Poder Executivo
Ministério da Educação
Universidade Federal do Amazonas
Faculdade de Ciências Agrárias
Programa de Pós-Graduação em Ciência Animal e Recursos Pesqueiros - PPGCARP

MORFOFISIOLOGIA DIGESTIVA DE *Potamotrygon wallacei* CARVALHO, ROSA E ARAUJO (2016): UMA ESPÉCIE DE ARRAIA ORNAMENTAL AMAZÔNICA

RUBIA NERIS MACHADO

Manaus, AM

2023

Poder Executivo
Ministério da Educação
Universidade Federal do Amazonas
Faculdade de Ciências Agrárias
Programa de Pós-Graduação em Ciência Animal e Recursos Pesqueiros - PPGCARP

MORFOFISIOLOGIA DIGESTIVA DE *Potamotrygon wallacei* CARVALHO, ROSA E ARAUJO (2016): UMA ESPÉCIE DE ARRAIA ORNAMENTAL AMAZÔNICA

Orientador: Professor Doutor Wallice Luiz Paxiúba Duncan

Dissertação submetida ao Programa de Pós-Graduação em Ciência Animal e Recursos Pesqueiros – PPGCARP da Universidade Federal do Amazonas – UFAM como requisito parcial para obtenção do grau de Mestre em Ciência Animal e Recursos Pesqueiros.

Manaus, AM

2023

Sinopse

Este trabalho teve como objetivo descrever e quantificar os componentes teciduais do tubo digestório, bem como as principais enzimas hidrolíticas (proteases, lipases e carboidratases de uma espécie de arraia de água doce da Amazônia Central.

Palavras-chave: arraia cururu, tubo digestório, morfologia quantitativa, estereologia, enzimas digestivas, proteólise, lipólise e carboidratases.

Ficha Catalográfica

Ficha catalográfica elaborada automaticamente de acordo com os dados fornecidos pelo(a) autor(a).

Machado, Rubia Neris
M149m Morfofisiologia digestiva de Potamotrygon wallacei Carvalho,Rosa e Araujo (2016) : uma espécie de arraia ornamental amazônica / Rubia Neris Machado . 2023
66 f.: il. color; 31 cm.

Orientador: Wallice Luiz Paxiúba Duncan
Dissertação (Mestrado em Ciência Animal e Recursos Pesqueiros) - Universidade Federal do Amazonas.

1. Potamotrygonidae. 2. digestive tract. 3. stereology. 4. quantitative morphology. 5. digestive enzyme. I. Duncan, Wallice Luiz Paxiúba. II. Universidade Federal do Amazonas III. Título

RUBIA NERIS MACHADO

MORFOFISIOLOGIA DIGESTIVA DE *Potamotrygon wallacei* CARVALHO, ROSA E ARAUJO (2016): UMA ESPÉCIE DE ARRAIA ORNAMENTAL AMAZÔNICA

Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal e Recursos Pesqueiros da Universidade Federal do Amazonas, como requisito para obtenção do título de Mestre em Ciência Animal e Recursos Pesqueiros, área de concentração em Produção Animal.

Aprovado em 27 de julho de 2023.

BANCA EXAMINADORA

Dr. Wallice Luiz Paxiúba Duncan - Presidente
Universidade Federal do Amazonas

Dr. Jaydione Luiz Marcon - Membro
Universidade Federal do Amazonas

Dra. Grazyelle Sebrenski da Silva - Membro
Universidade Federal do Amazonas

É com imensa gratidão e amor que dedico esta dissertação a você, meu amado pai, Nivaldo Machado. Cada palavra escrita aqui é um reflexo do seu apoio incondicional.

Desde os nossos momentos de conversas profundas até as brincadeiras descontraídas, você sempre esteve ao meu lado, compartilhando sabedoria e guiando-me com seus ensinamentos valiosos. Seu exemplo de determinação, dedicação e perseverança moldou quem eu sou hoje.

Mesmo quando a distância nos separa, sinto sua presença em meu coração. Obrigada por se fazer presente quando preciso.

Pai, amo você além das palavras e sinto uma profunda falta da sua companhia.

AGRADECIMENTOS

Agradeço a todas as pessoas que contribuíram para o sucesso desta dissertação de mestrado. Seu apoio e incentivo foram fundamentais ao longo deste percurso acadêmico.

Em primeiro lugar, não posso deixar de expressar minha profunda gratidão ao meu orientador, Professor Doutor Wallice Paxiúba Duncan. Ao longo de quase uma década de trabalho conjunto, ele tem sido uma figura fundamental em minha trajetória acadêmica e profissional. Gostaria de destacar a amizade e o apoio que recebi do Duncan. Sua dedicação em me guiar e me apoiar em cada etapa do caminho foi inestimável. Sua presença constante, tanto nos momentos de conquistas como nos desafios, fortaleceu minha confiança e determinação. Sou profundamente grata pela confiança que o Duncan depositou em mim desde o início. Seu constante encorajamento e incentivo foram essenciais para superar os desafios ao longo do caminho.

Gostaria de dedicar um momento para expressar minha profunda gratidão à minha mãe, Maria de F. Neris Machado. Seu amor incondicional ao longo dos anos tem sido uma bênção em minha vida. Mesmo nos momentos em que eu era insuportável e difícil de lidar, você permaneceu ao meu lado, mostrando compreensão, paciência e bondade. Sua dedicação em me apoiar e me amar incondicionalmente é verdadeiramente admirável. Seu apoio constante me deu coragem para enfrentar desafios e buscar meus sonhos. Nem todas as palavras podem expressar adequadamente o quanto sou grata por ter você como minha mãe. Seu amor é o alicerce sobre o qual construo minha vida.

Minha querida melhor amiga, Mahi, a *bat-frog ecologist*, quero dedicar algumas palavras para expressar minha gratidão por nossa longa e incrível amizade. Ao longo dos anos, você tem sido uma presença constante em minha vida, trazendo alegria, risadas e apoio incondicional. Nossas conversas intermináveis são verdadeiros tesouros. Desde as profundas reflexões sobre a vida até as fofocas mais divertidas, cada momento compartilhado com você é especial. Agradeço por estar ao meu lado em todas as situações, seja para comemorar as conquistas ou para me consolar nos momentos difíceis. Além disso, quero pedir desculpas pelas vezes em que fui rude ou agi de forma grosseira. Sua capacidade de ver além dos meus defeitos e continuar me amando é um presente precioso. Sou grata por cada risada compartilhada, por cada lágrima enxugada e por cada memória que construímos juntas.

Gostaria de expressar minha profunda gratidão às mulheres cientistas excepcionais que fizeram parte da minha jornada acadêmica: Ana Lúcia Gomes, Grazyelle Sebrenski, Maria Inês Braga e Akemi Shibuya. Não consigo expressar adequadamente a quão grata estou por tê-las tido ao meu lado ao longo desses anos. Elas foram fontes inestimáveis de incentivo e apoio, sempre demonstrando empatia e compreensão. Cada uma delas me inspirou de maneiras únicas e proporcionou conselhos valiosos, não apenas no âmbito acadêmico, mas também em aspectos pessoais, que certamente levarei para o resto da minha vida. Agradeço de todo o coração tudo o que fizeram por mim e por terem sido modelos tão admiráveis. Sou verdadeiramente

abençoada por tê-las conhecido e por ter tido a oportunidade de aprender com mulheres tão incríveis como vocês.

Às instituições públicas pela oportunidade para qualificação profissional, especialmente à Universidade Federal do Amazonas (UFAM) pela infraestrutura e ao Programa de Pós-Graduação em Ciência Animal e Recursos Pesqueiros (PPG-CARP) em nome das coordenadoras Flávia Kelly Siqueira de Souza e Marcela dos Santos Magalhães pela sensibilidade e compreensão nos momentos difíceis de nossas vidas. Às agências de fomento pelo suporte financeiro concedido: Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq (Processo: #484374/2011-7) e Fundação de Amparo à Pesquisa do Estado do Amazonas, FAPEAM (Processos: #PPP-209/2012 e #Universal-389/2012) e uma bolsa de estudo concedida por esta agência.

Um agradecimento a todos os pescadores de Barcelos nas áreas de coleta das arraias, dos quais destaco o "Alfredinho", "Santana", "Dona Maria", "Camarão" e "João". Sou grata não apenas pelo suporte nas capturas, mas pelo aprendizado para adquirir experiências em trabalhos de campo.

Por último, sou grata por ter tido a oportunidade de aprender com esses seres incríveis que são as arraias de água doce e de contribuir para a biologia e conservação das espécies. Que os resultados obtidos possam servir como base para futuras pesquisas e esforços de conservação, visando à proteção e o bem-estar de todos os Potamotrigonídeos e da vida aquática associada.

Abriu a porta, sorriu e pediu para entrar.

RESUMO GERAL

Este estudo é uma contribuição à morfologia e fisiologia digestiva da arraia de água doce *Potamotrygon wallacei*, uma espécie de elasmobrânquio neotropical endêmica da bacia do Rio Negro (Amazônia Central). Embora seja popular como peixe ornamental, a manutenção desta espécie em cativeiro frequentemente resulta em morte devido aos problemas alimentares e estresse do confinamento em ambiente artificial. O objetivo do estudo foi investigar detalhadamente a anatomia e a fisiologia do trato digestório (TD) desta arraia. Quanto aos aspectos morfológicos utilizou-se de técnicas em histologia convencional e ferramentas em estereologia para descrever e quantificar as diversas camadas presentes no estômago e intestino espiral (válvula espiral). Os aspectos funcionais do TD foram examinados por meio das atividades específicas das principais enzimas digestivas que atuam na proteólise, lipólise e hidrólise de carboidratos. Para compreender as interrelações entre a digestão dos macronutrientes (proteínas, lipídios e carboidratos), um modelo teórico foi avaliado por meio de modelagem de equações estruturais (MES). Morfologicamente, o esôfago possui pregas horizontais. As quatro camadas histológicas são distinguíveis: mucosa, submucosa, muscular (músculo estriado esquelético) e serosa. O epitélio estratificado da mucosa possui numerosas células mucosas PAS+ e azul de Alcian+, indicando secreção de mucinas neutras e ácidas, respectivamente. Atividades residuais de enzimas proteolíticas, lipolíticas e carboidratases foram encontradas no esôfago. Contudo, estas enzimas podem representar atividades endógenas intracelulares, o que sugere um limitado papel na digestão luminal dos macronutrientes ingeridos. O estômago tem formato de *U*. A porção descendente representa a região cardíaca, enquanto a ascendente é a pilórica. Independente da região, as camadas histológicas da parede estomacal incluem: mucosa, submucosa, muscular (com camadas interna e externa) e serosa. No estômago cardíaco, a camada mucosa representa 44,7% do volume total da parede do órgão. As glândulas gástricas (um componente da mucosa) ocupam quase metade do volume da camada. Enzimas proteolíticas tipo pepsina, tripsina, quimiotripsina e elastase, além das lipases, fosfatases ácidas, esterases e carboidratases também estão presentes. Isto indica que o estômago cardíaco tem elevado potencial para digerir proteínas, lipídios e alguns carboidratos. A densidade de glândulas gástricas diminui e tornam-se ausentes no estômago pilórico. Isto explica uma redução na capacidade das enzimáticas digestivas examinadas nesta região estomacal. Porém, a musculatura lisa da parede do estômago pilórico torna-se espessa devido a maior proporção de músculo liso na muscular interna. Tal mudança sugere um papel ativo do estômago pilórico no processo de mistura e auxílio na passagem do quimo para o intestino espiral. A válvula espiral da arraia *P. wallacei* possui 10-11 voltas histologicamente constituídas de epitélio colunar simples, lâmina própria (tecido conjuntivo frouxo) e músculo liso disperso. O epitélio é quase que exclusivamente formado por células absortivas (enterócitos) e raras células mucosas. A lâmina própria possui numerosos vasos sanguíneos. A parede da válvula espiral possui as quatros camadas teciduais: mucosa, submucosa, muscular e serosa. A densidade de tecido epitelial tende a diminuir, enquanto, aumenta-se a proporção de submucosa. Isto indica uma tendência na capacidade de transporte de nutrientes para os vasos sanguíneos. O

intestino espiral é a sede final da digestão das proteínas, lipídios e carboidratos no TD. As elevadas atividades das endopeptidases (tripsina, quimiotripsina e elastase), bem como da exopeptidase, leucina aminopeptidase, reforçam o potencial proteolítico da válvula espiral na digestão das proteínas. A capacidade lipolítica e hidrólise de carboidratos também são elevadas neste local. Os modelos baseados em MES sugerem forte interdependência entre estas três vias catabólicas nos diferentes órgãos do TD. Por fim, os dados morfológicos e das enzimas digestivas são consistentes com as informações sobre a dieta de *P. wallacei*, uma arraia carnívora que consome especialmente crustáceos, larvas de insetos e pequenos peixes. Os resultados deste estudo podem contribuir para o desenvolvimento de boas práticas de manejo na alimentação das arraias em cativeiro, visando a preservação da espécie e o bem-estar dos indivíduos em ambientes confinados.

Palavras-chave: arraia cururu, tubo digestório, enzimas digestivas, proteólise, lipólise e carboidratases.

ABSTRACT

This study is a contribution to the digestive morphology and physiology of the freshwater stingray *Potamotrygon wallacei*, a neotropical elasmobranch species endemic to the Rio Negro basin (Central Amazon). Although popular as an ornamental fish, keeping this species in captivity often results in death from feeding problems and stress from confinement. The objective of the study was to investigate in detail the anatomy and physiology of the digestive tract (DT) of this stingray. As for the morphological aspects, techniques in conventional histology and tools in stereology were used to describe and quantify the different layers present in the stomach and spiral intestine. The functional aspects of DT were examined through the activities of the main digestive enzymes that act in proteolysis, lipolysis and hydrolysis of carbohydrates. To understand the interrelationships between the digestion of macronutrients (proteins, lipids and carbohydrates), a theoretical model was evaluated using Structural Equation Modeling (SEM). Morphologically, the esophagus has horizontal folds. The four histological layers are distinguishable: mucosa, submucosa, muscular (skeletal striated muscle) and serosa. The stratified mucosal epithelium has numerous mucous cells reactive to PAS+ and Alcian blue+ staining, indicating secretion of neutral and acidic mucins, respectively. Residual activities of proteolytic, lipolytic and carbohydrase enzymes were found in the esophagus. However, these enzymes may represent endogenous intracellular activities, which suggests a limited role in the luminal digestion of ingested macronutrients. The stomach is *U*-shaped. The descending portion represents the cardiac region, while the ascending portion is the pyloric region. Regardless of the region, the histological layers of the stomach wall include mucosa, submucosa, muscular (with inner and outer layers) and serosa. In the cardiac stomach, the mucous layer represents 44.7% of the total volume of the organ wall. Gastric glands (a component of the mucosa) occupy almost half the volume of the mucous layer. Proteolytic enzymes such as pepsin, trypsin, chymotrypsin and elastase, in addition to lipases, acid phosphatases, esterases and carbohydrases are present. This indicates that the cardiac stomach has a high potential for digesting proteins, lipids and some carbohydrates. The density of gastric glands decreases, and they become absent in the pyloric stomach. This explains a reduction in the capacity of digestive enzymes in this segment. However, the smooth muscle of the pyloric stomach wall becomes thicker due to the greater proportion of smooth muscle in the muscularis interna. Such a change suggests an active role of the pyloric stomach in the mixing process and aid in the passage of chyme to the spiral intestine. The spiral valve has 10-11 turns histologically made up of simple columnar epithelium, lamina propria (loose connective tissue), and scattered smooth muscle. The epithelium is almost exclusively formed by absorptive cells (enterocytes) and rare mucous cells. The lamina propria has numerous blood vessels. The wall of the spiral valve has four tissue layers: mucosa, submucosa, muscularis, and serosa. The density of epithelial tissue tends to decrease, while the proportion of submucosa increases. This indicates a trend in the ability to transport nutrients into blood vessels. The spiral intestine is the final site of digestion of proteins, lipids and carbohydrates. The high activities of endopeptidases (trypsin, chymotrypsin and elastase), as well as exopeptidase, leucine aminopeptidase, reinforce the proteolytic

potential of the spiral valve in protein digestion. Lipolytic capacity and carbohydrate hydrolysis are also high at this site. SEM-based models suggest strong interdependence between these three catabolic pathways in different organs of the digestive tract. Finally, the morphological and digestive enzyme data are consistent with the information on the diet of *P. wallacei*, a carnivorous freshwater stingray that consumes mainly crustaceans, insect larvae and small fish. The results of this study can contribute to the development of good management practices in the feeding of stingrays in captivity, aiming at the preservation of the species and the well-being of individuals in confined environments.

Keywords: cururu stingray, digestive tract, digestive enzymes, proteolysis, lipolysis and carbohydrases.

LISTA DE FIGURAS

Capítulo 1

Figure 6. Details of the dissected spiral intestine of *P. wallacei* showing the organ wall and turns (spiral structure). Note in the image that the first segment corresponds to anterior intestine. The organ was sectioned into six segments to estimate the absolute volume (cm^3/100g) in each segment of the histological layers (mucosa, submucosa, muscularis and serosa) present in the wall of the spiral intestine. The scale bar corresponds to 0.5 cm.

Capitulo 2

Figure 1. Representative images of a specimen of the neotropical freshwater stingray, *Potamotrygon wallacei* (A) and the dissected gastrointestinal tract (B) showing the esophagus (ES), cardiac stomach (CS), pyloric stomach (PS) and spiral intestine (SI). The scale bars correspond: 5 cm (A) and 1 cm (B).

Figure 2. Conceptual diagram to be tested in the Structural Equation Model for the set of digestive enzymes analyzed in each organ of the gastrointestinal tract of the potamotrygonid stingray. The enzymes represent the observed variables in each organ, while "proteins", "carbohydrates" and "lipids" were the latent variables.

Figure 3. Pearson's correlation matrix for all the set of digestive enzymes detected in the esophagus of the freshwater stingray, *Potamotrygon wallacei.* The data of the enzymes trypsin and chymotrypsin used in the correlations were obtained from TAME and BTEE substrates (see material and methods for details).

Figure 4. Pearson correlation matrix (A) and structural model (B) including latent variables ("proteins", "lipids" and "carbohydrates") and digestive enzymes measured in the cardiac stomach of *P. wallacei*.

Figure 5. Pearson correlation matrix (A) and structural model (B) including latent variables ("proteins", "lipids" and "carbohydrates") and digestive enzymes measured in the pyloric stomach of *P. wallacei*.

Figure 6. Pearson correlation matrix (A) and structural model (B) including latent variables ("proteins", "lipids" and "carbohydrates") and digestive enzymes measured in the spiral intestine of *P. wallacei*. Lipase.M and lipase.P represent the unspecific lipases assayed with myristate and palmitate as substrates, respectively.

LISTA DE TABELAS

Capítulo 1 – Material suplementar

Capítulo 2

SUMÁRIO

INTRODUÇÃO GERAL

A linhagem mais antiga de animais mandibulados a possuir um trato digestivo verdadeiro e uma fisiologia complexa é a dos Chondrichthyes (Weatherbee et al. 2012). Os Chondrichthyes incluem tubarões, arraias e quimeras, e eles têm uma história evolutiva impressionante que remonta a centenas de milhões de anos. Os Chondrichthyes surgiram no registro fóssil há aproximadamente 400 milhões de anos, durante o período Siluriano. Nesse momento, esses animais já apresentavam mandíbulas, permitindo-lhes capturar e processar alimentos de forma mais eficiente do que seus ancestrais mais primitivos, que não tinham mandíbulas. (Inserir referência)

Os Elasmobrânquios (tubarões e arraias) são animais essencialmente carnívoros (Motta e Huber 2012), e ao longo da sua história evolutiva permaneceram como predadores de topo ou mesopredadores em seus ecossistemas (Weatherbee et al. 2012). Em termos de trato digestivo, os Elasmobrânquios possuem um sistema digestivo completo, semelhante a outros vertebrados incluindo boca, esôfago, estômago, intestino e cloaca (Theodosiou, 2012; Bucking, 2015). Esse sistema permite que eles processem e absorvam nutrientes de maneira eficiente. No entanto, em comparação com os teleósteos (peixes ósseos), os elasmobrânquios têm sido menos estudados em relação à sua morfologia e fisiologia digestiva. Há uma lacuna de informações nesse aspecto, especialmente quando se trata de arraias neotropicais de água doce. Essa falta de informações pode ser atribuída ao desafio de estudar esses animais em ambiente natural, bem como à complexidade de realizar experimentos em cativeiro.

Do ponto de vista evolutivo, acredita-se que os elasmobrânquios inovaram ao apresentar um compartimento para acidificar o alimento e utilizar-se de enzimas proteolíticas em meio ácido para digerir proteínas. Os elasmobrânquios são a mais antiga linhagem a apresentar glândulas gástricas verdadeiras e com células oxintopepticas capazes de secretar tanto HCl, quanto pepsinogênio (Ballantynes, 2015). No geral, a estrutura anatômica, histológica e o perfil das enzimas digestivas dos elasmobrânquios são semelhantes aos dos peixes teleósteos e dos grupos evolutivamente recentes. A despeito do longo período evolutivo dos elasmobrânquios, bem como dos diferentes ambientes onde estes habitam, em geral, há pouca variação morfofuncional no estômago dos diferentes taxa (Cortés et al., 2008).

O intestino dos elasmobrânquios, comumente chamado de intestino espiral/válvula espiral, passou por modificações ao longo da evolução provavelmente para maximizar o espaço da cavidade celomática levando em consideração o grande fígado desses animais (Holmgren & Nilsson 1999; Khanna, 2004). Apesar de o intestino espiral ser encontrado em outros grupos taxonômicos, os elasmobrânquios por pertencerem a uma linhagem basal, provavelmente foi o primeiro grupo a desenvolver tal característica (Weatherbee et al. 2012). Embora o intestino espiral seja compacto, sua área de superfície absortiva é compensada pelas espirais, o que aumenta significativamente a área de absorção, se comparada a teleósteos carnívoros (Parker, 1880; Khanna, 2004; Compagno et al., 2005; Leigh et al., 2017; Leigh et al., 2021; Bosi et al., 2022).

A anatomia do trato gastrointestinal (TGI) já foi bem descrita em várias espécies de tubarões e em algumas arraias marinhas, proporcionando um entendimento detalhado da estrutura morfológica nesses peixes cartilaginosos. A parede do trato gastrointestinal de todos os vertebrados, incluindo os elasmobrânquios, é constituída por quatro camadas básicas: mucosa, submucosa, muscular e serosa (Leigh et al., 2017).

A camada mucosa é formada por células epiteliais colunares apoiadas por tecido conjuntivo frouxo, que se organiza em grandes pregas. A camada submucosa fornece suporte à mucosa, enquanto a túnica muscular é composta por duas camadas de músculo que auxiliam no movimento do conteúdo intestinal: uma camada longitudinal externa e outra circular interna. A camada serosa é formada por tecido conjuntivo frouxo internamente e células epiteliais pavimentosas (mesotélio) externamente, que revestem o trato gastrointestinal. Todas essas características teciduais são descritas por Buddington et al. (1997), Buddington et al. (2000), Guillaume & Choubert (2001), Moraes e Almeida (2014), Theodosiou (2012) e Chatchavalvanich et al. (2006).

As células do epitélio mucoso se diferenciam ao longo do trato digestório de acordo com a função assumida. São encontrados três tipos principais de células epiteliais: as absortivas (intestino/válvula espiral), secretoras (estômago e intestino/válvulas espiral) e as mucosas (distribuídas ao longo do tubo) (Takashima & Hibiya, 1995). Inúmeras enzimas relacionadas com os processos digestivos e absortivos se distribuem nas células do epitélio ao longo do trato gastrointestinal. As informações disponíveis sugerem que os

peixes, de uma maneira geral, são semelhantes aos outros vertebrados quanto aos processos digestivos (Fange & Grove, 1979; Lovell, 1989).

O processo digestivo é a transformação do alimento no trato digestório em compostos mais simples (aminoácidos, ácidos graxos, glicerol, açucares) para que sejam transportados para os tecidos via corrente sanguínea (De Silva & Anderson, 1995). A digestão envolve uma série de eventos que requerem a coordenação de uma variedade de processos básicos do trato gastrointestinal, que é iniciada pela ingestão de alimentos, seguida pelas secreções de enzimas e muco, digestão mecânica, enzimática, absorção, motilidade (incluindo a evacuação) e, finalmente, a regulação destes diferentes processos (Zambonino-Infante & Cahu, 2001; Lazo et al., 2011; Rønnestad et al., 2013; Morais e Almeida 2014).

A digestão enzimática dos alimentos em nutrientes ocorre pela ação de diferentes tipos de enzimas produzidas no estômago, glândulas anexas e intestino, além disso, englobam a absorção e transporte dos nutrientes pelas células intestinais. Usualmente, as enzimas digestivas presentes no processo de digestão são classificadas de acordo com o seu papel catalítico. Temos três grupos de enzimas principais ao longo do trato digestivo responsável pela digestão de proteínas, lipídeos e carboidratos além de enzimas acessórias. Venkatesh et al. (2014) e Leigh et al. (2017) assumiram que os Elasmobrânquios (tubarões) geralmente apresentam o mesmo complexo de enzimas digestivas que os teleósteos.

Embora bastante estudados quanto à ecologia e dinâmica pesqueira (Cortés, 1999), existe uma escassez de estudos quanto a anatomia microscópica e fisiologia do trato digestório de elasmobrânquios. Mais raros ainda são os trabalhos realizados com arraias estritamente de água doce. Destacam-se os estudos publicados com *Flavitrygon signifer* (antes *Himantura signifer*), uma arraia de água doce do Sul da Ásia (Chatchavalvanich et al., 2006), e um estudo recente com *Potamotrygon amandae*, uma arraia neotropical da bacia do Paraná-Paraguay (Aquino et al., 2023). Contudo, apesar dos detalhes nas descrições, estes trabalhos apenas trazem descrições dos componentes teciduais e relatam as variações morfoanatômicas quando comparados com outros representantes. Por isso, nestes estudos, foi feita, além de uma descrição detalhada dos aspectos histológico, a quantificação de cada componente através da estereologia. Estereologia é uma área que utiliza conceitos em matemática, tais como geometria e

amostragens aleatórias sobre imagens bidimensionais para produzir dados tridimensionais, como estimativa de volume (Howard & Reed, 2010). Dessa forma, o método pode ser sensível para detectar variações quantitativas ao longo do tubo digestório.

Toda esta base teórica foi usada para compreender um dos maiores entraves associados à manutenção em cativeiro de uma espécie de arraia de água neotropical, *Potamotrygon wallacei* Carvalho, Rosa & Araújo (2016). Esta espécie é conhecida como arraia cururu, endêmica da bacia do Rio Negro (Amazônia Central). Alimenta-se de crustáceos, larvas de insetos e peixes (Shibuya, et al., 2009). Tem importância econômica no cenário regional, pois atualmente é a espécie de arraia mais explorada e comercializada como peixe ornamental na aquariofilia (Duncan et al., 2010). Contudo, quando mantida em cativeiro comumente não aceita ração comercial. Além disso, quando alimentos vivos (pequenos peixes, oligoquetas e crustáceos) são oferecidos ocorre boa aceitação inicial, porém, com o passar do tempo o animal desenvolve um quadro de desnutrição (perda de peso e, possivelmente deficiência nutricional), causado principalmente pela auto inanição e isso leva o animal à morte em poucos meses.

Existe claramente um problema associado à dieta/alimentação dessa espécie, que apesar de comercialmente importante, contrapõe-se à biologia da conservação, uma vez que, não se justifica retirar um recurso natural do seu habitat sabendo-se que, em pouco tempo, o animal irá morrer por inanição. Posto isso, este estudo apresenta dois trabalhos que podem ajudar a colocar uma peça neste quebra-cabeça: (i) o primeiro capítulo traz um olhar anatômico ao descrever e quantificar os aspectos morfológicos (anatomia macro e microscópica), cuja principal hipótese é que a organização das estruturas seja semelhante àquelas encontradas nas arraias marinhas. Porém, este estudo inova, uma vez que os componentes teciduais do tubo digestório (estômago e intestino espiral) foram quantificados por meio de métodos em estereologia. Em outras palavras, os constituintes anatômicos do tubo digestório foram quali e quantitativamente descritos. (ii) o segundo capítulo traz informações detalhadas do perfil dos principais grupos de enzimas digestivas, tais como proteases, lipases e carboidratases. A quantificação das enzimas foi feita tanto no estômago, quanto no intestino espiral (os dois órgãos mais importantes para a digestão dos alimentos).

Os achados apresentados no presente trabalho são inéditos para elasmobrânquios de água doce. Ao longo da discussão de ambos os capítulos deste estudo, relacionamos os dados quali-quantitativos da morfologia do tubo digestório com os dados das enzimas digestivas, de maneira a consolidar e compreender os diversos aspectos morfofuncionais do tubo digestório desta espécie de arraia. O objetivo foi produzir informações críticas para que se possa alcançar o sucesso do manejo alimentar desta espécie, dessa forma, contribuir para evitar a superexploração da pesca, bem como proteção do ecossistema, considerando o papel ecológico desta espécie na bacia do Rio Negro.

HIPÓTESES

H1: Os aspectos morfofuncionais do tubo digestório da arraia *P. wallacei* estão associados à capacidade digestiva para processar os macronutrientes: proteínas, lipídeos e carboidratos.

OBJETIVOS GERAL E ESPECÍFICOS

Geral

Descrever e quantificar os componentes teciduais e a fisiologia das enzimas digestivas de *P. wallacei*, uma arraia ornamental endêmica da bacia do Rio Negro, Amazônia Central.

Específicos

- Descrever e quantificar por meio de métodos estereológicos os componentes teciduais do tubo digestório de *P. wallacei*;
- Estimar as atividades das principais enzimas digestivas (proteolíticas, lipolíticas e carboidratases) do esôfago, estômago e intestino espiral de *P. wallacei.*

REFERÊNCIAS

Aquino, J. B., Melo, L. F., Rodrigues, R. F., Melo, A. P. F., Morais-Pinto, L. & Rici, R. G. R. 2023. Morphological aspects of the digestive system in freshwater stingray (*Potamotrygon amandae* Loboda and Carvalho, 2013): Myliobatiformes; Potamotrygoninae. Zoomorphology, 142, 181–191.

Ballantyne, J.S. (2015). Metabolism of Elasmobranchs (Jaws II). *Fish Physiology*, 34, 395-456. https://doi.org/10.1016/B978-0-12-801286-4.00007-1

Bosi, G., P. Merella, B.J. Maynard & B.D. Sayyaf. 2022. Microscopic Characterization of the Mucous Cells and Their Mucin Secretions in the Alimentary Canal of the Blackmouth Catshark *Galeus melastomus* (Chondrichthyes: Elasmobranchii). Fishes.

Buddington R.K., A. Krogdahl & A.M. Bakke-Mckellep. 1997. The intestines of carnivorous fish: structure and functions and the relations with diet. Acta Physiologica Scandinavica. Supplementum, v. 638:67-80.

Buddington, R.K. & V. Kuzmina. 2000. Digestive System. G. Ostrander, G.R. Bullock, and T. Bunton editors The Laboratory Fish: Handbook of Experimental Animals. Academic Press, Baltimore, USA, p. 173-179.

Bucking, C. 2015. Feeding and digestion in elasmobranchs: Tying diet and physiology together. In Physiology of Elasmobranch Fishes: Structure and Interaction with Environment; Shadwick, R.E., Farrell, A.P., Brauner, C.J., Eds.; Elsevier: London, UK. p. 347–394.

Carvalho, M. D., Rosa, R.S., & Araújo, M. L. (2016ª). A new species of Neotropical freshwater stingray (Chondrichthyes: Potamotrygonidae) from the Rio Negro, Amazonas, Brazil: the smallest species of Potamotrygon. *Zootaxa*, 4107, 566-586.

Chatchavalvanich K., R. Marcos, J. Poonpirom, A. Thongpan & E. Rocha. 2006. Histology of thedigestive tract of the freshwater stingray *Himantura signifier* Compagno and Roberts, 1982 (Elasmobranchii: Dasyatidae). Anatomy Embryology, v.211: 507–518.

Compagno, L., M. Dando & S. Fowler. 2005. Sharks of the world. Princeton, NJ: Princeton University Press.

Cortés E (1999) Standardized diet compositions and trophic levels of sharks. Journal Marine Science 56: 707–717.

Cortés, E., Papastamatiou, Y., Carlson, J., Ferry, L., & Wetherbee, B. (2008). An Overview of the Feeding Ecology and Physiology of Elasmobranch Fishes. (ed): Cyrino, J. E. P., Bureau, D., & Kapoor, B.G. In book: Feeding and Digestive Functions in Fishes. https://doi.org/10.1201/b10749-10

De Silva, S.S. & T.A. Anderson. 1995. Fish nutrition in aquaculture. London: Chapman e Hall, p. 319.

Fänge, R., & D. Grove. 1979. Digestion. Hoar, W.S.; D.J. Randall, and J.R. Brett, editors. Fish Physiology. Academic Press, New York, USA, p. 162-260.

Guillaume, J. & G. Choubert. 2001. Digestive physiology and nutrient digestibility in fishes. In: Nutrition and feeding of fish and crustaceans (Guillaume, P.J., Kaushirk, P.B., and Metiller, R. eds.) Springer-Praxis Publishing, Chichester. p. 27–56.

Holmgren S. & S. Nilsson. 1999. Digestive system. In Sharks, skates, and rays: the biology of elasmobranch fishes (ed. WC Hamlett). Baltimore, MD: The Johns Hopkins University Press, p. 144–173.

Howard, C. V., & M. G. Reed. 2010. Unbiased stereology: three-dimensional measurement in microscopy. 2nd. New York, Berlin, Heidelberg: Springer-Verlag.

Khanna, D.R. 2004. Biology of Fishes, Delhi, India: Discovery Publishing House. p. 56-63.

Lazo, J.P., M.J. Darias & E. Gisbert. 2011. Ontogeny of the digestive tract. In: Holt, I. Larval Fish Nutrition. Wiley Online Library. Oxford. p. 448.

Leigh, S.C., Y. Papastamatiou & D.P. German. 2017. The nutritional physiology of sharks. Reviews in Fish Biology and Fisheries, v. 27: 561-585.

Leigh, S.C., A.P. Summers, S.L. Hoffmann & D.P. German. 2021. Shark spiral intestines may operate as Tesla valves. Proceedings of the Royal Society of London, v 288.

Lovell, R.T. 1989. Nutrition and Feeding of Fish. Van Nostrand Reinhold, New York.

Moraes, G. & L.C. Almeida. 2014. Nutrição e aspectos funcionais da digestão de peixes. Biologia e fisiologia de peixes neotropicais de água doce. Jaboticabal: Funep/Unesp, Jabotical, Brazil. p. 233-252.

Motta PJ, Huber DR (2012) Biology of Sharks and Their Relatives. In: Carrier JC, Musick JA, Heithaus MR (ed) Prey Capture Behavior and Feeding Mechanics of Elasmobranchs, 2nd end. 58

Parker, T.J. 1880. On the intestinal spiral valve in the genus *raia*. Transactions of the Zoological Society of London, v. 11:49-61.

Ronnestad, I., M. Yúfera, B. Ueberschär, L. Ribeiro, O. Saele & C. Boglione. 2013. Feeding behaviour and digestion physiology in larval fish - current knowledge, and gaps and bottlenecks in research. Reviews in Aquaculture, v. 5: S59-S98.

Theodosiou, N.A. & A. Simeone. 2012. Evidence of a rudimentary colon in the elasmobranch, *Leucoraja erinacea*. Development Genes and Evolution, v. 222: 237-243.

Takashima, F. & T. Hibiya. 1995. An atlas of fish histology. Normal and pathological features. 2nd ed. New York: Fisher, p. 195

Venkatesh, B., A.P. Lee, V. Ravi, A.K. Maurya, M.M. Lian, J.B. Swann, Y. Ohta, M.F. Flajnik, Y. Sutoh, M. Kasahara, S. Hoon, V. Gangu, S.W. Roy, M. Irimia, V. Korzh, I. Kondrychyn, Z.W. Lim, B.H. Tay, S. Tohari, K.W. Kong, S. Ho, B. Lorente-Galdos, J. Quilez, T. Marques-Bonet, B.J. Raney, P.W. Ingham, A. Tay, L.W. Hillier, P. Minx, T. Boehm, R.K. Wilson, S. Brenner & W.C. Warren 2014. Elephant shark genome provides unique insights into gnathostome evolution. Nature, v. 505: 174-179.

Zambonino-Infante, J.L. & C.L. Cahu. 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacoly, v. 30(4): 477-487.

Weatherbee, B.M. & E. Cortes 2004. Food consumption and feeding habits. In J. C. Carrier, J. A. Musick & M. R. Heithaus (Eds.), Biology of sharks and their relatives (pp. 225–246). Boca Raton: CRC Press.

CAPÍTULO 1

"Digestive tract morphology of the Amazonian freshwater stingray, *Potamotrygon wallacei*"

Rubia Neris Machado[1] & Wallice Paxiúba Duncan[2]

Preparado para ser submetido ao periódico "*The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*", fator de impacto (2021): 2.227

Digestive tract morphology of the Amazonian freshwater stingray, *Potamotrygon wallacei*

Rubia Neris Machado[1] & Wallice Paxiúba Duncan[2]

[1]Programa de Pós-graduação em Ciência Animal, Faculdade de Ciências Agrárias, Universidade Federal do Amazonas.

[2]Laboratório de Morfologia Funcional, Departamento de Morfologia, Universidade Federal do Amazonas.

Ave. Rodrigo Octávio, 6200. Coroado I, 69.080-900, Manaus, Amazonas, Brazil.

ORCID: http://orcid.org/0000-0002-1279-7163

Running title: Quantitative morphology of the digestive tract of freshwater stingray.

Abstract

Potamotrygon wallacei is a neotropical freshwater stingray that feeds on crustaceans, insect larvae and small fish. This species has been exploited for decades as an ornamental fish. However, when kept in captivity, it tends to show stress due to confinement, does not feed properly and dies after a few months. Considering this, this work aimed to describe and quantify the tissue components of the digestive tract of this stingray. For this purpose, techniques in conventional histology and stereological methods were used to estimate tissue volume based on the principles of Cavalieri and Delesse. The volumes of the four fundamental layers in the stomach (cardiac and pyloric) and spiral intestine were estimated. The quantifiable layers were the mucosa (and its components), submucosa, muscularis (internal and external) and serosa. In the cardiac stomach, the mucous layer occupies 44.7% of the total volume of the organ wall. As the gastric glands are components of the mucous layer, only these structures represent 49.7% of the mucous layer. The large number of gastric glands suggests a high potential for processing items with high protein content. Methods in stereology were sensitive to show a reduction in the volume (and proportion) of the gastric glands from the cardiac region towards the pyloric in the stomach. These glands are absent in the pyloric region of the stomach. On the other hand, the muscularis layer becomes even thicker towards the pyloric region. This layer corresponds to 62.2% of the total volume of the pyloric stomach wall. The increase in smooth muscle thickness is due to thickening of the internal (circular) muscularis leaflet. This suggests that the role of the pyloric stomach may be related to the mixing and assisting in the passage of the chyme to the spiral intestine. We did not find any anatomical justification for dividing the stomach of *P. wallacei* into a fundic region. In the spiral intestine, data on the volume of the mucous layer (and lining epithelium)

suggest that the spiral valve has an absorptive area equivalent to the intestine of teleost fish. In several respects, the morphology of the digestive tract of *P. wallacei* is similar to that of other batoids. However, the small morphological variations may be related to the habitat specificity of this species.

Keywords: Potamotrygonidae, digestive tract, stereology, quantitative morphology.

1. INTRODUCTION

The Chondrichthyes represent the oldest lineage of jawed animals with the ability to acidify and then neutralize food within the digestive tract (Ballantyne, 2015). Therefore, they are the first groups to have gastric glands for acid secretion and production of proteolytic enzymes such as pepsin for protein digestion. The neutralization of chyme for the action of other digestive enzymes occurs in another compartment recognized as spiral intestine (Cortés et al., 2008). These two structural innovations increased the efficiency of food digestion and nutrient absorption, and possibly contributed to the evolution of >400 million ago as efficient carnivores. However, despite the phylogenetic relationships, in general, the small morphofunctional variations of the digestive tract are related to lifestyle and ecological diet.

Unlike teleost fish, the gross anatomy of the digestive tract of elasmobranchs does not have large morphological variations (Leigh et al., 2017). Anatomically, the gastrointestinal tract of elasmobranchs consists of several discrete structures and, associated organs. Some anatomical structures are common, even among different taxa, such as the oral cavity, pharynx, esophagus, stomach, spiral intestine, associated structures (pancreas and liver) and, cloaca (Bucking, 2015). Microscopic analysis of the digestive tract in elasmobranchs is limited compared to that of teleost fish. Most of the literature on this subject refers to DT descriptions of sharks and a few batoid representatives (Leigh et al., 2017), especially of the family Dasyatidae (Chatchavalvanich et al., 2006). These studies show that the tissue components of DT organs are similar to those of other vertebrates. For example, the walls of these organs are made up of four fundamental layers: mucosa, submucosa, muscularis, and serosa. The mucosa lined by stratified epithelium (in the mouth, esophagus and cloaca) or simple (stomach and spiral intestine). The function of the epithelial tissue is related to the

function of the organ. Beneath the epithelium is a lamina propria consisting of vascularized loose connective tissue. The submucosa is vascularized and apparently does not have glandular structures. The basic architecture of the muscularis layer is similar to that of other vertebrates. Consisting of two layers of smooth muscle, except in the esophagus (skeletal striated muscle). Whereas serosa layer is more external and extremely varied in tissue composition. (Está faltando a citação da referência aqui)

As far as we know, so far there is only one study describing the tissue architecture of the gastrointestinal tract (GIT) of a freshwater stingray from the subfamily Potamotrygoninae (Aquino et al., 2023). These authors showed that the basic anatomical plan, as well as the histological characteristics of the GIT organs of the stingray *Potamotrygon amandae*, as being similar to those described for marine and freshwater stingrays. The small differences found were related to the feeding habits and peculiar ecological specificity of each species.

The stingray *P. amandae*, a species studied by Aquino et al. (2023), consumes food items consisting of crustaceans and small fish (Shibuya, 2022). These food items are similar to those consumed by the Amazonian stingray *Potamotrygon wallacei* (Shibuya et al., 2009). Despite differences in size, *P. wallacei* is one of the smallest species of the subfamily Potamotrygoninae (Carvalho et al., 2016). Our hypothesis is that the tissue architecture of DT is highly similar, however, possible morphological differences may be associated with the peculiar environment where the stingray *P. wallacei* lives.

The stingray *P. wallacei* is endemic to the black and acidic waters of the Rio Negro basin (Carvalho et al., 2016). It is preferably found in flooded forests known as “igapós”, and in high population density in the Middle Rio Negro, Mariuá Archipelago, in the State of Amazonas, Brazil (Duncan et al., 2016). The diet of this stingray is preferably based on crustaceans (shrimps and crabs), insect larvae and small fish (Shibuya et a., 2009). Over several decades, this was the main species exploited for aquarium hobby as an ornamental fish (Araujo et al., 2004). Recently, population stocks in exploited areas have been reestablished, as there has been a reduction in demand over the years (Duncan et al., 2016). Other anthropic factors continue to threaten the preferred habitat of this species. They include tourism activities, leisure in the areas of igarapés (small streams) and negative fishing with mutilation or death of the animal (Shibuya, 2022). However, it is noteworthy that one of the most problems for the maintenance of this stingray in captivity

is the difficulty in feeding. In general, animals suffer stress from confinement, tend to starve, become malnourished and die within a few months. The reasons for this are still not well understood. Therefore, studies on the DT morphology of this species can become a piece in this puzzle.

Although the study by Aquino et al. (2023) provides information on the gross anatomy and describe in detail the histology of the GIT of a neotropical stingray of the family Potamotrygonidae, this study is only descriptive, as it was based on histological sections with two-dimensional images, like all other studies on the histology of the GIT of elasmobranchs. However, stereology method uses the same two-dimensional images, but with an impartial analysis and without bias to determine the three-dimensional (e.g., volume) of an organ or tissue (Weibel et al., 1966). Quantitative studies combined with histological descriptions can be robust to provide details of the variations in the different tissue layers of the digestive tract of vertebrates. Thus, the aim of the present study was to determine the volume (and the relative proportion) of each histological layer (and/or its tissue components) and to understand the morphological variations along the digestive tract of *P. wallacei*.

2. MATERIAL AND METHODS

Capture and animal handling

Juveniles (n=3; total weight 143.3 ± 9.4 g; disk width = 16.7 ± 1.8 cm) of the freshwater stingray, *Potamotrygon wallacei* (Fig. 1-A) were collected in the Cubá lake, Middle Negro River, in Barcelos city, State of Amazonas, Brazil (0°30'S/63°33'W; 0°27'S/63°09'W; 1°19'S/62°11'W; 1°14'S/61°55'W). The animals were captured through active search using wooden canoes, headlamps and nets. After collection, the stingrays were kept in the water of the same river to minimize post-capture stress. After capture, animals were euthanized with 0.5% benzocaine and then weighed and measured. Immediately, the digestive tract (DT) was completely removed and fixed in saline 10% formalin buffered for 24 hours, and then preserved in 70% ethanol. Captures were carried out with authorization from the Chico Mendes Institute for Biodiversity Conservation (ICMBio, Brazilian environmental agency, license #15068–6). All protocols involving the handling of animals were previously approved by the Ethics Committee for Animal

Experimentation/Federal University of Amazonas (CEUA/UFAM, protocol nº 007/2019) in accordance with the guidelines of the Brazilian Committee for the Control of Animal Experimentation.

Gross anatomy, stereology and histological processing

For the description of the gross anatomy, the DT was entirely photographed, dissected and each organ identified and measured. The esophagus was processed separately only to describe the tissue layers and identify the types of mucous cells present in the lining epithelium. This DT organ was not used for quantification through stereology. In the other regions of the DT (stomach and spiral intestine), each organ was processed to quantify the tissue components using stereological techniques. In the case of the stomach, a visual separation was made between the cardiac and pyloric regions. Each region was processed separately.

For each organ (cardiac stomach, pyloric stomach or spiral intestine) a linear measurement was obtained. After that, the organ was sectioned into six equidistant segments with similar dimensions (see Fig. 1). Each segment (1 to 6) was measured again to be used in the calculation of volume by Cavalieri's principle. Individually, each segment was embedded in methacrylate resin (Külzer-Heraues, Germany) for histological processing. After polymerization, the position of each section was recorded for later sectioning. The microtomy (3 µm) was performed using a microtome (SLEE MAINZ, Germany). The slides were stained in toluidine blue (0.25%), and each slice containing a complete cross-section of the organ was photographed in a stereomicroscope (Leica EZ4D, Germany). The images were examined in the Stepanizer software (Tschanz & Weibel, 2011) where a test counting system containing points was superimposed on each image obtained. Each time the points coincided with the lumen or the organ wall, they were counted separately. The total volume of the organ was calculated as the sum of these two components. The volume (Cavalieri) of the organ (lumen + tissue layers) was calculated using the following equation: $Cv = \sum_{i=1}^{n} Pi \left(\frac{a}{p}\right) \times \Delta s$

Where, *Cv* is the absolute volume of the organ, $\sum_{i=1}^{n} Pi$ is the total number of points over each section, $Pi \left(\frac{a}{p}\right)$ is the area represented by each point (90,000 µm2) and [Equation] is the distance (5,000 µm) between each section (Gundersen et al., 1988). As we do not know if the volume values scale with the size of the animal, it was preferable to

standardize the data for the approximate size of the examined animals (between 130-160 g). Thus, the absolute volume values of each segment or organ were expressed in cm^3/100 g.

Estimating the proportion (and volume) of each layer in the DT organs.

The percentage (volume density) of each component in the DT wall (mucosa, submucosa, muscularis mucosae, muscularis, and serosa) was obtained by Delesse's principle (Howard and Reed 2010). Additionally, the mucous layer of each organ was fractionated to quantify the tissue components present. Digitized images including all tissue layers (or just the mucosa when applicable) were analyzed using ImageJ software (RSB, NIH https://imagej.nih.gov/). Each component was quantified by counting the points and the result expressed in percentage, according to the following equation:

$$Fv = \frac{\sum_{i=1}^{m} LP}{\sum_{i=1}^{m} RP}$$

Where, *Fv* is the fractional volume (or relative density) of a component (mucosa, submucosa, muscularis and serosa) of the tissue; *LP* is the sum of points that touch the tissue layer (mucosa, submucosa, muscularis and serosa), while *RP* is the sum of points that touch the reference space (Howard & Reed, 2010). Percentage value (*Fv*, %) were transformed into absolute volume (*AV*) after multiplication by the Cavalieri volume (*Cv*) of the organ (or segment, if applicable), according to the following equation: *AV*= *Cv* x *Fv*. The absolute volume values of each segment or organ were expressed in cm^3/100 g.

3. RESULTS

Gross anatomy

The digestive tract of *Potamotrygon wallacei* (Fig 1A) consists of an orobranchial cavity, esophagus, stomach, spiral intestine and cloaca. Most of the organs are found inside the coelomic cavity (Fig. 1B). Although the pancreas and liver are part of the gastrointestinal tract as accessory organs, in this work, these organs were not analyzed. The outer wall of the cardiac stomach and anterior intestine (first segments in the spiral intestine) is well vascularized (Fig 1B). The esophagus is a thick, muscular organ that traverses the orobranchial cavity and projects into the coelomic cavity, connecting with

the stomach through the cardiac sphincter. The stomach has the usual siphonal *U*-shape. In this study, to facilitate quantification by stereological methods, we divided the stomach into two anatomical regions: cardiac and pyloric. The cardiac region of the stomach constitutes the descending segment, which is more dilated and has a redder color than the pyloric portion in fresh animals. The pyloric portion represents the ascending segment and apparently appears to be thicker than the anterior (cardiac) region. The luminal surface has folds with a granular pattern that gradually decrease towards the pyloric region. The anterior intestine originates just after the pyloric sphincter and ends at the spiral projections.

In the region close to the pyloric sphincter, there is a wide space that is considered as the anterior portion of the intestine. The posterior portion is very small and almost negligible, being considered here only as part of the spiral intestine. The luminal volume of the spiral intestine was estimated to be 0.5 cm^3/100g (see Supplementary Material, Table 2).

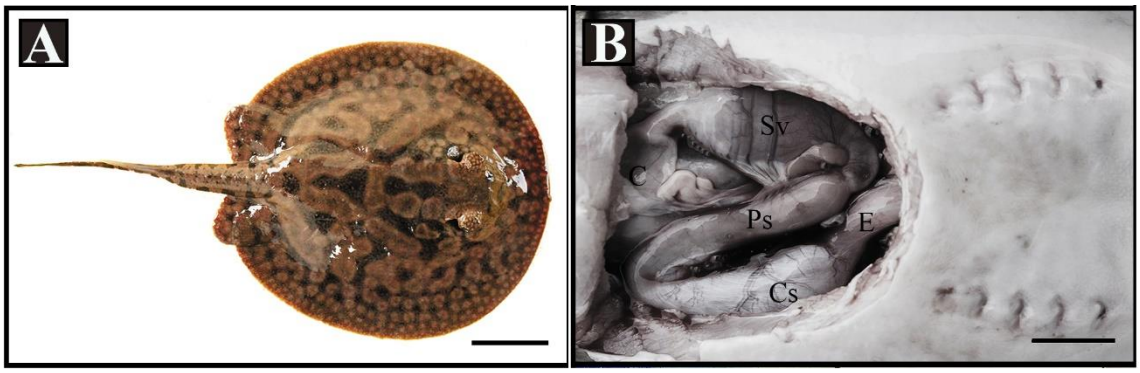

Figure 1. (A) Illustrative image of the Neotropical freshwater stingray, *Potamotrygon wallacei*. (B) Internal view of the coelomic cavity showing the anatomical arrangement of the digestive tract: esophagus (E), cardiac stomach (Cs), pyloric stomach (Ps), spiral valve (Sv) and cloaca (C). The bars represent 5 cm.

Microscopic anatomy and stereology of the digestive tract

Esophagus

As previously reported, it was not possible to quantify the tissue components of the esophagus of the stingray *P. wallacei*. Histological details of the esophagus are shown in figure 2. The esophagus has stratified epithelium containing PAS+ (periodic acid+Schiff's reagent-staining, which stain moderately magenta) and AB+ (Alcian blue-staining, which

stain intensely in cyanic color). Magnified images in figure 2 show that AB+ mucous-cells are larger and have several granules in the cytoplasmic space that strongly stain with Alcian blue, while PAS+ mucous-cells apparently are smaller and only the apical region is moderately reactive to PAS.

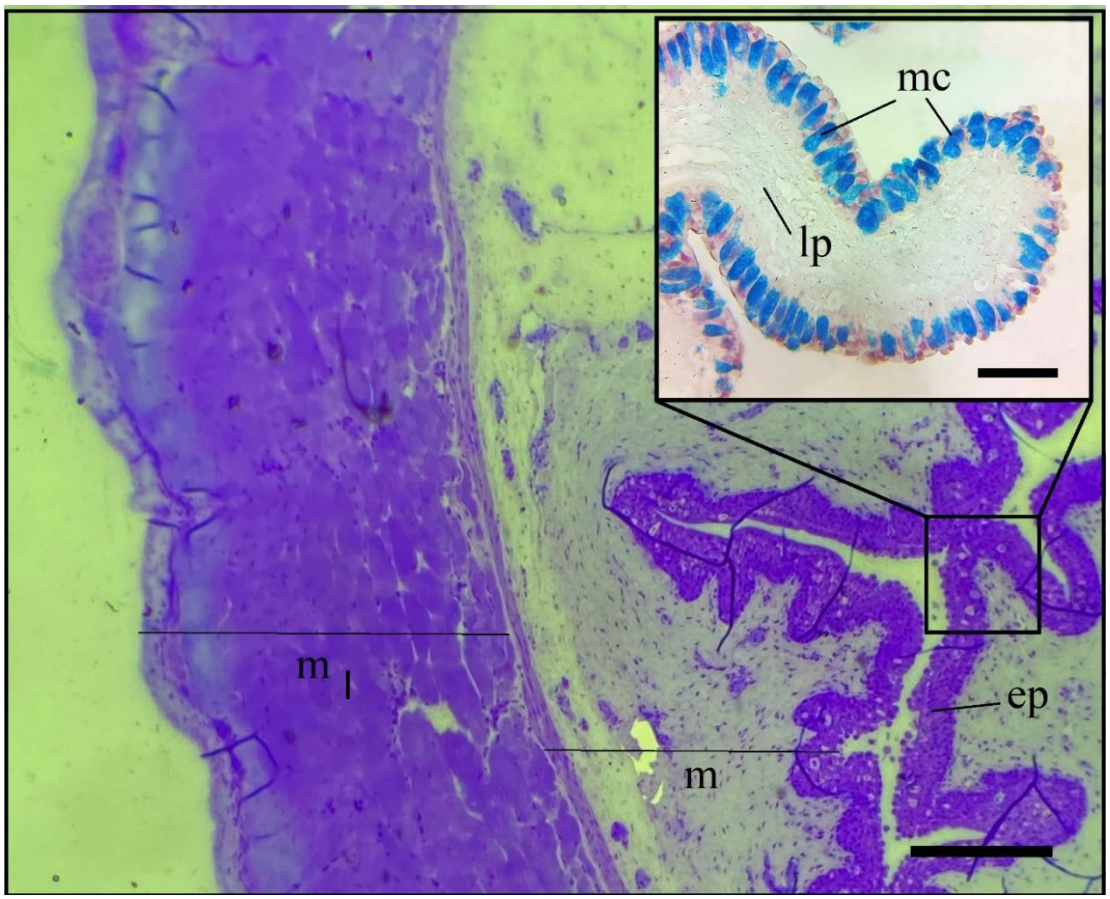

Figure 2. Photomicrograph of the esophagus of *P. wallacei*. The wall of the organ is thick consisting of skeletal striated muscle. The lining epithelium is stratified and presents mucous cells positive for PAS (magenta color) and Alcian blue staining (blue). The scales correspond: 200 µm and 50 µm (magnified image). The legends indicate: ml=muscular layer, m=mucosa, ep=epithelium, lp=lamina propria, mc=mucous cells.

The spiral intestine occupies a large part of the digestive tract and has 10 to 11 well-delineated helical projections. From the esophagus to the stomach, a transition is observed with profound changes in skeletal muscle to smooth muscle, as well as epithelium from stratified to unicellular columnar.

Stomach

The total volume estimated by the Cavalieri method for the stomach wall (cardiac and pyloric region), excluding the luminal region, was 0.60 cm^3/100 g. Only the cardiac

region the estimated volume was 0.31 cm^3/100 g, while the pyloric was 0.29 cm^3/100 g. In the cardiac stomach, the mucous layer occupies most of the organ wall (Fig 3A, B, E). It is also observed that along the six segments examined there is a clear variation in the volume of the four histological layers of the organ wall: mucosa, submucosa, muscularis (internal and external) and serosa (Fig 4). In the mucous layer is found the simple columnar epithelium of internal lining (Fig. 3A), consisting of cells with basally located nuclei and moderately PAS+ in the apical region, suggesting neutral mucosubstances production and secretion. Unlike epithelial cells, cells located in oxyntic glands show a moderate positive reaction to AB in the apical region, suggesting synthesis and secretion of acidic glycoproteins (Fig. 3A, enlarged image). The oxyntopeptic cells are present in the oxyntic gland (Fig. 3A). The oxyntic glands occupy the largest proportion of the mucous layer. The estimated volume for these structures was 0.074 cm^3/100 g (see supplemental material, Table 1). Clearly, the number of gastric glands decreases substantially towards the pyloric region. The decrease in the proportion of gastric glands consequently leads to a corresponding reduction in the volume of the mucous layer (see Supplementary Material, Table 1).

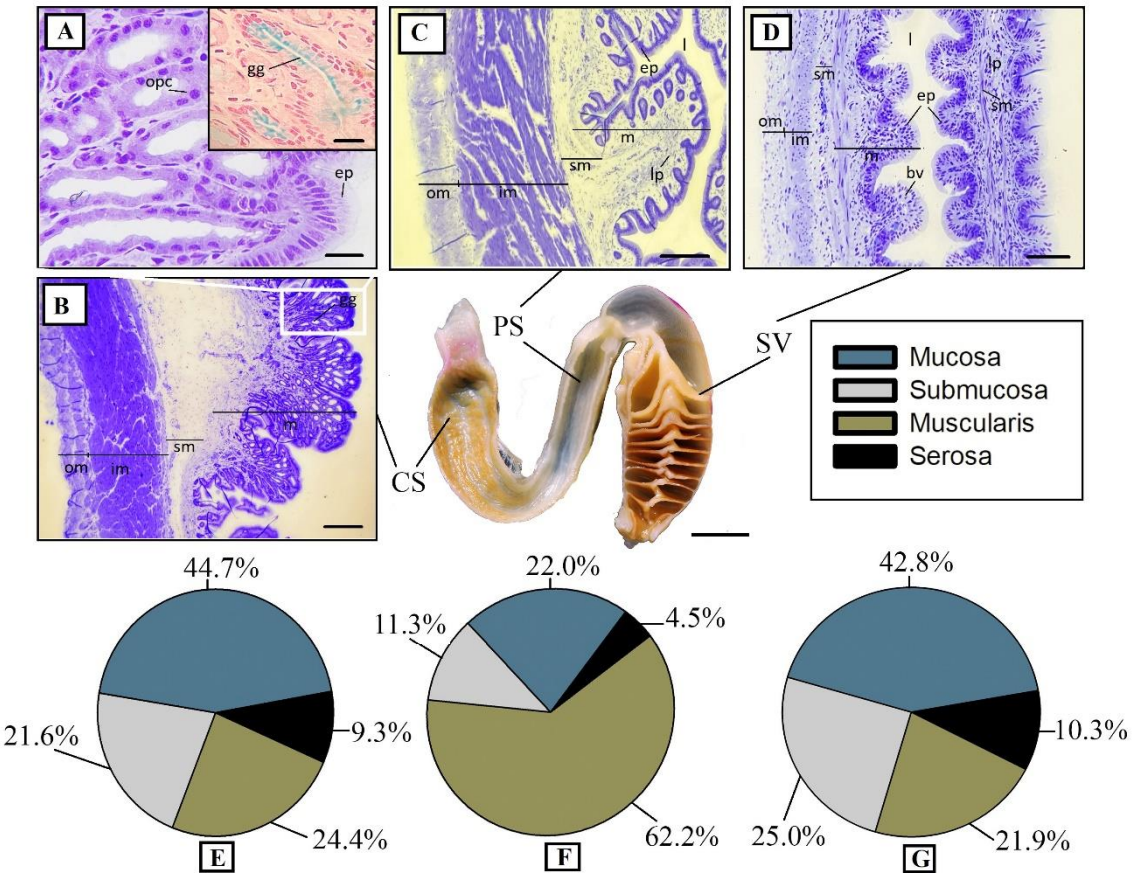

Figure 3. Photomicrographs showing the tissue layers in the cardiac stomach (A), with details of the oxyntic glands (B) with glandular epithelial cells with positive reactivity for Alcian blue. Tissue layers in the pyloric region of the stomach (C) and in the spiral intestine (spiral valve), showing a section of a spiral turn (D). Values in percentage of the wall components of the cardiac stomach (E), pyloric stomach (F) and spiral intestine (G) quantified using stereological methods. The legends in the figure represent: om=outer muscularis; im=inner muscularis; sm=submucosa; m=mucosa; gg=gastric gland; ep=epithelium; opc=oxyntopeptic cell; lp=lamina propria; l=lumen; sm=smooth muscle; CS= cardiac stomach; PC=pyloric stomach; SV=spiral valve. The bars indicate: 50 µm (A, C and D), 10 µm (B) and in the dissected image of the digestive tract = 1cm.

The submucosal layer has numerous fibroblasts and is highly vascularized. Unlike the mucous layer, the proportion of the muscular layer clearly increases towards the pyloric stomach. As in every digestive tract, the muscular layer is constituted by two leaflets (internal and external) of smooth muscle (Fig. 3B). In the cardiac stomach, the muscle layer volume was estimated at 0.073 cm^3/100 g. The serosa layer constitutes only a small fraction (0.03 cm^3/100 g) of the stomach wall.

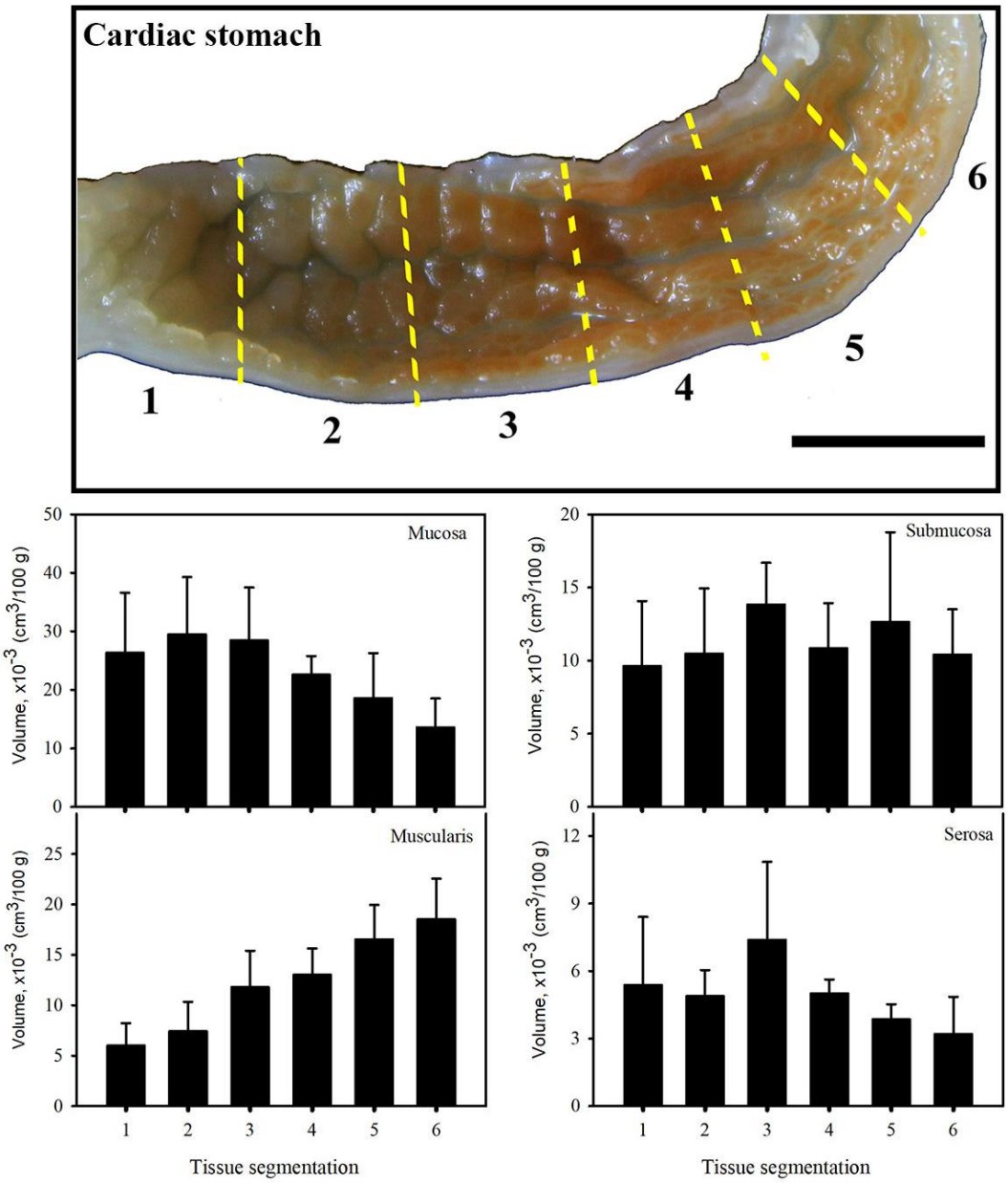

Figure 4. Representative image of the cardiac region of the stomach of *P. wallacei* showing segments 1 to 6 used to estimate tissue volume (cm^3/100 g of animal) according to Cavalieri's principle. The layers of the stomach wall analyzed were mucosa, submucosa, muscularis and serosa. The scale bar in the image corresponds to 0.5 cm.

In the descending portion of the stomach (pyloric) there are significant changes in the volume of the tissue layers of the organ wall (Fig. 5). In the mucosa layer, the gastric glands are already absent (Fig. 3C). The epithelial lining is still formed by a monolayer of columnar cells with the apical region strongly AB+ and has basally positioned nuclei (Fig. 3C). A special feature in the pyloric stomach is the thick smooth muscle layer (Fig.

3C). The quantification data in stereology show that the circular (internal) muscle increases significantly along the tract of the pyloric stomach (Fig 3F, supplementary material, Table 1).

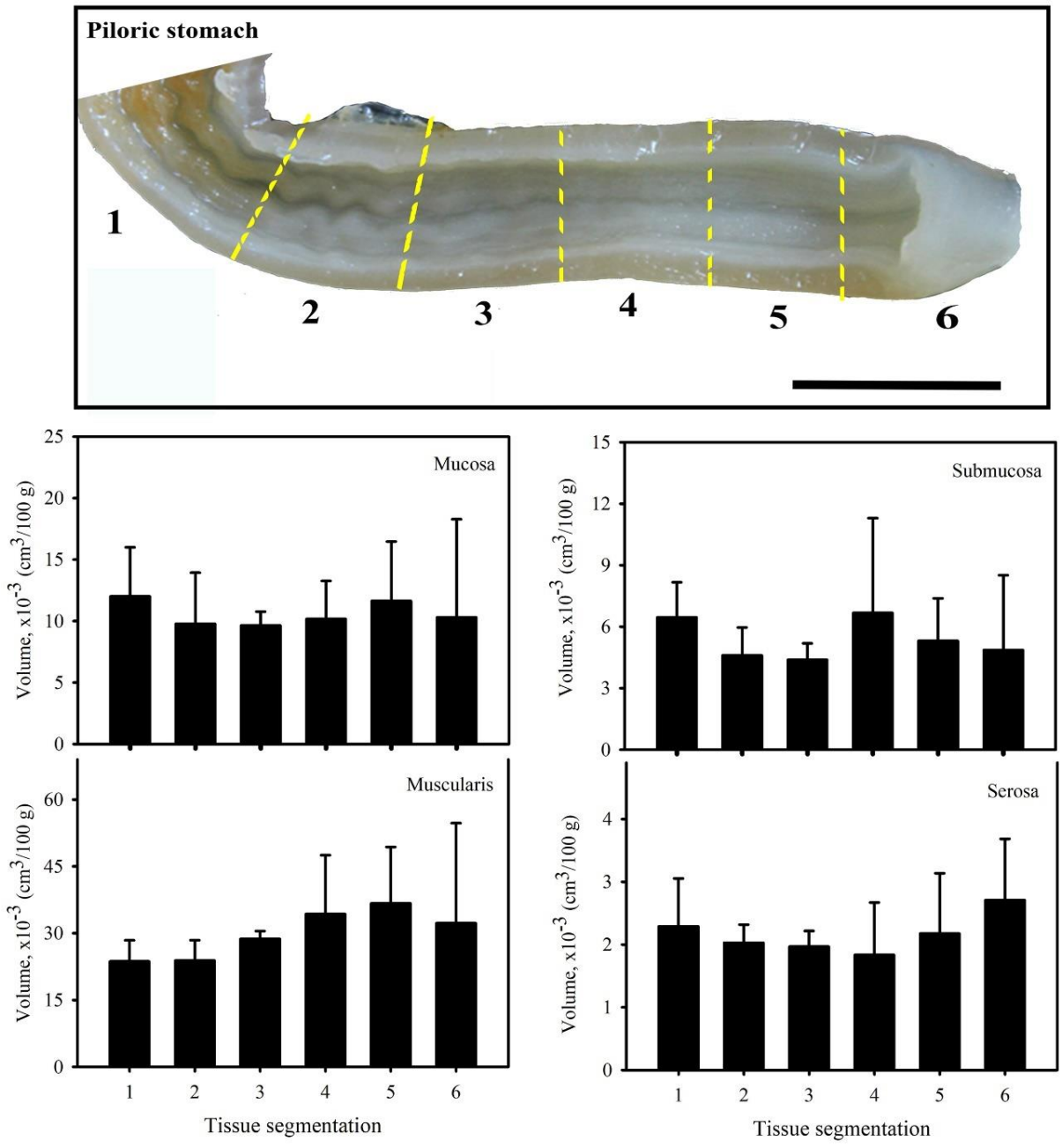

Figure 5. Image illustrating the segmentation of the pyloric region of the stomach of *P. wallacei*. The organ was sectioned into six segments to estimate the absolute volume (cm^3/100g) of the four histological layers: mucosa, submucosa, muscularis and serosa. The scale bar in the image corresponds to 0.5 cm.

Spiral intestine

Stereology data show that small variations in the volume of tissue layers occur, both in the wall and spiral (turns) of the intestine (Fig. 6, see Supplementary Material, Table 2). In our stereological analyses, it was not possible to separate the anterior from the rest of the spiral intestine. As described in materials and methods, the spiral valve was separated into six segments (Fig. 6). However, most of the first segment corresponds to the anterior intestine (see first segment in Fig. 6). The histological layers (mucosa, submucosa, muscularis and serosa) were quantified and the tissue volume of each one was estimated. The mucosa layer occupies the largest volume (Fig. 3D, G) of the wall of the spiral intestine. A gradual increase in the volume of the submucosa layer is observed towards the cloaca (Fig. 6). In contrast, the amount of mucosa layer tends to decrease in this same direction. When the mucosa layer was fractionated to quantify its tissue components, it was found that the decrease in mucosa layer volume was due to a decrease in the amount of epithelium (see Supplementary Material, Table 2). Epithelial cells show a weak reactivity for AB staining (Fig. 3D). The spiral turns inside the intestine are made up only of mucosa layer (Fig. 3D). These turns are specially formed by a large amount of epithelial tissue (0.06 cm^3/100 g) and lamina propria (0.04 cm^3/100 g). The epithelial cells are morphologically and histochemically similar to those found in the spiral intestine wall (Fig. 3D). Subjacent to epithelium is the lamina propria, which is richly vascularized and contains numerous smooth muscle fibers (Fig. 3D).

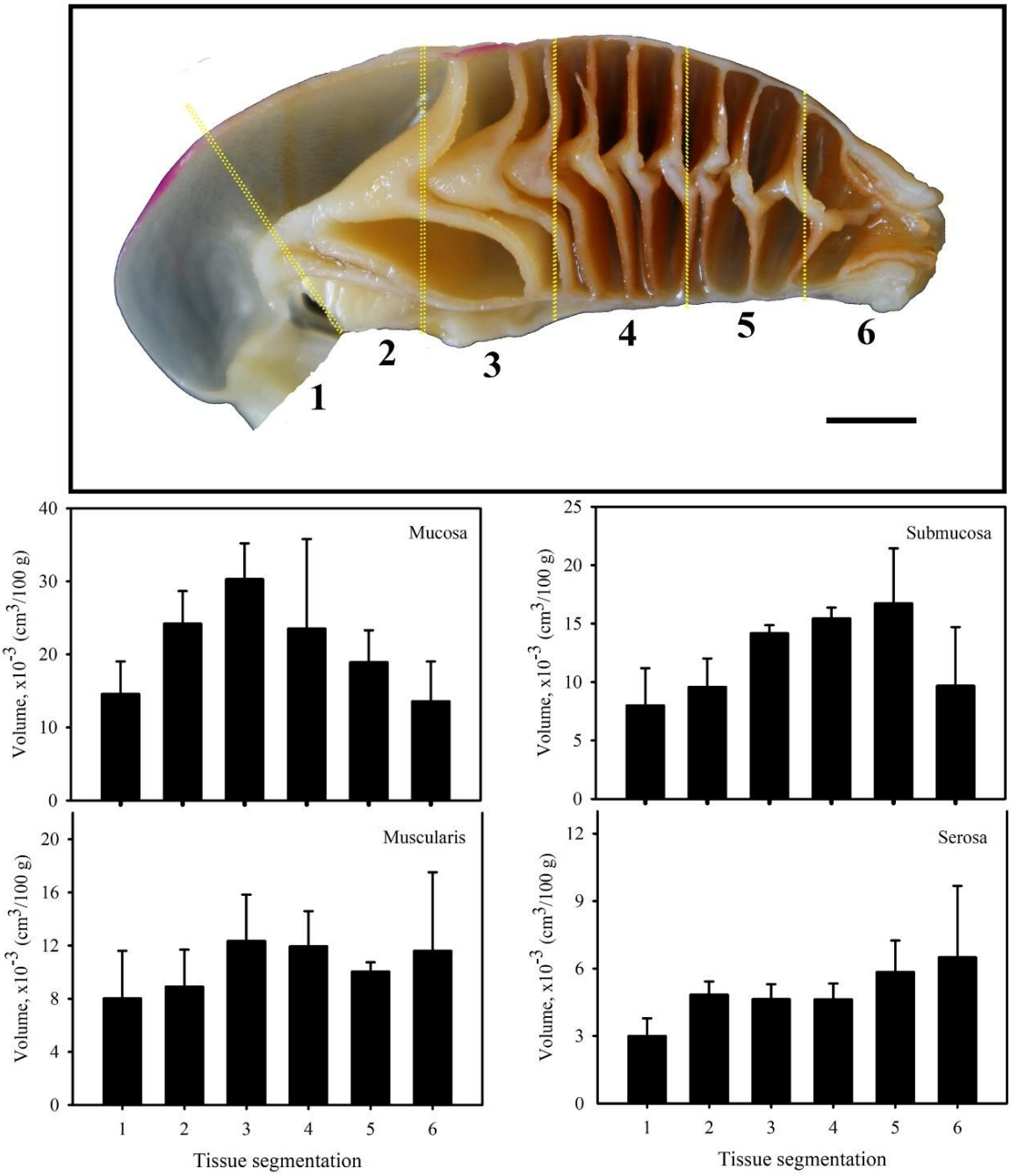

Figure 6. Details of the dissected spiral intestine of *P. wallacei* showing the organ wall and turns (spiral structure). Note in the image that the first segment corresponds to anterior intestine. The organ was sectioned into six segments to estimate the absolute volume (cm^3/100g) in each segment of the histological layers (mucosa, submucosa, muscularis and serosa) present in the wall of the spiral intestine. The scale bar corresponds to 0.5 cm.

4. DISCUSSION

Our study on the morphology of the digestive tract (DT) of *Potamotrygon wallacei* presents a qualitative and quantitative approach. Unlike most studies on the histology of the DT of fish, this work not only provides a detailed description of the morphology of the tissue layers and components, but also estimates the volume occupied by each layer (and each tissue component) of the digestive tract of a neotropical freshwater stingray. For this purpose, modern techniques in stereology were used to quantify these components with minimal bias. In addition, Cavalieri's method associated with Delesse's principle was sensitive to show the pronounced histological changes along the DT of the stingray. Obviously, these changes are related to the functions of the DT, such as digestion of food and absorption of nutrients.

The DT of *P. wallacei* is highly similar to that described for *Potamotrygon amandae*, a neotropical stingray found in the Paraná-Paraguay River basin, in southern Brazil (Aquino et al., 2023). These authors showed, using scanning and light microscopy, that the microscopic anatomy of the gastrointestinal tract of neotropical freshwater stingrays (family Potamotrygonidae) is similar to that of other freshwater and marine stingrays. According to these authors, small variations in the tissue morphology and architecture may be related to habitat and diet specificity. We believe that the close similarities in the digestive tract morphology of these two potamotrygonin species are due to the similarity of food items. Both species preferentially feed on crustaceans, fish and insect larvae (Shibuya, 2022). However, *P. wallacei* has habitat specificity. This species inhabits areas of flooded forests ("igapós") of small streams of black and acidic waters of the Rio Negro (Duncan et al., 2016). Therefore, it is possible that, despite the highly conserved morphology of the digestive tract, differences in morphological and functional aspects (e.g., activities of digestive enzymes) may occur due to the unique and peculiar environment ("*igapó*" forest) in the Amazon basin.

Histologically, the esophagus of *P. wallacei* has similar architecture to that described for *Fluvitrygon signifer* (Chatchavalvanich et al., 2006). The wall is composed of three tissue layers (mucosa, muscularis and serosa). As in other vertebrates, the thick layer muscularis is made up of striated skeletal muscle. Voluntary muscle contraction allows the animal to regurgitate food if it does not want to (Bucking, 2016). Unlike *P. amandae* (Aquino et al., 2023), longitudinal folds are present in *P. wallacei* and suggest the possibility of organ

distention during swallowing. This makes sense, because although potamotrygonins can crush their prey in a similar way to the chewing observed in mammals (Kolmann et al., 2016), *P. wallacei* consumes relatively large prey (crabs and fish), compared to it small body. The mucosa layer of the esophagus of *P. wallacei* is lined by a stratified mucous epithelium with the presence of numerous Alcian blue-staining mucous cells, similar to that described for *P. amandae* (Aquino et al., 2023), *F. signifer* (Chatchavalvanich et al., 2006) and other marine elasmobranchs (Bucking, 2015; Leigh et al., 2017). The mucous cells of *P. wallacei* can secrete neutral and acidic mucins, possibly acting in the pre-digestion of food, such as providing better viscosity for the transit of food towards the stomach, in addition to acting as a microbicidal agent (Abundour & El Bakary, 2018). Despite several authors suggesting a potential role for the esophagus in food digestion (Bucking, 2015; Leigh et al., 2017; Ballantyne, 2015), our studies on the activities of the digestive enzymes of this species suggest a secondary role for proteolytic, lipolytic and carbohydrase enzymes in this organ (see chapter 2 of this work).

The transition from the esophagus to the stomach is marked by profound changes in tissue architecture. The stomach of *P. wallacei* is typically *U*-shaped as in *P. amandae* (Aquino et al., 2023) and *F. signifer* (Chatchavalvanich et al., 2006) and in other batoides (Holmgren & Nilsson, 1999). In this study, we sectioned the stomach into six equidistant segments and quantified its tissue components using stereology. Unlike the work by Aquino et al. (2023) for *P. amandaei*, we divided the stomach of *P. wallacei* into just two regions: cardiac and pyloric. We did not find any anatomical justification for sectioning into three regions. The two main morphological differences between these regions are: the proportion of the muscularis layer increases substantially from the cardiac region (descending) towards the pyloric region (ascending), mainly due to the increase of the muscularis interna. In the cardiac portion, the thick mucosa layer is marked by the presence of numerous gastric glands, which decrease and, and consequently become absent in the pyloric portion, as occurs in other elasmobranchs (Holmgren & Nilsson 1999; Bucking, 2016). In the cardiac region, the mucosa contains gastric pit (foveola) that leads to the tubular gastric glands. This organization is similar to that found in the stingray *F. signifer* (Chatchavalvanich et al., 2006) and *P. amandae* (Aquino et al., 2023) and other batoids (Wilson & Castro, 2010). However, apparently, we can find only one unique type of acidophilic oxyntic cells, as found in the cardiac and fundic regions of the stomach of *P. amandae* (Aquino et al., 2023). The apical region has a positive reaction to acid

mucosubstances (Alcian blue-positive). We suggest a protective role of the mucins against high acidity inside these glands, as reported by Abumandour & El-Bakary (2018). The gastric glands of elasmobranchs are the site of secretion of hydrochloric acid and pepsinogen for protein digestion (Bucking, 2015). Under acidic conditions, pepsinogen is converted to pepsin, a proteolytic enzyme that acts in the first phase of gastric digestion (Leigh et al., 2017). An estimate based on stereological data indicates that the gastric glands of *P. wallacei* stingray correspond to almost 50% of the volume occupied by the mucosa layer in the cardiac portion of the stomach. This is consistent with the high specific activity of pepsin-like enzyme found in the cardiac stomach of this stingray (see chapter 2 of this work). Therefore, these two findings (large volume of gastric glands and high activity of pepsin-like enzymes) explain the high capacity to process proteins by *P. wallacei*. This is consistent with the carnivorous habit of this stingray (Shibuya et al., 2009).

While the cardiac stomach acts in the first phase of gastric digestion, producing the chyme, the pyloric region is muscular and must act by conducting the chyme to the spiral intestine. The thick musculature of the pyloric region aids in mixing the chyme further increasing the efficiency of endopeptidases and other digestive enzymes. In *P. wallacei*, the absence of gastric glands in the pyloric region explains the absence of pepsin-like enzyme activity (see chapter 2 of this study). In *P. amandae*, the large amount of mucus-producing columnar cells helps to protect the mucosa from gastric juice (Aquino et al., 2023). The same characteristic was found in *P. wallacei*. The chyme passes into the intestine controlled by the activity of the pyloric valve or sphincter (Bucking, 2015). It is assumed that the chyme produced in the stomach is neutralized in the spiral intestine by the action of bicarbonate produced by the pancreas (Ballantyne, 2015). The neutralization of chyme favors the action of numerous endo- and exopeptidases (alkaline proteases) that act in the intestine. In fact, high activity of nonspecific lipases, esterases and endopeptidases such as trypsin, chymotrypsin and elastase and exopeptidase such as leucine aminopeptidase were found in the spiral intestine of *P. wallacei* (chapter 2 of this work). Macronutrient digestion provides the micronutrients that will be absorbed by the spiral intestine mucosa. The intestine of *P. wallacei* is morphologically similar to that of *P. amandae* (Aquino et al., 2023) and *F. signifer* (Chatchavalvanich et al., 2006).

Unfortunately, the surface area of the mucosa of the spiral intestine of the stingray *P. wallacei* has not yet been quantified. However, estimation of epithelial tissue volume

suggests that this structure has a large surface area for nutrient absorption. As suggested by other authors, the turns and their secondary folds inside the spiral valve constitute an efficient strategy to increase the absorption area (Hassanpour & Joss, 2009). Stereology data suggest that along the intestine (from the initial segment to the final portion) there is a reduction in the proportion of the mucosa layer and, proportionally, an increase in the submucosa. This indicates that the initial segments of the spiral intestine may be associated with the chemical digestion of macronutrients (proteins, lipids and carbohydrates), while in the final segments, the simple columnar epithelium and high vascularization provide an increase in the efficiency of absorption and release of nutrients into the blood vessels of the submucosa.

5. CONCLUSION

Despite the long evolutionary history of elasmobranchs reported to >400 million years ago, many aspects of intestine morphology are still remarkably similar. As already reported for another potamotrygonin stingray, small variations are associated with habitat specificity and food ecology. In cases where the diet items are similar, the tissue components of the digestive tract are also similar. This is the case with *P. wallacei* and *P. amandae*. These two species of stingrays are part of a monophyletic lineage (family Potamotrygonidae, subfamily Potamotrygoninae) that evolved by vicariant event, at least 10 million years ago (Fontenelle et al., 2020). Although inhabiting different hydrographic basins (Amazon and Paraná-Paraguay), these two species consume similar food items (crustaceans and small fish). This explains the similarity in the architecture of the layers and tissue components of the digestive tract. However, this study shows details of the variations in tissue components throughout the digestive tract of *P. wallacei*. Here we interpret these findings with the functional aspect based on digestive enzyme assays examined in the same compartments of the digestive tract of the stingray *P. wallacei* (see chapter 2 of this work). The set of these results can help to clarify many obscure aspects of the morphophysiology of digestion of the Neotropical potamotrygonids.

ACKNOWLEDGMENT

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, process #484374/2011-7), Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM-PPP #209/2012 and FAPEAM-UNIVERSAL #389/2012) to WPD. RNM received master fellowship from FAPEAM.

AUTHOR CONTRIBUTIONS

Rubia Machado: Conceptualization; formal analysis; investigation; methodology; visualization; writing-original draft; writing-review & editing.

Wallice Duncan: Funding acquisition; Conceptualization; methodology; project administration; supervision; writing-original draft; writing-review & editing.

REFERENCES

Abumandour, M. M. A., & El-Bakary, N. E. R. (2018). Morphological Descriptions of the Esophagus of the Sea Bream (*Sparus aurata*, Linneaus 1758). *Russian Journal of Marine Biology* 44, 135–140. https://doi.org/10.1134/S1063074018020025

Aquino, J. B., Melo, L. F., Rodrigues, R. F., Melo, A. P. F., Morais-Pinto, L., & Rici, R. G. R. (2023). Morphological aspects of the digestive system in freshwater stingray (*Potamotrygon amandae* Loboda and Carvalho, 2013): Myliobatiformes; Potamotrygoninae. *Zoomorphology*, 142, 181–191. https://doi.org/10.1007/s00435-023-00592-w

Araújo MLG, Charvet-Almeida P, Almeida M, Pereira H (2004) Freshwater stingrays (Potamotrygonidae): status, conservation and challenges. In: AC 20 Informative 8.

Ballantyne, J.S. (2015). Metabolism of Elasmobranchs (Jaws II). *Fish Physiology*, 34, 395-456. https://doi.org/10.1016/B978-0-12-801286-4.00007-1

Bucking, C. (2015). Feeding and Digestion in Elasmobranchs: Tying Diet and Physiology Together. *Fish Physiology* 34(B), 347-394. https://doi.org/10.1016/B978-0-12-801286-4.00006-X

Chatchavalvanich, K., Marcos, R., Poonpirom, J., Thongpan, A., & Rocha, E. (2006) Histology of the digestive tract of the freshwater stingray Himantura signifer Compagno and Roberts, 1982 (Elasmobranchii, Dasyatidae). *Anatomy Embryology*,

211(5), 507-18. https://doi.org/10.1007/s00429-006-0103-03

Carvalho, M. D., Rosa, R.S., & Araújo, M. L. (2016ª). A new species of Neotropical freshwater stingray (Chondrichthyes: Potamotrygonidae) from the Rio Negro, Amazonas, Brazil: the smallest species of Potamotrygon. *Zootaxa*, 4107, 566-586.

Cortés, E., Papastamatiou, Y., Carlson, J., Ferry, L., & Wetherbee, B. (2008). An Overview of the Feeding Ecology and Physiology of Elasmobranch Fishes. (ed): Cyrino, J. E. P., Bureau, D., & Kapoor, B.G. In book: Feeding and Digestive Functions in Fishes. https://doi.org/10.1201/b10749-10

Duncan, W. P. (2016). Ecofisiologia das raias de água doce (Potamotrygonidae): mecanismos de interação organismo-ambiente. In: Lasso C. A., Rosa, R., Morales-Betancourt, M. A., Garrone-Neto, D., & Carvalho, R. C. Rayas de água Dulce (Potamotrygonidae) de Suramérica Parte II: Colombia, Brasil, Perú, Bolivia, Paraguay, Uruguay y Argentina. Bogotá: Instituto de Investigación de Recursos Biológicos Alexander von Humboldt. 45-64.

Fontenelle, J. P., Portella, F., Marques, L., Kolmann, M. A., & Lovejoy, N. R. (2021). Biogeography of the neotropical freshwater stingrays (Myliobatiformes: Potamotrygoninae) reveals effects of continent-scale paleogeographic change and drainage evolution. *Journal of Biogeography*, 48, 1406-1419. https://doi.org/10.1111/jbi.14086

Holmgren, S., & Nilsson, S. (1999). Digestive system. *Sharks, skates, and rays. The biology of elasmobranch fishes*, 144-173.

Gundersen, H., Bendtsen, T. F., Korbo, L., Marcussen, N., Møller, A., Nielsen, K., Nyengaard, J., Pakkenberg, B., Sørensen, F., Vesterby, A. (1988). Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. Apmis. 96(1-6): 379-394. https://doi.org/10.1111/j.1699-0463.1988.tb05320.x

Hassanpour, M & Joss, J. (2009). Anatomy and Histology of the Spiral Valve Intestine in Juvenile Australian Lungfish, *Neoceratodus forsteri*. The Open Zoology Journal. 2: 62-85. https://doi.org/10.2174/1874336601002009062

Howard, C. V., Reed & M. G. (2010). Unbiased stereology: three-dimensional measurement in microscopy. 2nd. New York, Berlin, Heidelberg: Springer-Verlag.

Kolmann, M. A., Welch Jr, K. C., Summers, A. P., & Lovejoy, N. R. (2016). Always chewyour food: freshwater stingrays use masticationto process tough insect prey. *Proc. R. Soc*, B283, 20161392. https://doi.org/10.1098/rspb.2016.1392

Leigh, S. C., Papastamatiou, Y., & German, D.P., (2017). The nutritional physiology of sharks. *Reviews in Fish Biology Fisheries*, 27, 561-585.

Shibuya, A. (2022). A review of the ecological role of the Neotropical freshwater stingrays (Chondrichthyes: Potamotrygoninae). *Food Webs*, 32. https://doi.org/10.1016/j.fooweb.2022.e00244

Shibuya, A., Araújo, M. L. G., & Zuanon, J. (2009). Analysis of stomach contents of freshwater stingrays (Elasmobranchii, Potamotrygonidae) from the middle Negro River, Amazonas, Brazil. *Pan-American Journal of Aquatic Sciences*, 4(4), 466-475.

Tschanz, S. A., Burri, P. H., & Weibel, E. R. (2011). A simple tool for stereological assessment of digital images: the STEPanizer. *Journal of Microscopy*, 243(1), 47-59. https://doi.org/10.1111/j.1365-2818.2010.03481.x

Weibel, E. R., Kistler, G. S., & Scherle, W. F. (1966). Practical stereological methods for morphometric cytology. *Journal Cell Biol*ogy, 30(1), 23-38. https://doi.org/10.1083/jcb.30.1.23

Wilson, J. M., & Castro, L. F. C. (2010). Morphological diversity of the gastrointestinal tract in fishes. *Fish Physiology*, 30, 1-55.

SUPPLEMENTARY MATERIALS

Table 1. Stereological quantification tissue components in the stomach of *Potamotrygon wallacei*. The data are as $x10^{-3}$ cm^3/100 g. The legend in the table indicates: EP=epithelium; GG=gastric; LP=lamina propria; OM=outer muscularis; IM= inner muscularis.

	Cardiac stomach			Pyloric stomach			
	Mucosa layer			Mucosa layer		Muscularis layers	
Segment	EP	GG	LP	EP	LP	OM	IM
1.1	0.34±0.31	1.17±1.15	0.40±0.43	0.54±0.16	0.67±0.24	0.41±0.04	1.96±0.44
1.2	0.49±0.14	1.84±0.63	0.62±0.23	0.48±0.20	0.50±0.23	0.44±0.07	1.94±0.39
1.3	0.58±0.02	1.63±0.73	0.63±0.22	0.48±0.02	0.48±0.10	0.60±0.13	2.27±0.06
1.4	0.62±0.21	0.95±0.15	0.69±0.15	0.52±0.17	0.50±0.15	0.62±0.29	2.81±1.06
1.5	0.47±0.04	0.85±0.46	0.54±0.30	0.58±0.25	0.59±0.23	0.63±0.30	3.04±1.09
1.6	0.37±0.09	0.47±0.24	0.52±0.18	0.50±0.39	0.53±0.40	0.69±0.63	2.53±1.62

Table 2. Stereological quantification of tissue components in the spiral intestine of *Potamotrygon wallacei*. The data are as $x10^{-3}$ cm^3/100 g. The legend in the table indicates: EP=epithelium; LP=lamina propria; SM= mucosal muscularis.

	Components				Mucosa layer (intestine wall)			Mucosa layer (intestine turns)		
Segment	Tissues	Lumen	Wall	Turns	EP	LP	SM	EP	LP	SM
1.1	6.44	28.39	3.36	3.08	0.56±0.03	0.62±0.28	0.28±0.14	1.01±0.91	1.30±1.43	0.36±0.39
1.2	10.57	98.52	4.75	5.81	1.32±0.34	0.76±0.16	0.34±0.07	3.15±1.26	1.97±0.54	0.70±0.10
1.3	13.68	134.66	6.15	7.54	1.53±0.32	1.01±0.40	0.49±0.05	3.88±0.70	2.61±1.18	1.05±0.15
1.4	11.05	136.27	5.49	5.56	1.02±0.61	0.86±0.46	0.47±0.17	2.70±0.72	1.95±0.47	0.91±0.11
1.5	12.42	100.30	5.16	7.26	0.68±0.14	0.84±0.20	0.38±0.12	3.22±1.52	2.73±1.19	1.31±0.75
1.6	8.17	31.84	4.14	4.03	0.56±0.24	0.51±0.17	0.30±0.16	1.52±0.81	1.70±0.92	0.81±0.52

CAPÍTULO 2

Digestive physiology of the Amazonian freshwater stingray, *Potamotrygon wallacei*: processing capacity for proteins, lipids and carbohydrates

Rubia Neris Machado[1] & Wallice Paxiúba Duncan[2]

Preparado para ser submetido ao periódico "*Fish Physiology and Biochemistry*", fator de impacto (2021): 3.014

Digestive physiology of the Amazonian freshwater stingray, *Potamotrygon wallacei*: processing capacity for proteins, lipids and carbohydrates

Rubia Neris Machado[1] & Wallice Paxiúba Duncan[2]

[1]Programa de Pós-graduação em Ciência Animal, Faculdade de Ciências Agrárias, Universidade Federal do Amazonas.

[2]Laboratório de Morfologia Funcional, Departamento de Morfologia, Universidade Federal do Amazonas.

Ave. Rodrigo Octávio, 6200. Coroado I, 69.080-900, Manaus, Amazonas, Brazil.

ORCID: http://orcid.org/0000-0002-1279-7163

Running title: Digestive enzymes of freshwater stingrays

Abstract

Freshwater stingrays (Potamotrygoninae) are part of an oldest lineage of marine elasmobranch that evolved exclusively in the freshwater environment of South America. Although diet composition was adjusted during evolution in the new environment, Potamotrygonin stingrays continued to be carnivorous mesopredators with high digestive capacity to process macronutrients (proteins, lipids and carbohydrates). Based on this, we used digestive enzyme activity values to understand the proteolytic, lipolytic potential and capacity to hydrolyze carbohydrates in the gastrointestinal tract (GIT) of the stingray *Potamotrygon wallacei*, an endemic species of the Rio Negro basin (Central Amazon). A hypothetical model based on the role of digestive enzymes in the digestion of each macronutrient was examined using a structural equation model (SEM). The results of this study showed that the stingray *P. wallacei* has high activity of endoproteases-like (pepsin, trypsin, chymotrypsin and elastase) and exopeptidases-like (leucine aminopeptidase) in the stomach and spiral intestine. This can be explained by the protein-rich diet (crab, shrimp, insect larvae and small fish). Based on the data of nonspecific lipases and esterases, this stingray also has a high capacity for lipolysis, possibly due to the high lipid

contents of the insect larvae. Although carbohydrates are not the macronutrients significantly found in the food items of this stingray, in our study it was observed that the carbohydrates were also upregulated. When all the results are examined using SEM, an integration between the main catabolic pathways is clearly seen, especially between proteolysis and lipolysis in the spiral intestine. This finding is in line with the two most important macronutrients (proteins and lipids) found in the diet items of this stingray. These results can help to understand one of the most intriguing aspects of the feeding of *P. wallacei*. Potamotrygonin stingrays are important resources for ornamental purposes, they suffer from stress due to confinement and usually die because of inadequate feeding/diet. Therefore, it is likely that the food items offered to captive animals contain insufficient protein and lipid contents, as well as derivatives of complex carbohydrates such as chitin.

Keywords: Potamotrygonidae, digestive enzyme, proteolysis, lipolysis, carbohydrates.

1. INTRODUCTION

The Chondrichthyes constitute the oldest extant group of jawed vertebrates. Therefore, elasmobranchs are the first known vertebrates to have a digestive tract with a stomach and intestine, in addition to complex digestive physiology (Weatherbee et al. 2012). This includes a gastric secretion that allows, in addition to producing and secreting pepsin and hydrochloric acid into the stomach; produce and secrete pancreatic enzymes such as trypsinogen, chymotrypsinogen, proelastase, and procarboxypeptidases within an intestine (Leigh et al. 2017). Furthermore, elasmobranchs express aminopeptidases anchored to the absorptive cell membrane to enhance protein digestion (Jhaveri et al. 2015).

As chimeras do not have a true stomach, it is likely that this structure and the associated digestive physiology arose in the ancestor between elasmobranchs and other vertebrates (Ballantyne 2015). If the evolutionary history of elasmobranchs is almost 400 million years ago, then we can argue that the oxyntic glands in the stomach were already present in the first representatives of the group (Weatherbee et al. 2012). In other words, they are the first groups known to compartmentalize digestion, while the stomach acidifies chyme to facilitate enzymatic hydrolysis, the spiral intestine alkalizes chyme to complete digestion and then absorbs them.

It cannot be ruled out that the origin and evolution of the gastrointestinal tract and associated physiology arose in elasmobranchs and in the ancestors of recent vertebrates. Therefore, it is likely that the common ancestor had many features in common with the chondrichthyans. In the context of digestive physiology, this allows for some comparisons, although with some limitations, as elasmobranchs have a long evolutionary history essentially as carnivorous predators (Motta and Huber 2012). While the teleost fish conquered different types of niches, thus expanding their trophic categories; elasmobranchs specialized their carnivorous diet and remained top predators or mesopredators (Weatherbee et al. 2012).

The carnivore diet has been maintained through hundreds of thousands of years of evolution, even in the only group that evolved exclusively in the freshwater environment, the stingrays of the subfamily Potamotrygoninae (Rosa et al. 2010). Obviously, no innovations in the anatomy of the gastrointestinal tract of potamotrygonins are expected (see chapter 1 of this work; Aquino et al. 2023). On the other hand, the conquest of a new

niche (freshwater environment) was the result of adaptations to new selective forces, such as types and availability of prey in the freshwater environment (Shibuya 2022). As the enzymatic machinery for digestion was already present in the marine ancestors of potamotrygonins, it is assumed that they were only adjusted to act on the types of prey existing in fresh water. Potamotrygonins occupy a variety of habitats in Neotropical rivers, where they feed mainly on benthic prey composed mainly of mollusks, decapods, insects and fish (Shibuya 2022). Among the 38 valid potamotrygonin species, *Potamotrygon wallacei* deserves attention because it is endemic to the Rio Negro basin (Central Amazon), a river with acidic waters (<pH 5.0), poor in black nutrients (Duncan and Fernandes, 2010), and it was once the freshwater stingray species most exploited by the ornamental fish trade in Brazil (Araújo et al. 2004). In recent years, local populations of this species have been threatened by high capture effort by ornamental fisheries, recreational fisheries, ecotourism (kill stingrays, as the poisonous stinger is a threat to tourist) and habitat deterioration and destruction by anthropogenic activities (Duncan et al., 2016). Unlike most potamotrygonin stingrays, *P. wallacei* is small and lives in the interior of the flooded forest, feeding preferentially on small crustaceans, insect larvae and small fish (Shibuya et al. 2009). In general, when kept in captivity (or aquariums for ornamental purposes) these stingrays suffer from stress due to confinement, do not feed properly, become malnourished and invariably die (Duncan et al. 2016). This reinforces the idea that we should be concerned with the well-being of this animal, starting with understanding its digestive physiology to develop an adequate diet/food that can meet the nutritional requirements of this species.

The scientific basis for this lies in the study of digestive enzymes and their compartmentalization. A detailed description of the gross and microscopic anatomy of the digestive tract of this species was presented in the previous chapter of this work (see chapter 1). Anatomical and histological details of the gastrointestinal tract of another species (*Potamotrygon amandae*) were recently described (Aquino et al. 2023). As expected, the morphology is similar to marine batoids. Unlike teleost fish, information on the activities of digestive enzymes in elasmobranchs is scarce. And, as far as we know, no studies have been published for neotropical freshwater stingrays.

Although scarce, we presume that the proteolytic capacity, through the action of endoptidases (e.g., pepsin, trypsin, chymotrypsin and elastase) and exopeptidases (e.g., carboxypeptidase A and leucine aminopeptidase) are similar to those found in marine

elasmobranchs that consume similar food types (at least crustaceans and fish) to that of stingray *P. wallace*i. However, insect larvae are important items in the diet of *P. wallacei* (Shibuya et al., 2009). Insects are rich in lipids and proteins (Tzompa-Sosa et al. 2014). Thus, our hypothesis is that *P. wallacei* also has a high lipolytic capacity. Due to the carnivorous diet, marine elasmobranchs have a high rate of gastric and intestinal lipolysis (). This can be confirmed by the high activity of nonspecific lipases (Jhaveri et al. 2015). However, carbohydrates are not the main constituents of the diet of elasmobranchs (Newton et al. 2015), but important enzymes such as amylase, maltase and sucrase, in addition to those that degrade chitin (chitinase and chitobiose) may play an important role in the digestion of carbohydrates for many elasmobranch species (Jhaveri et al. 2015).
Finally, we believe that carbohydrases (such as amylase, maltase and sucrase), lipases and proteolytic enzymes may be interrelated in order to increase the efficiency in the digestion of dietary components in elasmobranchs. This type of approach is innovative, as it brings a broad understanding of the role of each digestive enzyme (in each region of the GIT). To test this, we used structural equation modeling (SEM) with enzyme activity data in each digestive organ (stomach and spiral intestine). For this, the catabolic pathways related to digestion of the three most important food components (proteins, lipids and carbohydrates) were considered as latent variables in the SEM model, while enzymes were the observable variables. Our a priori hypotheses are that: (i) *P. wallacei* has a high proteolytic capacity due to its carnivorous diet, as proteolytic enzymes (endo and exopeptidases) are upregulated in the stomach and spiral intestine; (ii) as insect larvae constitute almost a third of the diet, then the lipolytic capacity is also upregulated in the GIT; (iii) assuming a low carbohydrate diet, we believe that carbohydrates may only play a minor role in the food digestion process. We believe that potamotrygonin stingray can vary widely in its ability to digest carbohydrates. However, the digestion of complex carbohydrates such as chitins has not been examined. Unfortunately, chitinolytic enzymes were not measured in this study, since crustaceans (crabs and shrimp) represent half of the items in the diet of this stingray.

2. MATERIAL AND METHODS

Animal capture and handling

The animals (N = 12; total weight = 385.2 ± 99.1 g; disk width = 21.3 ± 2.2 cm) were collected on the Middle Rio Negro, in Barcelos, State of Amazonas, Brazil

(0°30'S/63°33'W; 0°27'S/63°09'W; 1°19'S/62°11'W; 1°14'S/61°55'W). The stingrays were captured through active search using non-motorized wooden canoes, headlight and nets. After collection, the stingrays remained in the water of the same river to minimize post-capture stress. Between 3-6 hours after capture, stingrays were euthanized with 0.5% benzocaine, weighed and measured. Immediately, fragments of the esophagus, cardiac stomach, pyloric stomach and spiral intestine (Figure 1) were removed and frozen in liquid nitrogen. Captures were carried out with authorization from the Chico Mendes Institute for Biodiversity Conservation (ICMBio, the Brazilian environmental agency, license No. #15068–6). All protocols involving animal manipulation were previously approved by the Ethics Committee on Animal Experimentation/Federal University of Amazonas (CEUA/UFAM, protocol #007/2019) in accordance with the guidelines of the Brazilian Council for Control of Animal Experimentation.

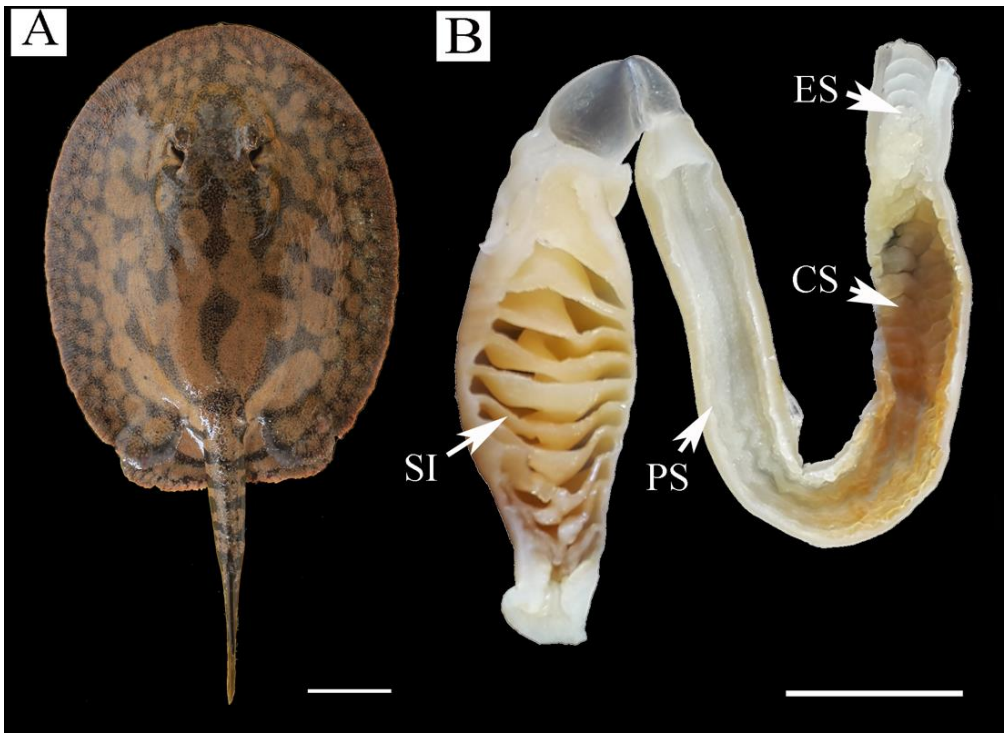

Figure 1. Representative images of a specimen of the neotropical freshwater stingray, *Potamotrygon wallacei* (A) and the dissected gastrointestinal tract (B) showing the esophagus (ES), cardiac stomach (CS), pyloric stomach (PS) and spiral intestine (SI). The scale bars correspond: 5 cm (A) and 1 cm (B).

Enzyme extraction and total protein concentration

The homogenates were prepared in distilled water, followed by centrifugation (10,000 g, 15 min, 4 ^{o}C). The pellet was discarded, while the supernatant (crude extract) was used as a source of digestive enzymes (nonspecific acid and alkaline proteases, trypsin, chymotrypsin, elastase, carboxypeptidase A, leucine aminopeptidase, nonspecific esterases and lipases, acid and alkaline phosphatases, amylase, maltase and sucrase) and incubated or added to the enzyme assay cocktail listed below. The quality of enzyme reagents and substrates were all Sigma-Aldrich grade. All enzymatic assays were performed in duplicate using a microplate reader (Multiskan Go, Thermo Scientific^{TM}, MA, USA) under constant temperature of 30 ^{o}C. Soluble protein of crude enzyme extracts was quantified by the Bradford method (Bradford 1976) using bovine serum albumin as a standard. Specific enzyme activities were expressed as U/mg protein, where 1U= 1 µmoles of substrate/min.

The activities of total acid proteases were measured using a nonspecific substrate according to Walter (1984) with adaptations to carry out the assays in a microplate reader. Assays were performed with 200 mM Glycine-HCl buffer (pH 2.0) and 1% casein incubated at 37^{o}C for 30 min. The reaction was stopped using 10% trichloroacetic acid and all readings were performed at 280 nm. Tyrosine was used as standard, and one unit of enzyme was defined as the amount of enzyme needed to catalyze the formation of 1.0 mg of tyrosine per hour per mg protein. Alkaline proteases were measured in 100 mM Tris-HCl buffer (pH 7.6) and 1% azocasein as substrate (Sarath et al. 1989). Readings were performed at 440 nm, one enzyme unit being equivalent to a change of 0.001 absorbance unit. Trypsin activities were performed using two different substrates: 1 mM BAPNA, N-α-benzoyl-DL-arginine p-nitroanilide (Erlanger et al. 1961) and with 1 mM TAME (Tosyl L-Arg Methyl Ester) substrates (Hummel 1959). On both substrates, assays were performed in 50 mM Tris-HCl buffer (pH 8.0), 10 mM $CaCl_2$, 2 mM NaCl, 100 µM tosyl L-phenylalanine-chloromethylketone (chymotrypsin inhibitor) with readings were performed at 410 nm (BAPNA) and 247 nm (TAME). Calculations were made considering the molar extinction coefficient of BAPNA (ε=8.8 cm/mM) and TAME (0.54 cm/mM). Assays for chymotrypsin-like activities were also performed using two different substrates: 1 mM GAPNA, N-Glutaryl L-Phe 4-paranitroanilide (Hummel 1959) and BTEE, Benzoyl L-Tyr Ethyl Ester (Asgeirsson and Bjarnason 1991) in 200 mM TRIS-HCl buffer (pH 8.0), 10 mM $CaCl_2$, 2 mM NaCl, 100 µM tosyl L-Lysine-

chloromethylketone (trypsin inhibitor). Readings were performed at 410 nm for GAPNA and 256 nm for BTEE. Calculations of chymotrypsin activities were made considering the molar extinction coefficients of GAPNA (ε=8.8 cm/mM) and BTEE (0.96 mM/cm). Data are expressed as 1 µmoles of substrate/min.

Elastase enzyme assays were performed in 100 mM TRIS-HCl buffer (pH 8.0) using 4 mM SucAla3-pNA (N-Succinyl-Ala-Ala-Ala-p-Nitroanilide) as substrate (Taylor and Crawford 1975). Readings were taken at 410 nm. To calculate the enzyme activities, the SucAla3-pNA molar extinction coefficient of ε=9.9 cm/mM was considered. Carboxypeptidase A activities were performed in 50 mM TRIS-HCl buffer (pH 8.0) and 100 mM NaCl using 2 mM hippuryl-L-phenylalanine as substrate (Folk and Schirmer 1963). Readings were monitored at 254 nm and calculations made using a molar extinction coefficient of 0.019 cm/mM. Leucine aminopeptidase activity assays were determined using 1 mM L-leucine p-Nitroanilide substrate (Appel 1974) in 100 mM TRIS-HCl buffer (pH 8.0), 10 mM $MnCl_2$ and 10 mM $MgCl_2$. Absorbances were monitored at 410 nm. To calculate the enzyme activities, the molar extinction coefficient of 8.8 cm/mM was considered.

Nonspecific esterases and lipases were assayed using 200 mM TRIS-HCl buffer (pH 8.0), 10 mM $CaCl_2$ and 10 mM NaCl. The substrates were previously diluted in isopropyl alcohol and then in a cocktail containing 200 mM TRIS-HCl, 2 mM sodium taurodeoxycholate, 0.4% Triton X-100 and 0.1% gum arabic. For esterases, 2 mM p-nitrophenyl-acetate was used as substrate (Innocenti et al 2008), while for lipases two different substrates were used: 5 mM p-nitrophenyl palmitate and 5 mM p-nitrophenyl myristate (Albro et al., 1985). For all enzymes, absorbances were monitored at 405 nm and activity calculations considered the molar extinction coefficient of 18 cm/mM.

Acid and alkaline phosphatases were assayed in 50 mM TRIS-HCl buffer (pH 3.0 and 8.0, respectively), 10 mM $CaCl_2$, 5 mM $ZnSO_4$ and 10 mM $MgCl_2$ according to Stauffer (1989). For these phosphatases, p-Nitrophenyl phosphate substrate (2 mM) was used and the readings monitored at 405 nm. The calculation of activities considered the molar extinction coefficient of 8.8 cm/mM.

Amylase activities were determined with two different substrates (1% starch and 1% glycogen). The reactions consisted of incubating (at 37ºC) the crude extracts with the substrate (starch or glycogen) in 20 mM sodium phosphate buffer (pH 8.0), 10 $CaCl_2$, 10

mM NaCl for 30 min. After that, 3,5-dinitrosalicylic acid was added at 100°C and again incubated for 10 min. Immediately after cooling, readings were taken at 570 nm (Junge et al. 2001). A calibration curve using maltose was used to calculate the released maltose produced.

For the activities of maltase and sucrase enzymes, the substrates 20 mM maltose and 20 mM sucrose were used, respectively (Worthington 1991). The assays consisted of incubating the crude extracts with the substrate (maltose or sucrose) in 100 mM sodium acetate buffer (pH 8.0), 20 mM $CaCl_2$, 10 NaCl. The reaction was stopped by boiling at 100°C for 5 min. One unit of enzyme activity (maltase or sucrase) was expressed as one mole of glucose released at 37°C. To quantify the concentration of glucose released, a commercial Labtest kit was used (Lagoa Santa, Brazil).

Statistical analyses

The values in the tables are expressed as mean±standard deviation (minimum – maximum). A structural equation model (SEM) was used to analyze the theoretical model (Figure 2) of the relationships between enzymes in the gastrointestinal tract of the potamotrygonid stingray. To examine this model, enzyme data were classified into three categories (latent variables), with causality flowing between them. The observed variables were the specific activity data of each digestive enzyme quantified in the three regions of the stingray digestive tract (cardiac stomach, pyloric stomach and spiral intestine): The latent variables included in the model were: (i) “Proteins” were included all the enzymes that participate in the hydrolysis of peptides and oligopeptides, such as: acid proteases (pepsin-like), alkaline proteases, trypsin, chymotrypsin-like, elastase and carboxypeptidase A; (ii) “Lipases”, including non-specific lipases and esterases; (iii) “Carbohydrates”, this component includes amylase, maltase and sucrase enzymes. All variables were log-transformed for analysis to achieve linearity and normality. The SEM model in Figure 2 was translated into structural equations, where linear relationships between variables were assumed. The parameters to be estimated are path coefficients (indicated by arrows in the figures). These path coefficients provide the magnitude of the effect of one variable on the other, keeping the other variables constant. To construct Pearson's correlation matrix, activity data of enzymes assayed with the following substrates were used: trypsin (TAME), chymotrypsin (BTEE) and lipase (4-nitrophenyl palmitate). The other enzymes were with the substrates described in the material and

methods section. The Pearson's correlation matrix was performed using the R software, while the SEM were tested using the R-package lavaan, semTools and semPlot (Rosseel 2012; Epskamp 2017; R Core Team 2014).

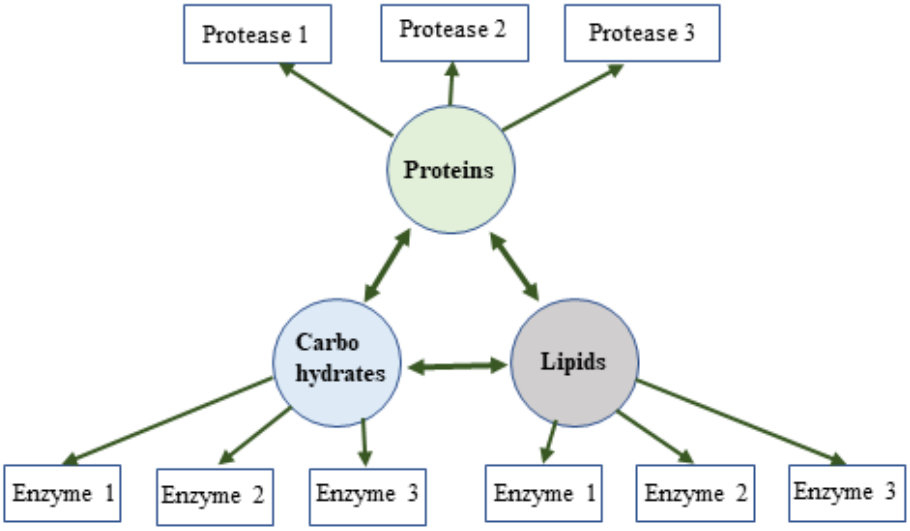

Figure 2. Conceptual diagram to be tested in the Structural Equation Model for the set of digestive enzymes analyzed in each organ of the gastrointestinal tract of the potamotrygonid stingray. The enzymes represent the observed variables in each organ, while "proteins", "carbohydrates" and "lipids" were the latent variables.

3. RESULTS

Digestive enzyme in esophagus

In the freshwater stingray, *Potamotrygon wallacei*, the esophagus is a muscular tract that presents quantifiable activities of proteolytic enzymes, such as: trypsin-like, chymotrypsin-like, elastase and carboxypeptidase A (Table 1). Detectable amounts of digestive enzymes related to the hydrolysis of polymeric carbohydrates (amylase) and disaccharidases such as maltase and sucrase. Acid phosphatase, although with low activity, was also detected. However, no measurable lipase activities were found, even using different substrates, such as palmitate and myristate. In Pearson's correlation matrix (Figure 3), the endopeptidases (trypsin, chymotrypsin and elastase) are positively ($p<0.05$) related to each other. Similarly, enzymes that catabolize carbohydrates, such as amylase, maltase and sucrase, also show positive relationships ($p<0.05$) with each other. However, amylase showed a significantly positive relationship with the endopeptidase's

trypsin and chymotrypsin. The other enzymes showed moderate and weak non-significant correlations.

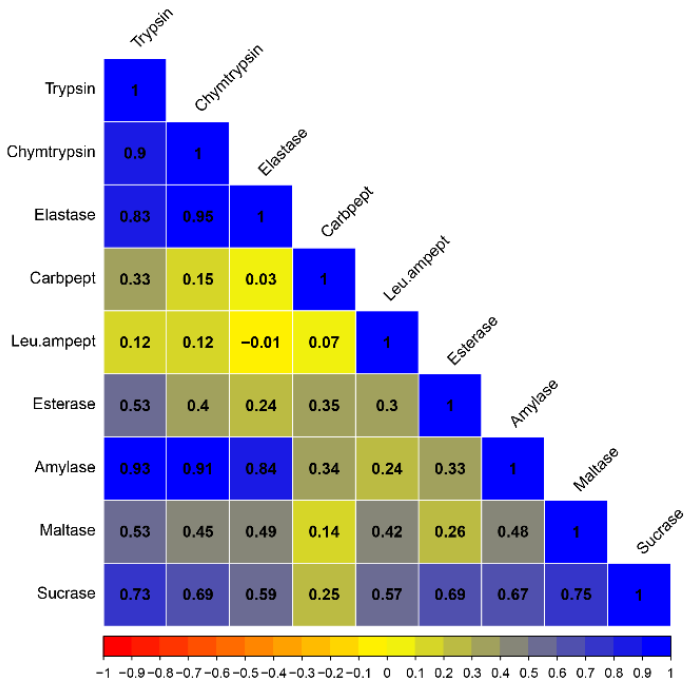

Figure 3. Pearson's correlation matrix for all the set of digestive enzymes detected in the esophagus of the freshwater stingray, *Potamotrygon wallacei.* The data of the enzymes trypsin and chymotrypsin used in the correlations were obtained from TAME and BTEE substrates (see material and methods for details).

Digestive enzyme in cardiac stomach

The cardiac stomach of the freshwater stingray represents the descending portion of the digestive stomach. In this portion are the oxyntic glands that secrete HCl and acid proteases, such as pepsin. In this region we found the highest specific activities of nonspecific acid proteases (pepsin-like activity). The activity of this enzyme is almost 7 times higher than in the pyloric region of the stomach. Fourteen enzymes were analyzed in this region of the stomach, only nonspecific lipase (assayed with the substrate myristate), alkaline phosphatase, amylase (assayed with the substrate glycogen) and carboxypeptidase A did not show detectable activities. Although moderate, the pepsin-like enzyme has a positive correlation ($p<0.05$) with trypsin and chymotrypsin (Figure 4A). Among the endopeptidases (trypsin, chymotrypsin and elastase), a strong positive relationship ($p<0.05$) is observed between them. The enzyme leucine aminopeptidase did

not show any relationship with endopeptidases. In the cardiac stomach, proteolytic enzymes (pepsin-like, trypsin, chymotrypsin and elastase) are strongly associated with carbohydrate hydrolases, such as amylase, maltase and sucrase. As expected, the hypothetical model (Figure 2) of the causal relationships between the digestive enzymes examined in the cardiac stomach was confirmed, as can be seen in the path diagram SEM-based showed in Figure 4B. The hydrolysis of peptides and oligopeptides (indicated by the latent variable "proteins") is strongly demonstrated by the relationship of endopeptidases (trypsin and elastase) and pepsin-like. Carbohydrases also showed strong association with each other, as shown by the "carbohydrates" catabolism pathway (one of the latent variables of the model). This pathway has a moderate (but significant) association with protein catabolism. Nonspecific lipases, esterases and acid phosphatase were grouped together to form the latent variable "lipids". Among them, lipase strongly contributes to lipid metabolism. Furthermore, a strong association between the hydrolysis of lipids and carbohydrates is clearly observed. However, the relationship between lipid and peptide catabolism is only moderate.

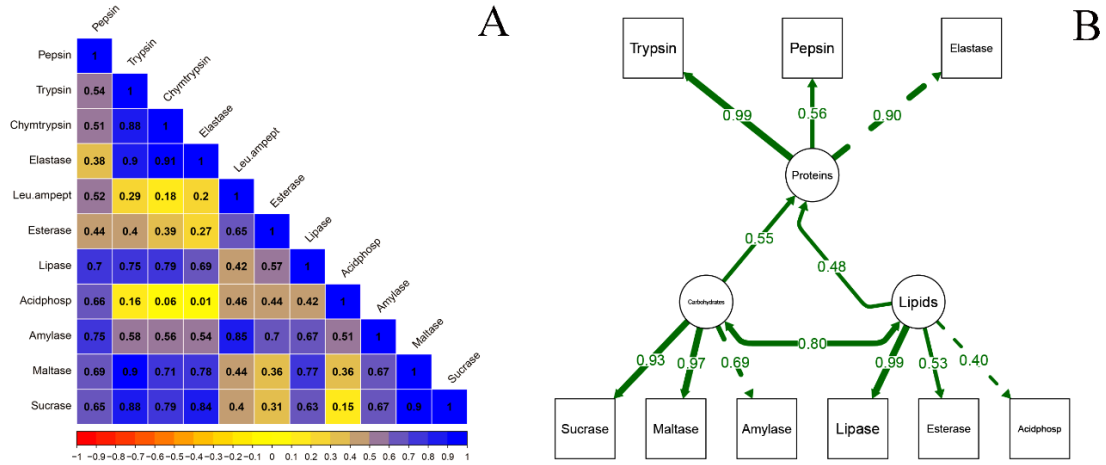

Figure 4. Pearson correlation matrix (A) and structural model (B) including latent variables ("proteins", "lipids" and "carbohydrates") and digestive enzymes measured in the cardiac stomach of *P. wallacei*.

Digestive enzyme in pyloric stomach

The ascending portion of the potamotrygonid stingray stomach corresponds to the pyloric portion of this organ. In this segment, three enzymes (acid and alkaline proteases and carboxypeptidase A) are not found in detectable activities. Significant activities of acid

protease and disaccharidases enzymes, such as maltase and sucrase, were found. The endopeptidases chymotrypsin and elastase showed a strong positive association ($p<0.05$) with each other (Figure 5A). Moderately, trypsin also showed a positive (but significant) relationship with lipase, acid phosphatase, and carbohydrate hydrolytic enzymes (amylase, maltase, and sucrase). A strong positive relationship was observed between lipase and acid phosphatase. In the SEM-based path analysis diagram, one can see the weak contribution of amylase and maltase enzymes to carbohydrate catabolism in the pyloric stomach (Figure 5B). Furthermore, nonspecific lipase and esterase enzymes show only a moderate contribution to lipid hydrolysis. The relationship between carbohydrates and lipid catabolism is very weak. On the other hand, although the specific activity of trypsin and chymotrypsin is low in the pyloric stomach (see Table 1). These enzymes are the most important endopeptidases that contribute to protein catabolism in this region of the stomach.

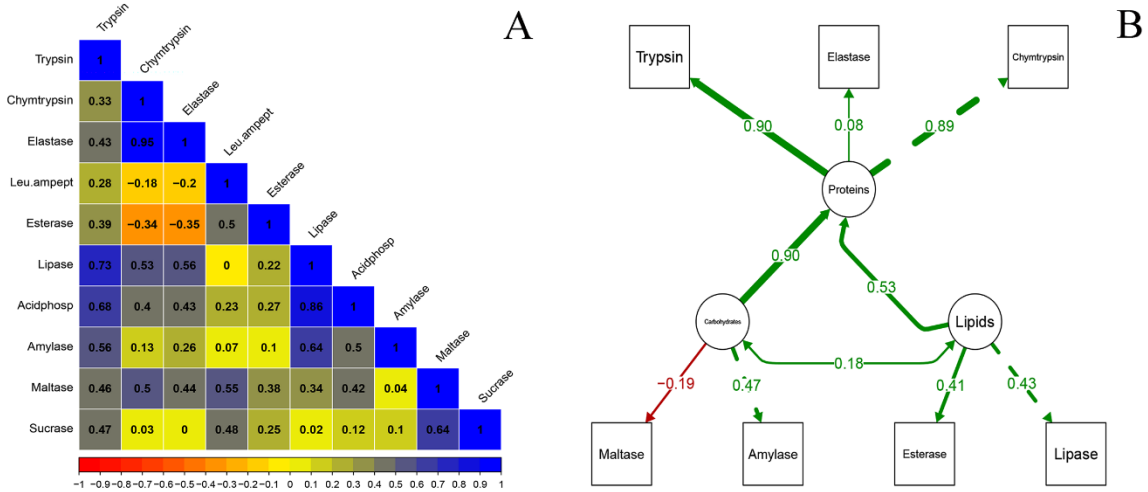

Figure 5. Pearson correlation matrix (A) and structural model (B) including latent variables ("proteins", "lipids" and "carbohydrates") and digestive enzymes measured in the pyloric stomach of *P. wallacei*.

Digestive enzyme in spiral intestine

The intestine of the stingray is like a spiral valve. Digestive enzymes were measured in the medial portion of this organ. There were no detectable activities of the enzymes, such as carboxypeptidase A, amylase (with the substrate glycogen) and acid phosphatase. On the other hand, the activities of alkaline proteases predominate. The activities of the

endopeptidases (trypsin, chymotrypsin and elastase) are quite high compared to the other organs of the gastrointestinal tract of the freshwater stingray. Leucine aminopeptidase exopeptidase was also found in detectable amounts in the spiral valve. It should also be highlighted the expressive activities of starch hydrolases (amylase) and disaccharides (maltase and sucrase). As for lipid catabolism, activities of lipases (using both substrates, palmitate and myristate), nonspecific esterases and alkaline phosphatases were detected.

Pearson's correlation matrix (Figure 6A) indicated that trypsin and chymotrypsin endopeptidases are positively associated ($p<0.05$) with each other. Unlike trypsin, chymotrypsin was positively ($p<0.05$) related to elastase. The endopeptidases trypsin and chymotrypsin were also positively related to exopeptidase leucine aminopeptidase. A positive relationship was found between lipases and alkaline phosphatase. Similarly, the same association was also found between carbohydrate hydrolases (amylase, maltase and sucrase).

The SEM-based model (Figure 2 And Figure 6A) confirmed the strong contribution of trypsin and chymotrypsin endopeptidases to protein catabolism. Similarly, amylase, maltase and sucrase also strongly contribute to carbohydrate catabolism. The same was observed for nonspecific lipases. Overall, the model showed a strong interaction between carbohydrate and lipid hydrolysis, although a moderate association between proteolytic enzymes and lipases could be observed. However, endopeptidases are weakly related to carbohydrate catabolism.

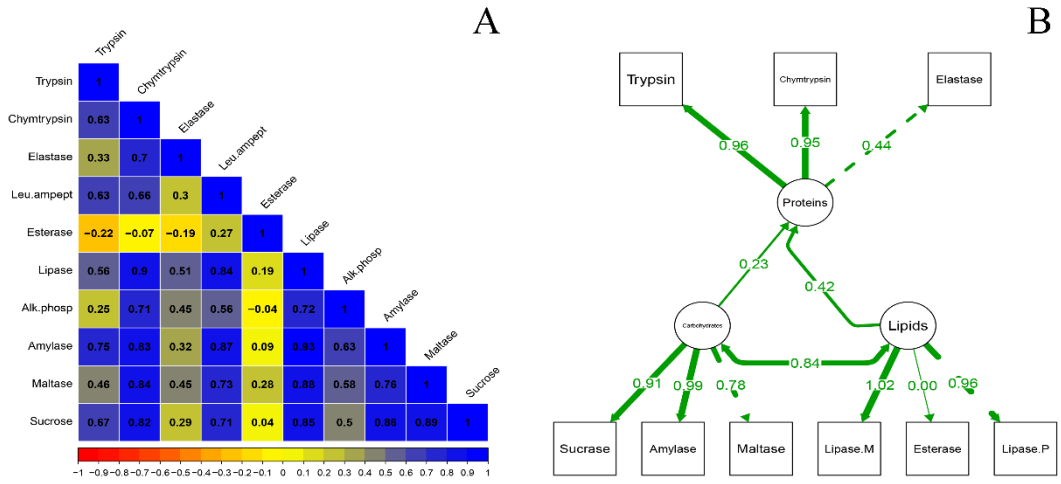

Figure 6. Pearson correlation matrix (A) and structural model (B) including latent variables (“proteins”, “lipids” and “carbohydrates”) and digestive enzymes measured in the spiral intestine of *P. wallacei*. Lipase.M and lipase.P represent the unspecific lipases assayed with myristate and palmitate as substrates, respectively.

Table 1. Enzyme activities (U/mg protein) of selected digestive enzymes in different organs of digestive tract of neotropical freshwater stingray, *Potamotrygon wallacei*. nd= non-detectable. See Material and methods for enzyme substrate acronyms.

Group	Class	Enzymes	Substrates	Esophagus (n=11)	Cardiac stomach (n=12)	Pyloric stomach (n=12)	Spiral intestine (n=12)
Protases	Endopeptidases	Acid protease (Pepsin-like)	Casein	Nd	111.09±133.60 (21.15-504.33)	nd	8.89±9.68 (0.00-30.79)
		Alkaline protease	Azocasein	Nd	3.69±2.72 (0.82-9.37)	nd	19.16±15.64 (2.56-47.54)
		Trypsin	TAME	0.20±0.12 (0.05-0.48)	0.55±0.07 (0.41-0.66)	0.13±0.08 (0.01-0.31)	0.60±0.05 (0.46-0.67)
			BAPNA	0.33±0.23 (0.08-0.90)	0.91±0.22 (0.59-1.28)	0.51±0.15 (0.35-0.81)	1.11±0.25 (0.71-1.58)
		Chymotrypsin	BTEE	0.06±0.04 (0.02-0.15)	0.22±0.05 (0.12-0.29)	0.13±0.06 (0.04-0.29)	0.28±0.02 (0.21-0.29)
			GAPNA	0.19±0.11 (0.05-0.46)	0.53±0.14 (0.27-0.74)	0.26±0.08 (0.14-0.39)	0.67±0.23(0.37-1.04)
		Elastase	N-Succinyl-Ala-Ala-Ala-p-Nitroanilide	0.25±0.17 (0.08-0.69)	0.56±0.23 (0.28-1.08)	0.26±0.16 (0.07-0.71)	0.76±0.15 (0.51-1.07)
	Exopeptidases	Carboxypeptidase A	Hippuryl-L-phe	0.12±0.05 (0.05-0.22)	Nd	nd	nd

		Leucine aminopeptidase	L-leu p-Nitroanilide	nd	0.07±0.02 (0.02-0.11)	0.03±0.01 (0.02-0.04)	0.29±0.15 (0.12-0.56)
	Ratio	Trypsin/Chymotrypsin	TAME/BTEE	3.18±0.81 (1.94-4.65)	2.63±0.34 (2.19-3.38)	1.03±0.62 (0.13-2.13)	2.16±0.07 (2.06-2.27)
			BAPNA/GAPNA	1.71±0.27 (1.37-2.20)	1.77±0.24 (1.33-2.23)	2.03±0.36 (1.53-2.87)	1.73±0.37 (0.89-2.18)
Carbohydrate-enzymes	Carbohydrate polymers	Amylase	Starch	0.78±0.62 (0.00-2.17)	3.33±0.68 (2.19-4.69)	1.75±0.27 (1.46-2.36)	3.35±1.37 (0.92-4.94)
			Glycogen	0.99±0.89 (0.00-2.34)	nd	0.68±0.54 (0.00-1.36)	nd
	Disaccharidases	Maltase	Maltose	167.73±73.39 (50.07-305.03)	209.72±26.78 (172.27-263.52)	92.59±23.09 (55.80-128.84)	216.67±46.08 (155.91-302.08)
		Sucrase	Sucrose	201.02±53.54 (80.54-261.39)	207.28±48.44 (145.33-302.39)	112.76±19.33 (89.07-153.89)	316.86±52.35 (207.03-376.30)
Lipases/Esterases	Lipases	Nonspecific lipase	Palmitate	nd	4.59±2.09 (0.97-9.09)	1.01±0.76 (0.13-2.87)	3.79±3.69 (0.00-11.19)
			Myristate	nd	nd	0.42±0.37 (0.00-1.23)	3.63±3.06 (0.00-7.33)

	Esterases	Nonspecific esterase	p-NP-acetate	1.99±0.78 (0.91-3.46)	1.24±0.19 (0.92-1.54)	0.76±0.29 (0.31-1.16)	1.86±0.26 (1.38-2.21)
Phosphatases	Acidic	Acid phosphatase	p-NP-phosphate	0.04±0.04 (0.00-0.11)	0.18±0.07 (0.11-0.27)	0.05±0.02 (0.02-0.08)	nd
	Alkaline	Alkaline phosphatase	p-NP-phosphate	nd	nd	0.05±0.03 (0.02-0.10)	1.62±0.93 (0.24-2.84)

4. DISCUSSION

Although no studies have confirmed a conclusive list of all digestive enzymes produced in the gastrointestinal tract of elasmobranchs (Leigh et al. 2017). Therefore, as well as several works that estimated the activity of digestive enzymes were based on studies of *in vitro* assays, both for elasmobranchs and for teleost fish.

To our knowledge, this is the most complete profile of digestive enzymes analyzed along the gastrointestinal tract (GIT) of a potamotrygonin stingray, neotropical freshwater elasmobranch. The activities of endopeptidases (acid and alkaline proteases, trypsin, chymotrypsin and elastase), exopeptidases (carboxypeptidase A and leucine aminopeptidase), nonspecific lipases and esterases, acid and alkaline phosphatases, amylase, maltase and sucrase were examined in the esophagus, cardiac stomach, stomach pyloric and spiral intestine.

We found high activities of acid proteases (pepsin-like) in cardiac stomach. This finding was expected, since large amounts of gastric glands were also observed in the mucosa (see Chapter I). The activities of endopeptidases, trypsin, chymotrypsin and elastase were found to be high in cardiac stomach and spiral intestine. In the spiral valve, the trypsin/chymotrypsin (T/Q) ratio indicates that tryptic activity is twice as active compared to chymotryptic. The spiral intestine also has measurable leucine aminopeptidase activity. Also, as we expected, acid phosphatase was more active in the cardiac stomach, while alkaline phosphatase was in the spiral intestine. Lipases and carbohydrases (both for polymers and disaccharides) are also higher in the spiral valve. The relationships between digestive enzymes and the interrelationships between the main digestive pathways, such as proteins, carbohydrates and lipids, indicate an organ-specific profile, however, strongly dependent on the catabolism of peptides and oligopeptides. Associations between digestive enzymes will be discussed within each organ of the GIT. However, its implications for the diet and feeding of this freshwater stingray species will be discussed holistically.

Esophagus

Elasmobranchs can use mechanical and chemical processes to break down their food and absorb their nutrients. Recently, potamotrygonin stingrays have been shown to perform asymmetric jaw movement, effectively chewing, to dismantle their prey like mastication

observed in mammals. (Shibuya et al. 2012; Kolmann et al. 2016; Laurence-Chasen et al. 2019). Thus, the mouth of stingrays becomes the initial mechanical site of digestion. The dismantling of prey can facilitate the attack of digestive enzymes on their substrates. Studies have shown that nearly 50% of *P. wallacei* diets consist of prey hard chitin-containing exoskeletons, such as trichodactylid crabs and euryrhynchid shrimps (Shibuya et al. 2009). The food is then moved down the esophagus. As described in Chapter 1 of this paper, the esophagus is a short tract that has a thick striated musculature. The mucosa has a stratified epithelium with several neutral and acidic mucin-producing cells. This morphological organization is similar to that recently described for the digestive system of *P. amandae* (Aquino et al. 2023).

Although it can be assumed that chemical digestion truly begins in the stomach, there are some works demonstrating the presence of digestive enzymes in the esophagus of fish (Nagase 1964; Borlongan 1990; Kozarić et al. 2004; Batista and Pires 2016). Some authors argue that in some fish species (e.g., *Chelidonichthys lucerna*) the entire digestive tract of the fish is involved in the digestion and absorption of dietary components (Bastiančić et al. 2023). However, we believe that the digestive enzyme activities detected in the esophagus of *P. wallacei* are due to the intracellular hydrolases and phosphatases. For example, the proteolytic activities attributed to trypsin-likes in the esophagus can be attributed to proteolysis carried out by lysosomal cathepsin serine proteases, such as cathepsin-like A and G (Patel et al. 2018). Several digestive enzymes were located by immunohistochemical methods in the esophagus of fish, among them we can mention acid phosphatase (epithelial localization), alkaline phosphatase (lamina propria) and non-specific esterases found both in the epithelium and in the lamina propria of *Chelidonichthys lucerna* (Bastiančić et al. 2023). However, the esophagus has an epithelial lining with a high density of mucous cells. Furthermore, it is a muscular tract with voluntary movement. Therefore, in the context of the mechanical processing of food, the esophagus must play an intermediary role by mixing the food previously dismantled in the mouth, improving the viscosity by adding mucosubstances (it also helps to hydrate the food) and, finally, transport food to the stomach. In the context of chemical food processing, the contribution of the digestive enzymes to the biochemical digestion of food in the esophagus should be minimal.

Stomach: cardiac and pyloric portion

As described in Chapter 1, in stingray *P. wallacei* the stomach has a siphonal shape (U-shaped), with the descending portion representing the cardiac region, while the descending portion is pyloric. Unlike the cardiac portion, the pyloric region lacks gastric glands. These glands are the sites of secretion of digestive fluids containing HCl and pepsinogen in elasmobranchs (Ballantyne 2015; Bucking 2015; Leigh et al. 2017). Therefore, as expected, acid secretion and pepsinogen synthesis occur in the cardiac portion of the stomach in this species.

In elasmobranchs, as in other vertebrates, the secretion of gastric juice is stimulated by the presence of food (Bucking, 2015). We do not know whether potamotrygonin stingrays show a continuous or sporadic pattern of HCl secretion (see Papastamatiou, et al. 2007). However, if we based on their nocturnal behavior (preferred feeding time), in theory, these stingrays could control gastric acidification. Regardless of the type, whether continuous or sporadic, under acidic conditions, pepsinogen is converted into pepsin (Ballantyne 2015). Concomitantly, acidity itself helps to denature many proteins, exposing internal peptide bonds to attack by proteolytic enzymes. Pepsin is the major digestive enzyme in the stomach, responsible for protein hydrolysis. It is a stomach endopeptidase that cleaves peptide bonds in the middle of the polypeptide that prefers to cleave aromatic amino acid such as phenylalanine, tyrosine and tryptophan, as well as attacking acidic amino acid, such as aspartic and glutamic acid (Ahn et al. 2013). The results of the present study with the stingray *P. wallacei* consider that acidic proteases (assayed at pH 2.0) are related to the proteolytic activities of pepsin (herein referred to as pepsin-like).

Unlike pyloric stomach, the proteolytic capacity of pepsin-like was found to be high in the cardiac stomach of this species. The pepsin-like is probably responsible for the initial stage of the protein hydrolyzation phase. However, other endopeptidases-like such as trypsin, chymotrypsin and elastase were also found in the cardiac stomach of *P. wallacei* stingray. However, only traces of exopeptidases (e.g., leucine aminopeptidase) were recorded in this organ. The gastric phase of proteolysis continues in the pyloric stomach but is aided by tryptic and chymotryptic activities in addition to elastase. Multivariate methods based on structural equation modeling (SEM) support the hypothesis that subsequent steps of peptide digestion continue to the formation of oligopeptides due to

the action of endopeptidases both in the cardiac and pyloric portions. One explanation for this may be due to the carnivorous feeding habits of potamotrygonin stingrays (Shibuya 2022). Furthermore, it is generally accepted that carnivorous fish have higher proteolytic activity than other fish with different dietary habits (Xiong et al. 2011).

Although crustaceans constitute almost half of the prey items of the stingray *P. wallacei*. Furthermore, chitin is the major structural component of the exoskeleton of arthropods, unfortunately, we did not measure the activity of chitinolytic enzymes, whose pH optimum usually occurs in the acidic region of the stomach (Matsumiya & Mochizuki 1996). Therefore, we cannot disregard the importance of this polymeric and complex carbohydrate for feeding this species of stingray. It is possible that the tendency to consume crustaceans can serve as a link in the sequential chain of carbohydrate digestion, from the most complex to the simplest, in the same way as in skipjack tuna (Dias et al. 2021) and some elasmobranchs that prey primarily crustaceans (Cortés 1999). Amylolytic activity was weakly recorded in the stomach (cardiac and pyloric portions) of the stingray *P. wallacei*. However, the disaccharidases (maltase and sucrase) have also high activity in the cardiac stomach, but not in the pyloric portions. Stomach carbohydrases were reported for several fish species (Munilla-Moran and Saborido-Rey 1996). However, our SEM-based model supports a structural relationship between protein and carbohydrate digestion in the cardiac stomach of *P. wallacei* stingray. This suggests a functional plasticity between these two major catabolic pathways into the stomach. Although this species is remarkably carnivorous, it has clearly been considered a generalist mesopredator, whose prey availability fluctuates due to the annual hydrological pulse of the Rio Negro basin (Shibuya 2022). In the context of the food chain, being a generalist also means having physiological plasticity to process different types of substrates and promoting an interchangeable flow of components between different catabolic pathways.

In addition to the proteins and carbohydrates present in the crustaceans that are part of the diet of the potamotrygonin stingray, lipids can be another important source of nutrients. Lipids can be present in large amounts in insect larvae, especially gomphid dragonflies, which constitute 30% of the items consumed by the stingray *P. wallacei* (Shibuya et al. 2009). Insects have a high lipid content, second only to proteins (dos Santos Aguilar 2021). The fatty acid profile indicates that oleic, linoleic, linolenic and palmitic acids are the most important, in addition to providing more calories and having excellent nutritional value (Tzompa-Sosa et al. 2014). The lipids present in the *P. wallacei*

stingray food items are initially emulsified in the stomach by mechanical action. After that, possibly some gastric lipase should promote lipid lipolysis. We found a moderate enzymatic activity of the gastric lipase and nonspecific esterases in both cardiac and pyloric portion. Lipases are triacylglycerol hydrolases that preferentially attack long-chain triglycerides, while "true" esterases are carboxyl ester hydrolases that prefer to hydrolyze short-chain triglycerides (Lopes et al. 2011). As both are present in the cardiac stomach of the potamotrygonin stingray, we believe that the stomach has a moderate to high lipolytic capacity. It is also suggestive that the stomach itself may be involved in the absorption of lipids. Our study demonstrated that acid phosphatase is present in this organ, but more active in the cardiac portion than in the pyloric. Acid phosphatase acts by dephosphorylating di- and tri-phosphorylated nucleotides, whose residual components will also be absorbed, but it also helps to regulate lipid absorption (Barka 1963) and probably acting on the demineralization of bone tissue (Bull et al. 2002) in prey such as small fish consumed by the stingray. Some authors also argue in favor of some absorptive capacity in the stomach (Bucking 2015; Leigh et al. 2017). Again, our SEM-based model showed a high association between lipolytic capacity and carbohydrate hydrolysis in the stingray stomach. Likewise, although moderate, the relationship between lipolysis and proteolysis can also be evidenced. Therefore, the chyme produced in the stomach is a complex mixture of peptides, oligopeptides, soluble carbohydrates, fibers, fractions of various types of fatty acids, di- and triglycerides, vitamins, mineral salts and other nutrients that can still be digested and subsequently absorbed in the spiral valve.

Spiral intestine

As in other vertebrates, chyme passes from the stomach to the intestine through the pyloric sphincter (c). The anatomical and histological details of the intestine of *P. wallacei* stingray, including the spiral valve, are presented in chapter 1 of this work. However, the activities of all digestive enzymes were examined only in the spiral intestine of this species. As expected, peptic activity was markedly reduced in this region. Possibly the alkaline pH of the spiral intestine reduces the activity of pepsin-like enzyme (Papastamatiou et al. 2007). The detection of acidic proteases in the homogenates of spiral valve is probably due to cathepsins, a group of lysosomal proteases that can also hydrolyze proteins (Natalia et al. 2004). Like other elasmobranchs, the spiral valve is the major site of alkaline proteases (Ballantyne 2015; Bucking 2015; Leigh et al. 2017). Unlike the other portions of the GIT studied, in the potamotrygonin stingray, the spiral

intestine was the only organ that showed detectable activity of alkaline proteases. In fact, the alkaline endopeptidases, such as trypsin, chymotrypsin and elastase were more active in this portion than in the others. Therefore, the spiral valve completes protein digestion. The chyme coming from the stomach is neutralized by the secretion of bicarbonate (Ballantyne 2015). While food is retained in the spiral valve by retrograde contractions (Leigh et al. 2017). Meanwhile, enterokinase activates trypsin, which in turn tends to amplify protein digestion through a cascade of activation of other proteolytic enzymes such as chymotrypsin and elastase (Ma et al. 2005). The ratio between tryptic/chymotryptic activity (T/C ratio) in the spiral intestine of *P. wallacei* is around 2:1. Generally, in carnivorous fish, trypsin activity is greater than chymotrypsin (Rungruangsak-Torrissen et al. 2006). The elevated T/C ratio has been associated with greater digestive efficiency (Rungruangsak-Torrissen et al. 2006). Mechanically, trypsin itself cleaves the derived peptides and oligopeptides, preferentially at arginine and lysine residues (Ma et al. 2005), whose hydrolysis products result in greater digestibility than other endoproteases. Arginine is the most potent stimulator of insulin secretion in fish, which results in an increase in the rate of absorption of other amino acids (Matty and Lone 1985). On the other hand, chymotrypsin hydrolyses peptides at tryptophan, phenylalanine, tyrosine and leucine residues (Appel 1986). However, some authors argue that the excess of aromatic amino acids such as phenylalanine and tyrosine have a negative impact on biological systems, similarly to aromatic hydrocarbon pollutants (Rungruangsak-Torrissen et al. 2006). Despite this, leucine is a ketogenic and branched-chain amino acid that in addition to stimulating the synthesis of muscle components (Harper et al. 1984), is also deaminated to produce acetoacetate, an important fuel for muscle metabolism (Ballantyne 2015). In turn, elastase preferentially attacks alanine, glycine, and serine residues (Gray and Cooper 1971). Although trypsin activates carboxypeptidases A (Ma et al. 2005), this enzyme was not detected in the spiral valve of *P. wallacei*. Carboxypeptidases A is an exopeptidase that cleaves hydrophobic amino acid residues at the C-terminal (Fricker 2007). As these residues had already been efficiently hydrolyzed in the stomach by the action of pepsin, the absence (or undetected enzymatic activity) of carboxypeptidase A makes sense. However, we detected moderate activity of an exopeptidase, leucine aminopeptidase in this species. It is an enzyme located on the brush border of absorptive cells located in the intestinal mucosal epithelium that act on N-terminal peptide of proteins (Taylor 1993). In some species of sharks, such as bonnethead sharks (*Sphyrna tiburo*), levels of aminopeptidase activities in the spiral

intestine are up to 10-fold higher than in the intestine of carnivorous teleost fish (Jhaveri et al. 2015). Thus, the high level of endopeptidases and exopeptidase activities suggests that potamotrygonin stingray is highly efficient at digesting proteins. The proposed conceptual model SEM-based corroborates the close relationship between tryptic and chymotryptic activities, indicating that these two enzymes, in addition to elastase and leucine aminopeptidase tend to efficiently complete protein digestion within the spiral intestine of *P. wallacei*. This model also supports a moderate association between protein digestion and lipid hydrolysis.

Lipids are an important component of the diet for elasmobranchs because they can be used as an energy source to save nitrogen (Ballantyne 2015), although saving nitrogen is not the case for potamotrygonins (Wood et al. 2002). Lipolytic activity into the spiral intestine suggests that potamotrygonin stingray likely readily digests lipids with great efficiency. Elasmobranchs have a long evolutionary history as carnivorous predators. Therefore, a strong dependence on the hydrolysis of proteins and lipids in the GIT was expected to sustain high metabolic demands for these substrates by the potamotrygonin stingray. As previously discussed, a large part of the diet of *P. wallacei* consists of insect larvae and fish, we can assume that proteins and lipids constitute most of the diet components of this stingray. The specific activities of non-specific lipases and esterases were found to be elevated in the spiral valve. However, regression analyzes showed no relationship between them. This suggests that lipases break ester bonds of long-chain fatty acids, while esterases act on short-chain ones. A nonspecific lipase secreted by the pancreas involved in intestinal lipolysis has been identified and found at high levels in *Triakis semifasciata* (Pattons et al. 1977). Elasmobranchs have unusual lipid metabolism. For example, striated muscles and heart are unable to oxidize fatty acids to produce energy (Ballantyne 2015). One explanation for this could be a lack of albumin or a similar protein to transport lipids. Instead, striated muscle and the heart use ketone bodies produced by the oxidation of fatty acids in the liver. High lipolytic activity in the stomach and spiral intestine relies especially on LDL and VLDL, low density and very low-density lipoproteins, respectively (Metcalf and Gemmell 2005) to transport lipids into the liver of the elasmobranchs.

Although carbohydrates (starch and other disaccharides) are not energetically significant in the diet of many elasmobranchs (Leigh et al. 2017). Even so, these animals have a high capacity to use glucose in different tissues (Ballantyne 2015). From an evolutionary point

of view, this group shows that catabolic mechanisms and carbohydrate absorption arose in ancestors before tetrapod. *P. wallacei* has a significant ability to hydrolyze carbohydrates, especially starch and disaccharides such as maltase and sucrase. However, as previously discussed, we believe that potamotrygonin stingrays have a high capacity to hydrolyze complex carbohydrates present in arthropod exoskeleton. The SEM-based model supports the weak interaction between carbohydrate hydrolysis and the two main catabolic pathways (proteins and lipids). Suggesting that the digestion of proteins and possibly lipids represent the two most important macronutrient degradation pathways for the stingray *P. wallacei*.

Implications for diet and nutrition

One of the most sensitive aspects for the conservation biology of the stingray *P. wallacei* is feeding. In captivity or kept in aquariums, this stingray does not eat. When still eating insufficiently on small fish and crustaceans (especially small shrimp) and, within a few months loses weight and dies of starvation. The causes of this are not known. Obviously, this is one of the biggest problems for the maintenance of this species in aquariums, which came to represent more than 60% of the species of stingrays exported by Brazil (<60% of the total number of stingrays for ornamental purposes). No studies have been conducted to understand the physiological mechanisms of nutrient digestion or attempts at a balanced diet to meet the metabolic demands of this species. In any case, we suggest that any attempt to elaborate a ration or food for this species should take into account the balanced quantity and quality of proteins, carbohydrates and lipids present in the ration and/or food offered for this species. In other words, one needs to carefully examine the centesimal composition of trichodactylid crustaceans, gomphid dragonflies' larvae and the main fish species consumed by this species of stingray. After all, it is a carnivorous species that depends on an adequate diet of proteins and lipids present in its prey.

ACKNOWLEDGMENT

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, process #484374/2011-7), Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM-PPP #209/2012 and FAPEAM-UNIVERSAL #389/2012) to WPD. RNM received master fellowship from FAPEAM.

REFERENCES

Ahn J, Cao Mj, Yu YQ, Engen JR (2013) Accessing the Reproducibility and Specificity of Pepsin and other Aspartic Proteases. Biochim Biophys Acta 1834(6): 1222–1229 https://doi.org/10.1016/j.bbapap.2012.10.003

Albro PW, Hall RD, Corbett JT, Schroeder J (1985) Activation of nonspecific lipase (EC 3.1.1.-) by bile salts. Biochim Biophys Acta 835: 447-490. https://doi.org/10.1016/0005-2760(85)90117-1

Appel W (1974) Leucine aminopeptidase determination with L-leucinamide as substrate. In: H.U. Bergmeyer (ed.). Methods of enzymatic analysis. Academics Press, New York, pp. 954-958.

Appel W (1986) Chymotrypsin: Molecular and catalytic properties. Clin Biochem 19(6): 317-322. https://doi.org/10.1016/S0009-9120(86)80002-9

Aquino JB, Melo LF, Rodrigues RF, Melo APF, Morais-Pinto L, Rici RGR (2023) Morphological aspects of the digestive system in freshwater stingray (*Potamotrygon amandae* Loboda and Carvalho, 2013): Myliobatiformes; Potamotrygoninae. Zoomorphology 142:181–191. https://doi.org/10.1007/s00435-023-00592-w

Araújo MLG, Charvet-Almeida P, Almeida M, Pereira H (2004) Freshwater stingrays (Potamotrygonidae): status, conservation and challenges. In: AC 20 Informative 8.

Asgeirsson B, Bjarnason JB (1991) Structural and kinetic properties of chymotrypsin from atlantic cod (*Gadus morhua*): comparison with bovine chymotrypsin. Comp Biochem Physiol 99B:327–335. https://doi.org/10.1016/0305-0491(91)90050-N

Ballantyne JS (2015) Metabolism of Elasmobranchs (Jaws II). Fish Physiol 34:395-456 https://doi.org/10.1016/B978-0-12-801286-4.00007-1

Barka T. (1963) Fat Absorption and Acid Phosphatase Activity in Intestinal Epithelium of Mice. JAMA. 183(9):761–764. https://doi.org/10.1001/jama.1963.63700090007013b

Bastiančić L, Vlahek I, Benko V, Lovrić M, Valić D, Kuž S (2023) Histochemical research of enzymes involved in cellular digestion in the digestive tract of tub gurnard, Chelidonichthys lucerna. Fish Physiol Biochem https://doi.org/10.1007/s10695-023-01188-3

Batista I, Pires C (2016) Comparative Studies of the Proteolytic Activity of Crude Extracts from the Digestive Tract of Three Shark Species. J Aquat Food Prod Technol 151-165 https://doi.org/10.1300/J030v11n03_12

Borlongan IG (1990) Studies on the digestive lipases of milkfish, *Chanos chanos*. Aquac 89: 315-325 https://doi.org/10.1016/0044-8486(90)90135-A

Bucking C (2015) Feeding and Digestion in Elasmobranchs: Tying Diet and Physiology Together. Fish Physiol 34(B): 347-394 https://doi.org/10.1016/B978-0-12-801286-4.00006-X

Bull H, Murray PG, Thomas D, Fraser AM, Nelson PN (2002) Acid phosphatases. Mol Pathol 55(2):65-72 https://doi.org/10.1136/mp.55.2.65

Cortés E (1999) Standardized diet compositions and trophic levels of sharks. J Marine Scie 56: 707–717 https://doi.org/10.1006/jmsc.1999.0489

Dias D, Dardengo GM, Engrola S, Navarro-Guillén C (2021) Characterization and comparison of the digestive physiology of two scombrids, *Katsuwonus pelamis* and *Sarda sarda*, in the Gulf of Cádiz. Plos One 16(4): e0249541. https://doi.org/10.1371/journal.pone.0249541

dos Santos Aguilar JG (2021) An overview of lipids from insects. Biocatal Agric Biotechnol 33 https://doi.org/10.1016/j.bcab.2021.101967

Duncan WLP, Fernandes MN (2010) Physicochemical characterization of the white, black, and Clearwater rivers of the Amazon Basin and its implications on the distribution of freshwater stingrays (Chondrichthyes, Potamotrygonidae). Pan-Am J Aquat Sci 5(3): 454-464

Duncan, WLP (2016) Ecofisiologia das raias de água doce (Potamotrygonidae): mecanismos de interação organismo-ambiente. In: Lasso CA, Rosa R, Morales-Betancourt MA, Garrone-Neto D, Carvalho RC. Rayas de água Dulce (Potamotrygonidae) de Suramérica Parte II: Colombia, Brasil, Perú, Bolivia, Paraguay, Uruguay y Argentina. Bogotá: Instituto de Investigación de Recursos Biológicos Alexander von Humboldt 45-64.

Epskamp S (2015) semPlot: Unified Visualizations of Structural Equation Models. Struct Equ Modeling 22: 474-483 https://doi.org/10.1080/10705511.2014.937847

Erlanger BFE, Kokowsky N, Cohen W (1961) The preparation and properties of two new chromogenic substrates of trypsin. Arch Biochem Biophys 95: 271–278. https://doi.org/10.1016/0003-9861(61)90145-X

Folk JE, Schirmer EW (1963) The porcine pancreatic carboxypeptidase A system. I. Three forms of the active enzyme. J Biol Chem. 238:3884-94.

Fricker L (2007) Carboxypeptidases. xPharm: The Comprehensive Pharmacology Reference 1-4 https://doi.org/10.1016/B978-008055232-3.60564-1

Gray GM, Cooper HL (1971) Protein Digestion and Absorption. Gastroenterol 61(1): 535-544 https://doi.org/10.1016/S0016-5085(19)33506-1

Harper AE, Miller RH, Block KP. (1984) Branched-chain amino acid metabolism. Annu Rev Nutr 4:409-54 https://doi.org/10.1146/annurev.nu.04.070184.002205

Hummel BCW (1959) A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. Can J Biochem Physiol 37:1393-1399.

Innocenti A, Scozzafava A, Parkkila S, Puccetti L, De Simone G, Supuran CT (2008) Investigations of the esterase, phosphatase, and sulfatase activities of the cytosolic mammalian carbonic anhydrase isoforms I, II, and XIII with 4-nitrophenyl esters as substrates. Bioorg Med Chem Lett 18:2267-2271. https://doi.org/10.1016/j.bmcl.2008.03.012

Jhaveri P, Papastamatiou YP, German DP (2015) Digestive enzyme activities in the guts of bonnethead sharks (*Sphyrna tiburo*) provide insight into their digestive strategy and evidence for microbial digestion in their hindguts. Comp Biochem Physiol Part A Mol Integr Physiol 189: 76-83 https://doi.org/10.1016/j.cbpa.2015.07.013

Junge W, Wortmann W, Wilke B, Waldenström J, Kurrle-Weittenhiller A, Finke J, Klein G (2001) Development and evaluation of assays for the determination of total and pancreatic amylase at 37 degrees C according to the principle recommended by the IFCC. Clin Biochem 34:607-615. https://doi.org/10.1016/s0009-9120(01)00278-8

Kolmann MA, Welch KC, Summers AP, Lovejoy, NR (2016) Always chew your food: freshwater stingrays use mastication to process tough insect prey. Royal Soc 283 https://doi.org/10.1098/rspb.2016.1392

Kozariæ Z, Kužir S, Nejedli S, Petrinec Z, Sreboèan E (2004) Histochemical distribution of digestive enzymes in hake, *Merluccius merluccius* L. 1758. Veterinarski Arhiv 74 (4): 299-308

Laurence-Chasen JD, Ramsay JB, Brainerd EL (2019) Shearing overbite and asymmetrical jaw motions facilitate food breakdown in a freshwater stingray, *Potamotrygon motoro*. J Exp Biol 222 https://doi.org/10.1242/jeb.197681

Leigh SC, Papastamatiou Y, German DP (2017) The nutritional physiology of sharks. Rev Fish Biol Fish 27:561-585

Lopes DB, Fraga LP, Fleuri LF, Macedo GA (2011) Lipase and esterase: to what extent can this classification be applied accurately? Food Sci. Technol 31(3): 608-613 https://doi.org/10.1590/S0101-20612011000300009

Ma W, Tang C, Lai L. (2005) Specificity of trypsin and chymotrypsin: loop-motion-controlled dynamic correlation as a determinant. Biophys J. 89(2):1183-93 https://doi.org/10.1529/biophysj.104.057158

Matsumiya M, Mochizuki A (1996) Distribution of Chitinase and β-N Acetylhexosaminidase in the Organs of Several Fishes. Fish Scie 62(1): 150-151 https://doi.org/10.2331/fishsci.62.150

Matty AJ, Lone KP (1985) The Hormonal Control of Metabolism and Feeding. In: Tytler P, Calow P. (eds) Fish Energetic. Springer, Dordrecht 185-209 https://doi.org/10.1007/978-94-011-7918-8_7

Metcalf VJ, Gemmell NJ (2005) Fatty Acid Transport in Cartilaginous Fish: Absence of Albumin and Possible Utilization of Lipoproteins. Fish Physiol Biochem 31: 55–64 https://doi.org/10.1007/s10695-005-5124-y

Motta PJ, Huber DR (2012) Biology of Sharks and Their Relatives. In: Carrier JC, Musick JA, Heithaus MR (ed) Prey Capture Behavior and Feeding Mechanics of Elasmobranchs, 2nd end. 58

Munilla-Morán R, Saborido-Rey F (1996) Digestive enzymes in marine species. II. Amylase activities in gut from seabream (*Sparus aurata*), turbot (*Scophthalmus maximus*) and redfish (*Sebastes mentella*). Comp Biochem Physiol 113(B): 827-834 https://doi.org/10.1016/0305-0491(95)02101-9

Nagase G (1964) Contribution to the physiology of digestion in *Tilapia mossambica* Peters: Digestive enzymes and the effects of diets on their activity. Z Verg Physiol 49: 270–284 https://link.springer.com/article/10.1007/BF00298199

Natalia Y, Hashim R, Ali A, Chong A (2004) Characterization of digestive enzymes in a carnivorous ornamental fish, the Asian bony tongue *Scleropages formosus* (Osteoglossidae). Aquac 233:305-320 https://doi.org/10.1016/j.aquaculture.2003.08.012

Newton KC, Wraith J, Dickson KA (2015) Digestive enzyme activities are higher in the shortfin mako shark, *Isurus oxyrinchus*, than in ectothermic sharks as a result of visceral endothermy. Fish Physiol Biochem 41: 887–898

Papastamatiou YP, Purkis SJ, Holland KN (2007) The response of gastric pH and motility to fasting and feeding in free swimming blacktip reef sharks, *Carcharhinus melanopterus*. J Exp Marine Biol Ecol 345: 129-140 https://doi.org/10.1016/j.jembe.2007.02.006

Patel S, Homaei A, El-Seedi HR, Akhtar N (2018) Cathepsins: Proteases that are vital for survival but can also be fatal. Biomed Pharmacot 105: 526-532 https://doi.org/10.1016/j.biopha.2018.05.148

Patton JS, Warner TG, Benson AA (1977) Partial characterization of the bile salt-dependent triacylglycerol lipase from the leopard shark pancreas. Biochim Biophys Acta Mol Cell Biol Lipids 486: 322-330 https://doi.org/10.1016/0005-2760(77)90028-5

Rosa RS, Charvet-Almeida P, Quijada CCD (2010) Sharks and Their Relatives II. In: Carrier JC, Musick JA, Heithaus MR (ed) Biology of the South American Potamotrygonid Stingrays, 1st end. 42

Rosseel, Y. (2012). lavaan: An R Package for Structural Equation Modeling. J Statist Softw 48(2): 1–36 https://doi.org/10.18637/jss.v048.i02

Rungruangsak-Torrissen K, Moss R, Andresen LH, Berg A, Waagbø R. (2006) Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (*Salmo salar* L.). Fish Physiol Biochem 32(1):7-23 https://doi.org/10.1007/s10695-005-0630-5

Sarath G, de la Motte RS & Wagner FW (1989) Protease assay methods. In: Proteolitic Enzymes. A Practical Approach (Beymon, R.J. & Bond, J.S. eds), pp. 25–55. IRL Press, Oxford.

Shibuya A (2022) A review of the ecological role of the Neotropical freshwater stingrays (Chondrichthyes: Potamotrygoninae). Food Webs 32 https://doi.org/10.1016/j.fooweb.2022.e00244

Shibuya A, Araújo MLG, Zuanon J (2009) Analysis of stomach contents of freshwater stingrays (Elasmobranchii, Potamotrygonidae) from the middle Negro River, Amazonas, Brazil. Pan-American Journal of Aquatic Sciences 4(4): 466-475

Shibuya A, Zuanon J, Tanaka S (2012) Feeding behavior of the Neotropical freshwater stingray *Potamotrygon motoro* (Elasmobranchii: Potamotrygonidae). Neotrop ichthyol 10 (1) https://doi.org/10.1590/S1679-62252012000100018

Stauffer C (1989) Enzyme assays for food scientists. Van Nostand Reinhold/AVI, Nueva York

Taylor A (1993) Aminopeptidases: structure and function. FASEB J. 7(2):290-8 https://doi.org/10.1096/fasebj.7.2.8440407

Taylor JC, Crawford IP (1975) Purification and preliminary characterization of human leukocyte elastase. Arch Biochem Biophys 169: 91-101. https://doi.org/10.1016/0003-9861(75)90320-3

Tzompa-Sosa DA, Yi L, Valenberg HJF, Boekel MAJS, Lakemond CMM (2014) Insect lipid profile: aqueous versus organic solvent-based extraction methods. Food Research Internat 62: 1087-1094 https://doi.org/10.1016/j.foodres.2014.05.052

Walter HE (1984) Proteinases: methods with hemoglobin, casein and azocoll as substrates. In: Methods of Enzymatic Analysis (Bergmeyer, H.U. ed.), Vol. 5. pp. 270–277. Verlag Chemie, Weinheim.

Weatherbee BM, Cortes E (2004) Food consumption and feeding habits. In J. C. Carrier, J. A. Musick, & M. R. Heithaus (Eds.), Biology of sharks and their relatives (pp. 225–246). Boca Raton: CRC Press.

Wood CM, Matsuo AY, Gonzalez RJ, Wilson RW, Patrick ML, Val AL (2002) Mechanisms of ion transport in *Potamotrygon*, a stenohaline freshwater elasmobranch native to the ion-poor blackwaters of the Rio Negro. J Exp Biol 205:3039-54 https://doi.org/10.1242/jeb.205.19.3039

Worthington C (1991) Worthington enzyme manual related Biochemical. Freehold, New Jersey.

Xiong DM, Xie CX, Zhang HJ, Liu HP (2011) Digestive enzymes along digestive tract of a carnivorous fish *Glyptosternum maculatum* (Sisoridae, Siluriformes). J Anim Physiol Anim Nut 95: 56-64 https://doi.org/10.1111/j.1439-0396.2009.00984.x VOLUME 95

CONCLUSÕES GERAIS:

- O epitélio de revestimento da camada mucosa no esôfago de *Potamotrygon wallacei* é estratificado e possui numerosas células mucosas que secretam mucinas ácidas e neutras para proteger e lubrificar a passagem do alimento para o estômago;
- Não encontramos nenhuma justificativa anatômica para dividir o estômago da arraia *P. wallacei* em região fúndica. A divisão deste órgão consiste em apenas duas regiões: cardíaca e pilórica;
- No estômago cardíaco encontram-se numerosas glândulas gástricas que ocupam quase a metade do volume da camada mucosa estomacal. As glândulas têm formato tubular onde se encontras as conspícuas células oxintopepticas secretoras de HCl e pepsinogênio. Isto sugere que o estômago de *P. wallacei* tem elevado potencial para digestão de itens proteicos;
- A quantidade de glândulas gástricas diminui em direção ao estômago pilórico. No segmento intermediário e final desta região, as glândulas estão ausentes;
- A espessa musculatura da camada muscular no estômago pilórico deve-se ao amento da muscular interna (circular). Isto sugere que o segmento descendente do estômago está relacionado à condução do quimo em direção ao intestino espiral;
- O intestino espiral possui cerca de 9 a 11 voltas. A estrutura na forma espiral, além das vilosidades proporcionam uma expansão da área superficial para absorção de nutrientes;
- Os dados de estereologia mostram que o volume do epitélio na camada mucosa tende a diminuir em direção à cloaca. Em contrapartida, o volume da camada submucosa aumenta na mesma proporção. Isto sugere que os segmentos iniciais do intestino espiral da arraia *P. wallacei* pode estar mais relacionado à digestão dos alimentos, enquanto os segmentos medial e final podem estar associados ao transporte de nutrientes para os vasos sanguíneos;
- Os dados de atividades das enzimas digestivas mostram que o esôfago possui baixa capacidade digestiva. As enzimas digestivas detectadas neste órgão podem ser atividades endógenas intracelulares;
- Baseado nos resultados das endopeptidases, tais como pepsina, tripsina, quimiotripsina e elastase, o estômago cardíaco possui elevada capacidade de proteolítica;

- Os níveis de atividades da fosfatase ácida, lipases e esterases inespecíficas no estômago cardíaco sugerem alto potencial para lipólise neste órgão;
- Os teores das carboidratases, tais como amilase, maltase e sacarase sugere um papel moderado do estômago cardíaco para hidrolisar carboidratos;
- Análise em modelagem de equações estruturais (análise de caminhos) sugerem uma moderada, porém, significativa associação em as três mais importantes vias catabólicas: proteica, lipídica e de carboidratos;
- Se considerarmos as atividades das enzimas proteolíticas, o estômago pilórico possui baixo potencial para digestão de proteínas. Isto pode ser justificado pela ausência de glândulas gástricas;
- Verificou-se moderada atividades das enzimas lipolíticas e carboidratases no estômago pilórico. O modelo baseado em equações estruturais sugere uma forte associação entre a digestão de proteínas e hidrólise de carboidratos;
- As mais importantes proteases alcalinas (tripsina, quimiotripsina, elastase e leucina aminopeptidase) estão presentes em elevados níveis no intestino espiral de *P. wallacei*. Isto sugere elevado potencial para a segunda fase da digestão proteica;
- Os baixos níveis ou atividade não detectada da carboxipeptidase A no tubo digestório desta arraia pode estar associada à eficiência da digestão proteica realizada pelas endopeptidases (pepsina, tripsina, quimiotripsina e elastase);
- O intestino espiral tem elevada capacidade lipolítica, pois apresenta detectáveis níveis de atividades de fosfatase alcalina, lipases e esterase inespecífica;
- Embora os carboidratos tenham um papel secundário no metabolismo digestório dos elasmobrânquios, em *P. wallacei* encontrou-se atividades enzimáticas moderadas para as carboidratases;
- O modelo baseado em equações estruturais sugere uma forte associação entre o processamento de carboidratos e lipídeos, e apenas uma relação moderada entre o catabolismo proteico e de carboidratos;
- Considerando a hipótese deste estudo, os resultados da morfologia quantitativa e os dados de atividades das enzimas digestivas de *P. wallacei* estão profundamente relacionados à dieta carnívora baseada em crustáceos, larvas de insetos e pequenos peixes. Os dados de modelagem enzimática mostram de uma moderada a forte interdependência de dois importantes macronutrientes: proteínas e lipídeos.