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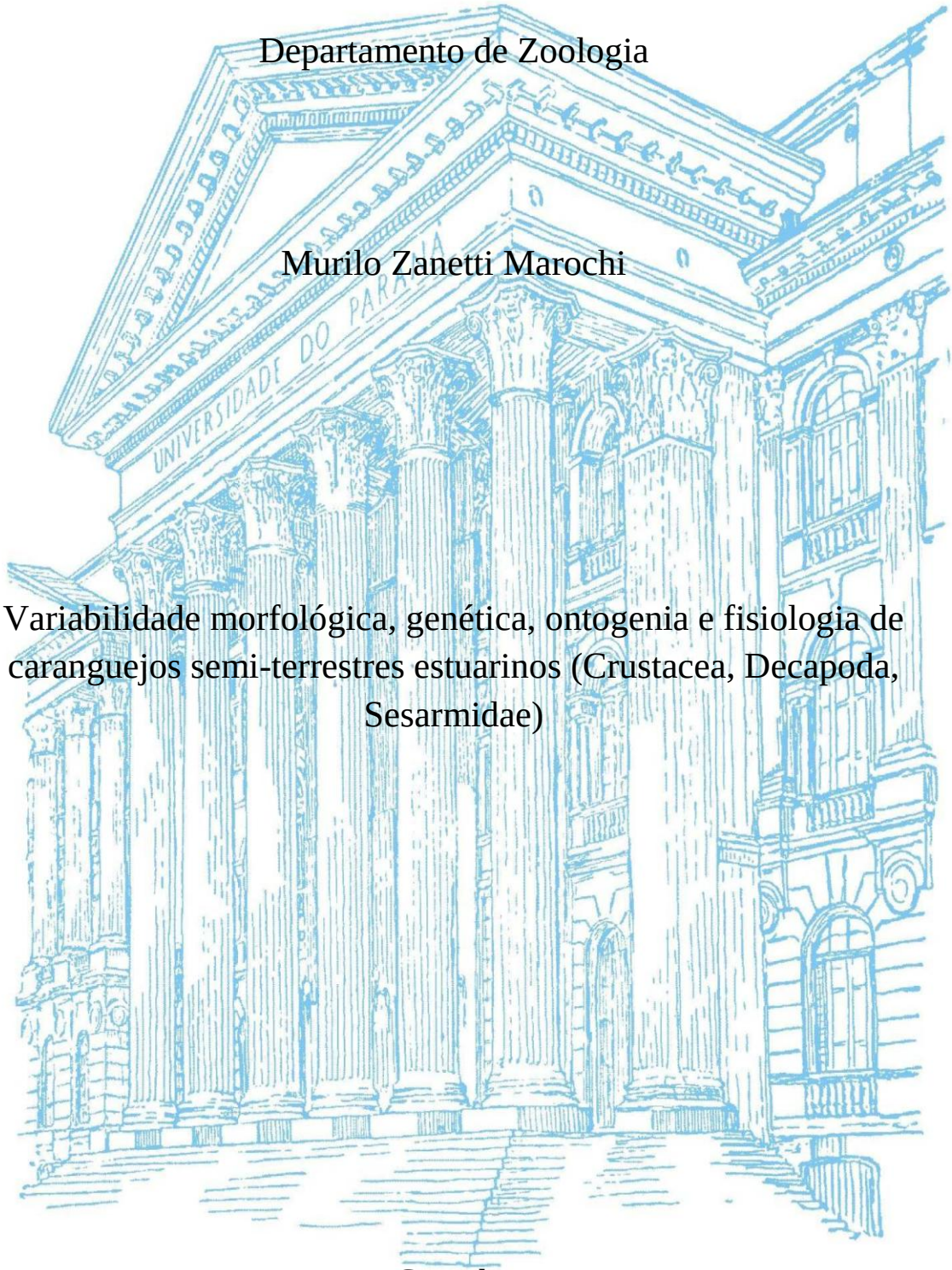

Variabilidade morfológica, genética, ontogenia e fisiologia de caranguejos semi-terrestres estuarinos (Crustacea, Decapoda, Sesarmidae)

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Murilo Zanetti Marochi

Variabilidade morfológica, genética, ontogenia e fisiologia de caranguejos semi-terrestres estuarinos (Crustacea, Decapoda, Sesarmidae)

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Orientadora: Dra. Setuko Masunari

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ZOOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **MURILO ZANETTI MAROCHI** intitulada: **Variabilidade morfológica, genética, ontogenia e fisiologia de caranguejos semi-terrestres estuarinos (Crustacea, Decapoda, Sesarmidae)**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua aprovação.

Curitiba, 21 de Fevereiro de 2017.

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“A frase mais deliciosa de se ouvir na Ciência, aquela que anuncia descobertas, não é Eureka, mas... Que engraçado"

Isaac Asimov

“Dedico este trabalho aos meus pais e irmão, Hamilton, Cláudia e Marcos, pelo apoio, carinho e compreensão; a todos os amigos pela ajuda e conselhos e a minha companheira Ariane pelo carinho e auxilio em todos os momentos.”

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Resumo Geral

Dentre os crustáceos decápodos, os caranguejos sesarmídeos possuem uma grande diversidade, expressa no alto número de espécies e grande variedade de ambientes que a linhagem habita. Outra característica marcante é a grande plasticidade fenotípica, exemplificada na gama de micro habitats em que uma única espécie ocorre. Esta diversidade é resultado da conhecida rápida radiação adaptativa que a linhagem apresenta. Em sua grande maioria, a família é composta por representantes semi-terrestres, terrestres ou dulcícolas com hábito de vida anfíbio. Muitas espécies apresentam adaptações morfológicas, comportamentais e ecológicas para tal. Possuem fase larval dependente do ambiente aquático, ao passo que os adultos possuem diferentes níveis de independência do meio aquático. Características essas que, associadas a uma distribuição pantropical/sub-tropical, tornam o grupo um bom modelo para avaliar padrões e processos nos mais variados níveis, inter/intra populacionais ou intra/inter específicos, com implicações evolutivas.

Diante disso, a presente tese foi estrutura em cinco capítulos com enfoques distintos, utilizando caranguejos sesarmídeos neotropicais como modelo. O primeiro capítulo teve como objetivo avaliar os padrões de dispersão e distribuição espacial em ambientes estuários com base no efeito da salinidade sobre a sobrevivência e a duração do desenvolvimento larval do estágio de zoea I até a fase de megalopa, e desta até o primeiro estágio juvenil, de *Aratus pisonii*. No segundo e terceiro capítulos, os objetivos foram avaliar as tendências de seleção sexual e do desenvolvimento ontogenético da fase juvenil para a adulta, nas espécies *Armases rubripes* e *Aratus pisonii*, respectivamente, através da analise do dimorfismo sexual e da variação ontogenética de forma e tamanho. O quarto capítulo teve como objetivo avaliar a estruturação genética e

morfológica do caranguejo *Armases angustipes* ao longo da costa brasileira, e a influência da divisão da Corrente Central Sul Equatorial (CSEC) sobre a dispersão larval. Enquanto o quinto capítulo teve como objetivo avaliar se a incubação dos ovos e a privação do contato com a água afetam a fisiologia materna e o desenvolvimento embrionários em crustáceos semi-terrestres, através da mensuração da atividade da enzima anidrase carbônica e a osmolalidade da hemolinfa da mãe, bem como o número de larvas eclodidas de fêmeas de *A. pisonii*.

Abstract

Among the decapod crustaceans, sesarmid crabs have a great diversity, expressed in the high number of species and the great variety of environments that the lineage inhabits. Another striking feature is the great phenotypic plasticity, exemplified in the range of micro-habitats in which a single species occurs. This diversity is a result of the known rapid adaptive radiation that the lineage presents. In your vast majority, the family consists of semi-terrestrial, terrestrial or freshwater species with amphibious life habit. Many species have morphological, behavioral and ecological adaptations. They have a larval phase dependent on the aquatic environment, while adults have different levels of independence from the aquatic environment. These characteristics, associated to a pantropical / sub-tropical distribution, make the group a good model for evaluating patterns and processes at the most varied levels, inter / intra-population or intra / inter-specific, with evolutionary implications.

Therefore, the present thesis was structured in five chapters with distinct approaches, using neotropical sesarmid crabs as a model. The first chapter aimed to evaluate the dispersion patterns and spatial distribution in estuarine environments based on the effect of salinity on the survival and duration of larval development of the stage of zoea I to the megalopa stage, and from this to the first juvenile stage of *Aratus pisonii*. In the second and third chapters, the objectives were to evaluate the sexual selection and ontogenetic development trends of the juvenile phase for the adult, in the species Armases rubripes and Aratus pisonii, respectively, through the analysis of sexual dimorphism and ontogenetic variation of shape and size. The fourth chapter aimed to evaluate the genetic and morphological structure of the spceies *Armases angustipes* along the Brazilian coast, and the influence of the division of the Equatorial South Central Current (CSEC) on the larval dispersion. While the fifth chapter aimed to

evaluate whether incubation of eggs and deprivation of contact with water affect maternal physiology and embryonic development in semi-terrestrial crustaceans, by measuring the activity of the carbonic anhydrase enzyme and the hemolymph osmolality of the as well as the number of hatched larvae of *A. pisonii* females.

Introdução geral

Os membros da infraordem Brachyura Linnaeus, 1758, compreendem cerca de 7.000 espécies de caranguejos e siris, pertencentes a 98 famílias. Esse grupo exibe complexidade morfológica devido ao processo de carcinificação (“carcinisation”), no qual a cabeça e os somitos abdominais são fusionados, o primeiro par de pereiópodos é “quelado” (possuí quela) e as pernas ambulatórias estão situadas nas laterais do corpo. São encontrados em quase todos os ecossistemas marinho-estuarinos, bem como no ambiente terrestre e dulcícola. Presentes em ambientes com características físicas, químicas e ecológicas muito distintas, como fossas abissais (a mais de 6.000 m de profundidade), regiões montanhosas (a mais de 2.000 m de altitude) e cavernas com solo constituído por lodo e guano em ambiente terrestre (Ng 1989, Ng et al. 2008, De Grave et al. 2009).

Guinot (1977,1978, 1979) dividiu Brachyura em três seções de acordo com a posição dos gonóforos: Podotremata, Heterotremata e Thoracotremata. O táxon é considerado monofilético pela maioria dos autores, apesar de não haver um consenso, com provável origem no começo do Jurássico (há aproximadamente 200 milhões de anos). Esta falta de consenso se deve principalmente a incertezas sobre a monofilia dos taxa mais basais (seção Podotremata) que retém caracteres presumivelmente ancestrais (ex. último par de pereiópodos rudimentares). De modo contrário, os Eubrachyura (Heterotremata e Throracotremata) apresentam monofilia suportada por filogenias moleculares e morfológicas, com consenso entre autores (Ahyong et al. 2007, Anger et al. 2008, Ng et al. 2008, De Grave et al. 2009, Scholtz & McLay 2009, Tsang et al. 2014).

Atualmente a família Sesarmidae é composta por 30 gêneros com pelo menos 255 espécies de caranguejos semiterrestres/ terrestres, sendo o táxon com maior número de espécies de Thoracotremata, representando 22% do total de espécies do taxa (Ng et al. 2008, De Grave et al. 2009). A família apresenta uma distribuição pan-tropical e subtropical, com raras exceções de ocorrência em regiões temperadas. De modo geral, os sesarmídeos possuem uma grande plasticidade e alto potencial de adaptação para ocupação de novos ambientes e ocorrem em uma ampla gama de habitats estuarinos (principalmente manguezais), dulcícolas e terrestres. Frequentemente habitam áreas de transição entre o ambiente marinho e regiões adjacentes de água doce ou zonas terrestres, representando uma parte importante da biodiversidade destes ecossistemas (Anger & Charmantier 2000, Schubart et al. 2000, Thiercelin 2015). Ocorrem em áreas com grande amplitude de salinidades como regiões ou corpos de água temporários em que a salinidade pode variar de próximas a 0 (durante períodos chuvosos) até situações de hipersalinidade com salinidades próximas a 60 (Anger 1996).

Nas Américas, os Sesarmidae são compostos por 34 espécies pertences a quatro gêneros: *Aratus* H. Milne Edwards, 1853, *Armases* Abele, 1992, *Metopaulias* Rathbun, 1856 e *Sesarma* Say, 1817 (Abele 1992, De Grave et al. 2009). Com exceção de *Armases elegans* (Herklots, 1851), as demais espécies são endêmicas da região Neotropical (Abele 1992). Segundo estimativas de Thiercelin (2015), a origem dos Sesarmidae Americanos data do final do Mioceno, a aproximadamente 10 milhões de anos, com o surgimento de dois grandes clados: 1) *Sesarma* e *Metopaulias* e 2) *Aratus* e *Armases*. A provável separação do gênero *Aratus* das demais espécies de *Armases* ocorreu a cerca de 6,7 milhões de anos, enquanto o gênero endêmico da Jamaica, *Metopaulias* divergiu das demais espécies de *Sesarma* a aproximadamente 2,4 milhões

de anos (para maiores detalhes sobre a filogenia de sesarmídeos americanos verificar Schubart et al. 1998, Thiercelin 2015).

Dentre os Sesarmídeos americanos algumas espécies se destacam devido a total independência de águas oceânicas ou estuarinas para a sobrevivência e reprodução. Outras possuem estratégias reprodutivas únicas, como algumas espécies endêmicas da Jamaica (Schubart et al. 1998). Possuem cuidado parental ativo com as larvas, como *Metopaulias depressus* Rathbun, 1896 que libera suas larvas na "axila" de bromélias contendo água e a mãe remove detritos, realiza a circulação e oxigenação da água, protege a área onde estão as larvas de possíveis predadores (aranhas e larvas de libélulas) e adiciona conchas vazias de moluscos para regulação do pH e fonte de cálcio (Diesel 1989, Diesel & Schuh 1993). Outro exemplo é o caranguejo de montanhas *Sesarma jarvisi* Rathbun 1914, que libera suas larvas em conchas (desocupadas) de gastrópodes coletando água da chuva ou levando água até a concha, até o fim do desenvolvimento larval (Diesel & Horst 1995).

O gênero *Aratus* é um dos mais comuns e abundantes grupos de invertebrados de florestas de manguezais nas Américas (Conde et al. 2000). Atualmente é constituído pelas espécies *Aratus pisonii* (H. Milne Edwards, 1873) e *Aratus pacificus* Thiercelin & Schubart 2014, que habitam áreas estuarinas principalmente a porção aérea de raízes, tronco e galhos de árvores de manguezais, especialmente da espécie *Rhizophora mangle* (Warner 1967, Diaz & Conde 1988). Indivíduos adultos são encontrados em um amplo regime de salinidades (0-35), de margens de rios próximos à desembocadura até áreas estuarinas com grande aporte de águas oceânicas. Apresentam dimorfismo sexual no tamanho da carapaça e própodo dos quelípodos, sendo machos maiores que fêmeas (Díaz and Conde 1988, Chiussi 2002).

Aratus pisonii ocorre na costa leste das Américas no Oceano Atlântico (da Florida até o estado de Santa Catarina no Brasil), enquanto *A. pacificus* ocorre na costa oeste Americana no Oceano Pacífico (do México ao Peru) e o provável evento de especiação ocorreu durante o Plioceno (aproximandamente a 3 milhões de anos) com o fechamento do Istmo da América Central e a mudança drástica de correntes oceânicas na região (Thiercelin & Schubart 2014). A separação dos Oceanos, que anteriormente era um sistema interligado, causou a formação de dois sistemas marinhos distintos, com condições de temperatura, salinidade, sazonalidade e produção primária distintas (Haugh & Tieldermann 1998, Steph et al. 2006, Rebolledo et al. 2015). Ocasionando variações genéticas e morfológicas (estrutura do gonópodo e a morfologia larval) entre os dois táxons (Thiercelin & Schubart 2014, Rebolledo et al. 2015).

O gênero *Armases* é formado por 13 espécies que habitam áreas estuarinas em substratos arenosos, lodosos e rochosos em margens de manguezais e rios (próximo à desembocadura), sob folhas secas na borda da vegetação (geralmente manguezais), bromeliáceas e margens de costões rochosos sob a vegetação. Algumas espécies podem ocorrer em áreas secas próximas a riachos a mais de 2 km da desembocadura de rios, bem como centenas de metros distante de qualquer corpo de água (Abele 1992, Melo 1996). Segundo estimativas de Thiercelin (2015), o gênero passou por distintos eventos de especiação durante o Plioceno (de 4,6 a 1,13 milhões de anos) com a separação das linhagens do Oceano Atlântico e Pacífico (entre 4,6 a 4,21 milhões de anos), provavelmente pelo fechamento do Istmo da América Central e mudanças drásticas de correntes oceânicas na região (Thiercelin & Schubart 2014).

Armases angustipes (Dana, 1852) é um caranguejo semi-terrestre pertencente a família Sesarmidae com ampla distribuição geográfica. Existem registros de ocorrência

para a espécie em Yucatan (México), Ilha Andros (Bahamas), Trinidade e Tobago e para o Brasil (do Maranhão até Santa Catarina) (Abele 1992, Melo 1996). *A. angustipes* ocorre em uma ampla variedade de habitats em áreas estuarinas: em substratos arenosos, lodosos e rochosos em margens de manguezais e rios (próximo à desembocadura), sob folhas secas na borda da vegetação (geralmente manguezais), bromeliáceas e margens de costões rochosos sob a vegetação (Abele 1992, Melo 1996). A espécie possui quatro estágios zoea com desenvolvimento em salinidades superiores a 20 ‰, e um estágio de megalopa que retorna para ambientes com salinidade menor que 20 ‰ antes da metamorfose para o primeiro estágio juvenil, sendo considerada uma espécie com estratégia de exportação larval (Anger et al. 1990, Cuesta & Anger 2001).

Armases rubripes (Rathbun, 1897) é um caranguejo semiterrestre pertencente à família Sesarmidae Dana, 1851 com uma ampla distribuição geográfica. Habita regiões costeiras tropicais e subtropicais na América Central e América do Sul, da Nicarágua até o estuário do Rio de la Plata (entre Uruguai e Argentina), sendo encontrado em manguezais sobre o sedimento e em tocas feitas por ele mesmo, em bromélias, entre raízes, em áreas próximas a desembocadura de rios principalmente próximos a pirizais *Scirpus californicus*, marismas próximo a gramíneas como *Spartina sp.* e também em áreas de restinga (Capítoli et al. 1977, Fischer et al. 1997 Lima et al. 2006). A espécie possui uma dieta herbívora consumindo detritos vegetais, principalmente raízes e caules de *Spartina sp.* bem como inflorescências de bromélias (Capítoli et al. 1977, Lima 2007).

Dimorfismo sexual

O dimorfismo sexual é definido como a diferenciação morfológica de machos e fêmeas adultos, sendo amplamente conhecido no reino animal. Por exemplo, em alguns grupos animais, os machos têm tamanhos maiores do que as fêmeas (mamíferos, aves, caranguejos), enquanto em outros grupos as fêmeas são maiores do que os machos (aranhas, insetos, peixes). Este dimorfismo tende a aumentar com o aumento do tamanho corpóreo. Isto está diretamente relacionado com o crescimento alométrico diferenciado. O desenvolvimento de caracteres sexuais secundários (que irão intensificar o dimorfismo sexual) é mais provável de ocorrer quando um aumento no tamanho relativo da característica (armas para combates macho-macho ou ornamentos de atração de parceiros) produz maiores benefícios de sucesso de acasalamento (Bonduriansky 2007). Hipóteses distintas são mencionadas para explicar os padrões de dimorfismo sexual, tais como seleção natural em traços reprodutivos, divergência de nicho ou outras causas ecológicas (Shine 1989, Abouheif and Fairbairn 1997, Fairbairn 1997). Assim como em vários grupos animais, em crustáceos o dimorfismo sexual é geralmente uma condição mediada pela ação de hormônios sexuais durante o desenvolvimento (Gopal et al. 2010).

Desenvolvimento larval

A maioria dos invertebrados marinhos apresenta um ciclo de desenvolvimento complexo com distintos estágios larvais até a metamorfose para o estágio juvenil (Anger et al. 2006). De modo geral crustáceos apresentam o desenvolvimento ontogenético constituído por três períodos: 1) desenvolvimento embriológico dentro dos ovos, 2) eclosão de alguma forma larval (ex. nauplius ou zoea) seguido por uma série de larvas planctônicas e 3) metamorfose para, na maioria dos casos, juvenis bentônicos que

crescem e posteriormente atingem a maturidade sexual (Vogt 2013). A fase larval representa uma grande variedade de características adaptativas que diferem das demais fases do desenvolvimento ontogenético e são geneticamente hereditárias e influenciadas por condições ambientais atuais e refletem as pressões seletivas sofridas pela linhagem (Anger et al. 2006, Giménez 2006).

Em Decapoda, o desenvolvimento larval planctônico ocorre durante um intervalo de tempo de alguns dias até algumas semanas antes da metamorfose para o ambiente bentônico (Anger 2001, Anger et al. 2006). Durante este período a larva é frequentemente exposta a variações de temperatura, salinidade, disponibilidade de alimento e predação. Dentre essas pressões ambientais, a salinidade é considerada um parâmetro ecológico chave para os decápodos costeiros e estuarinos devido ao fato da maioria dos processos metabólicos e fisiológicos necessitarem de condições osmóticas e iônicas estáveis (Anger 2001, Anger et al. 2006, Simith et al. 2012). O efeito do estresse salino em Decapoda pode atrasar o desenvolvimento, reduzir a sobrevivência, afetar as taxas de alimentação e crescimento, bem como alterar o ciclo de mudas, metabolismo e o comportamento (Anger 2003).

De modo geral, os Brachyura possuem duas estratégias de desenvolvimento larval: retenção larval no habitat dos pais ou exportação para habitats com condições mais estáveis, geralmente águas costeiras/oceânicas. A retensão larval exige uma tolerância maior para variações físico-químicas pelo fato das larvas realizarem todo o desenvolvimento em ambiente estuarino sujeito a grandes variações ambientais. Poucos braquiúros utilizam esta estratégia, como as espécies *Rhithropanopeus harrisii* (Gould, 1841) (Cronin, 1982) e *Neohelice granulata* (Dana, 1851) (Cervelline, 2001). A segunda estratégia de desenvolvimento é a mais comum entre caranguejos e siris

estuarinos, onde a larva é liberada em ambiente estuarino, migra para águas costeiras/oceânicas e somente após atingir o estágio de megalopa, retorna para ambientes estuarinos com salinidade reduzida para completar o desenvolvimento ontogenético até o primeiro estágio juvenil. Esta estratégia pode facilitar a dispersão e proporcionar um ambiente mais estável para o desenvolvimento das larvas (Anger 2001, Simith et al. 2012). Diesel & Schuh (1998) descreveram três padrões de "liberação" das larvas, para caranguejos com estratégia de exportação larval da família Sesarmidae: 1) espécies semiterrestres que repetidamente voltam para ao mar ou estuários para liberação das larvas, 2) espécies semi-dulcícolas que liberam suas larvas em rios e subsequentemente são transportadas até estuários ou oceanos, e 3) espécies semi-dulcícolas que migram para áreas estuarinas para liberar suas larvas.

A taxa de sobrevivência larval em relação a distintas salinidades pode ser usada como um indicador da estratégia de desenvolvimento larval adotada pela espécie. Para algumas espécies com dispersão larval para águas costeiras, a salinidade pode servir como indicadora de condições estuarinas, orientando as megalopas durante o recrutamento (O'Connor & Epifanio 1985, Charmantier et al. 2002).

Variabilidade populacional

Populações naturalmente ocorrem em ambientes heterogêneos. Em habitats marinhos/estuarinos esta heterogeneidade está relacionada com a salinidade, temperatura, força de marés e energia de ondas, predação, disponibilidade de alimento, competição, parasitismo, doenças e patógenos, entre outras forças que variam ao longo da escala temporal (de segundos a eras) e espacial (metros a graus/latitude). Em resposta, indivíduos de uma população podem exibir notáveis variações em seu

comportamento, aspectos do desenvolvimento. Estas variações surgem devido à adaptação ou plasticidade de determinadas características frente a estímulos ambientais ou ecológicos (Sotka 2012).

A variação fenotípica pode ser gerada através da adaptação local, na qual genótipos locais se "ajustam" melhor às necessidades ambientais locais em relação a genótipos oriundos de processos de emigração (de fora da população local) (Sotka 2012). Variações populacionais morfológicas geralmente refletem a adaptação ao meio, e podem ser direcionadas pela plasticidade fenotípica, uma vez que esta favorece a adaptação local (Kawecki & Ebert 2004, Sotka 2012). A plasticidade fenotípica é conceituada como a capacidade de um único genótipo exibir uma gama de fenótipos em resposta à variação ambiental (Fordyce 2006). Como mencionado anteriormente, fatores ambientais podem atuar seletivamente sobre o fenótipo, alterando a frequência genotípica ou produzindo genótipos únicos. Porém, a conclusão de quais destes fatores ambientais/ecológicos estão efetivamente agindo sobre o genótipo nem sempre é evidente e na maioria dos casos, as causas da plasticidade não são totalmente compreendidas.

A maioria dos invertebrados marinhos possui populações com grande número de indivíduos, períodos larvais de longa duração e estratégia de reprodução com milhares de larvas por geração. Isso facilita a dispersão por grandes distâncias, e reduz a variabilidade genética entre populações (Hedgecock 1986, Avise 2004, Fratini et al. 2011). Assim, a extensão da dispersão larval é frequentemente negativamente correlacionada com o nível de diferenciação genética destas espécies (Kyle & Boulding, 2000). A duração do estágio larval planctônico é comumente utilizada para justificar a estruturação genética ou a falta de, uma vez que a larva é "levada" por correntes

marinhas e isso influência fortemente a dispersão e consequentemente o “pool” genético de espécie (Dias et al. 2006).

Descrever as variações intra e interespecíficas e os fatores que as originam é fundamental para o conhecimento da variabilidade das populações e dos seus papeis no ambiente. Como reconhecido inicialmente por Darwin e Wallace, variações fenotípicas dentro e entre populações são o “estopim” para o processo de especiação (Endler 1976). Além disso, variações fenotípicas podem direcionar padrões existentes na distribuição, abundância, e papéis ecológicos de distintos organismos, incluindo respostas populacionais a perturbações naturais ou antropogênicas (Fox & Morrow 1981, Davis et al. 2005, Visser 2008, Bolnick et al. 2011, Sotka 2012). De modo geral, a variabilidade populacional representa uma resposta frente a diversas pressões seletivas que variam no espaço (região geográfica independente da escala) e no tempo (de segundos a eras) aos quais as linhagens estão submetidas. Esta variação pode ou não ser transmitida geneticamente e expressa fenotipicamente de maneira diferencial e plástica entre populações.

Objetivo Geral da Tese

Abordando aspectos diversos que caracterizam os caranguejos sesarmídeos e sua linhagem, a presente tese objetiva testar o potencial de disperção larval local, regional/continental e o nível de conectividade interpopulacional; a tendência de seleção sexual através da avaliação do dimorfismo sexual e alometria ontogenética; e a resistência à dessecação no ambiente terrestre.

Para tal, a tese foi dividida em cinco capítulos. O primeiro capítulo teve como objetivo avaliar os padrões de dispersão e distribuição espacial local em ambientes estuarinos com base no efeito da salinidade sobre a sobrevivência e a duração do desenvolvimento larval do estágio de zoea I até a fase de megalopa, e desta até o primeiro estágio juvenil, de *Aratus pisonii*. No segundo e terceiro capítulos, os objetivos foram avaliar a tendência de seleção sexual e do desenvolvimento ontogenético da fase juvenil para a adulta, nas espécies *Armases rubripes* e *Aratus pisonii*, respectivamente, através da analise do dimorfismo sexual e da variação ontogenética de forma e tamanho. O quarto capítulo teve como objetivo avaliar a possível estruturação genética e morfológica do caranguejo *Armases angustipes* ao longo da costa brasileira, e a influência da divisão da Corrente Central Sul Equatorial (CSEC) sobre a dispersão larval continental. Enquanto o quinto capítulo teve como objetivo avaliar se a incubação dos ovos e a privação do contato com a água afetam a fisiologia materna e o desenvolvimento embrionários em crustáceos semi-terrestres, através da mensuração da atividade da enzima anidrase carbônica e a osmolalidade da hemolinfa da mãe, bem como o número de larvas eclodidas de fêmeas de *A. pisonii*.

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Chapter I

The salinity during larval development affects the dispersion in adults of the tree-climbing crab *Aratus pisonii*

Abstract

Adults of the tree-climbing crab Aratus pisonii occurs in estuarine regions with a range of salinity of 0 to 35. However the larvae cannot complete the development in a wide range of salinities. A study about the effect of salinity on the larval development was conducted in order to infer about the local dispersion of the species. Two experiments were conducted: 1) from zoae I to megalopae stage and 2) from megalopa to juvenile stage. Larvae from eight females from Paranaguá Bay, Paraná, Brazil were obtained. After eclosion, for the first experiment, 100 zoea larvae from five females were acclimated, individualized and transferred to small aquariums with five distinct salinity treatments (S0, S5, S15, S25 and S35). For the second experiment, 50 megalopae from three females were individualized in aquariums in the same salinity treatments. Both experiments were conducted in incubator BOD with constant temperature of 25°C and photoperiod of 12 hours (light/dark). Fed daily with eggs and naupli newly hatched of Artemia sp. The salinity affects the survival rate of development in both experiments. The larger survival percentage from zoea I to megalopa stage occurs in salinities 25 and 35 PSU, while from megalopa to juvenile stage in salinities 15 and 25 PSU. In both experiments the larvae did not tolerate low salinities as adults, with total mortality in S0 and S5. Our results confirm the larval export strategy of zoae larvae to oceanic regions (higher salinities), and the return of megalopae to estuarine areas with intermediate salinities. The occurrence of adults in estuarine areas with low (<5 PSU) or high (>25 PSU) salinities must occur after the metamorphosis to the juvenile stage by dispersion or at the stage of megalopa during favorable weather events.

Key-words: Larval dispersion, Survival rate, Ontogenetic development, Sesarmidae, Estuarine areas.

Introduction

In general estuarine/marine brachyuran crabs have the ontogenetic development constituted by three periods: 1) embryological development inside the eggs; 2) larval form hatching (usually zoea) followed by a series of planktonic larvae and a megalopae stage; 3) metamorphosis, in most cases, to benthic juveniles that grow and subsequently reach sexual maturity (Vogt 2013). The larval stage represents a wide variety of adaptive features that differ from other phases of the ontogenetic development, being genetically inherited, influenced by environmental conditions and reflecting the selective pressures suffered by lineage (Anger 2006, Giménez 2006).

The larval development can last a few days or a few weeks until the migration to the benthic environment, during this period the larva is exposed to abiotic (e.g., temperature and salinity) and biotic (e.g., food availability and predation) changes (Anger 2006). Among the abiotic variables, the salinity is considered a key parameter because it affects larval biology, such as survival, morphology, moult cycle, feeding, metabolism, behavior and development (Anger 2003). The development is specially affected in species that live in environments with wide range of salinity, as estuaries and coastal ecosystems. Although the majority of juveniles and adults of estuarine crabs are osmoregulators, tolerating a wide daily range of salinity, the larvae are less tolerable (especially during zoea stage) and can exhibit high rates of mortality in this critical period of development (Morgan 1995, Anger 2001, Luppi et al. 2003, Simith & Diele 2008).

The marine/estuarine crabs have two main larval development strategies: larval retention in the parental habitats or larval export to habitats with more stable conditions, coastal/ocean waters generally. The second strategy of development can facilitate the

dispersion and provides a more stable environment for the development of the larvae, while the first requires a greater tolerance to physical and chemical variations. Larval survival rate in different salinities can be used as an indicator of larval development strategy and fitness. For some species with larval export to coastal waters, salinity can indicate estuarine conditions, guiding the megalopae during recruitment (O'Connor and Epifanio 1985, Anger 2001, 2003, 2006, Charmantier et al. 2002, Simith et al. 2012).

Aratus pisonii (H. Milne Edwards, 1873) is an aboreal sesarmid crab found on branches and trunks of mangrove trees (Warner 1967; Diaz and Conde 1988). It has a wide geographic distribution occurring from Florida (EUA) to Santa Catarina (Brazil) and Caribbean islands (Melo 1996; Chiussi 2002). During development *A. pisonii* has four zoeal stages (which develop best on 25 PSU) and one megalopae (Diaz and Bevilacqua 1986; Cuesta et al. 2006). The adults are found in habitats with a wide salinity regime (0-35 PSU). However, is common for species with export larval strategy that the megalopae return to waters with intermediate salinity (10-25 PSU) to complete the development (Anger 2001). Thus, the occurance of adults in a wide salinity regime still has unsolved questions.

Despite zoea of *A. pisonii* has experimental data showing the best salinity it does not occur to megalopa. Information about the best salinity in different stages of development can provide important information about the dispersal and spatial distribution within an estuarine system and among populations. We evaluate experimentally the effect of salinity on the survival rate and the duration of larval development (1) from zoea I stage to megalopa stage and (2) from megalopa to first juvenile stage, inferring about dispersion of *A. pisonii* and occurance of adults in wide salinity regime.

Material and Methods

Collection area and conditioning of females

Eight ovigerous females of *A. pisonii* were sampled (F1-F8), with carapace width ranging from 16.03 to 20.61 mm, in Paranaguá Bay, Brazil (25°30'57,65''S - 48°29'57,31''W). All ovigerous females had egg mass of dark color, indicating advanced stage of embryonic development (embryos with formed eyes) (Simoni et al. 2011). After sampled, the females were placed in plastic boxes with water from filed (salinity = 25 PSU) and carry to the Laboratory of Crustacean Ecology of the Federal University of Paraná. Ovigerous females were acclimatized individually in aquariums (18x18x18cm) containing 300 ml of water (salinity 25 PSU and 25°C temperature) and a small rock, under constant aeration and photoperiod of 12:12 (light/dark) until the hatching of the larvae. The females were fed with two leaves of *Rhizophora mangle* every day morning (8 a.m.) and observed three times per day (8:00, 13:00 and 18:00 hours) until larvae hatching.

Acclimation and experimental design for zoea larvae

After hatching (the females hatched their eggs with maximum interval of 7 days), the larvae in zoea I stage were removed with a sterile pipette of hatching aquariums and transferred to 1L polyethylene containers to be acclimated in the experimental salinity. To prevent osmotic stress, the larvae that hatched in water with salinity 25 PSU were transferred to containers with salinity increased or decreased 5 units, until the salinity of 35 PSU (maximum) or zero (minimum), gradually, every 60 minutes. This period of acclimatation was established based on previously works (Foskett 1977, Charmantier & Charmantier-Daures 1991, Anger 1996).

After acclimatization, 100 zoea larvae from five females were placed individually in 50 ml polyethylene containers with water in the following treatments of salinity (20 larvae per treatment): salinity 0 PSU (S0), salinity 5 PSU (S5), salinity 15 PSU (S15), salinity 25 PSU (S25) and salinity 35 PSU (S35), totalizing 500 zoea larvae. Only active larvae were chosen (indicating healthy conditions). These treatments were chosen because they contemplate the variations of salinity in an estuarine system and oceanic waters. All containers containing the larvae were kept in an incubator BOD (ELETROLAB 122FC) with the photoperiod of 12 hours (light:dark), and constant temperature of water (25°C). The laboratory room and the water for experiment were also maintained in the same temperature of the BOD to avoid any influence of temperature stress during maintenance of the larvae. Daily the water in the container was changed and the larvae were fed with eggs and newly hatched larvae (nauplius) of *Artemia sp* (0.6 ind.ml^{-1}) (Diaz and Bevilacqua 1986). Also, it was carried a daily inspection of the survival of the larvae under a stereoscopic microscope until the megalopa stage. Those larvae that reached the megalopae stage were fixed and preserved in 75% ethanol.

The water of the experiments was obtained by dissolving artificial refined sea salt without iodine in deionized water, in desire proportion for salinities of treatments (0, 5, 15, 25 and 35 PSU) and confirmed with a refractometer.

Acclimation and experimental design for megalopae larvae

After hatching, 200 zoea larvae from each of three ovigerous females (distinct those used in the previous experiment), totaling 600 larvae, were removed from the maternal aquarium, following the same procedure from the previous experiment, were

transferred to 2L polyethylene aquariums with 1L of water with salinity 25 PSU. These aquariums, were placed in an incubatior BOD (ELETROLAB 122FC) with constant temperature of 25°C and a photoperiod of 12 hours (light:dark). The salinity 25 PSU was chosen to represent the highest rate of survival for the species until the megalopa stage (Diaz & Bevilacqua 1986). Every day, the water was renewed and the zoea I larvae fed with eggs and newly hatched larvae (nauplius) of *Artemia* sp. (0.6 ind.ml^{-1}).

When the larvae reached the megalopa stage, they were removed and acclimated gradually in higher or lower salinities, in the same way as the previous experiment. After acclimation, 50 megalopae of each female were individually placed in polyethylene containers of 50 ml in five salinity treatments (S0, S5, S15, S25 and S35) and in the same physical conditions of the previous experiment. The feeding and the maintenance of containers followed the protocol used previously. Survival was checked daily under stereoscopic microscope until the first juvenile stage. The megalopae that reached the first juvenile stage were fixed and preserved in ethanol 75%.

Analysis statistics

To evaluate the effect of salinity on the survival rate and duration of development of zoea I stage to megalopae, and from megalopae until the first juvenile stage was used a Poisson Generalized Linear Model (GLM) (Zuur et al. 2009). The rate of larval survival (number of larvae alive), time for metamorphosis and maximum time for mortality were used as response variable and the salinity treatments as orthogonal/random to the response variables. Survival curves were obtained to estimate the probability of survival using Kaplan-Meier test. All analyses were performed on the R environment (R Development Core Team 2016) with the packages lme4 (Bates et al.

2015) and survival (Terry 2015), respectively, and the differences were considered significant when $p > 0.05$.

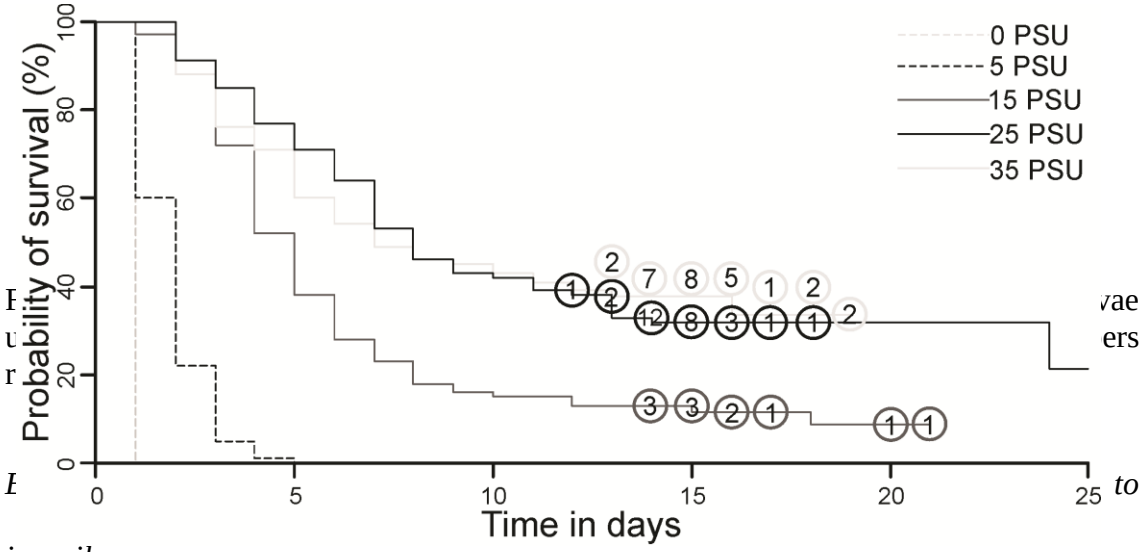
Results

Effect of salinity on the survival and development duration of the zoea larvae

Salinity affected the survival of zoea larvae until the megalopa stage ($p < 0.001$, df = 495). The zoea larvae reached the megalopae stage in the treatments S15, S25 and S35, while in S0 and S5 all larvae died before (Figure 1). The salinity also affect the number of larvae that reach the megalopae ($p < 0.001$, df = 495). The highest rate of metamorphosis were observed for salinities 25 PSU (28%) and 35 PSU (27%) followed by salinity 15 PSU (11%). The development duration of zoea until the megalopae stage in the treatments that the metamorphosis occurred (S15, S25 and S35), was not affected by salinity ($p = 0.08$, df = 76) (

Figure 1). However, the salinity affect the maximum time (in days) for mortality of the larvae ($p < 0.001$, df = 416). In S0, mortality was complete in less than 24 hours, while in S5, the maximum survival time was five days (

Figure).



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The salinity affected the survival of megalopa until the juvenile stage ($p < 0.001$, $df = 140$). The megalopae reached juvenile stage only in S15 and S25. The salinity also affect the number of larvae that reach the megalopae ($p < 0.001$, $df = 145$). The highest rate of metamorphosis was observed for salinities 15 PSU (17%) followed by the salinity 25 PSU (3%) (Figure 2). In S0, S5 and S35 all megalopae died before reaching the juvenile stage (Figure 2). There was a difference in the maximum time (in days) for mortality of the larvae ($p < 0.001$, $df = 140$). In S0, the mortality was complete in less than 24 hours, while for S5 and S35 maximum survival time was respectively 28 and 22 days. However the development duration until juvenile stage, in the treatments that metamorphosis occurred (S15, S25), was not affected by salinity ($p = 0.48$, $df = 4$) (Figure 2).

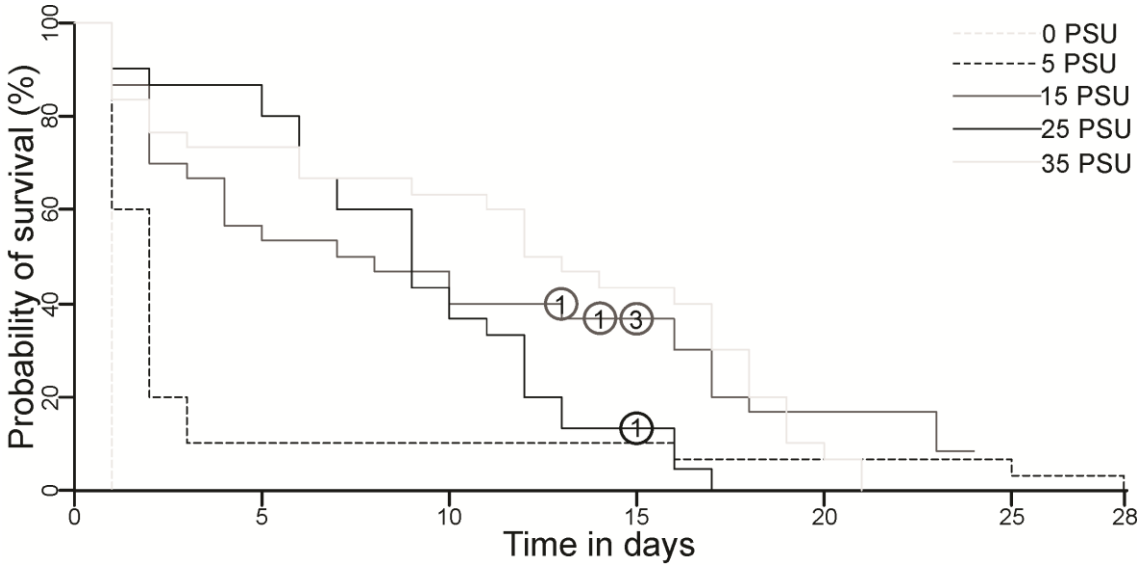

Figure 2. Survival analysis of *Aratus pisonii*. Survival percentage of 30 megalopae until juvenile metamorphosis or death in five salinity treatments. Circles with numbers represent events. Events represent metamorphosis to juvenile stage.

Discussion

The survival and duration of development of zoea I to the first juvenile stage of *A. pisonii* is affected by salinity. Our results suggest a need of zoea I export to oceanic waters, or estuary portions, and the return of megalopae to estuarine regions (with intermediate salinities) for completes the development. This is the first time that the optimum salinity for megalopae settlement is described. Our results also appoint implications for the occurence of adults in regions of not favorables salinities to the settlement.

Salinity also affected larval survival in other Sesarmidae species, as noted for *Armases angustipes* (Dana, 1852) (Anger et al. 1990), *Armases cinereum* (Bosc, 1802) (Costlow et al. 1960), *Armases miersii* (Rathbun, 1897) (Anger 1996), *Armases rubripes* (Rathbun, 1897) (Luppi et al. 2003), *Selatium brockii* (De Mann, 1887) (Kannupandi et al. 2000) and *Sesarma curacaoense* De Man, 1892 (Schuh & Diesel 1995).

Due to the wide variation in salinity in estuaries and other coastal marine environments, it can regulate larval dispersal of crustaceans. This influence can occur on local, regional or continental scales due the capability of larvae be carried by currents (Anger 2001, 2003, Simith et al. 2012). The highest survival percentages of *A. pisonii* zoea larvae observed in treatments with high salinities (S25 = 28% and S35 = 27%) indicating a need for export the larvae to oceanic or estuarine regions with marine influence. These data corroborate the results obtained by Díaz & Bevilacqua (1986) for populations from Venezuela, in which the optimum salinity was 25 PSU, followed by salinity 35 PSU. The need of high saline waters can favor the dispersion at regional and continental level, facilitating gene exchange among populations by increasing the

probability of the larvae be carried to long distances through marine currents, as well as reduce the predation risk that is generally higher in estuaries than oceanic waters (Strathmann 1982, Morgan 1990, 1995). However, as observed for the larvae that reach the megalopae stage in S15, is likely that a small percentage of the larvae perform the entire development within estuarine environments.

On the other hand, the best survival rate of megalopae stage until the first juvenile stage observed in S15 (17%) followed by S25 (3%), indicates the need of return to estuarine habitats, where salinities of intermediate values are favourable to complete development. The total mortality of the megalopae in less than 24 hours in S0 and the lack of metamorphosis in S5 and S35, despite the long survival (up to 21 and 28 days, respectively), confirm this inference. The return of the megalopae to parental environments can occur due the sensory capacity which enables the recognition of physical and biological stimuli that guide the navigation (Kingsford et al. 2002). Despite the small size of the larvae and megalopae of Decapoda, they may swim more than 10 cm s^{-1}, which can be enough to progress against the currents, according to Chiswell & Booth (1999). The combination of mobility and the ability to respond to sensory signals allow the larvae to direct the movement to increase the probability of achieving suitable habitats for settlement (transition from pelagic to benthic habit) (Yednock & Neigel 2011).

The variation of osmoregulatory capacity during the ontogenetic development has been evidenced in many crabs, especially for species with larval export strategy (Anger et al. 1990, Charmantier 1998, Anger & Charmantier 2000, Charmantier et al. 2002, Simith et al. 2012). The long period of survival without megalopae metamorphosis in salinities unfavorable for development (<15 and >25 PSU) that we

observed, indicates a resistance of species that live in environments with large fluctuations of abiotic variables such as estuarine areas. This may favor the displacement to areas with favorable salinity and increasing the probability of finding a suitable substrate for metamorphosis (Jackson & Strathmann 1981, Anger et al. 1990, Simith et al. 2012).

In the Paranaguá estuarine complex, salinity can vary from 0 PSU in the portion more internal up to salinities greater than 25 PSU closer to oceanic waters, with seasonality (rainy summers and dry winters) (Lana 1996, Netto 1996, Marone et al. 2005). Adults of *A. pisonii* can be found in all salinity regimes (near 0 to 35 PSU) in vegetable formations (especially *R. mangle*), as observed in other estuaries along American coast (Díaz & Bevilacque 1986, Leme 2002). Due to the high mortality rates of megalopae and inability to complete the development into juvenile in salinities ≤ 15 PSU and ≥ 25 PSU, we purposed two hypotheses for the wide salinity regime occurance of adults. The first is that the dispersal of juvenile to estuarine regions with low salinities (≤ 15 PSU) occurs after the settlement of the megalopae in regions with favorable salinity (15-25 PSU), whereas juveniles and adults have mobility enough for disperse hundreds of meters. The species is considerate an agile tre-climbing crab with morphological characters that favour a rapid displacement through the trees. The overall body shape (carapace) is conspicuously flattened and the walking legs have relatively long propodi (second most distal segment) and short dactyli (most distal segment) which provides more adherences to the substratum (Vanini et al. 1997, Rader and Reed 2005). Thus, is possible that individuals (especially adults that live in trees canotopy) disperse by walking from tree to tree.

The second hypothesis is that the colonization of areas with low salinities should be performed during events that provide favorable salinity as long periods of drought, when the influence of the sea is more pronounced and provides a higher salinities, as noted during the dry seasons in Paranaguá Bay where the supply of freshwater can be reduced up to 30% and rang of salinity can vary from 0-35 PSU in summer (wet season) and 3-35 PSU in winter (dry season) (Marone et al. 1995, Marone et al. 2005). Strong winds, can also help a rapid dispersion (for several days), as suggested by Luppi et al. (2003) for *A. rubripes* in the Rio De La Plata. Horizontal variations in salinity (salinity variations at different depths) can also help the dispersal as proposed by Acha et al. (2001) for the fish *Micropogonias furnieri* (Desmarest, 1823) and proposed as unlikely hypothesis for the rare occurance of *A. rubripes* in Samboromón Bay (Argentina) from the Uruguayan coast and adjacent areas (Luppi et al. 2003).

Our results demonstrate that the salinity exerts great influence on the different stages of larval development in *A. pisonii*, confirming the larval export strategy of the species. The occurrence of adults in estuarine areas with low (<5‰) or high (>25‰) salinities must occur after the metamorphosis to the juvenile stage by dispersion or at the stage of megalopa during favorable weather events.

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Chapter II

Sexual dimorphism and ontogenetic allometry in *Aratus pisonii* (Crustacea: Brachyura)

Abstract

The crab *Aratus pisonii* presents sexual dimorphism on chelipeds and abdomen. It is useful model to infer about sexual selection, evaluating the shape variation between sexes and life stages, due the presence of structures that allows precise placement of landmarks. We used ten bidimensional anatomical landmarks on the carapace of adult and juveniles of both sexes and nine landmarks in cheliped propodus of adults, to describe the sexual dimorphism and ontogenetic allometry. Size variation was analyzed through a T test, while shape variation between sexes through Discriminant Function Analysis and shape variation between life stages through a multivariate regression of symmetric components and size, using the configurations aligned by Procrustes fit. There was no sexual dimorphism on adult carapace size, but there was in the cheliped propodus of adults and carapace of juveniles. On the other hand, carapace shape differed between sexes in juveniles and adults, but not for cheliped propodus. Females showed a larger posterior region of carapace. In both genders, the growth generates a comprehension of the anterior region and an elongation of the posterior region of carapace, with stronger allometric influence in males. Our results bring new information about the shape development between genders and life stages.

Key-words: Geometric morphometry, morphological variation, Sesarmidae, shape variation, sexual selection.

Introduction

Sexual dimorphism and allometry are two sources of phenotypic variation and are widely known in the animal kingdom (Shine 1989, Weckerly 1998). Sexual dimorphism can be defined as morphological differentiation of adults, and can display distinct evolutionary trends in each taxa. For example, in some animal groups males have larger body sizes than females (mammals, birds) while in other groups females are larger than males (spiders, insects, fishes). Distinct hypothesis are mentioned to explain the patterns of sexual dimorphism, such as natural selection on reproductive trait, niche divergence or other ecological causes (Abouheif & Fairbairn 1997, Fairbairn 1997). Hypotheses regarding the selective processes have the common property that the pattern (relative dimorphism of different traits), direction (which sex is larger), and magnitude of sexual dimorphism can be predicted from components of reproductive success. On the other hand the niche divergence or ecological sexual dimorphism hypothesis, proposes that sexual dimorphism evolves to reduce intraspecific competition for food and is not associated directly with selection on reproductive traits (Fairbairn 1997).

The differences between sexes can also be influenced by the growth rate during the development and the level of allometry. In a general view, allometry can be defined as differences in proportions correlated with changes in the absolute magnitude of the total organism (Fairbairn 1997, Klingenberg 1998). There is three allometric frameworks. The ontogenetic allometry (the association between size and shape across different age stages) frequently is used as an estimate of a population´s ontogenetic trajectory. Static allometry (the association between size and shape within the same species and developmental stage) is often used to explain the coevolution of size and shape, and evolutionary allometry (the covariation between size and shape among

species) as a model for function and behavioral adaptations (Cock 1966, Klingenberg 1996).

Studies on the ontogenetic allometry and sexual dimorphism in Brachyura are very common, especially those using relative growth to determine when the transition from juvenile to adult occur and also, to describe the differences between males and females (Hartnoll 1978, Hartnoll 2001, Marochi et al. 2013). However, these studies are related to linear measurements, which have both size and shape components. To test if shape changes during development and how it scales to size on needs a geometric approach. The geometric morphometric (GM) approach is based on the use of anatomical landmarks to describe shape-related variations between species, populations, sexes or ontogenetic (Rosenberg 2002, Adams et al. 2004, Trevisan et al. 2014). Crustaceans are particularly suitable to GM studies because exoskeleton facilitates the use of anatomical landmarks (Rosenberg 1997, Clark et al. 2001, Trevisan et al. 2012). Geometric morphometrics have improved the resolution of comparisons between the sexes and other aspects, by identifying minimal morphological differences in body structures where traditional morphometrics were not capable of doing so (Abelló et al. 1990, Rufino et al. 2004, Barría et al. 2011).

Aratus pisonii (H. Milne Edwards, 1873) is a Sesarmidae crab that lives in estuarine areas in the aerial structures (stems, branches and roots) of mangrove trees, especially in *Rhizophora mangle* (Warner 1967, Diaz & Conde 1988). The species has a wide distribution, from Florida (U.S.A.) and most of Antilles to Santa Catarina (Brazil) (Thiercelin & Schubart 2014). *A. pisonii* has a sexual dimorphism in size with males larger than females. The species also show variation on the behavior between juveniles and adults, with juveniles occupying lower arboreal status and adults all status (Díaz &

Conde 1989, Chiussi 2002). The sexual morphological maturity is reached with 10.47 mm of carapace width for males and 11.52 mm for females (Pescinelli et al. 2015).

Although the sexual size dimorphism has been previously described for *A. pisonii* only adults were studied. To understand how sexual dimorphism is generated requires understand the whole development because dimorphism can be produced by different developmental strategies of males and females or by differences in adult energy allocation. This kind of feature can clarify the evolutionary trait for the species and lineage. In this sense, our aims were: 1) evaluate the sexual dimorphism in shape and size of the carapace and cheliped propodus of adults, 2) evaluate the sexual dimorphism in shape and size of the carapace of juveniles and 3) evaluate the allometric ontogenetic trajectory between and within sexes of *A. pisonii*.

Material and Methods

Sampling of A. pisonii and acquisition of morphometric data

We sampled 77 females (31 juveniles and 46 adults) and 70 males (33 juveniles and 37 adults) of one population of *A. pisonii* located in Paranaguá Bay, Paranaguá, Brazil (25°30’58’’S, 48°29’58’’W). Samples were made manually and the individuals were fixed in 75% ethanol. Only intact carapaces and cheliped propodus were included on the morphometric analyses.

For the sexual dimorphism of adults we used 46 females and 37 males for the analyses of carapace, 35 females and 25 males for the rigth cheliped propodus and 32 females and 25 males for the left cheliped propodus. The differences in the number of cheliped propodus analysed and carapaces are related to the loss of structure during the life or during the preparation for morphometric geometric analyses. For the sexual dimorphism analyses of juveniles we used the carapace of 31 females and 33 males. The

cheliped propodus were not analysed because the cheliped of juveniles did not have the same landmarks as adults. All animals were used in the carapace allometry analyses (70 males and 77 females). Were considerate juveniles individuals with less than 10 mm of carapace width for both sexes (Pescinelli et al. 2015).

Pictures from the structures were obtained in dorsal view with aid of a Fujifilm Finepix HS10 camera with a resolution of 10 megapixels, from the same distance and zoom. We established 10 anatomical bidimensional landmarks on the carapace and nine on the left and right cheliped propodus (Fig. 1). The landmarks were digitalized three times for the same person in different days to avoid landmark position errors, using the TPS Dig2 software, version 2.16 (Rohlf 2010).

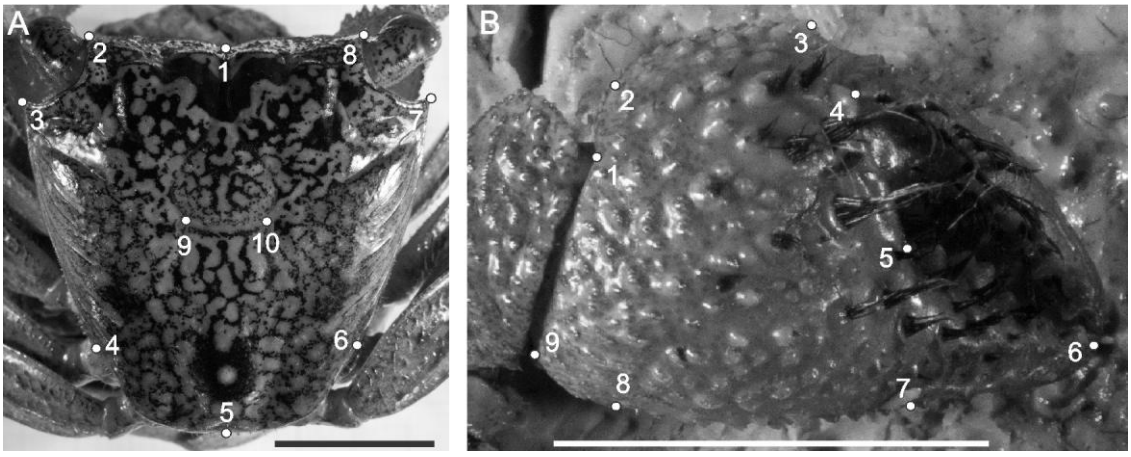

Figure 1. *Aratus pisonii*. Position of anatomical landmarks on the carapace (A) and cheliped propodus (B). Scale 10 mm. (A) 1: Extreme of protogastric region; 2 and 8: End of antero-lateral line; 3 and 7: Tip of antero-lateral tooth; 4 and 6: Beginning of the lateral margin; 5: Posterior margin of intestinal region; 9 and 10: Extremes of cardiac line. (B) 1: Inner base of the articulation carpo-propodus; 2: Proximal tip of the cheliped propodus; 3: Distal tip of the cheliped propodus; 4: Suture in the intersection between "pré-dactilar" lobe and the base of cheliped propodus; 5: Base of the fixed finger of the cheliped propodus; 6: Tip of the fixed finger; 7: limit of fixed finger in the margin of cheliped propodus; 8: Vertical line through the tip of the cheliped propodus; 9: Outer base of the articulation carpo-propodus.

After the digitalization a Procrustes Generalized Analyses (GPA) was performed (Adams et al. 2004). The GPA consists in overlapping the configuration through the centroid (the mass center of the configuration), escalating the centroid size of each configuration to the value of one and, rotate the configurations, resulting in landmarks adjusted by the least square distance possible (Monteiro & Reis 1999).

As the carapace has right and left side separated by a central axis (symmetrical object), the shape components (GPA aligned coordinates) can be separated in symmetrical and asymmetrical components (Klingenberg et al. 2002). The symmetrical components represent shape variation amog individuals, thereby, to analyse the carapace shape, only the symmetrical components were used. The size of each structure was estimated through the centroid size, which is calculated as the square root of the sum of the square distances of a group of points to that centroid (Monteiro & Reis 1999). Morphometric analyses were performed in the MorphoJ 1.06d software (Klingenberg 2011).

Sexual dimorphism in size (SSD) and shape (SShD) of adults and juveniles

The SSD of carapace and cheliped propodus of adults and carapace of juveniles of *A. pisonii* was analyzed between sexes through Student T test using the centroid size (log standardized). The analyses were performed in the R environment (R Development Core Team 2011).

The SShD on the carapace and cheliped propodus of adults and carapace of juveniles was analyzed through Discriminant Function Analysis (DFA) using the Procrustes shape coordinates (Viscosi & Cardini 2011). The correct percentage of classification was determined by cross-validation and the difference between the

Procrustec distance means of groups was test by permutation (Klingenberg 2011). When SSD was detected, a multivariate regression of symmetric components of Procrustes coordinates (carapace) or Procrustes coordinates (cheliped propodus) on Log-transformed centroid size was made and the regression residuals used in ShSD analyzes (Klingenberg 2016). This procedure performs a size correction of shape data. The analysis was performed in the MorphoJ (Klingenberg 2011).

Ontogenetic Allometry

The shape variation during the development (ontogenetic allometry) was described through a multivariate regression of symmetric components of Procrustes coordinates regarding the Log-transformed centroid size separately in each sex.

The significant level of the multivariate regression was evaluated through permutation test considering the null hypothesis of independency between variables. To avoid the interference of static allometry, a multivariate regression of symmetric components of shape variables on Log-transformed centroid size was made separately for juveniles and adults of both sexes (Monteiro & Reis 1999, Klingenberg 2016). To test the allometric trajectories of males and females, the angle between the regression vectors were compared between sexes. The allometry was tested in both life stages (juvenile and adult) within each sex and between sexes.

Results

Sexual size dimorphism (SDD)

There was no dimorphism in carapace size (centroid size) adults. However, the size of both cheliped differed between adults (larger in males) (Table 1). For juveniles

there are significant differences in carapace size (centroid size) between sexes, with females larger than males (Table 1).

Table 1 – T test results comparing body parts size (centroid size) of females and males of *Aratus pisonii*.

Life stages	Body part	Females size (mean ± SD)	Males size (mean ± SD)	DF	t
Adults	Carapace	38.6 ± 4.9	37.9 ± 4.8	81	0.646
	Right cheliped	7.5 ± 0.1	11 ± 0.2	59	-9.412**
	Left cheliped	7.4 ± 0.1	10.8 ± 0.2	40	-8.588**
Juveniles	Carapace	1.10 ± 0.26	0.93 ± 0.24	62	2.580*

*P<0.05, **P<0.001.

Sexual shape dimorphism (SShD)

The carapace shape of adults differed between sexes (Procrustes distance = 0.036, p <0.001) with a correct classification of 96.3%. The diference occurs mainly on landmarks 4 and 6, and 5, at the posterior margin of the carapace. Females showed a wider posterior margin than males, and a variation on the hepatic region (landmarks 9 and 10). Males showed broader antero-lateral margin (landmarks 3 and 7) than females (Fig. 2A).

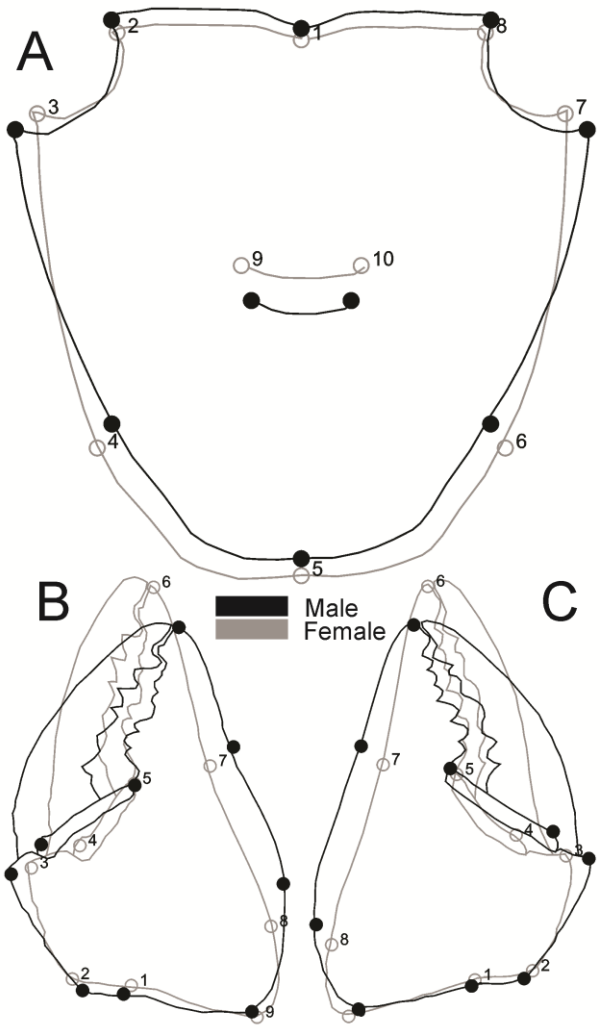

Figure 2. *Aratus pisonii*. Sexual dimorphism in adults, on the shape of carapace (A), right (B) and left (C) cheliped propodus. Mangification: A - 3 times, B and C - 2 times.

The shape of the propodus of chelipeds also differs between the sexes, both in the right propodus (Procrustes distance = 0.026, p <0.01), with a correct classification of 92.3%, as in the left propodus (Procrustes distance = 0.044, p <0.001), with a correct classification of 76.9%. The cheliped propodus of both right and left sides showed to be more robust in males (wider in landmarks 8, 7, 3 and 4) and long/thin in females (narrow in landmarks 8, 7, 3 and 4). On the other hand the shape of both chelipeds did not differed between sexes after the size correction (Fig. 2B, C) (right cheliped

propodus: Procrustes distance = 0.018, p = 0.21; left cheliped propodus: Procrustes distance = 0.024, p = 0.22). This shows that the difference of male and female chelipeds shape are derived from allometry.

The carapace shape of juveniles also differed between sexes (Procrustes distance = 0.018, P = 0.03), with a correct percentage of reclassification of 80%. The variation on shape is mild and occurs on landmarks 4 and 6, and 5, at the posterior margin of the carapace. Females showed a wider posterior margin than males (Fig. 3). Even after the size correction the ShSD of juvenile carapace showed the same dimorphism pattern (Procrustes distance = 0.018, p = 0.01).

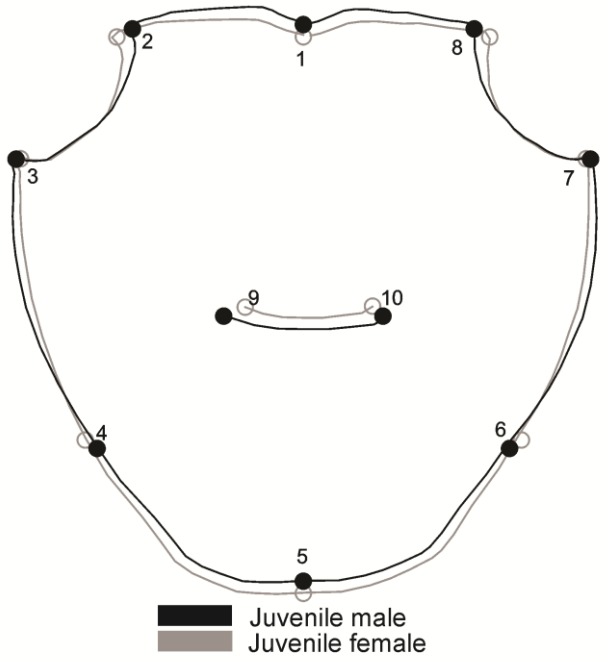

Figure 3. *Aratus pisonii*. Sexual dimorphism on the shape of carapace of juveniles. Mangification: 3 times.

Ontogenetic Allometry

The ontogenetic allometry occurs in the shape of the carapace of females ($p < 0.001$) and males ($p < 0.001$). The size is responsible for 61% of the shape variation in

females and 62% in males. In both sexes an increase in size, produces a comprehension of the anterior region and an elongation of the posterior region (Fig. 4 and 5).

The angle between the regression vectors of the ontogenetic trajectory between males and females was 23° and differ significantly from the expected for the pairs of random vectors ($p < 0.001$), indicating shape variation in both sexes during ontogeny.

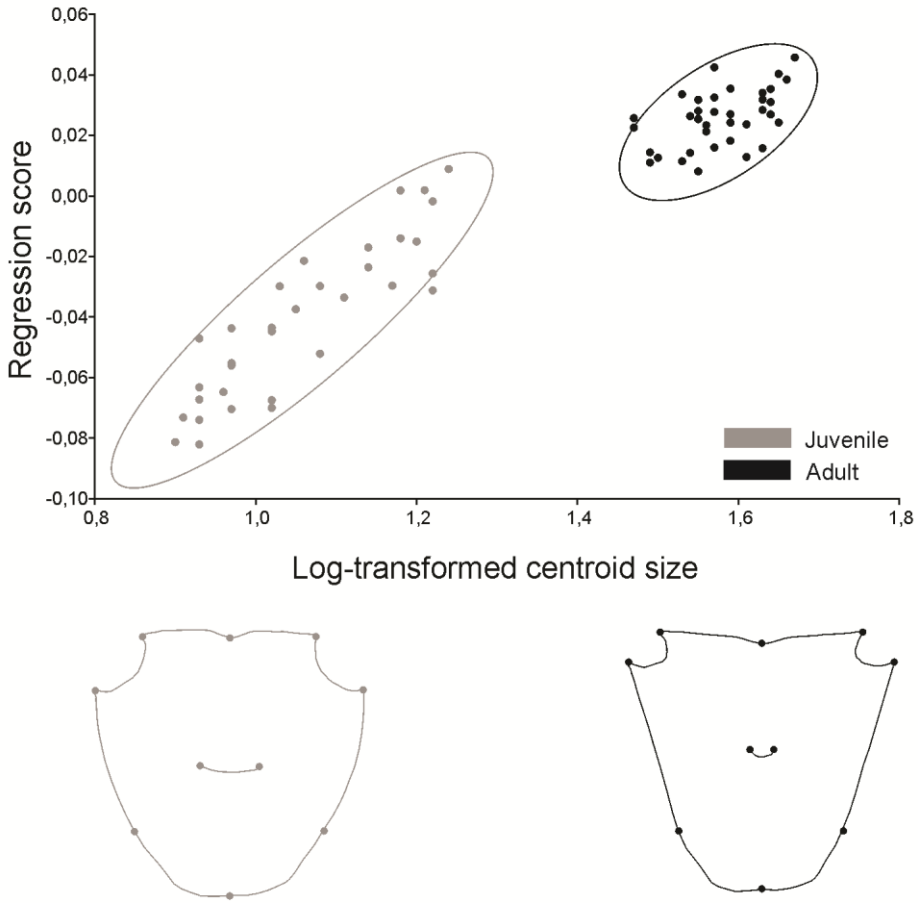

Figure 4. *Aratus pisonii*. Ontogenetic allometry of carapace shape, based on multivariate regression of symmetrical components on log-transformed centroid size. The two drawings show the shapes expected for changes by 0.8 and 1.8 units of log-transformed centroid size from the mean shape (the extremes at the left and right of the plot) in males.

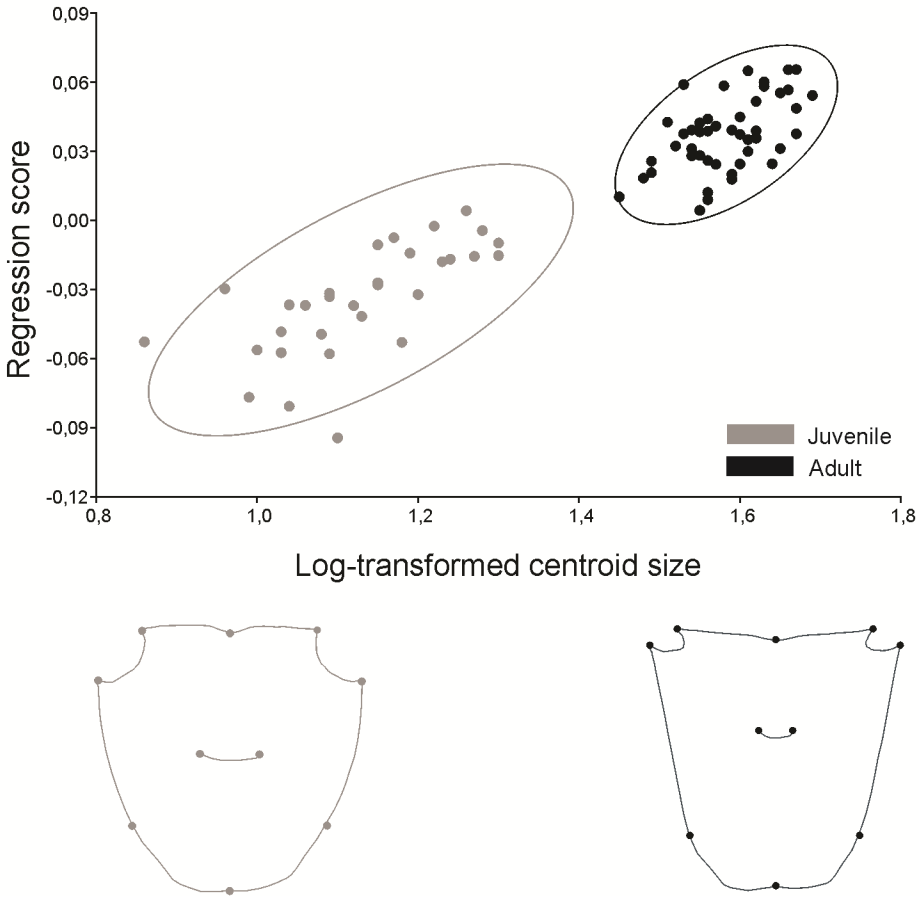

Figure 5. *Aratus pisonii*. Ontogenetic allometry of carapace shape, based on multivariate regression of symmetrical components on log-transformed centroid size. The two drawings show the shapes expected for changes by 0.8 and 1.8 units of log-transformed centroid size from the mean shape (the extremes at the left and right of the plot) in females.

In males, stactic allometry occurs in both life stages, juvenile ($p < 0.001$) and adult ($p < 0.001$). The size was responsible by 42% e 17% of shape variation in each life stages, respectively. The angle between the regression vectors of males juvenile and adults was 29° and differ significantly from the expected for the pairs of random vectors ($p < 0.001$) (Fig. 4). For females, the allometry also occurs in both juvenile ($p < 0.001$) and adult ($p < 0.001$) stages. The size is responsible for 21% and 11% of the shape variation, respectively. The angle between the regression vectors of juveniles and adults of females was 33° and differ significantly from the expected for the pairs of random vectors ($p < 0.001$) (Fig. 5). This result indicates that the allometric trajectory is similar

in juveniles and adults in both sexes, in other words, the allometric effect of size on carapace shape follow the same trajectory throughout life in males and females. On the other hand, the carapace shape differs between the life stages in both sexes (Female: Procrustes distance = 0.072, $p < 0.001$; male: Procrustes distance = 0,066, $p < 0.001$), The difference in shape between juveniles and adults is related to the allometric effect and the size correction eliminates this effect of shape variation between life stages in females (Procrustes distance = 0.0024, $p = 0.98$) and males (Procrustes distance = 0.0068, P =0.36).

The angle between the regression vectors of juvenile males and juvenile females is 19° ($p < 0.001$) while between adult females and males was 36° ($p < 0.001$), indicating that males and females have a similar static allometry during juvenile phase and it is less expressive during the adult phase.

Discussion

Sexual dimorphism

The size variation found in *A. pisonii* is a common feature in Brachyura, as a consequence of the different sexual strategies for the group, since the energy to growth has different uses in males and females (Hartnoll 1974, Adams et al. 1985). The fact of males and females have similar sizes (with females somewhat larger than males, in both juvenile and adult phases) contradicts the expected for brachyuran crabs (Hartnoll 1974). Larger females can carry/generate more eggs, and some studies have demonstrated a positive correlation between egg number and female size (Hines 1982). In this sense, populations where females have similar body size to males can generate more larvae than populations with females smaller than males. For an taxa with an R

reproductive strategy, as Brachyura, the increment in number of individuals generate per broods can increase the probability of reproductive success. The absence of SSD in mean carapace width of adults was also observed for some populations of São Paulo (Conde et al. 2000, Leme 2002), but the presence of SSD was registered for other populations of São Paulo, Rio de Janeiro and Venezuela (Díaz & Conde 1989, Nicolau & Oshiro 2007, Leme et al. 2014). This size similarity between sexes can also be an advantage to avoid intraspecific competition between genders for resources or predation between them.

The sexual dimorphism in carapace shape in both juvenile and adults phase brings new information about the dimorphism during development. Both adult and juvenile females showed the posterior region of carapace wider than males. This increasing in posterior region of female´s carapace concomitantly with the increase of the abdomen can be explained by the necessity of space increase to accommodate and protect the egg mass during incubation, as well as protect the gonophores (Hartnoll 1974). This assumption was also observed using geometric morphometrics for the Anomura *Aegla marginata* Bond-Buckup and Buckup, 1994 (Trevisan et al. 2012). This sexual pattern (wider posterior region in females) was also documented for insects and lizards (Anholt 1991, Braña 1996), and in our species females can exhibit a more peripheral distribution of fat in early adulthood (Wells 2007).

On the other hand, the presence of sexual dimorphism in size and absence in shape of cheliped propodus, after size correction, indicates that the cheliped more robust (larger and wider) in males is a consequence of increasing size of *A. pisonii*, because when only shape components are compare, there is no sexual differences. The larger cheliped propodus in males confirm the pattern for the species, which is characterized

by a positive allometric growth in males and an isometric or negative allometric growth in females, for this structure (Pescinelli et al. 2015). Distinct authors have suggest that this size variation can provide advantages during direct male-male competition and the sexual selection acted on male traits to increase their efficacy as functional weapons (e.g., the closing force of claws; Lailvaux et al. 2009). In distinct taxa, larger weapon-like male traits are favored during male fighting just like female mate choices (Judge & Bonanno 2008, Milner et al. 2010, Callander et al. 2013). The probability of success during fighting is a strong sexual selection because, usually, win a fight provide direct benefits in the form of shelter, feeding territory, mating sites and quantity of mates (Smith & Miller 1973, Backwell & Passmore 1996, Koga et al. 2001). This size variation can also provide advantage in inter and intraspecific interactions like defense against predators and distinct process related to reproduction and communication (Warner 1970, Leme et al. 2014).

In some families of crabs, like Sesarmidae and Grapsidae, there is a competition for females (ritual agonistic behavior) in which, the chelipeds are usually used (Warner 1970). *Aratus pisonii* exhibit territorial and agonistic (fight for females) behavior with a "display" performance composed by vibration, lifting and lateral positioning of the cheliped to intimidate the opponent (for more details see Warner 1970). Differentiation of reproductive roles leads directly to morphological differentiation of organs and structures associated with mating success, and such selection is frequently associated with hyperallometric growth of organs and structures associated with combat or display in males (Fairbairn 1997). Larger chelipeds can promote success in male-male combat (Mariappan et al. 2000) and likely are an evolutionary sexual tendency, presumably increasing opportunities for mating.

The sexual dimorphism traits (wider posterior region in females and larger cheliped propodus in males) represent the specialization results of reproductive roles for the breeding success, hence, the evolutive success of sexual selection. The sexual dimorphism traits can also be result of ecological sexual dimorphism (intersexual niche divergence) (Punzalan & Hosken, 2010). This hypothesis assume that the sexual dimorphism evolve to reduce intraspecific competition for food and is not associated with reproductive selection. But empirical tests of this hypothesis, with different taxa, showed that even when the conclusions are positive related, it is difficult to exclude the hypothesis that trophic dimorphism evolved as a consequence of pre-existing sexual dimorphism (for more details see Fairbain 1997).

Although the sexual dimorphism in carapace shape is present in the juvenile phase, the level of differentiation between sexes was higher in adults (Procrustes distance: juvenile - 0.018, adults - 0.036). Therefore, juveniles have morphological characters more similar between sexes than adults, indicating that, during juvenile phase, males and females have similar growth rate in all body structures and, after puberty moult (Hartnoll 1974), those structures related with the respective reproductive activities show variation in this rate, culminating in a marked morphological differentiation among adults.

Ontogenetic Allometry

Males and females of *A. pisonii* have ontogenetic allometry on the carapace. In both genders, the growth (from juvenile to adult phase) generates a comprehension of the anterior region and an elongation of the posterior region. The same ontogenetic shape variation is observed for *Armases rubripes* (Rathbun 1897) (unpublished data),

and this variation seems to be a pattern for Sesarmidae. In both species, during the first juvenile stages, the orbital region occupies a proportionally larger area in carapace, to accommodate the eyestalk, than in adults. In the first juvenile stages in Brachyura, the eyestalk and the eye are propocionaly larger than in adults, and this proportion decreases progressively during the development (Guerao et al. 2004, Guerao et al, 2007).

The ontogenetic differentiation mentioned above, can be caused for distinct factors during the ontogenesis related with the development, as well as can represent an adaptation to occupy distinct habitats during the juvenile and adult phase, respectively. Although adults and juveniles of *A. pisonii* occupy the same biome of mangroves a spatial distribution was observed between these two demographic categories: adults occur in abundance in upper arboreal strata (branches and upper trunk), while juveniles are frequently observe in bigger abundance in trunks and exposed roots (personal observation). However, it is unlikely that this spatial distribution, even indirectly, influence the differential proportion of the orbital region during the ontogeny.

Males and females of *A. pisonii* have similar ontogenetic morphological trajectories of the carapace with 23° of angular relation ($p = 0,001$). However, the size of carapace influences the shape in males and females in a different way. The males shape are influenced more by size variation (juveniles = 42% and adults = 17%) than females (juveniles = 21% and adults = 11%). This indicates that, during the ontogeny, the size increase of carapace and structures directly related to it, influence more the shape of males than females. This fact can be related with the sexual selection for the species, where males who reach the size and shape required to display cohort behavior

earlier may have advantages in competition for females, as well as during fights against other males.

The smaller angle of comparison observed in the ontogenetic trajectory of juvenile males and females (19°) than adults (36°), refers to similar ontogenetic trajectories during the immature stages, follow by more divergent trajectories during adulthood. In Decapoda species with indeterminate growth (for more details see Hartnoll 1982), the moulting frequency is higher during the early juvenile stages and the percentage of moult increment decline and the intermoult periods increase with the ageing (Vogt 2012). These results added with the data of minor shape variation amog juveniles than adults, reinforce the hypothesis of similar morphologies during juveniles stages, and after puberal moult, there is an intensification of morphological diversification between sexes influenced by size increase.

Conclusions

Our results bring new information about the development of secondary sexual characters on the carapace and cheliped propodus shape in males and females of *Aratus pisonii*. This observation makes it possible to infer that variations the on carapace shape and cheliped propodus reflect the evolutionary sexual trend for lineage. Although juveniles and adults of both sexes showed variation on the carapace, this variation is related to size increase with a stronger allometry influence in males than females. There is a pattern of carapace variation shape between juveniles and adults in both sexes. With the aging there was a comprehension of the anterior region and an elongation of the posterior region of carapace. Further studies on the static allometry and evolucionary allometry can provide usefull information about the sexual selection of the lineage.

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Chapter III

Relative growth, ontogenetic allometry and sexual dimorphism of *Armases rubripes* (Rathbun, 1897) (Decapoda: Sesarmidae)

Abstract

Growth and reproduction are antagonistic processes and constitute a trade-off during development. The stepped growth of crustaceans combined with the calcified exoskeleton facilitates morphometric comparison between sexes and life phases (juvenile and adult). To understand the energy use during the development, we infer the onset of morphological sexual maturity through relative growth analysis, evaluate the sexual dimorphism in shape and size of the carapace and cheliped propodus of juveniles and adults, and evaluate the allometry ontogenetic of the semi-terrestrial crab *Armases rubripes*. Crabs (247 males and 253 females) were obtained in the Guaratuba Bay (25°51'29.82"S - 48°44'0.14"O), Paraná, Brazil. To estimate the onset of sexual maturity the carapace width (CW) and length (CL), left and right cheliped propodus length (RPL and LPL) and height (RPH and LPH) for all individuals and abdomen width in females were recorded. Ten anatomical landmarks on the carapace of adult and juveniles of both sexes and eigth landmarks in cheliped propodus of adults were used to describe the sexual dimorphism and ontogenetic allometry. Size variation was analyzed through a T test, while shape variation between sexes through Discriminant Function Analysis and shape variation between life stages through a multivariate regression of symmetric components and size, using the configurations aligned by Procrustes fit. The relationships that better indicate the onset of sexual maturity were RPL, LPL and LPH for males (inflection point 7.17 mm CW), and AW for females (inflection point 5.52 mm CW). There was sexual dimorphism on carapace size and shape of adults, but there was not on the carapace of juveniles. The cheliped size varied between adult males and females but did not in shape. Females showed a larger posterior region of carapace. In both genders, the growth generates a comprehension of the anterior region and an elongation of the posterior region of carapace. Our results bring new information about the shape development and the energy use in different life stages of each gender.

Key-words: Geometric morphometry, morphological variation, ontogeny, sexual shape dimorphism, sexual selection.

Introduction

Growth can be defined as a measurable increase in weight or length of an organism which is result of the balance between the processes of anabolism and catabolism that occur in all animal taxa (Bertalanffy 1938, Hartnoll 1982). In crustaceans the growth is asymptotic because of the molt increment and growth seasonality (Pinheiro et al. 2005). Distinct factor such as hormones, temperature, food availability and intra and interspecific competition can affect the growth rate. The hormonal control of moulting is the main aspect that regulates growth in Crustacea. It is important to determining when a moult will occur, to control the onset of the terminal ecdysis and also, determining when the puberty moult will be initiated (Hartnoll 1982, 2001).

In the majority of crustaceans, the growth (from first juvenile stage to adult) initially involves a series of immature instars of similar morphology until the moult in which they became mature. This moult is characterized by a higher morphological change and has been termed as "puberty moult" (Perez 1928, Hartnoll 2001). The puberty moult may, or may not, be also the terminal moult (for more details see Hartnoll 1985).

Size regulation in crustaceans still not fully understood, but is performed by two major hormone categories: Moult inhibiting hormone (MIH) and moult hormones known as "crustecdysone", "β-ecdysone", ecdysterones, ecdysteroids or 20-hidroxyecdisone (Hampshire & Horn 1966, Hartnoll 2001, Hopkings 2009). While the metamorphosis, that occur from zoea stage until the first juvenile stage, with remarkable shape changes is probably regulated by juvenile hormones (JH) and ecdysteroids. The JH and ecdysteroids act regulating metamorphosis and gametogenesis in insects, but the

effective regulation of these hormones in crustaceans is not well defined and may be distinct from regulation in insects (Laufer et al. 1987, Hopkings 2009).

Growth and reproduction are antagonistic processes and constitute a trade-off during development (Roff 2000, Koene & Maat 2004). In crustaceans the demand of energy for reproduction can for example interrupt the necessary moult sequence for somatic growth or direct the energy of growth to certain body structures. This is an event that occurs in females and males differently, resulting in sexual dimorphism (Hartnoll 1985).

The stepped growth of crustaceans combined with the calcified exoskeleton facilitates the morphometric comparison between sexes and life stages (juvenile and adult). The morphometric data can be used to identify sexual dimorphism, ontogenetic allometry and to estimate the onset of the maturity size (Dalabona et al. 2005). Information regarding the size of maturity, sexual dimorphism and the ontogentic allometry can be useful to understand the energy use during the development, reproductive process and sexual selection (Stearns & Koella 1986, Dalabona et al. 2005). In this sense, our aims were: 1) infer the onset of morphological sexual maturity through relative growth analysis, 2) evaluate the sexual dimorphism in shape and size of the carapace and cheliped propodus of juveniles and adults using geometric morphometrics techniques, and 3) evaluate the allometry ontogenetic trajectory between and within sexes of the semi-terrestrial crab *Armases rubripes* (Rathbun, 1897).

Material and Methods

Sampling of Armases rubripes

Armases rubripes (Rathbun, 1897) is a sesarmid crab that occurs from Nicaragua to Argentina in tropical and sub-tropical estuaries. The species can be found in estuarine marginal vegetation (*Spartina sp.* and *Scirpus californicus*) as well as in morgrove forest between roots and branches, direct in the mud substrate (or in bromeliads (Capítoli et al. 1977, Fischer et al. 1997, Lima et al. 2006). The species is also characterized by a continuos growth after puberty moult (personal observation). Individuals of *A. rubripes* were sampled from Guaratuba Bay, Paraná, Brazil (25°51'29.82"S - 48°44'0.14"O) at March 2010 and February 2013. Samples were collected by hand and individuals were preserved in 75% ethanol.

Relative growth: laboratory procedure and statistical analysis

Linear measurements were performed in 247 males, 186 females and 67 ovigerous females, using a digital caliper (0.01 mm precision). Measurements included: carapace width (CW) and length (CL), right cheliped propodus length (RPL) and height (RPH), left cheliped propodus length (LPL) and height (LPH) for both sexes, and the abdomen width (AW) at the basis of the 4th somite only for females (Fig. 1). The classification of right and left cheliped was used due the homochely for the species (chelipeds with similar size and shape). The body dimensions choice was based on Hatnoll (1974).

Changes in the proportion of body structures regarding the independent variable (CW) were tested using the allometric equation $y = ax^b$. This equation was linearized ($log\ y = log\ a + b\ log\ x$), where x represent the independent variable (CW), y the dependent variables (all other body measurements) (Huxley 1950).

The allometric coefficient (*b*) was tested with a Student *t* test, against the null hypothesis of isometry (b = 1). The slope of the lines and the intersections between the lines for juveniles and adults were tested with the aid of an analysis of covariance (ANCOVA), using a 95% confidence interval (Sokal & Rohlf 1979). To adjust the lines the software REGRANS (Pezzuto 1993) was used, while the other analysis were performed in the software BioEstat 5.0 (Ayres et al. 2007).

Sexual dimorphism: laboratory procedures, geometric morphometric procedures and statistical analysis

To evaluate the sexual dimorphism all individuals were used (adults: 194 males and 228 females) (juveniles: 53 males and 25 females). Pictures from the carapace in dorsal view of all individuals and from the right and left cheliped propodus were obtained with a Fujifilm Finepix HS10 camera with a resolution of 10 megapixels. Ten anatomical bidimensional landmarks and semi-landmarks on the carapace and eight of the right and left cheliped propodus were established using the TPS Dig 2 software, version 2.16 (Rohlf 2010). For juveniles the same landmarks were used, except those regarding the cardiac line (9 and 10) due the difficulties of visualized in juveniles. Only individuals with appendices and carapace intact and without sign of regeneration were used.

For differentiation of juveniles and adults of both sexes, was considered the average size of the onset of sexual maturity obtained in the relative growth analysis of this study (males: 7.17 mm CW and females: 5.52 mm CW, see results figure 3).

From the initial configurations, a generalized Procrustes analysis (GPA) was performed. This analysis consists in overlapping the configurations through the centroid (the mass centre of the configuration), escalating the centroid size of each configuration

to the value of ‘one’ and, lastly, rotating the configurations so that corresponding landmarks are adjusted by the least square distance possible (Klingenberg & Monteiro 2005). The GPA overlapping removes the effect of position, orientation and size of the configurations of anatomical landmarks, and the aligned configurations now correspond to the shape of the structures only (Adams et al. 2004). As the carapace is a symmetrical object, the shape components can be separated in symmetrical and asymmetrical components (Klingenberg et al. 2002). For the analysis of shape variation, only the symmetrical components of the carapace were used. The size of each structure was estimated through the centroid size (square root of the sum of the square distances of a group of points to that centroid) (Monteiro & Reis 1999). The size sexual dimorphism was evaluated with Student´s t test using the centroid size. The analyses were performed in R environment (R Development Core Team 2011).

When sexual size dimorphism was detected, a multivariate regression of symmetric components on Log-transformed centroid size was made and the regression residuals were used in the analyzes of shape sexual dimorphism (Monteiro & Reis 1999, Klingenberg 2016). This procedure performs a size correction of shape data. The shape sexual dimorphism was analyzed using GPA residuals, after size correction, with a a discriminant analysis with permutation test. The analyses were performed in MorphoJ 1.06d (Klingenberg 2011).

Ontogeny: laboratory procedures and statistical analysis

All individuals were used (males: 194 adults and 53 juveniles; females 228 adults and 25 juveniles). After the GPA, as described in the analysis of sexual dimorphism, the variation in shape during the ontogenetic development of each gender

was evaluated through a multivariate analysis of symmetrical components of Procrustes coordinates and the logarithmized centroid size. A multivariate regression in this case was used to test if there is influence of size in shape, and if positive, to categorize the allometry (Klindenberg 2016).

The significance of the multivariate regression was tested by permutation. To test whether the allometric trajectories of males and females differ, the angles between the regression vectors were compared between sexes. For allometric comparisons, the same semi-landmarks and landmarks established for the sexual dimorphism of juveniles were used.

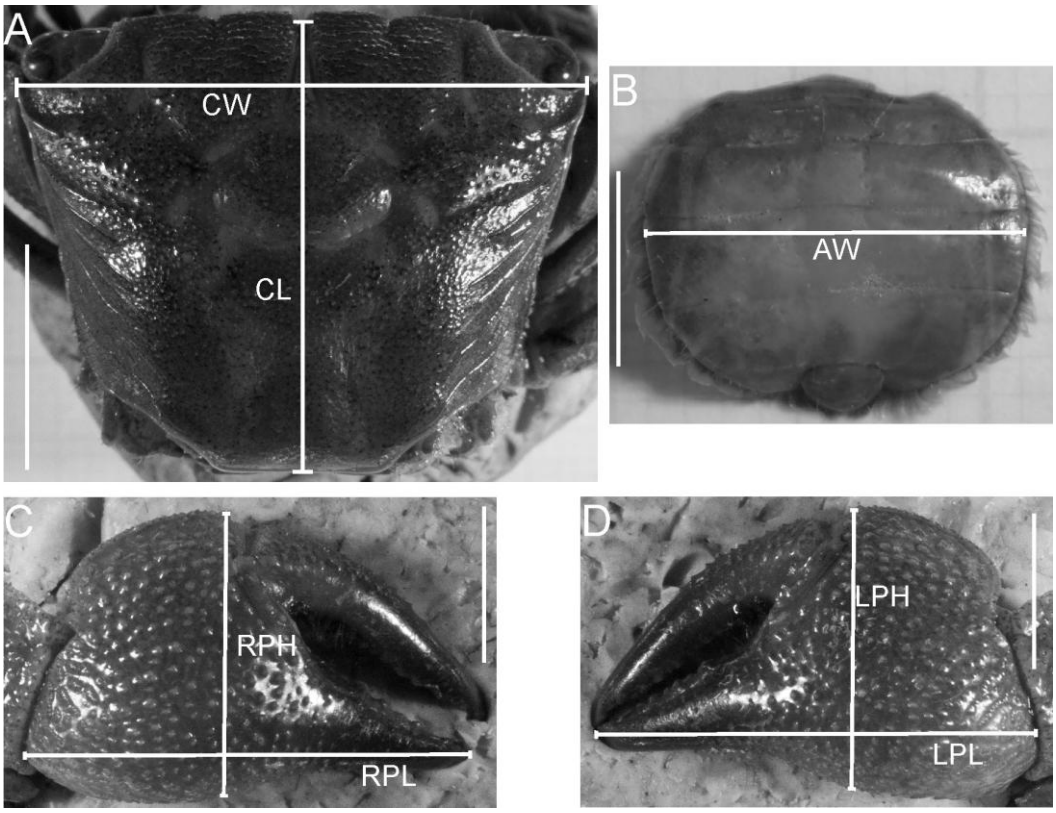

Figure 1. *Armases rubripes*. Measured body dimensions for relative growth analysis. CW: carapace width, CL: caparace length, RPL: right cheliped propodus length, RPH: right cheliped propodus height, LPL: left cheliped propodus length, LPH: left cheliped propodus height, AW: abdomen width at the basis of the 4th somite. Scale: A= 10 mm; B, C and D = 5 mm.

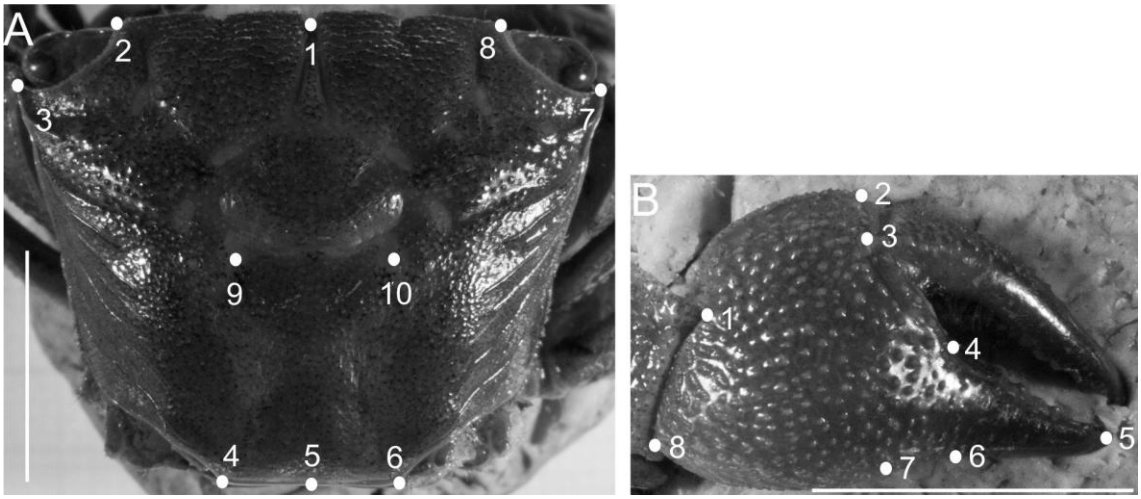

Figure. 2. *Armases rubripes*. Position of anatomical landmarks on the carapace (A) and right cheliped propodus (B) for sexual dimorphism and allometric ontogeny comparisons. (A) 1: Extreme of protogastric region; 2 and 8: End of antero-lateral line; 3 and 7: Tip of antero-lateral tooth; 4 and 6: End line of intestinal region; 5: Posterior margin of intestinal region; 9 and 10: Extremes of cardiac line. (B) 1: Inner base of the articulation carpo-propodus; 2: Distal tip of the cheliped propodus; 3: Suture in the intersection between "pré-dactilar" lobe and the base of cheliped propodus; 4: Base of the fixed finger of the cheliped propodus; 5: Tip of the fixed finger; 6: limit of fixed finger in the margin of cheliped propodus; 7: Vertical line through the distal tip of the cheliped propodus; 8: Outer base of the articulation carpo-propodus. Scale 10mm.

Results

Relative Growth

The carapace width (CW) of males ranged from 2.80 to 20.39 mm and of females from 3.62 to 18.23 mm. The CW of ovigerous females ranged from 6.1 to 16.34 mm.

The regression between CW x CL has a negative coefficient, indicating allometric growth in juveniles and isometric growth in adults for both sexes. This indicate that the CW growth faster than CL in juvenile phase, resulting in wider carapaces in adults when compare to juveniles (Table I).

The cheliped propodus have positive allometric growth for the majority of demographic categories (for both sexes), having the slope greater than 1. However, the

relative growth of RPL in juvenile females was isometric. There was significant difference between the intercepts (a) and the slope (b) of the lines for juveniles and adults in majority of comparisons ($p < 0.05$), except for CW x LPH (0.13) and CW x AW in females (0.59). These exceptions showed distinct intercepts ($p < 0.05$), but similar slopes (Table II). In this way, the cheliped propodus of males grow faster than CW throughout the development, resulting in larger dimensions of the propodus than the CW. The inflection point was 7.17 mm for three (RPL, RPH and LPL) of four dimensions of cheliped propodus of males, indicating the mean size for the onset of sexual maturity in males (Fig. 3 B,C and D).

On the other hand, the inflection point values obtained for the dimensions of cheliped propodus of females were not related to sexual maturity, although like males, they grew faster than CW. The dimension that showed a point of inflection that separated the juvenile females from the adults was AW, with a positive allometric growth in both phases of the development, with an estimate value of 5.52 mm CW (Fig. 3 A). The size of the smallest female (6.1 mm CW) was closer to our estimate value for the onset of sexual maturity what can indicate a good quality in the estimative.

Table I. *Armases rubripes*. Regression analyses of morphometric data.

Variable	Group	N	Inflection point (mm)	Linearized equation LogY= loga+blogx	r²	T (b=1)	Allometry
CL	MJ	56	7.21	LogCW= log-0.02 + 0.91logCL	0.98	52.42	-
	MA	235		LogCW= log-0.16 + 1.05logCL	0.99	161.23	0
	FJ	115	7.79	LogCW= log-0.08 + 0.98logCL	0.98	91.99	-
	FA	138		LogCW= log-0.18 + 1.08logCL	0.97	73.97	0
RPL	MJ	53	7.17	LogCW= log-0.54 + 1.19logRPL	0.95	31.47	+
	MA	194		LogCW= log-0.59 + 1.32logRPL	0.93	53.52	+
	FJ	50	7.64	LogCW= log-0.48 + 1.05logRPL	0.90	21.15	0
	FA	176		LogCW= log-0.62 + 1.23logRPL	0.95	59.63	+
LPL	MJ	53	7.17	LogCW= log-0.52 + 1.16logLPL	0.95	34.31	+
	MA	194		LogCW= log-0.59 + 1.31logLPL	0.93	54.12	+
	FJ	65	8.29	LogCW= log-0.52 + 1.10logLPL	0.92	28.55	+
	FA	158		LogCW= log-0.62 + 1.23logLPL	0.95	59.74	+
RPH	MJ	53	7.17	LogCW= log-1.12 + 1.52logRPH	0.92	25.44	+
	MA	194		LogCW= log-0.95 + 1.43logRPH	0.89	40.29	+
	FJ	122	9.84	LogCW= log-0.98 + 1.25logRPH	0.94	46.61	+
	FA	104		LogCW= log-1.07 + 1.35logRPH	0.94	40.12	+
LPH	MJ	66	7.62	LogCW= log-1.14 + 1.58logLPH	0.94	32.73	+
	MA	181		LogCW= log-0.88 + 1.36logLPH	0.86	34.24	+
	FJ	56	5.56	LogCW= log-1.02 + 1.31logLPH	0.96	17.4	+
	FA	214		LogCW= log-1.09 + 1.37logLPH	0.97	87.87	+
AW	FJ	25	5.52	LogCW= log-0.91 + 1.59logAW	0.85	7.89	+
	FA	228		LogCW= log-0.48 + 1.27logAW	0.88	41.71	+

CW: carapace width, CL: carapace length, RPL: right cheliped propodus length, RPH: right cheliped propodus, LPL: left cheliped propodus length, left cheliped propodus height, AW: abdomen width at the basis of the 4th somite. MJ= juvenile males, MA= adult males, FJ= juvenile females, FA= adult females. N= number of individuals, r^2= determination coefficient.

Table II. *Armases rubripes*. Comparisons of the straight line slopes (b) and intercepts (a) for juveniles and adults of both sexes based on a covariance analysis (ANCOVA). CW: carapace width, CL: carapace length, RPL: right cheliped propodus length, RPH: right cheliped propodus, LPL: left cheliped propodus length, left cheliped propodus height, AW: abdomen width at the basis of the 4th somite. M= males, F= females.

Sex	Comparison	F(a)	F(b)	α (a)	α (b)
M	CW X CL	118.51	12.69	<0.0001	0.0007
	CW X RPL	15.23	22.77	0.0003	<0.0001
	CW X RPH	14.31	17.22	0.0004	0.0002
	CW X LPL	16.22	25.73	0.0002	<0.0001
	CW X LPH	13.51	16.52	0.0006	0.0002
F	CW X CL	37.94	21.02	<0.0001	<0.0001
	CW X RPL	25.02	9.10	<0.0001	0.0032
	CW X RPH	4.94	50.55	0.0255	<0.0001
	CW X LPL	14.72	18.36	0.0004	0.0001
	CW X LPH	52.88	2.23	<0.0001	0.1326
	CW x AW	5.60	0.69	0.0177	0.59

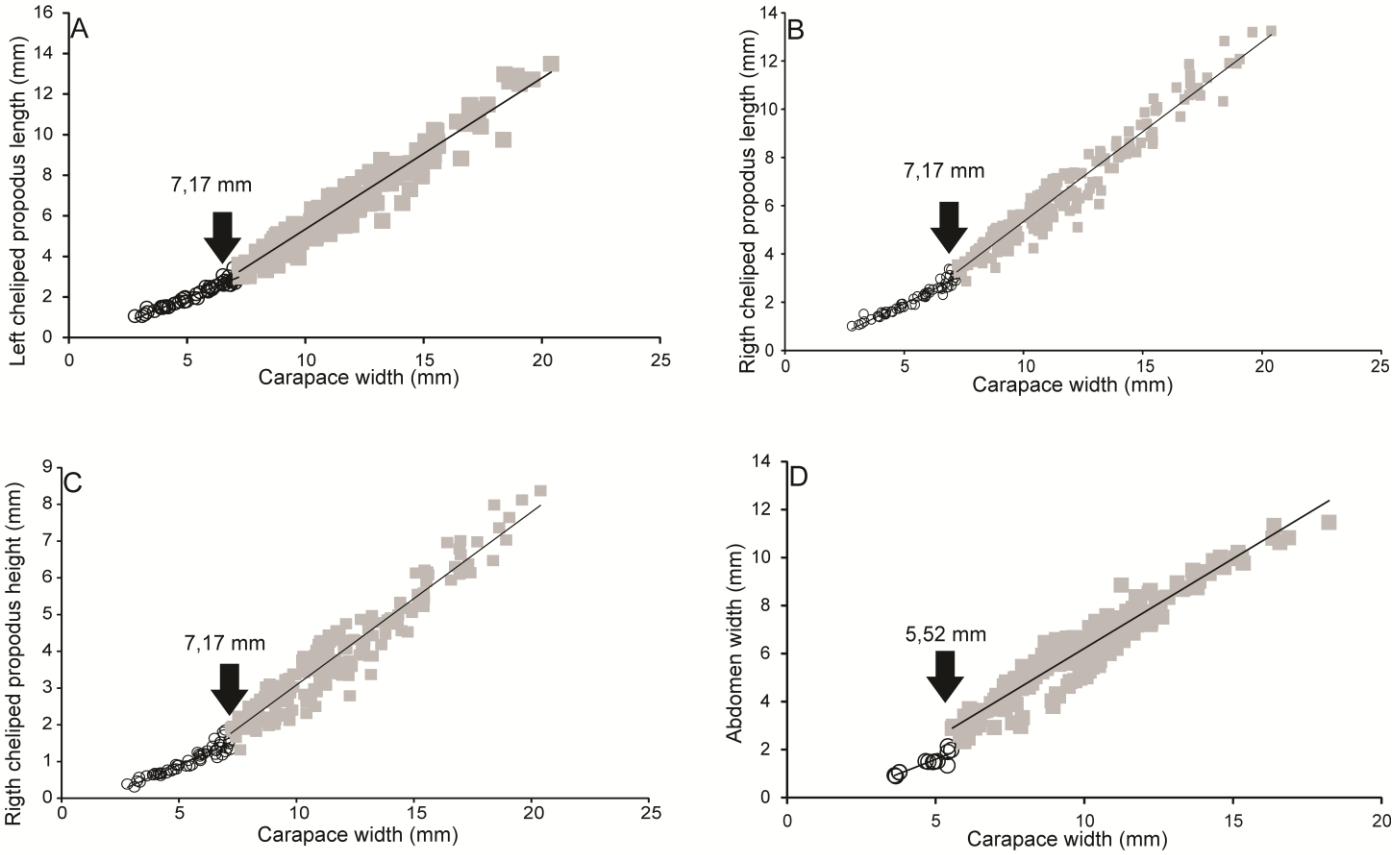

Figure 3. *Armases rubripes*. Regression between CW and cheliped propodus dimensions (males) and CW e AW (females). The circles represent juveniles and squares the adults. A: CW x LPL, B: CW x RPL, C: CW x RPH and D: CW x AW.

Sexual dimorphism

There was sexual size (centroid size) dimorphism of the carapace (t = -12.27; df = 190; p = < 0.001), right (t = -25.42; df = 142; p = < 0.001) and left cheliped propodus (t = -22.60; df = 141; p = < 0.001) between adults (males: carapace = 23.10 ± 2.4 mm, rigth cheliped propodus = 9.80 ± 1.4 mm, left cheliped propodus = 9.60 ± 1.6 mm; females: carapace = 18.50 ± 2.7 mm, right cheliped propodus = 5.1 ± 0.8 mm, left cheliped propodus = 5 ± 0.9 mm). However, there was no difference in the carapace size (centroid size) (t = -0.05; df = 44; p = 0.95) between juveniles (males: 5.47 ± 1.16 mm; fêmeas: 5.45 ± 0.75 mm).

There was also a significant variation in carapace shape between sexes of adults (Procrustes distances: 0.012, p: 0.04), with a correct percentage of classification of 60% for females and 62,5% for males. The shape variation occurred mainly in the landmarks 3-4 and 6-7, in the posterior margin of carapace. Females showed a wider posterior margin than males, which exhibit the anterior margin wider than females (landmarks 3 and 7) (Fig. 4A). On the other hand, there was no shape sexual dimorphism for the carapace of juveniles (Procrustes distance: 0.012, p: 0.067), with a correct percentage of classification of 53% for females and 62% for males.

The shape of both cheliped propodus showed no differences, after size correction, between sexes in adults (Fig. 4B and C) (right cheliped propodus: procrustes distances = 0.016, p = 0.83; left cheliped propodus: procrustes distances = 0.015, p = 0.85), with a correct percentage of classification for right cheliped propodus of 38% for males and 43% for females, and for the left cheliped propodus of 41% for males and 46% for females. Before the size correction, males showed the shape of both propodus more robust (higher and wider) than females.

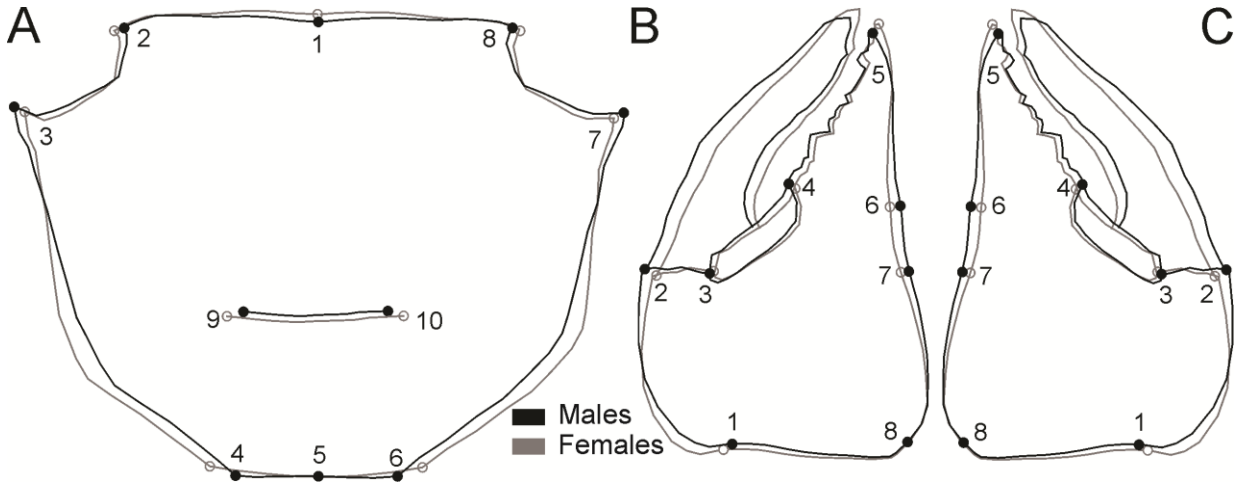

Figure 4. *Armases rubripes*. Sexual dimorphism in carapace (A), right (B) and left cheliped propodus in adults. Magnification: A = 5 times, B and C = 4 times.

Ontogeny

Males and females showed ontogenetic allometry in the shape of carapace ($p < 0.001$). Size was responsible for 34% of shape variation in males and 46% in females. In both sexes, the increase in size generates an elongation of the frontal region of the carapace and shortening of the orbital region in adults (Fig. 5 and 6).

The angle between the vectors of regression of ontogenetic trajectories among males and females was 8° and differed significantly from the expected for pairs of random vectors ($p = 0.001$), indicating that both sexes follows a similar ontogenetic trajectory.

In males, the stactic allometry occurs in both life stages, juvenile ($p < 0.001$) and adult ($p < 0.001$). The size was responsible by 21% e 9% of shape variation in each life stages, respectively. The angle between the regression vectors of males juvenile and adults was 31° and differ significantly from the expected for the pairs of random vectors ($p = 0.015$). For females, the stactic allometry also occurs in both juvenile ($p < 0.001$) and adult ($p < 0.001$) stages. The size is responsible for 33% and 32% of the shape

variation, respectively. The angle between the regression vectors of juveniles and adults of females was 61° and did not differ significantly from the expected for the pairs of random vectors ($p < 0.17$), indicating that adults follows a distinct trajectory than juveniles. These results indicate that the allometric trajectory is similar in males but distinct in females juvenile and adult.

On the other hand, the carapace shape differs between the life stages in both sexes (male: Procrustes distance = 0.033, $p < 0.001$; female: Procrustes distance = 0,056, $p < 0.001$). However, after size correction males and females showed distinct patters. The difference in shape between males juvenile and adult is related to the allometric effect and the size correction eliminates this effect of shape variation between life stages (Procrustes distance = 0.002, $p = 0.17$). While in females, the difference in shape between life stages reflects the distinct trajectories (Procrustes distance = 0.011, $p < 0.001$).

The angle between the regression vectors of juvenile males and juvenile females is 25° ($p = 0.006$) while between adult females and males was 23° ($p = 0.001$), indicating that males and females have a similar static allometry during juvenile and adult phase, respectively.

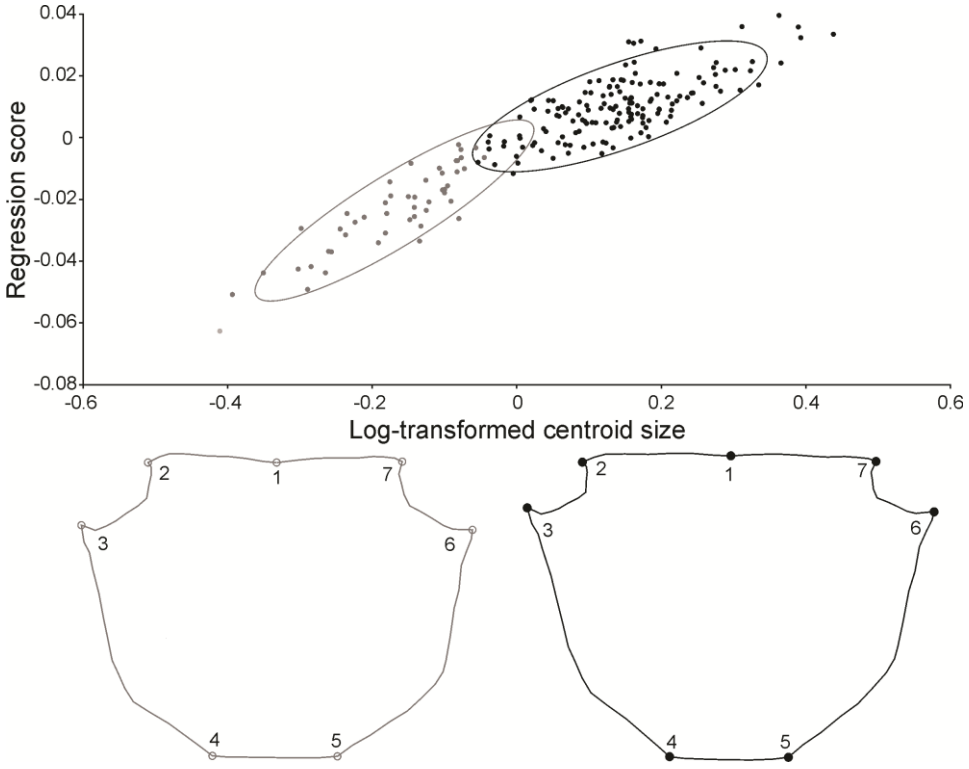

Figure 5. *Armases rubripes*. Ontogenetic allometry of carapace shape, based on multivariate regression of symmetrical components on log-transformed centroid size. The two drawings show the shapes expected for changes by -0.4 and 0.4 units of log-transformed centroid size from the mean shape (the extremes at the left and right of the plot) in males.

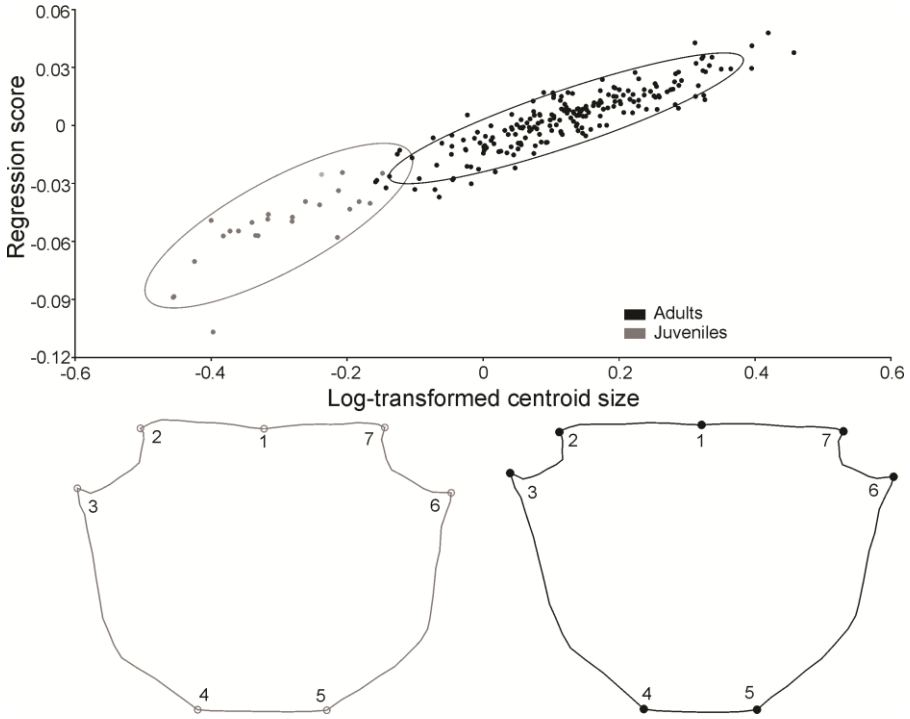

Figure 6. *Armases rubripes*. Ontogenetic allometry of carapace shape, based on multivariate regression of symmetrical components on log-transformed centroid size. The two drawings show the shapes expected for changes by -0.4 and 0.4 units of log-transformed centroid size from the mean shape (the extremes at the left and right of the plot) in females.

Discussion

Relative growth

The positive allometric growth observed in the majority of the analyzed body dimensions, especially in cheliped propodus in males and abdomen width in females, indicates a common pattern related to sexual selection. For distinct taxa such as lizards, insects and crabs, in which is hypothesized that exaggerated male traits have evolved under sexual selection in ornaments to attract mates and weapons to face/combat rivals, the directional sexual selection leads to the evolution of positive allometry (Petrie 1992, Green 2000, Kodric-Brown et al. 2006). The same pattern occurs in distinct animal taxa for females. The positive allometric growth in the abdomen or posterior region is observed in females of lizards, insects and crustaceans is also commonly associate with directional sexual selection to an increase of fat reserve or in area to accommodate the offspring/eggs (Hartnoll 1974, Anholt 1991, Braña 1996). In both sexes, the positive allometric growth of secondary sexual characters can be an evolutive trend drive by directional sexual selection. This directional selection is likely related to an increase in reproductive success (Kodric-Brown et al. 2006).

Since males showed the mean CW larger than females (10.36 and 9.56 mm, respectively, with a difference of 0.8 mm) it was expected that the CW values for the onset of morphological sexual maturity between sexes were also closer. However, there is a difference of 1.65 mm, larger than populations from São Paulo and Rio de Janeiro (Castiglioni et al. 2004, Lima & Oshiro 2006). This variation can be partially explained by the distinct reproductive strategies of each sex. When males reach the sexual maturity later than females they can increase the size and weapons (cheliped propodus) to fight for a mate, thus get advantages in intraspesific competition and copulation. In the other hand, females that reach the sexual maturity earlier can invest energy in egg

production as well as for abdomen development (Hartnoll 1982, 1985). This is likely the case of the study. The pattern of females become sexually mature with smaller size than males observed in the present study, was similar to São Paulo population but distinct from Rio de Janeiro population (males become sexually mature with smaller size than females) (Castiglioni et al. 2004, Lima & Oshiro 2006, Lima et al. 2006). This indicates that this parameter is a characteristic of population and not a pattern of the species.

The main size of the onset of morphological sexual maturity of *A. rubripes* of the present study was distinct from the populations of São Paulo (SP) and Rio de Janeiro (RJ). In SP there was a variation in the onset of sexual maturity from 9.1 to 11.6 mm CW for males and from 7.6 to 9.8 mm CW for females. In RJ it varied from 6.6 to 7 mm CW for males and from 8.2 to 9 mm CW for females (Castiglioni et al. 2004, Lima & Oshiro 2006). In Guaratuba Bay (present study), the individuals become sexually mature earlier (7.17 and 5.52 mm CW, respectively) than the populations mentionated. Distinct ecological and physical factors can be responsible for this populational difference in the onset of maturity like: predator pressure, temperature and photoperiod, food availibity, kind of substrate and population density, as observed for other brachyurans (Kuris 1971, Wenner et al. 1974, Hines 1989). All those factors would act on the size of the onset of morphological sexual maturity through the modulation of growth rates and longevity (Hines 1989). The temperature and photoperiod can act in large scales (latitudinal) reducing the growth rate in low temperatures (higher latitudes) for example, as observed for the crab *Panopeus herbstii* H. Milne Edwards, 1834 (Hines 1989). This can be occurring with Guaratuba and Rio de Janeiro populations (far more than 500 km), but not explain the variation found between Guaratuba and São Paulo populations (far around 100-200 km). Other factors

can be occurring in small scale (predator pressure, food availibity, kind of substrate and population density) that can influence the growth rate and longevity locally. This can also be occurring for Brazilian populations of *A. rubripes*, reflecting in local characteristic of sexual maturity.

Beyond the possible ecological and physical factors mentioned, the sampled methods and the number of juveniles and adults sampled from each sex can also influence the onset of sexual maturity.

Sexual dimorphism

In *Armases rubripes*, males are larger (carapace width and cheliped propodus length) than females. This sexual size dimorphism has been suggest to have an adaptive function against predation of females, since males can protect them during and after intercourse when the exoskeleton is decalcified in crab species (Hartnoll 1969, Pinheiro & Fransozo 1999, Pinheiro et al. 2005). Larger chelipeds in males can provide advantages in intraspecific interactions, such as territorial defense, agonistic behaviors and reproduction-related processes, such as the cohort (Warner 1970). In both cases (carapace width and cheliped legth), the SSD reflects adaptations for the reproductive success of the species, and it commonly observed in Brachyura (Hartnoll 1974).

The sexual shape dimorphism (SShD) in carapace was marked especially by a wider posterior margin of carapace in females than males. As discuss previously, females show postitive allometric growth for the abdomen, and consequently females show a wider abdomen than males. This allometric growth seems to affect the posterior margin of carapace too, as a consequence of the abdomen growth. Since the abdomen is a closer anatomical region to the posterior carapace margin, the abdomen growth can also affect the growth of structures in the closer area modifying the shape in adult

females. This pattern is also observed in males of fiddler crabs that exhibit an asymmetry in carapace due the growth of the asymmetrically-size claw. It has been interpreted as a biomechanical response during the development (Crane 1975). The increase in size of the abdomen can generate a biomechanical response to support this weigth increase during the development. This response, would leading to an increase of musculature and consequently to the posterior carapace area. But, it also can be a consequence of the abdomen growth simply because the posterior carapace margin is located closer to the abdomen and would be influenced by a similar growth rate. Since the posterior region of the carapace is not a region with a develop musculature, the more likely reason for this pattern seems to be proximity of the carapace margin and abdomen. The same pattern was observed for the Anomura *Aegla marginata* Bond-Buckup and Buckup, 1994 (Trevisan et al. 2012).

Adult males showed the shape of cheliped propodus more robust (higher and wider) than females. But after the size correction this variation was not significant. Thus, the sexual dimorphism of cheliped is a consequence of size and not shape. The differences of cheliped size are in the growth rate (higher in males), which is influencied by sexual hormones. In experiments develop by Nagamine et al. (1980a, 1980b) with the shrimp *Macrobrachium rosenbergii* de Man, 1879 demonstrated high level of feminization (beginning of oogenesis and development of oviducts and gonopores) in males who have androgenic gland removed at the beginning of ontogenetic development. On the other hand, females who had the same gland implanted developed male morphological features, as more robust chelipeds and the start of spermatogenesis in the ovaries. This cheliped size difference is a sexual selection trait in some Brachyura lineages evolution and the same sexual dimorphism

pattern is described for other species (Hartnoll 1974). Crabs with more robust claws have advantages in the sexual selection by females, since this males will have more chances to win fights against other males during the process of cohort or agonistic behavior in general (Lee 1995, Mariappan et al. 2000).

The absence of sexual dimorphism in the body structures of juveniles, but with expressive variation in adults, supports the hypothesis of differential growth of males and females occur, or be intensified, only after puberty moult when the individuals become sexually active. This pattern is common for Crustacea, whose growth involves a series of immature stages of similar morphology until the puberty moult with more expressive morphological changes (Hartnoll 1974, 2001). In both data (relative growth and geometric morphometrics), there are evidences of changes in size and shape of the structures analysed during the development of juvenile to adult phase in both sexes in distinct way. These changes culminate in marked sexual dimorphism that may represent the evolutive sexual tendency of the lineage.

Ontogeny

Males and females of *A. rubripes* have ontogenetic allometry on the carapace. In other words the carapace shape is not isometric from juvenile to adult phase. Males have a similar trajectory from juvenile to adult phase and the shape differences are mainly relate to the influence of size over shape (responsible for 34% of variance). When the effect of size is removed from the comparison (after size correction) there is no shape difference. On the other hand, females have distinct trajectories from juvenile to adult phase and the shape differences, even after size correction, reflect these differences. Females have influence of the development of secondary sexual characters (abdomen) over the posterior carapace area. While, for males the development of

secondary sexual characters is located especially in the cheliped propodus (Hartnoll 1974). This gender rule in reproduction, and consequently the development of the secondary sexual characters, reflects the differences in the ontogenetic trajectories between males and females. Since just the carapace shape was evaluated, only the development of secondary sexual characters of females was detected by shape analysis.

Males and females of *A. rubripes* have similar ontogenetic morphological trajectories of the carapace with 8° of angular relation (p = 0,001). However, the size of carapace influences the shape in males and females in a different way. The females shape are influenced more by size variation (juveniles = 32% and adults = 33%) than males (juveniles = 9% and adults = 21%). This indicates that, during the ontogeny, the size increase of carapace and structures directly related to it, influence more the shape of females than males. This fact can be related with the sexual selection for the species, where females who reach the size and shape required to accommodate the egg mass can reproduce earlier. The greater size/area increment of the abdomen during the ontogenesis is intensified in the puberty moult can also affect the carapace size increment in Brachyura, as discuss previously (Hartnoll 1974).

In both sexes, the growth generates an elongation of the frontal region of the carapace and shortening of the orbital region in adults. When the size of the orbital region is compare to the CW, it correspond to 37% in the first juvenile stage (Negreiros-Franzoso et al. 2011) and to 20% in adult phase in *A. rubripes* of the present study. This ontogenetic variation can be related to a higher rate of growth or growth relate hormones production in the orbital region, consequently a higher area occupy by this gland in juvenile than adults, since the moult (consequently the use of growth hormones) occur more frequently in juvenile than adult phase. The eyestalk in

crustaceans is recognized as a region related to direct and indirect production of moult hormones (e.g. "crustecdysone") and its antagonistic (Hartnoll 2001). But, it is more likely that the orbital region differences are a consequence of the growth without any specific functionality in juvenile phase.

The similar angle of comparison between the ontogenetic trajectory of male and female juveniles (25°) and adults (23°), refers to similar ontogenetic trajectories in each life stage (juvenile and adults) in males and females. Despite this, the carapace shape show variance only in adult phase. This fact reinforce the hypothesis of similar morphologies during juveniles stages, and after puberal moult, there is an intensification of morphological diversification between sexes influenced by size increase.

Conclusions

The present study demonstrated that the abdomen width for females and the length and height of the male cheliped propodus are the best dimensions for estimate the onset of morphological sexual maturity using allometric techniques for the species. New information on the development of secondary sexual characters and their consequences in the carapace and of the cheliped propodus shape in males and females of *A. rubripes* are presented. The shape of the carapace tends to be similar during the juvenile stage in males and females, and only after pubertal moult the sexual morphological variation becomes significant. The size increase (of one molt in relation to the other) tends to influence the shape of the carapace more in females than in males, although males present larger sizes than females. Based on the results and observations it is possible to infer that variations in the shape of the carapace and at the cheliped propodus reflect the evolutionary sexual tendency of the species and in general for Sesarmidae.

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Chapter IV

Genetic and morphological differentiation of the semi-terrestrial crab *Armases angustipes* (Brachyura: Sesarmidae) along the Brazilian coast

Abstract

The genetic and morphometric population structures of the semi-terrestrial crab *Armases angustipes* were examined along the Brazilian coast, and the influence of the Central South Equatorial Current on larval dispersal was evaluated. Six populations were sampled from estuarine areas in São Luis, Natal, Maceió, Ilhéus, Aracruz and Guaratuba. Patterns of genetic differentiation were assessed with DNA sequence data corresponding to parts of the mitochondrial cytochrome *c* oxidase subunit I. Geometric morphometric techniques were used to evaluate morphological variation of the carapace and right cheliped propodus shape and size. Our results reveal low genetic variability, lacking phylogeographic structure, while geometric morphometrics showed statistically significant morphological differentiation and geographic structuring. Our data indicate the absence of possible barriers to gene flow for this mobile species and no clear correlation of morphological and genetic variation with ocean currents and/or geographic distance. Our results also suggest that historical geological and climatological events and/or possible bottleneck effects influenced the current low genetic variability among the populations of *A. angustipes*.

Key-words: Crab populations, gene flow, geometric morphometrics, larval dispersal, mtDNA COI gene, population structure

Introduction

Natural populations have to cope with heterogeneous environments. In marine/estuarine habitats this heterogeneity is related to biotic (e.g., food availability, predation, and inter/intraspecific competition) and abiotic (e.g., marine currents and temperature) forces that vary across temporal (seconds to ages) and spatial (meters to latitudes) scales. In response, individuals and populations can display variation in distinct aspects of their life histories, possibly reflected in genetic and morphological traits. This variation arises due to the different responses to local environmental pressure (via genetic frequencies or phenotypic processes) (Sotka 2012). Such patterning of diversity can result in distinct local adaptation and structuring among populations along a geographic range.

Oceanic currents are one of the features of the world's marine enviroments with most fundamental impact on animal movements and dispersal, especially for those with planktonic larval phases (Chapman et al. 2011). The coast of Brazil is influenced by different marine currents. The Central South Equatorial Current (CSEC) splits into the North Brazil/Guiana Current (NBC) and the South Brazil Current (SBC) (Fig. 1). This area of current splitting varies annually with a mean occurrence between 10° - 14° S near the surface (for more details see Rodrigues et al. 2007). It has been classified as a biogeographical barrier for species with planktonic larval phases by some authors (Shanks 2009, Weersing & Toonen 2009). Based on this splitting of CSEC, the Brazilian coast can be divided into three regions influenced by coastal currents: 1) north of CSEC split; 2) split region (between 10°-14°) and 3) south of the CSEC split.

Dispersal of marine organisms is recognized as a complex process, especially in marine invertebrates with larval phases (Fratini et al. 2011). The absence of evident

physical barriers, a reproductive strategy with thousands of larvae per spawn per female, large population sizes, and extensive larval phases potentially allow the dispersal of invertebrate larvae over wide distances and consequently reduce the genetic variability among populations (Hedgecock 1986, Avise 2004, Fratini et al. 2011). However, some studies of animals with dispersing larvae show different amounts of genetic variability over different geographic scales (Avise 2004, Fratini et al. 2016).

The Brazilian coast extends over approximately 8500 km of shoreline and shows the third largest mangrove area in the world, accounting for 7% of global distribution. Mangroves in Brazil occur from the border with French Guiana to near Laguna (Santa Catarina state) (4°30'N to 28°30'S) with an approximate surface cover of 25,000 km² of mangrove forests (Saenger et al. 1983, Schaeffer-Novelli et al. 2000). Larval dispersal can be a complex process for species thriving in estuarine habitats, such as mangroves, marshes and near polyhaline zones, since larvae released from the parental areas must often be flushed into the sea to undergo the larval developmental in higher salinities and subsequently return to estuarine areas to complete the juvenile development (Bilton et al. 2002, Ituarte et al. 2012). Estuarine environments are fragmented and patchy habitats, so that the larval dispersal depends on the proximity between estuaries and the time in the plankton.

Ten species of fiddler crabs show intraspecific variation of the carapace shape among populations distributed along the Brazilian coast (Hampton et al. 2014). On the other hand, the mangrove crab *Ucides cordatus* (Linneaus, 1763) does not show any geographical structure in genetic variation, implying high connectivity among populations separated as far as 2700 km (Oliveira-Neto et al. 2007). The estuarine crab *Neohelice granulata* (Dana 1851) shows strong genetic differentiation between

populations of Argentina and Brazil, but does not show a geographical pattern of morphological differentiation (Ituarte et al. 2012). These results suggest that there is no general pattern for potential differentiation in coastal crabs, and the population variability of each species must be investigated separately.

Armases angustipes (Dana, 1852) is a semi-terrestrial crab of the family Sesarmidae with a wide distribution. This species has been reported to occur in Yucatan (Mexico), Andros Island (Bahamas), Trinidad and Tobago and Brazil (from the state of Maranhão to Santa Catarina) (Abele 1992, Melo 1996). However, the presence of the morphologically highly similar species *Armases miersii* (Rathbun, 1897) in the Caribbean, makes the exact distribution and possible sympatry in Central America difficult to determine (Cuesta et al. 1999, Cuesta & Anger, 2001). *Armases angustipes* occurs in a large variety of habitats in coastal areas, such as sandy, muddy and rocky margins of mangroves and adjacent areas, under dried leaves in border vegetation, in bromeliad leaf axils and along the terrestrial margins of rocky shores. This species has four planktonic zoeal stages that develop best in salinities >20 ‰, and one megalopa stage that returns to environments with lower salinity (<32 ‰) before the metamorphosis to the first fully benthic juvenile stage (Anger et al. 1990, Cuesta & Anger 2001). Thus it can be considered a species with an export larval strategy and a preference for estuarine systems during its late development.

In the larval export strategy the zoea larvae are released in estuarine areas. After the released, the zoea larvae migrate to coastal/oceanic waters (higher salinity) where they suffer successive moults until megalopa stage (last larval stage). During the megalopa stage, they tend to return to estuarine areas (lower salinity) to complete the ontogenetic development to first juvenile stage. This strategy may facilitate the

dispersion and provide a more stable environment for larval development in the early larval stages (Anger 2001, Simith et al. 2012).

In order to evaluate the population genetic and morphological structures of this semi-terrestrial crab and to test the influence of the CSEC as a geographic barrier to gene flow we here present: 1) haplotype data from the cytochrome c oxidase subunit 1 (Cox1) mitochondrial gene, 2) data on shape variation of carapace and chelar propodus, and 3) evaluate the correlation between the morphological and genetic distances from six populations along the Brazilian coast. The Cox1 gene comprises a coding sequences and has been efficient for evaluate population genetic variations in decapods (Terosii & Mantelatto 2012, Laurenzano et al. 2013; Thiercelin & Schubart 2014). Both sets of data were used to determine the extent of gene flow and the phenotypic variation among geographically separated populations. These combined data will help to understand the population biology and microevolution of this species, as well as the connectivity between estuarine faunas in general, which may also contribute to our comprehension of the biogeography and intraspecific diversity of intertidal organisms and the connectivity among the patchily distributed faunas (Ragionieri et al. 2009; Laurenzano et al. 2013).

Material and Methods

Sampling of Armases angustipes

Individuals of six Brazilian populations of *A. angustipes* were sampled from: São Luis do Maranhão, Maranhão (MA) at 09/06/14; Natal, Rio Grande do Norte (RN) at 08/28/14; Maceió, Alagoas (AL) at 08/27/14; Ilhéus, Bahia (BA) at 08/23/14; Aracruz, Espírito Santo (ES) at 08/20/14 and Guaratuba, Paraná (PR) at 12/07/13 (Fig.

1) (Table 1). Samples were collected by hand and individuals were preserved in 75% ethanol.

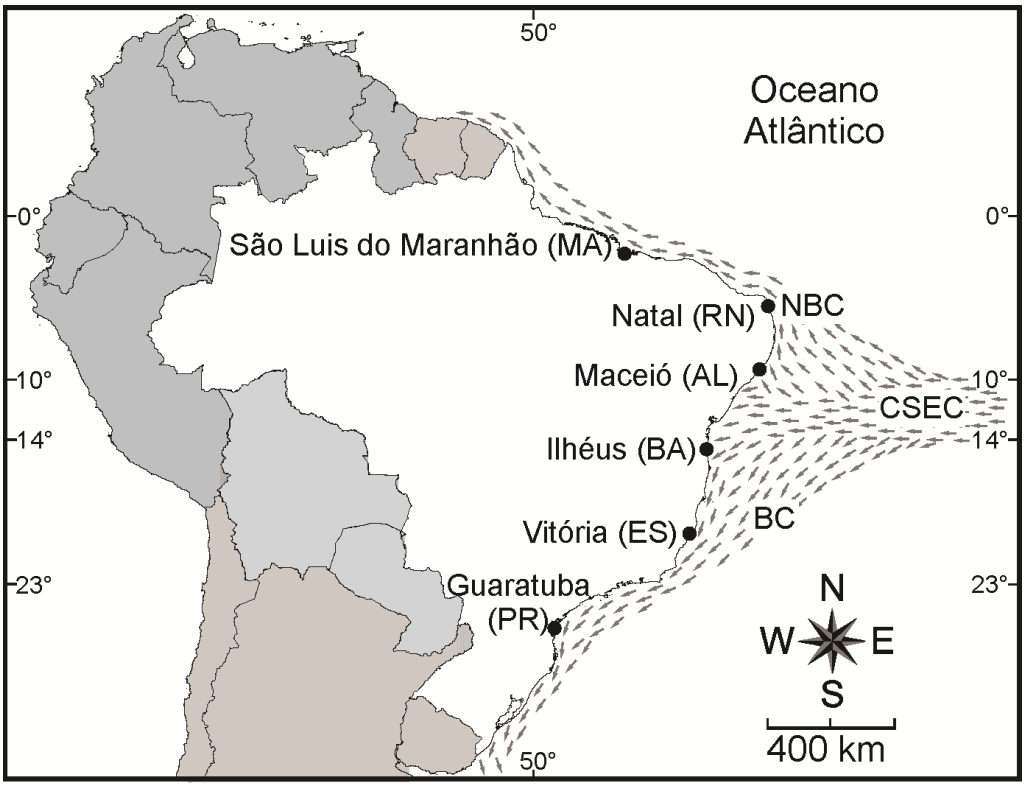

Figure 1. Sampling localites of *Armases angustipes* along the Brazilian coast (MA = São Luís, RN = Natal, AL = Maceió, BA = Ilhéus, ES = Aracruz, PR = Guaratuba). Pertinent surface ocean currents are indicated by arrows (CSEC = Central South Equatorial Current, NBC = North Brazil Current, SBC = South Brazil Current). The 10°-14° latitude indicates the seasonal surface variation area of the split of the ocean current.

Table 1. Samplig coordinates, number of individuals per sex and museum collection numbers in Museu de História Natural do Capão da Imbuia (MHNCI), Curitiba, Paraná, Brazil of *Armases angustipes*.

Locality	Coordinates	Male	Female	Description of habitat	Type of substrate	Collection number
São Luís, MA	2°28'26.3"S–44°12'54.5"W	42	13	Between rocks and under marginal vegetation near mangrove areas	Sandy soil	C5413
Natal, RN	5°45'5.1"S–35°14'9.26"W	24	25	Under leaves and marginatal vegetation near mangrove areas	Forest soil (organic rich)	C5415
Maceió, AL	9°42'1.99"S–35°47'26.49W	5	5	Under leaves and marginatal vegetation near mangrove	Sandy soil	C5414
Ilhéus, BA	14°40'28.9"S–39°4'49.3"W	16	9	Under leaves, trunks between mangrove and Atlantic forest	Forest soil (organic rich)	C5412
Aracruz, ES	19°57'0"S–40°9'18.1"W	10	17	Under leaves and marginatal vegetation near mangrove	Sandy soil	C5411
Guratuba, PR	25°51'41.2"S–48°35'23.5"W	7	8	Under leaves, rocks and bromeliad leaf axils between mangrove area and Atlantic Forest	Forest soil (organic rich)	C5160

Morphometrics

Pictures from 63 carapaces (MA: 26, RN: 13, AL: 5, BA: 7, ES: 8 and PR: 4) and 59 right cheliped propodi (MA: 24, RN: 12, AL: 4, BA: 7, ES: 8 and PR: 4) in dorsal view of adult males were obtained with a Fujifilm Finepix HS10 camera with a resolution of 10 megapixels (Table 2). In general, studies regarding shape variance in crabs use a minimum number of 20 individuals per group or population studied (Silva et al. 2010; Wieman et al. 2014). However, other studies regarding less abundant species use a reduced number of individuals (less than 5) (Figueirido et al. 2011). *Armases angustipes* is a less abundant species, when compare to other brachyurans species. Some localities the abundance of the species is naturally reduced when compare to others. In the few studies that evaluate the effect of sample size and error in geometric morphometrics, the authors conclude that the mean size, standard deviation of size and variance of shape are found to be fairly accurate even in relatively small samples (Cardini & Elton, 2007). Even that some populations of the present study have a relative small number of individuals, the accuracy of the analyses is not affect by this.

Table 2. Number of male specimens, mean size ± standard deviation of the carapace, and cheliped propodus used in each population of *Armases angustipes*. MA: Maranhão, RN: Rio Grande do Norte, AL: Alagoas, BA: Bahia, ES: Espírito Santo and PR: Paraná.

Locality	Population	*N* carapace	Mean ± SD	*N* cheliped	Mean ± SD
São Luís	MA	26	2.44 ± 0.37	24	0.91 ± 0.20
Natal	RN	13	2.47 ± 0.68	12	0.87 ± 0.37
Maceió	AL	5	2.37 ± 0.16	4	0.84 ± 0.11
Ilhéus	BA	7	2.29 ± 0.57	7	0.82 ± 0.29
Aracruz	ES	8	2.47 ± 0.38	8	0.90 ± 0.17
Guaratuba	PR	4	2.56 ± 0.31	4	1 ± 0.22

The differences in the number of sampled individuals and those used in the morphometric analyses are due to the fact that only intact carapaces and right chelipeds were used, and females and juveniles were not included in the analyses due to the sexual dimorphism and allometry that could influence the data. We considered only adult individuals with carapace widths of more than 10 mm to be adults, based on a previous study by Kowalczuk & Masunari (2000). We defined eleven two dimensional landmarks on the carapace and eight on the propodus of the right cheliped (Fig. 2), using the TPS Dig 2 software, version 2.16 (Rohlf 2010). We performed a generalized Procrustes analysis (GPA) with raw landmark coordinates, which consists in superimposition the configurations through the centroid (the mass centre of the configuration), scaling the centroid size of each configuration to the value of 1 and rotating (Monteiro & Reis 1999). The GPA overlapping removes the effect of position, orientation and size of the configurations of landmarks, and the aligned configurations then exclusively correspond to the shape of the structures (Adams et al. 2004). As the carapace is symmetrical, the shape components can be separated in symmetrical and asymmetrical components (Klingenberg et al. 2002). Only the symmetrical components of the carapace were used for the analysis of shape variation. The size of each structure was estimated through the centroid size (root of the sum of the square distances of a group of points to that centroid) (Monteiro & Reis 1999).

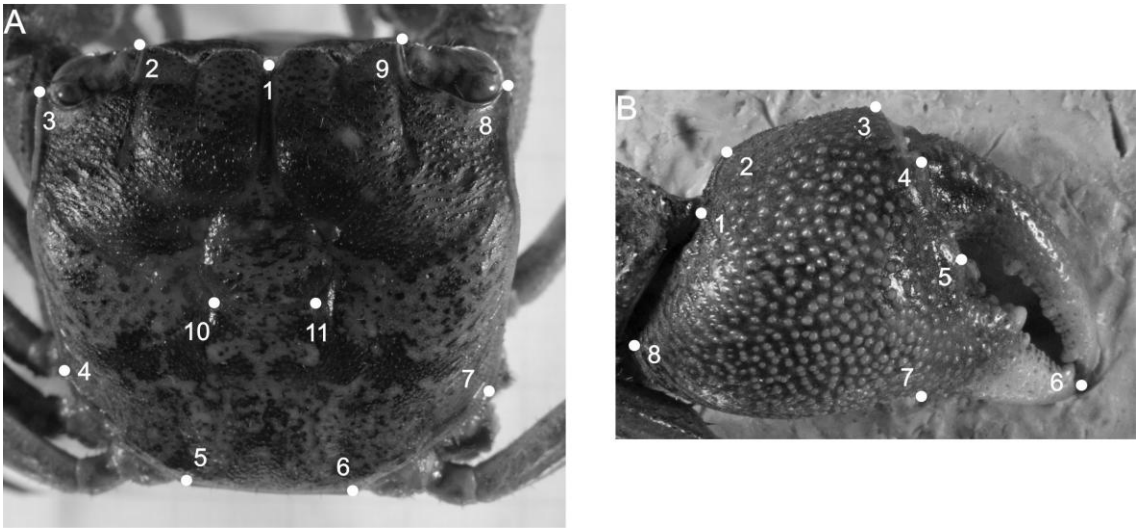

Figure 2. *Armases angustipes*. Position of anatomical landmarks on (A) the carapace and (B) right cheliped propodus. (A): 1: frontal end of protogastric region; 2 and 9: frontal end of antero-lateral line; 3 and 8: tip of antero-lateral tooth; 4 and 7: anterior bent to vertical lateral margin; 5 and 6: posterior end of vertical lateral margin and beginning of posterior margin of intestinal region; 10 and 11: distal points of sutures of cardiac line. (B) 1: inner base of the articulation carpo-propodus; 2: proximal dorsal tip of the cheliped propodus; 3: distal dorsal tip of the cheliped propodus; 4: suture between predactylar lobe and the base of cheliped propodus; 5: dorsal base of the fixed finger of the cheliped propodus; 6: distal tip of the fixed finger; 7: ventral base of fixed finger along the margin of cheliped propodus; 8: Outer base of the articulation carpo-propodus.

The size variation of the evaluated structures among the populations was analyzed through a one-way analysis of variance (ANOVA), using the centroid size as response variable and the populations as the predicting variable. A principal component analysis (PCA) was performed on the variance–covariance matrix of the GPA, and the scores were used as new variables with a reduced dimensionality of the data (Klingenberg & Monteiro 2005). These PCA scores were used to test if there is variation of shape among each populations and comparing three geographic groups: (1) MA and RN populations (north of CSEC split); (2) AL and BA (split region between

10°-14°); (3) ES and PR (south of the CSEC), through a Multivariate Analysis of Variance (MANOVA). This grouping was based on prior expectations, caused by the split of CSEC as a possible barrier to larval dispersal. Shape differences between groups were visualized using a Canonical Variables Analysis (CVA). The relationship among the populations was visualized, and an UPGMA grouping analysis was carried out using the Mahalanobis distances corresponding to each analyzed structure. The influence of the habitats types over shape variation was evaluated with a MANOVA. The habitats were divided into sandy and forest soil (organic rich) bottoms based on the specificity of each environment (Tab. 1). Statistical analyses were performed with the MorphoJ 1.06d software (Klingenberg 2011) and the R environment (R Development Core Team 2011).

DNA extraction, amplification and sequencing

A total of 66 individuals from six sample sites were used for the genetic analyses. We included at least ten or more representatives of each population, in order to apply statistics to the population genetic analyses (Table 3). Mitochondrial DNA was extracted from muscle tissue of pereiopods or chelae using the Puregene buffer system (Gentra Systems). A 941 basepair (bp) region of the mitochondrial cytochrome oxidase subunit I (Cox1) gene, encoding the 3' end was amplified by means of polymerase chain reaction (PCR) (40 cycles; 45 sec 95 °C/ 1 min 48 °C/ 90 sec 72 °C denaturing/ annealing/ elongation temperatures) in a volume of 25 µl with the primers COL8 5′-GAY CAA ATA CCT TTA TTT GT-3′ and COH16 5′-CAT YWT TCT GCC ATT TTA GA-3′ (Schubart 2009). Amplification results were checked by running 4 µl PCR product on 1.5% TBA agarose gel electrophoresis. PCR products were purified and

sequenced in the forward direction with COL8 by Macrogen Inc. Obtained sequences were proofread with Chromas Lite 3.01 (Technelysium Pty Ltd. 2005), corrected manually when required, and then aligned with BioEdit 7.2.5 (Hall 1999). Sequences were submitted to GenBank under the reference numbers KX906606 to KX906671.

Table 3. Genetic diversity indices and neutrality tests for each population of *Armases angustipes* based on 941bp of the Cox1 gene: *p < 0.05, **p < 0.01, ***p < 0.001, except for Fu´s Fs test *p < 0.02. N: number of individuals; *h*: number of haplotypes; *S*: number of polymorphic sites; *Hd*: haplotype diversity; π: nucleotide diversity.

Locality	Population	*N*	*h*	*S*	*Hd*	π	Tajima´s *D*	Fu´s *Fs*
São Luís	MA	10	7	10	0.933	0.00475	1.168	-1.01
Natal	RN	11	11	11	1	0.00213	-2.011**	-12.36***
Maceió	AL	10	6	13	0.778	0.00409	-0.7435	-0.228
Ilhéus	BA	11	8	16	0.891	0.00533	-0.362	-1.451
Aracruz	ES	12	7	11	0.773	0.00253	-1.443	-2.026
Guaratuba	PR	12	6	6	0.758	0.00147	-1.167	-2.358
MA + RN Group		21	16	20	0.971	0.00394	-1.265	-10.14***
AL + BA Group		21	13	20	0.829	0.00464	-0.8218	-4.166
ES + PR Group		24	10	14	0.746	0.00327	-1.7562*	-4.2080**
A. angustipes all sequences		66	31	36	0.86	0.00345	-1.858**	-24.07***

Genetic data analyses

To assess the levels of genetic differentiation among populations, pairwise ΦST values were calculated using Arlequin 3.11 (Excoffier et al. 2005). The variance between tested groups was assessed by an Analyses of Molecular Variance AMOVA using Arlequin 3.11, comparing the same three geographic groups defined by current described for the morphometrics analyses: 1) MA and RN (north), 2) AL and BA (intermediate) and (3) ES and PR populations (south).

To examine the population history and to evaluate whether the populations follow the neutrality model at the sampling sites, Tajima's *D*, Fu's *Fs* and Mismatch Distribution Analyses (Tajima 1989, Fu 1997, Schneider & Excoffier 1999) were carried out using Arlequin 3.11. The software DnaSp 5.10.1 (Librado & Rozas 2009) was used to assess the nucleotide (π) and haplotype (*h*) diversities. A statistical parsimony haplotype network was constructed with PopART (Leigh & Bryant 2015).

Comparative Analysis

The influence of the genetic differentiation and geographic distance on the body shape were tested with a three-way Mantel test, using the distance matrices of carapace and right cheliped propodus, genetic differentiation and geographic distances among populations (Mantel & Valand 1970). The morphometric matrices (carapace and cheliped propodus) were created using Mahalanobis distances, while the genetic matrix consisted of the pairwise ΦST values and the geographic matrix of the distances in kilometers between populations. The Mantel tests were run using the vegan package in R (Oksanen et al. 2010, R Development Core Team 2011).

Results

Morphometric Analysis

The six populations of *A. angustipes* did not differ significantly in the mean centroid size of the carapace ($F = 0.23$, $p = 0.94$) and right cheliped propodus ($F = 0.31$, $p = 0.89$), which ranged from 2.29 ± 0.57 cm to 2.56 ± 0.31 cm and 0.82 ± 0.29 cm to 1 ± 0.22 cm, respectively (Table 2).

The carapace shape differed among populations of *A. angustipes* (Pillai's trace = 1.87, $p < 0.0001$) (Table 4). The first canonic axis explained 50.81% of the variation among groups and was mainly related to frontal region of carapace, landmarks 1 (frontal end of protogastric region) and landmarks 2 and 9 (frontal end of antero-lateral line) (Fig. 3). The majority of individuals from populations of the north and northeast of Brazil (MA, RN, AL, and BA) occupied the positive quadrant of shape space for the first canonical axis and had an advanced carapace frontal region, while the majority of individuals from south and southeast Brazil (ES and PR) showed negative scores for this axis and a receding carapace frontal region. The second canonical axis explained 31% of the variation among groups and was mainly related to the cardiac line (Fig. 3). For the cluster analysis of carapace shape, the PR population can be distinguished from the other populations (ES, BA, AL, RN and MA) (Fig. 4 A). High co-phenetic correlation values were obtained ($r = 0.89$) in the carapace cluster analysis, indicating that the grouping satisfactorily reflects the structure of the morphometric data.

Table 4. Mahalanobis distances (below diagonal) and corresponding p-values (above diagonal) referring to the carapace shape between the populations of *Armases angustipes*. The p-values were adjusted for multiple comparisons by false discovery rates (Benjamini and Hochberg, 1995). MA: Maranhão, RN: Rio Grande do Norte, AL: Alagoas, BA: Bahia, ES: Espírito Santo and PR: Paraná.

	MA	RN	AL	BA	ES	PR
MA	-	<.0001	<.0001	<.0001	0.002	<.0001
RN	2.316	-	0.001	0.0206	<.0001	0.003
AL	2.591	2.985	-	0.0027	0.0019	0.0047
BA	3.717	2.316	4.047	-	0.0002	0.0025
ES	1.985	2.958	4.015	4.570	-	0.0016
PR	4.587	4.631	6.102	6.199	3.841	-

Source of Variation	Mahalanobis distance	*p* value
Among north (MA, RN) X northeast (AL, BA) populations	1.8899	0.0002
Among north (MA, RN) X south (ES, PR) populations	2.3804	<.0001
Among northeast (AL, BA) X south (ES, PR) populations	3.9858	<.0001

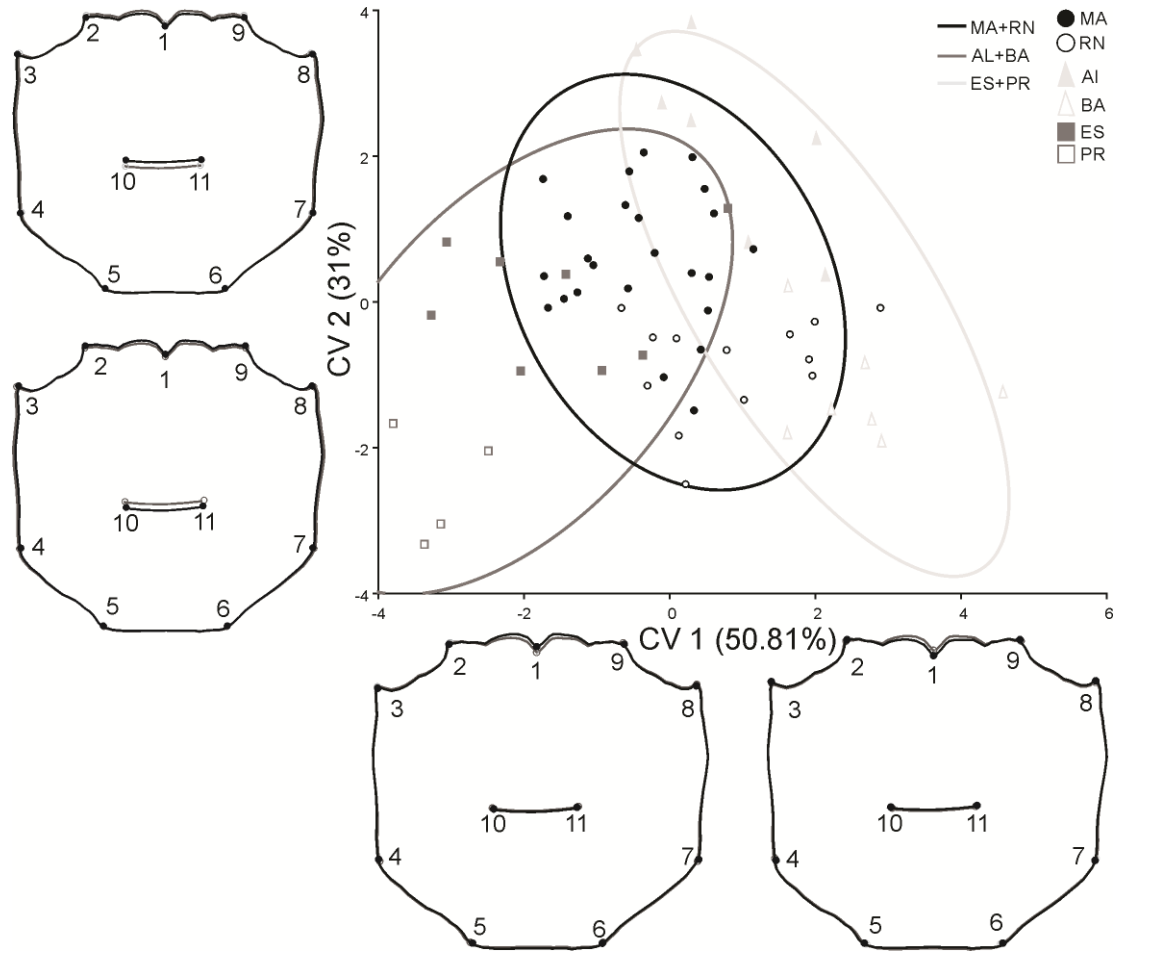

Figure 3. *Armases angustipes*. Canonical variables analysis (CVA) of the carapace among populations. MA: São Luis, RN: Natal, AL: Maceió, BA: Ilhéus, ES: Aracruz and PR: Guaratuba. Dark lines denote mean morphological deformation on the axis; clear lines denote maximum morphological deformation on the axis. Left drawings in CV1 correspond to negative deformation. Right drawings in CV1 correspond to positive deformation. Upper drawings in CV2 correspond to positive deformation. Lower drawings in CV2 correspond to negative deformation. Landmarks 10 and 11: cardiac line.

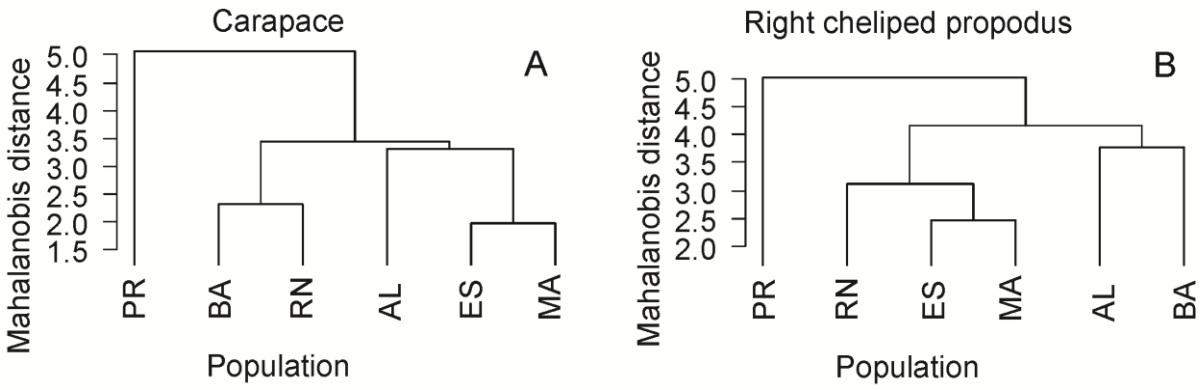

Figure 4. *Armases angustipes*. Cluster analysis (UPGMA) using the Mahalanobis distance matrix of the carapace shape (A) and right cheliped propodus (B) of the populations of MA: São Luis, RN: Natal, AL: Maceió, BA: Ilhéus, ES: Aracruz and PR: Guaratuba.

The right cheliped propodus shape also differed significantly among populations (Pillai's trace = 2.39, $p < 0.0001$) (Table 5). The first canonical axis explained 41.96% of the data variation and was mainly related to landmarks 4, 5 and 6, corresponding to the predactylar lobe and the fixed finger area (Fig. 5). The PR and RN population showed negative scores for the first canonical axis and had a receding fixed finger and predactylar lobe compared to the other populations (ES, BA, AL, MA). The second canonical axis explained 30.96% of the data variation and was mainly related the upper portion of the cheliped (landmarks 1, 2, 3 and 4) (Fig. 5). For the cluster analysis of the shape of the right cheliped propodus, the result was similar as with the carapace shape, with PR population being distinguishable from the other populations (ES, BA, AL, RN and MA) (Fig. 4 B). High co-phenetic correlation values were obtained ($r = 0.87$), indicating that the grouping satisfactorily reflects the structure of the morphometric data.

Table 5. Mahalanobis distance referring to the right cheliped propodus shape among the populations of *Armases angustipes*. The p-values were adjusted for multiple comparisons by false discovery rates (Benjamini & Hochberg, 1995). MA: Maranhão, RN: Rio Grande do Norte, AL: Alagoas, BA: Bahia, ES: Espírito Santo and PR: Paraná.

	MA	RN	AL	BA	ES	PR
MA	-	0.001	0.001	< 0.001	0.001	0.001
RN	2.768	-	< 0.001	< 0.001	0.001	< 0.001
AL	3.772	4.817	-	0.003	0.005	0.015
BA	4.008	3.853	3.760	-	< 0.001	0.001
ES	2.479	3.502	3.909	4.543	-	0.002
PR	4.645	3.520	6.325	5.255	5.382	-

Source of Variation	Mahalanobis distance	*p* value
Among north (MA, RN) X northeast (AL, BA) popultions	3.4310	<.0001
Among north (MA, RN) X south (ES, PR) popultions	1.9039	0.0002
Among northeast (AL, BA) X south (ES, PR) popultions	3.7398	<.0001

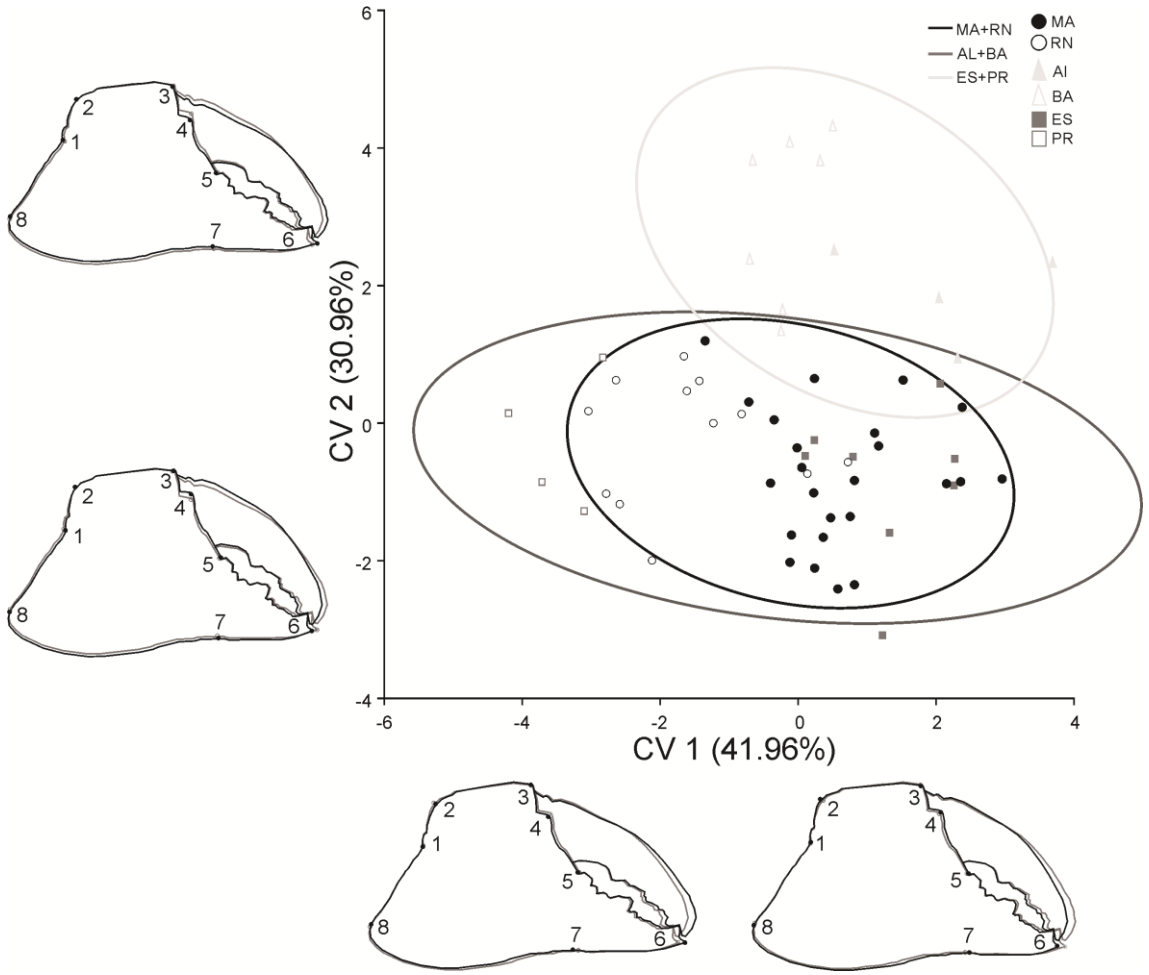

Figure 5. *Armases angustipes*. Canonical variables analysis (CVA) of the right cheliped propodus between populations. MA: São Luis, RN: Natal, AL: Maceió, BA: Ilhéus, ES: Aracruz and PR: Guaratuba. Dark lines denote mean morphological deformation on the axis; clear lines denote maximum morphological deformation on the axis. Left drawings in CV1 correspond to negative deformation. Right drawings in CV1 correspond to positive deformation. Upper drawings in CV2 correspond to positive deformation. Lower drawings in CV2 correspond to negative deformation. Fixed finger area: region comprising the landmarks 5,6 and 7. Predactylar lobe: region closer to landmark 4.

There is evidence for an influence of the type of habitat, based on the specificity of each environment (sandy and organic rich soil), over the shape of the carapace ($f = 7.36$, $p < 0.001$) and right cheliped propodus ($f = 7.56$, $p < 0.001$).

Genetic Analysis

A dataset of 66 mtDNA sequences with an alignment length of 941 base-pairs (bp) was obtained from six Brazilian populations, resulting in 31 different haplotypes

with 36 variable sites (Table 3). The highest number of haplotypes (*h*) within populations (n = 11) was observed in the RN population, and the lowest (n = 6) in the AL and PR populations.

The haplotype network (Fig. 6) confirms the presence of five haplogroups (I, II, III, IV and V) with the highest haplotype diversity (*Hd*) in the northern populations of MA and RN (0.933 and 1) and highest nucleotide diversity in the northernmost population MA (0.00475), while the lowest haplotype (0.758) and nucleotide (0.00147) diversities were found in the southernmost population PR (Table 3). Haplotype I was the most common haplotype and recorded in over 37% of the analyzed individuals. It was found in all populations, while haplotype II was found in one individual of BA, ES and PR population, haplotype III in one individual of MA, RN, AL, ES and two of PR, haplotype IV in two individuals of MA and one of BA and haplotype V in one individual of MA, AL and ES.

The demographic history of Brazilian populations of *A. angustipes* was reconstructed by means of mismatch distribution and neutrality tests. The mismatch distribution showed a bimodal distribution of pairwise differences (Fig.7). The values of the neutrality tests (Table 3), when combining all populations (Tajima's D = -1.858, Fu's FS = -24.07), were negative.

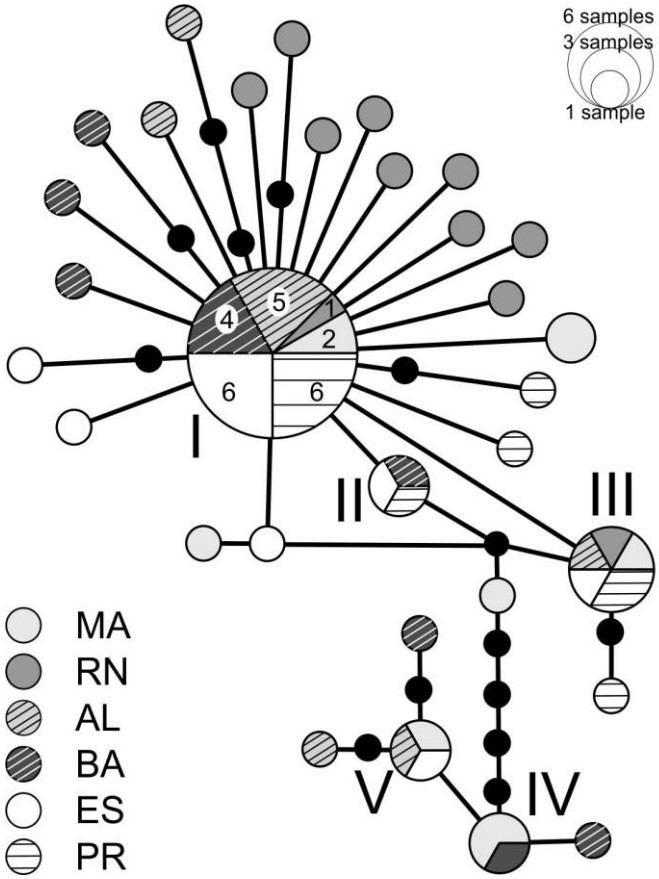

Figure 6. *Armases angustipes*. Maximum parsimony spanning networks of the Cox1 gene on a 941 bp constructed with PopArt. Black dots represent missing haplotypes (one step edges). TR = Trinidad and Tobago, MA = São Luis, RN = Natal, AL = Maceió, BA = Ilhéus, ES = Aracruz and PR = Guaratuba. Arabic numbers = number of individuals within a haplogroup. Roman numbers = haplogroups.

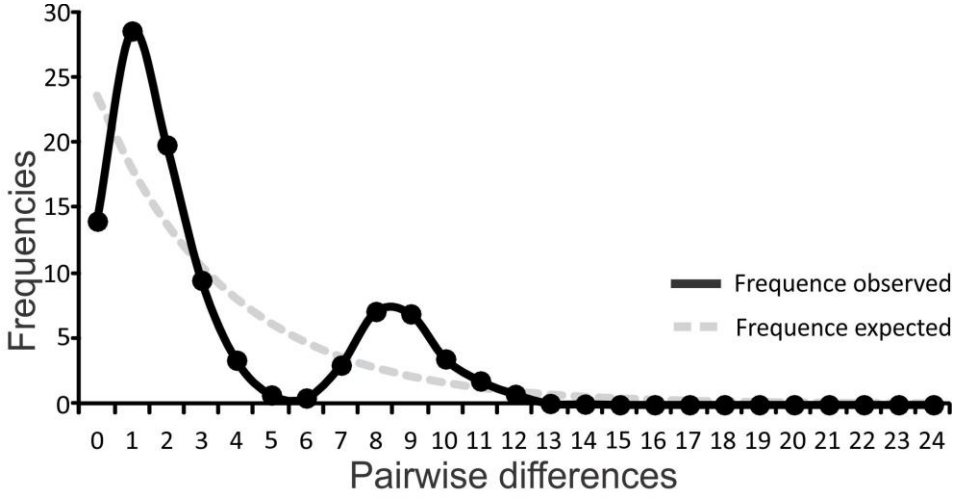

Figure 7. Mismatch distribution for six Brazilian populations of *Armases angustipes.*

The mean pairwise ΦST value among populations is low (0.045) (Table 6). The majority of pairwise differences between populations were not significant, except for the comparison between MA x RN, and MA x PR, i.e. the most distant populations (around 4100 km) (Table 6).

The AMOVA results showed no significant differences between the three tested groups (p = 0.6). No significant differences were found within populations (p = 0.07), although the highest amount of variation (95%) was in this category. The variation among populations within groups was also not significant (p = 0.055) and responsible for 7.41% of the total variation (Table 6).

Table 6. Estimation of pairwise difference of the individual populations *of Armases angustipes*. ΦST-values below diagonal, corresponding p-values above diagonal. Number of permutations: 1023. Numbers in brackets give sample size of the population. **p < 0.01, ***p < 0.001. MA: São Luís, RN: Natal, AL: Maceió, BA: Ilhéus and PR: Guaratuba.

	MA (10)	RN (11)	AL (10)	BA (11)	ES (12)	PR (13)
MA	-	<0.001***	0.33	0.6445	0.1035	0.0068**
RN	0.23762	-	0.2285	0.163	0.1582	0.2207
AL	0.01527	0.0497	-	0.6884	0.7734	0.375
BA	-0.05008	0.1117	-0.0538	-	0.3857	0.1464
ES	0.09841	0.0129	-0.0515	0.00038	-	0.6904
PR	0.21039	0.0104	0.02353	0.08203	-0.0095	-

Source of Variation	df	Sum of squares	Variance components	Percentage of variation	*p* value
Among groups	2	3.639	-0.0481 Va	-2.97	0.6002
Among populations within groups	3	8.628	0.1204 Vb	7.41	0.0547
Within populations	60	93.158	1.5526 Vc	95.55	0.0703
Total	65	105.424	1.695		
Fixation Indices					
FSC: 0.0720					
FST: 0.0044					
FCT:- 0.0296					

Comparative Analysis

The Mantel tests reveal no significant correlation among morphological, genetic and geographic distances for both carapace (r = 0.29, p = 0.22) and right cheliped propodus (r = -0.008, p = 0.49), validating the null hypothesis that the distances matrices are independent from each other (Table 7).

Table 7. One-way and three-way Mantel tests comparing morphological, genetic and geographic distances of populations of *A. angustipes*. r-Value: correlation coefficient.

Comparison	r-Value	p-Value
Simple Mantel test		
Carapace		
Shape x geographic locality	0.22	0.26
Shape x genetic data	-0.23	0.76
Rigth cheliped propodus		
Shape x geographic locality	-0.001	0.48
Shape x genetic data	-0.16	0.71
Genetic data x geographic data	-0.03	0.5
Three-way Mantel test Carapace		
Shape x genetic data x geographic	0.29	0.22
Three-way Mantel test rigth cheliped propodus		
Shape x genetic data x geographic	-0.008	0.49

Discussion

This study investigates the extent of gene flow and phenotypic variation among geographically separated populations of *A. angustipes* and tests the influence of marine currents (CSEC) as a possible barrier for larval dispersal, using mtDNA sequence variation and anatomic landmarks describing the shape of carapace and cheliped propodus. Our results give evidence of a high degree of genetic homogeneity among

populations and significant shape variations over a wide geographic area in Brazil (around 4000 km), suggesting a high level of gene flow and confirming that the split of CSEC is not a barrier for larval dispersal in *A. angustipes*.

Genetic analysis

The absence of a clear spatial genetic structure pattern, with shared haplotypes in populations separated by more than 4000 km and without significant ΦST values between the majority of populations, endorses the hypothesis that this coastal marine species has a high intraspecific connectivity. The same pattern was obtained for South American populations of the sesarmid tree-climbing crab *Aratus pisonii* (H. Milne Edwards, 1853) (Thiercelin 2015) and for other estuarine crabs, like *Leptuca uruguayensis* Nobili, 1901 (Laurenzano et al. 2012), *Minuca rapax* (Smith, 1870) (Laurenzano et al. 2013), *Uca maracoani* (Latreille, 1802) (Wieman et al. 2014), and *Ucides cordatus* (Linneaus, 1763) (Oliveira-Neto et al., 2007). This could be due to the extended planktonic larval phase that often results in a low level of genetic differentiation (Crisp 1978, Gooch 1975) or to movement along the coasts by adults.

Some authors have suggested that genetic connectivity increases with larval dispersal ability of the organims (Neethling et al. 2008). As it is highly unlikely that adults disperse over long distances, the only possible dispersal option is by planktonic larvae, as observed for *Paraleptuca annulipes* (H. Milne Edwards, 1837) along the African coast (Silva et al. 2010). *A. angustipes* has an export larval strategy with long development (around 20 days) in the plankton, consisting of four zoeal stages, before the magalopae return to estuarine waters to complete the development (Anger et al. 1990). This long period can help the species to disperse over large distances forming a

widespread metapopulation, at least for Brazilian populations, similar as assumed by Fratini et al. (2011) for the intertidal crab *Pachygrapsus marmoratus* (Fabricius, 1787) in the Mediterrain Sea.

The AMOVA results also suggest that the split of CSEC is not a strong barrier for larval dispersal in *A. angustipes*. A similar pattern was observed for Brazilian populations of *U. maracoani* (see Wieman et al. 2014). Populations south and north of the split seem to be able to exchange individuals between populations (shared haplotypes). The influence area, effective area and speed of the CSEC can vary seasonally and over the years (Rodrigues et al. 2007). Probably this temporal and seasonal variation are enabling the gene flow among distant populations or the sharing of haplotypes are occurring among regional and "neighboring" populations, resulting in relative homogeneity among regional populations. The reproductive period combined with the seasonally of the CSEC can also influence the gen flow. Ovigerous females are more abundant from January to July (Araújo et al. 2014). The CSEC bifurcation reaches its southernmost position in July, increasing the NBC transport, while the northernmost position is reached in November, increasing the SBC transport. In both cases the seasonal variation is associated with changes in the local wind stress curl (Rodrigues et al. 2007). In this sense, likely the larval dispersal from south to north will be facilitate near July, while the dispersal from north to south near November. Thus, the probability of larval dispersal success will vary seasonally.

The demographic history of Brazilian populations of *A. angustipes* indicates a recent population bottleneck, followed by demographic expansions, as denoted by Tajima´s and Fu´s negative values and the shape of the mismatch distribution. The high haplotype diversities and low nucleotide diversities is also a residual effect of a recent

evolutionary history. Beyond this, individuals from all populations share the most common haplotype, which could also hint towards a recent bottleneck effect. Similar results were obtained for Brazilian populations of the mangrove climbing crab from the family Sesarmidae, *Aratus pisonii* (see Thiercelin 2015) and the Ocypodidae *Minuca rapax* (see Laurenzano et al. 2013), *Leptuca uruguayensis* (see Laurenzano et al. 2012) and *Uca maracoani* (see Wieman et al. 2014).

The recent expansion events and bottleneck effect reflected by the significantly negative values of neutrality test (Tajima's D = -1.85, Fu's FS = -24.07) can be related with recent geological and glaciation events. The closure of the Central American Isthmus (approximately 3.5-2.8 Mya) affects the ocean currents and sundered the range of marine species severing their gene flow (Lessions 2008), as observed for the sister species *Aratus pisonii* and *A. pacificus* by Thiercelin & Schubart (2014) in the Atlantic and Pacific oceans, respectively. Beyond a restricted area of possible occurrence of tropical species due the lower temperatures in northern South America, the glaciation cycles caused a fluctuation of sea levels in the Atlantic subpolar region (approximately 3.2 – 2.7 Mya), also affecting the habitats and the evolution of tropical and shallow water marine organisms (Coates et al. 2004, Lessions 2008). The north part of the South American continent was affected by the Andean uplift, reorganizing the drainage systems of the continent, changing the outflows of Orinoco and Amazon rivers as well as increasing the amount of discharge waters (Hoorn et al. 1995, 2010). These events could have restricted the occurrence of Sesarmidae and other mangrove-related crabs to the Caribbean region and posteriorly, with the increase of temperatures, this species possibly colonized South America in one or multiple events. But this assumption has not been tested so far and further studies are necessary to support it.

Although there is no clear genetic spatial structure, there is a tendency of higher haplotype and nucleotide diversities in northern populations of the present study (closer to the Caribbean region). This trend suggests a possible lineage origin in northern South American or Central America populations, and can be related with historical geological and climatological events that influence the current species distribution from isolated populations of the Caribbean. In crabs, expansion events have been correlated following changes in sea level and temperature during interglacial cycles (Ituarte et al. 2012, Favier & Scartascini 2012). In a population genetic study by Laurenzano et al. (2013) with Caribbean and mainland (South American) populations of *Minuca rapax* the authors did not detect differentiation among South American populations, but between Caribbean populations and between Caribbean and South American populations, suggesting a genetic population structure with South American homogeneity and Caribbean heterogeneity.

Morphometric analyses

In opposition to the genetic data, the six Brazilian populations of *A. angustipes* exhibit significant intraspecific variation in shape, but not in size, of carapace and right cheliped propodus with morphological spatial structure, especially for carapace. A similar pattern of morphological population variation shape for Brazilian populations was obtained for *Uca maracoani* (see Wieman et al. 2014), and for eight additional species of fiddler crabs (Hampton et al. 2014), for fiddler crabs of Caribbean and eastern North America (Hopkins & Thurman 2010), and for the sesarmid crab *Perisesarma guttatum* (A. Milne-Edwards, 1869) along the East African coast (Silva et al. 2010). There was a morphological population structure with three main groups: (1)

MA and RN, (2) AL and BA, (3) ES and PR, based on the MANOVA result. Probably the main factors grouping the morphology of the populations are the similar selective pressures, like environmental conditions that are driving the phenotypic plasticity regionally in the same direction and diverge from each other. However, the cluster shows a distinct grouping. This analysis was based on the mean Mahalanobis distances among populations, while the MANOVA uses the first and second PC scores to evaluate the differences among groups of populations. The groups were morphologically distinct among each other, but some populations can have a closer morphological distance to populations belonging to other groups than those from the same group.

The most important morphometric differences observed concerned the frontal region of the carapace and the fixed finger area of the right cheliped propodus. The morphological variance among populations can be related to phenotypic plasticity driven by environmental differences between localities (Hampton et al. 2014, Sanford & Kelly 2011).

A. angustipes inhabits very different habitats in estuarine areas with very different physical, chemical (e.g., salinity, temperature, tidal regimes) and ecological (e.g., intra and interspecific interactions, food availability) conditions throughout its geographical range. This amplitude of habitats may account for the local morphological divergence, as well as phenotypic plasticity. In general, the populations sampled live in marginal regions of mangroves with distinction in the substrate, being sandy or organic rich soil. The populations of AL, ES and MA occupy habitats with sandy bottoms and all of them were grouped in the same branch. Individuals from BA and RN that occupy forest beyond mangrove areas with organic rich soil, formed a sister branch in the

cluster analyses of carapace (Fig. 4A) and showed morphological variance related to the kind of habitat in the MANOVA analysis. This morphological similarity among populations from similar substrate may reflect similar pressures that the individuals are submitted to (e.g., predators, food source and availability) and that are influencing the carapace shape, since phenotypic plasticity will be favored over local adaptation (Sotka 2012).

The sandy bottoms can be a more stressful environment to desiccation than forest soil, when humidity is high. Hampton et al. (2014) showed that the intraspecific morphological variation found in eight species of fiddler crabs can be related to humidity variation, to its relationship to water conservation in the gill chambers. The authors infer that humidity differences can influence gene expression and morphological variation via an unidentified epigenetic mechanism. The same pattern can happen with the species in the present study. But no known factor is driving the morphological variation in populations of *A. angustipes*.

The southernmost population (PR) was also the most distinct in both carapace and cheliped analyses and show the larger morphological distances among populations (Table 3 and 4). This fact may be related to distinct physical conditions, for example lower mean temperatures and higher mean annual rainfall, when compared to the other populations localities (Alvares et al. 2014) or other ecological factors as discussed previously.

The differences of morphological similarity among populations when comparing carapace and right cheliped propodus are probably related to the plasticity in the shape of the carapace and cheliped propodus. In general, the carapace is considerate a more conservative character than chelipeds in Brachyura (Harrison & Crespi 1999). In this

sense, is expected that the cheliped propodus exhibit a higher level of shape variation caused by factors distinct from genetic sources than the carapace. This is supported by the greater correlations (even if not statistically significant) between genetic and morphological distances based on the carapace than the chelipeds (Table 7). In Brachyura, the size and shape of cheliped are strongly influenced by sexual selection (especially in males) due the cohort behavior and male-male combat (Callander et al. 2013). Distinct population size or sexual proportion in a population may affect the cheliped shape locally. Another factor affecting the cheliped shape can be the prey/food type, quality and availability (Smith & Palmer, 1994). Both hypotheses may affect the cheliped shape in a short time period, reflecting a divergent morphological intraspecific variation when compare to other anatomical structure.

Combined analyses

In the present study there are no correlations between morphological versus genetic and geographic distances (Mantel test results). The incongruence between genetic and morphological results suggests that processes responsible for the pattern of mtDNA are different from those impacting morphology. Contrasting patterns of morphological and genetic variation among populations are relatively common and reflect the contrasting effects of gene flow and local adaptation. This incongruence was also observed for species of fiddler crabs and *Neohelice* from the South American coast (Ituarte et al. 2012, Wieman et al. 2014, Hampton et al. 2014).

The morphological and genetic incongruence can be derived from phenotypic plasticity, incomplete lineage sorting, or very recent and ongoing genetic divergence, as well as environmental factors (as previously discuss) (Vogt 2008, Sotka 2012, Wieman

et al. 2014). The most likely explation for this incongruence is the phenotypic plasticity. Concerning the plasticity, different phenotypes can arise from the same genotype, but submitted to different environmental conditions. The large variety of biological factors (previously discuss) that may act over the shape "operate" on a recent scale of time. On the other hand, the genetic influence over population variability acts on a large scale of time. The incomplete lineage sorting can occur when the effective population is large (probably not the case of *A. angustipes*) or the coalescence time of genetic marker is not sufficient to observe variation on a certain scale (Wieman et al. 2014). In this sense, the morphological and genetic incongruence are probably related to the necessary amount of time that the body shape and genetic markers need to show intraspesific variation.

Conclusions

Our study revealed no clear correlation between morphological and genetic variation to ocean currents and geographic distances in Brazilian populations of *A. angustipes*. We reject the hypothesis of the CSEC acting as a barrier for larval dispersal. The considerable panmixia among populations from different estuaries with concurrent phenotypic divergence indicates that local populations are demographically interdependent, with larval exchange among adjacent estuaries. Our results also indicate that historical geological and climatological events as well as possible bottleneck effects influenced the present low genetic variability. The established morphological differentiation can be a plastic response driven by distinct environmental selective pressures, and not a direct cause of genetic differentiation. Future studies using more sensitive markers such as microsatellites probably can be useful in highlighting recent demographic dispersal events. The morphological and genetic data have brought complementary information about population variability for the specie

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Chapter V

Does water deprivation affect mother physiology or embryonic development in semi-terrestrial crabs?

Abstract

Female crabs carry their eggs under the abdomen, which are fixed through filaments to the pleopods during the embryonic period. In semi-terrestrial species the incubation is a critical period for both, females and embryos, due to the costs and effort necessary for maintaining the egg mass during incubation and avoids dehydration. In this sense, the aims of the present study were: evaluate the effect of water deprivation on the osmolality of hemolymph and gills carbonic anhydrase activity of non-ovigerous and ovigerous females, and on the egg volume and larvae survival rate using the semi-terrestrial species *A. pisonii* as a model. Twelve ovigerous females were divided in three treatments of water deprivation: six (6H), twelve (12H) and eighteen hours (18H) perday without access to water. Four females with free water contact were used as positive control. Four non-ovigerous females were used as negative control with free water contact. The experiment was carried out until the larvae hatching, which lasted 14-19 days. The water deprivation of ovigerous females for 18 hours affect the embryonic development, the egg volume and percentage of not hatched larvae, hence, the larvae survival. The osmolality was higher in ovigerous females shortly after eclosion, while the carbonic anhydrase activity was higher in non ovigerous females. There were differences between anterior and posterior gills weigth only in 18H of water deprivation. The main physiological variance found in ovigerous semi-terrestrial crabs can be an indirect effect of the weight increased that the egg mass entails over mother´s body, and possibly associated with the action of hormones rate during hatching period.

Key-words: Carbonic anhydrase, Osmolality, Osmotic stress, dehydration, larval survival, Guaratuba bay.

Introduction

The evolution from marine to terrestrial and semi-terrestrial life has been a recurrent process in crustacean radiation (Little 1990). This shift requires a transitional phase in which the lineages gradually adapt to the new environmental requirements for maintenance of body homeostasis with a gradual conquest via semi-aquatic or semi-terrestrial zones (Simoni et al. 2011). This process is relatively recent in Crustacea, with fossil records dated the initial of semi-terrestrial life style in brachyuran crabs to the Cenozoic period (Naruse et al. 2003). Information about the physiological, morphological and behavioral traits of semi-terrestrial and terrestrial species aids to the understanding of this evolutionary process (Little 1989, Powers & Bliss 1983).

As a consequence of the recent adaptive process of evolution, in most land-adapted brachyurans, unlike the juvenile and adult stages, the embryonic and larval stages are strongly dependent to marine or other aquatic environments (Anger 1995a, Simoni et al. 2011). Adults and juveniles of semi-terrestrial crab species evolved integrated strategies, encompassing morphological (e.g. plumose setae at the base of pereiopods), respiratory (e.g. unsophisticated lungs), excretory (e.g. ammonia and urate stored in the body and reduction in the metabolic rate in absence of water for long periods) and behavioural (e.g. directly drink water by the use of chelipds) adaptations to overcome the constraints of aerial and terrestrial environments (Greenaway 1988, Little 1990, Linton & Greenaway 1995, Greenaway 1999, Morris 2002, Weihrauch et al., 2004). On the other hand, although embryos have ability to extract oxygen from air, they have water dependence especially for vital metabolic pathways, such as the excretion of ammonia and CO2, as well as to avoid desiccation (Simoni et al. 2011).

This amphibious life-cycle affects the life-history patterns and requires active parental behavior care by females.

Female crabs carry their eggs under the abdomen, which are fixed through filaments to the pleopods during the embryonic period, and periodically relocate the position of eggs in the egg mass to provide water contact to all embryos (Simoni et al. 2011). In semi-terrestrial estuarine crabs the contact with water is common and frequent due the proximity to water sources, but ovigerous females tend to be closer to water sources or go towards more often than non-ovigerous females (Wolcott 1988). This strategy allows an active protection of the brood, but result in a great energy costs by the mother (Ruiz-Tagle et al. 2002, Fernández & Brante 2003, Simoni et al. 2011).

In same species there is also a behavior change during the egg incubation. This strategy is observed in ovigerous females of aquatic crabs such as *Cancer pagurus* Linnaeus, 1758 that is less active than non-ovigerous, move to deeper and calmer water to incubate their eggs and show lower average rates of oxygen uptake (Nichols et al. 1982, Naylor et al. 1997). Despite the well-known behavior changes of ovigerous females the likely physiological changes in the mother body are poorly known.

Among the physiological mechanisms, the enzyme carbonic anhydrase (CA) are considered a key element in crustaceans because the role in acid–base regulation, respiration, calcification and ion regulation (Vitale et al 1999, Skaggs & Henry 2002, Lionetto et al. 2012). This enzyme catalyzes the hydration reaction of CO_2, and regulate the Na^+/H^+ e Cl^-/HCO_3^- íon exchange (for more details see Henry 1996, Henry et al. 2012), being a useful tool to evaluate physiological patterns and process.

The tree climbing crab *Aratus pisonii* (H. Milne Edwards, 1837) occurs on the surface of branches and trunks of mangrove trees, mainly on *Rhizophora mangle*,

spending most of its time out of water. There are a variety of crabs that climb trees occasionally, but *A. pisonii* is the only one which spends almost whole life in trees (von Hagen 1977, Wolcott 1988). The species is considerate a semi-terrestrial species with amphibious life-cycle (Warner 1967, Diaz & Conde 1988, Wolcott 1988). The specialization to semi-terrestrial life described for the species are related to the long tufts of setae between the bases of the walking legs, which function to transport water into the branchial chamber, and a dense array of geniculate setae on the pterygostomial regions of the carapace which function is related to the circulation of water from the gill chamber over the outer surface of the carapace in order to oxygenate it (Wolcott 1988). The species do not have specialized lungs like terrestrial crabs to breath, instead it obtain oxygen of the air by flowing a thin film of water over the branchiostegites (a reticulated expansion of the carapace covering the gills) (Warner 1977). The ovigerous females (OF) incubate the eggs for around 16 days, tending to migrate toward to the fringe of the mangroves (Wolcott 1988). The OF are observed in lower arboreal strata (closer to the water level) (personal observation). This behavior change during the incubation period can be an indicative of adaptation for arboreal life and the physiology of the mother body is also likely to be affected.

The incubation period is a critical life stage in semi-terrestrial crabs for females and embryos. The mother has to carry an excessive weight and likely it affects their physiology. On the other hand, the survival rate of embryos depends on physical conditions during the development and variations on “optium conditions” (ex: periods without water contact, temperature of water or air) can reduce the viability of the egg mass. In this sense, the aims of the present study are: to evaluate the effect of water deprivation on the osmolality of hemolymph and gills CA activity of non-ovigerous and

ovigerous females, and on the egg volume and larvae survival rate using the semi-terrestrial species *A. pisonii* as a model.

Material and Methods

Sampling of Aratus pisonii and experiment procedures

We sampled manually 31 adult females. Among them, 27 ovigerous in initial stage of egg embryonic development and four non-ovigerous from mangrove trees of Guaratuba Bay, Paraná State, Brazil (25°51'41.22"S – 48°35'23.56"W). The crabs were transported to the laboratory. They were acclimated to laboratory conditions in photoperiod of 12h light: 12h dark and constant temperature of 25 ± 2°C for 2 days. Each female was maintained in individual aquariums of five liters containing a small rock, 150 ml of water (25 ‰) and plastic tubes glued on the wall of aquariums to simulate tree branches. The aquarium water was obtained by dissolution of artificial refined sea salt without iodine into deionized water. The crabs had free access to food (leaves of *Rhizophora mangle*, carrots and *Tenebrium sp*. larvae) before and during the experiments, but free water contact only during acclimatization.

Experimental design

The model species occur in estuarine areas with semi-diurnal tides (Angulo et al. 2006). This tide regime naturally exposes the crabs to water deprivation of 6h to 12h (depend on wind and other physical forces). To evaluate the effect of water deprivation on larval development and on mother's phisiollogy we purpose an experiment comparing natural periods of water deprivation (6h and 12h) and a period beyond the normal (18h).

Twelve ovigerous females were divided in three treatments of water deprivation: six (6H), twelve (12H) and eighteen hours (18H). Four females with free water contact were used as positive control. Four non-ovigerous females were used as negative control with free water contact. The water deprivation was performed by moving the ovigerous females to another similar aquarium but without water for 6, 12 or 18 hours, every day. The experiment was carried out until the egg hatching, which lasted 14-19 days. The initial and final egg volumes were accessed removing 15 eggs from each ovigerous females in two occasions: 1) one day before the beginning of experiments in initial stage of development (embryos in stage II: gastrulation) and 2) on the eighth day of experiment (stage IV: embryos with developed eyes). These categories were based on Simoni et al. (2011). After egg hatching, the numbers of alive and dead larvae as well as not hatched eggs were counted. Crabs were then quickly killed by cutting the cerebral glanglion on the frontal region of the carapace. The anterior and posterior gills (of both sides of caparace) of these females were removed and immediately frozen at −80 °C for the carbonic anhydrase (CA) assay.

The egg mass in initial stage of embryos development of another eleven ovigerous females were weighed, as well as the total weight of the females. These data were used to calculate the relative weight of the egg mass in percentage (weight of egg mass/weight of female). After weigthed, the egg mass of these females were frozen at -20°C for osmolality assay.

Egg volumes

Photos of the eggs were obtained with aid of a digital microscope Dino-Lite Pro AM413 (AnMo Electronics Corporation, Hsinchu, Taiwan). The egg volume was

calculated from measurements of the minor and major axis of the eggs, using the formula for oblate spheroids: $V=1/6(\pi.d^2.D)$, where d=smaller diameter, D=larger diameter (Simoni et al. 2011).

Osmolality dosage and braquial carbonic anhydrase activity

The osmolality of the water from aquariums, of the hemolymph samples and from eggs was measured in a micro-osmometer of vapor pressure (Wescor VAPRO 5520) in undiluted samples.

The gills were weighted and homogenized with buffer (approximately 10% of tissue weigh). The resulting solution was centrifuged at 2000xg by five minutes at 4°C, and the supernatant was aliquoted for analyses. The carbonic anhydrase activity (CA) was assayed using the method established by Vitale et al. (1999) and described by Souza-Bastos & Freire (2009). As a negative control for this technique, the same protocol was used with buffer added acetazolamide that inhibit the CA action. The final concentrations were 100 nM and 100 µM respectively. These concentrations was based on Henry et al. (2012). The homogenization buffer consists of mannitol 225 mM, saccharose 75 mM, Tris-phosphate 10 mM, with pH adjusted to 7.4. The total concentration of proteins of each homogenization was obtained by the method of Bradford (1976), in order to calculate the CA activity.

Statistical analysis

The effect of water deprivation treatments on the larvae survival rate and egg volume was evaluated through a Kruskal-Wallis test and analysis of variance (ANOVA), respectively, with a *post hoc* test of Tukey. The difference in the osmolality

and CA activity between treatments and females hatching eggs was evaluated through an ANOVA with *post hoc* of Holm-Sidak. The difference between the hemolymph of females hatching eggs and their respective eggs was evaluated through a t test. The osmolality of aquarium water was compare with hemolymph of females from each treatment by confidence interval. The CA activity and size differences between anterior and posterior gills were evaluated through a t test. Statistical analyses were performed in R environment (R Development Core Team 2011; available at: www.R-project.org).

Results

The carapace width (CW) of female ranged from 12.96 to 19.3 mm and did not vary between treatments ($p = 0.26$). The egg mass weight ranged from 0.21 to 1.12g, and the relative weight of egg mass relative ranged from 9.92 to 15.47% with an average of 12.11% in blastula stage (I), 12.23% in gastrula stage (II) and 14.97% in stage III (Table 1).

Table 1. *Aratus pisonii*. Carapace width (CW), embryonic stage, female´s total weight (Fweight), egg mass weight and relative weight of egg mass (IET). The categorization of the embryonic stages was based on Simoni et al. (2011).

CW (mm)	Embryonic stage	Fweight (g)	Egg mass weight (g)	IET (%)
16.25	I: Blastula	1.82	0.24	13.73
18.99	I: Blastula	2.60	0.33	12.69
19.09	I: Blastula	2.82	0.27	9.92
16.12	II: Gastrula	1.90	0.25	13.15
16.72	II: Gastrula	1.93	0.22	11.39
16.84	II: Gastrula	2.13	0.25	11.73
16.97	II: Gastrula	2.25	0.32	14.22
17.38	II: Gastrula	2.14	0.26	12.14
21.80	II: Gastrula	4.09	0.44	10.75
14.73	III: Appearance of eyes	1.45	0.21	14.48
18.10	III: Appearance of eyes	2.52	0.39	15.47

Egg volume and hatching success

The initial egg volumes did not differ between ovigerous females ($p = 0.07$). These indicate that any variation of the final volume would be a consequence of the treatments and not of the maternal origin. The treatments of water deprivation affected the final egg volumes ($p < 0.001$). The larger volumes were observed for 6H and 12H treatments follow by control and 18H treatment (Fig.1).

The deprivation on water contact of ovigerous females affected the larvae survival and the embryonic development ($p = 0.01$). The larval survival percentage in control (92%), 6H (87%) and 12H (89%) differed from 18H (0.04%) (Fig. 2). In the same way, the percentage of not hatched eggs was also influenced by water deprivation ($p = 0.03$), being the treatment 18H distinct from the others (Fig. 2).

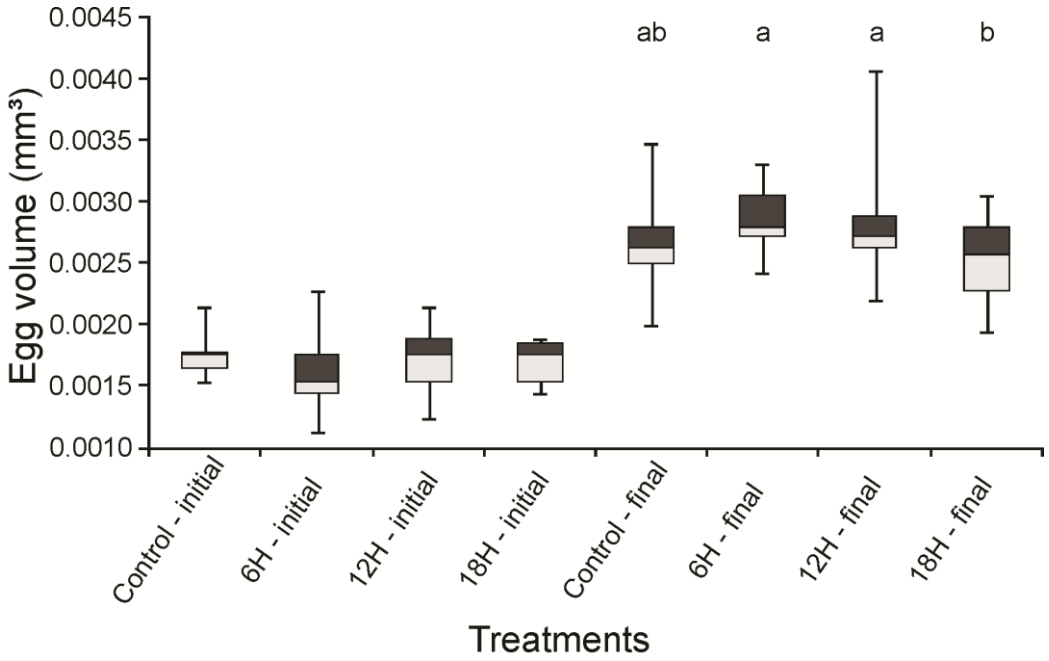

Figure 1. *Aratus pisonii*. Median (horizontal line), maximum/minimum values (vertical lines in each box) and range of 50% of data (boxes) of egg volumes at initial and final embryonic development stages in the three water deprivation treatments.

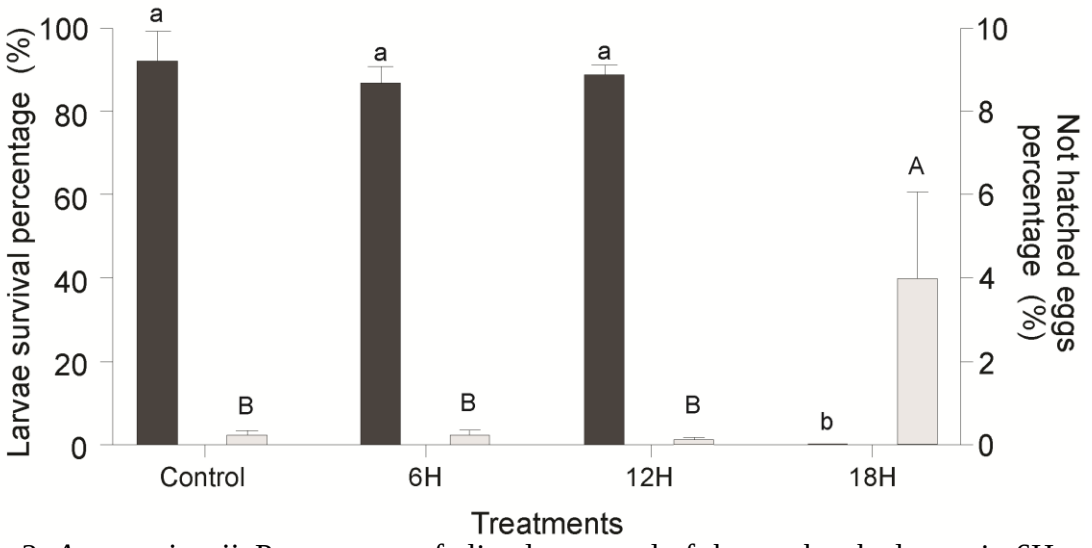

Figure 2. *Aratus pisonii*. Percentages of alive larvae and of the not hatched eggs in 6H, 12H, 18H treatments of water deprivation and with free water contact (control). Dark grey bars indicate alive larvae and light grey, not hatched eggs. Lowercase letters: *post hoc* test of Tukey for larvae data. Capital letters: *post hoc* test of Tukey for egg data.

Hemolymph osmolality, gill carbonic anhydrase activity (CA) and gills relative weight

The hemolymph osmolality of non-ovigerous females were significantly lower than ovigerous females ($p < 0.001$, $f = 15.35$) (Fig. 3). The osmolality of aquarium water was inferior to all ovigerous females but similar to non-ovigerous and eggs in initial stage (Fig. 3). The osmolality of the females hatching eggs (initial stage of development) were higher than their eggs ($t = -5.03$, $p < 0.001$) (Fig. 3).

The CA activity was greater in the posterior gills than in the anterior ones in all females ($p < 0.001$) (Fig. 4). The CA activity in posterior gills of non-ovigerous was superior to ovigerous females, while the CA activity in anterior gills varied between non-ovigerous and females incubating eggs compared to females that have their eggs hatched ($p = 0.001$) (Fig. 4).

The relative weight of the gills was similar among treatments (anterior: $p = 0.53$, posterior: $p = 0.57$) and between posterior and anterior gill weight within in each

treatment ($p > 0.05$), except for 18H treatment that show significant difference between posterior and anterior gill weight ($p = 0.006$) (Fig. 5). Although the differences among treatments were not significant, there was a trend towards an increase of the relative weight of posterior gills in ovigerous females under treatments. On the other hand, there is also a trend towards an increase of the relative weight of anterior gills in non-ovigerous females (Fig. 5).

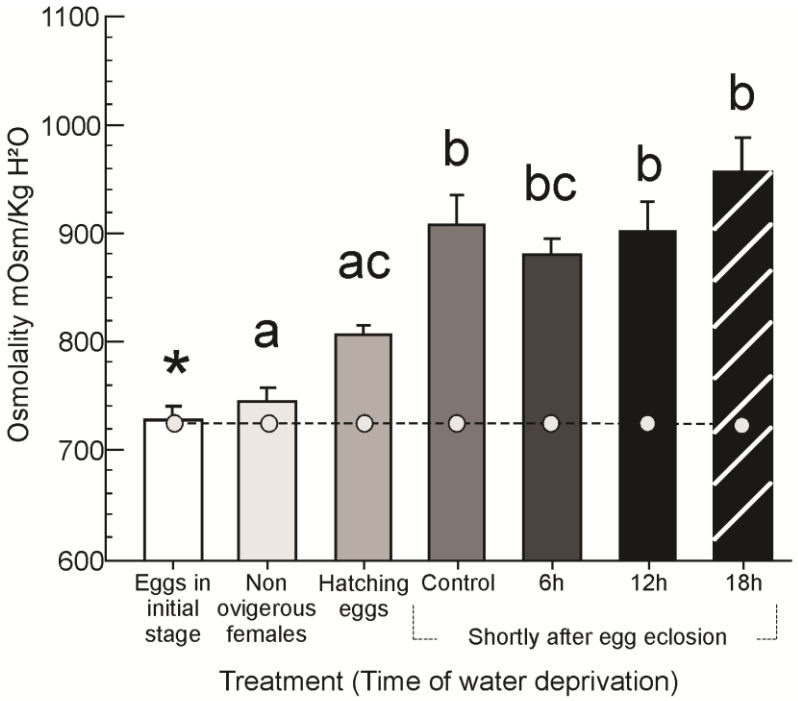

Figure 3. *Aratus pisonii*. Osmolality of the aquarium water (dashed line), eggs (initial stage of development) and of the hemolymph of non-ovigerous and ovigerous females under treatments. Letters: differences among treatments, non-ovigerous and females hatching eggs. Asterisk: difference between females haching eggs and their eggs.

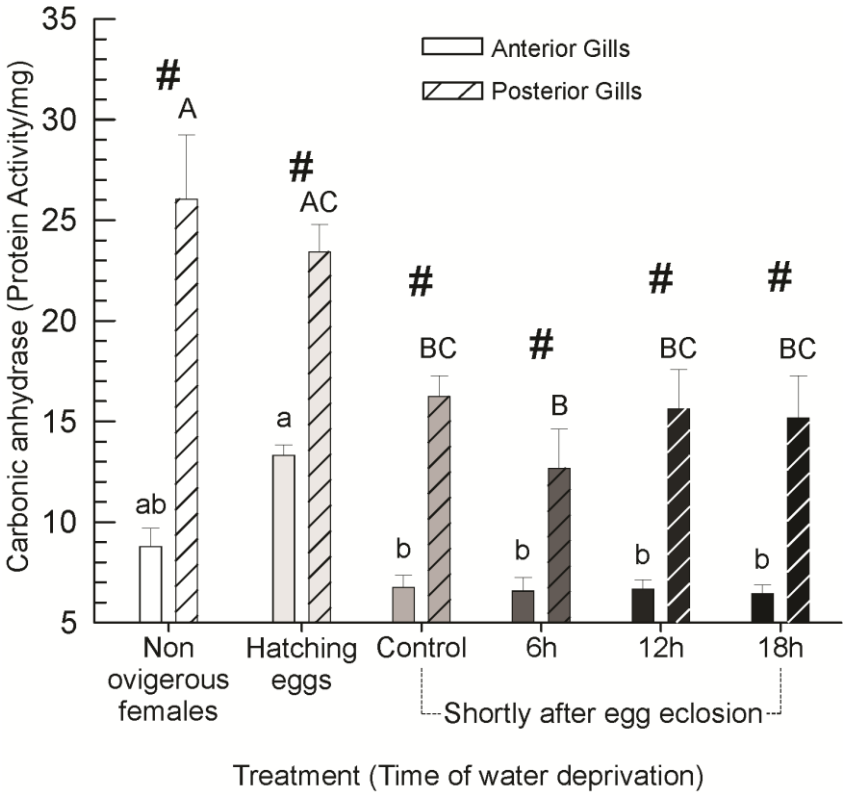

Figure 4. *Aratus pisonii*. Carbonic anhydrase activity in non-ovigerous and ovigerous females under experimental conditions. Bars: carbonic anhydrase activity. #: significant difference in carbonic anhydrase activity between anterior and posterior gills in the same treatment. Capital letters: differences in posterior gills anhydrase activity among treatments. Lowercase letters: differences in anterior gills anhydrase activity among treatments.

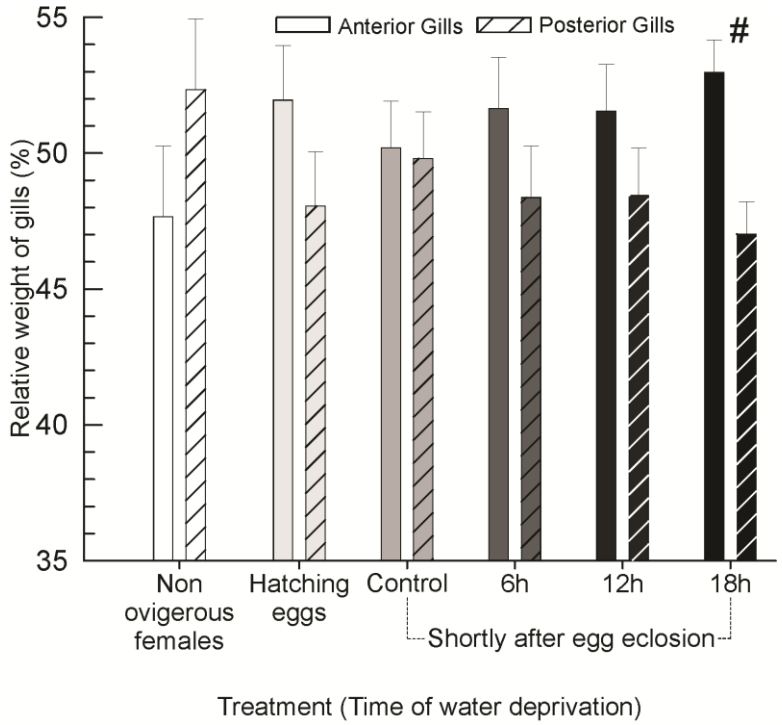

Figure 5. *Aratus pisonii*. Relative weight of anterior and posterior gills in non-ovigerous and ovigerous females. #: differences in the relative weight of anterior and posterior gills within the same treatment.

Discussion

Egg volume and hatching success

Our data suggest that the limit time of water deprivation to *Aratus pisonii* embryos successfully develop, and consequently the maximum time intervals that the ovigerous females should get in contact with water, is between 12 -18h. The limited access to water for ovigerous females in our experiments resulted in egg volume variation (18h<control<12h<6h), low hatching success with almost total larvae mortality and increase in unhatched embryos in the most extreme treatment (18h). These results can be explained by two hypotheses: (1) metabolic rate is limited by reduced oxygen extraction from air or (2) is a consequence of water-dependence of other metabolic processes such as ammonia excretion, acid–base an ionic balance of embryos, consequently affecting the development in both hypothetic cases.

During the development, embryos of semi-terrestrial crabs, are capable to breathing from both air and water but the respiration rate is higher in water than in air, despite the higher oxygen content and faster diffusion in the air than in the water, as observed by Simoni et al. (2011) for the sesarmid crab *Armases miersii* (Rathbun 1897). This reduced ability of embryos to extract oxygen from the air and the combined effects of surface tension and gravity may produce a collapse of the interstices within the egg mass, generating anoxic regions in the egg mass by preventing oxygen diffusion as observed in invertebrate and vertebrate species with gelatinous egg masses (Seymour, 1999, Strathmann & Chaffee 1984, Strathmann & Hess 1999, Simoni et al. 2011). The reduced oxygen uptake at the tissue level for long periods, could induce a metabolic depression, causing e.g. bradycardia or other abnormalities related with low level of oxygen as observed in embryos of crustaceans experiencing anoxic conditions in water (Reiber 1997, Spicer 2001).

If is assumed that the metabolic rate is not limited by reduced oxygen extraction from the air, once the embryos are capable of extracting the necessary amount of oxygen through the thin layer of water on the egg surface, other metabolic pathways would still need a constant supply of water. In this situation the water evaporation from eggs could increase the osmotic concentration due to dehydration, or the disappearance of the water layer on the egg surface could hamper the ammonia excretion hence cause a disturbance of the acid–base and ionic balance, as inferred by Simoni et al. (2011), for the variation on the egg volume and hatching success.

Our egg volume data support the disturbance of osmoregulation and excretion in embryos exposed to the air. The egg volume in the stage IV (with eight days of experiment) was higher in treatments 6h and 12h than control (6.2% and 3.2% larger, respectively). This pattern can be a consequence of a continuous accumulation of catabolic end products but without affecting the development of the embryo, as observed for the similar larvae survival when compare to control. However, in the treatment 18h the volume was 8.22% minor than control, suggesting that the accumulation of metabolic excreta affect the embryos development, as observed by the lower hatching success and rate of larvae survival. A similar pattern was reported also for the semi-terrestrial crabs *Neohelice granulata* (Dana, 1851) and *Armases miersii* (Bas & Spivak 2000, Simoni et al. 2011) when embryos were exposed to periods of dehydration. Since the embryos are capable to obtain oxygen in air, even in a minor rate than in water, it is more likely that the main cause of low larvae survival rate and increased unhatched eggs are the accumulation of metabolic excreta or combined, but not exclusively, with reduced oxygen extraction.

Hemolymph osmolality and gill carbonic anhydrase activity

The hemolymph osmolality was higher in ovigerous (hatching eggs and shortly after eclosion) than non-ovigerous females. This pattern can be an indirect effect of the larger weight entailed by the egg mass (corresponding for 12-15% of total weight of ovigerous females) hence, resulting in a great energy cost by the expenditure of the mother. If it is assumed that the ovigerous females of *A. pisonii* did not reduce their activity, as aquatics brachyuran species, the higher energy cost will lead to an increase in metabolic excreta, such as NH_4^+. In fact, the ovigerous females tend to increase the frequency of water contact and move towards to the trees located on the edges of mangrove areas (closer to the water) (Wolcott 1988). The increase in excreta leads to an activity increase of excretory enzymes. However, the CA activity was higher in non-ovigerous than ovigerous females. The role of excretory ion activity can be performed by another enzyme as Na^+/K^+ATPase, the most important enzyme relate to ion regulation in crustaceans (Henry et al. 2012), and likely was used by ovigerous females. This enzyme accepts NH_4^+ in addition to K^+ as substrate (Skou 1960), thus excreting ammonia ions outside of gills epithelium and absorbed Na^+ in the hemolymph, what can explain the higher osmolality in ovigerous females. This change of ammonia instead of K^+ in the Na^+/K^+ATPase have been already observed for the freshwater shrimp *Macrobrachium olfersii* (Wiegmann, 1836), in which high levels of ammonia stimulates ATP hydrolysis to rates higher than those for K^+ ions (Furriel et al. 2004). For air-breathing crabs the NH_4^+ excretion is commonly associated with sodium transport in the Na^+/K^+ATPase in gills membrane (Weihrauch 2004). High hemolymph osmolality can facilitate the water conservation in ovigerous female due the higher hemolymph

osmolality favor the water absorption during water contact (Bliss 1979, Henry 1984). Since the water contact exposes the females to predators and during the hatching period the mothers have to do this more frequently (Wolcott 1988), this physiological adaptation can facilitate for obtaining of the necessary water provisions faster than "normal" hemolyph osmolality.

The higher hemolymph osmolality of ovigerous females can also be an indirect effect of hormones related to, or that play a role in, reproduction. The occurrence of steroid hormones similar to vertebrate hormones (estradiol 17β and progesterone) are described in decapods, although their precise role in female reproduction is not clear (Warrier et al. 2001). Theses hormones are involved especially in the vitellogenesis until the release of mature eggs from the gonad, decreasing in the female during the embryonic development and are present in eggs of some decapods species likely play a role in the embryogenesis (Quinitio et al. 1991, Warrier et al 2001). Since the present experiment begun with females with eggs extruded in the pleopods, is unlikely that these hormones influenced the osmolality during the experiment.

The ecdysteroids are hormones especially related with growth in crustaceans, with a strong effect in ionic balance. Generally molting and female reproduction are antagonistic events in crustaceans (Gunamalai et al. 2004). However, the body size influences the patterns of growth and reproduction. In large-bodied decapods the female reproduction blocks the growth, while other small-bodied species like isopods, shrimps and anomurans exhibit overlapping reproductive and molting activities (allowing both new cuticle synthesis and ovarian maturation to occur synchronously) (Blanchet 1972, Crocos 1991, Vafopoulou & Steel 1995, Gunamalai et al. 2004). The growth hormone 20-hydroxyecdysone is negatively regulated by two neuropeptides: molt-inhibiting

hormone (MIH) and crustacean hyperglycemic hormone (CHH) (Webster et al. 2012). The CHH is involved in hyperglycemic activity and in the control of branchial ionic transport. In experimental conditions the CHH significantly stimulates the Na^+ influx in perfuse posterior gills of *Pachygrapsus marmoratus* (Fabricius, 1787), indicating a direct effect of this neuropeptide on the posterior gills, the main osmoregulatory organ in crabs (Spanings-Pierrot et al. 2000). If *A. pisonii* did not exhibit overlapping reproductive and molting activities, the inhibitory hormones of growth are present in the hemolymph of females. In this sense, the CHH can stimulate the Na^+ influx in a higher rate in ovigerous (that are under influence of CHH) than non-ovigerous females, thus increasing the osmolality.

The hemolymph osmolality in females shortly after eclosion was higher than those hatching eggs. This pattern can be a consequence of the start of pre-moulting period. In crustaceans the hemolymph ionic concentrations are higher in pre-moult than inter-moult and after-moult periods. This increase occurs mainly due the calcium reabsorption from the old cuticle (Sàrda et al. 1989, Chang 1995). This calcium reabsorption can increase the hemolymph osmolality, but is unlikely that it is occurring after some minutes to few hours after egg eclosion. Another possible hypothesis is related to a new oviposition. In the majority of brachyurans, females posses an cavity used to store sperm (spermatheca) who allows some oviposition without a new mating (Guinot & Quenette 2005). In this sense a new oogenesis could be occurring after the eggs eclosion. The oogenesis is an energetically expensive reproductive process and the main precursor molecule of vitellin (a nutrient source for developing embryos) is transported through the mother´s hemolymph to developing oocytes (Tsukimura 2004). Thus, the oogenesis for a new oviposition can increase the hemolymph osmolality of

mother. Both hypotheses can be occurring, but one is antagonistic to another, since the hormones involved in these processes are antagonistic and have inhibitory effect over the other one.

The osmolality of the egg mass were very similar to the osmolality of water. This was a expect pattern, since the embryos have none osmorregulatory capability in the early embryonic stage and tend to be isosmotic to water (Charmantier and Charmantier-Daures 2001).

The CA activity was distinct in anterior and posterior gills in all females with higher activity in posterior gills. This pattern is occurring due the position of gills influence their functionality, hence the necessary quantity of enzyme to assist these functions. The anterior gills are commonly associated with respiration and acid-base balance functions, while the posteriors perform an osmoregulatory function in crustaceans (Henry 1996, Henry et al. 2012, Rivera-Ingraham et al. 2016).

In the anterior gills the CA activity was higher in females during incubation period than those that had hatched their eggs (independent of the time of water deprivation analyzed). The observed pattern can be an indirect consequence of the higher respiratory demand that the eggs weight increase causes to the mother, with higher rates during egg incubation when compare to females that had hatched their eggs. With the increase of weight the oxygen consumption of brooding females also increased throughout embryonic development (Baeza & Fernández 2002). Higher oxygen consumption also increases the CO_2 excretion. As a consequence of higher CO_2 excretion the hemolymph tends to alkalosis, due to the reversible reaction of hydration and dehydration of CO_2 (H^+ and $HCO3^-$) (Henry et al. 2012). Another molecule that can vary the hemolymph pH is ammonia (Henry et al. 2012), as mentioned above. With a

variance in pH, the CA activity tend to be higher in the sense of maintain the acid-base balance. This increase of CA activity during the egg incubation seems to be a physiological adaptation to the maintained of mother body homeostasis.

On the other hand, in the posterior gills the CA activity was higher in non-ovigerous, intermediate in ovigerous females hatching eggs and lower in females after hatched the eggs. The low CA activity can be a consequence of the enzyme inhibition by high hemolymph osmolality levels during and shortly after the hatching of the eggs in ovigerous females. As discussed previously, if ammonia is in high concentration in the hemolymph it will cause high hemolymph osmolality (due the action of Na^+/K^+ATPase). In high osmolality levels (especially NaCl levels) osmoregulatory branquial CA activity in decapods is inhibited, as observed for aquatic and terrestrial species (Henry & Cameron 1982, Henry 2001). This mechanism of inhibition facilitates the body homeostasis without increasing the energy costs. Since this pattern is common for all individuals, independently of the sex or life stage, it is not direct related with hatch of eggs.

Despite the gills weight did not vary among non-ovigerous, hatching females, control and treatments there was a tendency of anterior gills with more weight in ovigerous females and posterior gills with more weight in non-ovigerous females. The gills epithelium of semi-terrestrial crabs is generally cover by a thin film of water (Warner 1977, Greenaway 1988), and counts for a high percentage of gills weight. For a good functionality of the gills it is essential that their epithelium remain hydrated (Taylor et al. 1987). In this sense, the physiological process that involves the use of branchial water, as osmoregulation and acid-base balance, will influenced the gills weight. The higher anterior gills weight of ovigerous females (when compare to non-

ovigerous) can be related with high respiratory and ion-balance activities (higher CA activity), while higher posterior gills weight in non-ovigerous females can be related with high osmoregulatory activities (higher CA activity), as observed in the present study.

The relative weights of anterior and posterior gills were significantly distinct only in the treatment 18h. This pattern can represent a dehydration suffered by the gills (especially posterior ones, with osmoregulatory function) of females submitted to 18 hours per day of water deprivation for 14-19 days. In amphibious crabs, the ions excrete occurs mainly by the gills epithelium when in contact with water (Greenaway 1988, 1999). The long daily period of water deprivation likely affect the osmoregulatory function of the posterior gills, due the presence of metabolic excreta for long periods, leading to their dehydration. Although there was no difference among the osmolality levels of hatched females those submitted to 18 hours of water deprivation showed the higher osmolality levels. This also can be an indirect indicative of the beginning of failure of osmorelatory functions.

Conclusion

The water deprivation of ovigerous females for periods equal or superior to 18 hours affect the embryonic development, hence, the larvae survival in *Aratus pisonii*. The physiology of mother´s body show indicatives of the start of osmotic stress and gill dehydration when submitted daily to 18 hours of water deprivation. Likely, this is the maximum health period of water deprivation that the species can tolerate. As observed in many other animal taxa, the reproductive period, comprising the oviposition, embryos development in the pleopods and the zoea larvae eclosion, affects the normal

physiological patterns of the mother. Both physiological parameters evaluated (osmolality of hemolymph and carbonic anhydrase activity) showed variance between non-ovigerous and ovigerous females. The main physiological variance found in ovigerous semi-terrestrial crabs can be an indirect effect of the weight increased that the egg mass entails over mother´s body, and possibly associated with the action of hormones rate during hatching period. The physiological variance combined with behavior changes can increase the reproductive success, and thus one “step” forward to arboreal/semi-terrestrial life style.

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Conclusões Gerais

Larvas em estágio final de desenvolvimento (megalopas) possuem alto nível de tolerância a variações abióticas (principalmente salinidade) em regiões estuarinas e propiciam a ocupação dessas regiões caracterizadas por grandes amplitudes de salinidades (0-35 ‰), demonstrando alta capacidade de disperção dentro de estuários. Ao passo que os níveis intermediários de conectividade entre as populações demonstram a grande capacidade de disperção regional/continental que larvas em estágio inicial (zoea) possuem. Esta conectividade é expressa de modo distinto no genótipo e fenótipo das populações.

O dimorfismo sexual tende a ser intensificado somente após a muda puberal, que representa a mudança da fase juvenil para adulta, afetando a forma e tamanho geral do organismo, principalmente, através do desenvolvimento de caracteres sexuais secundários: quelípodos em machos e abdômen em fêmeas. Crescimento este, que reflete a tendência sexual evolutiva da linhagem.

A privação do contato com a água afeta o desenvolvimneto de embriões e sua taxa de sobrevivência em caranguejos anfíbios, porém não apresenta efeito severo na fisiologia de adultos. A fisiologia da mãe provavelemtne é afeta pelo aumento de peso que a massa de ovos acarreta, e combinado com mudanças comportamentais pode aumentar o sucesso reprodutivo no ambiente semiterrestre.

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