ROSIELY FELIX BEZERRA

ABORDAGENS BIOQUÍMICAS E BIOTECNOLÓGICAS DOS PEIXES AMAZÔNICOS PIRARUCU (*Arapaima gigas*) E TAMBAQUI (*Colossoma macropomum*)

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Dedico ao meu querido irmão Jailton

in memoriam

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“Ninguém ignora tudo. Ninguém sabe tudo. Todos nós sabemos alguma coisa. Todos nós ignoramos alguma coisa. Por isso aprendemos sempre.”

Paulo Freire

RESUMO

A piscicultura é uma atividade promissora no mundo e principalmente no Brasil devido, sobretudo, a sua extensa malha hidrográfica. Entre as espécies nativas de destaque está o tambaqui, *Colossoma macropomum.*O pirarucu, *Arapaima gigas*, tem um grande potencial para fortalecer a piscicultura nacional nos próximos anos devido a características que o tornam importante para a piscicultura, tais como: grande resistência, alto valor de mercado, excelente sabor da carne e extraordinário desenvolvimento ponderal. O sistema de cultivo intensivo, usualmente praticado pelas pisciculturas industriais, é caracterizado pelas altas densidades de estocagem e elevado nível de arraçoamento, fatores que resultam em peixes susceptíveis à infecção e consequentemente perdas econômicas. Por esta razão o conhecimento do sistema imune em peixes é de grande importância para a piscicultura uma vez que possibilita a prevenção de doenças. O sistema imune dos peixes pode ser dividido em imunidade inata e adaptativa; a imunidade inata é considerada a mais importante no estudo de resistência a doenças em peixes. Entre as moléculas efetoras da imunidade inata estão as lectinas, proteínas ou glicoproteínas que ligam especificamente e de maneira reversível a mono, oligo ou polissacarídeos. Lectinas são importantes ferramentas biotecnológicas e têm sido isoladas dos mais diversos organismos, tais como, microrganismos, fungos superiores, liquens, plantas e animais. Essas proteínas têm sido purificadas de ovos, soro, muco da pele de várias espécies de peixes desempenhando importante papel na defesa contra microorganismos, processo de fertilização, embriogênese e morfogênese. Exposições dos peixes a diferentes estressores ambientais tem sido a principal causa de prejuízos na piscicultura. O efeito da variação de temperatura sazonal (estresse crônico) em indicadores secundários de estresse foi avaliado em pirarucus criados em cativeiro. Níveis séricos de glicose, triglicerídeos, colesterol total e frações bem como parâmetros de osmorregulação foram analisados; todos esses indicadores, com exceção da osmorregulação, mostraram diferenças sazonais em seus níveis sugerindo que alterações nos parâmetros metabólicos são extremamente importantes para a manutenção da homeostase do pirarucu submetidos a estresse crônico. O efeito de pluviosidade e temperatura sobre indicadores bioquímicos (atividade de lectina, atividades de lactato desidrogenase e fosfatase alcalina) e hematológicos (contagem total de células vermelhas do sangue, hematócrito, hemoglobina e índices hematimétricos de Wintrobe) de estresse bem como sobre o crescimento de *A.gigas* também foram analisados; este trabalho foi conduzido em três períodos (abril-julho 2010, agosto-novembro 2010 e, dezembro 2010 – março 2011) definidos de acordo com pluviosidade e temperatura médias. Todos os indicadores bioquímicos e hematológicos de estresse mostraram variações sazonais; o crescimento dos peixes foi alométrico positivo e os valores elevados do fator condição indicaram bom estado de salubridade no cultivo. Estes resultados reforçam a característica robusta do pirarucu e representam um ponto de partida para a compreensão da fisiologia do estresse durante o cultivo. O tambaqui é um peixe de grande importância econômica devido ao elevado padrão de crescimento, qualidade da carne e rusticidade. A primeira lectina do soro do tambaqui (ComaSeL) foi purificada e mostrou atividade antibacteriana para Gram-negativas. ComaSeL reconhece os carboidratos D-galactose, 1-*O*-methyl-D-galactopyranosídeo e D-fucose. Esta proteína foi estável em valores de pH entre 4,0 e 9,0 e perdeu 100% de sua atividade hemaglutinante (AH) a 70 °C. AH mostrou variação sazonal sendo maior no verão. Com estas informações novas ferramentas podem ser desenvolvidas para o melhor entendimento do papel das lectinas no sistema imune do tambaqui. Diferentes

abordagens bioquímicas e biotecnológicas do pirarucu e tambaqui contribuem para o conhecimento biológico das espécies e também podem ser úteis na melhoria das técnicas que aumentam o sucesso da cultura e da produtividade em piscicultura.

Palavras-chave: *Arapaima gigas*, *Colossoma macropomum*, lectinas, purificação, sazonalidade, estresse.

Abstract

Fishculture is a promising activity in the world and especially in Brazil, mainly due to its extensive water network. Among the native species highlighted is the tambaqui, *Colossoma macropomum*. The pirarucu, *Arapaima gigas*, has great potential to strengthen the national fishculture in the coming years due to characteristics that make it extremely important for fish farming, such as: high resistance, high market value, excellent beef flavor and extraordinary weight development. The intensive cultivation system, usually practiced by industrial fish farms, is characterized by high stocking densities and high level of feeding, factors that result in fish susceptible to infection and consequently economic losses. The knowledge of the immune system in fish then is very important for fish farming since it allows disease prevention. The immune system of fish can be divided in innate and adaptive immunity; innate immunity is considered the most important in the study of fish disease resistance. Among the effector molecules of innate immunity are lectins, proteins or glycoproteins that bind specifically and reversibly to mono-, oligo- or polysaccharides. Lectins are important biotechnological tools and have been isolated from various organisms, such as microorganisms, upper fungi, lichens, plants and animals. These proteins have been purified from egg, serum, mucus, skin of various fish species playing an important role in defense against microorganisms, fertilization process, embryogenesis and morphogenesis. Exposures of fish to different environmental stressors have been the main cause of loss in fish farming. The effect of seasonal temperature variation (chronic stress) on secondary indicators of stress was evaluated in pirarucu developed in captivity. Serum levels of glucose, triglycerides, total cholesterol and fractions as well as osmoregulation parameters were analyzed; all these indicators, with the exception of osmoregulation, showed seasonal differences in their levels suggesting that changes in metabolic parameters are extremely important for maintaining homeostasis of pirarucu subjected to chronic stress. The effect of rain and temperature on biochemical (lectin activity, lactate dehydrogenase and alkaline phosphatase activities) and hematological (total count of red blood cells, hematocrit, hemoglobin and hematimetric indexes of Wintrobe) stress indicators as well as on growth of *A. gigas* has also been analyzed; this study was conducted in three periods (April-July 2010 August-November 2010 and December 2010 -March 2011) defined according to rainfall and medium temperatures. All hematological and biochemical indicators of stress showed seasonal variations; fish growth was allometrically positive and high values of condition factor indicated good state of healthiness in cultivation. Tambaqui is a fish that has great economic importance due to the high standard of growth, meat quality and rusticity. The first *C. macropomum* serum lectin (ComaSeL) was purified and showed antibacterial activity for Gram-negative bacteria. ComaSeL recognizes the carbohydrate D-galactose, 1-*O*-methyl-D-fucose and D-galactopyranoside. This protein was stable between pH values of 4.0 and 9.0 and lost 100% of its hemagglutinanting activity (HA) at 70 °C. The HA showed seasonal variation and was higher in summer. With these new information tools it can be developed a better understand of the lectin role in tambaqui immune system. Different biochemical and biotechnological approaches of pirarucu and tambaqui contribute to the biological knowledge of species and may also be useful in improving techniques that increase the success of culture and productivity in fish farming.

Keywords: *Arapaima gigas*, *Colossoma macropomum*, lectins, purification, seasonality, stress.

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LISTA DE ABREVIATURAS

AH - Atividade Hemaglutinante
ANOVA – Analysis of the Variance
AP - Alkaline Phosphatase Activity
ATP - Adenosine triphosphate
BmoLL - Lectin from leaves of *Bauhinia monandra*
BmoRoL - Lectin from *B. monandra* secondary roots
CC – Competitor Carbohydrate
CD - Circular Dichroism
CDR - Carbohydrate Recognition Domain
CF – Condition Fator
cMoL - Lectin from *Moringa oleifera* seeds
ComaSeL - *Colossoma macropomum* serum lectin
Con A - Concanavalin A
Cramoll - Lectins from *Cratylia mollis*
CrataBL - Lectin from bark of *Crataeva tapia*
CRP – Pentraxins membrane associated
CTL - C-type lectins
CTLD - C-type lectin-like domain
DNA - Deoxyribonucleic Acid
EmaL - Lectin from *Eugenia malaccensis* seeds
EMC - Erythrocyte Membrane Carbohydrate
EPN - Motif Glu-Pro-Asn
ESC - Erythrocyte Surface Carbohydrate
ESI - Electrospray Ionization
EST - Expressed Sequence Tag
FAO - Organização das Nações Unidas para Agricultura e Alimentação
FCN - Ficolins
FPLC - Fast Protein Liquid Chromatography
HA - Hemagglutinating Activity
HAI - Hemagglutinating Activity Inhibition
Hb - Hemoglobin
HDL - High Density Lipoproteins
HPLC - High Pressure Liquid Chromatography
Htc - Hematocrit
IAH - Inibição da Atividade Hemaglutinante
IEF - Isoelectric Focusing
Ig - Imunoglobulinas
IPP - Inositol Pentaphosphate
LA – Lectin Activity
LCAT - Lecithin-Cholesterol Acyltransferase
LDH- Lactate Dehydrogenase Activity
LDL - Low Density Lipoprotein
LDL - Low-Density Lipoprotein
LPS - Lipopolysaccharide
MALDI - Matrix-Assisted Laser Desorption/Ionization
MBL - Mannose-binding lectin
MCH - Mean Corpuscular Hemoglobin

MCHC - Mean Corpuscular Hemoglobin Concentration
MCV - Mean Corpuscular Volume
MHC - Main Histocompatibility Complex
MIC – Minimum Inhibitory Concentration
MR - Mitochondria-Rich
mtDNA - Mitochondrial DNA
MvRL - *Microgramma vaccinifolia* rhizome lectin
NB – Nutritive Broth
NC – Negative Control
NMR - Nuclear Magnetic Resonance
OniL - Lectin from *Oreochromis niloticus* serum
P1- Period 1
P2 – Period 2
P3 – Period 3
PAGE - Polyacrylamide Gel Electrophoresis
PAMP - Pathogen-Associated Molecular Patterns
PBS - Phosphate Saline Buffer
pI - Isoelectric Point
PMF - Peptide-Mass Fingerprinting
pNPP - p-nitrophenyl phosphate
PRPs – Receptor-Recognizing Pathogens
PSA - Prostate-Specific Antigen
QPD - Motif Gln-Pro-Asp
RBC - Total Count of Red Blood Cells
RBL - Lectin Binding to L-Rhamnose
rDNA – Ribosomal DNA
RNA – Ribonucleic Acid
rRNA – Ribosomal RNA
RT-PCR – Reverse Trascriptation PCR
SAP – Pentraxins component of the extracellular matrix
SC - Specific Carbohydrate
SDS - Sodium Dodecyl Sulfate
SHA – Specific Hemagglutination Activity
Siglecs - Sialic Acid-Binding Immunoglobulin Superfamily Lectins
TEPP - Tetraethyl Pyrophosphate
TOF - Time-of-flight
tRNA - Transfer RNA
TSB – Trypticase Soy Broth
UFPE - Universidade Federal de Pernambuco
UFRPE - Universidade Federal Rural de Pernambuco
VHSV - Rhabdovirus Causer of Haemorrhagic Septicaemia
VLDL - Very Low Density Lipoproteins
WSMoL - Water-Soluble *Moring oleifera* lectin

SUMÁRIO

CAPÍTULO I

1. INTRODUÇÃO

A piscicultura, ramo da aquicultura voltada para criação de peixes em cativeiro, está sendo apontada por especialistas como uma atividade promissora no mundo e principalmente no Brasil, em decorrência da malha hidrográfica e do clima propício. A produção mundial hoje é da ordem de 126 milhões de toneladas e a previsão é de que até 2030 a demanda internacional de pescado aumente em mais 100 milhões de toneladas por ano, de acordo com a Organização das Nações Unidas para Agricultura e Alimentação (FAO). O Brasil é um dos poucos países que tem condições de atender à crescente demanda mundial por produtos de origem pesqueira, sobretudo por meio da piscicultura (MPA 2011). Nosso país destaca-se por possuir imenso potencial para o desenvolvimento da piscicultura por meio dos 8,4 mil km de litoral e 5,5 milhões de hectares de reservatórios de águas doces, representando aproximadamente 12% da água doce disponível no planeta (FAO 2004). A piscicultura brasileira teve uma elevação de 60,2% nos últimos três anos; até 2010 a produção de pescado passou de 990.899 para 1.240.813 toneladas anuais (Sebrae 2012).

Nos próximos anos espécies nativas devem fortalecer a piscicultura nacional como é o caso do pirarucu *Arapaima gigas*. O pirarucu, nativo da Bacia do Rio Amazonas, é o maior peixe de escamas do mundo. Este peixe possui características que o tornam extremamente importante para a piscicultura, tais como: grande resistência, alto valor de mercado, excelente sabor da carne e extraordinário desenvolvimento ponderal (Fogaça et al. 2011). O Tambaqui (*Colossoma macropomum*, Cuvier 1818), é um peixe nativo da Região Norte e é uma das espécies aquáticas de maior expressão na alimentação nessa Região (Val e Almeida-Val 1995); também é um peixe que possui grande importância econômica no nosso país devido ao elevado padrão de crescimento, qualidade da carne e rusticidade ocupando a segunda posição no *ranking* na produção piscícola brasileira com 46.454 toneladas/ano (Sebrae 2012).

A piscicultura pode ser uma alavanca de desenvolvimento social e econômico, possibilitando o aproveitamento efetivo dos recursos naturais locais, principalmente os hídricos e a criação de postos de trabalhos assalariados. O sistema de cultivo intensivo, usualmente praticado pelas pisciculturas industriais, é caracterizado pelas altas densidades de estocagem e elevado nível de arraçoamento (Araripe et al. 2013). O manejo inadequado propicia uma série de problemas nutricionais como também enfermidades infecciosas e parasitárias. A criação de peixes susceptíveis à infecção em

sistemas intensivos tem levado a grandes perdas econômicas; por esta razão a prevenção de doenças é muito importante tanto para piscicultores como para a indústria. O maior problema sanitário de origem bacteriana em sistemas de cultivo intensivo é a septicemia causada por *Streptococcus spp*. (Salvador et al. 2003) e ainda *Aeromonas spp*. (Hirsch et al. 2006). Dentre as diferentes espécies responsáveis por infecções em peixes estão *Streptococcus iniae, Aeromonas hydrophila, A. sobria, A. veroni, Pleisiomonas shigelloides* e *Edwardsiella tarda* (Buller 2004).

O Brasil possui, talvez, a maior e mais variada ictiofauna do planeta. Só na Bacia Amazônica brasileira calcula-se que existam cerca de 2.000 espécies de peixes muitas das quais são de extrema importância para a alimentação (Sebrae 2012). Peixes constituem um grupo heterogêneo de organismos que inclui os agnatas (lampréias e enguias de casulo), condríctios (tubarões e raias) e teleósteos (peixes cartilaginosos) (Nelson 1994). Estes animais são os vertebrados mais primitivos e constituem um importante elo entre os invertebrados e os vertebrados superiores, possuem os mecanismos não específicos de defesa dos invertebrados, como a fagocitose desenvolvida por macrófagos e leucócitos granulares, e também são os primeiros animais a desenvolver resposta celular e humoral através de linfócitos (Scapigliati 2013).

O sistema imune de peixes ao contrário de vertebrados superiores é comparativamente simples e diferenciado. Os maiores órgãos linfomielóides de peixes são timo, rim anterior e baço (Muleroet al. 2007). Semelhante ao descrito em mamíferos pode ser dividido em sistema imune inato e sistema imune adaptativo, possui também órgãos centrais cuja principal função está envolvida na defesa imune (Rauta et al. 2012).

1.1 Sistema Imune Inato em Peixes

O sistema imune inato tem recebido atenção como sendo da maior importância no estudo de resistência a doenças em peixes (Magnadottir et al. 2010); isto pode ser um reflexo da facilidade de estudo ou do fato que este sistema proporciona a primeira linha de defesa. Como um organismo aquático, o corpo do peixe é submetido a contínuo contato com muitos tipos diferentes de microorganismos. A primeira barreira contra os agentes patogênicos, a superfície tegumentar, está equipada com mecanismos de proteção contra a entrada do patógeno. Em teleósteos, o tecido linfóide associado a

tegumentos está distribuído em torno da pele, brânquias e intestino, complementando assim a proteção física e química fornecida pela estrutura. Entre os importantes mecanismos tegumentares estão a secreção de muco, e um grupo diverso de moléculas antibacterianas que atuam, direta e indiretamente, sobre os componentes da parede celular bacteriana resultando em lise. Entre as secreções epidérmicas, complemento, proteínas antimicrobianas, lisozima, fosfatases e tripsina são frequentemente encontradas, além de células imunocompetentes, tais como leucócitos intra-epiteliais e células plasmáticas (Holopainen et al. 2012).

Peptídeos com atividade antibacteriana têm sido encontrados em superfícies de peixe, porém, os mecanismos de ação ainda não foram especificamente determinados (Smith et al. 2000). Várias enzimas líticas, agindo individualmente ou em cascata, são elementos de defesa especialmente importantes contra as bactérias; essas são hidrolases como a lisozima e a quitinase, as catepsinas, as da via lítica do sistema complemento e outras enzimas bacteriolíticas / hemolíticas encontradas nos tecidos e fluidos corporais dos peixes (Alexander e Ingram 1992). Vários inibidores de protease estão presentes no soro de peixes e outros fluidos corporais; inibidores de cisteíno proteases têm sido descritos em ovos de algumas poucas espécies (Kudo 1998), mas seu papel na defesa inata é desconhecido. Por outro lado, um inibidor de protease de soro de tilápia, *Oreochomis niloticus*, apresentou atividade antibacteriana tanto para bactérias Gram-positivas quanto para Gram-negativas (Leite et al. 2012).

Em teleósteos, o sistema complemento desempenha um papel na resposta imune inata estando envolvido na quimiotaxia, opsonização, fagocitose e degradação de patógenos (Aoki et al. 2008). Além disso, também desempenha um papel crucial em vários outros processos não-imunológicos que vão desde a fecundação (Llanos et al. 2000), a regeneração (Kimura et al. 2003) transdução de sinal (Bohana-Kashtan et al. 2004), metabolismo energético (Chrast et al. 2004), bem como organogênese (Lange et al. 2004).

Lectinas são proteínas ou glicoproteínas, importantes mediadores imunológicos em vertebrados e invertebrados. As lectinas estão envolvidas na imunidade inata sendo por isso, consideradas como a primeira linha de defesa imunológica dos peixes (Castellana et al. 2007). Vários tipos de lectinas têm sido isolados do soro, muco da pele e ovos de várias espécies de peixes (Jimbo et al. 2007, Silva et al. 2012, Zhang et al. 2012).

1.2 Sistema Imune Adaptativo em Peixes

Os peixes podem mostrar respostas imunes adaptativas típicas de vertebrados caracterizadas por imunoglobulinas, receptores de células T, citocinas e moléculas de complexo de histocompatibilidade principal (Tort et al. 2003). No entanto, tem sido demonstrado que apesar dos teleósteos possuírem uma completa composição de parâmetros imunes adaptativos a imunidade inata tem um papel decisivo na proteção deste grupo de vertebrados. Alguns representantes de teleósteos são ainda considerados com uma baixa resposta em relação à produção de anticorpos (Solem e Stenvik 2006).

Acreditava-se que os teleósteos sintetizavam apenas uma classe de imunoglobulinas (Ig), a IgM; no entanto, a presença de outros isotipos de Ig como IgD, quimeras IgT/IgZ e IgM–IgZ também tem sido registrada em uma grande variedade de espécies (Bag et al. 2008), IgD em *Ictalurus punctatus* (Bengten et al. 2002), *Salmo salar* (Hordvik et al. 1999), *Gadus morhua* (Stenvik e Jorgensen 2000), *Paralichthys olivaceus* (Hirono et al. 2003) e IgT/IgZ em *Siniperca chuatsi*, *Danio rerio*, e *Oncorhynchus mykiss* (Sakai e Savan 2004, Tian et al. 2009). Além desses isotipos de Ig, quimera IgM–IgZ é também relatada em *Cyprinus carpio* (Savan et al. 2005). Recentemente, novos genes IgH teleósteos-específicos foram identificados (Danilova et al 2005, Hansen et al. 2005).

1.3 Temperatura e Imunidade

O efeito da temperatura sobre a resposta imune dos animais ectotérmicos, como teleósteos, é de particular interesse especialmente porque os peixes são incapazes de regular sua temperatura interna. A temperatura tem sido o princípio de sinalização ambiental estimulando mudanças na resposta imune de muitas diferentes espécies de peixes. Atualmente se aceita que ambos os sistemas imune inato e adquirido sejam significativamente afetados pela temperatura; em salmão-vermelho (*Oncorhynchus nerka*) a resposta imune dos peixes criados em 8 °C é mais dependente da resposta imune inata e não da resposta imune adquirida em comparação com os peixes cultivados em 12 °C (Alcornet al. 2002). Embora os peixes possam ter uma maior tolerância a baixas temperaturas, uma redução severa de temperatura poderá resultar em

imunossupressão, até mesmo para complemento e proteínas de fase aguda (Vasta e Lambris 2002).

A temperatura tem um efeito variado sobre a hematologia em diferentes espécies de peixes, aumentando ou diminuindo os níveis de células específicas (Bowden 2008). Se essa mudança é acompanhada por eventos ambientais, como sazonalidade, migração ou desova, os efeitos podem ser severamente agravados devido ao aumento do cortisol e esteróides sexuais (Magnadottir et al. 2010).

1.4 Sazonalidade e Atividade Biológica em Peixes

Ciclos sazonais podem afetar diversas atividades biológicas, tais como comportamento, alimentação, metabolismo, imunidade e reprodução em peixes (Herrero et al. 2005). Os peixes apresentam uma forte associação com a sazonalidade, especialmente no que diz respeito às suas estratégias de reprodução; o ciclo reprodutivo da truta arco-íris é controlado pelo padrão anual de fotoperíodo (Davies et al. 1999).

O complexo mecanismo que permite que os peixes euritérmicos sincronizem esses eventos para mudança de estação requer ao animal o senso de perceber mudanças físicas no ambiente (por exemplo, temperatura e fotoperíodo), comum a correspondente transdução de sinais moleculares. Este mecanismo ainda não está completamente esclarecido, embora se saiba que os peixes euritérmicos dependem de sinais do ambiente externo para alcançar essa sincronização (Molinaet al. 2002). A determinação de estímulos ambientais específicos e da influência do sistema neuroendócrino são atualmente áreas de especial interesse no estudo dos efeitos da variação sazonal sobre a resposta imune de vertebrados (Reppert e Weaver 2002).

Variações sazonais tais como temperatura, pluviosidade e fotoperíodo são consideradas fatores ambientais de estresse crônico. Geralmente, em peixes, a resposta ao estresse tem três níveis: primário, secundário e terciário. A liberação de catecolaminas e cortisol em vários órgãos-alvo tem as consequências bioquímicas e fisiológicas conhecidas como resposta primária ao estresse (Wendelaar Bonga 1997, Castro e Fernandes 2009). As respostas secundárias incluem efeitos metabólicos como a hiperglicemia sintomática e depleção das reservas de glicogênio assim como a lipólise e a inibição da síntese de proteínas (Milligan 2003; Martins da Rocha et al 2004). O estresse em aquicultura é inevitável desde que em todas as fases do processo de

produção, ocorrem procedimentos considerados adversos aos peixes, entretanto, quando o agente estressor é crônico, o valor adaptativo à resposta pode ser comprometido e efeitos deletérios podem tornar-se aparentes. Os fatores estressantes têm sido a principal causa das perdas de lucros na piscicultura, pois afetam o metabolismo e, consequentemente, o crescimento dos peixes, bem como aumentam a suscetibilidade a diferentes doenças infecciosas (Herrero et al. 2005).

A fim de atenuar os surtos de doenças na aquicultura e diminuição da produtividade é necessário desenvolver estratégias de controle com base numa melhor compreensão dos efeitos dos métodos de criação e estressores ambientais sobre o estado de saúde dos peixes cultivados. Abordagens bioquímicas possibilitam o estudo de moléculas envolvidas na imunidade inata de peixes, tais como lectinas, bem como a análise da interferência da sazonalidade na biologia desses animais através de indicadores bioquímicos e hematológicos de estresse. Desta forma, pode ser possível realizar práticas de produção animal (vacinação, transferência, classificação) no momento em que o animal não esteja sazonalmente imunocomprometido e ainda possibilitar o surgimento de novas ferramentas biotecnológicas que contribuam para os programas de melhoramento genético visando aumentar a resistência dos peixes.

2. OBJETIVOS

2.1 Objetivo Geral

Reunir conhecimentos sobre a biologia de *A. gigas* bem como avaliar a influência da sazonalidade frente a indicadores de estresse e crescimento em pirarucu cultivado em cativeiro. Investigar as estratégias de obtenção de lectinas e variedade dessas proteínas em peixes bem como purificar e caracterizar a lectina do soro de tambaqui *C. macropomum*.

2.2 Objetivos Específicos

I- Avaliar informações sobre as estratégias utilizadas para obtenção de lectinas de distintas fontes, destacando suas funções e aplicações biológicas

II- Explorar informações relacionadas à diversidade de lectinas em peixes, sua classificação geral, aplicações biotecnológicas e funções biológicas

III- Reunir informações relacionadas à biologia do pirarucu

IV- Analisar a influência da temperatura sazonal em indicadores de estresse e crescimento do pirarucu em condições de cativeiro

- Obter sangue total, soro e plasma de pirarucu nos diferentes períodos do ano;
- Analisar a influência da temperatura sazonal em indicadores secundários de estresse (glicose, triglicerídeos, colesterol total e frações, eletrólitos e osmolalidade plasmática) nas amostras obtidas;
- Avaliar a influência da temperatura sazonal no estresse por meio de indicadores bioquímicos (lectina, lactato desidrogenase, fosfatase alcalina) e hematológicos (contagem total de células vermelhas do sangue - RBC, hematócrito, hemoglobina e índices hematimétricos de Wintrobe) nas amostras obtidas;
- Determinar tamanho e peso de pirarucus em diferentes períodos do ano;

- Identificar o tipo de crescimento de pirarucus criados em cativeiro;
- Analisar a influência da sazonalidade no crescimento (constante de regressão linear “b”) e grau de bem estar (fator de condição) de pirarucus criados em cativeiro.

V- Caracterizar a lectina do tambaqui quanto à atividade antibacteriana

- Obter o soro de tambaqui e pré-purificar a lectina do soro por fracionamento com sulfato de amônio;
- Detectar a lectina através da atividade hemaglutinante (AH) e inibição da atividade hemaglutinante (IAH) das diferentes frações obtidas;
- Purificar a lectina por processos cromatográficos a partir da fração de maior rendimento;
- Caracterizar a fração eluída através da inibição da AH com carboidratos e glicoproteínas, estabilidade térmica, influência de íons e eletroforese em gel de poliacrilamida;
- Avaliar a atividade da lectina do soro do tambaqui frente a bactérias Gram-negativas patógenas de peixes.

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CAPÍTULO II

Strategies to Obtain Lectins from Distinct Sources

Andréa F. S. Santos, Thiago H. Napoleão, Rosiely F. Bezerra, Elba V. M. M. Carvalho, Maria T. S. Correia, Patrícia M. G. Paiva and Luana C. B. B. Coelho

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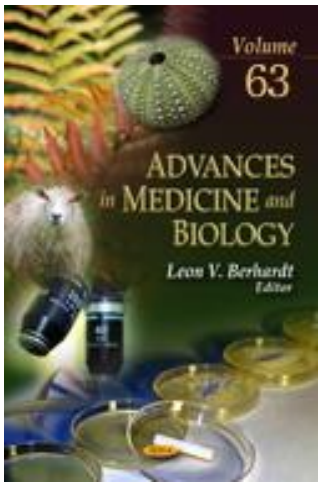

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Chapter 2

Strategies to Obtain Lectins from Distinct Sources

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Abstract

Lectins are proteins or glycoproteins that bind specifically to mono, oligo or polysaccharides. They are important biotechnological tools and have been isolated from microorganisms, higher fungus, lichens, plants and animals. Different purification strategies are used to obtain pure lectins. It is important to approach the principles of each method to be used in protein purification before start to work in this important field. The initial purification step is the protein extraction followed by saline fractionation and dialysis. Hemagglutinating activity (HA) is the assay to follow each protocol step. The prepared sample is submitted to chromatographic methods, such as, affinity, ion exchange and molecular exclusion chromatographies to purify the lectins. High resolution techniques for instance the fast protein liquid chromatography (FPLC) and high pressure liquid chromatography (HPLC) are also used to obtain lectins with high purity. A large number of lectins characterized by specific techniques are available with different carbohydrate specificities. Lectins or lectin molecular forms have structural characteristics making them unique proteins. The pure molecules can contribute to unravel its structure, potential biological function, as well as biotechnological and biomedical uses. Lectins can have applications in different areas of knowledge; they can be used to explore cell surface, be applied as mitogenic, cytotoxic and antimicrobial agents, and also be used as antiproliferative molecules for cancer cells. These proteins may be useful in water treatment due to their coagulant and antibacterial properties. Insecticidal

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lectins with potential biotechnological application for control of agricultural pests have also been described. There are pitfalls in lectin purification; among them is the structural similarity between contaminant proteins or other lectin molecular forms promoting difficulties to the purification process. A common problem is to establish the ideal protocol that ensures high level of purity and good recovery in few steps.

Keywords: Lectins, purification strategies, characterizations, applications

1. Introduction to Lectin Purification Strategies

Lectins are proteins of non-immune origin that bind specifically and reversibly to different types of carbohydrates or glycoproteins [1]. These proteins can be purified from different sources such as microorganisms [2,3], fungi [4], lichens [5], plants [1,6,7] and animals [8,9]. The presence of a lectin in a sample is evidenced through hemagglutinating activity (HA) assay followed by a HA inhibition assay with a solution containing specific carbohydrates or glycoproteins [10].

The HA assay is characterized by connection between the lectin binding sites and the carbohydrates present in the erythrocytes surface, forming a network; in the HA inhibition assay the lectin binding sites are occupied by free carbohydrates thus preventing the network formation (Figure 1).

The purification is a key step for the studies of lectin structural and functional unravel besides biotechnological applications. The purification process starts with strategies to remove contaminants that may be incompatible with chromatographic methods. The first step to purify these proteins, in general, involves an extraction with saline or buffer solution (Figure 2a), a salt fractionation (Figure 2b), and dialysis (Figure 2c).

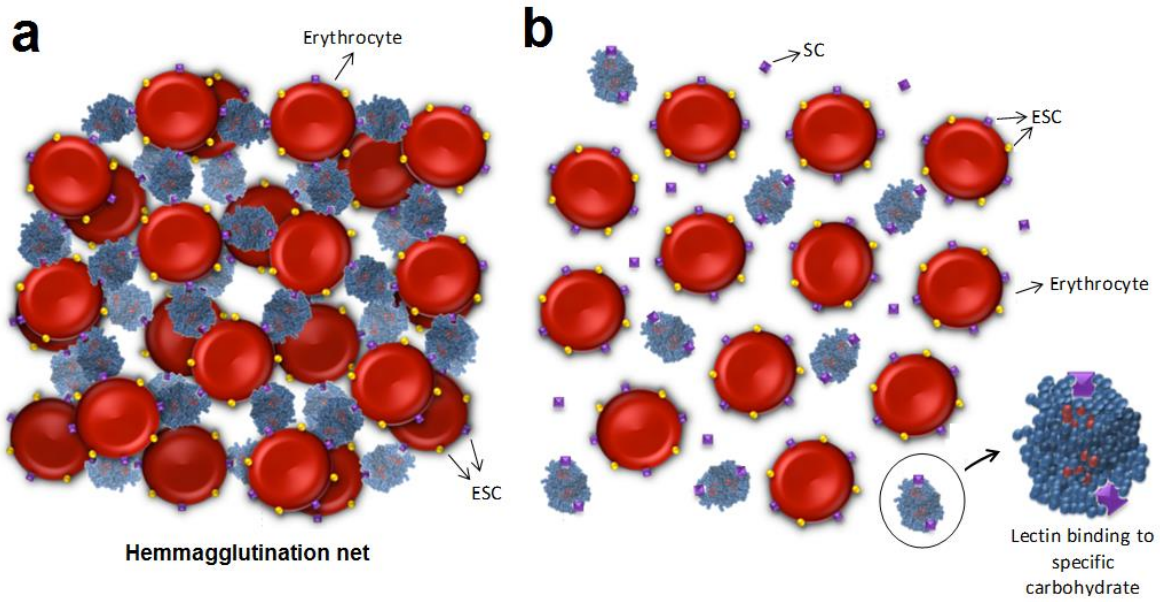

Figure 1. Scheme of hemagglutinating activity (HA) and inhibition of HA assays. (**a**) The lectin sample induces hemagglutination due to lectin linkage to erythrocyte surface carbohydrate (ESC). (**b**) HA inhibition occurs when lectin sample is incubated with carbohydrate prior to addition of erythrocytes; binding of a specific carbohydrate (SC) to lectin sites extinguish net formation.

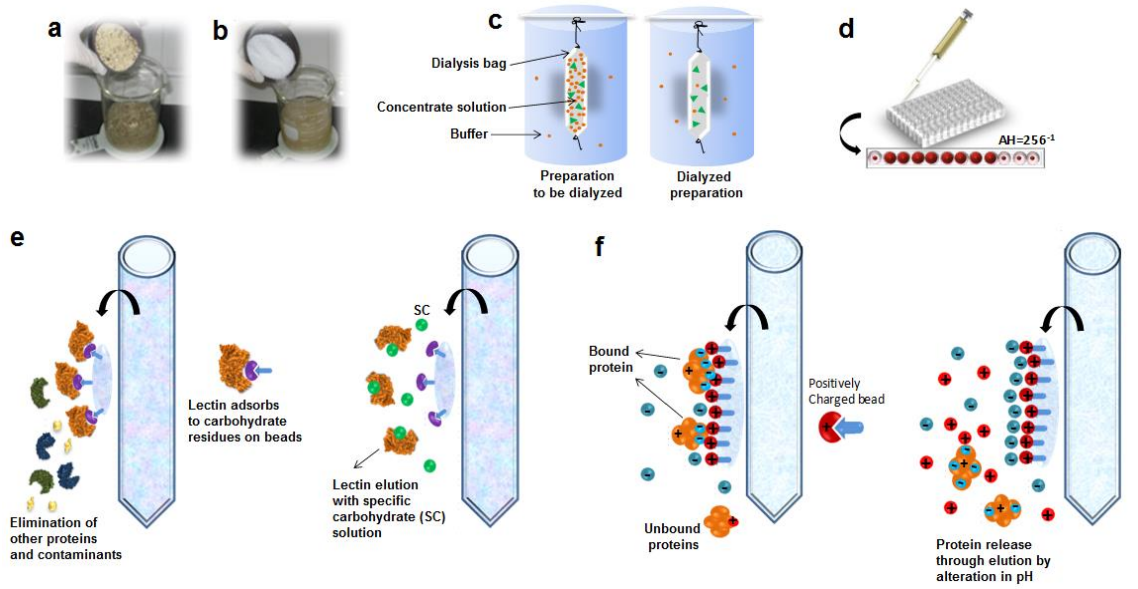

Figure 2. Potential approaches to obtain pure lectins. The protein extraction (a) is followed by salt fractionation (**b**) and dialysis (**c**). Hemagglutinating activity assay (HA) is made in 96-wells microtiter plates. Lectin sample (50 µL) is serially two-fold diluted in 0.15 M NaCl or buffer; erythrocyte suspension (50 µL, 2.5% v/v) is added to each well. Plates are incubated (28 °C for 40 min) and activity (256^{-1}) corresponds to last dilution in which hemagglutination is observed (**d**). Affinity (**e**) and ion exchange chromatographies (**f**) are also represented.

All protocol steps are followed by HA (Figure 2d). Chromatographic strategies to obtain pure lectins are based upon lectin charge, size and specific carbohydrate interactions (Figures 2e and 2f).

The lectin preparation, during and after purification process, is subjected to characterization procedures such as one- and two-dimensional polyacrylamide gel electrophoresis, molecular mass determination and sequencing, among others. Examples of lectin characterization steps are showed in Figure 3. Lectin molecular forms even from the same tissue may show distinct biotechnological applications. Structurally characterized lectins have been applied as insecticide agents [11] and to disease diagnosis [12]. Anticoagulant and antiplatelet aggregating properties [1], as well as coagulant activity [6], are among lectin applications.

2. Methods and Concepts to Obtain and Characterize Pure Lectins

The following distinct methods have been used to obtain and characterize pure lectins from virus, microorganisms, plants and animals.

The concepts of lectin purification and characterization techniques are explained below.

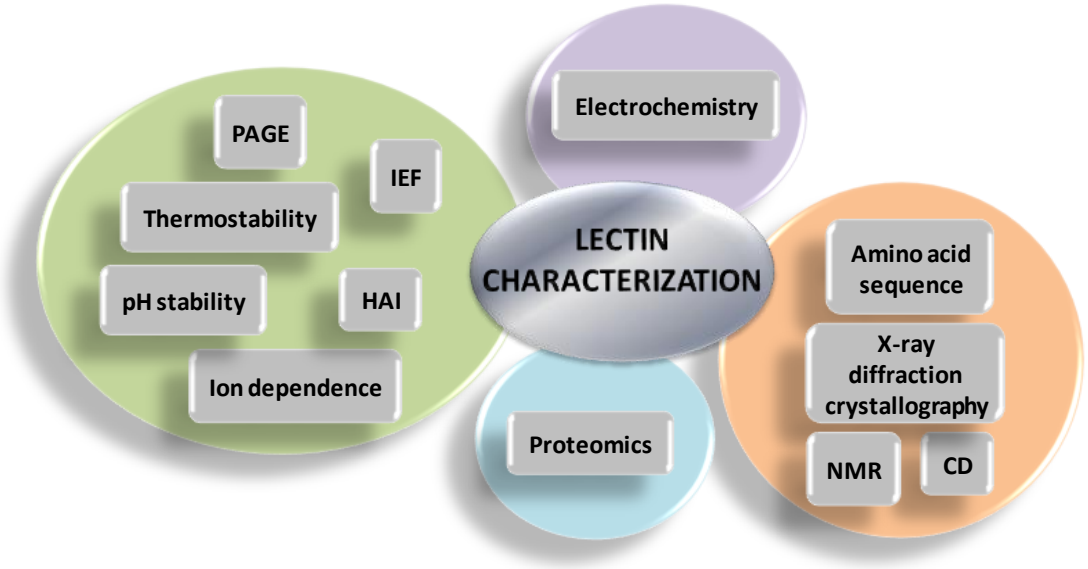

Figure 3. Some techniques and assays for lectin characterization. PAGE, polyacrylamide gel electrophoresis [7]; IEF, isoelectric focusing [13]; thermostability [6]; pH stability [14]; HAI, hemagglutinating activity inhibition [10]; ion dependence [15]; electrochemistry [47,94]; amino acid sequence [16]; X-ray diffraction crystallography [16]; NMR, Nuclear Magnetic Resonance [17]; CD, circular dichroism [18,19]; proteomics [20,21].

2.1. Protein Extraction and Fractionation

The first step for most lectin purification is the obtention of an extract. To obtain a protein extract is necessary defining some conditions such as selection of the extraction medium, temperature and time. Aqueous solutions are suitable solvents for extraction, since lectins are soluble in water and saline solutions [11, 22]. The preparation obtained, namely crude extract, is then evaluated for protein concentration and HA before used as a starting material for lectin isolation. Soluble proteins from the crude extract can be fractionated by precipitation with salts [11]. High concentration of salts removes the water of hydration from the proteins, reducing its solubility [23]. Thus, protein aggregates and this phenomenon are called salting-out. Ammonium sulfate is the most commonly used salt due to its high solubility. Dialysis is then performed to remove salt and the fraction is ready to be introduced in a chromatographic column.

2.2. Protein Quantification

The quantification of proteins in samples containing lectins can be made using different methods. The method of Lowry et al. [24] is a colorimetric assay that combines the reaction between cupper salts and peptide bonds (Biuret reaction) with the oxidation of aromatic amino acid residues in the protein. The method of Bradford [25] is also a colorimetric assay which involves the conversion, in acid medium, of the reddish form of Coomassie Brilliant Blue to its blue form. The blue form is stabilized when binds to a protein. Also, lectin concentration can be quantified by measuring the absorbance at 280 nm.

2.3. Chromatographies

The aim of a chromatographic process is the separation of a mixture which is dissolved in a mobile phase that carries the sample through a stationary phase. It can also serve to separate denatured and native forms of the same protein [26]. Affinity chromatography is one of the most used techniques to obtain highly purified lectins and depends on the interaction between the molecule to be purified and the solid phase that will allow the exclusion of contaminants [26]. The application of this chromatographic technique to lectin purification is mainly based on the ability of these proteins to bind carbohydrates in a specific and reversible way. Different affinity matrices are used according to the lectin carbohydrate specificities. Chitin [27], agarose [28], guar gel [6,9], Affi-gel blue gel [29], among others, are examples of affinity matrices.

Proteins are also purified by ion exchange chromatography; the molecules are adsorbed to the matrix mainly according to their charges. Lectins have been successfully purified through exchangers such as DEAE-cellulose [30], DEAE-A 50 [2], CM-cellulose [11] and CM Sephadex [30].

Molecular exclusion or gel filtration chromatography is a technique based on filtration of a protein sample through the pores of inert matrices with controlled porosity and with subsequent separation of the components by differential elution, according to the molecular size [31]. Sephadex G-100 [32], Superdex 75 [1,9] and Sephacryl S200 [29] have been used as molecular exclusion matrices for lectin purification. FPLC (fast protein liquid chromatography) and HPLC (high pressure liquid chromatography) are also high resolution techniques used to purify lectins [3,9]. These types of chromatographies can be associated to obtain homogeneous lectins. Lectins from leaves of *Phthirusa pyrifolia* (PpyLL) [32] and *Sebastiania jacobinensis* bark [33] were purified using two consecutive chromatographic steps on Sephadex G-100 and ion exchange on CM-cellulose.

2.4. Principles of High Pressure Liquid Chromatography and Fast Protein Liquid Chromatography

HPLC constitutes a type of liquid chromatography where the solvent is not dripped through the column under gravity but forced under high pressure. When the pressure produced by the pump is increased, the speed at which the liquid passes through the columns also increases and performance of the chromatographic column is enhanced. The type of material in the column is also related to the high performance of the chromatographic method, since a very much smaller particle size for the column packaging material gives a greater interaction surface, allowing a better separation. The stationary phase can be an absorbent or a material impregnated with a high boiling point of liquid. The mobile phase used can be water, aqueous buffers, salt solutions or organic solvents. HPLC equipments are usually constituted of five components (recorder, detector, column, pump and a solvent) with varying degrees of sophistication; the method becomes extremely sensitive. The detectors used in this type of column monitor are the ultraviolet and visible lights or fluorescence (for substances that absorb light); for transparent substances, the refractive index is used [23].

FPLC is also a high-performance chromatography type where the solvent velocity is controlled by pumps allowing high resolution in purification. The main difference between FPLC and HPLC is the standard working pressure (much lower in FPLC). All chromatographic systems can become a FPLC automated system by using high pressure pumps (100-400 bar) and columns with materials that withstand high pressures. This system significantly reduces the time of purification using stationary phases (usually cross-linked agarose beads packed into a cylindrical glass or plastic column) with higher holding power, increasing the yield percentages and purification. The high resolution is possible due to the use of small-diameter stationary phases. There are various columns available according to desired separation type (e.g., ion exchange, gel filtration, hydrophobic interactions, reversed phase, affinity, and chromatofocusing). The high automated features (such as fraction collection, sample injection, and gradient control) of FPLC systems increase the reproductibility and allow the recovering of proteins even at very low concentrations, from micrograms to picograms [34,35].

2.5. Ultrafiltration

Ultrafiltration can be used to obtain more concentrated protein solutions [3]. Lectin can be obtained by an affinity purification strategy which combines affinity binding with ultrafiltration separation. In this approach, a macromolecular ligand is retained on one side of a membrane and is allowed to interact with a crude extract. The substance specifically binds to the ligand forming a high-molecular-weight complex; whereas other compounds are washed out through the membrane pores. With the addition of free ligands or other dissociation media, the complexes dissociate and the formerly bound material is liberated, passing through the membrane and being collected in a purified state [36]. This method is less efficient than the size exclusion chromatography, since it provides only two fractions: larger and smaller proteins. The great advantage is that it concentrates diluted protein solutions with the minimum denaturation [23].

2.6. One- and Two-Dimensional Polyacrylamide Gel Electrophoresis

Electrophoresis is an analytical method based on the migration of charged particles in a medium under the influence of an electric field. This technique is widely used for protein structural characterization establishing the purity degree of these molecules. The polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions is used to analyze the purity degree of native molecular structures. One example using *Cratylia mollis* seed lectin containing Cramoll 1,4 is showed in Figure 4. Native Cramoll 1,4 was obtained through chromatography on Sephadex G-75 and two bands with distinct migrations were revealed (Figure 4a). These two isoforms can be separated by ion-exchange chromatography on CM-cellulose column (Figure 4b; lane 1, Cramoll 1 and lane 2, Cramoll 4).

PAGE under denaturing conditions (presence of sodium dodecyl sulfate, SDS-PAGE) and reducing conditions (presence of β-mercaptoethanol) reveals the subunit

composition or polypeptide apparent molecular mass of the protein [37].

Also, the presence of glycosylated moiety can be indicated by a glycoprotein staining [38].

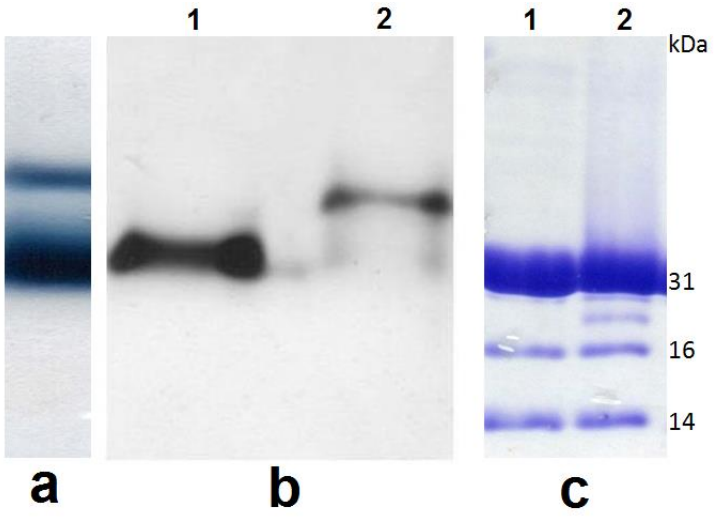

Figure 4. One-dimensional polyacrylamide gel electrophoresis (PAGE) for native basic Cramoll 1,4 (a), Cramoll 1 (b1) and Cramoll 4 (b2); SDS-PAGE under denaturing and reducing conditions of Cramoll 1 (c1) and Cramoll 4 (c2) is also showed.

Under denaturing and reducing conditions, Cramoll 1 and Cramoll 4 showed a similar profile: a main polypeptide band with 31 kDa and two bands with 16 and 14 kDa, which correspond to a fragmentation of the 31 kDa polypeptide chain (Figure 4c, lanes 1 and 2). The lectin Cramoll 1,4 exists as dimers of two intact subunits or as a dimer of one intact subunit with one subunit constituted by fragments.

Two-dimensional electrophoresis is an important tool in proteomics analysis and characterization of proteins. This technique allows separating proteins according to both the isoelectric point (pI) and the apparent molecular mass (Figure 5). In the case of lectin preparations, it is used to characterize a purified sample and can be useful to reveal the presence of isoforms or protein contaminants with similar mass but different pI values. The first dimension step, which is known as isoelectric focusing, is performed using gel strips containing ampholyte mixtures that create a pH gradient throughout the gel (A). The material of interest is then applied on gel and the strip is submitted to an electrophoretic run to migrate according to protein liquid charge (B). The migration will stop when the protein cannot move beyond the pH at which it reaches the pI (C). Next, the gel strip is placed on the top of a SDS-PAGE gel and the protein migrates according to its apparent molecular mass (D). At the end of two-dimensional electrophoresis and after staining, the protein will be visualized in a position that horizontally corresponds to its pI and vertically to its molecular mass (E).

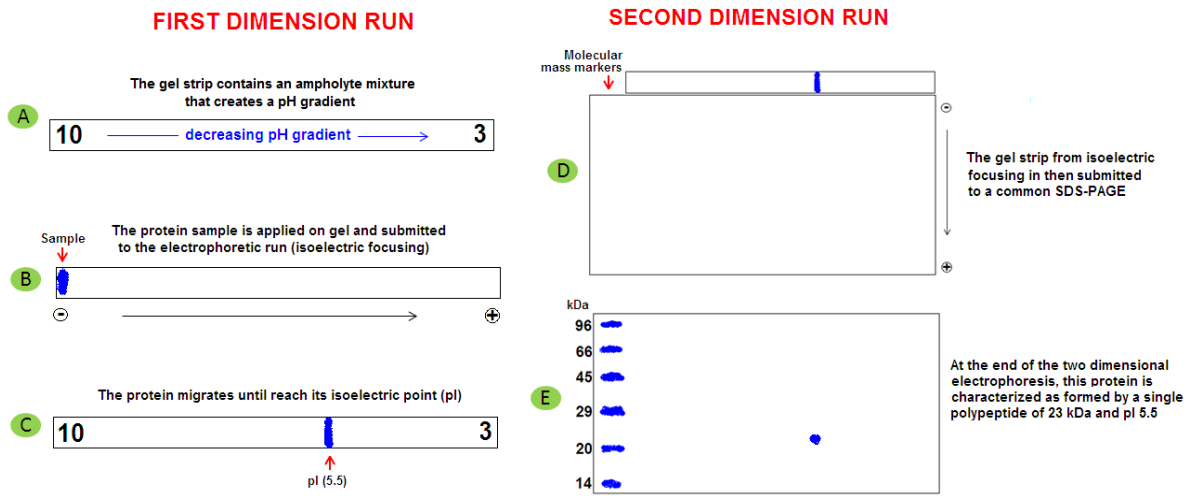

Figure 5. Schematic representation of steps in a two-dimensional electrophoresis used to characterize a protein through isoelectric point (pI) and apparent molecular mass.

Two-dimensional electrophoresis showed that a lectin from *Fenneropenaeus merguiensis* hemolymph is constituted by two subunits with 32.3 and 30.9 kDa, both with pI 6.0 [39]. The technique was also able to reveal the presence of two isoforms of the collectin CL-43 with pI 4.9 and 5.3, which differed in the number of amino acid residues [40]. A Western-blot analysis combined with two-dimensional electrophoresis revealed that a fish-egg lectin from *Cyprinus carpio* occurs as three isoforms with pI 4.9 which migrated closely in the second dimension step and as another isoform with pI 4.5 [41].

2.7. Protein Sequencing and Identification

The Edman degradation, one of the main methods used for protein sequencing, consists in the cyclic degradation of polypeptide chains; the amino acids are removed one at a time. The technique involves the reaction between phenylisothiocyanate and the free amino group of the N-terminal residue followed by incubation at slight acidic conditions for peptide bond cleavage. The N-terminal residue is released as a phenylthiohydantoin derivative and can be identified by HPLC. The polypeptide chain, now with one amino acid less, is submitted to another degradation cycle [42]. Nowadays, the Edman degradation is automated and performed in sequencers, which considerably reduce the time needed for total protein sequencing.

The mass spectrometry is, above all, the better way to determine the molecular mass of a protein. In this technique, the protein in study must first be ionized. There are two main methods used for protein ionization: electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). The ionization in ESI is reached by submitting a liquid containing the protein to an electric field so that the ionized samples are dispersed as an aerosol. The protein in MALDI technique is mixed with a matrix solution which is composed by crystallizable molecules such as the α-cyano-4-hydroxycinnamic acid. Following crystallization, a laser is focused on the sample and the matrix molecules are ionized and desorbed or transfer the proton received to a protein molecule, which is then charged. Once ionized by any of these techniques, the protein is introduced into a mass analyzer and accelerated by an electrical field. The

velocity of an ionic species is function of its mass-to-charge ratio, then the time-of-flight (TOF) of the ionized protein is used to measure the velocity and consequently it's mass.

Mass spectrometry can be used to identify a protein or to detect similarities between proteins. The peptide-mass fingerprinting (PMF) technique allows protein identification by comparing the experimental and theoretical masses of known peptides found in databases with the molecular mass of polypeptide fragments generated after digestion of protein by an endoprotease [43]. The polypeptide spot from a two-dimensional SDS-PAGE is excised from gel, washed several times and then treated with trypsin, which is the most used enzyme for this purpose. The hydrolysis of peptide chains by trypsin occurs by cleavage of peptide bonds involving lysine or arginine residues; the tryptic digestion yields a specific number of peptides with definite length, sequence, and mass. The mass of generated peptides is determined by mass spectrometry. According to the comparison between the peptides generated and the informations about peptides obtained from known proteins in databases, the protein under study can be found to be similar to other protein or even identified. On the other hand, if this protein was not previously studied, no similarities would be detected. PMF revealed that the water-soluble lectin from *Moringa oleifera* seeds (WSMoL) has similarity (score 70%) with flocculating proteins also found in the seeds of this plant [27].

If there is no information on the sequence of a protein in the databases or if the objective is to obtain the protein sequence, *de novo* sequencing can be performed by mass spectrometry. For this, the protein is enzymatic degraded to peptide fragments, which have their mass determined. The use of different proteases will produce distinct fragments. In addition, the fragmentation events are somewhat random. Some peaks in mass spectrum will appear to differ only by the approximate mass of an amino acid residue. Thus, the sequence of the peptide can be determined by computer analysis of the peak mass differences, which corresponds to a respective amino acid. The primary sequence of a lectin from *Bauhinia forficata* seeds was obtained after analysis of peptide fragments resulting from hydrolysis by trypsin, chymotrypsin, Asp-N and Lys-C [1].

2.8. Lectin Electrochemistry

Eletrochemistry has been used to characterize and study the behaviour of lectins in different media as well as to evaluate these proteins as components in potential biosensors. Electrochemical techniques are important to give a direct insight into the interface containing electrically charged groups adsorbed to the electrode surface. The kinetics behaviour and redox potential of adsorbed molecules depend of the pH near the electrode, the temperature in the system and properties of a double layer at the interface. This potential is important for the application of cyclic voltammetry techniques in the kinetics investigation of a biological interface [44].

Electrochemistry was used to evaluate the interaction between *Microgramma vaccinifolia* rhizome lectin (MvRL) and cations. The study revealed that stimulatory effect of Ca^{2+} and Mg^{2+} on hemagglutinating activity of MvRL was linked to changes in lectin surface charge. Also, the system using free MvRL was able to detect changes in the electrochemical potentials of lectin in presence of Ca^{2+} and Mg^{2+} even in presence of human serum, a complex environment [45].

Other important technique to investigation the bulk and interfacial electrical properties and processes of electrode systems is the electrochemical impedance spectroscopy (EIS). It is very valuable for characterizing biomaterial films and following the kinetics and mechanisms of bioelectrocatalytic reactions in order to detect biorecognition events [12,46]. An electrical double layer exists around each particle and the liquid layer surrounding the particle exists as two parts: an inner region called the stern layer, where the ions are strongly bound, and an outer and diffuse region where they are less firmly attached. Within the diffuse layer there is a boundary inside which the ions and particles form a stable entity. When a particle moves (e.g. due to gravity), ions within the boundary move with it, but any ions beyond the boundary do not travel with the particle. This boundary is called the surface of hydrodynamic shear or slipping plane. The potential that exists at this boundary is known as the zeta potential. Andrade et al. [46] studied *Eugenia uniflora* and *Triticum vulgaris* lectins using EIS and zeta potential; the interfacial properties of both lectins were strongly dependent upon the pH of bulk phase. The authors also determined the pI (6.5) of *E. uniflora* lectin using impedance spectroscopy. Santos et al. [47] studied the zeta potential of a protein extract from *M. oleifera* seed and humic acids to understand the interaction between these molecules.

2.9. Immobilized Lectins as Protein Purification Strategies

Lectins, recognizing and specifically binding carbohydrates, can be immobilized in different supports and the affinity matrices obtained have been used to purify glycoproteins [11], polysaccharides [48], trypsin inhibitor [49] and enzymes like peroxidase [50]. Lectin immobilization techniques can vary from reversible and non-covalent attachments to covalent immobilization onto various substrates (e.g., agarose, silica, and polymeric materials) [51].

The most commonly used support for lectin immobilization is Sepharose (a tradename of a registered product), which consists in a beaded form of agarose cross-linked through lysine side chains. Immobilized *Anacardium occidentale* bark lectin was effective in isolate the glycoprotein fetuin [52] and the immobilized *Crataeva tapia* bark lectin was also used as affinity matrix for purification of casein, fetuin and ovalbulmin [11]. A trypsin inhibitor from *Echinodorus paniculatus* seeds and a lectin from Nile tilapia (*Oreochromis niloticus*) serum were purified by chromatography on matrices containing the *C. mollis* and *Canavalia ensiformis* (concanavalin A, Con A) seed lectins, respectively, immobilized on Sepharose 4B [49,53]. Affinity supports containing lectins were also employed in isolation of a lectin from *Colossoma macropomum* serum, a cytotoxic protein from the scyphozoan *Cyanea lamarckii* as well as the major surface coat glycoprotein from *Trypanosoma brucei brucei* [54–56].

Lectin affinity chromatography has also been successfully used for purification of glycoproteins with medical relevance; fractionation of human plasma proteins allowed obtaining a preparation rich in potential disease-specific glycoprotein markers [57]. Affinity chromatography of urine voids on different supports containing immobilized lectins revealed that four urinary prostate-specific antigen (PSA) isoforms could be identified by distinct binding profiles to columns containing lectins from *Ulex europaeus, Aleuria aurantia* or *Phaseolus vulgaris* [58].

The *C. mollis* seed lectin immobilized on Sepharose CL-4B was used to isolate a glycoprotein from *Glycine max* seeds with antiplatelet aggregation and anticoagulant

activities [59]; lecithin-cholesterol acyltransferase (LCAT, EC 2.3.1.43), a serum glycoprotein has also been isolated and characterized [60].

3. Purification of Virus Lectins

Enveloped viruses expose lectins at their surface whose structure plays a crucial role in virus attachment and entry into the cells [61]. Lectin from influenza virus was purified by gel filtration chromatography with Sephadex G- 150 [62]. A lectin, named glycoprotein G, from a fish rhabdovirus causer of haemorrhagic septicaemia (VHSV) was purified using affinity chromatography on immobilized Con A [61]. Lectins from varicella-zoster virus were purified by immune affinity chromatography using monoclonal antibodies [63]. Two glycoproteins from *Sendai* virus, a hemagglutinin neuraminidase and a fusion protein, were purified by affinity chromatography on a *Lens culinaris* lectin-Sepharose column [64]. Structure prediction methods and structural modeling of the protein A33 from *Vaccinia* virus, which is a type II membrane protein found in the outer envelope of extracellular and cell-associated virus particles, revealed that it evolved from a C-type lectin-like protein [65].

4. Purification of Bacterial Lectins

Lectins are also present in bacteria displaying several important roles. Bacteria adherence to the surface of host intestinal mucosa is been established by lectins promoting enteric infections [66]. A cell-associated mannose/glucose-specific lectin from *Vibrio cholerae* O1 strain was purified by chromatography on a chitin column followed by affinity purification on Sephadex G100 [66]. This lectin was revealed as a single polypeptide band of 40 kDa by SDS-PAGE, exhibited high affinity towards D-mannose and D-glucose and showed globular protein form under electron microscope. This protein reacted strongly with sera from convalescent cholera patients in immunodiffusion tests. Alam et al. [67] isolated two distinct lectins from enterotoxigenic strain E-33 of *Vibrio mimicus* by ultrafiltration followed by gel filtration and anion-exchange chromatographies. The hemagglutinating activities of both lectins were inhibited by glycoproteins, including mucin. The opportunistic human pathogen *Pseudomonas aeruginosa* produces two lectins in close association with virulence factors PA-IL and PA-IIL, which bind to galactose- and fucose/mannose-containing glycoconjugates, respectively. PA-IL was the first bacterial lectin to be purified by affinity chromatography [68].

5. Purification of Fungus Lectins

Lectins are found in fungus but their physiological roles in these microorganisms are still not well understood. Various chromatographic techniques have been used to purify lectins from fungus. Vranken et al. [69] isolated an *N*-acetylgalactosamine specific lectin from *Rhizoctonia solani* mycelium by affinity chromatography on gum arabic-Sepharose. This is a dimeric protein composed by two identical subunits of 13 kDa and with high content of asparagine/aspartic acid, valine, glycine,

glutamine/glutamic acid and lysine. Francis et al. [3] purified a mannose-specific lectin from *Penicillium chrysogenum* using gel-filtration chromatography column associated with a FPLC system; this lectin represent a very promising protein to control aphid pest damages in crops.

Lectins are also purified from higher fungus (mushrooms) and exhibit various biological activities such as mitogenic, antiproliferative, antitumor, immunomodulatory, and hypotensive activities [70]. Otta et al. [71] purified a lectin from the mushroom *Ciborinia camelliae* on a hydroxyapatite column, an ion-exchange chromatography type [72]. Amino acid analysis revealed two cysteines and no methionine. The *N*-terminal sequence was determined up to residue 21, and no homologous proteins were found [71]. A lectin from *Clitocybe nebularis* mushroom was isolated by affinity chromatography and exerted antiproliferative activity specific to human leukemic T cells [73]. A lectin was isolated from mycelia of *Ganoderma lucidum* using affinity chromatography on BSM-Toyopearl [74]. Jung et al. [70] purified a lectin from ascomycete *Cordyceps militaris*, one of the most popular mushrooms in Eastern Asia used as a traditional Chinese medicine; this lectin showed mitogenic activity on mouse splenocytes.

6. Purification of Lichen Lectins

Lichens are symbiotic associations between a fungus and a cyanobacterium (cyanolichens) or a green alga (phycolichens). Investigations on lichen properties have greatly contributed to knowledge of general mycology and symbiosis. These organisms represent a rich lectin source. Lichen lectins may play important role as recognition factors for the association of compatible fungi and algae in lichen symbiosis [75]. Lichens also have been a source of other bioactive compounds. Insecticidal activity on larvae of *Aedes aegypti* [76] and *Culex pipiens* mosquitoes [77], induction of genotoxic and oxidative damages in cultured lymphocytes [78], induction of apoptosis in cancer cell lines [79] as well as antioxidant, antimicrobial and antiproliferative activities [80] have been reported for lichen compounds.

A lectin from *Cladonia verticillaris* lichen was purified in milligram quantities through Sephadex G-100 gel filtration chromatography and characterized through AKTA-FPLC and HPLC systems. This lectin showed insecticidal activity on termite *Nasutitermes corniger* [5]. A D-galactose specific lectin was isolated from *Peltigera aphthosa* lichen using Sephadex G-100 gel filtration and showed hemagglutinating activity Ca^{2+} and Mg^{2+}dependent; differences in the hemagglutinating activity were observed in extracts from different parts of the thallus reflecting differences in lectin roles [75]. Lectin specific for *N*-acetylgalactosamine from *Dictyonema glabratum* lichen was purified using ammonium sulphate fractionation at 50-70% saturation followed by chromatographies on DEAE-Sepharose and Sulfopropyl (SP)-Sepharose, a strong cation exchanger; a molecular mass of 33.39 kDa and native conformation as a dimer formed by two identical subunits was estimated using gel filtration chromatography and SDS-PAGE as well as ESI mass spectrometry [81]. Two isolectins of *Xanthoria parietina* lichen, secreted and algal-binding proteins, were purified by ammonium sulphate fractionation at 50% saturation followed by Sephadex G-150 column. These proteins also showed arginase activity and the same molecular mass (58.6 kDa), being identified as isoforms by capillary electrophoresis, which revealed two acidic proteins with isoelectric points of 3.53 and 4.54 [82].

Amino acid sequences of *Evernia prunastri* and *X. parietina* lectins were determined by MALDI TOF/TOF allowing the identification of similarities between lectins of phylogenetically unrelated lichen species. Very similar conformation could also be found in regard to the active center of yeast and soybean arginase; N-terminal sequence could not be assigned, suggesting that the N-terminal amino acid is possibly blocked and involved in the glycosylation of the protein [83].

The recognition mechanisms used by chlorolichens and cyanolichens are based on the production and secretion of fungal lectins which act as signaling molecules and develop arginase activity (Figure 6a). When the specific carbohydrate is present, the lectin shows complete loss of enzymatic activity (Figure 6b). The loss of arginase activity must be due to a change of protein tertiary structure produced after binding [84,85]. The importance of arginase activity in lichen lectins is the fact that the enzyme secreted by the fungi increases polyamine levels which can induce ultrastructure damages such as disorganization of algae chloroplasts with chlorophyll degradation, hydrolysis of cell wall and, finally, cell death [82].

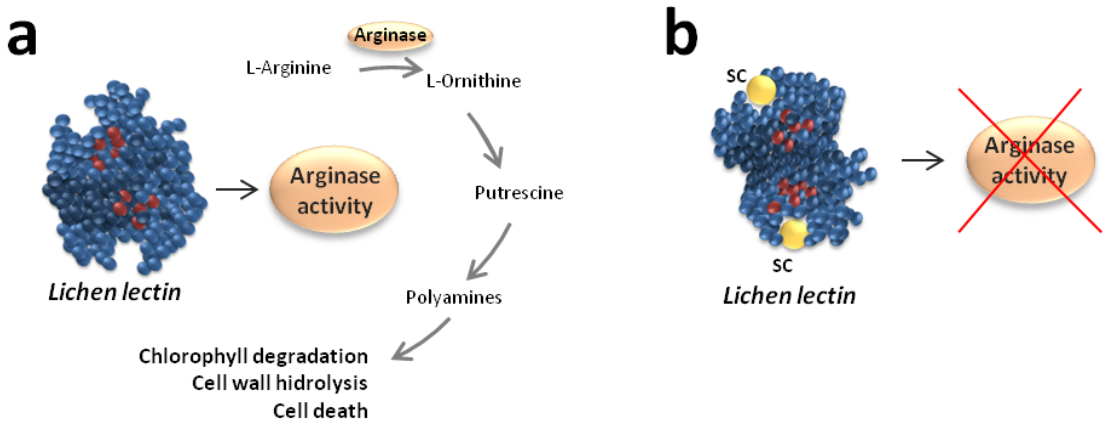

Figure 6. Lichen lectin with arginase activity. In the absence of specific carbohydrate the lectin develops arginase activity (a); with specific carbohydrate enzymatic activity is abolished (b). SC- Specific carbohydrate.

A galactose specific lectin from the *E. prunastri* lichen develops arginase activity and binds to the homologous algae that contain polygalactosilated urease in their cell walls. Hydrolysis of the galactoside moiety of urease in intact algae releases high amount of D-galactose and impedes the binding of the lectin to the algal cell wall. The enzyme bound to its ligand shows to be inactive to hydrolyze arginine.

The production of glycosylated urease might be coincident to the season in which algal reproduce assuring the recognition of new phycobiont produced after cell division by its fungal partner [83].

7. Purification of Plant Lectins

Plant lectins can be isolated from different tissues and have distinct functions including carbohydrate transportation, packaging and/or mobilization of stored proteins, elongation of cell wall, interactions between plants and microorganisms as well as defense against fungi, viruses, bacteria and insects [6,86,87]. The initial steps in lectin purification include preparation of a crude extract in buffer or saline solution followed by precipitation with ammonium sulfate. Affinity chromatography is quite common to obtain plant lectins [26]. Various biotechnological applications are assigned to plant lectins; these proteins can be used as cytotoxic [1], antimicrobial [7,32,88], immunomodulatory [89], insecticidal [11,27,90–96] and antitumoral [97] agents. The relationship between structure and function of plant lectins can be carried out by gene synthesis and cloning [20].

Seeds are rich sources of lectins. The main molecular form of *C. mollis* lectin, Cramoll 1, was purified by affinity chromatography on Sephadex G-75 followed by ion exchange chromatography on CM-cellulose, as previously mentioned [13]. Paiva and Coelho [98] purified two distinct molecular forms of lectins from *C. mollis* seeds by chromatography on Sephadex G-75 and Bio-Gel P-200 (Cramoll 2) as well as on CM-Cellulose and Sephadex G-75 (Cramoll 3).

Cramoll 1,4 or Cramoll 1 molecular form revealed mitogenic activity [99]. Cramoll 1,4 showed anti-helminthic activity against *Schistosomiasis mansoni* infection in mice [100]; an isolectin hydrogel (Cramoll 1,4) can be used as therapeutic applications in the treatment of thermal burns [101]. Some biotechnological properties and biological activities of *C. mollis* lectin structurally represented [16] are shown in Figure 7.

A lectin from *Eugenia malaccensis* seeds (EmaL) was purified by affinity chromatography on Sephadex G-50 column, used as antimicrobial agent and repairing process of cutaneous wounds [102]. A basic coagulant lectin from *M. oleifera* seeds (cMoL) was purified after saline extraction and guar gel column chromatography [6]. cMoL showed coagulant activity, similar to aluminium sulphate, the most widely used coagulant in water treatment. Also, this lectin showed affinity to humic acids and could be used in water treatment to remove these compounds [47]. cMoL showed significant negative impact against *Anagasta kuehniella*, a polyphagous pest that feeds on a wide variety of stored products, particularly in stored grains [94]. WSMoL (water-soluble *M. oleifera* lectin), isolated from *M. oleifera* seeds using a chitin column, showed potential as natural biocoagulant for water, reducing turbidity, suspended solids and bacteria [22].

WSMoL also showed larvicidal activity against *A. aegypti* as well as stimulate oviposition by *A. aegypti* gravid females and impaired the hatching of eggs by killing the embryos inside the eggs [27,103]. A galactose-specific lectin was purified in milligram quantities from leaves of *Bauhinia monandra* (BmoLL) by guar gel affinity chromatography [38]. BmoLL showed inseticidal activity against *Anagasta kuehniella*, *Zabrotes subfasciatus* and *Callosobruchus maculatus* [90]. A basic lectin was purified from *B. monandra* secondary roots (BmoRoL), also in milligram quantities, by affinity chromatography on guar gel and showed antifungal activity against phytopathogenic species of *Fusarium* and termiticidal activity on *Nasutitermes corniger* workers and soldiers. A lectin from bark of *Crataeva tapia* (CrataBL) was purified by ion exchange chromatography; CrataBL was an insecticide agent against *N. corniger* workers [11]. Chitin-binding lectins from *Myracrodruon urundeuva* bark, heartwood and leaves also showed termiticidal activity on *N. corniger* workers and soldiers, being also able to kill symbiotic bacteria from termite gut, as well as larvae of *A. aegypti* [88,92,93,96].

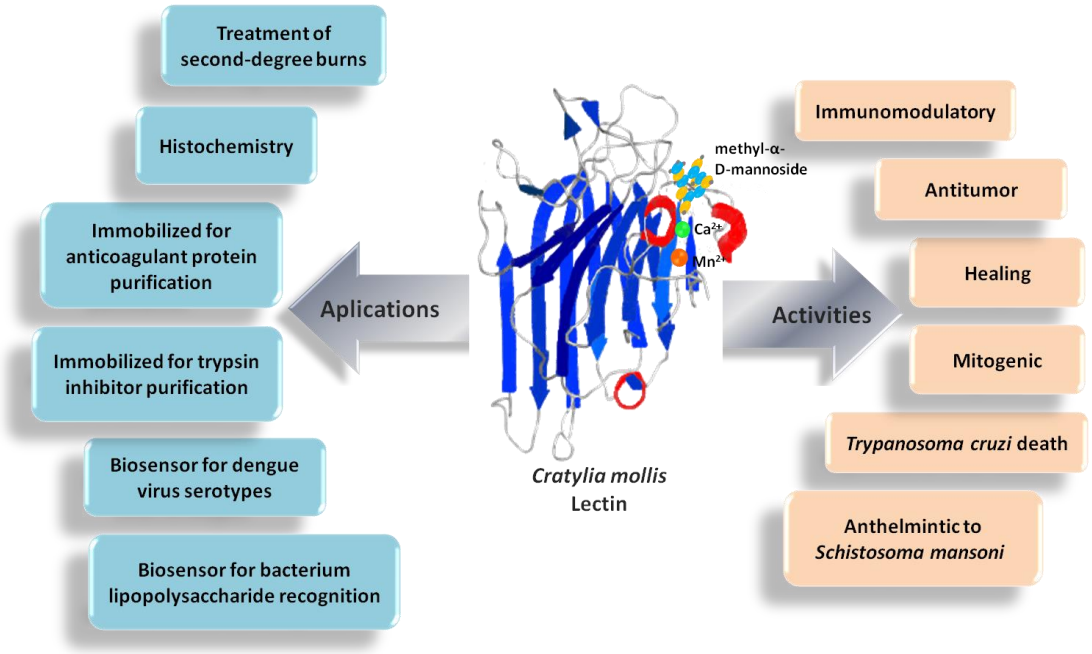

Figure 7. Applications and activities of *Cratylia mollis* lectin [12,49,59,89,99–101,126–130].

8. Purification of Animal Lectins

Lectins, among other animals, have been obtained from fishes [56], snakes [9], shrimps [104], jellyfishes [105], scallops [106] and humans [107]. Nunes et al. [9] purified a lectin from *Bothrops leucurus* snake venom using a combination of affinity and gel filtration chromatographies. This lectin showed antibacterial activity and mediated cytotoxic effect and apoptosis induction on tumor cell lines [108].The γ-radiation can lead to significant changes in the *B. leucurus* venom lectin structure, which may promote the loss of its binding property and toxic action [109].

It is well recognized the importance and roles of mammal lectins in the lectin complement pathway, especially ficolins (FCN) and mannose-binding lectin (MBL) [110]. Relationship between levels of human serum MBL and the leptospirosis pathogenesis was recently suggested [111]. Some animal lectins may be present in the intracellular space mediating processes, such as splicing of RNA to protein folding and trafficking proteins [112].

Lectins are found in several fish species participating in events such as fertilization, embryogenesis and morphogenesis [112], opsonization and phagocytosis [113]. Recently it was identified a *Rachycentron canadum* serum lectin with specificity for methyl-α-D-mannopyranoside [114]. A lectin from *Oreochromis niloticus* serum (OniL) was isolated by affinity chromatography; OniL showed highest affinity for methyl-α-D-mannopyranoside and D-mannose as well as immunomodulatory activity in mice, preferentially Th1-type immune response [53]. *Colossoma macropomum* serum lectin (ComaSeL) was obtained by two steps: affinity chromatography in Con A-Sepharose 4B followed by DEAE-Sepharose 4B, an ion exchange column. ComaSeL recognized the carbohydrates D-galactose, 1-O-methyl-D-galactopyranoside, and D-

fucose, showing antibacterial activity against Gram-negative bacteria, which suggests its participation in fish immune system [56].

Animal lectins may also be obtained by molecular cloning through the use of recombinant DNA technology. Atlantic salmon (*Salmo salar*) serum lectin produced by recombinant expression in *Escherichia coli* has been used in studies of oligomerization [115]. The C-type recombinant lectin from bay scallop *Argopecten irradians* was able to bind lipopolysaccharide of Gram-negative bacteria inducing agglutination [116]. Many animal lectins have been characterized in terms of amino acid sequence; the deduced amino acid sequence allows the determination of similarity with proteins of other species [117]. Gene sequences of certain lectins, such as MBL gene from channel catfish, *Ictalurus punctatus,* have been obtained allowing the analysis of tissue expression of these proteins front to challenges for bacteria [21].

9. Pitfalls in Lectin Purification

The optimal protein purification strategy attains high level of purity in fewer steps. Purification protocols quickly allowed obtaining milligram quantities from *Cratylia mollis* [13] and *Crataeva tapia* [11] lectins. However, tambaqui fish lectin [56] and *Cladonia verticillaris* lichen lectin [5] required several distinct chromatographic approaches taking years to achieve purity.

As discussed above, the process of lectin purification includes the steps usually applied to isolate proteins. In an effective purification procedure, the passage through the distinct steps should be accompanied by continuous increase in the specific hemagglutinating activity. This increase indicates that the target protein is present and being concentrated while undesired proteins are excluded. The lectin should be active and homogeneous at the end of purification, which can be confirmed using different techniques. To reach the lectin homogeneity with a good yield in both milligram amount and biological activity, in practice, can be a simple or substantially laborious procedure. These variations occur due to the great diversity and versatility of these proteins, so that there is not a single and inflexible protocol to be followed. It is necessary to diverse adjusts according to the sample to overpass or avoid pitfalls in the lectin purification.

Special adjustments in lectin purification planning are needed even in sample preparation before extraction. The maceration or fragmentation of the plant tissue is important to promote a high solubilization of protein due to the increase of contact surface [118]. The tissue should be completely dried before transformation in a meal or powder, which usually results in a high yield in protein amount after extraction.

However, the total removal of water as well as the maintenance of the tissue at room temperature for several days or in incubators at high temperatures may lead to loss of lectin activity. It is frequently a reason for absence or low activity even when a high protein concentration is obtained. Alternatively, these lectins can be extracted immediately after collection by rapid homogenization of fresh plant tissue in a blender [119]. Animal lectins can be extracted by manual homogenization of tissue on ice while extraction of microbial lectins can need an initial step for disrupting the cells [120]. The extraction can also be made in liquid nitrogen to obtain a maximum conservation of lectin activity [121].

The extraction medium can be a saline solution [6,11,45,93], a buffer solution [5,7,52] or distilled water [27,122]. Saline solutions are commonly used since at low salt concentrations there is an increase in ionic strength resulting in highest protein

solubization. However many times a low activity is detected due to inadequate pH value.

The use of a buffer solution is recommended when the biological activity of target protein is sensitive to alterations in pH value during extraction. Other condition that can be modulated in extracts is the temperature since some lectins are thermo-sensitive while others are thermo-resistant. In the first case, it is imperative that extraction occurs at low temperature. If part of the target lectin has already been lost due to an inadequate sample preparation, the recovery in extraction using inadequate temperature will worsen. On the other hand, the use of high temperature or low/high pH conditions can be desirable. For example, if it is known that the target lectin is stable to heating at 80 °C, the extract can be prepared at this temperature and thus all thermo-sensitive proteins are denatured to be excluded.

Santi-Gadelha et al. [123] used a buffer solution at pH 2.6 to extract the lectin from *Araucaria angustifolia* seeds since the authors were already aware that this lectin is able to resist in medium with high acidity.

It is also necessary to bear in mind that some proteins require the presence of metallic ions to exert their biological activity and thus these ions should be included in the extraction solution in order to preserve and recover the maximum of activity [9,53]. Also, in the case of tissues rich in proteolytic activity, protease inhibitors should be included in extraction medium to prevent that the target lectin is degraded during and after extraction [124].

The adequate adjust of extraction protocol according to the characteristics of lectin source is then essential for success in lectin isolation and to save time in the subsequent steps, which may not work well due to errors before or during the extraction.

It is important to highlight that the absence of lectin activity in an extract is not a safe indicative that this protein is not present in the tissue; the conditions used could not be adequate for the extraction. Also, protein quantification methods possess limitations or can suffer interference of other compounds present in the sample; it is then important to evaluate protein concentration by another method even if the presence of proteins has not been revealed.

The detection of lectin activity is mainly achieved by HA followed by carbohydrate inhibition, as already described. The absence of HA inhibition by used carbohydrates is not an indicative of pseudo-hemagglutination. Several lectins did not have their HA inhibited by monosaccharides but are strongly inhibited by more complex structures, such as glycoproteins, and able to interact with polysaccharides [93]. A possible explanation is that the correct geometry for lectin-carbohydrate recognition occurs more easily when the monosaccharide is attached to protein or lipid or present in a polymeric structure.

A correct interaction within the carbohydrate-binding site in the case of these lectins could be more difficult or even avoided when the monosaccharide is free. An increase of monosaccharide molecular movement possibilities and geometric conformations of lectin recognition sites allow specific binding when the carbohydrate is covalently linked to a more complex structure. The carbohydrate affinity matrix may also have a spacer chemical arm between ligand and matrix to facilitate and promote lectin binding.

Proteins can be concentrated by different strategies after extraction. The researcher working with lectins should be precautious on the use of certain methods, such as organic solvent precipitation, that can lead to irreversible protein denaturation. The thermal precipitation can also impair lectin recovery if heating promotes

precipitation due to loss of protein structure levels that cannot be restored. The isoelectric precipitation and ammonium sulphate precipitation are safer since the protein can be frequently recovered more active. The precipitation with ammonium sulphate is the most used approach since it does not affect protein structure or function; salt can be easily removed by dialysis [125].

In the chromatographic step, one or more techniques may be necessary to achieve lectin homogeneity. The presence of contaminant proteins with structural and/or physical-chemical characteristics similar to those of the lectin in study can difficult its isolation using a single chromatographic step. The affinity chromatography is certainly the technique of choice for isolating lectins. However the presence of more than one lectin with similar or distinct carbohydrate specificity, multiple isoforms or isolectins, makes imperative other chromatographic steps. In addition, it is imperative to adjust the relation between the column size and the amount of lectin loaded. The loading of a high lectin amount can led to saturation of adsorption capacity of the matrix and thus substantial amount of protein will be lost, besides of impure lectin preparation obtention. This problem can be solved or minimized by decreasing of total protein amount to be loaded. Recycling of non-adsorbed material could also be used in order to recover the maximum of lectin.

Conclusion

The different strategies to obtain lectins from distinct sources such as microorganisms, fungus, lichens, plants and animals, involve several purification methodologies which are followed by characterization with the purpose to explore the structural, functional and a broad field of applications.

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CAPÍTULO III

Fish Lectins: A Brief Review

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Chapter 4

Fish Lectins: A Brief Review

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Abstract

Studies on fish lectins in recent decades are motivated to unravel the role of these proteins in the innate immune system of these vertebrates. Lectins are carbohydrate-binding proteins found in viruses, prokaryotes and eukaryotes. These proteins can agglutinate cells, and precipitate polysaccharides, glycoprotein or glycolipids mediating different biological processes such as cell-cell interactions, glycoprotein traffics and clearance, induction of apoptosis, antibacterial, antiviral, mitogenic and antitumor activities. Lectins are believed to mediate pathogen recognition in fish immune system with important roles in innate immune response. In addition to the function of defense against microorganisms, there is evidence that these fish lectins have also an important role in fertilization, embryogenesis and morphogenesis. Mitogenic and antiproliferative activities have also been identified in various fish species. The classification of animal lectins is diversified and is based on structural, functional and evolutionary studies. Currently there are a very large number of animal lectin families that have already been identified; most of them occur in fish such as galectins, C-type, Pentraxins, Calnexins, I-type, F-type and L-rhamnose lectins. The purification, structural and functional characterization of fish lectins has been approached; most of these studies reinforce the role of lectins in innate immune system in these animals. This review deals with different families of animal lectins found in body fluids, cells and tissues of fish. Some properties, functions, and biological events of such proteins will also be presented. The analysis of lectin roles are mainly related to the innate immune response in fish of economic economic importance and certainly will contribute to increasing knowledge on the subject and generate appropriate technologies to improve the development of aquaculture.

Keywords: Fish lectins; Classification; Lectin purification; Lectin function.

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1. Introduction

Lectins are groups of proteins characterized by their ability to bind carbohydrates with considerable specificity (Nilsson, 2007). These proteins are present in virus, bacteria, cyanobacteria and yeast (Loris, 2002; Loris, 2009; Veelders et al., 2010; Huskens et al., 2010; Xu et al., 2012), plants and animals (Ferreira et al., 2011; Nunes et al., 2012). Lectins can agglutinate cells and precipitate polysaccharides, glycoproteins or glycolipids (Lis and Sharon, 1998; Zhang et al., 2009). These properties enable lectins to mediate different biological processes such as cell-cell interactions (Gabor et al., 2004), induction of apoptosis (Perillo et al., 1995; Vervecken et al., 2000), cytotoxic activity (Kawsar et al., 2010; Silva et al., 2012), antibacterial and antiviral activity (Araújo et al., 2012; Napoleão et al., 2012), antiproliferative activity for cancer cells (Bah et al., 2011), mitogenic activity (Maciel et al., 2004; Bah et al., 2011) and antitumor activity (Andrade et al., 2004). Lectins have been investigated in marine bioresources by their various pharmacological applications to develop new drugs (Ogawa et al., 2011).

The lectin term, from Latin *lectus*, which means chosen, was introduced by William Boyd and Elizabeth Shapleigh in 1954 reflecting etymologically their property to agglutinate selectively blood cell groups (Boyd and Shapleigh, 1954). Initially the study of lectins was focused only on plants, since it was believed that these proteins occurred only in the plant kingdom. The first agglutinin was identified in the crude extract from *Ricinus communis* by Stillmark, in 1888, when searching effects of plant toxicity (Sharon and Lis, 1989, 2004). The first plant lectins have been isolated and characterized from *Phaseolus vulgaris* (Takahashi et al., 1967), *Phaseolus lunatus* (Galbraith and Goldstein, 1970; Gould and Scheinberg, 1970) and *Canavalia ensiformis* (Lloyd, 1970). In the late twentieth century the field of glycobiology had a major breakthrough due to the recognition of the lectin presence in animal tissues (Sharon, 2008). Probably the first mammalian lectin was identified by Stockert et al. (1974) in rabbit liver and, also, the first serum lectin was identified by Ashwell and Morell (1974) as the hepatic asialoglycoprotein receptor.

Animal lectins provided great advances in the field of Glycobiology decoding the glycocode and contributing to the development of various areas of basic and applied bioscience (Sharon, 2008; Varki et al., 2009; Kumar and Mittal, 2011).

The association of lectins with carbohydrates is a primary event in some biological processes such as infection and metastasis (Bouwman et al., 2006; Rambaruth and Dwek, 2011). In addition to this important role in cellular recognition, the interaction of lectins with carbohydrates has been explored in various fields of research where saccharide specificity is essential. These proteins are invaluable tools for the study of simple or complex carbohydrates, in solution or on cell surface, as well as for cell characterization (Sharon, 2008) due to the ability to discriminate among the myriad of complex carbohydrate structures (Drickamer and Taylor, 1993).

Lectins have been classified based on specific carbohydrates which they recognized, but with the growing number of lectins being discovered, classification is also based on structural information. Ultimately, with the increasing interest, a burst of

information about structures, properties and biological functions of lectins are available and can be accessed in different databases (Krengel and Imberty, 2007; Frank and Schloissnig, 2010; Kumar and Mittal, 2011).

This article reviews the different families of animal lectins found in body fluids, cells and fish tissues. Some properties, functions, and biological events of such proteins will be presented. This study intended to gather general information about lectins occurring in several fish's species, their purification, characteristics, immunological roles, among other biological functions, and potential biotechnological applications for aquaculture.

2. Fish Lectins and Function

Lectins are proteins that recognize cells through carbohydrate binding sites. Each year the understanding of lectins has grown and various fish lectins have been characterized (Vasta et al., 2011; Ogawa et al., 2011). This diversity and characterization led to an expansion of its definition to any protein that has a non-catalytic carbohydrate recognition domain (CDR). There are effective lectins that do not agglutinate cells, such as membrane-bound proteins, and have only a carbohydrate recognition domain (Ewart et al., 2001). The lectin binding to carbohydrate in the CRD occurs through weak interactions such as hydrogen bonding, ionic attractions, hydrophobic and van der Waals forces; these interactions can make the specific and transient nature of protein-ligand bonds (Nelson and Cox, 2011) (Figure 1).

The lectins of animal origin have been classified according to CRD comparison. The CRD found in each particular lectin type share a pattern of amino acid residues highly conserved and invariable (Ewart et al., 2001; Loris, 2002; Suzuki et al., 2003). Furthermore, different properties such as a requirement of divalent cations or reducing environment for ligand binding led to its classification in several major families (Vasta et al., 2011).

The number of studies investigating the role of fish lectins using both immunological and molecular biology techniques has been growing (Shiina et al., 2002; Magnadóttir et al., 2010; Bah et al., 2011). Humoral and membrane-associated lectins from host are critical recognition molecules that may facilitate the establishment of favorable mutualistic interactions with colonizing microbes, or initiate innate and adaptive responses against potentially pathogenic microorganisms (Vasta et al., 2011). In addition, fish lectins mediate other functions, such as agglutination, fertilization, immobilization with complement-mediated opsonization and death of pathogens (Ewart et al., 2001, Dong et al., 2004; Russell and Lumsden, 2005).

Some fish lectins may be present in the intracellular compartments mediating processes such as splicing of RNA to protein folding and trafficking proteins (Vasta et al., 2011). Fish lectins can still be released to extracellular compartment having two destinations: cell surface or soluble components in biological fluids (Hébert, 2000; Vasta et al., 2011).

The immune system of vertebrates involves the innate and adaptive immune response. The innate immune system possesses several proteins acting, such as lysozyme and lectins. The lysozyme acts by attacking and disrupting the cell wall polysaccharides of different bacterial species killing microorganisms. Major events in innate immune defense include the recognition of microbial targets for lectins, such as collectins. These proteins recognize foreign cells as "non-self" through the carbohydrates expressed on the surface acting as opsonins and encouraging their destruction by complement and/or phagocytic cells (Fock et al., 2001; Dutta et al., 2005; Battison and Summerfield, 2009; Imamichi and Yokoyama, 2010).

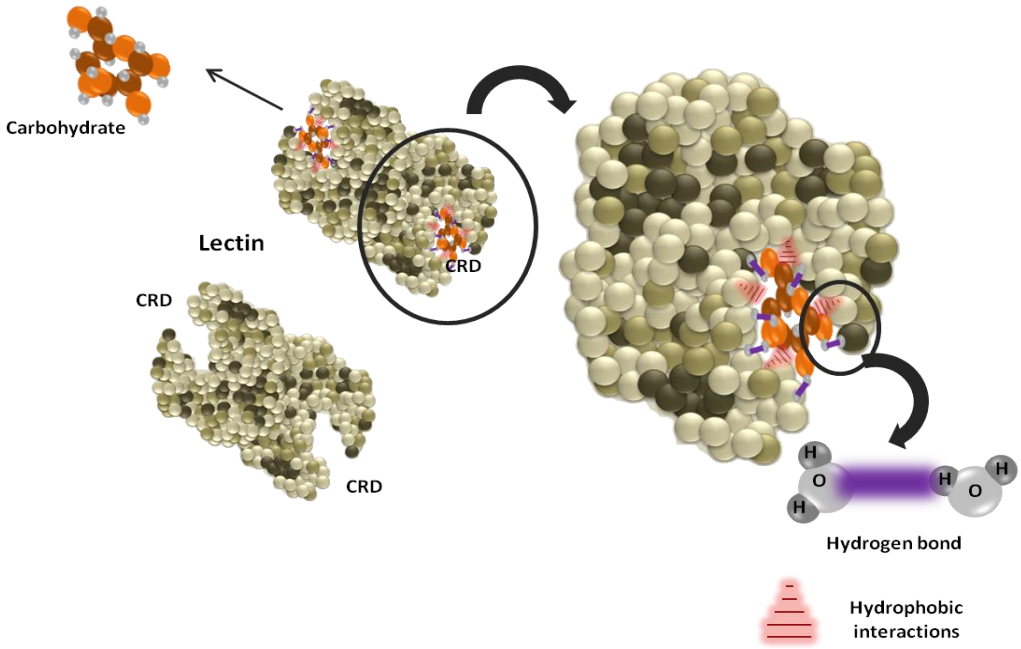

Figure 1. Representation of carbohydrate lectin bind through carbohydrate recognition domains (CRD). The carbohydrate-lectin interactions involve, among other non-covalent forces, the formation of hydrogen bonds and hydrophobic interactions.

The importance and roles of innate immune components such as circulating lectins is well recognized in other vertebrates such as mammals (Fock et al., 2001; Russell and Lumsdem, 2005). Fishes differ from mammals in relation to the acquired immune response; then lectins and other innate immune effectors may have much more important roles.

There are several studies on the role of lectins in the immune system of fish. Several fish lectins are believed to mediate pathogen recognition in the immune system. There is a mannose binding lectin isolated from the serum of Atlantic salmon that has antibacterial activity against *Aeromonas salmonicida*; and according to Ottinger et al. (1999) this lectin has similarity in structure and activity with the mannose-binding lectin of mammal which in turn plays a key role in innate immunity. A lectin isolated from the ovaries of cobia (*Rachycentron canadum*) showed antibacterial activity against *Escherichia coli* and no antifungal activity for *Coprinus comatus*, *Fusarium oxysporum*,

Mycosphaerella arachidicola and *Rhizoctonia solani* (Ngai and Ng, 2007). Studies with mucus isolated from lectin catfish (*Silurus asotus*) showed that the gene of this lectin, in RT-PCR assay was not induced by bacterial stimulation in vivo; agglutination activity against the pathogenic bacteria *A. salmonicida* suggested that this lectin plays an important role in self-defense against bacteria on the skin surface of the catfish (Tsutsui et al., 2011). A lectin isolated from egg chum salmon (*Oncorhynchus keta*) showed different patterns of hemagglutinating activity inhibition to rabbit erythrocytes when Gram-negative bacteria lipopolysaccharides were used, such as *A. salmonicida*; *E. coli* and *Bacillus subtilis* bacteria were agglutinated (Shiina et al., 2002). Another lectin from Chinook salmon roe (*Onchorhynchus tshawytscha*) showed no antifungal activity or agglutination towards *Valsa mali*, *Helminthosporium. maydis*, *Mycosphaerella arachidicola*, *Setospaeria turcica* and *Bipolaris maydis* (Bah et al., 2011). In addition to the role of defense against microorganisms, there is evidence that these lectins have are important in fertilization, embryogenesis (Dong et al., 2004; Vasta et al., 2011) and morphogenesis (Ahmed et al., 2004; Dutta et al., 2005).

Opsonization activity has also been reported in fish. The salmon serum lectin was found to be an opsonin for *A. salmonicida*. It enhanced the phagocytosis of heat-killed *A. salmonicida* by macrophages in a dose-dependent manner (Ottinger et al., 1999). Other lectin isolated from serum of sea bass (*Dicentrarchus labrax*), whose localization and expression occurs in hepatocytes and intestinal cells, also revealed that the exposure of *E. coli* formalin-killed to this lectin enhanced their phagocytosis by *D. labrax* peritoneal macrophages relative to unexposed controls (Salerno et al., 2009). The opsonization assay with another lectin isolated from serum of the gilt head bream (*Sparus aurata*) showed that this lectin binds to formalin-killed *E. coli* and enhances their phagocytosis by peritoneal macrophages (Cammarata et al., 2012). A C-type lectin isolated from conger eel (*Conger myriaster*) showed that when it was bound to microspheres significantly enhanced their phagocytosis in conger eel macrophages (Tsutsui et al., 2007). These findings suggest that these lectins act as opsonins and may play an important role in innate immunity.

There are lectins that have other bioactivities such as mitogenic and antiproliferative activity. Some lectins induced lymphocyte proliferation or modulated several immune functions; these mitogenic lectins are useful as reagents to study lectin interactions with lymphocyte cells in vitro (Maciel et al., 2004). The grass carp (*Ctenopharyngodon idellus*) roe lectin exhibited mitogenic activity toward murine splenocytes with a potency lower than that of the plant lectin Con-A (Ng et al., 2003). The lectins isolated from the ovary of cobia (*Rachycentron canadum*) also showed mitogenic activity toward mouse splenocytes (Ngai and Ng, 2007). The lectin of salmon (*Oncorhynchus tshawytscha*) showed no mitogenic activity towards murine splenocytes, but it showed antiproliferative activity, which reduced the proliferation of human breast tumor (Bah et al., 2011).

Acquired immunity is reduced at low temperatures even in eurythermal fish; studies suggest that components of the fish innate immune system can be less affected by temperature (Ewart et al., 2001; Magnadottir et al., 1999). If these findings could be extended to the components of the innate immune system increasing of innate immunity would be the route of choice for the generation of higher disease resistance in fish. In addition, fish lectins play important roles in many biological systems. The knowledge

gained from the study of these lectins as a bioactive compound with activity to human tumor cells points towards its potential use in biotechnological applications (Lam and Ng, 2011).

3. Classification and Diversity of Fish Lectins

Lectins are structurally diverse molecules (Shirai et al., 2009); this structural complexity, inherent to these proteins reflects in a large number of families. The structures of lectins are important for describing the characteristics of glycan classes found in several species and currently, animal lectins are incorporated into different categories, grouped by shared evolutionary origin and/or similarity of structural folds (Russell and Lumsden, 2005; Lin et al., 2009). In this section we try to summarize the main animal lectins that also occur in fish, highlighting their main characteristics and general functions.

The first classification of animal lectins divided these proteins into two categories S-type and C-type based on structural information of the protein portion responsible for interaction with carbohydrate, CRD (Drickamer, 1988). New lectin groups have then emerged based mainly on structural information of the CRD. The variety of functions of animal lectins could be considered in general terms to be recognition molecules within the immune system. More specifically, lectins have been implicated in a direct first-line defense against pathogens, cell trafficking, immune regulation and prevention of autoimmunity (Kilpatrick, 2002). Table 1 shows the main categories of animal lectins with their relevant features and examples of function and/or biological activities.

The S-type lectins are designated as thiol-dependent proteins of intra-and extracellular localization recognizing mainly β-galactosides (Drickamer, 1988). However the need for thiol reducing agents is not very clear for some lectins of this group; a consensus of the term galectin to this group is due to common characteristics, such as ability to binding beta-galactosides and Ca^{2+}-independent activity (Arason, 1996; Kilpatrick, 2002). The galectins abundant in cytosol can be divided into three types, the proto-type (galectin 1, 2, 5, 7, 10, 11, 13 and 14), chimaera-type (galectin 3) and tandem-repeat type (galectin 4, 6, 8, 9 and 12). Galectin-1 containing two CRD as a homodimer is bound by a high-affinity receptor and acts as an autocrine inhibitor of cell growth. Galectin-4 has two CRD connected by a link peptide, whereas galectin-3 has one CRD connected to two domains which causes the molecule to form multimers (Dumic et al., 2006). All three major galectin types, proto, chimera, and tandem-repeat are present in teleost fish. Galectin-3 sequences have been determined in pufferfish genome *Tetraoodon nigroviridis* (AL301540) and zebrafish EST *Danio rerio* (BM034940) been used in comparative studies with human galectin-3 (HSPC159) (Cooper, 2002). Homology screening of fish databases, in addition, reveals many other galectin-like sequences in catfish (*Ictalurus punctatus*), flounder (*Paralichthys olivaceus*), trout (*Oncorhynchus mykiss*), medaka (*Oryzias latipes*), zebrafish (*D. rerio*), and two species of pufferfish (*Takifugu rubripes* and *T. nigroviridis*) (Cooper, 2002).

The C-type superfamily includes the C-type lectins (CTL) and proteins containing C-type lectin-like domain (CTLD). CTL require calcium ions in binding to carbohydrate. The calcium domain is highly conserved in all members of the family; however, usually differ in the types of recognized carbohydrates and within the CRD. Key conserved residues that bind sugars, include the Glu-Pro-Asn (EPN) or Gln-Pro-Asp (QPD) motifs, in vertebrates (Zelensky and Gready, 2005; Cummings and McEver, 2009).

Table 1. Main categories of animal lectins, features, functions and biological activities.

Categories	Main features	Function/Biological activity
S-type lectins (Galectins)	Binding β-Galactosides; Ca^{2+} independent activity	Inflammatory responses; development, differentiation, morphogenesis, tumor metastasis, apoptosis; cell growth control and apoptosis (Fukumori et al., 2007)
C-type lectin	Ca^{2+} dependent activity, conserved Ca^{2+} biding site	Innate immunity (collectins); promote phagocytosis, complement activation (MBL); Cell adesion (selectins): Lymphocyte homing (L-selectin); Leukocyte trafficking to sites of inflammation (E- and P-selectins); Cell growth control and apoptosis (Kerrigan and Brown, 2009; Arnold et al., 2006; Ourth et al., 2008)
Pentraxins	Ca^{2+} dependent, exist in serum as acute phase protein	Recognition of foreign or aberrant cell glycosylation (Endocytosis or initiation of opsonization or complement activation) (Kilpatrick, 2002; Magnadóttir et al., 2010)
Calnexin	Intracelular lectin	Folding mechanism and misfolded protein retention in endoplasmic reticulum (Williams, 2006); stress-induced apoptosis (Takizawa et al., 2004)
I-type lectins	Structural similarity to the immunoglobulin superfamily, affinity for sialic acid	Immune and neural system; Cell-cell interactions; Cell routing (Varki and Angata, 2006)
F-type lectins or Fucolectin	Affinity for L-fucose, Ca^{2+} independent, non glycolsylated	Molecular recognition in innate immunity (Salerno et al., 2009)
L-rhamnose binding lectins	Binding L-rhamnose, two or three homologous CRD in tandem of about 95 at 100 amino acids residues	Carbohydrate metabolism regulation, fertilization, cell proliferation, cytotoxicity, and opsonisation, respiratory burst stimulation, microbicidal activity (Terada et al., 2007; Watanabe et al., 2009; Franchi et al., 2011)

CTLD refer to protein domains that are homologous to CRD of the C-type lectins, or which have structure resembling the structure of the prototypic C-type lectin CRD, regardless of their ability to bind sugars. Many CTLD have evolved to specifically recognize a variety of ligands, including carbohydrates, inorganic ligands (Ca_2CO_3), lipid, specific snake venoms, fish antifreeze and bird egg-shell proteins (Zelensky and Gready, 2005). C-type domains are normally found in animal lectins from serum, extracellular matrix, and membranes (Vijayan and Chandra, 1999). C-type lectins have been identified in various fish species such as sea lamprey *Petromyzon*

marinus (Ourth et al., 2008), japanese flounder *Paralichthys olivaceus* (Kondo et al. 2007), venomous fish *Thalassophryne nattereri* (Lopes-Ferreira et al., 2011) and grass carp *Ctenopharyngodon idellus* (Liu et al., 2011), among others.

C-type lectins are functionally diverse and have been implicated in various processes including cell adhesion, tissue integration and remodeling, platelet activation, complement activation, pathogen recognition, endocytosis, and phagocytosis, cytotoxic effect, mitogenic and antibacterial activities, besides of specific antibody production (Ngai and Ng, 2007; Kerrigan and Brown, 2009; Komegae et al., 2011; Saraiva et al., 2011).

Selectins and collectins are members of the C-type lectin superfamily. MBL can also be classified within the superfamily of C-type lectin according to characterization with CTL or CTLD. Mannose-binding lectin (MBL) is an important component of innate immunity in mammals, extensively studied (Turner, 2003; Gadjeva et al., 2004). This lectin has affinity for mannose, fucose and *N*-acetyl glucosamine (GlcNAc); MBL is calcium-dependent to sugars that have hydroxyl groups on carbon-3 and carbon-4 orientated in the equatorial plane of the pyranose ring (Turner, 1996). MBL is an acute-phase protein produced by hepatocytes and increases in response to an infection or inflammatory response. The protein acts directly as an opsonin promoting phagocytosis of foreign material to which it has bound triggering the lectin pathway of complement activation via MBL associated serine proteases (Arnold et al., 2006; Ourth et al., 2008). MBL, identified in several fish species, may play important immune functions. Channel catfish *Ictalurus punctatus* showed up-expression with Gram-negative bacterium infection (Zhang et al., 2012), African catfish *Clarias gariepinus* with antimicrobial activity (Argayosa et al., 2011) and tilapia fish *Oreochromis niloticus* inducing cytokine production (Silva et al., 2012).

Pentraxins are composed of multiple subunits with size varying between 20–25 kDa and one CRD per subunit. These lectins show Ca^{2+}-dependent binding to saccharides on bacterial cell surfaces and exist in serum as acute phase proteins indicating their role in defense system (Magnadóttir et al., 2010). They may also be membrane associated, CRP, with preference for phosphorylcholine; or exist as a component of the extracellular matrix, SAP, with preference to phosphoethanolamine (Kilpatrick, 2002). Pentraxins showed opsonin activity in snapper *Pagrus auratus* displaying a functional role in the host defense fish (Cook et al., 2005); they were also detected in serum of pangasius *Pangasianodon hypophthalmus* (Huong-Giang et al., 2010) and Atlantic cod, *Gadus morhua* (Gisladottir et al., 2009).

Calnexin and calreticulin are related proteins that represent a group of intracellular lectins, proteins of the endoplasmic reticulum that interacts transiently with glycoproteins and might participate in the folding mechanism but more probably acts to retain misfolded proteins in the endoplasmic reticulum. They possess a lectin site that recognizes an early oligosaccharide processing intermediate on the folding glycoprotein, Glc1Man9GlcNAc2 (Williams, 2006). Calnexin and calreticulin have a high-affinity Ca^{2+}-binding site and bind Zn^{2+} at sites within the globular domain; both bind ATP, although no ATPase activity has been detected (Leach et al., 2002). They have been identified in mammals, plants, fish salmonids as rainbow trout, *O. mykiss*, cyprinids (Kales et al., 2004; Kales et al., 2007; Bielek, 2008). Calreticulin in mammals has numerous physiological and immunological functions in the eukaryotic cell such as

regulation of intracellular calcium homoeostasis, lectin binding and oxidative stress responses (Michalak et al., 1999; Kales et al., 2004). The calreticulin gene is little studied in teleost fishes but has been characterized in channel catfish *I. punctatus*; *e*ven with 72% identity with mammalian calnexins some characteristics indicate that assembly of class II molecules MHC in the catfish probably proceeds via different steps than occur in mammals (Fuller et al., 2004).

Lectins with structural similarity to the immunoglobulin superfamily were called I-type lectin; they constitute a category of lectins that mediates cell-cell interactions through the recognition of specific sialylated glycoconjugates (Powel and Varki, 1995). The sialic acid-binding immunoglobulin superfamily lectins (Siglecs) are a structurally distinct subclass of I-type lectins. They are integral membrane proteins, preferentially expressed on the plasma membrane (Angata and Brinkman-Van der Linden, 2002). Structurally different than the Siglecs, but also apparently recognizing sialic acids are CD83 (Scholler et al., 2001) and cell adhesion molecule L1 (Kleene et al., 2001). A genomic sequence of a Siglec-4 was identified in two fishes Fugu, *T. rubripes* and zebrafish, *D. rerio* (Lehmann et al., 2004). Several lines of evidence suggest important roles of Siglec-4 in the maintenance of myelin integrity and the regulation of neuronal growth (Spencer et al., 2003).

F-type is a lectin group specific for α L-fucose, Ca^{2+}-independent and non-glycosylated. This protein category was named fucolectin by Honda et al., (2000). The ell fucolectin shows a structure unique among the known lectins. The northern blot analysis revealed the presence of seven types of clones, three of which from the liver, and coding for similar but distinct proteins with 180 amino acid residues (Honda et al., 2000). Fucolectins have been identified as immunorecognition molecules in invertebrates and vertebrates such as pearl oyster, *Pinctada martensii* (Chen et al., 2011), rock bream, *Oplegnathus fasciatus* (Park et al., 2012) and gilt head bream *S. aurata* (Cammarata et al., 2012). Tandem-repeated types of F-type lectins are found in modern teleosts such as Nile tilapia (*O. niloticus)*, Japanese sea perch (*Lateolabrax japonicus*) and striped bass (*Morone saxatilis*) (Argayosa and Lee, 2009; Bianchet et al., 2010; Qiu et al., 2011). F-type lectin CRD motifs are absent in genomes of higher vertebrates such as reptiles, birds, and mammals (Ogawa et al., 2011).

The group of lectin binding to L-rhamnose (RBL) was proposed based on their binding specificity characteristic of the carbohydrates and molecular structure, which consists of two or three homologous CRD in tandem of about 95 at 100 amino acid residues with characteristic topology and a series of conserved motifs (Tateno et al., 2002; Terada et al., 2007). The RBL are classified into five subgroups based on the domain architecture, hemagglutinating activity for human erythrocytes and carbohydrate specificity (Nitta et al., 2007). These proteins have been identified in various types of fish fat-east dace, *Tribolodon brandti* (Jimbo et al., 2007), spanish mackerel, *Scomberomorus niphonius* (Terada et al., 2007) and sweet fish (ayu), *Plecoglossus altivelis* (Watanabe et al., 2008) interacting with various types of bacteria, such as *Staphylococcus epidermidis*, *E. coli* and *Pseudomonas aeruginosa* (Franchi et al., 2011). It is also suggested to be involved in inflammatory reactions by functioning as agents of recognition and trafficking of cells to sites of inflammation as well as activate the inflammatory cascade by regulating the expression of cytokines (Watanabe et al., 2009).

4. Purification and Characterization of Fish Lectins

Isolation and characterization of lectins are of paramount importance for elucidation of the basic properties and biological functions of these proteins. A considerable number of lectins from fish has been isolated from serum (Carvalho et al., 2012; Silva et. al., 2012), plasma (Mitra and Das, 2002), mucus (Tsutsui et al., 2011), gill (Pan et al., 2010) and eggs (Shiina et al., 2002; Ngai and Ng, 2007).

Serum samples containing lectins can be subjected to partial purification by methods such as fractionation dependent on pH (Suzuki et al., 1979) or salt, especially ammonium sulfate, followed by exhaustive dialysis (Sage and Green, 1972). Currently, almost all lectins are purified by affinity chromatography (Correia and Coelho, 1995; Coelho and Silva, 2000; Santos et al., 2009; Leite et al., 2012; Nunes et al., 2011; Souza et al., 2011), a technique that relies on the ability of lectins to bind carbohydrates specifically and reversibly (Lis and Sharon, 1981). Often other chromatographic methods are needed to obtain a lectin with high purity such as ion exchange chromatography (Bah et al., 2011; Dutta et al., 2005) and molecular exclusion (Silva et al., 2009).

Lectins have the ability to agglutinate cells such as erythrocytes (hemagglutination), lymphocytes and bacteria, being also able to precipitate glycoconjugates (Correia and Coelho, 1995; Silva et al., 2011). Thus a useful assay for identifying a lectin in a given sample such as serum or tissue homogenate is performed using a hemaglutination assay with human or other animal erythrocytes (Figure 2a and 2b). To be considered a lectin, the hemagglutination activity should be inhibited (Figure 2c) when added to the assay environment a mono or oligosaccharide (Coelho et al., 2012).

The gene evolution or expression of fish lectins has been investigated to elucidate their origin and their occurrence in different tissues (Shirai et al., 2009; Mistry et al., 2001). In certain experiments, the fish are defied against pathogens and observed in vivo, and subsequently, leads to gene expression analysis comparing healthy and infected animals under experimental conditions (Jorgensen et al., 2008).

Our group has been working with lectins besides of protease inhibitors of exotic and Brazilian autochthonous fish. The first protease inhibitor of tilapia (*O. niloticus*) was identified and showed antibacterial activity (Leite et al., 2012).

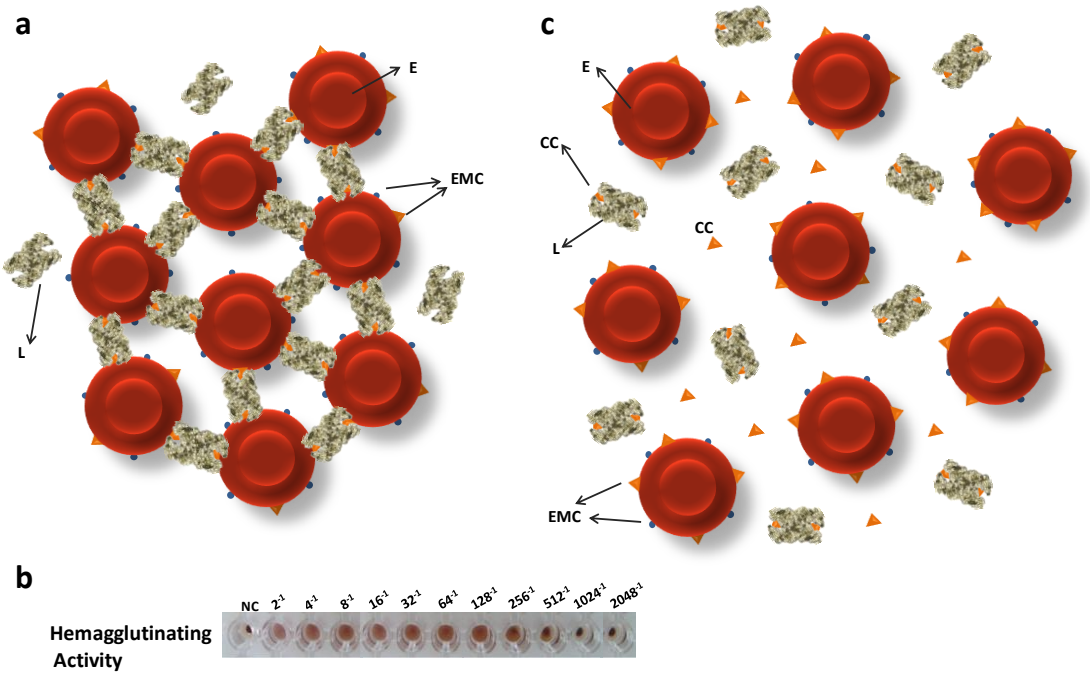

Figure 2. Hemagglutinating and inhibition of hemagglutinating activity assay (HA). The presence of lectin is revealed by the formation of a hemagglutination net due to lectin binding to erythrocyte surface carbohydrate (a). HA is performed in 96-wells microtiter plates. Lectin preparations (50 µL) are serially two-fold diluted and an equal volume of erythrocyte (2.5%) suspension is added to each well. Plates are incubated at room temperature for 40 min. Activity corresponds to the last dilution in which hemagglutination is visualized; in this case 512^{-1} (b). HA inhibition is revealed when lectin sample is incubated with carbohydrate prior to erythrocytes. Specific carbohydrate binding to lectin abolishes net formation (c). E – Erythrocyte, EMC - erythrocyte membrane carbohydrate, L – lectin, CC – competitor carbohydrate, NC – negative control.

A mannose recognizing lectin from *O. niloticus* serum was purified and partially characterized. This lectin (OniL) did not show cytotoxicity against splenocytes and induced higher IFN-γ production. OniL is a potencial immunomodulator which has preferentially Th1-type immune response (Silva et al., 2012). Another lectin was identified from serum of cobia (*Rachycentron canadum*) with specificity for methyl-α-D-mannopyranoside (Coriolano and Coelho, 2012). Lectin was identified in the serum of the Amazonian fish tambaqui (*Colossoma macropomum*). This lectin (ComaSeL) showed antimicrobial activity against pathogenic bacteria to freshwater fish. ComaSeL is seasonal and its serum concentration in cold periods is extremely low; high mortality of these fish for fungal and bacterial infections occurs in cold seasons (Carvalho et al., 2012). The temperature is the main environmental factor that stimulates changes in the immune response of many species of fish, affecting both innate and acquired immunity. The effects of seasonality on the immunological and hematological parameters of the Amazonian fish pirarucu (*Arapaima gigas*) are under investigation.

Perspectives

The fish innate immune system includes lectin(s) which could increase innate immunity efficiency. Pathogens are present in aquatic environments; the generation of fish with enhanced disease resistance would be of great value to the industry to increase productivity and therefore economic gains.

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CAPÍTULO IV

PIRARUCU, *Arapaima gigas*, THE AMAZONIAN GIANT FISH IS BRIEFLY REVIEWED

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PIRARUCU, *Arapaima gigas*, THE AMAZONIAN GIANT FISH IS BRIEFLY REVIEWED

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CONTENTS

PREFACE

Pirarucu, *Arapaima gigas*, is the largest freshwater fish with scales in the world, so called "giant of the Amazon River". It is a highly rustic fish of primitive origin, which can reach 200 kg body weight and approximately 3 m length in natural environment. The fish has a high quality of meat without thorns and with low fat, in addition to great flavor; the leather represents 10% of the weight being used as feedstock in the footwear and clothing industry. Pirarucu, native from the Amazon hydrographic basin, has strategies to adapt to daily and annual variations in water level and to distinct environments of the floodplain. Genetic variability studies have been conducted with the purpose to approach pirarucu conservation. *A. gigas* is considered an air-breathing mandatory fish and shows peculiar characteristics regarding reproduction. Most studies involving this fish are concentrated on nutrition and reproduction in an attempt to further improve its meat yield as well as to understand the species metabolism. Physiological responses to acute and chronic stress have enhanced the feature of a robust fish. A greater stress loads promotes more susceptibility to disease emergence in pirarucu semi-intensive and intensive creation systems. The survey of several parasite species in different pirarucu tissues has given information to prevention of infectious diseases. Great advances have been achieved in *A. gigas* genetics such as the complete sequence of the mitochondrial genome; on the other hand there were no differences associated with sex chromosome by cytogenetics. Pirarucu studies promise discoveries since the animal metabolism has many questions to be answered. In this book we gathered knowledge about pirarucu biology as well as analysis that may contribute to increase interest and a better understanding of the specie.

Chapter 1

PIRARUCU AMAZONIAN FISH (*Arapaima gigas*) OVERVIEW

The pirarucu (*Arapaima gigas*, Shinz, 1822) is a noble fish described as highly rustic and originated in the Jurassic period. *A. gigas* is the largest scale fish of the world and has been known as the "Amazon giant" reaching 200 kg body weight and 3 m long in its natural environment. *A. gigas* is one of the most important species to development of intensive aquaculture in the Amazonian Region; it is a robust fish with excellent taste of meat, despite its carnivorous diet. This fish exhibits a high growth rate with extraordinary weight development resulting in high market value; in semi intensive creation system this fish may reach 10 kg body weight during the first year fattening (Ono et al. 2003).

The name pirarucu, as called in Brazil, comes from the Tupi linguistic phylum meaning red fish, due to the color of scales, tail and abdomen. The pirarucu has the advantage of being handled out of water; as an air-breathing fish the intense vascularization from *A. gigas* swimming bladder allows it to stay out of water for about thirty minutes, while handled. During mentioned period fish is constantly irrigated with water. Juvenile pirarucu (Figure 1) is the unique fish in the world which development in captivity can fatten up over 1 kg per month; generally this fish is ready for slaughter weighing between 14-15 kg (Fogaça et al. 2011).

Figure 1. Aspects of juvenile pirarucu developed in captivity (1.40 m length, 36 kg weight). Fish was taken from nursery to biometrics.

The creation of pirarucu in captivity constitutes an economically viable practice due to the high yield of fillet with skin, a product of great commercial value. The yield of animal leather represents 10% of fish weight; in the industry it can be used as feedstock for the production of bags, shoes, belts and clothing. Fish scales are used as sandpaper or handmade typical ornaments (Gandra et al. 2007). The intense commercial exploitation of fish in their natural environment induced environmental disturbances and *A. gigas* now compose the list of endangered animals; then creation of pirarucu in captivity for commercial exploitation have substantially reduced the pressure on stocks (Castello 2004). Thus, fish farming has become an alternative to increase pirarucu production in domestic market.

Efforts have been focused to understand biological aspects of pirarucu, among them dietary habits, reproductive pattern and behavior in natural and captivity environment, however, the fish metabolism still possesses several facets to be investigated. This book reports some information obtained from pirarucu over the years in order to increase fish interest which certainly could contribute to a better understanding of the species.

Chapter 2

TAXONOMIC CLASSIFICATION

Osteoglossiformes are one of the oldest teleosts living groups, which emerged in the Jurassic Period (165 million years ago) possibly in the supercontinent Pangea, covering 6 families, 29 genera and about 217 species. Current Osteoglossiformes are endemic in freshwater Tropical Regions, such as *Notopterus*, *Papyrocranus*, *Xenomystus*, *Clupsudis*, *Gymnarchus* and *Pantondon* (India and Africa), *Scleropages* (Indo-Australian Region), *Arapaima* and *Ostoglossum* (South America), as well as *Hidan* (North America) (Nelson 2006). Osteoglossiformes order comprises less than 1% of teleost living species; this order has a wide geographic distribution due to characteristics that lead this group to the basal position in general fish classification (Lavoué and Sullivan 2004).

The genera *Arapaima* and *Osteoglossum* occur in the Amazon Basin and Tocantins-Araguaia from Amazon Region. Nelson (2006) described the genus *Arapaima* among the 4 genera of the family *Osteoglossidae* (Figure 2). *A. gigas* is considered an important species due to its location in the base of phylogenetic tree from teleosts and represents a link between old teleosts and derived from teleost fish.

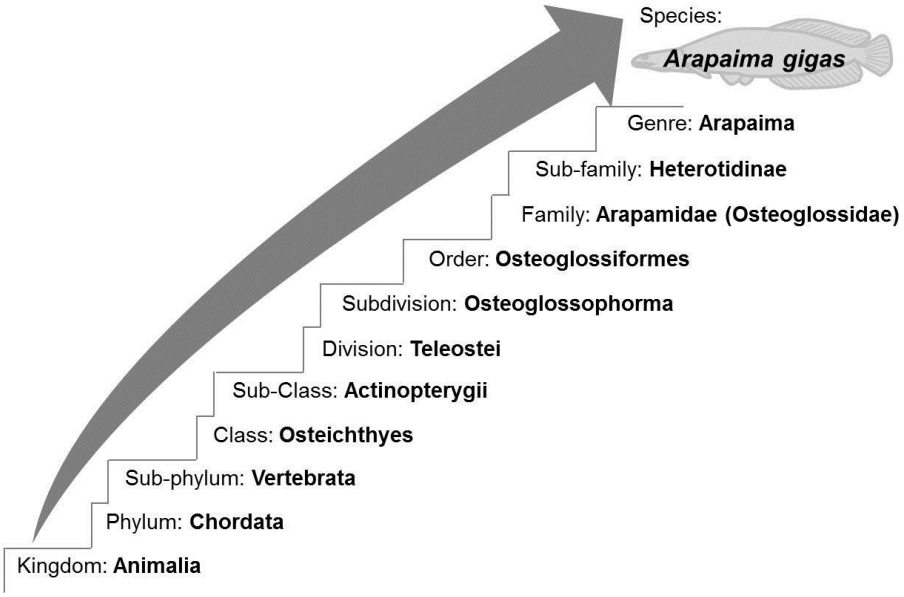

Figure 2. Taxonomic classification of *A. gigas*.

Chapter 3

HABITAT AND GENETIC VARIABILITY

Pirarucu is a natural habit fish from tropical freshwater of South America, with temperatures ranging among 14 and 31 °C; the fish can be found in Peru, Bolivia, Guyana and Brazil. It is a native fish from the Amazon hydrographic basin, preferentially found in lowland regions, where it undergoes the influence of the hydrological cycle of seasons from drought and flood periods (Goulding et al. 2003). *A. gigas* has strategies to adapt to daily and annual variations in water level and to various environments of floodplain, as well as other Amazonian species (Junk et al. 1997). *A. gigas* lives mainly in white water in the Amazonian floodplains, the lowland *Várzea* Region (Figure 3) including flooded forests, rivers, lakes, and coastal drainages, usually up to the first major rapids or waterfall on a river. The main physical factors of lateral migration of pirarucu seem to be the low depth and low current velocity (Castello 2008). During drought they are mostly found in lakes and in the main channel that traverses the floodplain system; some are also found in shallow areas of the river and lake pipes (Goulding et al, 2003).

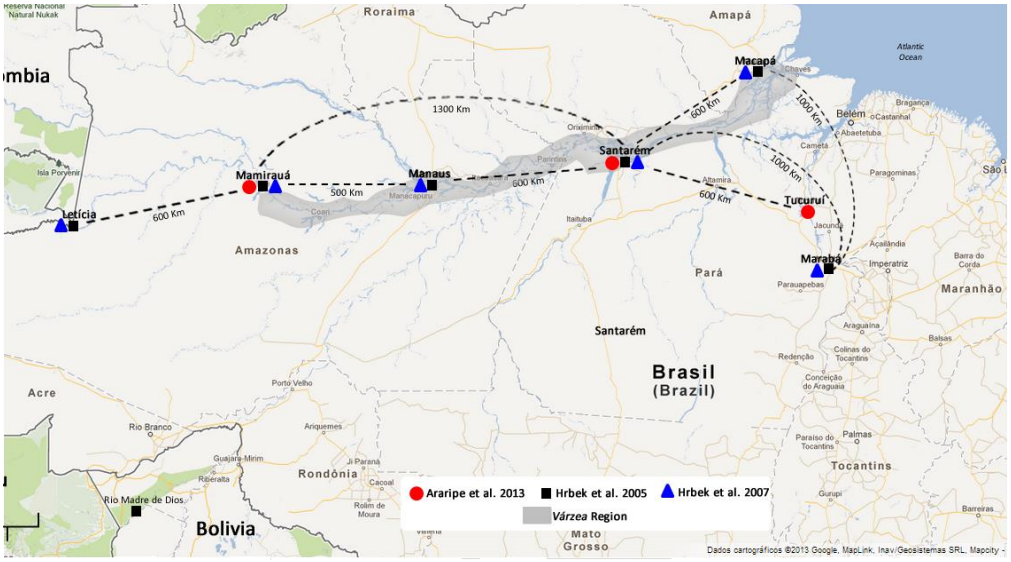

Figure 3. The Amazonian Region with localities sampled for three genetic variability studies. Dashed lines represent the approximate distances among locations. Grey area corresponds to *Várzea* Region with the highest exploitation of *A. gigas*. Adapted from 2013 Google MapLink, Inav/Geosistemas SRL, Mapcity.

The use of microsatellite DNA to determine levels of genetic variability of *A. gigas* populations from the Amazon Basin recorded a high level of fish intra-population variability, which means that the species has satisfactory genetic conditions to maintain itself in environment (Farias et al. 2003).

Development of a multiplex panel of eight microsatellites proved to be a rapid method capable of quantifying the genetic variability from pirarucu population (Hamoy et al. 2008). A pioneering study of genetic population from *A. gigas* mitochondrial DNA sequences of 139 individuals was used from localities spanning the Amazon basin from Iquitos, Peru to Macapá, Brazil (Figure 3). Higher haplotype diversity was observed in these areas far from major urban centers of the Amazon. No significant association was detected between geographical distance and genetic differentiation suggesting intense gene flow among pirarucu populations located throughout the Amazon basin (Hrbek et al. 2005). Analysis of hyper variable microsatellite markers using 14 microsatellite loci and 2347 bp of mtDNA from 126 individuals sampled in seven localities within the Amazon basin suggests that the *Arapaima* genus forms a continuous population with genetic exchange among localities. Yet the analysis of spatial autocorrelation of genetic data and geographic distribution suggest that genetic exchange is significantly restricted at distances greater than 2500 km (Hrbek et al. 2007).

Evaluation of microsatellite markers allowed the analysis of dispersal capacity of *A. gigas* on fine, meso, and large geographic scales evidencing an asymmetric gene flow. The fine scale analysis (distances of up to 25 km) indicated a marked genetic similarity among lakes, with low genetic differentiation, and significant differences between only a few pairs of sites. Low to moderate genetic differentiation was observed between pairs of sites on a meso scale (100 km), which could be explained by the

distances between sites. The distribution of pirarucus over short distances indicates a process of lateral migration within the *Várzea* floodplains, which may be the principal factor determining the considerable homogeneity observed among *Várzea* lakes. Finally, a high degree of genetic differentiation was recorded in the large scale analysis, that is, among stocks separated by distances of over 1300 km (Figure 3). The analysis indicated that differentiation was not related solely to distance, but, may be related primarily to historical bottlenecks in population size and the sedentary behavior of the species (Araripe et al 2013).

Chapter 4

MORPHOLOGICAL AND PHYSIOLOGICAL ASPECTS

The pirarucu has a sub cylindrical and extended body that is gradually flattened from the origin of the dorsal fin; the flat head with the space between the eyes is small relative to the body corresponding to approximately 10% of total weight. There are 58 bone plates of different sizes distributed on the surface of the head; each of these plates has 6-8 pores in its rear end. These pores, in males, secrete a substance of mucoid aspect. According to jungle natives and pisciculturists from the Amazonian Region it is popularly called “milk” that nourishes small fishes which swim in shoals near the head of the male parent. However, there is no evidence about the constitution of pore secretion. Structure and composition of the ventral portion from the occiput skull region from *A. gigas* is unique among teleost fishes; comparative anatomical studies interpreted as containing only the basioccipital region or the basioccipital fused with one or two vertebral centers (Hilton et al. 2007).

Pirarucu neurohypophysis is well differentiated into the median eminence and the neural lobe; it is characterized by the presence of the paraphysis, saccus dorsalis, and velum transversum. The pineal is found and the paraphysis is relatively large and consists of many saccules (Tsuneki 1986). Adenohypophysis cells from pirarucu were identified by immunohistochemistry revealing some baseline characteristics of the ancient Actinopterygii and newer teleosts (Borella et al. 2009).

The fish upper mouth is large and oblique, with lower jaw prognathism. Dorsal fin originates on the posterior third of the body and its last rays longer pass of the caudal origin. The anal fin starts next two rows of scales behind the origin of the dorsal and the caudal fin is rounded; abdominal and ventral fins are much later. The body is coated by cycloid and granular large scales formed by thicker layers of collagen fibers; the collagen of pirarucu scales has unique properties which confer greater resistance performing, for example, the important role in protection against predators (Lin et al. 2011, Torres et al. 2008, 2012). The celiac-mesenteric artery of pirarucu is responsible for the flow of the digestive tract, as described by Santos et al. (2007).

Respiration

Pirarucu has the need to rise to the surface in an average interval of 15 min to capture the atmospheric air. About 90% of the oxygen they need is acquired by breathing air; this feature makes the pirarucu be considered an obligatory air-breathing fish. Transition from breathing in water to breathing air results in striking morphological alterations in gill physiology; in small fish (10 g), the gills are qualitatively similar in appearance to another closely related water-breathing fish, *Osteoglossum bicirrhosum*; however, as fish grows (100–1000 g), the inter-lamellar spaces become filled with cells, including mitochondria-rich (MR) cells, leaving only column-shaped filaments (Brauner et al. 2004). Changes also occur in the swim bladder allowing efficient gas exchanges (Figure 4). Numerous capillaries are found in the inner surface of swim bladder; these capillaries are separated from the air inside the body only by thin cytoplasmic epithelial extensions, lamina propria and endothelium slender (Brauner and Rombough 2012).

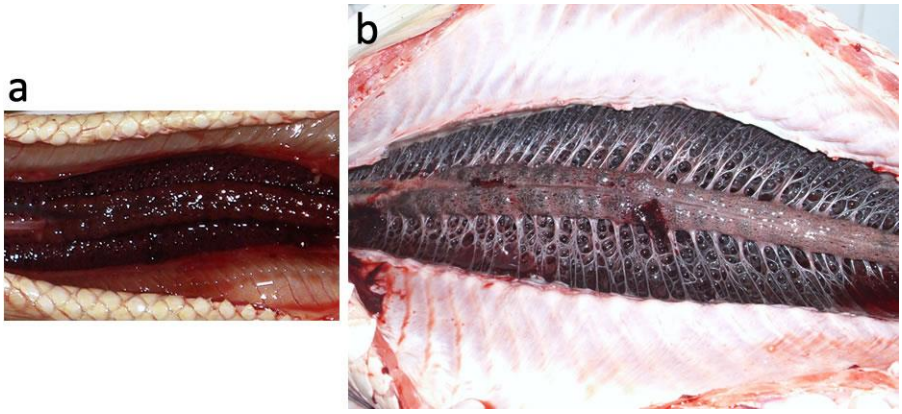

Figure 4. Aspects of *A. gigas* swim bladder. Post larval stage (21 cm, 52 g) at the beginning of the transition breathing in water to breathing air (a). Juvenil pirarucu (122 cm, 17 kg) adapted to air breathing; development of numerous capillaries in swim bladder inner surface may be observed (b).

There are variations in the mechanisms of oxygen transport in *A. gigas* gills; these variations were observed by Fernandes et al. (2012) for both gills and swim bladder. The area of respiratory average surface from gas bladder (2173 cm^2 kg^{-1}) is superior to the gills (780 cm^2 kg^{-1}) by a factor of 2.79, as well as the capability of diffusion of oxygen and carbon dioxide is 88 times higher in the swim bladder than in pirarucu gills. Therefore, the initial function of the gills is mainly iono-regulatory and only secondarily respiratory (Brauner and Rombough 2012). There are few studies related to the mechanisms of oxygen transport in the pirarucu blood stream, but it is known that inositol pentaphosphate (IPP) is the main modulator of hemoglobin-oxygen affinity in erythrocytes of this fish (Isaacks et al. 1977).

Food and Nutrition

Pirarucu showed a wide trophic plasticity in the initial phase of life; however, it seems to have food preference for aquatic invertebrates. It is a harmless fish despite of topping-sized, devoid of thorns and sharp teeth (Oliveira et al. 2005). Adult fish is essentially piscivorous capturing its prey by a strong suction; their prey is generally abundant and small-bodied. Jaw is provided with a row of few conical teeth with less than 2 mm in length. The bony tongue is very mobile and quite resistant, an anatomical peculiarity of the family Arapaimidae. Pirarucu still has bony plates on the palate and in the two other sides, with the role of real teeth that help compress the prey before swallowing it (Fontenele 1948). Pirarucu is a visual predator, that is, although it uses other senses, almost always the final decision on whether or not to attack a prey passes by visual analysis; this quality was developed probably because the fish needs precise attacks. Pirarucu is in the highest trophic level of the food chain; carnivorous fish are elements that increase the stability of the ecosystem, in measure that regulate the abundance of forage different species. Thus, fish eating habits reinforces an ecological importance, since predatory species participate in the environmental balance as regulators of other species populations. Despite being a carnivorous fish, there are no records of aggression or cannibalism between juveniles pirarucu bred in captivity (Cavero et al. 2003a).

Several studies have been conducted to improve nutrition and growth from pirarucu development in captivity (Fracalossi et al. 2001; Ituassú et al. 2005; Menezes et al. 2006; Andrade et al. 2007; Ono et al. 2008). Pirarucus respond successfully to training food for accepting pelleted diet from the earliest phases of development; study using pirarucu under different food schemes revealed that the fish have greater weight gain when fed a pelleted diet containing 40% crude protein twice daily (Gandra et al. 2007). Feeding schedule for farmed fish can also affect on weight gain; pirarucu diurnal feeding performs better feed conversion than nocturnal feeding (Crescêncio et al. 2005). Diets with high vitamin C levels contributed for the synthesis of plasma proteins, improving O2 carrying capacity and, probably, inducing more efficient immune response in pirarucu (Menezes et al. 2006).

Feed conversion (food consumption / biomass gain) of 1.12 was reported to pirarucu reared in cages of small volume for 200 days (Cavero et al. 2003b), while in earth pond it was obtained a feed conversion of 1.51 for 12 months (Pereira-Filho et al. 2003). *A. gigas*, like other teleosts, is unable to synthesize ascorbic acid due to lack of activity of the L-gulonolactone oxidase enzyme in kidney and liver tissues; this enzyme is responsible for the final step of the ascorbic acid synthesis (Fracalossi et al. 2001).

Fish stomach, in general, has a low capacity of storage, but with strong capacity of contraction, some may use sand and stones in order to triturate the food and facilitate their absorption. Recently, small stones were detected in juvenile pirarucu stomach developed in captivity (Figure 5).

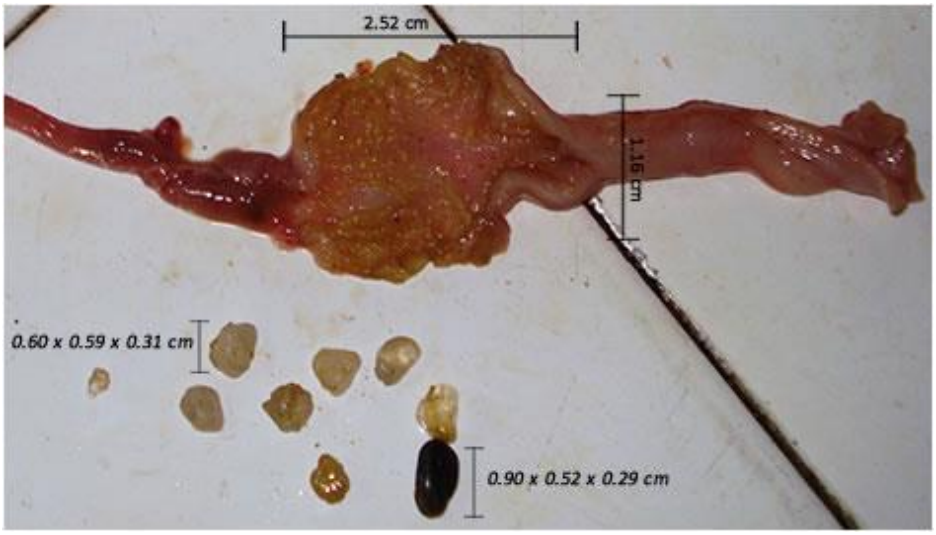

Figure 5. Presence of gastroliths in pirarucu (18.9 cm, 47 g, post-larvae) developed in captivity.

Stones ingested by certain animals are called gastroliths; these gastroliths serves as a stomach wall cleaner and lightens hunger sensitivity during long period of feed withdrawal. The gastroliths found inside the stomach of pirarucus are quite similar to stones from the pond bottom. Gastroliths presence has also been reported in ornamental fish (Yoon et al., 2004) and in primitive fish by palaeobiological studies (O'Gorman et al. 2013). This is the first information concerning to occurrence of gastroliths in pirarucu's stomach. Additional studies will be required to determine the composition and potential physiological variations related to depositions of stones in the mentioned organ.

Growth and Reproduction

Pirarucus are relatively long-lived fishes and of fast body growth. These fish grows to 70–100 cm in length and reach 10 kg weight in their first year of life; about 160 cm and 45 kg in 3–4 years (Ono et al. 2003). Total lengths of up to 285 cm have been confirmed. Female pirarucu are sexually mature at about 168 cm in total length; data indicate that *A. gigas* populations show great growth potential when juveniles and individuals engaged in reproduction are protected (Castello 2008). This is a lentic species, which do not perform reproductive migration and make their nest in low water movement place. This fish has peculiar habits, forming couples, selecting the spawning area, building nests and releasing eggs and sperm. Electrophoretic patterns were used to analyze transferrin and esterase gene loci in the same pirarucu population; detected patterns did not vary, as an indicative of monogamy (Teixeira, 2008).

Pirarucu life cycle can be divided into four different periods (Coutinho et al. 2010) summarized in Figure 6.

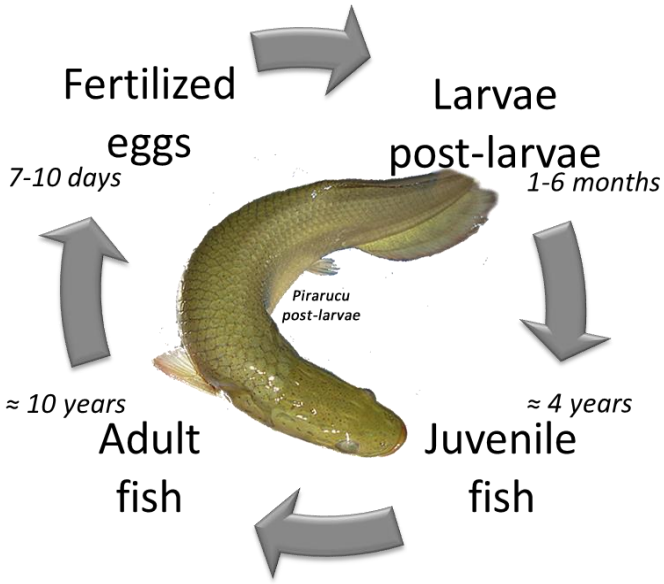

Figure 6. Four periods of *A. gigas* life cycle according to biological characteristics.

The first period of pirarucu life cicle constitutes the fertilized eggs after spawning. Lack of interest in food has been registered as a precursor signal of reproduction and fights were reported as a process of isolating the posture area (Monteiro et al. 2010); reproductive behavior of pirarucu is also characterized by demarcation of the territory by the male through blows on the water with its tail (Franco-Rojas and Peláez-Rodríguez 2007). The fish inhabits lakes during the dry season, where adults develop gonads, perform the cortege, build nests and intersect; during the rainy season, water previously concentrated on rivers, lakes and canals, runneth over, forming flooded areas (so-called *Várzeas*), the favorite environment to this species. Pirarucu produces nests with about 47 cm in diameter and 15 to 20 cm deep on hard ground, with no vegetation and no organic material (Franco-Rojas and Peláez-Rodríguez 2007). The choice of the partner is a female initiative; mating is determined by male's color intensity which is somehow associated with the capacity for nest building and the ability to protect newborns (Queiroz and Sardinha, 1999). Non-copulatory spawning is observed among this species (Coutinho et al. 2010).

A.gigas present only one functional gonad, the left; ovary has foliaceus format, absence of ovarian capsule on the ventral portion and absence of oviduct. The coelomic cavity is funnel-shaped, characteristics that indicate that the oocytes are released into the coelomic cavity before being poured into the aquatic environment through a genital papilla. Testicle is similar to a cable with a maximum diameter of 1-1.5 cm connected at genital papilla through a spermatic duct (Godinho et al. 2005). The eggs generally are larger and in fewer quantities than in rheophilic species. Performs spawning in installments and has low fertility, which is matched by the parental care given to offspring. Approximately after seven to ten days fertilized eggs hatch into larvae. Larvae and post-larvae, second period of pirarucu life cicle, remain under male protection till they become young fishes strong enough to protect themselves from external threatens (Fontenele, 1948).

The males do guard the nest, and after the eggs hatch, remains near larvae, defending them to avoid predation by other fish. *A. gigas* seemingly migrates to lowland forests where males perform parental care which extends for three months, during which time the fingerling becomes independent from its parent. The dependence of pirarucu fingerlings from the parent decreases already in the third week (Franco-Rojas and Peláez-Rodríguez 2007). The pirarucu juvenile phase corresponds to third life cycle period characterized by total length with less than 165 cm and sexual immaturity (Figure 6).

Sexual dimorphism recognition in fish is difficult; in most cases it is only possible in the period before reproduction. With pirarucu there is no difference; fish exhibits extragenital secondary sexual characteristics that differentiate them just in a few days before and after spawning. The feature that most attracts attention during the reproductive period of these fish is the color change in certain regions of the body; pirarucu reaches maturity around five years old corresponding to fourth life cycle period (Figure 6) (Queiroz and Sardinha, 1999). Male acquires sharp dark color on top of the head, extending to dorsal region until nearly the enrollment of dorsal fin; lower region of head has yellow spots, while the flanks, venter and caudal part acquires a red color (Figure 7a). In female, the color change is barely noticeable; the whole fish takes a light brown color (Figure 7b). This change occurs with greater emphasis in the rainy season, which coincides with the breeding period of this species, however, these characteristics are not always observed in all specimens (Monteiro et al. 2010).

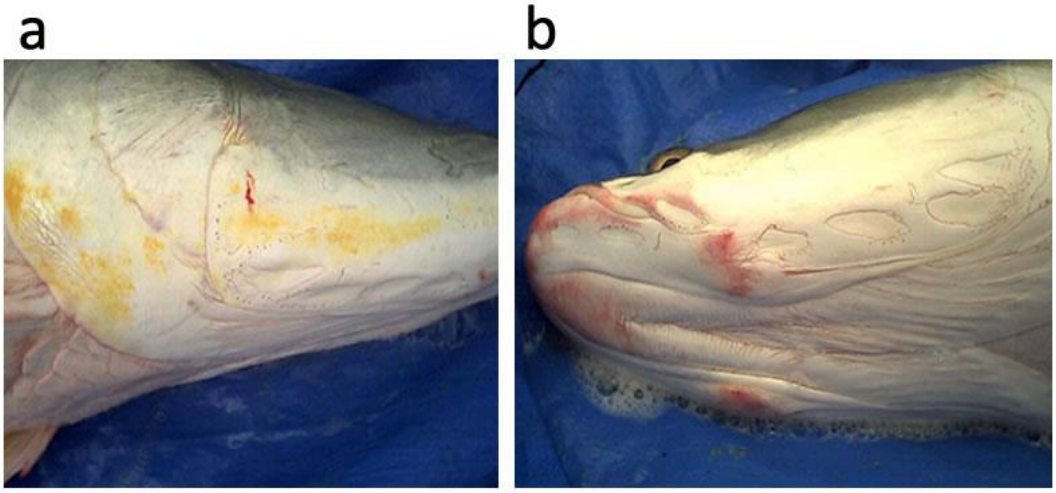

Figure 7. Secondary sexual characteristics of *A. gigas*. Male specimen of pirarucu yellow colored in head (a); female fish without yellow stain (b).

Sexual determination of adult pirarucu is possible through the use of enzymatic immunoassay for the detection of vitellogenin with 100% accuracy; the relationship between 11-ketotestosterona and estradiol levels can also be used with an accuracy of 95% (Chu-Koo et al. 2009). These techniques probably allow sex determination in fish even younger. Laparoscopic examination was used to differentiate between male and female pirarucu showing to be an efficient method for sex differentiation and causing

minimal stress to the fish. This method allowed observation and differentiation of fish reproductive organs (Carreiro et al. 2011).

Although it cannot yet be said definitively, the sexual maturity age of this species analyzing hormone profile in *A. gigas* reared in captivity concluded that females of 3-4 years have a greater physiological preparation for reproduction than females over the age of 4 (Monteiro et al. 2010).

Chapter 5

STRESS PHYSIOLOGICAL RESPONSES

Inadequate handling of fish in cropping systems, such as poor water quality and, great amount of ration giving provide stress which can compromise the adaptive capacity of the environment and even result in a temporary interruption of growth, a series of nutritional problems as well as increased susceptibility to infectious and parasitic diseases (Shoemaker et al. 2000; Goulding et at. 2003). *A. gigas* has shown to be a very resistant species to various environmental stressors and to acute stress resulting from common practices of cultivation system; however, the physiological responses of pirarucu subjected to stressors are different in their intensity and duration. The transport as a management practice in intensive aquaculture may cause stress levels in fish. Pirarucu submitted to transport for 96 h in plastic bags (closed system) showed no mortality. Some stress indicators such as cortisol and hematocrit unchanged; even dormant, after transport, there was an increase in glucose levels and decreased levels of lactate (Brandão et al. 2006). The use of commercial table salt (NaCl, 97%) during pirarucu juvenile transportation should be avoided since there is no reduction in the magnitude and intensity on stress responses causing osmoregulatory disturbances (Brandão et al. 2008; Gomes et al. 2006); however has mitigating effect of stress for some fish like tambaqui and matrinxã (Carneiro and Urbinati 2001; Gomes et al. 2003).

Stocking density can be considered as one of the most acute, severe, and aggressive steps of management in pisciculture. The potential from *A. gigas* to cage culture is showed by high survival ranged between 100.0% and 94.7 ± 5.0% in cages, at 10 and 12.5 fish/m^3, respectively. Density significantly affected ($P < 0.05$) final mean weight (2630.4 ± 213.7 and 2138.0 ± 148.2 g) and weight gain (2516.9 ± 202.0 and 2043.1 ± 142.9 g). In contrast, specific growth rate (2.25 ± 0.09 and 2.22 ± 0.06% / day), feed conversion ratio (1.2 ± 0.1 and 1.2 ± 0.2) and production (26.3 ± 2.1 and 25.4 ± 2.6 kg/m3) were not significantly ($P > 0.05$) affected by stocking density (Oliveira et al. 2012). The responses of cortisol, glucose, lactate and hematocrit of pirarucu subjected to densification were more acute than those of transport (Brandão et al. 2006). High concentrations of ammonia can cause fish growth retardation and facilitate the emergence of diseases. It was reported that in captive conditions pirarucu showed tolerance to increased ammonia concentration, till 25 mg / l of total ammonia, obtaining 100% survival (Cavero et al. 2004). Pirarucu subjected to concentration of 20 mg / l of

ammonia showed no variation in levels of cortisol, but there was a significant increase in glucose concentrations after exposure to ammonia and reduction of lactate, with the notable decrease of swimming rhythm; already for hematocrit no change was observed (Brandão et al. 2006).

Evaluation of genotoxic and mutagenic effect of ultraviolet radiation on tambaqui specimens and young pirarucu showed that both UVA and UVB induced genotoxicity oxidative damage in both species. However, tambaqui showed higher sensitivity to ultraviolet radiation and higher DNA damage than that observed in pirarucu; this difference between the two species can be related to their different evolutionary characteristics. Tambaqui, an Actinopterigean more recent than pirarucu, may have poorer adaptive capacity to ultraviolet radiation than that primitive species (Groff et al. 2010). It has been suggested that pirarucu exhibits physiological stress responses to handling similar in magnitude to those previously documented for many teleostean fishes, including salmonids. Pirarucus subjected to air exposure for 75 min demonstrated a rapid physiological response (plasma cortisol, lactate and hematocrit increased after handling) accompanied of quick recovery to baseline levels. Glucose increased after handling and increase remained even after 24 h, suggesting that *A. gigas* did not use its glycogen stores in this situation (Gomes 2007).

Secondary indicators of stress, such as serum glucose, triglycerides, total cholesterol and fractions showed seasonal variation in pirarucu indicating that seasonal stress can trigger side effects in this fish, such as changes in the metabolism of glucose and lipids. On the other hand, the osmoregulation represents a highly effective mechanism in maintaining the physiological balance during fish chronic stress (Bezerra et al. 2013). The exposure of pirarucu to pH 3.5 water led to net ion loss; when it was transferred from pH 5.7 water to pH 7.0 or vice-versa induced only minor changes in net ion fluxes. Therefore, any osmoregulatory difficulties encountered by *A. gigas* during changes between these latter two waters can be easily overcome (Baldisserotto et al. 2008). This high osmorregulation capacity from pirarucu is closely related to their natural habitat in which it is exposed to extreme changes in water pH.

Chapter 6

OTHER *A. gigas* CONSIDERATIONS

Henneguya arapaima was described as a new species parasitic of *A. gigas* gill arch and gallbladder (Feijó et al. 2008). Five other species were also found in pirarucu, as following, *Dawestrema cycloancistrium* (Monogenea) on the gills; *Nilonema senticosum* and *Goezia spinulosa* (Nematoda) in the swim bladder and stomach, respectively; *Caballerotrema brasiliense* (Digenea) and *Polyacanthorhynchus rhopalorhynchus* (Acanthocephala) in the intestine (Santos et al. 2008). *A. gigas* in semi-intensive cultivation was parasitized by *D. cycloancistrioides, D. cycloancistrium, Trichodina sp., Ichthyobodo sp.* (Protozoa), *Camallanus tridentatus, Terranova serrata, Goezia spinulosa* (Nematoda) and *Argulidae* (Araújo et al. 2009). In cropping systems, where the fish is subjected to greater stress load, these parasites can develop into different organs and impair the health of the host.

HDL (high density lipoprotein) dominates the lipoprotein profile in some species of *Agnatha* and *Osteichthyes* (Babin and Vernier, 1989). It has been suggested a different profile of pirarucu lipoproteins for juveniles with the predominance of LDL (low density lipoprotein); this difference may be due to the origin of the most primitive pirarucu compared with other living teleost species (Bezerra et al. 2013). Studies of pirarucu were performed in semi-intensive farming in the Amazon demonstrating that some biochemical and hematological blood parameters of *A. gigas* are influenced by the age of the fish pointing to the need for additional studies (Tavares-Dias et al. 2007; Drumond et al. 2010).

A. gigas acetylcholinesterase in the presence of pesticides (the organophosphates: dichlorvos, diazinon, chlorpyrifos, temephos, tetraethyl pyrophosphate - TEPP and the carbamates: carbaryl and carbofuran) retained 81% of the activity after incubation at 50 °C for 30 min. Thus, it was suggested as a biomarker for organophosphorus and carbamate detection in routine environmental and food monitoring programs for pesticides (Assis et al. 2012). Trypsin purified from pirarucu pyloric caeca has characteristics that suggest potential applications in food and detergent industry (Freitas-Junior et al. 2012).

With respect to genetic studies, analysis by *in situ* hybridization using 18S rDNA probes revealed *A. gigas* karyotype, 2n=56 chromosomes, being 14 pairs meta-submetacentric and 14 subtelo-acrocentric. The presence of a sexual heterochromatin would be of great importance in sexing pirarucu by cytogenetics, however, no

differences were observed associated with sex chromosome (Marques et al. 2006; Rosa et al. 2009). A great advance in *A. gigas* genetics was to define the complete sequence of the mitochondrial genome which has 16433 bp, 2 rRNA genes, 22 tRNA genes and 13 protein-coding genes; from these genes, 12 are encoded in the heavy chain, while nad6 is encoded in the light chain. The control region sequences show common characteristics with other osteoglossiformes species (Hrbek and Farias 2008).

The production of EST libraries from pituitary *A. gigas* in natural environment allowed the sequencing of 3857 clones. One hundred thirty nine genes found were differentially expressed among the four libraries analyzed; 8 of these genes were directly related to reproduction and growth of pirarucu by acting as hormone receptors in target tissues, with role in regulation of individual homeostasis and connecting to various neurotransmitter hormones. The differences observed by the ontology of found genes allow us to affirm that gene expression in *A. gigas* occurs in different forms among adults and young's, but with very similar forms between males and females (Lima et al. 2012).

There are some *A. gigas* biological aspects studied, however several gaps remain to be filled till the knowledge of its complex biology be unraveled; it is a primitive species of great importance in the current aquaculture scenario besides still being in danger of extinction. Among the hiatus to be fulfilled are the reproductive aspects of this fish; there are still several obstacles to breeding the fish in captivity. Also, the biochemical and molecular aspects of pirarucu immune response has been shown to be an obscure field, since until now only few studies have been conducted on the subject.

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CAPÍTULO V

Secondary Indicators of Seasonal Stress in the Amazonian Pirarucu Fish (*Arapaima gigas*)

Rosiely F. Bezerra, Maria do Carmo F. Soares, Athiê J. G. Santos, Elba V. M. Maciel Carvalho, Luana C. B. B. Coelho

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Chapter 11

Secondary Indicators of Seasonal Stress in the Amazonian Pirarucu Fish (*Arapaima gigas*)

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Abstract

The pirarucu (*Arapaima gigas*), native to the Amazon River Basin, is the largest fish with scales in the world possessing characteristics that make it extremely important for fish farming such as: great hardiness, high market value, excellent taste of meat and extraordinary weight development This fish may reach 10 kg body weight during the first year fattening in semi-intensive creation system. Adult pirarucu is the unique fish in the world which bred in captivity can fatten up over 1 kg per month; generally this fish is ready for slaughter weighing between 14-15 kg. *A. gigas* can reach 200 kg and 3 m length in his natural environment. The exposure of fish to different environmental stressors, such as seasonal events, has been the main cause of lost profits in fish farming affecting the balance that endanger the health of animals. Seasonal cycles can affect various fish biological activities, such as behavior, nutrition, metabolism, immunity and reproduction.

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This study aimed to evaluate the effect of seasonal temperature variation on secondary indicators of stress in pirarucu under captivity. The work was conducted in the State of Pernambuco, Northeastern Brazil in two seasons, summer and winter, with average temperatures of 32 °C and 24 °C, respectively. Juvenile pirarucu fishes developed in semi-intensive creation system were used. The values of some biochemical parameters were estimated as secondary indicators of stress such as glucose serum levels, measured by enzymatic photometric method, triglycerides, total cholesterol and fractions (high density lipoproteins-HDL, low-density lipoprotein-LDL and very low density lipoproteins-VLDL) performed by enzymatic colorimetric method. Osmorregulation parameters, electrolytes and plasma osmolality obtained by means of ion selective electrodes were also evaluated.

The parameters, with the exception of osmorregulation, showed seasonal differences in their levels. Glucose was higher in winter; triglyceride levels were about 4 times lower in winter than in summer. Total cholesterol, HDL and LDL levels were higher in winter but VLDL showed a different standard variation of serum lipoproteins with higher levels in summer.

The results suggest that changes in metabolic parameters are extremely important for the maintenance of pirarucu homeostasis in chronic stress. The study of stress physiology in pirarucu will contribute to the biological knowledge of the species and also may be useful in the improvement of techniques to increase the success of cultivation and productivity in fish farming.

Keywords: Seasonality; Stress; Pirarucu; *Arapaima gigas*

1. Introduction

Stress can be defined as a condition in which the dynamic equilibrium or homeostasis of a given organism is disturbed or influenced by an internal or external stimulus, called stressor (Pickering 1981).

The stress is always present in fish farming as a result of management which the animals are subjected, and also to the imbalance of the environment. In any case, the stress response can be considered as organic balance disorders that endangers the health of animals (Wedemeyer, 1996).

The exposure of fish to different environmental stressors, such as temperature, photoperiod, rainfall and other, with the possibility of different interactions can lead to an increase in susceptibility to infectious agents (viruses, bacteria and fungi) and parasites (protozoa, monogenes, nematodes and crustaceans) (Tavares-Dias et al., 2007; Araújo et al., 2009). Seasonal events, in addition to these stressors, especially the temperature factor, are capable of triggering secondary responses to stress with changes in metabolism and osmorregulation (Herrero et al., 2005). The stressful situation in fish farming is constantly present and may affect the adaptive capacity of fish, negatively affecting the productive performance, impairing the health state, increasing susceptibility to diseases and even may result in a temporary interruption of growth (Araújo et al., 2009).

The pirarucu, *Arapaima gigas*, Shinz 1822 (Figure 1), native from the Amazon basin, have characteristics which makes it particularly attractive for fish farming. In the first year of fattening can reach 10 kg body weight and in its natural environment can reach 200 kg and 3 m length; adult pirarucu is the single fish developed in captivity in the world that fattening up over 1 kg per month (Fogaça et al., 2011).

Figure 1. Juvenil pirarucu in Prof. Johei Koike Continental Aquaculture Station, *Departamento de Pesca da Universidade Federal Rural de Pernambuco* (UFRPE).

The pirarucu enchants by the size, flavor of their meat and fillet yield, besides being appreciated by the leather industry and, scales to crafts. This fish has become the target of fishermen, due to the occurrence in lakes and isolated environments, which enabled an intense disorganized exploration. Thus, *A. gigas* began to compose the list of endangered species; the creation of the fish in captivity for commercial exploitation has substantially reduced the pressure on natural stocks, being therefore, an alternative to reducing the risk of extinction (Castello, 2004). Pirarucu, in the system of cultivation, is subjected to transport, handling, high stocking densities, poor water quality and high level of feeding (Arantes et al., 2011).

The physiological information under stress caused by environmental factors may be useful in developing new and better techniques to increase the success of cultivation thus improving economic fish production.

A. gigas is a rustic fish with origins in the Jurassic age. It is the largest freshwater fish with scales in the world being also known as the "Amazon Giant" (Nelson 1994). *A. gigas* constitutes a true document of the evolution of life and is the only osteoglossiform fish living representative of the remote Arapaimidae family, a relative of *Scleropages sp.* from Asia and Oceania, and *Heterotis sp.* which occurs in Africa (Nelson, 1994). The pirarucu is a noble fish, with a high quality meat devoid of spines, with low fat and good taste. The fish scales are used as nail file or handmade typical ornaments; the leather which represents 10% of the weight, can be used in the industry for production of shoes, handbags, belts and clothing contributing significantly to the increase in cultivation of this species (Gandra et al., 2007). *A. gigas* has the need to rise to the surface in average interval of 15 min to capture the atmospheric air. The aerial respiration of this fish is possible due to a change in the swimming bladder. Although *A. gigas* is considered an obligatory air-breathing fish, 10% of its breath still depends of oxygen dissolved in water (Brauner et al., 2004). Fish essentially piscivorous, the pirarucu capture their prey by a powerful suction. Pirarucu, in captivity, can receive training for food rations of high protein content with good conversion levels (Menezes et al., 2006; Andrade et al., 2007, Ono et al., 2008). *A. gigas* is a lentic specie with peculiar habits, forming pairs, selecting the spawning area, building the nest and releasing eggs and sperm (Imbiriba, 2001). Pirarucu have extragenital secondary sexual characteristic that differentiate them only a few days before and after spawning; the feature that stands out during the reproductive season of these fish is the red coloration of the posterior edge of the scales in certain regions of the body (Monteiro et al., 2010).

2. Materials and Methods

2.1. Obtaining Fish

Youth pirarucus were kindly provided by Prof. Johei Koike Continental Aquaculture Station, Departamento de Engenharia de Pesca da Universidade Federal Rural de Pernambuco (UFRPE), Recife City, State of Pernambuco, and Northeastern Brazil. The fishes were kept under the same diet throughout the experiment and fed with a diet containing 40% crude protein. The blood was withdrawn in winter and summer.

2.2. Serum and Plasma Collection

Blood was collected from caudal vein (Figure 2) using 5 mL syringes and a 21G, 23G or 25G needle (BD Precision Glide®, PN, Brazil), depending on fish size. To obtain serum tubes without anticoagulant were used; after the formation of clot, the blood was centrifuged at 3000 x *g* for 10 min at 4 °C. To obtain plasma tubes contained sodium citrate 3.2% as an anticoagulant were used (Vacuette®, Greiner bio-one, Brazil). Samples of serum and plasma were transported on ice to be processed.

Figure 2. Blood collected from caudal vein of pirarucu (*A. gigas*).

2.3. Glucose and Triglyceride

Glucose serum levels were estimated by enzymatic photometric method using the Glucose kit Pap Liquiform (Labtest Diagnostica S/A, Brazil) following the manufacturer's instructions. The determination of serum triglycerides was performed by enzymatic colorimetric method utilizing the Triglycerides FS kit (DiaSys Diagnostic, Germany) following the manufacturer's instructions.

2.4. Total Cholesterol and Fractions

Total cholesterol, low density lipoprotein (LDL) and high density lipoproteins (HDL) were estimated from the serum by enzymatic colorimetric method using kits, Cholesterol FS (DiaSys Diagnostic, Germany), LDL-C Select FS (DiaSys Diagnostic, Germany) and HDL LE (Labtest Diagnostica S / A, Brazil) following the manufacturer's instructions. Very low density lipoproteins (VLDL) were obtained by the method of Lowenstein et al. (1984).

2.5. Osmorregulation

Plasma chloride, sodium and potassium were obtained by means of ion selective electrodes (Electrolyte Analyzer 9180 Roche Diagnostics, Brazil). Urea was estimated using Kit Urea CE (Labtest Diagnostica S/A, Brazil) following the manufacturer's instructions. Plasma osmolality was obtained by calculating the plasma osmolality:

$P_{osm} = ([(Na + 10) \times 2] + (Ur \times 0.16651) + (Glu \times 0.055)) \times 0.4$

where:

P_{osm} = Plasma osmolality, mOsm/l H_2O
Na = Sodium, mEq/l or mmol/l
Ur = Urea, mg/dl
Glu = Glucose, mg/dl

2.6. *Statistical Analysis*

Statistical significance of data between groups was determined with analysis of variance (ANOVA) and Tukey test using OriginPro 8.0 (OrginLab Corporation, USA). A value of $P < 0.05$ was considered significant.

3. Results

3.1. Glucose and Triglyceride

A significant difference ($P < 0.05$) was obtained in serum glucose between winter and summer.

The serum glucose levels in winter were 77.0 ± 1.8 mg/dl, while in summer was 57.6 ± 4.8 mg/dl. Triglyceride levels showed a more pronounced seasonal variation; in

the winter triglyceride levels were 61.3 ± 2.88 mg/dl reaching 257.2 ± 3.5 mg/dl in summer (Figure 3a).

3.2. Total Cholesterol and Fractions

The serum total cholesterol levels were significantly ($P < 0.05$) higher in winter than in summer, 497 ± 6.3 mg/dl and 387.3 ± 24.5 mg/dl, respectively. The HDL and LDL also had higher serum levels in winter (107.1 ± 8.2 mg/dl for HDL, 386.5 ± 2.9 mg/dl for LDL) than in summer (91.24 ± 5.6 mg/dl for HDL, 282.4 ± 25.8 mg/dl for LDL). However, VLDL showed a different variation standard of lipoproteins with serum levels higher in summer, 43.13 ± 5.11 mg/dl, than in winter, 13.3 ± 1.5 mg/dl (Figure 3b).

3.3. Osmorregulation

There was no significant difference ($P < 0.05$) in plasma levels of chloride ions (winter: 91 ± 1.7 mmol/l, summer: 95.3 ± 2 mmol/l), sodium (winter: 146 ± 4.1 mmol/l, summer: 155 ± 1.26 mmol/l) and potassium (winter: 10 ± 0.6 mmol/l, summer: 5.54 ± 0.38 mmol/l) between winter and summer. There was also no significant seasonal variation ($P < 0.05$) to the plasma osmolality (winter: 298.94 ± 03.02 mOsm/l H_2O, summer: 311.40 ± 2.7 mOsm/l H_2O), figure 3c.

4. Discussion

Fishes are sensitive to both chronic and acute environmental changes showing a classic response to stress (Barton and Iwama 1991; Wendelaar Bonga 1997). Generally, in fish, the stress response has three levels: primary, secondary and tertiary; the primary, secondary responses were introduced by Mazeaud et al. (1977). The release of catecholamines and cortisol in many target organs has the biochemical and physiological consequences known as a primary response to stress (Wendelaar Bonga 1997, Castro and Fernandes 2009). The secondary responses include metabolic effects as hyperglycemia, hyperlactatemia, and depletion of glycogen reserves as well as lipolysis and inhibition of protein synthesis (Milligan 2003; Martins da Rocha et al. 2004).

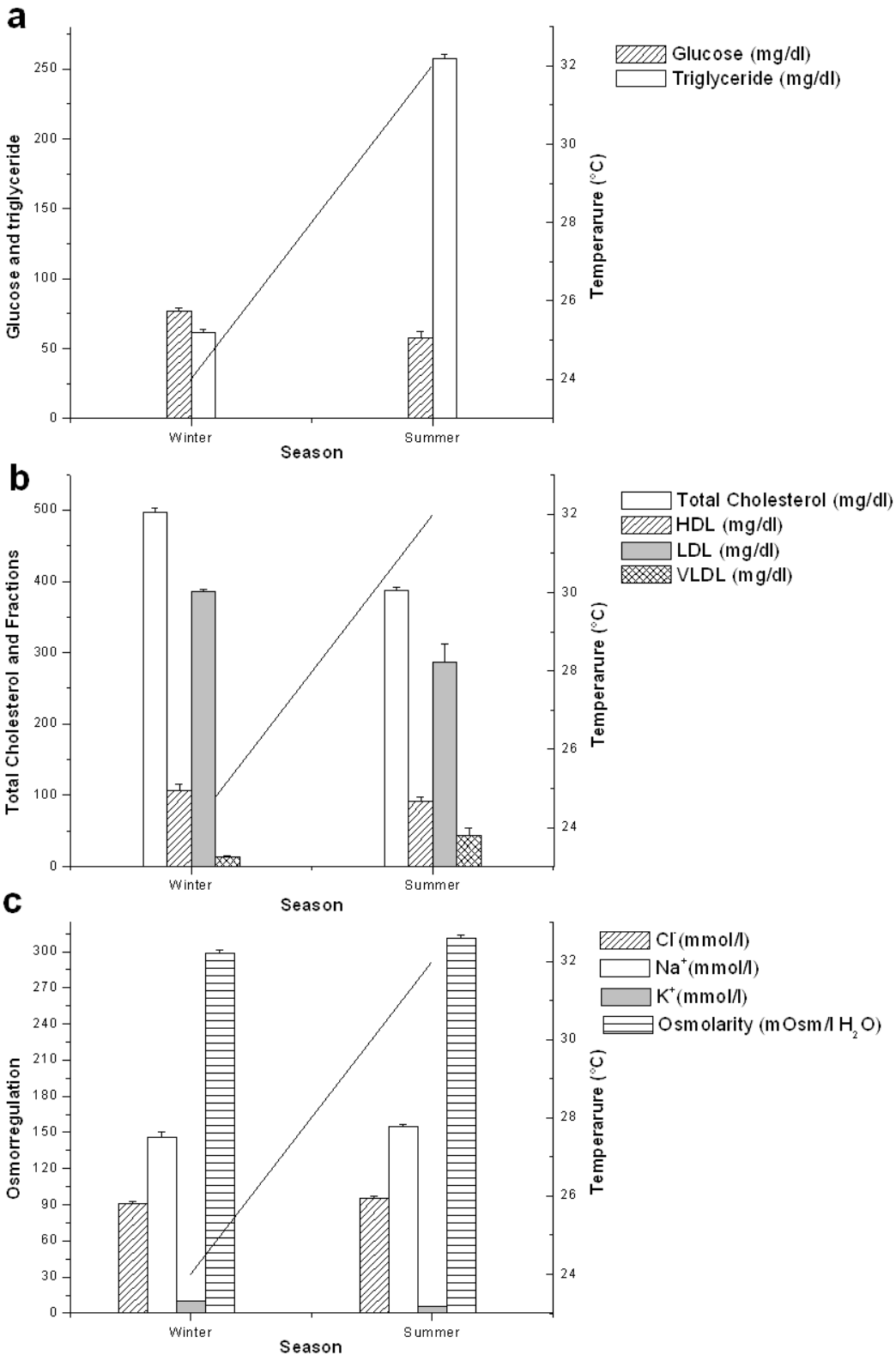

Figure 3. Variations in serum of *A. gigas* in winter (24.0° C) and summer (32.0° C) (n=5; mean ± s.e.), (*) significant (P<0.05) to glucose and triglyceride (**a**); total cholesterol as well as HDL, LDL and VLDL fractions (**b**); and osmorregulation with Cl^-, Na^+, K^+ determination and plasma osmolality (**c**).

The inclusion of a third level of stress related to disease or resistance, the tertiary effects, includes changes in behavior, reduced growth, reproductive capacity and increased susceptibility to disease (Qiu et al., 2009).

There is clear evidence that ectothermic vertebrates respond differently to temperatures of several year seasons (Ali 1992). The metabolic changes that occur in fish are direct responses of alterations in environmental conditions during the seasons. Similar to other vertebrates, fish produces an organized set of responses at different levels of organization, to deal with stressors, tolerate temperatures different of the ideal using a metabolic adjustment and behavioral thermoregulation. For example, when the temperature rises above the ideal, feed rate can be reduced and completely inhibited (Le Morvan et al., 1997; Person-Le et al., 2004). Stressors such as seasonal change in temperature can result in the necessity to modify the lipid composition of fish. Teleost fish are hyperlipidemic and hypercholesterolemic compared with mammals (Stoletov et al. 2009), which was also observed for *A. gigas* in this work. Similar to higher vertebrates, fish stores lipids, mostly as triacylglycerols (Stoletov et al. 2009).

The fish, in general, show variation in lipid content in their natural environments, according to the seasons. The metabolism of fish rises in summer; much of the body fat reserve is used to provide energy for the animal to reproduce and go in search of food. The animal becomes lighter, the storage of fat is limited to 2% of the dry weight, and growth accelerates, the fish becomes more active searching for food in order to accumulate reserves to be burned in winter (Ali 1992). This study revealed that serum levels of triglycerides in pirarucu were much higher in summer (257.2 mg/dl) than in winter (61.3 mg/dl), which may be due to increased lipolysis in the hot season. It is known that during the winter, the metabolism of fish becomes slower, lipogenesis is stimulated and the percentage of body fat increases, from 10 to 50% dry weight of the fish (Ali 1992).

The majority of plasma cholesterol is present in an esterified form in most species of fish (Babin and Vernier 1989). The concentration of serum total cholesterol is significantly related to fish mortality due to bacterial or viral infections; diseased fish have levels of total cholesterol concentrations below normal (Fukuda et al. 1997). Therefore, the total cholesterol is a good indicator of overall health status of fish (Yoneyama et al. 2009). The pirarucu showed lower serum cholesterol levels in summer; this seasonality may occur in response to increased levels of specific potential pathogens in the environment. The occurrence of pathogens is generally greater with increasing temperature, as in summer (Tavares-Dias et al. 2007). One possible explanation for serum total cholesterol to increase in the winter can be related to the endocrine system; cholesterol is the precursor of various steroid hormones, and in winter, the weight of gonads and the production of testosterone also increase in fish (Ali 1992). Thus, a greater mobilization of cholesterol would be required for the biosynthesis of steroid hormones.

Lipids are transported by lipoproteins in blood and their associated apoproteins with various combinations of lipids and proteins produce particles of different densities, ranging from chylomicrons, VLDL, LDL and HDL. In some classes of fish such as Agnatha and Osteichthyes HDL dominates the lipoprotein profile (Babin and Vernier, 1989), however, there are no comparative data with the Osteoglossiformes Order or with the family Arapamidae with the species *A. gigas*. The largest LDL lipoprotein fraction in both seasons obtained in the present study (386.5 ± 2.9 mg/dl in winter and 282.4 ± 3.8 mg/dl in summer) suggests a different lipoprotein profile in pirarucu compared to more recent species of teleosts in the phylogenetic scale.

Glucose is one of the most widely used secondary responses to quantify metabolic stress in fish (Li et al. 2010). It is believed that hyperglycemia reported in several species of teleosts, under stress, would be mediated primarily by the effect of catecholamine stimulation of glycogenolysis and mobilization of liver glycogen reserves (Wendelaar Bonga 1997; Castro and Fernandes 2009). However, corticosteroids may contribute to the maintenance of hyperglycemia by stimulating gluconeogenesis from amino acids (Pickering 1981; Vijayan et al. 1991; Wendelaar Bonga 1997; Castro and Fernandes 2009) and this may contribute to weight loss during chronic stress. The increase in the levels of glucose in response to stress is documented in several studies (Benfey and Biron 2000; Sadler et al. 2000; Carneiro et al. 2002; Urbinati et al. 2003) and evidence suggests that cortisol can help to keep high blood glucose levels after stress, although the mechanisms involved in this process are not yet clear (Wendelaar Bonga 1997). Pirarucus subjected to transport stress presented a pattern of hyperglycemia without changes in cortisol levels (Gomes et al. 2003, 2006). Pirarucus subjected to prolonged air exposure showed significantly increased glucose, but there was no change in glycogen levels suggesting that this fish does not use its glycogen stores in this situation (Gomes 2007).

A. gigas showed different responses when compared with some temperate species. The salmonids revealed common responses to a stressor such as release of adrenaline and cortisol followed by secondary changes in blood and tissue metabolites inducing hyperlactatemia, hyperglycemia and reducing glycogen content in the liver (Wendellaar Bonga 1997; Barton 2000). The present study revealed that pirarucu glucose serum levels were highest in winter (77 ± 1.8 mg/dl) than in summer (57.6 ± 4.8 mg/dl) in response to seasonal variation in temperature. These results confirm the hypothesis that fish response to stress is proportional to severity and duration of the stressor (Barton 2000). Stressful situations can occur after a decrease in plasma concentration of ions such as sodium and chloride. Therefore, the elevation of catecholamines induces increased permeability of the gills, resulting in changes in blood levels of electrolytes as a function of external environment gradients (Centeno et al., 2007; Castro and Fernandes 2009). The osmorregulatory variation induced by seasonal stress was not significant in this study. To restore the osmorregulatory balance, the fish spends of extra energy, depressing its resistance (Carneiro and Urbinati 2001). The osmorregulatory balance of *A. gigas* in relation to seasonal stress reveals a feedback system highly efficient what strengthens its characteristic of a robust species. The results also suggest that changes in lipid and glucose metabolism, as well as the profiles of total cholesterol and fractions can be extremely important for the maintenance of homeostasis front to pirarucu chronic stress. Further studies are needed to understand the regulatory mechanisms and metabolic integration in pirarucu, as well as in fish in general.

Conclusions

The results indicate that seasonal stress can trigger secondary effects in the pirarucu such as changes in the metabolism of glucose and lipids. The osmoregulation represents a highly effective system in maintaining the physiological mechanisms during seasonal stress in pirarucu. This work also showed that *A. gigas*, of more primitive origin than other teleosts species, has a lipoprotein profile with dominating LDL.

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CAPÍTULO VI

Seasonality Influence on Biochemical and Hematological Indicators of Stress and Growth of Pirarucu (*Arapaima gigas*), an Amazonian Air-Breathing Fish

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SEASONALITY INFLUENCE ON BIOCHEMICAL AND HEMATOLOGICAL INDICATORS OF STRESS AND GROWTH OF PIRARUCU (*Arapaima gigas*), AN AMAZONIAN AIR-BREATHING FISH

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ABSTRACT

Environmental factors such as seasonal cycles are the main chronic stress cause in fish increasing incidence of disease, mortality and affecting productive performance. *Arapaima gigas* (pirarucu) is an Amazonian air-breathing and largest freshwater fish with scales in the world. The captivity development of pirarucu is expanding since it can fatten up over 1 kg per month reaching 10 kg body mass in the first year of fattening. This work was conducted in three periods (April to July 2010, August to November 2010, and December 2010 to March 2011) defined according to rainfall and medium temperatures. Seasonality effect analysis was performed on biochemical (lectin activity, lactate dehydrogenase and alkaline phosphatase activities) and hematological (total count of red blood cells, hematocrit, hemoglobin and hematimetric Wintrobe indexes) stress indicators, as well as on growth and wellbeing degree expressed by pirarucu condition factor developed in captivity. All biochemical and hematological stress indicators showed seasonal variations. However, the fish growth was allometrically positive; condition factor high values indicated good state of healthiness in cultivation. These results reinforce the robust feature of pirarucu and represent a starting point for understanding stress physiology and environmental changes during cultivation enabling identification and prevention of fish adverse health conditions.

Keywords: *Arapaima gigas*; pirarucu; air-breathing fish; seasonality; stress indicators.

1. Introduction

Pirarucu, *Arapaima gigas* (Shinz 1822) is the largest Amazonian and freshwater fish with scales in the world. It is considered an air-breathing fish and constitutes a species with great potential for farming due to interesting features such as excellent taste of meat and high growth rate with extraordinary weight development [1]; in captivity the fish can fatten up over 1 kg per month reaching 10 kg body mass in the first year of fattening [2]. Pirarucu has been a target of fishermen, due to its occurrence in lakes and isolated environments, which allows a disorderly intense exploration depreciating their natural stocks [3]. The expansion of the creation of pirarucu in captivity around the world, for commercial exploitation, was too an alternative to reduce the risk of extinction.

The increase of fish farming has developed a growing interest by producers in respect to the search for solutions to avoid the losses caused by mortality and production problems. Stress caused by common farming practices (acute stress) as well as environmental factors (chronic stress) such as seasonal changes, increase the incidence of disease and mortality affecting the productive performance of animals [4]. The seasonal cycles can affect fishes in several biological activities, such as behavior, nutrition, metabolism, immunity and reproduction [5]. The immunocompetence is often affected by seasonal variations and in general, biochemical, hematological and immunological parameters such as levels of various blood cells, hematocrit percentages [6], lysozyme activity [7], respiratory burst levels of anterior kidney macrophages [8], and lectin activity [9]. The biochemical and hematological parameters are useful tools to determine the characteristics of fish blood in different situations such as stress or normality. The relative robustness, or degree of wellbeing from a fish is expressed by the coefficient of condition or condition factor (CF), considered basically as the quotient

between observed mass and theoretical mass estimated through the length-mass relationship. Variations in fish's coefficient of condition primarily reflect the state of sexual maturity and degree of nourishment; they provide relevant information about physiological and health characteristics of individual or population, which are very important in captive fish to their management and maintenance [10].

The aim of this work was to analyze the effects of seasonality (temperature and rainfall) on biochemical (lectin activity; lactate dehydrogenase and alkaline phosphatase activities) and hematological parameters (hematocrite, hemoglobin and hematimetric indexes of Wintrobe) that can be used as physiological indicators of stress. Also, the influence of seasonality on growth of pirarucu developed in captivity as well as the state of wellbeing of the fish was analyzed. This is a first study that relates chronic stress with biochemical and hematological indicators of seasonal stress as well as health and growth in *A. gigas* fish farming.

2. Material and Methods

2.1. Weather data obtention: rainfall and temperature

Weather data was provided by the *Instituto Nacional de Meteorologia* (INMET, Brazil), collected from weather station 82900 (08°03'S 34°57'W) Recife-PE, Brazil according to international standards of the World Meteorological Organization. Medium rainfall (mm^3) as well as medium temperature (°C) was calculated to each period, respectively: 9.99 mm^3 and 26.14°C to period 1 – P1 (April to July 2010); 2.74 mm^3 and 25.46°C to period 2 – P2 (August to November 2010); 5.8 mm^3 and 26.94°C to period 3 – P3 (December 2010 to March 2011) (Figure 1).

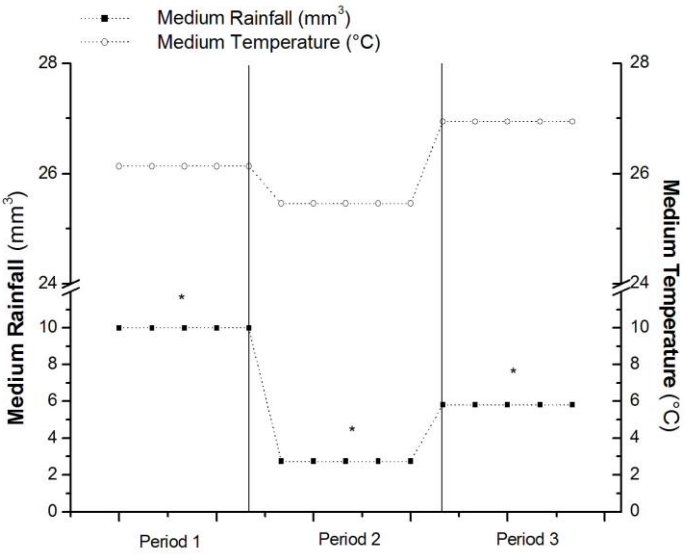

Figure 1. Medium rainfall and medium temperature for each period. Period 1 (April-July, 2010); Period 2 (August-November, 2010); Period 3 (December 2010 to March 2011). Data were obtained from *Instituto Nacional de Meteorologia* (INMET, Brazil). (*) Significant difference in rainfall among the periods ($P<0.05$).

2.2. Obtaining fish blood

Fish were provided by the *Estação de Aquicultura Continental Prof. Johei Koike, Departamento de Pesca da Universidade Federal Rural de Pernambuco* (UFRPE) and developed in earth pond. The animals (n=6) were anesthetized by hypothermia on ice; immediately after blood collection procedure the fishes returned to earth pond. Blood was obtained from caudal with syringes 5 mL, 21G, 23G or 25G needles (BD Precision Glide®, PN, Brazil), depending on fish size. To obtain whole blood tubes containing EDTA 1.8 mg/ml as anticoagulant (Vacuette®, Greiner bio-one, Brazil) were used. Serum was collected from tubes without anticoagulant; the blood was centrifuged at 3000 x *g* for 10 min at 4 °C.

2.3. Lectin activity and Protein evaluation

Serum lectin activity (LA) was evaluated as specific hemagglutinating activity in microtiter plates with 96 wells [11]. Specific hemagglutinating activity was defined as the ratio between titer and protein concentration (mg/mL) and expressed with hemagglutinating activity units for protein milligrams (HAU/mg). Protein concentrations were determined by Bradford [12].

2.4. Lactate dehydrogenase activity

Lactate dehydrogenase activities (LDH) were determined following the oxidation of NADH (340 nm, 25 °C). The reaction mixture contained a total volume of 1 ml, 50 mM imidazol, 1 mM KCN buffer pH 7.4 at 25 °C, 0.13 mM of NADH and different concentrations of pyruvate for LDH saturation plots. One unit of enzyme activity is defined as the amount of enzyme using 1 μmol of substrate per min (340 nm, 25 °C). Each value represents the mean of three measurements.

2.5. Alkaline phosphatase activity

The serum alkaline phosphatase activity (AP) was performed with modifications [13]. Briefly, enzyme activity was measured using p-nitrophenyl phosphate (pNPP) as substrate (5.0 mM) in 1 M diethanolamine (pH 9.8) containing 1 mM $MgCl_2$ (405 nm, 25 °C, 1 min). One unit of enzyme activity is defined as the amount of enzyme using 1 μmol of substrate per min (405 nm, 25 °C). Each value represents the mean of three measurements.

2.6. Total count of red blood cells

To determine the total count of red blood cells (RBC), a 1 in 1000 dilution was made in 0.02 M phosphate saline buffer (PBS, pH 7.3). Counts were carried out using a Neubauer haemocytometer (INLAB, Brazil) and expressed as cell/mm^3 [14].

2.7. Hematocrit, hemoglobin and hematimetric indexes of Wintrobe

The hemoglobin (Hb) levels were obtained using a kit for determination of hemoglobin in whole blood (Doles, Brazil) following the manufacturer's instructions. The hematocrit (Htc) was determined by the micro hematocrit technique and result was expressed as percentage of erythrocytes compared to whole blood. Hematimetric indexes of Wintrobe was calculated as following: MCV (Mean Corpuscular Volume) = Htc/RBC x 10 (fl); MCH (Mean Corpuscular Hemoglobin) = Hb/RBC x 10 (pg); and MCHC (Mean Corpuscular Hemoglobin Concentration) = Hb/Htc x 100 (g/dL).

2.8. Condition factor

Mass (g) and length (cm) were used to determine CF and constant regression (b), which reveals the rate of growth in mass. Empirical point ratios M/L (mass/length) for each period were submitted to regression analysis, adjusted by power function, M = aLb, where M is the dependent variable, L the independent variable, "a" the CF, and "b" the constant associated with the type of growth in mass of animals. These constants were estimated by linear regression of the transformed equation: M=log a + b x log L, where M= mass (g), L= total length (cm), a = constant, b= growth exponent or constant regression [10].

2.9. Statistical analysis

Data shown represent the mean values of each parameter in the specified periods. Statistical significance of data between groups (mean ± s.e.) was determined

with analysis of variance (ANOVA) and Tukey Test using OriginPro 8.0^{TM} (OrginLab Corporation, U.S.A). A value of $P<0.05$ was considered significant.

3. Results

Significant difference was observed to serum lectin in the periods (P <0.05); activity increased in P2 (28.45 HAU/mg) while in P1 (8.26 HAU/mg) and P3 (12.8 HAU/mg) values were lower, indicating some seasonal influence on LA (Figure 2a). The activity of the serum enzyme LDH was significantly higher in P3 (444.0 U/L) than in P1 (184.0 U/L) and P2 (115.0 U/L); in addition, there was a greater decrease in LDH activity in P2 than in P1 (Figure 2b). Serum AP activity in the studied periods showed a gradual increase (5.83 U/L P1, 13.0 U/L P2 and 18.0 U/L P3), and, was significantly different from one period to another (Figure 2b).

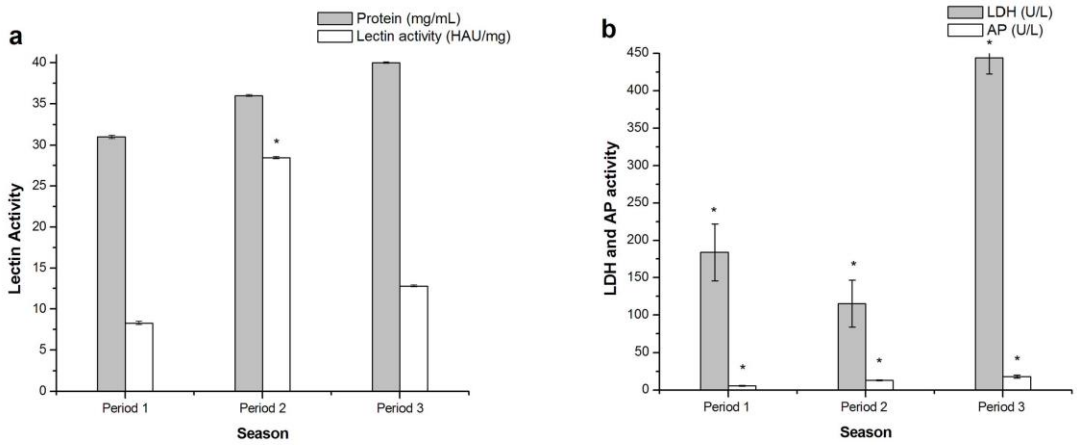

Figure 2. Variations in serum of *A. gigas* in Period 1 (9.99 mm^3, 26.14 °C), Period 2 (2.74 mm^3, 25.46 °C) and Period 3 (5.8 mm^3, 26.94 °C) to Lectin Activity (hemagglutinating activity) and Serum Protein Concentration (**a**); LDH- Lactate Dehydrogenase Activity and AP- Alkaline Phosphatase Activity (**b**). (*) Significant ($P<0.05$).

RBC (1.24 x10^6 /mm^3 P1, 1.14 x10^6 /mm^3 P2 and 1.48 x10^6 /mm^3 P3), Hb levels (10.6 g/dl P1, 8.55 g/dl P2 and 9.7 g/dl P3) and Htc percentages (24.6% P1, 18.75% P2 and 27.9% P3) revealed values decreasing in P2, however this decrease was significant to Hb and Htc and not to RBC (Figure 3a). MCV showed seasonal variation with values significantly lower in P2 (198.4 fl P1, 164.4 fl P2 and 188.5 fl P3); no significant difference was showed between the other periods. MCH decreased gradually from P1 to P3 (85.5 pg P1, 75.05 pg P2 and 65.5 pg P3). MCHC (43.08 g/dL P1, 45.6 g/dL P2 and 34.75 g/dL P3) was significantly lower in P3, while P1 to P2 did not reveal significant difference (Figure 3b).

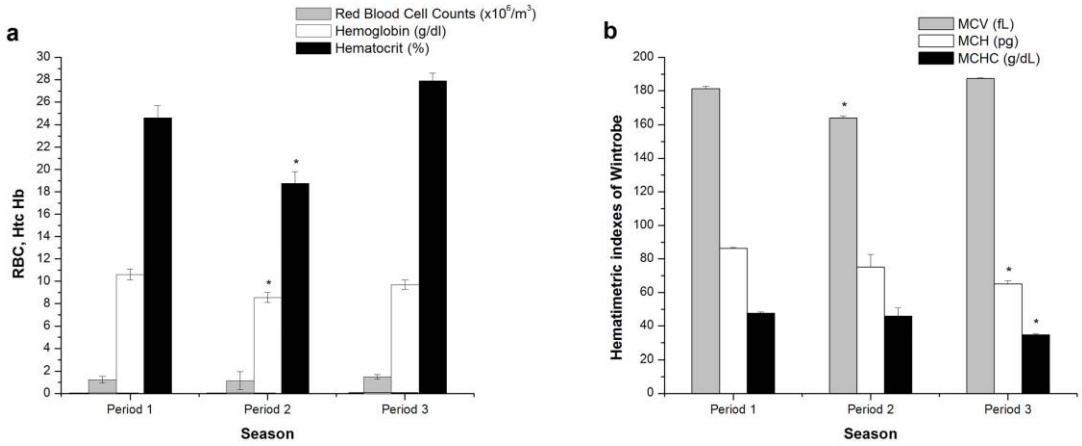

Figure 3. Variations in serum of *A. gigas* in Period 1 (9.99 mm^3, 26.14 °C), Period 2 (2.74 mm^3, 25.46 °C) and Period 3 (5.8 mm^3, 26.94 °C) to RBC-Red Blood Cell Counts, Hb-Hemoglobin and Htc-Hematocrit (a); Hematimetric indexes of Wintrobe - MVC, MCH and MCHC (b). (*) Significant (P<0.05).

The constant values of linear regression "b" obtained in P1 (b = 3.12), P2 (b = 3.33) and P3 (b = 3.46) revealed a positive allometrically growth to pirarucu; this can be observed by a progressive increase in body mass in grams (9.36 x 10^3 ± 0.24 P1, 12.48 x 10^3 ± 1.27 P2 and 17.96 x 10^3 ± 1.41 P3, mean values per period) throughout the periods (Figure 4a). The values of the constant linear regression "b" may vary from 2.50 to 3.50

[15]. This wide variation of "b" is a function of biotic and abiotic factors; when b = 3 growth is isometric, b> 3 positive allometric and b <3 negative allometric. Isometric growth (b = 3.00) suggests an increase in mass and length at the same rate which is theoretically ideal for fish, especially in cultivation. CF average showed high values, 0.891 ± 0.038 in P1, 0.909 ± 0.058 in P2 and 0.926 ± 0.124 in P3; was observed progressive increase in fish length (cm) over the periods (101.6 ± 0.94 P1, 109.8 ± 2.67 P2 and 115.5 ± 2.98 P3, mean values per period) (Figure 4b).

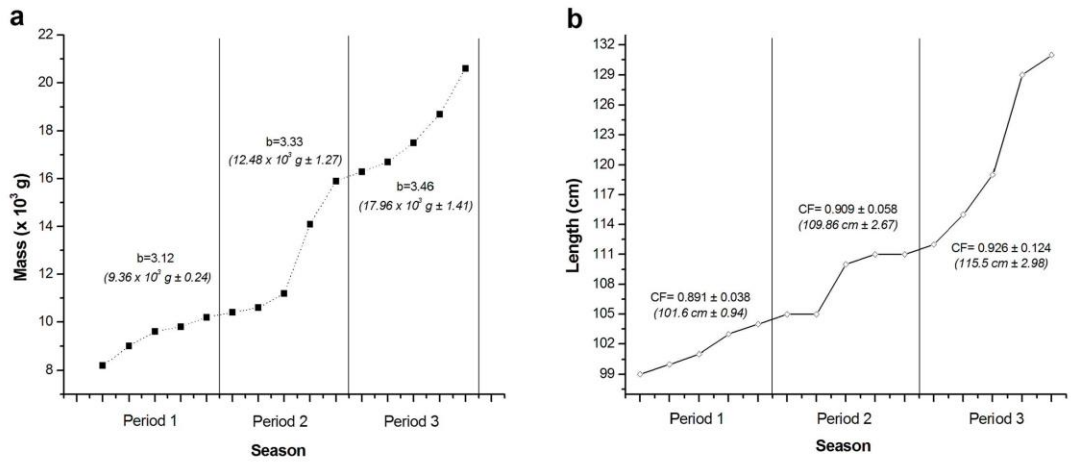

Figure 4. Variations in mass and estimated values for the regression constant "b", rate of growth in mass (**a**); variations in length and condition factor - CF (**b**) to *A. gigas* in Period 1 (9.99 mm^3, 26.14 °C), Period 2 (2.74 mm^3, 25.46 °C) and Period 3 (5.8 mm^3, 26.94 °C).

4. Discussion

Lectins are proteins or glycoproteins involved in innate immunity and are therefore considered as the first line of immune defense of fish [16]. These proteins have been found in serum, mucus skin, and eggs of several fish species [17, 18].

The seasonal variation found for *A. gigas* LA in P2 can be displayed in response to increased concentrations of specific potential pathogens in the environment due to

lower rainfall and lowering temperature. Some pathogens have higher occurrence in winter, although the presence of pathogens generally is higher with increasing temperature, in summer [19]. Stress situations increased the levels of lectin; in this case it can function as an acute phase protein [20]. Seasonal variation too was found in activity of lectin present in serum of *Colossoma macropomum*, showing higher activity in summer and lower activity in winter [9].

The enzyme lactate dehydrogenase (LDH, EC: 1.1.1.27) has been approached in several studies with fish since it is directly linked to the glycolytic pathway and anaerobic metabolism, responsible for environmental stress responses. In this work the levels of LDH in pirarucu serum were significantly lower in P2 (P <0.05) that demonstrates the relation with environmental change processes, such as changes in oxygen levels, even for air breathing fish. The highest LDH levels found in P3 showing that, probably, the enzyme activity in pirarucu is more sensitive to variation on temperature than on rainfall. Alkaline phosphatase (EC 3.1.3.1; AP) has been approached as a potential indicator of stress in the epidermal mucus of Atlantic salmon (*Salmo salar*); high levels of AP were observed in the mucosa of Atlantic salmon infected with the ectoparasite *Lepeophtheirus salmon* [21]. However, AP activity in pirarucu could be related with the fish growth. Human serum AP may provide an index of bone formation rate and probably plays a role in the mineralization of newly formed bone [22].

The seasonal variation observed in the RBC, Hb and Htc in pirarucu may be due to a compensatory effect between rainfall and temperature. Thus, with more oxygen available in water, fewer red blood cells are needed to carry oxygen around the fish body; consequently, the hematocrit and amount of hemoglobin available is lower [23]. Similar results were obtained for RBC of *Oncorhynchus mykiss* [23], however with higher rates, probably due to higher seasonal variations in temperature experienced in

the northern hemisphere, as well as the type of *A. gigas* breathing. Hematocrit values for *O. mykiss* were low in the summer (higher temperatures) and high in winter (lower temperatures) [25] demonstrating the differences in the physiological response to environmental stimuli into distinct species. Decrease in MCV in P2 is strongly related to low hematocrit percentage at this period indicating the possible development of anemia in response to seasonal variation, a chronic stress situation. The low Htc levels exhibit hemoconcentration as a help to cope with the stress related to oxygen demand, corroborating with the results obtained by Gomes [26]. Lower levels of dissolved oxygen should not be detrimental to the creation of pirarucu, especially juveniles, which during its development should provide breathing air, due to a change in its swim bladder [27]. This may be explained since *A. gigas* is considered an obligatory air-breathing fish; however, 10% of its breath still depends on the oxygen dissolved in water [25].

The type of growth observed for pirarucu was allometric positive ($b > 3$) indicating a greater increase in mass than in length. Growth-type isometric was observed to *A. gigas* in semi-intensive fish farming in central Amazonia [28]. Studies suggest that allometry should be used to characterize the different growth strategies of fish associated with ecological, behavioral and physiological characteristics [29]. CF is an index widely used in the bio-ecology of fish; it reflects the physiological status of the animal conditioned to interaction of biotic and abiotic factors [30]. The values of CF, in this work, did not show seasonal variation, however, the values were high indicating a good state of fish healthiness in cultivation. Furthermore, the progressive increase in CF having the highest value in P3 may be an indicator of onset gonadal development in *A. gigas* since it can also be used as an indicator of sexual maturation [10].

5. Conclusions

Lectin activity decrease in period of lower medium rainfall and temperature. LDH activity is more sensitive to changes in temperature than rainfall. AP could be related to bone growth of fish since length (cm), body mass (g) and enzyme activity increased progressively. Hb, Htc and RBC decreased in response to dissolved oxygen in water and reflect changes in hematimetric indexes of Wintrobe. Pirarucu growth was allometrically positive; good healthiness in cultivation was indicated by CF.

Regulation of seasonal effects in fish has not yet been satisfactorily elucidated, but surely constitute a complex defense mechanism in these animals. *A. gigas* physiological informations under stress caused by environmental factors, besides expanding the biological knowledge of the species may also be useful in developing better techniques to increase the success of cultivation and improve fish production in different periods of the year.

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CAPÍTULO VII

Detection of the First Lectin with Antimicrobial Activity Present in Serum of the Amazonian Fish Tambaqui *Colossoma macropomum*

Elba V. M. Maciel Carvalho, Rosiely F. Bezerra, Ranilson S. Bezerra, Janete M. Araújo, Athiê J. G. Santos, Maria Tereza S. Correia, Luana C. B. B. Coelho

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ORIGINAL ARTICLE

Chemistry and Biochemistry

Detection of the first lectin with antimicrobial activity present in serum of the Amazonian fish tambaqui *Colossoma macropomum*

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Abstract Tambaqui *Colossoma macropomum* is the most important Amazonian native species in South American aquaculture. Innate immunity at least partially depends on the recognition of pathogen-associated molecular patterns by receptor-recognizing pathogens (PRRs). Some PRRs have been characterized in fishes, and several studies have focused on the role of lectins in the immune system of various fishes. Lectins are proteins that specifically recognize carbohydrates and which have important biological functions. Tambaqui serum lectin (ComaSeL), which was identified on the basis of its hemagglutinating activity, was pre-purified, biochemically characterized, and used in assays of antibacterial activity against pathogenic bacteria in freshwater fishes. A study of the seasonality of this lectin was performed. Comasel activity was stable at a pH between 4.0 and 9.0 and lost 100 % of its activity at 70° C. It recognized the carbohydrates D-galactose, 1-O-methyl-D-galactopyranoside, and D-fucose, showing antibacterial activity for Gram-negative bacteria. Its activity showed significant differences between the summer and winter ($p < 0.05$, Tukey test), thereby corroborating observations that tambaqui becomes more susceptible to mortality from diseases caused by bacteria and fungi during the winter. With this information, new tools may be developed for gaining a better understanding of the role of these proteins in the immune system of the tambaqui, ultimately resulting in the improved management of this fish by pisciculturists.

Keywords Tambaqui fish *Colossoma macropomum* Serum lectin Antibacterial activity

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Introduction

The tambaqui or Black Pacu *Colossoma macropomum* is one of the most important fishes in Brazilian aquaculture and is the second largest scale fish found in the Solimões river in the Amazon Region. It has been introduced in other tropical countries in both Africa and Asia. Due to its rustic nature and excellent meat quality and because it is able to reach a length of 1 m and a body weight of 30 kg in its natural environment [1], tambaqui is of special interest to fish culturists in many South American countries. The great importance of this fish in the Brazilian pisciculture has also resulted in it being the focus of attention of researchers.

Although it is possible to cultivate tambaqui throughout Brazil, it suffers from high mortality risks during the winter months in the colder regions, which has discouraged its cultivation in the south and southeast regions where water temperature can reach −17° C. The ideal temperature for growing tambaqui is around 25–30° C [2]. Thus, the cultivation of tambaqui has been concentrated in the north, northeast, and midwest regions of the country.

Lectins are proteins or glycoproteins with the ability to bind, selectively, to free or conjugated saccharides in a reversible way through the mediation of carbohydrate-recognition domains (CRD) [3, 4]. Lectins are ubiquitous in nature, and due to their unique property to recognize carbohydrates they can bind to the sugars present on the cell surface where they function in various cellular processes, such as cellular recognition, symbiosis, opsonization, and apoptosis [5–7]. This characteristic has been utilized in various biotechnological applications, such as studies on lymphocytes proliferation, antimicrobial activity, and wound healing [3, 8, 9]. The discovery that these proteins can be isolated from fish serum, plasma, skin mucus, and eggs [10–12] has added a new dimension in the study of immunology in such animals.

Lectins can be classified according to the carbohydrates that preferentially bind [11, 13]. Among some animal lectins, galectin (lectin that recognizes galactose) comprises one of the largest families found in fishes, including *Myriaster Conger*, *Arius thalassinus*, and *Oncorhynchus mykiss* [11, 14, 15]. Fucose-binding proteins, also known as fucolectins, have been isolated and characterized from *Morone saxatilis* and *Sparus aurata* [16, 17].

Several soluble proteins are effectors in the innate immune response of fishes, and some have been well characterized as lysozymes. Major events in the innate immune defense include the recognition of microbial targets by lectins. These proteins identify foreign cells as "non-self" through the recognition of carbohydrates expressed on the cell surface acting as opsonins and subsequently encourage their destruction by complement and/or phagocytic cells [7, 18–21].

Vertebrate lectins play an active role in the innate immunity system, particularly in pathogen-associated molecular pattern (PAMP) recognition, opsonization, phagocytosis, and complement activation [7, 10, 22, 23]. The importance and roles of circulating lectins in the innate immune system is well recognized in vertebrates, such as mammals [18, 24]. In fishes, which differ from mammals in aspects of the acquired immune response, lectins and other innate immune effectors may plays much more important roles. However, characterization of the structures and activities of immune-active fish lectins has only recently been initiated [10].

In the study reported here, we partially purified and characterized the lectins present in the serum of the tambaqui fish and then evaluated their functional properties against bacteria causing high incidences of disease in freshwater fishes in order to investigate their antibacterial properties for future application in fish farming. This study is the first to report the presence of lectins in the serum of the Amazonian fish tambaqui.

Materials and Methods

Fish serum extraction

Blood samples from the caudal vein of an adult tambaqui were collected using a 5-ml syringe at the Estação de Aqüicultura Continental Prof. Johei Koike, Departamento de Pesca e Aquicultura da Universidade Federal Rural de Pernambuco (UFRPE). The collected blood was kept in glass tubes for approximately 3 h at room temperature. After the blood was coagulated, the serum was removed with the aid of a Pasteur pipette and subjected to centrifugation to remove some red blood cells at 1,300g for 5 min at 4° C. Following the centrifugation, the serum was distributed in aliquots of 5 ml and kept at −20° C until use.

Saline fractionation

Ammonium sulphate (Sigma-Aldrich, St. Louis, MO) precipitation of the serum, according to Green and Hughes [25], using a saturated salt at concentrations of 0–50 % (0–50F) and 50–80 % (50–80F), respectively, for 4 h at 4° C. These fractions, including the final supernatant of fraction 50–80 % (50–80SF), were dialyzed extensively against Tris buffered saline (TBS; 20 mM Tris–HCl, 150 mM NaCl, 20 mM $CaCl_2$, pH 8.0).

Hemagglutination activity

To verify the presence of lectins in the serum of tambaqui we performed a hemagglutination activity (HA) assay in microtiter plates with 96 wells according to Correia and Coelho [26]. Lectin preparations (50 µl) were serially twofold diluted in 0.15 M NaCl before the addition of 50 µl of a 2.5 % (v/v) suspension of glutaraldehyde-treated rabbit erythrocytes. Titer was expressed as the highest dilution exhibiting hemagglutination. Specific HA (SHA) was defined as the ratio between the titer and protein concentration (mg/ml).

Study of seasonality on HA and SHA

The serum of tambaqui was obtained as described above for each month of the year covering the major seasons in north and northeast Brazil (summer and winter). The HA [26] and protein concentration [27] were performed on the same day as the blood collections.

Purification of lectins from serum

The fraction of 0–50 % (0–50F) was loaded onto a *Canavalia ensiformis* lectin [Concanavalin A (Con A)]

Sepharose 4B (Sigma-Aldrich) column pre-equilibrated with 10 mM $CaCl_2$ and 10 mM $MnCl_2$. The column was washed with equilibrating buffer (TBS), and bound protein was eluted with equilibrating buffer containing 200 mM methyl a-D-mannopyranoside (Sigma-Aldrich). Active fractions with HA, denoted ComaSeL (*Colossoma macropomum* serum lectins), were pooled and dialyzed against TBS. The protein concentration was estimated [27], and protein homogeneity was checked by 7.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [28] under reducing conditions and staining with Coomassie Blue. The molecular weight of the proteins was calculated by comparison with molecular-weight markers (BioRad, Hercules, CA).

Inhibition of HA

The results of the lectin inhibitory assays using the 0–50F fraction and ComaSeL were evaluated with solutions of carbohydrates according Correia and Coelho [26]. The concentrations of carbohydrates and glycoproteins used in the test ranged from 200 to 50 mM for mono- and disaccharides, and 0.5 mg/ml for glycoproteins. The inhibitory assay was similar to the HA assay, with the exception of an extra incubation step (room temperature, 20 min) prior to erythrocyte addition. The presence of hemagglutination activity inhibition (HAI) was established by the HA sample in the presence of carbohydrates, which was compared to the HA of each sample tested in the absence of carbohydrates.

Heat stability

The heat stability test was performed by incubating 250 µl(1 mg/ml) of serum diluted 1:2 (v/v) at 30, 40, 50, 60,70, 80, or 90° C for 30 min. In sequence, samples were immediately cooled and centrifuged at 3,000g for 20 min. The HA assay was performed with the supernatant of each heated sample. The HA assay value for the lectin obtained at room temperature was defined as 100 % activity [26].

Effect of pH on HA

The pH effect was measured with serum (50 µl) diluted in 50 µl of citrate–phosphate buffer (pH 3.0–6.0), sodium phosphate buffer (pH 6.5–7.5) and Tris–HCl buffer (pH 8.0–9.0) at different molarities (10, 20, 30 and 40 mM). The HA was measured as described above.

Antibacterial activity assay

Bacteria growth inhibition by ComaSeL was examined using Gram-negative bacteria *Aeromonas hydrophila* (IOC/FDA 11036), *Aeromonas sobria* (ATCC 43979), and *Edwardsiella tarda* (ATCC 15947) in an assay performed according to Amsterdam [29]. *A. hydrophila* was cultured in nutritive broth (NB, 30 C, 24 h), *A. sobria* in trypticase soy broth (TSB, 30° C, 24 h), and *E. tarda* (ATCC 15947) in NB (37° C, 24 h). Serial dilutions of ComaSeL (at concentrations of 200–3.125 µg/ml, 180 µl) containing TBS (20 mM Tris–HCl, 20 mM $CaCl_2$, 15 mM NaCl, pH 8.0) were placed in NB or TSB medium in wells of a 96-plate microtiter plate, following which 20 µl of the bacterial suspension (1.5 x 10^9 cells) was added to each well. TSB or NB containing only TBS buffer was used as the negative control for bacterial growth (Control 1), and NB or TSB with bacterial suspension was used as the positive control for bacterial growth (Control 2). All treatments were incubated, and the optical density at 490 nm (k 490) was measured in a microplate photometer. Maximum growth of bacteria in Control 2 was taken as 100 % of bacterial viability and used as a baseline for calculating the antibacterial activity. The experiments were done in triplicate.

Inhibition of antibacterial activity

This test was performed with the CRD of ComaSeL inhibited by specific carbohydrates (fucose). TSB or NB containing only TBS buffer, pH 8.0, with D-fucose was used as the negative control for bacterial growth (Control 1), and NB or TSD with bacterial suspension was used as the positive control for bacterial growth (Control 2). All treatments were incubated, and optical density at 490 nm (k490) was measured in a microplate photometer. Maximum growth of bacteria in Control 2 was taken as 100 % of bacterial viability and used as the baseline for calculating the antibacterial activity inhibition. The experiments were done in triplicate.

Statistical analysis

Statistical significance of data between groups was determined by analysis of the variance (ANOVA) and the Tukey test using OriginPro ver. 8.0 statistical software (OrginLab Corp, Northhampton, MA). The Tukey test was used to study the seasonality of HA and SHA. A value of $p < 0.05$ was considered to be significant.

Results

The HA assay was performed with all serum collected during all months of the year covering all seasons (spring, summer, autumn, and winter). There were significant differences in HA and SHA between the summer and winter

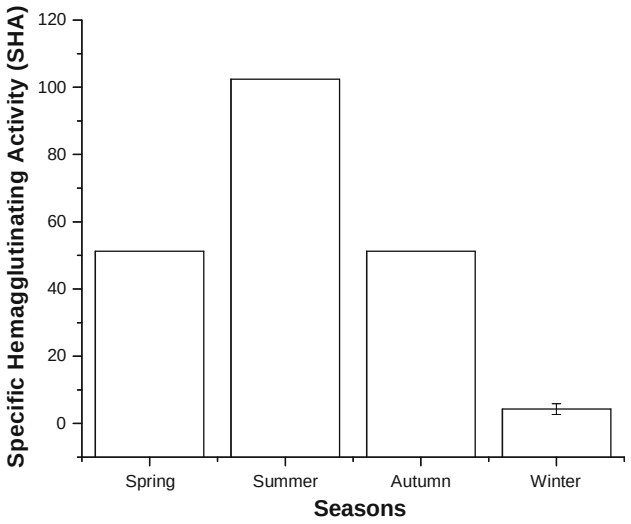

Fig. 1 Seasonality effect on specific hemagglutination activity (SHA) of lectins present in tambaqui serum ($p < 0.05$, Tukey test)

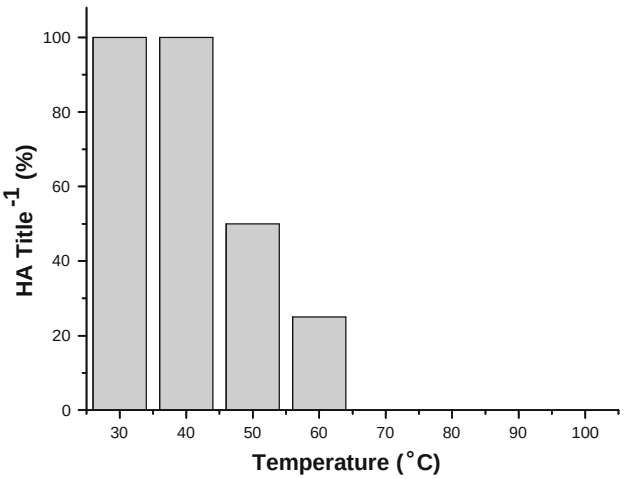

Fig. 2 Effect of temperature on the HA of tambaqui serum lectins

Table 1 Protein concentrations and specific hemagglutinating activities of serum and fractions

Samples	HA (titer^{-1})	Protein concentration (mg/ml)	SHA (HA/protein concentration)
Serum[a]	512^{-1}	20.98	24.4
0–50F	$2{,}048^{-1}$	19.65	107.78
50–80F	0	8.74	0
50–80FS[a]	0	0.527	0

HA hemagglutination activity, SHA specific hemagglutination activity

[a] Data obtained from serum collected during spring

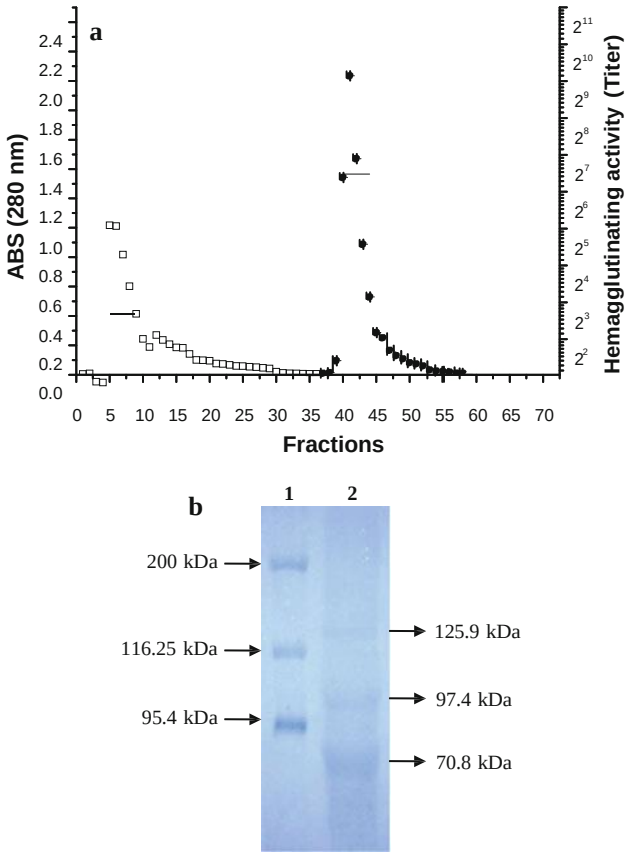

Fig. 3 a Chromatography on Con A–Sepharose 4B. A single peak was eluted with N-methyl-mannopyranoside (filled circles), corresponding to ComaSeL. Unadsorbed (open squares), eluted with 1 M NaCl (jagged line, triangles) and HA (solid lines). b Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5 %) products, stained with Coomassie blue: lanes: 1 protein molecular weight marker, 2 ComaSeL

($p < 0.05$, Tukey test) in which there are sudden changes in temperature in the region (north and northeast Brazil) (Fig. 1). The temperature test showed that lectins present in tambaqui serum lost 100 % of their activity at 70 C (Fig. 2). The HA of the serum was stable at pH ranging from 4.0 to 9.0 and molarity ranging from 10 to 30 mM.

At the pre-purification step by salt fractionation, two fractions (0–50 and 50–80F) and a final supernatant (50–80SF) were obtained; the 0–50F fraction was the only fraction showing HA and SHA (Table 1). Therefore, the 0–50F was the fraction of choice for the next steps of purification.

The partial purification of lectins present in the serum of tambaqui was performed using the Con-A Sepharose 4B affinity matrix. A single peak of material with HA was obtained during elution with the carbohydrate methyl a-D-mannopyranoside (specific for Con-A) (Fig. 3a). Each 20.0 mg/ml of the 0–50F produced 1 mg/ml of ComaSeL.

Table 2 Hemagglutination activity inhibition (HAI) of ComaSeL

Carbohydrates	HAI (titer^{-1})
D-Galactose	TI
1-O-methyl-a-D-galactopyranoside	TI
D-Fucose	TI
D-Mannose	NI
Methyl-a-D-mannopyranoside	NI
D-Glucose	NI
Methyl-a-D-glucopyranoside	NI
N-acetyl-glucosamine	NI
Xylose	NI
Arabinose	NI
Trehalose	NI
Fructose	NI
Lactose	32
L-Rhamnose	NI
D-Raffinose	32
Maltose	NI
D-Cellobiose	NI
Fetuin	NI

TI totally inhibited at the minimum concentration of 50 mM, NI not inhibited

SDS-PAGE revealed three polypeptide bands with apparent molecular weights of 125.9, 95.4, and 70.8 kDa, respectively (Fig. 3b). Analysis of ComaSeL and 0–50F showed that HA was completely inhibited by D-galactose, 1-O-methyl-a-D-galactopyranoside, and D-fucose and that it was partially inhibited by the glycoprotein fetuin and rabbit serum glycoproteins (Table 2).

The antibacterial activity assay was carried out against pathogenic bacteria of freshwater fishes. ComaSeL showed antibacterial activity against *E. tarda*, *A. hydrophila*, and *A. sobria*, with a minimum inhibitory concentration (MIC) of 50, 200, and 12.5 **l**g/ml, respectively (Fig. 4). The inhibition test of antibacterial activity was conducted to show the performance of this lectin on antibacterial activity. During this test, the CRD of ComaSeL was inhibited by D-fucose, with the highest inhibition of antibacterial activity by ComaSeL occurring at a carbohydrate concentration of 200 mM (Fig. 4). D-Fucose was able to significantly inhibit lectin antibacterial activity for all tested bacteria.

Discussion

Several studies have reported that lectins are molecules widely distributed in almost all living organisms. Fish lectins have been found mainly in serum, mucus, skin, and eggs. These lectins are known to react with a diverse range of pathogens and are believed to confer immunity to the individual host [11, 18, 23, 30, 31]. This study is the first to report the presence of lectins in the serum of the Amazonian fish tambaqui C. macropomum.

Lectins can be used to recognize or purify glycoproteins [3, 32–34]. ComaSeL was obtained using the Canavalia ensiformis lectin (Con A) immobilized on Sepharose 4B (Con-A Sepharose 4B; Sigma-Aldrich). As this latter lectin has an affinity for D-glucose or D-mannose, ComaSeL may possess the carbohydrate D-glucose or D-mannose motifs that are recognized by Con-A Sepharose [7, 26]. SDS-PAGE of ComaSeL resulted in three polypeptide bands with apparent molecular weights of 125.9, 95.4, and 70.8 kDa, respectively. The presence of high-molecular-weight bands have been found in purified serum galectins from Indian catfish [7], and a low-molecular-weight lectin has been found in purified carp gill fucolectin [35].

The HA inhibition assay showed that the 0–50F fraction and ComaSeL had the same carbohydrate specificity, but that the HA was only completely inhibited by D-galactose, 1-O-methyl-α-D-galactopyranoside, and D-fucose in ComaSeL. Lectins isolated from fishes commonly have a specificity for these carbohydrates, such as those from *Myriaster conger*, *Arius thalassinus*, *Oncorhynchus mykiss*, *Clarias batrachus*, *Morone saxatilis*, and *Sparus aurata*, which recognize the carbohydrates galactose and fucose [7, 11, 14–17].

Lectins with an affinity for galactose appear to have important roles in modulating immune responses in fishes [11, 36, 37]. The antibacterial activity was assessed with ComaSeL, a preparation obtained from Con A–Sepharose with the property to inhibit the bacterial growth of the three bacteria utilized in this experiment. Serum and mucus lectins are able to recognize and bind to carbohydrate patterns on the surface of pathogens [10, 38, 39], thereby appearing to be important defense molecules. It has been proposed that proteins with antibacterial activity, including lectins, have different mechanisms than antibiotics in that they form a channel in the cell membrane, with the result the cell die due to the loss of the cell contents [40].

The recognition of non-self PAMPs by soluble pattern recognition receptors (PRRs) constitutes a first line of defense against infection in vertebrates and invertebrates [41–43]. In fishes, serum lectins from several fishes, including the Atlantic salmon *Salmo salar* serum lectin [29, 44], rainbow trout *Oncorhynchus mykiss* ladder lectin [45, 46], blue gourami Trichogaster trichopterus serum lectin [18], and the Indian catfish *Heteropneustes fossilis*, have been found to have similar functions, which may indicate the capacity to recognize microbial PAMPs [7].

The specific antimicrobial activities of lectins may depending on the characteristics of the bacteria. Gram-positive bacteria have a thick outer layer containing

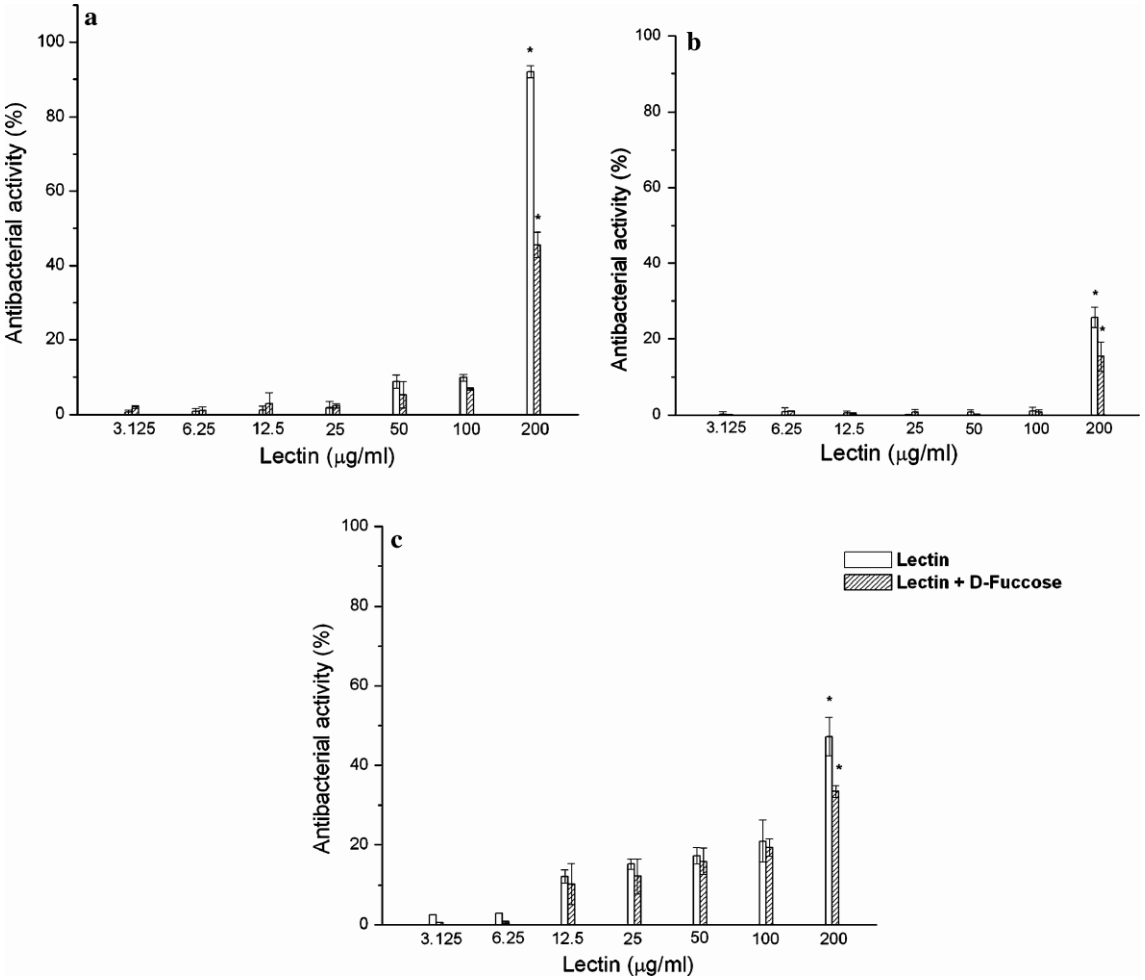

Fig. 4 Antibacterial activity and antibacterial activity inhibition assays of ComaSeL. Serial dilutions of lectin (at concentrations of 200–3.125 μg/ml, 180 μl) with Tris buffered saline (TBS; 20 mM Tris–HCl, 20 mM $CaCl_2$, 15 mM NaCl, pH 8.0) for antibacterial activity and TBS with D-fucose for antibacterial activity inhibition were placed in NB or TSB medium in wells of a 96-plate microtiter plate, and 20 μl of the bacterial suspension (1.5 x 10^9 cells) was added to each well. All treatments were incubated, and optical density at 490 nm (k490) was measured in a microplate photometer. a Edwardsiella tarda, b Aeromonas hydrophila, c A. sobria. The data shown represent the mean percentage ± standard deviation of three experiments of the antibacterial activity and antibacterial activity inhibition. Asterisk indicates significance at $p < 0.05$ between antibacterial activity and inhibition of the three bacterial species with D-fucose

peptidoglycan, while Gram-negative bacteria have a thin layer of peptidoglycan and an outer cell wall acting as an additional barrier to the entry of some antibacterial agents. Lipopolysaccharide (LPS) has been frequently found to be responsible for the pathogenicity of several bacterial diseases, especially that of Gram-negative origin in fishes [47]. Several studies suggest that the binding of lectins to the surfaces of Gram-negative bacteria occurs through LPS, especially by means of the O-antigen or O-polysaccharide core region [42].

ComaSeL had a different MIC for each bacterial species tested in our experiments. The O-antigen is the immunodominant structure exposed to the environment and it is highly variable among bacterial strains. In ayu Plecoglossus altivelis, skin mucus lectin has a high affinity for the LPS of Vibrio anguillarum [48]. Rainbow trout ladder lectin [45] binds to the purified LPS of *Aeromonas salmonicida* coupled to a synthetic matrix [46], while steelhead trout egg lectins bind to distinct serotypes of LPS [31]. Tateno et al. [31] showed that rhamnose-binding lectins from O. mykiss eggs recognize the LPS of Gram-negative bacteria through their O-antigens and core polysaccharide. The ability of tambaqui serum lectin to bind to *A. hydrophila*, *E. tarda*, and *A. sobria* surface may be due to the D-galactose residue of the LPS. Although a significant antibacterial activity was observed, the difference between binding affinities against the Gram-negative bacteria used may be due to the variable spatial arrangement of the multivalent binding sites. Also, the interaction between lectins and carbohydrates is highly dependent on

structural features; binding avidity for some lectins can be weak [49, 50].

ComaSeL antibacterial activity was concentration dependent for the bacteria *E. Tarda* and *A. sobria*. For the bacterium *A. hydrophila*, ComaSeL showed antibacterial activity at a concentration of 200 µg/ml. The antimicrobial role of soluble lectins depends on various genetic and acquired characteristics, but susceptibility to infection is often influenced by serum concentration [31]. In vitro assays have shown that the antibacterial activity of the Atlantic salmon serum lectin occurs through its binding to both *Vibrio anguillarum* and *A. salmonicida* [30], thereby generating a concentration-dependent increase in A. salmonicida phagocytosis by macrophages in the presence of graded concentrations of the purified lectin [44].

A lectin isolated from the marine *sponge Halichondria okadai* was found to have antibacterial activity against Gram-positive bacteria, such as *Bacillus megaterium* and *B. subtilis*. However, his lectin does not inhibit the growth of Gram-negative bacteria, such as *Salmonella typhi* and *Escherichia coli* [51]. Lectins isolated from the skin of the amphibian *Bufo arenarum* [52] showed activity against Gram-positive *Enterococcus faecalis* and Gram-negative *E. coli* bacteria.

Despite the presence of protein co-eluents, the lectin antibacterial activity against the Gram-negative bacteria tested in our study was confirmed through inhibition of the lectin antibacterial activity assay using the binding carbohydrate D-fucose. Since some lectins are known to have multiple sugar-binding affinities, they are likely to be polyspecific [31]. The carbohydrates D-galactose and D-fucose have the same spatial configuration, with the exception of carbon-6. According to Correia and Coelho [26], some lectins do not accept variations in position C-4 of the monosaccharide, which is critical for lectin recognition. Additionally, some lectins bind D-fucose and D-galactose [53, 54].

It is not surprising that being equipped with such diverse recognition abilities to a range of carbohydrate groups on microbial surfaces, lectins play a very important role in the innate immune system of fishes by enhancing their disease resistance. Our study is the first report of tambaqui serum lectins (ComaSeL) able to significantly affect the viability and pathogenicity of a bacterial pathogen. Our results support the hypothesis that serum lectins play a protective function against microbial infection in fishes.

The lectins present in tambaqui serum have antibacterial activity; the HA detected in tambaqui serum varied depending on the time of blood sampling, with a significant decrease in HA activity during the winter (May and June). Differences in HA according to seasonality corroborates the observation that tambaqui during winter becomes more susceptible to mortality from diseases caused by bacteria and fungi [2, 22]. As winter in Brazil is of short duration, it should be an interesting challenge to devise strategies to avoid handling these fishes during this period.

The aim of researchers in the field of pisciculture is to develop approaches that will produce disease-resistant fishes for the aquaculture industry. The identification of lectins in the serum of the tambaqui and its potential application to antimicrobial activity can contribute to an unraveling of the role of these lectins in the fish immune system. It can also improve the management of tambaqui by pisciculturists from the north and northeast regions of Brazil and other countries in South America.

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CAPÍTULO VIII

CONCLUSÕES

Para o pirarucu, *A. gigas*:

- O estresse sazonal desencadeia alterações no metabolismo da glicose e dos lipídeos;
- A osmorregulação representa um sistema altamente eficaz na manutenção dos mecanismos fisiológicos durante o estresse sazonal;
- A lipoproteína de baixa densidade (LDL) domina o perfil de lipoproteínas nos pirarucus criados em cativeiro;
- A atividade de lectina diminuiu no período de menor pluviosidade e temperaturas médias;
- A atividade de lactato desidrogenase (LDH) é mais sensível às variações de temperatura que de pluviosidade;
- A fosfatase alcalina pode estar relacionada com o crescimento ósseo do peixe desde que, assim como o comprimento (cm) e massa corporal (g), a atividade dessa enzima aumentou progressivamente;
- Hb, Htc e RBC diminuíram em resposta aos níveis de oxigênio dissolvido na água refletindo em alterações nos índices hematimétricos de Wintrobe;
- O crescimento do pirarucu foi alométrico positivo e o CF indicou bom estado de saúde no cultivo.

Para o tambaqui, *C. macropomum*:

- A lectina do soro de tambaqui (ComaSeL) foi purificada e caracterizada parcialmente;
- A AH foi estável nos valores de pH 4,0 a pH 9,0 e apresentou termoestabilidade;
- A atividade hemaglutinante foi maior no verão;
- ComaSeL reconheceu os carboidratos D-galactose, 1-*O*-methyl-D-galactopyranosideo e D-fucose;
- ComaSeL apresentou atividade antibacteriana para bactérias Gram-negativas.