuals	88	0.0034	<0.0001		
	Caudal Peduncle length	Sex	1	<0.0001	<0.0001	0.375	0.542
		Site	2	0.0054	0.0027	13.870	**<0.0001**
		Sex:Site	2	0.0017	<0.0001	0.447	0.641
		Residuals	87	0.0170	0.0002		
Shape	Procustes cordinates	Sex	1	0.0044	0.0044	5.641	**0.0001**
		Site	2	0.0060	0.0030	4.000	**0.0001**
		Sex:Site	2	0.0020	0.0010	1.330	0.0788
		Residuals	90	0.0700	0.0008		
		Total	93	0.0781			

Table 3 SSD index analysis. The mean value of centroid size (log) for each sex was used to estimate the sexual size dimorphism in *Bryconamericus iheringii* from lentic and lotic habitats.

	All habitats	Dam	Downstream	Upstream
Mean females log(CS)	0.7449	0.7933	0.7172	0.7457
Mean males log(CS)	0.7091	0.7467	0.6575	0.7044
SSD index	0.0480	0.0590	0.083	0.055
% of size difference between sexes	4.81	5.87	8.33	5.54

Figures

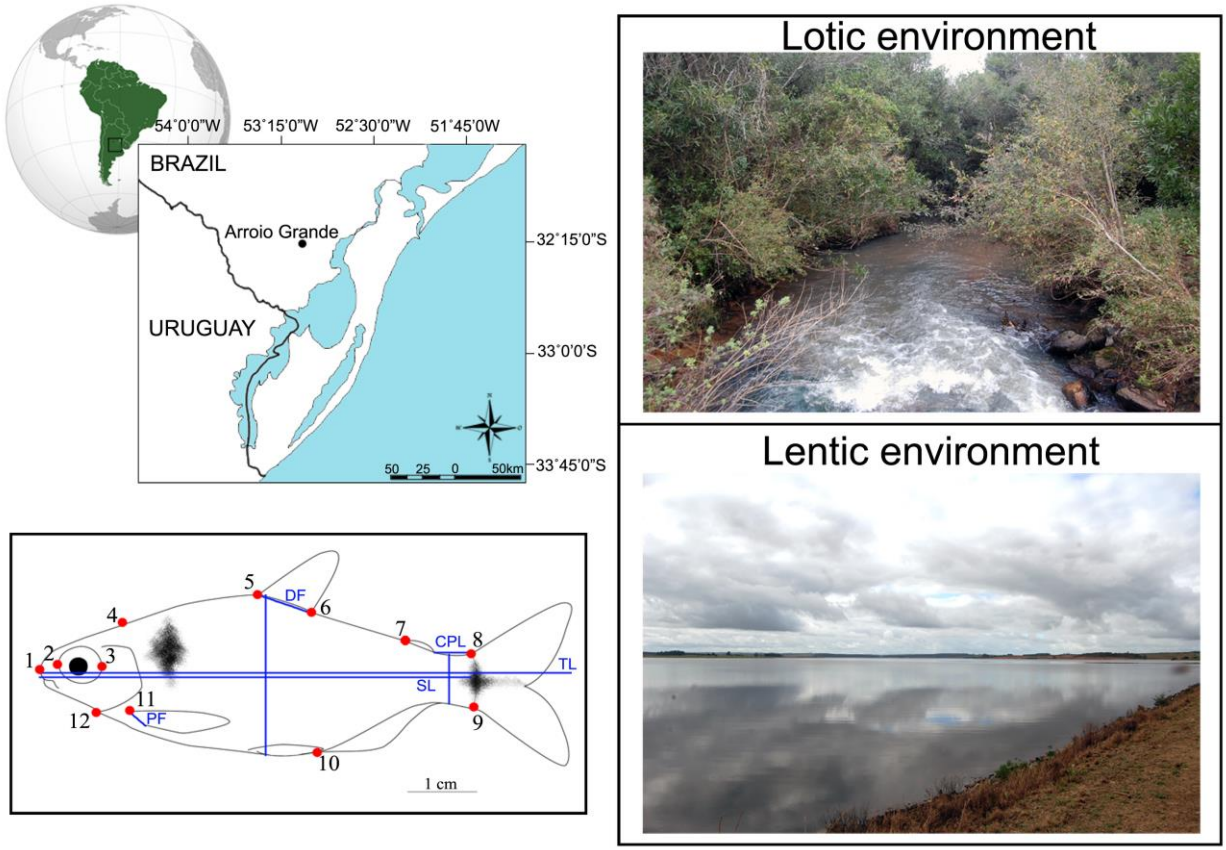

Fig. 1 Sampling site (map and illustrative images from lotic environment: stream - upstream and downstream; and lentic environment: reservoir) and landmarks plus linear measurements of *Bryconamericus iheringii* in left view. Landmarks (red dots): 1 – snout anterior margin upper jaw; 2 - eye anterior most margin; 3 - eye posterior most margin; 4 - supraoccipital process posterior margin; 5 - dorsal fin base origin, 6 - dorsal fin base posterior margin; 7 – adipose fin base origin; 8 - caudal fin base dorsal margin; 9 - caudal fin base ventral margin; 10 - anal fin origin; 11 – pectoral fin base anterior margin; 12 – ventral margin of gill opening. Linear measurements (blue lines): Total length (TL), standard length (SL), dorsal fin base length (DF), body width (BW), pectoral fin base length (PF), caudal peduncle length (CPL) and caudal peduncle width (CPW).

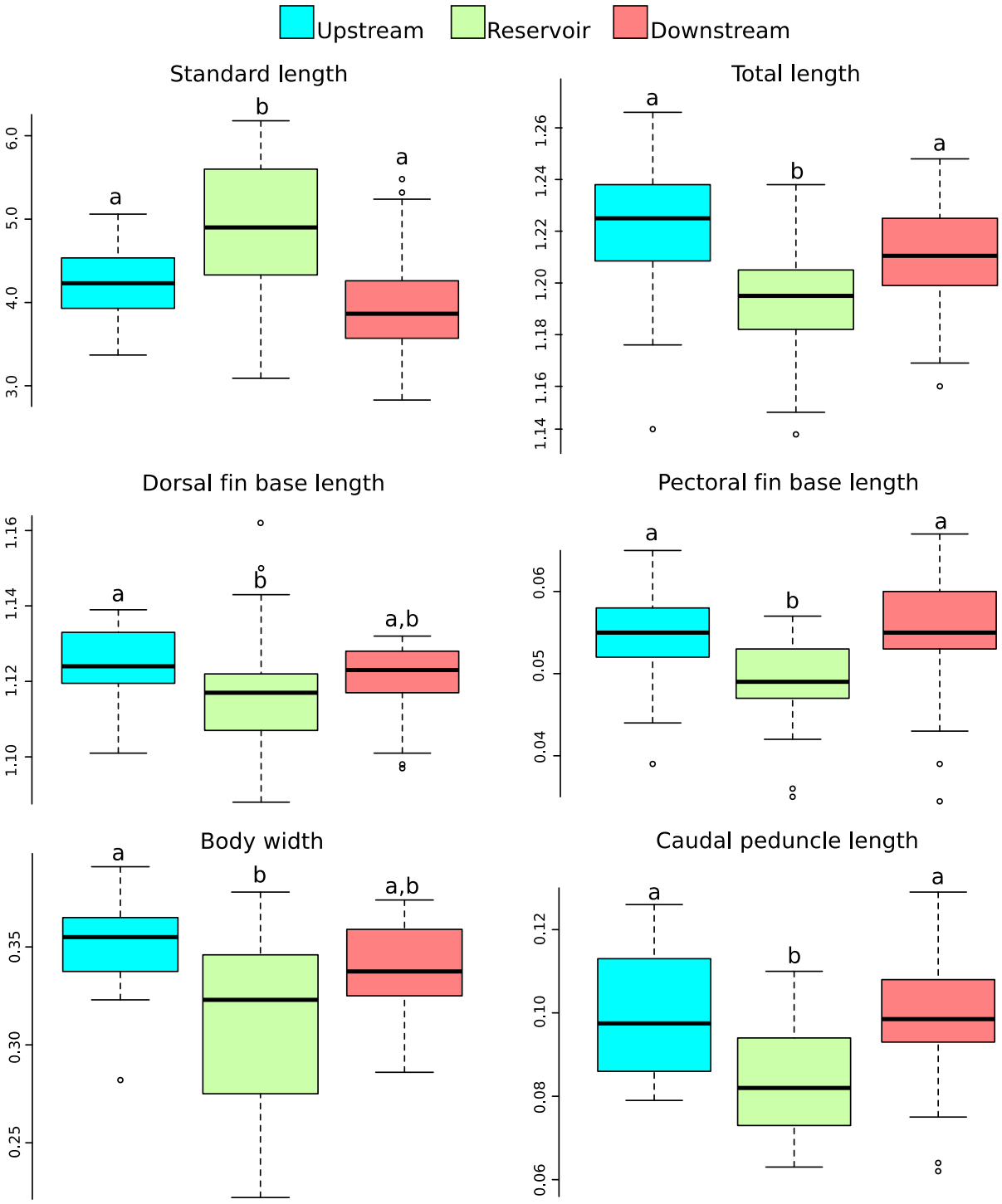

Fig. 2 Boxplot of linear measurements comparisons between the three environments. Different letters represent statistical difference for each comparison.

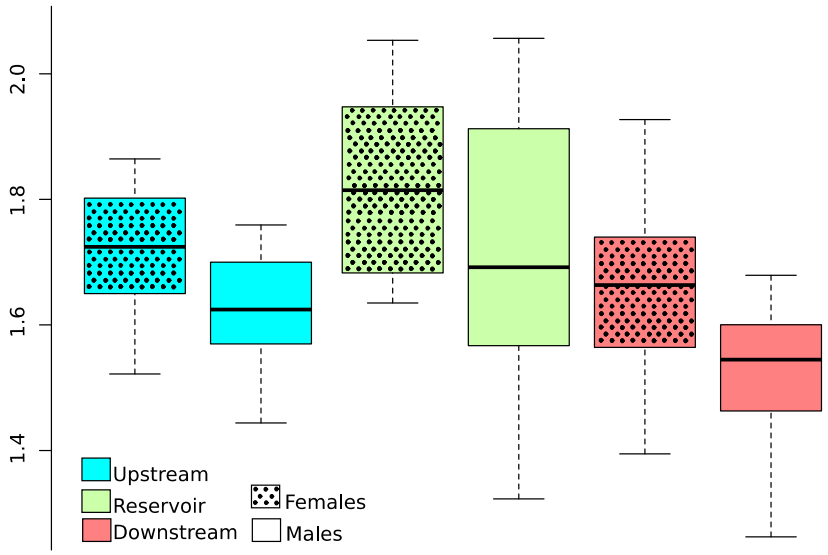

Fig. 3 Boxplot of centroid size comparisons between environments and sex. There is no significant difference between sexes. However, there is statistical difference between males and females of downstream and the reservoir.

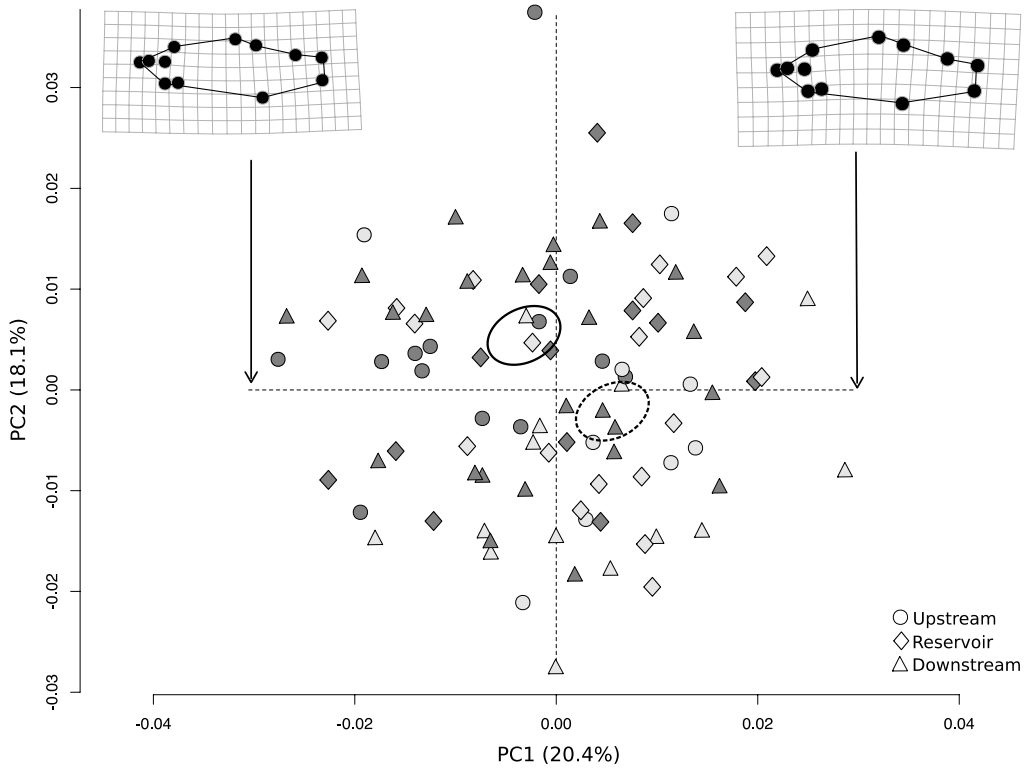

Fig. 4 PCA with all samples. Females: dark grey; males: light grey. Circles are the mean shape for each sex (continuous line: females; dotted line: males). Above the PCA are plotted the shape through warped drawings on grid of deformation for the maximum and minimum PC1.

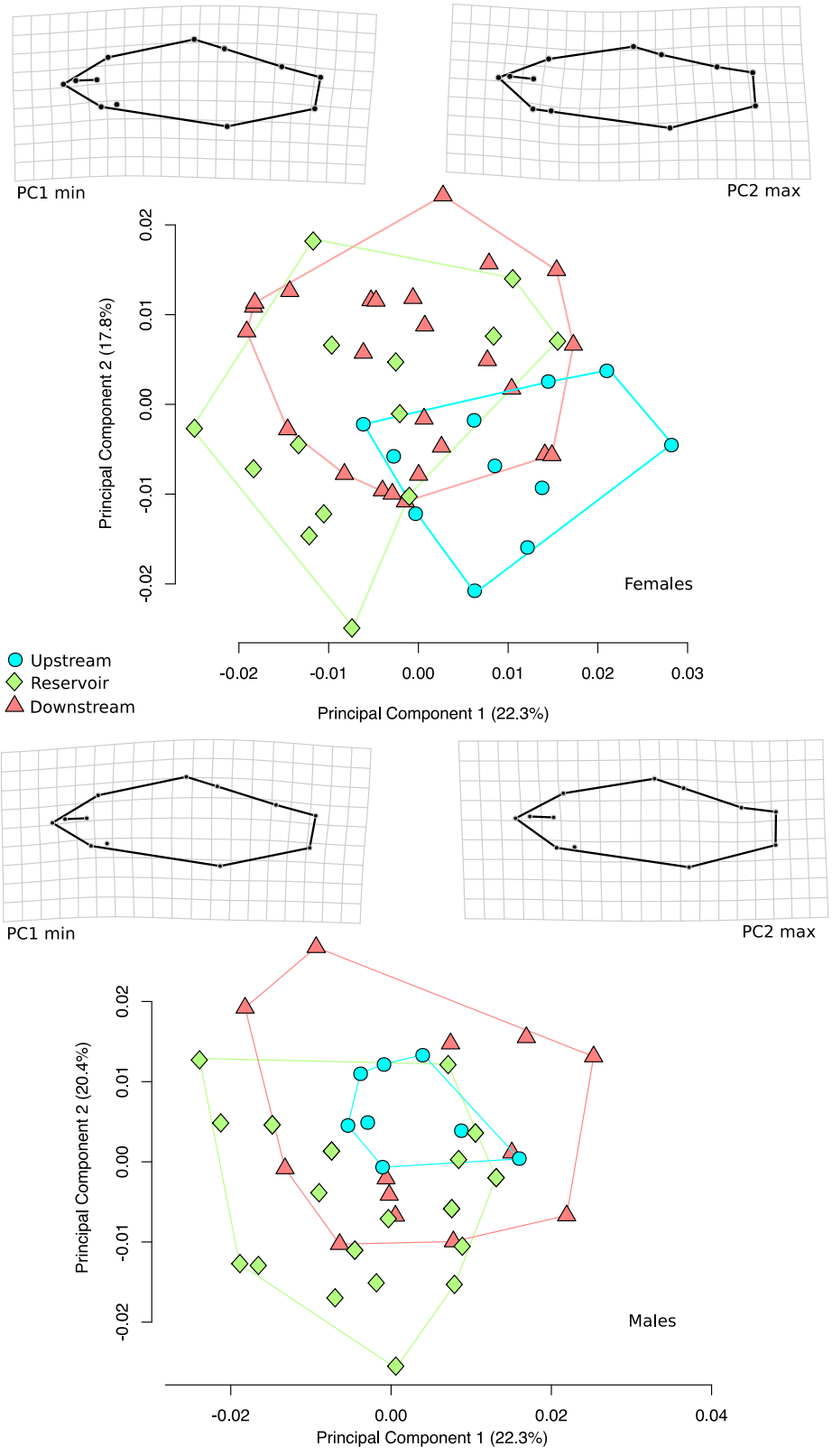

Fig. 5 PCA performed for females and males separately. Above each PCA graph are plotted the shape through warped drawings on grid of deformation for the maximum and minimum PC1.

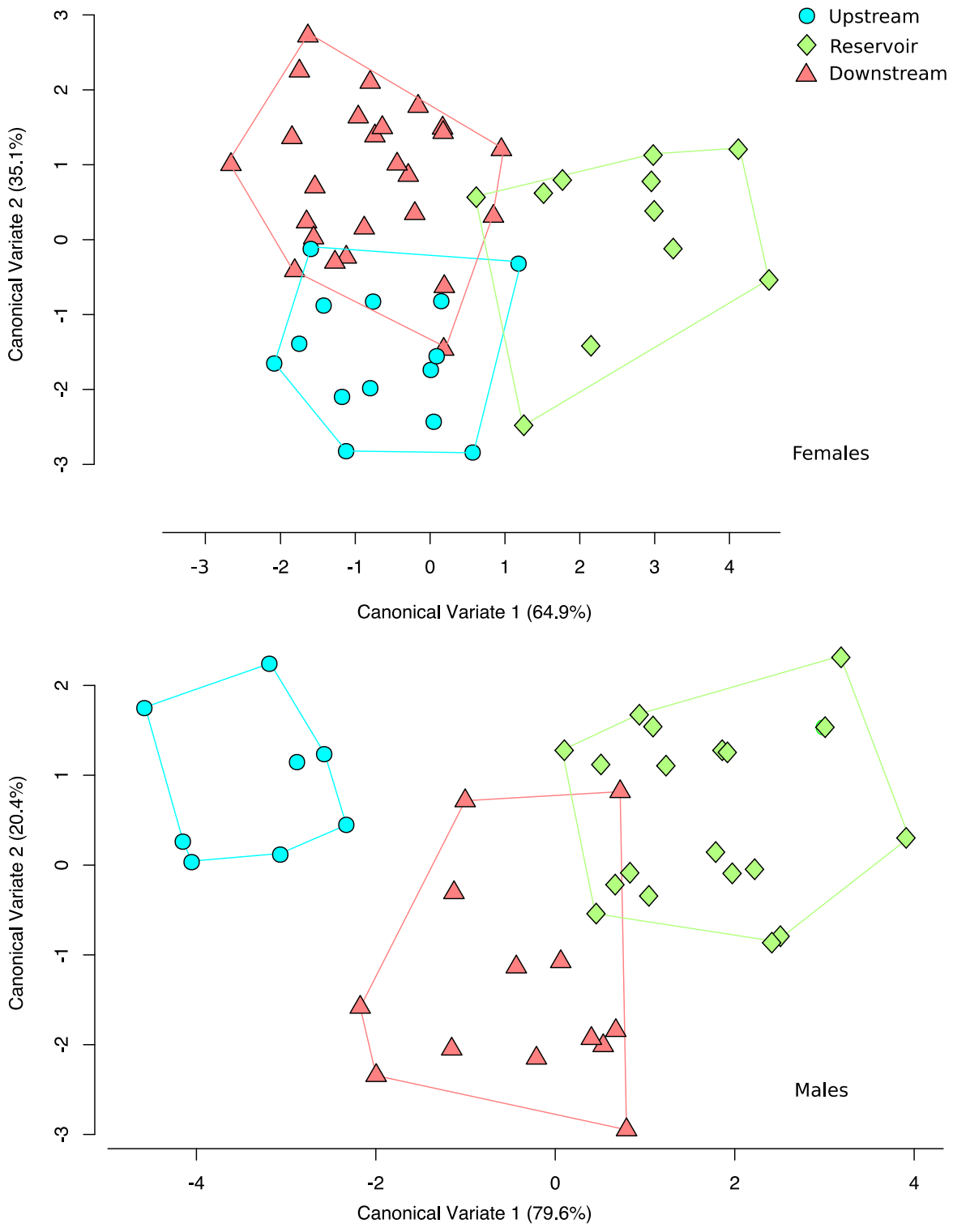

Fig. 6 CVA performed for females and males, exploring the differences among the environments.

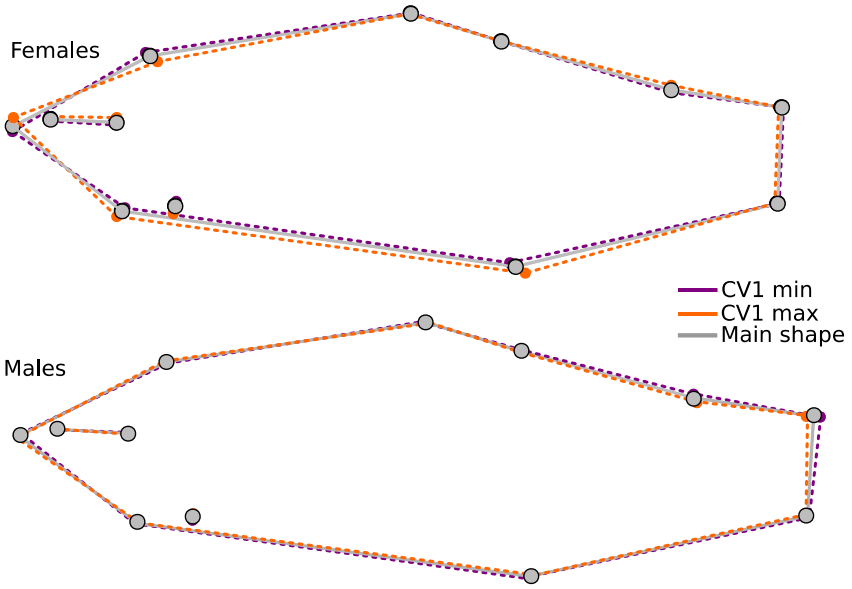

Fig. 7 Representation of shape variation along the first canonical variate, for females and males.

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CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A proposta principal desta Tese foi estudar adaptação intraespecífica em peixes expostos a ambientes heterogêneos, tanto naturais como antropizados. Como exposto ao longo do trabalho, este tipo de abordagem tem relevância tanto para o entendimento dos processos evolutivos desses organismos (KAWECKI; EBERT, 2004), como também na compreensão das interações entre organismos aquáticos e o seu meio, cada vez mais sujeito às alterações antrópicas (FLORENTINO; SÚAREZ, 2014). Grande aporte do conhecimento que temos atualmente nesta área é de estudos realizados no hemisfério norte (BAKER et al., 2015; BERNER; GRANDCHAMP; HENDRY, 2009; CURETON; BROUGHTON, 2014; DEFAVERI; MERILÄ, 2014; FOSTER et al., 2015; FRANSSEN; STEWART; SCHAEFER, 2013; GASTON; LAUER, 2015; HAAS; BLUM; HEINS, 2010; JACQUEMIN; PYRON, 2016; JØRGENSEN et al., 2008; MARCHINKO; SCHLUTER, 2007; TORRES-DOWDAL et al., 2012, entre outros), além dos estudos com os ciclídeos dos grandes lagos africanos (revisado por SALZBURGER, 2018). Na América do Sul, alguns trabalhos têm sido desenvolvidos nessa área, principalmente com poecilídeos (ARAÚJO; MONTEIRO, 2013; ARAÚJO et al., 2014; GOMES; MONTEIRO, 2008; MISE et al., 2015), assim como com outros táxons (FLORENTINO; SÚAREZ, 2014; KOKUBUN et al., 2018; SANTOS; ARAÚJO, 2015). A maioria deles realizados no Brasil, especificamente na região costeira do Rio de Janeiro, bacia do rio Paraná e campos sulinos.

Observa-se, portanto, que nosso conhecimento sobre adaptação local em peixes da região Neotropical ainda é incipiente, especialmente na região compreendida pelo estudo conduzido nesta Tese. Como apresentado pelos nossos resultados, bem como pelo suporte das referências bibliográficas utilizadas, diferentes ambientes exercem diferentes pressões seletivas aos organismos a eles expostos. Logo, é importante que diferentes ambientes sejam estudados a fim de que se obtenha melhor compreensão sobre o processo. Nesse sentido, os resultados desta Tese contribuem com dados iniciais para o conhecimento sobre adaptação local em peixes da região costeira do Rio Grande do Sul e do Uruguai.

Cabe ressaltar que encontramos variação morfológica, tanto para *J. lineata* de ambientes naturalmente distintos, como para *B. iheringii* de ambientes alterados por ação humana. Em ambos os casos, o principal componente ambiental que parece estar selecionando variantes morfológicas é o fluxo de água, como visto para outras espécies em variados ambientes (CURETON; BROUGHTON, 2014; GASTON; LAUER, 2015; HAAS; BLUM; HEINS, 2010; MISE et al., 2015; RAJKOV et al., 2018). É bastante provável que estas variações estejam associadas à

plasticidade fenotípica, a qual está relacionada com a capacidade de um genótipo produzir mais de um fenótipo sob diferentes condições ambientais, sem que haja mudança adicional nas gerações subsequentes (GARLAND; KELLY, 2006). Contudo, não se pode descartar a possibilidade de que a seleção destes fenótipos ao longo do tempo promova a distinção genética entre estas populações. Como visto na análise de loci polimórficos para *J. lineata*, populações de distintos habitas são diferentes geneticamente, e apresentam SNPs *outliers* indicando a seleção de genes relacionados com o desenvolvimento corporal, entre outras funções. De fato, para testar se a variação fenotípica se deve a plasticidade fenotípica ou a variação alélica de loci codificantes, experimentos do tipo *common garden* devem ser implementados a fim de se comprovar ou não a herança da variação fenotípica observada.

Destaca-se, ainda, que em recente estudo com *B. iheringii* de 22 riachos dos campos sulinos, KOKUBUN et al. (2018) não observaram variação na forma e tamanho do corpo, o que reforça a hipótese de que a variação morfológica que observamos para esta espécie provavelmente está associada com o recente represamento do Arroio Chasqueiro. Represamento e canalização dos corpos de água alteram substancialmente paisagens naturais criando novos desafios ecológicos e evolutivos para organismos aquáticos capazes de responder à alteração de fluxo (HAAS; BLUM; HEINS, 2010). Sabe-se que aquelas espécies capazes de responder rapidamente a essas mudanças por meio da plasticidade fenotípica possuem a habilidade intrínseca necessária para sobreviver em ambientes onde mudanças ambientais dinâmicas estão ocorrendo, podendo ocasionar, inclusive, alteração da estrutura da assembleia de peixes destes locais (GASTON; LAUER, 2015). Portanto, futuros estudos devem considerar os efeitos da construção de barragens na assembleia de peixes, especificamente considerando espécies com co-ocorrência em córregos e reservatórios. Assim será possível analisar se a variação observada para *B. iheringii* também ocorre em outras espécies, o que indicaria a existência de uma regra de modificação morfológica induzida por represamento de ambientes lóticos.

No caso do estudo com *J. lineata*, cabe ainda destacar mais dois pontos importantes dos resultados obtidos nessa Tese. Primeiramente é a distinção, tanto a nível morfológico como genético, da população marinha de Punta del Este. Os espécimes dessa população apresentaram corpo maior com morfologia atípica (curvatura) quando comparado com os demais, além da presença de um número consistentemente maior de SNPs privativos, quando comparados com espécimes de outras populações. Estes resultados provavelmente estão relacionados com as condições ambientais incomuns que as piscinas rochosas impõem para a espécie, que além de representarem um ambiente extremamente variável (em função da composição por água marinha, variação no volume de água ocasionado pelas marés, movimentação de água sem sentido definido

pela entrada e saída de água das piscinas, variada composição alimentar), também está isolado de outras ambientes comuns para a espécie (como córregos, lagoas, banhados – ambientes dulcícolas).

O segundo dado que merece atenção foi a descoberta de que machos e fêmeas respondem morfologicamente de maneira diferente, considerando-se um mesmo ambiente. Ou seja, *J. lineata* apresenta dimorfismo sexual relacionado ao habitat: para fêmeas, o fluxo de água é um fator relacionado com a variação na forma do corpo; já em machos não existe tal relação. Esta condição pode estar intimamente relacionada com o papel da seleção natural no dimorfismo sexual, tão evidente em espécies como *J. lineata*. É possível que machos e fêmeas não ocupem o mesmo nicho dentro de um mesmo ambiente, que explicaria a diferente resposta para cada sexo obtida em nosso trabalho. Além disso, a evidente diferença no tamanho entre machos e fêmeas também pode propiciar que ocorra a segregação sexual, que ocorre quando cada um dos sexos de uma espécie apresentam um uso diferencial do espaço (PETERSON; WECKERLY, 2017). Este fenômeno comportamental ocorre em vários grupos do reino animal, contudo é pouco documentado para organismos aquáticos (WEARMOUTH; SIMS, 2008).

Finalizo esta Tese apresentando um apanhado geral com algumas perspectivas para futuros estudos sobre adaptação intraespecífica em ambientes heterogêneos, considerando os achados com *J. lineata* e *B. iheringii* nos ambientes estudados:

- Seria interessante realizar experimentos, como de transplante recíprocos (troca de ambientes entre populações) ou de *common garden* (populações de distintos ambientes são criadas em um determinado ambiente), para testar o papel de um particular fator ambiental como agente de seleção divergente que está direcionando a adaptação local, como por exemplo a salinidade e o fluxo de água. Por meio de experimentos também é possível verificar se a variação fenotípica observada é herdada (tem base genética) ou se deve a plasticidade fenotípica;
- O dimorfismo sexual dependente de habitat relatado para *J. lineata* abre portas para estudos sobre evolução do dimorfismo sexual em peixes utilizando uma espécie abundante na região, que podem envolver a análise tanto das bases genéticas para tal variação, como também comportamentais, a fim de se comprovar a suposição de distinta ocupação de nichos entre machos e fêmeas.
- Por último, ressalta-se a importância de estudos sobre o impacto da construção de barragens com uma abordagem na comunidade de peixes que habitam tanto a porção original dos riachos/rios como o reservatório. Utilizando ferramentas

genéticas, é possível traçar o caminho evolutivo das espécies que estão enfrentando as alterações causadas pela construção de reservatórios, e contribuir com o entendimento dos impactos causados por este tipo de alteração ambiental.

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ANEXOS

Anexo 1: Publicação extra produzida pela discente no período de doutoramento

1. **PERAZZO, G.**; NOLETO, R.; VICARI, M.R.; GAVA, A.; CESTARI, M.M. 2018. B chromosome polymorphism in South American cichlid. Neotropical Biodiversity, 4: 3-9. https://doi.org/10.1080/23766808.2018.1429164
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3. **PERAZZO, G.**; Garcez, D.K; Trindade, C; Massia, K; Tozetti, A.M. 2018. Is the presence of eggs a relevant cue for predators of freshwater chelonian nests? Neotropical Biology and Conservation, 13(4): 350-355. http://10.4013/nbc.2018.134.10
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6. Desenvolvimento do website de divulgação dos estudos sobre cromossomos realizados pelo Laboratório de Genética, da FURG, fruto da aprovação do Edital 01/2015 - Inovação na Graduação, da FURG. Acesso disponível em: http://cromossomosfurg.wixsite.com/cromossomos-furg

Anexo 2: Certificado do Comitê de Ética em Uso Animal

COMISSÃO DE ÉTICA EM USO ANIMAL
Universidade Federal do Rio Grande
Pró-Reitoria de Pesquisa e Pós-Graduação - PROPESP
ceua@furg.br http://www.propesp.furg.br

CEUA

CEUA - FURG

CERTIFICADO Nº P023/2016

Certificamos que o projeto intitulado "Variabilidade genética e morfométrica de peixes do Pampa brasileiro", protocolo nº 23116.008829/2015-43, sob a responsabilidade de Adriana Gava - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi APROVADO pela COMISSÃO DE ÉTICA EM USO ANIMAL DA UNIVERSIDADE FEDERAL DO RIO GRANDE (CEUA-FURG), em reunião de 23 de março de 2016 (Ata 004/2016).

A CEUA lembra aos pesquisadores que qualquer alteração no protocolo experimental ou na equipe deve ser encaminhada à comissão para avaliação e aprovação. Um relatório final deve ser enviado à CEUA no término da vigência do seu projeto.

CEUA Nº	Pq042/2015
COLABORADORES	Giselle Xavier Perazzo
VIGÊNCIA DO PROJETO	31/12/2019
ESPÉCIE/ LINHAGEM	*Jenynsia multidentata*; *Bryconamericus iheringii*; *Astyanax jacuhiesis*; *Hyphesobrycon luetkenii*; *Geophagus brasiliensis*; *Crenicichla lepidota*; *Australoheros facetus*
NÚMERO DE ANIMAIS	40 animais de cada espécie em cada ponto de coleta
PESO/ IDADE	*Jenynsia multidentata* 6 g; *Bryconamericus iheringii* 5 g; *Astyanax jacuhiesis* 4 g; *Hyphesobrycon luetkenii* 3,5 g; *Geophagus brasiliensis* 200 g; *Crenicichla lepidota* 150 g; *Australoheros facetus* 100 g/Adultos
SEXO	Indeterminado
ORIGEM	Pampa gaucho (SISBIO: 49103-1)
ENVIO DO RELATÓRIO FINAL	Janeiro/2020

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Rio Grande, 28 de março de 2016

Med. Vet. Márcio de Azevedo Figueiredo
Coordenador da CEUA-FURG

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