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Biochemical and biological characterization of the *Hypanus americanus* mucus: A perspective on stingray immunity and toxins

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ABSTRACT

Stingrays skin secretions are largely studied due to the human envenoming medical relevance of the sting puncture that evolves to inflammatory events, including necrosis. Such toxic effects can be correlated to the biochemical composition of the sting mucus, according to the literature. Fish skin plays important biological roles, such as the control of the osmotic pressure gradient, protection against mechanical forces and micro-organism infections. The mucus, on the other hand, is a rich and complex fluid, acting on swimming, nutrition and the innate immune system. The elasmobranch's epidermis is a tissue composed mainly by mucus secretory cells, and marine stingrays have already been described to present secretory glands spread throughout the body. Little is known about the biochemical composition of the stingray mucus, but recent studies have corroborated the importance of mucus in the envenomation process. Aiming to assess the mucus composition, a new non-invasive mucus collection method was developed that focused on peptides and proteins, and biological assays were performed to analyze the toxic and immune activities of the *Hypanus americanus* mucus. Pathophysiological characterization showed the presence of peptidases on the mucus, as well as the induction of edema and leukocyte recruitment in mice. The fractionated mucus improved phagocytosis on macrophages and showed antimicrobial activity against *T. rubrumç. neoformans* and *C. albicans in vitro*. The proteomic analyses showed the presence of immune-related proteins like actin, histones, hemoglobin, and ribosomal proteins. This protein pattern is similar to those reported for other fish mucus and stingray venoms. This is the first report depicting the *Hypanus* stingray mucus composition, highlighting its biochemical composition and importance for the stingray immune system and the possible role on the envenomation process.

1. Introduction

Fish skin plays important biological roles, such as the control of the osmotic pressure gradient, protection against physical forces and microorganism infections [1]. It is also important for gas exchanges, nutrition and nitrogen waste excretion [2]. Stingrays are cartilaginous fish belonging to the Chondrichthyes class, Elasmobranch subclass (together with the sharks), Myliobatiformes order. The elasmobranch epidermis is characterized as a tissue composed mainly by secretory cells: goblet cells, skein cells and club cells, with the presence of exocytotic activity [3].

Fish mucus is a viscous semipermeable fluid that makes the fish skin surface smoother, thus improving swimming [4]. Moreover, it is essential to the protective activities of fish skin [5], being composed by a mixture of (glyco)proteins, lipids, DNA, RNA and secondary metabolites. Proteins may be originated from different metabolic pathways, but they are mainly synthesized via the endoplasmic reticulum and secreted by the Golgi complex. Cellular debris of epidermal cells could be a

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source of mucus components, too [6].

The mucus is considered to be the first physical/biochemical barrier between the fish and its environment, acting against infections through epidermis, trapping pathogens and eliminating these microorganisms into the water [7]. In addition to this mechanism, the mucus layer contains a wide variety of proteins: antimicrobial peptides [8], lysozyme, histones, peptidases, ribosomal proteins, hemoglobin, actin, lectin, complement factors, immunoglobulins that participate in the innate immunity of fishes [6,7,9].

Peptidases are presents in fish secretions, and their profile varies according to the fish species [10,11]. Stingrays also display a variable peptidase profile and toxic activities, depending on the animal habitat [12], age [13] and gender [14].

The marine stingrays mucus layer is produced by secretory cells dispersed over their epidermis [15], and contributes to epidermal healing [16] in a similar process to other fish [17,18]. There are reports describing antimicrobial activities against *Klebisiella pneumoniae*, two strains of *Escherichia coli* and three strains of *Candida sp.* [19]. Freshwater stingrays mucus also presents antimicrobial activity, as described for a hemoglobin-derived protein that was effective against *Micrococcus luteus*, *Escherichia coli* and *Candida tropicalis* [20].

The stingrays comprise a group characterized by animals displaying a calcified spine ("sting") on the tail, covered by an epithelium that secretes molecules for chemical defense and skin maintenance [21], which the literature associates to a venomous glandular system [12,22,23]. Such similarities, however, could be explained by a common ancestor, since it is proposed that the marine ancestor of the freshwater stingrays invaded continental waters about 18 million years ago [24,25].

Human accidents involving marine stingrays are less common than those involving freshwater stingrays, once human-ray encounters are more likely to happen in rivers than in the oceans, where the animals lie deeper and only divers actually encounter them [26]. Recent researches focusing on the toxic activities of the freshwater stingrays mucus described that the secretion contains toxic components that directly acts on the envenomation process [27–32], but the information about the biochemical profile of stingrays mucus is still poor, and there is no description of the immune role or toxic activities in marine stingray mucus.

The Brazilian shores house many marine stingrays, to which the mucus biochemical characterization and biological activities remain unknown. Therefore, the present study aims to standardize the mucus collection - via a non-invasive method - of the marine ray *Hyapnus americanus* (*Dasyatis americana, Hildebrand & Schroeder, 1928)*, biochemically characterize it and asses its pathophysiological features and immune role.

2. Material and methods

2.1. Biological samples attainment

H. americanus mucus was collected *in situ* at the aquarium "Aqcua Mundo" (Guarujá, SP, Brazil), of a living adult female specimen, by using the new proposed approach. The 'immersion' method was employed to collect the viscous secretion from the stingray, much like it has already been employed in the attainment of amphibian's skin secretions [33]. This method consists in removing the animal from the marine-water tank and placing it in another tank containing freshwater for 40 s (a process commonly used to treat parasitic infections at the aquarium). The resulting solution was then collected; half of it (~3 L) was acidified with 0.1% acetic acid, while the other half was not. Both solutions were filtered through 0.22 μm Millipore filter.

2.2. Biochemical charcterization

Preparative binary RP-HPLC (6AD Prominence HPLC, Shimadzu Co. Kyoto, Japan) was performed to fractionate the acidified solution. Four liters, approximately, of the acid mucus solution were directly infused into the chromatographic column (C18, Phenomenex, Luna 100 Å 250 × 21.20 mm/15 μm), via pump A (no actual sample injection). After complete sample loading, the chromatographic fractionation was performed by a linear gradient of B (acetic acid:acetonitrile; 1:1000) over A (acetic acid:water; 1:1000), for 45 min, under a constant flow rate of 15 mL min^{-1}. Eluates were monitored at 214 nm (SPD 20A UV monitor, Shimadzu), and the fractions were manually collected and analyzed by MALDI-TOF.

For MALDI-TOF/MS analyses, (Axima Performance, Shimadzu, Japan), samples were mixed with a saturated solution of CHCA (Sigma) matrix (1:1, v:v) and dried at room temperature. Spectra were obtained in positive mode, under linear acquisition mode.

The lyophilized non-acidified mucus and the fractions from the preparative chromatography were processed for proteomic analyses. Briefly, 50 μg protein, as estimated by spectrophotometry (BioDrop, BioDrop, UK) were resuspended (0.4 mg mL^{-1}) in 100 mM of NH_4HCO_3 containing 8 M urea and heated at 30 °C for 15 min. After that, 1,4-dithiothreitol (10 mM, final concentration) was added to solution and reacted for 60 min at 30 °C, following incubation with iodoacetamide (25 mM, final concentration) for 30 min, protected from light. The reagents were diluted into NH_4HCO_3 100 mM pH 8 for a final concentration of 0,8 mol L^{-1} urea. Then, trypsin (Trypsin Singles, Proteomic Grade) was added (1 μg trypsin:50 μg mucus proteins ratio) and the reaction was carried out overnight, at 30 °C due the presence of urea. The reaction was stopped by the addition of trifluoroacetic acid 5%.

The tryptic peptides were extracted by zip tip (Merck Millipore, Germany), dried and then dissolved into 0.1% acetic acid for LC-MS/MS analysis, performed in LTQ-XL mass spectrometer (Thermo Fisher Scientific, EUA). Sample aliquots were separated by a C-18 column, on a NanoLC-1D system (Eksigent). The elution was performed by a linear gradient of B over A, from 0 to 30% in 45 min, 30–80% in 10 min and 80% of B in 5 min, under a flow rate of 600 nL per minute. The solvents were: A - water containing 0.1% acetic acid and B - acetonitrile containing 0.1% acetic acid.

The MS^2 profiles were analyzed by MS/MS ion search algorithms by PEAKS studio 7.0 for matches with known proteins sequences deposited on the public UniProt database (subset Elasmobranchi: taxon identifier 7778 combined with Teleostei: taxon identifier 32443). The MS and MS/MS tolerances were fixed as 0.1 Da. In parallel, *de novo* sequences were obtained by PEAKS studio 7.0 and analyzed by BLAST (Basic Local Alignment Search Tool) [34] for the identification of proteins, by peptide sequence similarities.

2.3. Pathophysiological and immune characterization

2.3.1. Zymography

The gelatinolytic activity assay was performed with 20 μg non-acidified mucus. The sample was diluted into semi-native sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 15% (v/v) glycerol, and 0.02% (w/v) bromophenol blue). The samples were loaded on a 9% SDS-PAGE gel co-polymerized with 0.1% (w/v) gelatin (from porcine skin). Ten μg *Bohtrops jararaca* venom was used as positive control. In order to observe the positive protein bands, gels were submitted to four washes of 15 min each with 2.5% (v/v) Triton X-100 to remove traces of SDS. Next, the gels were washed with deionized water, to remove excess of Triton X-100. Then, they were incubated at room temperature for 18 h with Tris-HCl (pH 7.4), 0.5 mM $CaCl_2$ buffer. Finally, the gels were stained with Coomassie Blue R-250, following de-staining. Hydrolysis bands can be observed as clearer bands over a dark (blue) background [35].

2.3.2. Edema assay

Male mice (Swiss, 25 g) were kept in a housing system equipped

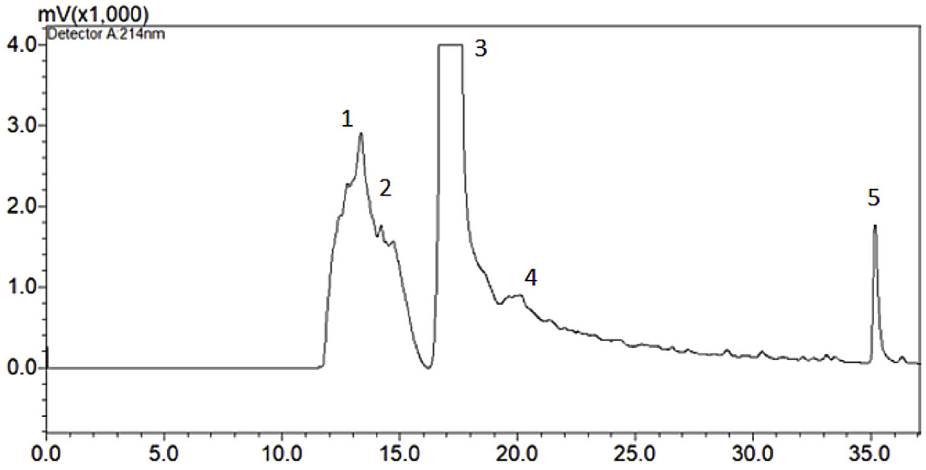

Fig. 1. Preparative C18-RP-HPLC profile of the acidified *H. americanus* mucus solution, obtained by whole animal immersion in fresh water. The numbers indicate the manually collected fractions. Detection performed at 214 nm.

with micro-isolators. The room was set at a 12 h/12 h, light/day cycle, with 70% air humidity and constant temperature of 22 °C. All the experiments were approved by the Institutional Animal Care Committee of the Instituto de Pesquisas Energéticas e Nucleares (IPEN) and followed the Conselho Nacional de Controle de Experimentação Animal (CONCEA) determinations.

Edema was induced by injecting, via the intraplantar route, carrageenan (300 μg/30 μL), no-acidified mucus solution (1, 10 and 50 μg/30 μL) or sterile saline, into one of the hind paws of Swiss mice (n = 5). The thickness of paws was measured using a digital paquimeter (Mitutoyo, CD-6″ CSX-B model, Brazil) before and 3 h after the carrageenan administration, or 1 h after mucus administration. Differences between measurements (before and after the treatment) were expressed as percentual edema increase.

For each experiment, five animals were used. Data were evaluated by GraphPad Prism to verify statistical differences between groups. One-Way ANOVA test, followed by Tukey post-test were applied and differences were considered when $p < 0.05$.

2.3.3. Histological analysis

For histological analysis, the Swiss mice injected hind paw (mucus solution 1, 10 and 50 μg/30 μL) or control (sterile saline), were removed and fixed in 10% formalin. Samples were embedded in paraffin, sectioned into 5 μm sections by a manual microtome (Leica - RM 2145), and stained with hematoxylin and eosin for light microscopic observation.

2.3.4. Antifungal activity

The biological activity of the RP-HPLC fractions was evaluated against *Trichophyton rubrum*, *Cryptococcus neoformans*, *Candida albicans* by using microplate assay [36]. Samples were assayed in concentrations ranging from 0.5 up to 500 μg mL^{-1} in three independent experiments, and the minimal inhibitory concentrations (MICs) were determined using the resazurin dye. Amphotericin B was used as the positive control.

2.3.5. Peritoneal macrophages phagocytic activity

In order to collect macrophages, male adult rats were submitted to euthanasia in a CO_2 chamber. Thereafter, the skin of the abdominal region was removed, and 10 mL of PBS were injected into the peritoneal cavity. After gentle compression of the abdomen, the peritoneal fluid was collected with the help of a polyethylene Pasteur pipette and the cells were submitted to the phagocytosis test.

Macrophages (2×10^5/100 μL) were placed on 13 × 13mm round glass coverslips, packed in 24-well plates for 1 h at 37 °C with 5% CO_2. After adherence of the cells to the coverslips, they were incubated with different concentrations of the fractions added to the RPMI 1640 medium, for 24 h at 37 °C with 5% CO_2. After that, the coverslips were washed and incubated with zymosan for 40 min at 37 °C in an atmosphere containing 5% CO_2, and 1 mL RPMI 1640 medium containing the particles to be phagocytosed. After incubation, the coverslips were washed with PBS and fixed in Rosenfeld. The percentage of phagocytosis was determined in each sample by counting 100 cells under light microscopy (Standart 25, Carl Zeiss - Germany), considering the number of macrophages that phagocytosed more than three particles [37].

3. Results

3.1. Biochemical characterization

The standardization of the mucus collection targeted mainly the retrieval of secreted water-soluble molecules, versus cellular contents. The method (immersion of the animal in a freshwater tank), efficiently extracted mucus from the stingray without detectable cellular debris (data not shown). The collected material was divided into two batches: one 'natural' (for pathophysiological assays) and one acidified (to avoid proteolysis). After the acidified sample was completely loaded into the column, the gradient phase of the separation begun, and fractions were obtained according to their elution profile (Fig. 1: 1–5 indicate the manually collected fractions, λ = 214 nm). All fractions were submitted to MALDI-TOF/MS. Fraction 3, the largest according to the UV 214 nm absorbance, showed to contain peptides, with *m/z* values ranging from 2300 to 9000 Da (Fig. 2).

3.2. Biological characterization

The zymogram shows that the aqueous mucus solution ('natural') displayed proteolytic activity over gelatin (Fig. 3), in a concentration-dependent manner. *B. jararaca* venom, a known gelatinolytic venom, was used for comparison and positive control.

The crude aqueous mucus was able to induce significative paw edema in mice compared to saline injection, in both 10 and 50 μg, as shown in Table 1.

Histopathological analysis (Fig. 4) show a leukocyte influx for both 10 and 50 μg mucus, confirming its pro-inflammatory effect. The same leukocyte influx can be observed for the carrageenan administration (positive control). Saline and 1 μg mucus did not induce edema.

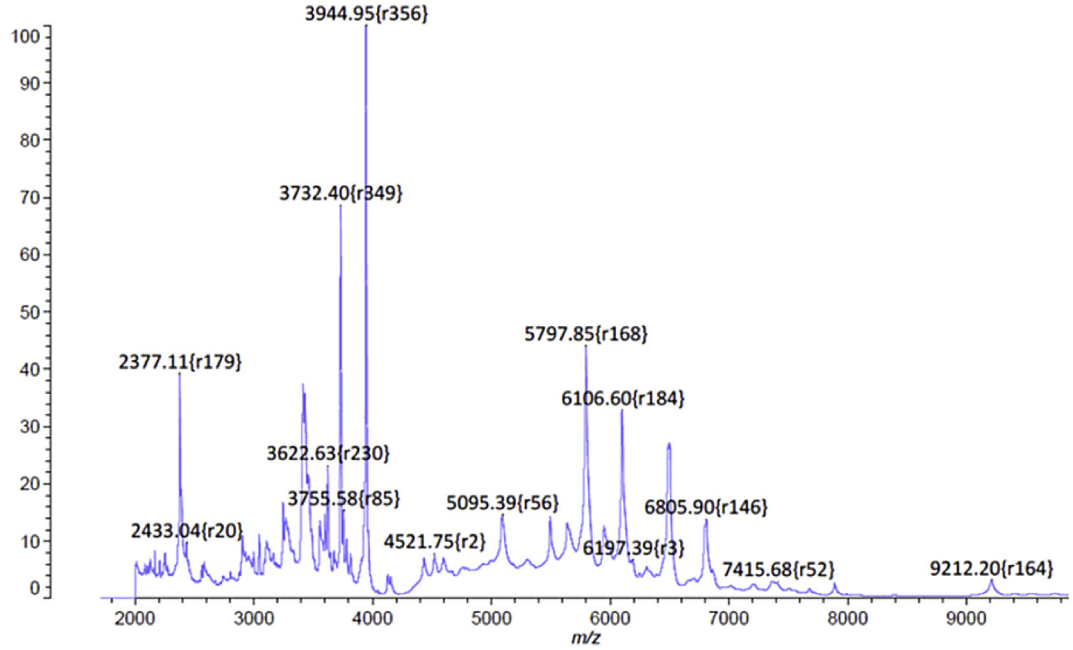

Fig. 2. MALDI-TOF/MS spectrum of 'fraction 3' collected from the preparative C18-RP-HPLC separation of the acidified *H. americanus* mucus solution.

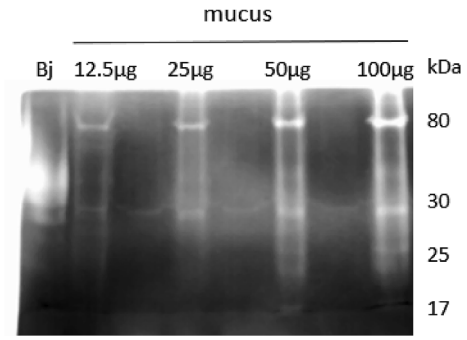

Fig. 3. Gelatin-zymography gel of the aqueous *H. americanus* mucus solution, at 4 different protein concentrations. Bj = *Bothrops jararaca* venom (control +).

Table 1
Percentual mice paw edema induction by *H. americanus* mucus, carrageenan (300 µg) or saline administration. Data are expressed by mean ± SEM. *p < 0.05.

Sample	Edema induction (%)	Standard error
Saline	1.2	0.2
Carragenan	26.6	1.4
1 µg mucus	1.4	0.2
10 µg mucus	2.1*	0.9
50 µg mucus	20.4*	1.9

3.3. Antimicrobial activities

All fractions from the preparative chromatography were tested against 4 different fungi. Fraction 3 was able to inhibit *T. rubrum* and *C. neoformans* growth. Fraction 5, on the other hand, was active only against *C. albicans*; however, presenting a MIC value of 30 µg mL^{-1} (Table 2). No antibiotic effect was observed for the other fractions.

Regarding the phagocytosis assays, only fraction 3 (peptide rich) was selected for testing, and an increased phagocytic capacity of macrophages could be observed at the highest tested dose (Table 3).

3.4. Proteomic analyses

The proteomic analysis was initially performed over an Elasmobranchi (taxid 7778) database. Due to poor identification, the search was expanded to include the Teleostei (taxid 32443), yielding 26 proteins, which could be identified as actin, histones, ribosomal proteins, hemoglobin, galectin and ATP synthase, as presented in Table 4, together with the chromatographic fraction of origin of the sample.

4. Discussion

Mucus sampling was the most critical step for the current study. Handling viscous secretions requires the development of efficient sample preparation techniques in order to allow proper analysis of their biochemical composition [33]. Viscous matrices behave differently when compared to other fluid matrices, like scorpions [38], snakes [39] or spider venoms [40]. In this work, the extraction was performed so that only the water-soluble mucus layer would be gathered, avoiding the interference of the animal epidermidis [41].

The 'issue' of the high initial volume of the sample collection (the minimal amount of water necessary to submerge the whole animal in the tank), was solved by directly submitting the whole sample to a preparative column via pump A and letting the molecules interact to the column as the solvent (freshwater), ionic and polar molecules did not bind to the column. The chromatographic elution was not intended to yield a resolved chromatogram; rather, it was meant to allow the concentration and fractionation of the main soluble components in the mucus, as presented in Fig. 1.

According to our methodological rational, the total skin secretion solution was separated into two batches: one containing 0.1% acetic acid that was used for mass spectrometry analysis (proteomics and peptides evaluation) and another without acid, used for biological assays, to keep peptides and proteins structure in their native conformation. In the *H. americanus* mucus it is possible to observe the presence of peptides (Fig. 2) and the presence of several proteins (Table 4).

In the zymography assay, the activity of proteolytic enzymes could be detected. This activity is well described for stingrays, mainly from freshwater [42,43], which have been described as more proteolytic and toxic than sea stingrays [12]. Accordingly, our zimographic profile

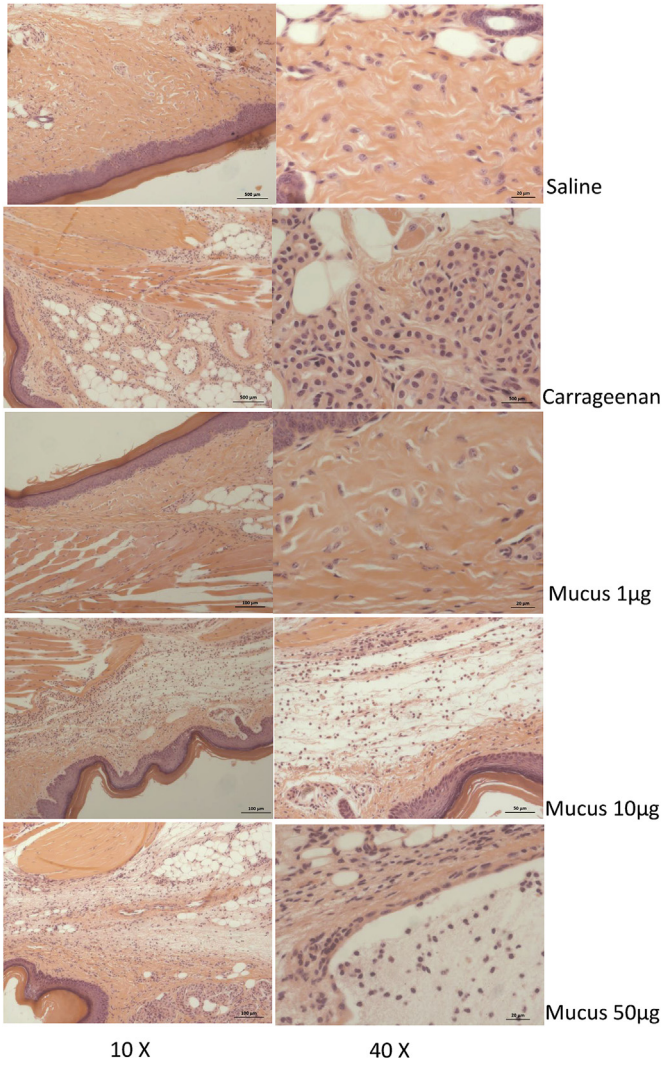

Fig. 4. Histopathological analysis of paws injected with *H. americanus* mucus (1, 10 and 50 µg), carrageenan (300 µg, control +) or saline (control -).

Table 2
Fungi growth inhibitory activity of the fractionated *H. americanus* mucus. Only active fractions are listed.

Fungi	Fraction 3	Fraction 5
	MIC ($\mu g.mL^{-1}$)	
Trychophyton rubrum	**433**	-[a]
Cryptococcus neoformans	**144**	–
Candida albicans	–	**30**
Aspergillus fumigatus	–	–

[a] No effect observed.

Table 3
Macrophage phagocytic activity modulation induced by *H. americanus* mucus fraction 3. Data are expressed by mean ± SEM. *p < 0.05

Phagocytosis activity		
Sample	*Score (%)*	*Standard error*
Saline	62	0.5
0.09 ug	67	0.9
5.20 ug	122*	0.7

(Fig. 2) is similar to the one obtained by Barbaro in 2007, for freshwater stingrays. Metallopeptidases are more abundant in freshwater fish than in marine ones [10], that present high levels of serinopeptidases. Such proteolytic mucus could explain why the Mayas used fresh stings of marine rays on bloodletting rituals [44].

The *H. americanus* mucus was able to induce edema 1 h after injection, indicating its pro-inflammatory effect. It is important to point out that the percentage of edema caused by 50 µg mucus was similar to 300 µg carrageenan (positive control) suggesting the very potent inflammatory action induced by the mucus (Table 1). Moreover, the mucus was able to induce edema after 1 h, faster than carrageenan (that induces inflammation after 3 h). Besides paw edema, we could observe the presence of leukocyte influx, by histopathological analysis, with 10 µg of mucus. Interestingly, 300 µg carrageenan (positive control) induced edema comparable to 50 µg of mucus.

Similar results have already been described for *P. cf. henlei* venom samples, obtained from the sting, from mucus of the region close to the sting and from mucus of a distal region, by Monteiro dos Santos et al. (2011). Authors showed that both mucus and venom displayed edematogenic activity [29]. Magalhães et al. (2006) observed edematogenic, necrotizing and nociceptive activities, with the sting venom from two of different species of freshwater. They also observed that the biological activities increased 2-fold when the venom was co-injected with mucus [30]. Mechanisms regarding inflammatory events have been studied, e.g, *P. motoro* venom was able to promote cytokines/chemokines release (Interleukin 1, Keratinocyte chemoattractant, Interleukin 6 and Monocyte chemoattractant protein-1), which peak occurred 1 h after treatment, besides inducing edema [45].

Molecules like 'porflan' that has the property to increase the number of leukocytes adhered in post capillary venules [46], isolated from *P. gr. Orbigny* sting cellular extract, could be acting on the leukocyte recruitment caused by *H. americanus* mucus.

Marine stingrays mucus exhibit antimicrobial activities against gram-negative bacteria and fungi [19], showing the importance of the stingrays mucus in the immune system. Fish mucus acts directly on immune innate system [6,7,9], and our results showed that the peptide rich fraction (fraction 3) and fraction 5 were active against fungi of human health importance. Fraction 5 displayed high activity against *C. albicans*, but its purity and chemical nature still requires further characterization. We supposed that it is a low molecular mass compound, due the absence of results in the MALDI/TOF and proteomic analyses. Freshwater [20] and marine stingrays have already been described to present antifungal activities [19], and this work was the first to show that the mucus can positively modulate phagocytosis on peritoneal macrophages against *C. albicans*.

This scenario points out to the role of *H. americanus* mucus as an agent of innate immunity. Moreover, mucus can be a source of cryptides [47]. One example is histone H2A from the fish *Atlantic halibut*, a cryptein for hipposin, an antimicrobial peptide [48]. Histone may genarate peptides with antimicrobial action [49]. Conversely, in the present work, we were able to proteomically identify Histone 1 and 4 (Table 4).

Proteomic analyses also revealed the presence of hemoglobin beta subunit, and hemoglobin subunits are largely described in fish mucus [50–53] and their peptides present important antimicrobial activities [20,54]. Metabolic enzymes were also identified, such as ATP synthase [6,55–57], malate dehydrogenase [53,57] and alpha enolase [53,55,58], as well proteins that participate in cell cycle regulation like Enlogation-factor [53,58] and translation initiation fator [53].

H. americanus mucus also contains Actin, a protein important for processes like phagocytosis and it is a very common protein in fish mucus [53,55–57,59]. As ribosomal proteins are [52,53,55,57,58,60–62], to which derived peptides are known to display antimicrobial effects [63]. Peptides have previously been isolated from other stingrays, such as 'orpotrin', which displays vasoconstrictive effects [64]. Other protein previously described in the mucus is

Table 4
Proteomic identification of the proteins present in the RP-HPLC chromatographic fractions of *H. americanus* acidified mucus solution. [1]UniProt accession code. [2]Peaks studio combined identification score.

Protein accession[1]	−10lgP[2]	Peptide(s)	Description	Organism	Fraction
A0A387IGC1	125.67	KDSYVGDEAQSKR KDSYVGDEAQSKRG KSYELPDGQVITIGNERF RHQGVMVGMGQKD K.EITALAPSTMK.I KQEYDESGPSIVHRK K.DSYVGD.E	Beta-actin (Fragment)	*Atelomycterus marmoratus*	**2**
A0A0P7UUX5	111.15	K.LVLVGDGGTGK.T R.HLTGEFEKK.Y R.VCENIPIVLCGNK.V K.FNVWDTAGQEK.F K.NLQYYDISAK.S	GTP-binding protein	*Scleropages formosus*	**3**
A0A3S2UCN1	104.93	R.VVDALGNAIDGK.G K.AVDSLVPIGR.G K.ISEQSDAK.L R.STVAQLVK.R R.FNDGSDEK.K R.ELIIGDR.Q	ATP synthase subunit alpha	*Oryzias javanicus*	**2**
B9ELB4	99.20	R.DYQDNKADVILK.Y K.AYGELPEHAK.I K.EDGQEYAQVIK.M	Eukaryotic translation initiation factor 1A, X-chromosomal	*Salmo salar*	**3**
Q7T334	85.65	R.ANTFVAELK.G	Malate dehydrogenase	*Danio rerio*	**2**
A0A1A8QIT0	83.59	K.EQIVPKPEEEVAQK.K R.YSLDPENPTK.S K.NAESNAELK.G	Ribosomal protein L17	*Nothobranchius rachovii*	**3**
A0A1A7WWD2	79.05	K.KGQGGAGGADDEEED R.RLFEGNALLR.R K.LGLAK.S R.KTYVTPR.R	Ribosomal protein S9	*Iconisemion striatum*	**3**
A0A3B4DA50	76.01	R.TLYGFGG R.DNIQGITKPAIR.R R.DAVTYTEHAK.R	Histone 4	*Pygocentrus nattereri*	**3**
Q4G0B1	75.59	R.LAPDYDALDVANK.I K.VNTLIRPDGEK.K K.FPLTTESAMK.K	Ribosomal protein L23a	*Danio rerio*	**3**
C1BY87	67.86	R.TICSHVQNMIK.G	Ribosomal protein L9	*Esox lucius*	**3**
C1BY87	67.86	R.TICSHVQNMIK.G	Ribosomal protein L9	*Esox lucius*	**3**
A0A3B4U2H	66.66	K.VVDLLAPYAK.G K.ALVGSPA.V K.ILQDYK.S K.AHGGY.S	ATP synthase subunit beta	*Seriola dumerili*	**2**
A0A146NKB6	64.66	KSNLGANAILGVSLAVCKA	Alpha-enolase	*Fundulus heteroclitus*	**1**
E9QF69	63.11	L.TAVVVGTITDDVR.I	Ribosomal protein L18	*Danio rerio*	**3**
B5XEA6	57.50	R.LVQAFQFTDK.H R.GLFIIDDK.G	Peroxirredoxin-1	*Salmo salar*	**3**
A0A3B4DA50	55.84	R.SGVSLAALKK.A K.GTLVQTK.G K.GTGASGSFK.L	Histone 1	*Astyanax paranae*	**3**
A0A401PIW4	54.82	K.FAAATGATPIAGR.F	Unkown BLAST = 40S Ribosomal protein Sa	*Scyliorhinus torazame*	**2**
A0A0P7UKI5	47.47	EAPAPRG	Galectin	*Scleropages formosus*	**1**
B5DG78	47.24	RNVQAEEMVEFSSGL KGKAVDSLVPIGRG	ATP synthase subunit alpha	*Salmo salar*	**1**
A0A146Y312	44.11	PEEQRRK	Chromodomain-helicase-DNA-binding protein 2	*Fundulus heteroclitus*	**1**
G3NT84	42.78	RYADDSFTSAFVSTVGIDFKV	RAB3D, member RAS family	*Gasterosteus aculeatus*	**1**
A0A3Q3MCQ1	42.60	R.HTTDLDASK.V	Creatine-kinase	*Labrus bergylta*	**2**
A0A1S3LLQ8	40.26	KDVNAAIATIKT	Tubulin alpha chain	*Salmo salar*	**1**
P56692	38.20	R.VFVVYPW.T	Hemoglobin beta subunit	*Dasyatis akeji*	**3**
A0A3Q3IPY9	34.51	K.IGGIGTVPVGR.V	Enlongation factor 1-alpha	*Salmo salar*	**2**
D1M7K1	33.91	K.VLEQLTGQTPVFSK.A	Annexin	*Ictalurus punctatus*	**1**

hyaluronidase, isolated from *P. motoro* sting cellular extract [43].

In this work we were able to identify 26 proteins. The proteome presented here bares similarities with those described in the literature for fish mucus [20,50,51,55–63,65–69]. Therefore, by comparing the proteome obtained here with the one obtained by Baumann, from t *Neotrygon khulli* marine stingray sting extract [22], we can observe some similarities among them.

Some authors stand on the presence of actual venom glands in the stings of stingrays [14,23]. For example, in a work that studied the proteome of the sting of an Asian marine stingray, authors described a purification method based on ammonium sulfate and ketone precipitation to remove the 'mucus', referred to as a sting venom 'contaminant'. With such method, authors stated that they have performed the analysis of the unique and exclusive proteins of the sting [22]. Nevertheless, when comparing to the literature, we could observe that, among 16 proteins reported on this sting proteome, 15 had already been described on fish mucus [20,50,51,53,55–63,65–69].

Other authors, on the other hand, understand that the 'venom' would be the mucus from the sting. Such conclusions are a consequence of the immunogenic similarity between proteins from the mucus and

Table 5
Comparison of protein identified on fish mucus and with the venom from Neotrygon khulii marine stingray. Legend: (1) = (Bergsson et al., 2005); (2) = (Raeder. et al., 2007); (3) = (Easy et al., 2009); (4) = (Easy et al., 2012); (5) = (Li et al., 2013[a];(6) = Li et al., 2013b); (7) = (Rajan et al., 2011) (8) = (Provan et al., 2013); (9) = Rajan et al., 2013); (10) = (Valdengero-Vega et al., 2014); (11) = (Cordero et al., 2015); (12) = (Jurado et al., 2015); (13) = (Sanahuja et al., 2015); (14) = (Cordero et al., 2016); (15) = (Patel and Brinchmann, 2017); (15) = (Cordero et al., 2017); (16) = (Conceição et al., 2012); (17) = (Nigam et al., 2017); (18) = (Sanahuja et al., 2019); (*) = Present work.

Protein	Description in fish mucus
60S ribosomal protein	✓ (1,2,8,9,11,12,13,15,18,*)
ATP synthase	✓ (2,8,12,13,15,16,*)
Coronin	✓ (5)
Cystatin	✓ (7,10,15)
Cytochrome C	
Ferritin	✓ (10)
Galectin	(3,5,6,7,8,*)
Ganglioside GM2	✓ (10)
Glutathione-S-transferase	✓ (5,7,11,13,14,15,16)
Hemoglobin subunit	✓ (10,12,17,*)
Leukocyte elastase inhibitor	✓ (7,11,14)
Nucleoside diphosphate kinase	(7,9,11,12,13,15,17)
Peroxirredoxin 6	✓ (5,9,13,14,15,*)
Transaldolase	✓ (7,11)
Type III intermediate filament	✓ (18)
Voltage-dependent anion channel	✓ (9)

the sting 'venom' [32]. Lameiras et al. showed that the dorsal mucus and stinger extracts of *Potamotrygon* species are very similar in terms of protein profile, proteolytic activity and immunogenicity [27,31]. Those results demonstrate that both epithelia could be related, and all skin can be the source of the stingray's immunity and toxicity.

Table 5 summarizes the comparison among the data obtained in the present work, and those already described in the literature, also indicating whether or not those proteins have already been described in fish mucus.

Then we get to a critical point of this work: the biochemistry of stingray mucus is very similar to other fish mucus secretions, and clearly participates on the innate immunity of these animals. But, when we look to the proteins described for stingray venom, we also observe a high degree of similarity among the described protein classes.

That lead us to think from another perspective on fish venomous secretions: are these molecules found in fish stings really toxins, belonging to an active defense mechanism? Or are they molecules that constitute the innate immunity of these animals and belong to the passive defense system, much like the amphibians? Has the sting of stingrays evolved to be toxic, or solely a mechanical defense apparatus?

A study that analyzed the sting of *H. sabinus*, quantitatively evaluated the metabolic cost associated to the sting. The reported low metabolic balance suggests that active defense would not be the primary purpose of the sting apparatus [70].

A good example of this are the Warm acclimation proteins, present in mucus of different fish species [53,55,58] that participate in fish stress response and presents anti-inflammatory activities (for fish) by inhibiting the leukocyte migration [71]. The same protein is classified as a toxin for *Cathorops spixii* sting. On the other hand, in mammal physiopathology this protein is proinflammatory, enhancing the rolling and adhesion of leukocytes [72], i.e., the opposite effect when compared to the fish model. Another example is the natterins, first identified in *Thalassophryne nattereri* venom [73], that has also been described in non-venomous fish mucus [55,74], kidney and gills [75], and in the blood [76].

5. Conclusions

We have demonstrated that the *H. americanus* mucus is proinflammatory. Probably due the presence of peptidases, but also due to the presence of peptides derived from immune-related proteins. Moreover, these peptides may be acting directly against microorganisms or modulating immune responses like phagocytosis, showing the importance of mucus for immune system of stingrays. The review of literature associated with our proteomic results showed that the proteomic profile of stingray mucus and venom, are very similar to other fish mucus, and opened a new window to discuss the relationship between immune and toxic secretions of venomous and non-venomous fish.

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Conflicts of interest

The authors declare that there is no conflict of interests.

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