Proteomic profile and protease activity in the skin mucus of greater amberjack (*Seriola dumerili)* infected with the ectoparasite *Neobenedenia girellae* — an immunological approach

Álvaro Fernandez-Montero, Silvia Torrecillas, Daniel Montero, Felix Acosta, María-José Prieto-Álamo, Nieves Abril, Juan Jurado

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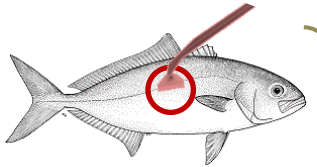
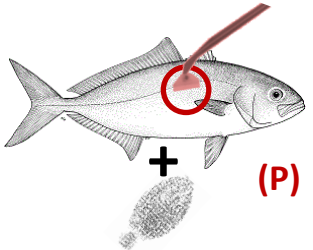
CRediT authorship contribution statement

Á. Fernandez-Montero: Investigation**,** Formal analysis, Resources, Writing – Original Draft, Review & Editing. **S. Torrecillas:** Resources, Writing – Review & Editing **D. Montero:** Conceptualization, Writing – Review & Editing, Project administration, Funding acquisition. **F. Acosta:** Resources, Writing – Review & Editing. **M-J Prieto-Álamo:** Conceptualization**,** Investigation, Formal analysis, Writing – Review & Editing. **N. Abril:** Conceptualization**,** Writing – Review & Editing **J Jurado:** Conceptualization, Investigation, Formal analysis, Writing – Original Draft, Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.



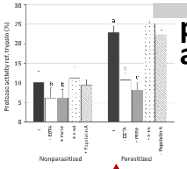
S. dumerili (NP)



S. dumerili + *N. girellae* (P)

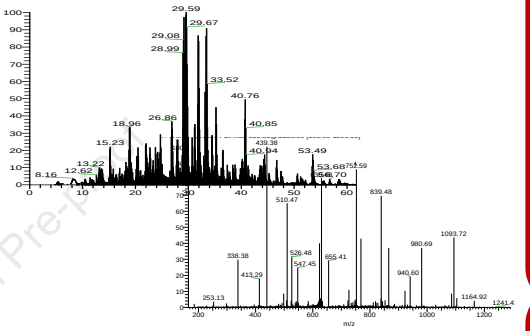
skin mucus samples

protein solubilization & purification

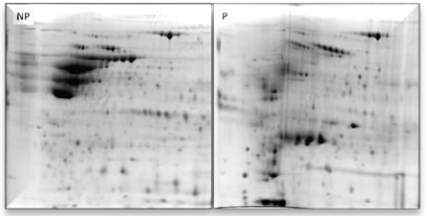


protease activity

gel-free LC-MS/MS



2-DE



PMF-MS/MS

Database search
protein identification
(MASCOT; BLAST)

Fish skin mucus
proteome
microbiota

Proteomic profile and protease activity in the skin mucus of greater amberjack (*Seriola dumerili)* infected with the ectoparasite *Neobenedenia girellae* — an immunological approach

Álvaro Fernandez-Montero[a], Silvia Torrecillas[a], Daniel Montero[a], Felix Acosta[a], María-José Prieto-Álamo[b], Nieves Abril[b], Juan Jurado[b,*].

[a] Grupo de Investigación en Acuicultura (GIA), Instituto Universitario Ecoaqua, Universidad de Las Palmas de Gran Canaria, Crta. Taliarte s/n, 35214, Telde, Las Palmas, Islas Canarias, España.

[b] Departamento de Bioquímica y Biología Molecular, Campus de Excelencia Internacional Agroalimentario CeiA3, Universidad de Córdoba, Campus de Rabanales, Edificio Severo Ochoa, E-14071 Córdoba, España.

*Corresponding author: Tel.: +34 957 218139; fax: +34 957 218856. E-mail address: jjurado@uco.es (J. Jurado).

Keywords: skin mucus; greater amberjack (*Seriola dumerili*); proteome; *Neobenedenia girellae*; protease; microbiota.

Highlights

- The skin mucus proteome of *Seriola dumerili* was analyzed for the first time.
- The effect of *Neobenedenia girellae* on the proteome, proteases, and the microbiota was assessed.
- Ribosomal proteins were overrepresented in the skin mucus of parasitized fish.
- 2-DE proteomics reveals that specifically keratins were cleaved in parasitized fish.
- The mucus of infected fish showed high metal-dependent protease and serine protease activities.

Abstract

Skin mucus is considered the first barrier against diseases in fish. The skin mucus protein profile of the greater amberjack (*Seriola dumerili*) and its changes due to experimental infection with *Neobenedenia girellae* were studied by combining 2-DE-MS/MS and gel-free LC-MS/MS proteomic approaches. The 2-DE results led to the identification of 69 and 55 proteins in noninfected and infected fish, respectively, and revealed that keratins were specifically cleaved in parasitized fish. Therefore, the skin mucus of the infected fish showed a higher protease activity due to, at least in part, an increase of metal-dependent protease and serine-type protease activities. Additionally, through a gel-free LC-MS/MS analysis, 1377 and 1251 different proteins were identified in the skin mucus of healthy and parasitized fish, respectively. The functional analysis of these proteins demonstrated a statistical overrepresentation of ribosomal proteins (a well-known source of antimicrobial peptides) in *N. girellae*-infected fish. In contrast, the components of membranes and protein transport GO categories were underrepresented after infection. Immune system process-related proteins constituted 2.5% of the total skin mucosal proteins. Among these skin mucosal proteins, 14 and 15 proteins exclusive to non-parasitized and parasitized fish were found, respectively, including specific serine-type proteases and metalloproteases in the parasitized fish. Moreover, the finding of tryptic peptides exclusive to some bacterial genera, obtained by gel-free LC-MS/MS, allowed us to construct a preliminary map of the microbiota living in the mucus *of S. dumerili*, with *Pseudomonas* and *Paracoccus* the most represented genera in both noninfected and infected fish.

1. Introduction

The mucus of different vertebrates has been studied from diverse points of view to determine its function, composition, and variations. However, because of its environment, mucosal surfaces play relevant roles in fish as the first barrier against a wide variety of chemical, physical, and biological stressors [1, 2]. Fish mucus is produced by goblet cells, which are scattered throughout mucosal tissue with epithelial cells [3, 4]. Although most fish mucus studies have been focused on the gut [5], knowledge of skin mucus is increasing because of its biomechanical and immunological properties [6, 7].

Skin mucus is composed mainly of water (95%) and mucins, which constitute a family of high-molecular-weight glycosylated proteins. Mucins are structural proteins, which play key roles in mucus viscosity, providing the surface of the fish body with rheological, viscoelastic, and adhesive characteristics that can be modified with the types and quantity of mucin glycosylation [8, 9]. Keratins are also important structural proteins in fish skin mucus, although in a different way than in other vertebrates, since in aquatic species, the absence of a specialized matrix and corneous cell envelope proteins prevent the cornification necessary for creating a barrier against loss of water in amniotes, and therefore, fish require fewer specialized epidermal keratins with a specific mechanical role than are required by terrestrial vertebrates [10]. Similar to other mucosal tissues, the microbiota of the skin mucus constitutes a key component of the host mucosal barrier defenses and can influence the functionality of the host mucosa. Nevertheless, information about skin mucus microbiota interactions with hosts in aquaculture is still limited (reviewed in [11]. To date, most of the studies addressing fish skin mucus proteins have been focused on their role in the innate immune response [4, 12], with biostatic or biocidal enzymes, such as lysozyme, phosphatases, proteases, cathepsins, and esterases, being the most-studied mucus components [12, 13]. Fish skin mucus proteases are secreted in response to bacterial and, especially, ectoparasite infections [14, 15]. Similarly, parasites produce proteases for the attachment necessary for feeding or disrupting the immune system of the host [14]. Interactions between parasite proteases and hosts have been specially studied in salmonids infected with sea lice (*Lepeophtheirus salmonis*) [15]. Moreover, some

studies have been conducted on other isopod [16] and monogenean ectoparasites, such as *Gyrodactylus sp*. [17].

Currently, monogenean infections are considered important bottlenecks in farming some interesting aquaculture species such as *Seriola spp.* Indeed, the prevalence of this infection in sea farms can reach 70% of the cultured population [18].

Neobenedenia girellae is a monogenean ectoparasite with a wide host range distributed in warm waters worldwide, with greater amberjack especially susceptible to this infection when reared in sea cages [19]. High parasite loads in greater amberjack induce fasting, stress-related changes in color appearance, erratic swimming, and a scratching tank fixtures, which results in the development of ulcers and subsequent opportunistic bacterial infections [20]. Some studies about the mechanical damage of *N. girellae* to greater amberjack skin [21] and how this infection affects the mucus glycoproteins and serine proteases profile [22, 23] are available. However, as far as we know, this is the first work addressing the study of the proteome, the protease characterization, and the microbiota of skin mucus of greater amberjack juveniles before and after an experimental *N. girellae* infection.

2. Material and methods

2.1. Experimental fish and skin mucus collection

Sixteen greater amberjack juveniles (150 ± 11.4 g body weight) reared in facilities at the University of Las Palmas de Gran Canaria were placed in four 500-L cylindroconical tanks. The fish were fed with a commercial diet (Europe 22%, Skretting, Stavanger, Norway) to apparent satiety 3 times a day. Skin mucus was obtained as described elsewhere [24]. Briefly, after 7 days of acclimation, the fish were anesthetized with clove oil (5mL/L; Guinama S.L; Spain, Ref. Mg83168), and the skin mucus was obtained by carefully scrapping the left dorsolateral side of the fish with sterile microscopy slides, introduced into sterile 2ml Eppendorf tubes and frozen in liquid nitrogen. The infection of greater amberjack with *Neobenedenia girellae* was carried out as previously described [24]. An experimental tank with greater amberjack previously infected with *N. girellae* was used for collecting parasite eggs in a 5 mm mesh in a 24 hour period. These eggs were introduced into a tank with uninfected greater

amberjack juveniles. After 15 days, all the fish were parasitized at the same level and used to enable a cohabitation challenge. For this purpose, two infected fish marked with a visible implanted elastomer (VIE) [25] were included in each tank. After 30 days of cohabitation, all fish were infected at a high level (between 32 and 65 adult parasites per fish). The fish were sampled (4 fish per tank) to obtain parasitized greater amberjack skin mucus as described above while avoiding collecting adult parasites. Then, the samples were centrifuged before being processed to exclude possible oncomiracidia and other insoluble material. All mucus samples were immediately frozen by immersion in liquid nitrogen and stored at -80°C.

To ensure the maintenance of animal welfare standards, anesthesia (clove oil, 5ml/L) was used in all sampling procedures. All animal experiments described in this manuscript fully complied with the recommendations in the Guide for Care and Use of Laboratory Animals of the European Union Council (2010/63/EU).

2.2. Sample preparation for proteomic analyses

Mucus samples were solubilized in an equal volume of buffer (8 M urea, 2% CHAPS, 60 mM DTT, and 1% protease inhibitor mixture) and centrifuged at 15000 *g* for 15 min at 4˚C. The resultant supernatant was used for determining the mucus proteome and the precipitate for the microbiota analysis. The protein concentration in the supernatant was determined using a Bradford assay [26], and bovine serum albumin was used as the standard. The mean and the standard deviation of the protein concentration measured was 14.8 ± 5.8 mg/ml for healthy fish, and 18.8 ± 4.8 mg/ml for infected fish. The precipitate was resuspended in 200 µl of buffer (50 mM Tris-HCl pH 7.6, 60 mM DTT, and 2% SDS) and treated on ice with three 30 s ultrasonic pulses (90 W) separated by 30 s intervals. After centrifugation (15000 *g*, 15 min, 4˚C) the protein concentration was determined as described above.

For all proteomic procedures, samples from nonparasitized (NP) and parasitized (P) fish were pooled into two respective groups (NP1, NP2, P1, and P2) using an equal amount of protein per fish. To reduce the conductivity and levels of interfering substances, the samples were processed with the 2-D Clean-Up Kit (GE Healthcare). After cleaning, the proteins were resuspended in 6 M urea and 200 mM ammonium

bicarbonate for use in gel-free LC-MS/MS or in rehydration buffer (8 M urea, 2% CHAPS, 12 µl/ml DeStreak reagent, 2% ampholyte solution pH 4-7, and 1% protease inhibitor mixture) for the 2-DE experiments.

2.3. Two-dimensional gel electrophoresis and MS analysis

Skin mucus proteins suspended in the rehydration buffer (340 µl) were first separated by isoelectric point in 18 cm, pH 4-7 IPG strips, and then by SDS-PAGE. The gels were stained with SYPRO Ruby dye for scanning with a Molecular Imager FX (Bio-Rad). Analytical and preparative gels were prepared using 200 µg and 400 µg of proteins, respectively. The most abundant spots in the preparative 2-DE gels were excised using an Investigator ProPic station (Genomic Solutions). To confirm that the desired protein spots were accurately obtained, the gel was rescanned after excision. The selected spots were destained and digested with trypsin using an Investigator Progest (Genomic Solutions). The peptide mixture was purified with a C18 microcolumn (ZipTip, Millipore) and spotted with matrix solution (3 mg/ml α-cyano-4-hydroxycinnamic acid in 70% acetonitrile and 0.1% trifluoroacetic acid) onto an Opti-TOF 96-well MALDI plate and analyzed using a 4800 Plus MALDI-TOF/TOF Analyzer (AB Sciex). Spectra were obtained using the reflector positive acquisition mode in the mass range of 800 to 4000 Da, with a precision ± 20 ppm, and 20kV of acceleration voltage. The eight strongest precursors from the MS scan were isolated and fragmented by the collision-induced dissociation system. Protein identification was performed by combining MS and MS/MS spectra and comparing the data with those in the public NCBInr database, subset Actinopterygii (taxid:7898), using MASCOT v2.0 (MatrixScience) integrated into GPS Explorer software (AB Sciex) and the following parameters: parent ion mass tolerance at 100 ppm, MS/MS mass tolerance of 0.2 Da, carbamidomethylation of cysteine selected as a fixed modification, and methionine oxidation as variable modification. The probability score (95% confidence level) was calculated by the software and used as a criterion for protein identification. Mass spectrometry procedures were performed at the Proteomics Unit, SCAI (Central Facilities for Research Support), University of Córdoba (Spain).

2.4. Gel-free LC-MS/MS analysis

The samples were cleaned, reduced, alkylated, and digested with trypsin using standard protocols. All analyses were performed at the Proteomic Unit, SCAI, University of Córdoba using a Dionex Ultimate 3000 nano UHPLC system (Thermo Fisher Scientific) connected to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ionization interface. The peptide mix was previously concentrated and cleaned up on a 300 µm x 5 mm Acclaim Pepmap precolumn (Thermo Scientific) with 2% acetonitrile and 0.05% trifluoroacetic acid for 5 min at 5 µl/min. The trapping column was switched to be on-line with the separation column, and the gradient was started at 40 °C, using mobile phase buffer A (0.1% formic acid) and mobile phase B (80% acetonitrile, 0.1% formic acid). Peptides were separated at 300 nL/min according to the following elution conditions: 4–45% buffer B for 60 min; 45–90% buffer B for 3 min followed by 8 min washing with 90% solution B, and re-equilibration for 15 min with 4% solution B. The mass spectrometer was operated in the positive mode. Survey scans of the peptide precursors from 400 to 1500 m/z were performed at 120K resolution (at 200 m/z) with a 5×10^5 ion count target. For tandem MS, precursor ions were first isolated in the quadrupole at 1.6 Da, and then CID-fragmented in the ion trap with 35% normalized collision energy. Monoisotopic precursor selection was turned on. The parameters for the ion trap were an automatic gain control of $2x10^3$, and a maximum injection time of 300 ms. Only precursors with charge state 2–5 were sampled for a second in-tandem mass analysis. The dynamic exclusion time was set to 15 s with a 10-ppm tolerance around the selected precursor and its isotopes to avoid redundant fragmentation. For protein identification, mass spectrometry raw data were processed using Proteome Discoverer v2.1.0.81 (Thermo Fisher Scientific). MS2 spectra were searched with the SEQUEST HT engine against the UniprotKB database restricted to *Seriola dumerili* (taxid: 41447) for the study of the mucus proteome or restricted to bacteria (taxid:2) in the microbiota analysis. Theoretical peptides were generated from tryptic digestion with up to two missed cleavages. Methionine oxidation was set as variable modification and carbamidomethylation of cysteines as a fixed modification. A value of 10 ppm was set for the mass tolerance of precursor ions, and 0.1 Da tolerance was set for the product

ions. The identification of a peptide was accepted when it exceeded the filter parameter Xcorr score versus charge state with SequestNode probability score (+1 = 1.5, +2 = 2.0, +3 = 2.25, +4 = 2.5). Peptide spectral matches (PSM) were validated using a percolator based on the *q*-values obtained with a 1% false discovery rate (FDR).

2.5. Protease activity analyses

Protease activity was quantified using the azocasein hydrolysis assay according to the method of Ross et al. [15]. Skin mucus samples were diluted 1:1 with 100 mM ammonium bicarbonate buffer, pH 7.8, containing 0.7% azocasein and incubated for 19 h at 30˚C. To stop the reaction, trichloroacetic acid (TCA) was added to a final concentration of 4.6 %. The reaction tubes were centrifuged (10 min, 10.000 *g*) and 100 µl of the supernatants were poured onto a 96-well plate containing 100 µl of 0.5 M NaOH per well. The absorbance was read at 450 nm. All determinations were carried out, at least, in triplicate. For the positive and negative controls, skin mucus was replaced by trypsin (5 mg/ml, Sigma) and 100 mM ammonium bicarbonate, respectively. Protease activity in each sample was expressed as the percentage of activity relative to the trypsin positive control (100%). For protease characterization, azocasein hydrolysis assays were conducted using protease inhibitors: 10 mM EDTA, 1 mM PMSF, 10 µM E-64, or 1 µM pepstatin A. Ethanol and DMSO were used for the solubilization of PMSF and pepstatin A, respectively. The concentration of these solvents in the working reaction was 85.6 mM for ethanol and 1.4 mM for DMSO. Controls containing these concentrations of solvent were included.

2.6. Statistical analyses

Gene ontology categories were compared with Fisher exact test (two-tailed comparison) in combination with FDR (false discovery rate) correction for multiple testing by using *Blast2GO* software (v5.2.5) [27]. The non-parasitized set of sequences was used as the reference set and the parasitized set was used as the test set. The cutoff threshold for statistical significance was established at FDR<0.05.

Statistical analyses of protease activity followed the methods outlined by Sokal and Rolf [28]. The means and standard deviations (SD) were calculated, and *t*-Student tests

were conducted. Differences were considered significant when $P < 0.05$. Data were analyzed with SPSS software (SPSS for Windows 10).

3. Results and discussion

The fish skin mucus is a physical and biochemical barrier crucial in the innate immune system. In this defensive barrier, proteins play key roles, and therefore, the identification of these proteins constitutes a first step in the design of preventive therapeutic strategies used to prevent infections that currently hinder and limit the development of intensive aquaculture. To our knowledge, this is the first work focused on obtaining a comprehensive view of the proteome of *S. dumerili* in skin mucus. In addition, we have investigated the effect caused by the experimental infection with the ectoparasite *Neobenedenia girellae,* which threatens the development of the aquaculture of this fish in farms.

In this study, the proteome of the greater amberjack was mapped using two different proteomic methodologies that previously have been shown to be complementary: conventional 2-DE and a gel-free LC-MS/MS approaches [29].

3.1. Two-dimensional gel electrophoresis reveals specific cleavage of keratins in parasitized fish

A 2-DE study was performed to compare the proteomic profiles of skin mucus of parasitized and nonparasitized fish qualitatively and quantitatively. We ran three gels per pool of NP and P samples (a total of 12 gels). The 12 gel images obtained are shown in Supplementary File 1. The dramatic differences found between the gels from NP and P samples made a quantitative analysis of spot intensity differences impossible mainly because of the difficulty in matching the spots among the 12 gels. Therefore, the most abundant spots were selected and identified using LC-MS/MS and database retrieval. A total of 69 and 55 spots were identified in the NP and P gels, respectively. A representative gel for each experimental condition, including the position of the identified spots, the symbol of the protein, and a reference number, is shown in Figure 1. Tables 1 and 2 list the proteins identified in the gels from the NP and P conditions, respectively, and several parameters related to protein identification. In these tables,

the proteins are manually classified into three groups according to their main function: structural, stress response, and metabolism proteins.

The most relevant observation in the gels from the NP group was the four trains of acidic proteins, with p*I* ranges from 4.6 to 5.6 and MWs between 40 and 60 kDa, corresponding to different species of keratins. These groups of proteins, which quantitatively account for more than 40% of the total fluorescence signal of the NP sample, were not present in the P group. In contrast, in the gels with proteins from the parasitized fish, numerous spots with lower MW (20-30 kDa) were identified as keratins. Proteolytic fragmentation of keratins explains the arrangement of these proteins in the P group. This phenomenon was not described in any previous studies of infected mucosal [30, 31] or experimentally wounded skin [32], even in studies in which the 2-DE technique was used. The keratin fragmentation can be explained not only as a host response to infection due to the role in immunity attributed to keratin fragments [33] but also as the protease activity of this parasite, which feeds on the mucus and skin of the fish.

Given their abundance in the NP samples, keratins seem to constitute a major component of the skin mucus of healthy greater amberjack. These proteins have previously been identified in mucus samples of different fish species (reviewed in [13]), but such an abundance has not previously been reported. The structural role of keratins is well known, but a protective function of this protein has also been described. Thus, pore-forming activity against bacteria of a glycosylated protein, similar to type II cytokeratin, has been described in rainbow trout (*Oncorhynchus mykiss*) skin mucus [33]. Moreover, an increment of epidermal keratin after sea lice infection in Atlantic salmon (*Salmo salar*) skin mucus has been associated not only with cellular damage and tissue regeneration but also with a specific response against this pathogen [30]. Additionally, in mammals, cytokeratin has been described as producing antimicrobial peptides (AMPs) after proteolysis by extracellular proteases [34, 35]. Therefore, the fragmentation of keratins found in the parasitized greater amberjack may be a response of the host to prevent bacterial infections. Fragmentation of the keratins does not correspond to a general degradation of proteins in the P samples but to a specific fragmentation of this type of protein. Evidence of this specificity is found with other proteins such as Trfe, Enoa, Capg, Hsc70 or Grp78, which appear in gels

312 from both experimental conditions at the same coordinates and, apparently, in a
313 similar amount. Other structural proteins common to both the NP and P experimental
314 groups were also found in the present study: beta-actin (Actb), capping protein (Capg),
315 destrin (Dstn), and cofilin-2 (Cfl2).
316 As we discussed previously, keratins are a major component of the skin mucus
317 proteome in *S. dumerili* but in other species, different structural proteins, such as β-
318 actin, hold this top position in Atlantic salmon [30]. Because of their high degree of
319 cross-linking and posttranslational modifications, mucins must be analyzed through
320 specific methods [36, 37], and consequently, reports of the mucin content are absent
321 in most of the proteomic studies.
322 Stress response proteins were also found in gels, a total of 18 spots in the healthy fish
323 skin mucus compared with 13 spots found in the parasitized fish (Tables 1 and 2).
324 Several spots of heat shock and warm temperature acclimation proteins were
325 identified under both experimental conditions while others, related to oxidative stress,
326 such as glutathione *S*-transferases (Gsto2 and Gste) and peroxiredoxin 1 (Prdx1), were
327 found only in NP samples, and protein disulfide-isomerase (Pdi), was identified only in
328 the gels of the P samples.
329 Intermediate enzymes of glycolysis, nucleotide, and amino acid metabolism were
330 found in parasitized and nonparasitized fish skin mucus with the 2-DE technique
331 (Tables 1 and 2).
332

333 **3.2. Gel-free LC-MS/MS shows an overrepresentation of ribosomal proteins in**
334 **infected fish**

335 The same samples used for the 2-DE approach were used for gel-free LC-MS/MS
336 proteomic analysis. Both pools of mucus samples from each experimental condition
337 (NP1, NP2, P1, and P2) were run in triplicate. Only proteins identified in at least two
338 out of the three replicates were considered for further analysis. A total of 1377 unique
339 proteins were identified in the NP group, and 1251 were identified in the P samples,
340 1017 of these unique proteins were found in both groups. The selected proteins and
341 the details of protein identification are listed in Supplementary File 2. To obtain a
342 global functional view of the proteome, Gene Ontology (GO) annotations were

obtained from *UniProt* database and the analysis was performed using *Blast2GO* (v5.2.5) [27]. The full set of functional annotations is presented in Supplementary File 3. The quantitative comparison of the main biological processes (those having at least 0.5% of the total number of protein sequences) did not show apparent differences between the NP and the P groups (Figure 2, A), including immune system process proteins, which represent approximately 2.5% of the sequences in both NP and P experimental groups. Similarly, the comparison of the 25 major GO names of biological processes, molecular functions, and cellular component categories at level 3 did not show marked differences between the two groups of the samples studied (Figure 3). Nevertheless, significant differences were observed when a statistical assessment of GO term enrichment was performed using *Blast2GO* (including all levels of GO terms) by a Fisher exact test in combination with a false discovery rate (FDR) correction [27]. The test displayed significant differences (FDR<0.05) between the NP and P groups in six GO terms (Figure 4). The biological process of translation (GO:0006412), the molecular function of structural constituent of ribosome (GO:0003735), and the cellular component of ribosome (GO:0005840) were overrepresented in the skin mucus of the parasitized fish. In contrast, the biological process of protein transport (GO:0015031), and the cellular component GO terms of bounding membrane of organelle (GO:0098588) and integral component of membrane (GO:0016021) were underrepresented in the P samples.

Overrepresentation in the parasitized fish of translation, structural constituents of ribosome, and ribosome GO terms reflects a significant increase in ribosomal proteins (RPs) in the P group. Nevertheless, despite the abundance of RPs in the mucus samples (Figure 3), RPs were not identified by the 2-DE approach (Tables 1 and 2). We have previously observed the same outcome when studying the proteome of the skin mucus of gilthead seabream [29]. Numerous studies have reported RPs in skin mucus samples of Atlantic salmon, Atlantic cod, gilthead seabream, and European sea bass (reviewed in [13]) but most of these proteins were found using gel-free approaches. This issue arises because RPs have extreme p*I* values. In fact, only five out of the 109 ribosomal proteins from *S. dumerili* annotated in the Uniprot database (including mitochondrial ribosomal proteins) have a theoretical p*I* included in the working pH range of the gels used.

In addition to the key role in translation, secondary immune functions have been attributed to RPs or fragments thereof, which can act as AMPs in fish skin mucus [13, 38, 39]. The high number of RPs in parasitized fish skin mucus (Supplementary File 4) may be due to the cell damage caused by the parasite, and once in the mucus, their secondary function as AMP may facilitate the onset of the innate immune response. In addition to ribosomal proteins, many other typical intracellular proteins appear in the skin mucus. In fact, according to the functional analysis (Figure 3), only a small percentage, less than 4%, of the identified proteins correspond to extracellular proteins (extracellular region, extracellular space, and extracellular matrix GO terms in the cellular component category at level 3). Several routes to deliver extracellular material, including the classical secretory/exocytic pathway and so-called "unconventional" secretion, which includes direct transport of molecules across the membrane by transporters or channels and secreted microvesicles, have been described [40]. Nevertheless, other origins for mucus proteins may be the contents of dead cells in the epidermal surface as Brinchmann suggested in [13]. This suggestion may explain the major presence of typical membrane and intracellular proteins in the skin mucus of fish. Proteomic approaches, particularly gel-free methodologies, have greatly increased the number of proteins identified in fish skin mucus. Although many of these proteins have been attributed to an immunological purpose, either due to the recognized role of the mucus in the defense of fish or inferred by the role of these proteins in other investigated systems, the functions of most proteins in the skin mucus of fish remain unknown. Clarifying the role of this huge number of proteins in the mucus is an important research challenge.

3.2.1. A global view of immune system GO terms

A specific analysis of greater amberjack skin mucus proteins classified under the immune system process GO term (GO:0002376) revealed 13 out of the 22 immune system process GO terms at level 3. Nevertheless, three of these terms, immune system development, immune response, and regulation of the immune system processes were the most abundant, and together, they constitute more than 50% of all the immune proteins (Figure 2, B). Other terms, including positive regulation of the

406 immune system process, immune effector process, activation of the immune response,
407 and myeloid cell homeostasis had a medium representation, between 7 and 10%.
408 Although some apparent variations can be observed between nonparasitized and
409 parasitized fish, the statistical analysis did not reveal any difference in the GO terms
410 distribution between healthy and infected fish. Nevertheless, this GO analysis revealed
411 the presence of 75 and 74 proteins related to the immune system process in the NP
412 and P groups, respectively, (Table 3). Among these proteins, we identified some of the
413 hallmarks of the mucosal immune response [12, 13] including complement-related
414 proteins, immunoglobulins, the numerous ribosomal proteins discussed above, and
415 several proteins related to the proteolytic activity such as proteasome subunits,
416 aminopeptidase, metallopeptidase, and serine peptidase inhibitors. Approximately
417 80% of these proteins (60 proteins) were found under both experimental conditions,
418 but 15 proteins were found only in the NP samples, including the inflammasome
419 complex related proteins (A0A3B4VAY3, A0A3B4UXT2, and A0A3B4URJ5), MHC
420 complex interacting protein (A0A3B4TZF2) cytokine (A0A3B4V008), retroviral
421 restriction factor (A0A3B4UZ11) and an interferon-induced protein (A0A3B4T6N4). In
422 contrast, 14 were identified only in samples from parasitized fish, such as some
423 complement components (A0A3B4TA33, A0A3B4VEZ1, and A0A3B4VIA0),
424 immunoglobulins (A0A3B4TL02 and A0A3B4UH18), peptidase-related proteins
425 (A0A3B4U7K2 and A0A3B4TAH0), actin (A0A3B4UV44) and tropomyosins
426 (A0A3B4U618 and A0A3B4U5X8), among others. The complement system is known for
427 playing a key role against ectoparasite infections in fish skin [41, 42]. Immunoglobulins
428 have also been related to skin mucus response against ectoparasites, specially IgT [43].
429 In contrast, some authors have pointed out that resistance to *N. girellae* infection is a
430 process primarily involving the innate immune system not the antibody-mediated
431 response; however, recent studies show the importance of this Ig for the protection of
432 *Seriola spp.* against *Neobenedenia sp*.[24, 44]. Nevertheless, the nonsignificant
433 differences in the immune-related proteins between parasitized and nonparasitized
434 skin mucus samples may be related to the low immune responsiveness of the greater
435 amberjack against *N. girellae*, and more research must be conducted for elucidating its
436 immune response against this ectoparasite.
437

3.2.2. Full set of protease activity-related proteins

Proteases are among the main humoral parameters of the immune system and by 2-DE analysis, we observed specific proteolytic activity in samples from parasitized fish. Some protease-related proteins were discovered in our previous analysis of the functional annotations in the immune system process, but other proteins were not elucidated. A precise search for protease-related proteins in the gel-free LC-MS/MS data was performed. Specifically, a search was conducted for the GO terms in the AmiGO2 database using “protease” as a filter. The 95 GO terms retrieved by this search were used to extract the protease-activity-related-proteins from the full gel-free LC-MS/MS dataset (Supplementary File 3). The proteins obtained are listed in Table 4. A total of 118 and 121 protease-activity related proteins were found in the NP and P samples, respectively. These proteins include several proteasome subunits, ubiquitin carboxy-terminal hydrolases, cathepsins, calpains, caspases, metallopeptidases, carboxypeptidases, aminopeptidases, and endopeptidases. Protease inhibitors were also detected in the skin mucus. Most of these proteins (approximately 70%) are found in the two experimental conditions studied, 20 proteins appear only in the samples of mucus from healthy animals including a variety of peptidase such as cysteine-type endopeptidases (A0A3B4TG30, A0A3B4UKC4, and A0A3B4V8Q2), aspartic-type endopeptidases (A0A3B4VFE7and A0A3B4V2Q5), serine-type endopeptidases (A0A3B4V640, A0A3B4UQ15, and A0A3B4TG57), threonine-type endopeptidase (A0A3B4VLM4), and metalloendopeptidase (A0A3B4V3V7), but also protease inhibitors (A0A3B4UIT0, A0A3B4ULR2, A0A3B4T5Z8, A0A3B4T2V3, and A0A3B4UTG8). In contrast, 23 proteins were found exclusively in samples from the *Neobenedenia*-parasitized fish. Among these proteins, a greater presence of serine-type proteases (A0A3B4TCH1, A0A3B4THA8, A0A3B4TKS5, A0A3B4UNL2, A0A3B4VDW8, A0A3B4VJ61, A0A3B4T3P6, and A0A3B4TQX4) and metalloendopeptidases (A0A3B4V967, A0A3B4U7K2, and A0A3B4VBW1) were notable stands out. A greater number of proteasome subunits (threonine-type endopeptidases) unique to the P samples were also found.

3.3. Protease activity in the skin mucus samples

Proteases in fish skin mucus are implicated in the resistance to infection because they directly cleave proteins of pathogens or modify properties of the mucus to prevent parasite attachment and facilitate its removal. The results obtained by the 2-DE experiments showed that skin mucus proteins of infected fish, particularly keratins, are affected by protease activity. Therefore, several protease-related proteins were identified by gel-free LC-MS/MS in the skin mucus of greater amberjack (Table 4), and some of these proteases appeared only in the P samples. Following these results, the protease activity in the skin mucus samples of the nonparasitized and parasitized fish was assessed by azocasein assay. The results are shown in Figure 5 and indicate that the protease activity was more than 2-fold higher in the P samples than in the healthy fish samples ($P < 0.001$). The addition of EDTA, an inhibitor of metal-dependent proteases (metalloproteases and proteases stabilized by calcium) to the protease assay triggered a decrease in the protease activity of approximately 60% in the parasitized samples, while this activity decreased by only approximately 30% in the nonparasitized samples. Similar results were obtained when PMSF, a serine-type protease inhibitor, was added to the azocasein assay. Neither E-64, a cysteine protease inhibitor, nor pepstatin A, an inhibitor of aspartyl peptidases, had any effect on the protease activity in the mucus sampled from the nonparasitized or parasitized fish. These results agree with those of Firth *et al*. [14] that did not find these types of proteases in the mucus of Atlantic salmon sampled from noninfected fish or fish infected with the salmon louse. These data suggest that metal-dependent proteases (metalloproteases and calcium-stabilized proteases) and serine proteases are responsible, at least in part, for the enhanced protease activity in parasitized fish. It should be noticed that PMSF also inhibits some cysteine proteases but the contribution of cysteine proteases to the total protease activity detected may be discarded by the absence of effect of E-64, an irreversible and highly selective cysteine protease inhibitor. These results agree with those showing the prevalence of serine-type proteases and metalloproteases in the P samples, as discussed above.

Parasites have previously been described as producing serine proteases for the attaching onto, feeding from, and disrupting the immune response of the host [14]. Nevertheless, proteases produced by the host are also known to modify structural proteins, *i.e.*, mucins, in skin mucus to change mucus consistency or viscosity to

facilitate the removal of pathogens [45]. In addition, host serine protease production is related mainly to a defensive process as part of the innate immunity against pathogens [46]. [46]. Serine proteases are also related to the enhancement and activation of innate immune components common in fish skin mucus and that we have found in the skin mucus of greater amberjack (Table 3), such as complement, immunoglobulins, and AMP [47, 48]. Moreover, serine proteases also activate metalloproteases [49]. In mammals, the role of metalloproteases in the wound-healing process for re-epithelialization and leukocyte infiltrations is well known [50]. This activation can explain the high level of metalloprotease activity in infected fish, considering the wounds on the skin due to both the feeding behavior of the parasite and the scratching of the parasitized fish on tank fixtures. On the other hand, the higher metalloprotease activity observed in greater amberjack parasitized with *N.girellae* is in accordance with the feeding behavior of these ectoparasites and the attachment damage produced in the epidermis of infected fish [21]. Knowing the source of the highest protease activity observed in the mucus from the skin of infected fish requires further investigation.

3.4. A proteomic approach to the microbiota composition of greater amberjack skin mucus

The bacterial community living in the skin mucus of fish can extend to 10^4 bacteria/cm^2 [51]. Intestinal microbiota, which is the more abundant community, has been more widely studied in teleosts and has been related to processes that promote host health, including the improvement of nutrient metabolism and the stimulation of the immune response [52]. In the same way, a correct balance between commensal and opportunistic bacteria in the skin mucus is thought to play a key role to preserve fish health and justifies the study of the microbiota of the skin mucus of the greater amberjack.

During sample preparation for the proteomic analysis reported here, skin mucus was diluted in solubilization buffer and centrifuged to precipitate any insoluble material that might interfere with proteomic protocols. Treatment for protein solubilization is not harsh enough to induce the lysis of bacteria, so bacterial populations living in skin

mucus can be recovered from the precipitate. The precipitate was processed by combining SDS and ultrasonic treatments as indicated in section 2.2, to break the bacterial wall and recover the proteins, which were analyzed by gel-free LC-MS/MS against the bacteria database. A total of 78 and 119 bacterial proteins were unambiguously identified from the NP and P samples, respectively. A selection of peptides used to identify these proteins was individually employed to perform a BLAST search against the full database, without any organism restriction. Peptides shared by bacteria and other taxa were discarded. One hundred and eight peptides displayed complete matches exclusively with bacterial sequences (Supplementary File 5). Some of these peptides matched no more than one genus of bacteria, namely, *Pseudomonas, Paracoccus, Acinetobacter, Serratia, Clostridium, Bartonella, Escherichia, Streptomyces,* and *Thermotoga*, indicating that species of the identified genera are living in greater amberjack skin mucus (Figure 6). According to the frequency of occurrence, the most abundant genus was *Pseudomonas*, since 12 and 14 peptides in the NP and P samples matched no other genus, respectively, followed by the genus *Paracoccus,* with 5 and 7 peptides in the NP and P samples. No remarkable differences were observed in the bacterial genera distribution between the parasitized and nonparasitized fish. Two previous studies have been focused on the gut microbiota of yellowtail amberjack (*S. lalandi*) [53, 54]. One study investigated differences in the microbiota between two growth stages, and the other study was interested in differences between wild and aquaculture specimens. Both studies showed that *Pseudomonas* was a significant genus being the most abundant in the gut of the juveniles weighing 50 g [53], and wild yellowtail amberjacks [54] but the genus *Paracoccus* was not found in these investigations. To our knowledge, this is the first approach to the microbiota of the skin mucus of *S. dumerili* but a more precise quantitation of the differences between the parasitized and nonparasitized samples needs further investigations.

4. Conclusions

Ectoparasite infections are one of the most important challenges for aquaculture sustainability, and particularly for the culture of the greater amberjack. Unfortunately, information about skin structure, skin-associated lymphoid tissue (SALT), and proteins

associated with the skin mucus of *S. dumerili* are still scarce. In this study, the skin mucus proteome of the greater amberjack was characterized for the first time. Our study shows that ribosomal proteins were overrepresented in infected fish while proteins related to membranes and transport were underrepresented in infected animals. In addition, a variety of immune system process proteins were present in the mucus. Despite this fact, the infection generally progresses unstoppable, highlighting the need for functional diets to stimulate the immune system of the greater amberjack. Nevertheless, the most remarkable difference between parasitized and nonparasitized fish was the specific cleavage of keratins in the mucus of the infected fish, as revealed by the 2-DE approach, implying the presence of a specific set of proteases in the mucus of parasitized fish. The higher protease activity in the mucus of parasitized fish was due, at least in part, to metal-dependent proteases (metalloproteases and proteases stabilized by calcium) and serine-type proteases, but not to cysteine proteases or aspartyl proteases, as confirmed by enzymatic assays in the presence of protease inhibitors. Thus, a set of serine proteases and metalloproteases was found only in the parasitized samples. The differences observed may be the result of a response from the host to the infection or a strategy of the ectoparasite to feed or to evade the host immune system. The characterization of this specific protease activity should be investigated to better understand parasitism by *N. girellae* as the results may provide a potential target for the development of specific new drugs to treat or prevent infection. Moreover, proteomic data have provided information to design a preliminary map of microbial communities associated with skin mucus of the greater amberjack. However, no differences were observed in genera distribution when healthy and parasitized fish were compared. This study is the first proteomic approach to define the microbiota of greater amberjack skin mucus and shows new insights for understanding the relationship between parasite and host.

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758
759

760 **Figure captions**

761

762 **Figure 1. Representative 2-DE gel images of skin mucus proteins from the**
763 **nonparasitized (NP) and parasitized (P) greater amberjack (*Seriola dumerili*).**
764 Identified proteins are indicated by their UniProt entry names and reference spot
765 number. The spots identified as keratins are labeled in white.

766

767

768 **Figure 2. Functional classification of the proteins identified by gel-free LC-MS/MS**
769 **according to biological process and immune system process categories.** Mucus
770 proteins of the nonparasitized (NP) and parasitized (P) fish are classified according to
771 biological process category of the Gene Ontology system (GO) at level 2 (panel A) and
772 immune system process at level 3 (panel B). The classification was performed using
773 *Blast2GO* software [27]. For a better comparison of the NP and P groups, the
774 percentage of sequences in each GO term is represented on the Y-axis. In panel A only
775 GO categories having more than 1% of the sequences were individually assessed.

776

777

778 **Figure 3. Functional classification of the proteins identified from greater amberjack**
779 **(*Seriola dumerili*) skin mucus.** Mucus proteins of the nonparasitized (NP) and
780 parasitized (P) fish are classified according to cellular component, molecular function,
781 or biological process categories at level 3 of the Gene Ontology (GO) system using
782 *Blast2GO* software [27]. For the comparison of the NP and P groups, the percentage of
783 sequences in each GO term is represented on the Y-axis. Only the 25 most abundant
784 GO categories were considered individually.

785

786

787 **Figure 4. GO terms showing significant enrichment in the parasitized (P) fish**
788 **compared with those in the nonparasitized (NP) greater amberjack (*Seriola dumerili*).**
789 All levels of GO terms in biological process, molecular function and cellular component

790 categories were compared using a two-tailed Fisher exact test (FDR < 0.05) with
791 *Blast2GO* software [27].
792
793
794 **Figure 5. Protease activity of skin mucus of the nonparasitized (NP) and parasitized**
795 **(P) greater amberjack (*Seriola dumerili*).** The data are presented as means ± SD of
796 protease activity expressed as the percentage of positive control (trypsin 5mg/ml)
797 from at least three determinations for each group. Comparisons were made by the
798 Students's t-test. Statistical significance: (a) $P < 0.001$, non-parasitized *vs* parasitized in
799 the absence of inhibitors; (b) $P < 0.05$, without inhibitors *vs* with inhibitors in the
800 nonparasitized group; (*c*) $P < 0.001$, without inhibitors *vs* with inhibitors in the
801 parasitized group. Solvents did not affect the protease activity in the nonparasitized or
802 parasitized mucus samples.
803
804 **Figure 6. Clade organization of the bacterial genera identified using gel-free LC-**
805 **MS/MS in the skin mucus of non-parasitized (NP) and parasitized (P) greater**
806 **amberjack (*Seriola dumerili*).** Genus names are highlighted in bold when at least one
807 peptide matched this genus uniquely. The total number of these peptides is indicated
808 for NP and P samples.

Table 1 - Proteins identified by 2-DE and combined PMF + MS/MS in the skin mucus of healthy *S. dumerili*.

SN[a]	Protein[b]	Organism[b]	UniProt ID[b]	Symbol[c]	Score[d]	Expect[e]	PM[f]	PF[f]	SC %[f]	Mass (kDa)	p*I*
	Structural proteins										
127	Beta-actin	*Oncorhynchus mykiss*	Q9I8X4	Actb	575	3.2E-52	19	6	49	42.0	5.4
128	Beta-actin	*Lepisosteus oculatus*	W5MB87	Actb	728	1.6E-67	19	8	44	42.2	5.3
131	Capping protein (actin filament), gelsolin-like b	*Oreochromis niloticus*	I3KRY6	Capg	136	2.6E-08	9	3	18	38.9	5.4
132	Capping protein (actin filament), gelsolin-like b	*Oreochromis niloticus*	I3KRY6	Capg	188	1.6E-13	9	4	19	38.9	5.4
302	destrin	*Seriola dumerili*	UPI000BBEF16B	Dstn	68	4.20E-01	13		41	22.7	6.0
169	cofilin-2-like	*Seriola lalandi dorsalis*	A0A3B4WDY5	Cfl2	125	7.4E-07	22		57	22.7	6.0
150	Tropomyosin alpha-4 chain	*Larimichthys crocea*	A0A0F8D2I6	Tpm4	101	8.1E-05	10	2	28	28.8	4.7
154	tropomyosin alpha-4 chain-like isoform X4	*Oryzias melastigma*	A0A3B3C047	Tpm4	79	1.20E-02	7		32	28.2	4.8
155	rho-related GTP-binding protein RhoF-like	*Stegastes partitus*	A0A3B5A5L2	Rhof	76	2.80E-02	7		27	24.4	6.5
170	coiled-coil domain-containing protein 71 (Fragment)	*Xiphophorus maculatus*	UPI000293C9A0	Ccdc71	43.5	2.00E-05					
110b	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	206	2.6E-15	11	2	27	54.1	5.6
112	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	407	2.0E-35	16	4	29	54.1	5.6
113	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	452	6.5E-40	17	4	31	54.1	5.6
114	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	490	1.0E-43	16	4	30	54.1	5.6
115	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	477	2.0E-42	15	4	29	54.1	5.6
116	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	372	6.5E-32	20	3	34	54.1	5.6
117	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	355	3.2E-30	19	3	29	54.1	5.6
130	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	396	2.6E-34	13	4	27	54.1	5.6
135	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	442	6.5E-39	22	3	35	54.1	5.6
136	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	468	1.6E-41	15	4	30	54.1	5.6
138	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	499	1.3E-44	17	4	29	54.1	5.6
156	Keratin 8	*Takifugu rubripes*	H2UIV7	K2c8	86	2.7E-03	9	1	26	50.2	5.4
137	Keratin 17	*Astyanax mexicanus*	W5K0S9	K1c17	206	2.6E-15	13	4	27	49.1	5.6
122	keratin, type I cytoskeletal 13-like	*Seriola dumerili*	A0A3B4UWE7	K1c13	176	5.90E-12	24		52	49.1	4.9
123	Type I cytokeratin, enveloping layer	*Xiphophorus maculatus*	M4AX75	Cyt1	79	1.2E-02	4	2	10	43.2	5.0
124	keratin, type I cytoskeletal 13-like	*Seriola dumerili*	A0A3B4UTI0	K1c13	101	1.90E-04	22		31	47.7	5.6
125	Type I cytokeratin, enveloping layer	*Xiphophorus maculatus*	M4AX75	Cyt1	88	4.1E-03	6	2	12	43.2	5.0
120	Keratin, type II cytoskeletal 8	*Ictalurus punctatus*	W5ULL9	Krt8	169	1.3E-11	9	2	21	57.0	5.5
129	Type II keratin E3	*Oncorhynchus mykiss*	Q8JFG4	Krte3	284	4.1E-23	12	3	22	55.2	5.3
134	Keratin, type II cytoskeletal 73	*Fundulus heteroclitus*	A0A146R0L3	K2c73	83	5.5E-03	5	2	10	53.2	5.2
140	Keratin, type II cytoskeletal 8	*Fundulus heteroclitus*	A0A146VV26	K2c8	79	1.2E-02	7	2	15	57.7	4.6
160	Keratin type II (Fragment)	*Epinephelus coioides*	F6KMG4	Krt	76	2.3E-02	5	2	18	26.6	7.8
	Stress response										
101	Glucose-regulated protein	*Larimichthys crocea*	A0A0F8AHC2	Grp78	431	8.1E-38	24	8	31	82.5	5.4
103	Heat shock cognate 71 kDa protein	*Oryzias latipes*	Q9W6Y1	Hsc70	240	1.0E-18	15	5	22	76.6	5.8
104	heat shock cognate 70 kDa protein	*Xiphophorus maculatus*	M3ZHB7	Hsc70	124	9.3E-07	21		31	71.0	5.2
106	Stress protein HSC70-2	*Seriola quinqueradiata*	B6F134	Hsc70	321	8.1E-27	18	7	30	71.4	5.3
107	warm temperature acclimation protein (hemopexin)	*Dicentrarchus labrax*	D5A7I0	Wap65	253	5.1E-20	9	4	21	49.7	5.4
108	Warm temperature acclimation protein (hemopexin)	*Seriola dumerili*	A0A3B4U472	Wap65	141	1.9E-08	21	1	40	49.3	5.7
109	warm temperature acclimation protein (hemopexin)	*Dicentrarchus labrax*	A1YTM9	Wap65	224	4.1E-17	10	3	20	49.7	5.4
110a	Warm temperature acclimation protein (hemopexin)	*Seriola dumerili*	A0A3B4U472	Wap65	141	1.9E-08	21	1	40	49.3	5.7
111	Warm temperature acclimation protein (hemopexin)	*Seriola dumerili*	A0A3B4U472	Wap65	155	7.4E-10	26		40	49.3	5.7
153	Glutathione *S*-transferase omega 2	*Xiphophorus maculatus*	H3D718	Gsto-2	118	1.6E-06	10	2	30	27.9	7.6

159	Glutathione *S*-transferase epsilon	*Tetraodon nigroviridis*	Q4RZP8	Gste	78	1.7E-02	6	1	22	23.2	4.9
161	Lactoylglutathione lyase	*Oreochromis niloticus*	I3KR87	Lgul	77	4.0E-02	5	1	22	21.0	5.2
162	Lactoylglutathione lyase	*Oreochromis niloticus*	I3J4P3	Lgul	119	1.30E-06	9	2	44	20.4	5.1
163	Peroxiredoxin 1	*Trachinotus ovatus*	A0A0H3W6U1	Prdx1	326	2.60E-27	6	6	29	22.2	5.9
157	Rho GDP dissociation inhibitor (GDI) alpha	*Tetraodon nigroviridis*	Q4S9L2	Arhgdia	89	1.2E-03	8	3	24	23.5	5.2
165	DELTA-stichotoxin-Hcr4a-like	*Seriola dumerili*	A0A3B4TN03	Shtx	93	1.1E-03	17		44	21.0	5.8
166	DELTA-stichotoxin-Hcr4a-like	*Seriola dumerili*	A0A3B4TN03	Shtx	98	3.6E-04	13		50	21.0	5.8
168	Glia maturation factor, beta	*Oreochromis niloticus*	I3KU52	Gmfb	76	2.40E-02	4	2	13	21.5	5.9
	Metabolism										
	Iron metabolism										
102	Serotransferrin	*Epinephelus coioides*	G9I0G6	Trfe	240	1.0E-18	10	3	12	75.9	5.7
105	Serotransferrin	*Poeciliopsis prolifica*	A0A0S7LCB5	Trfe	170	1.0E-11	9	1	16	71.6	6.4
301	Serotransferrin-like	*Seriola dumerili*	UPI000BBE0EAD	Trfe	333	1.20E-27	40	4	41	76.7	6.0
	Glycolysis and central metabolism										
118	Alpha-enolase	*Larimichthys crocea*	A0A0F8CJR2	Enoa	247	2.0E-19	11	5	24	47.3	6.2
119	Alpha-enolase	*Larimichthys crocea*	A0A0F8CJR2	Enoa	260	1.0E-20	14	5	31	47.3	6.2
121	alpha-enolase isoform X1	*Seriola dumerili*	A0A3B4TYP6	Enoa	162	1.50E-10	22		48	47.3	3.1
133	Fructose-bisphosphate aldolase C	*Fundulus heteroclitus*	A0A147AR05	Aldoc	123	5.1E-07	9	2	17	39.7	7.1
139	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	*Fundulus heteroclitus*	A0A146MHA0	Gapdh	162	6.5E-11	7	4	19	36.3	6.4
141	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	*Fundulus heteroclitus*	A0A146MMD9	Gapdh	200	1.0E-14	8	4	23	36.2	6.6
143	Pyruvate dehydrogenase (lipoamide) beta	*Oreochromis niloticus*	I3IV89	pdhb	102	6.5E-05	7	2	24	39.9	5.9
	Nucleotide metabolism										
142	Cytosolic 5'-nucleotidase 1A-like isoform X6	Seriola dumerili	UPI000BBF09A1	Nt5c3a	114	9.30E-06	23		45	33.3	6.1
144	Cytosolic 5'-nucleotidase 1A-like isoform X6	*Seriola dumerili*	UPI000BBF09A1	Nt5c3a	111	1.9E-05	20		45	33.3	6.1
145	Cytosolic 5'-nucleotidase 1A-like isoform X6	*Seriola dumerili*	UPI000BBF09A1	Nt5c3a	135	7.4E-08	21		52	33.3	6.1
147	Cytosolic 5'-nucleotidase 1A-like isoform X6	*Seriola dumerili*	UPI000BBEA090	Nt5c3a	135	7.4E-08	22		49	33.4	6.1
148	Cytosolic 5'-nucleotidase 1A-like isoform X6	*Seriola dumerili*	UPI000BBF09A1	Nt5c3a	120	2.30E-06	19		45	33.3	6.1
149	Cytosolic 5'-nucleotidase 1A-like isoform X6	*Seriola dumerili*	UPI000BBF09A1	Nt5c3a	113	1.20E-05	20		45	33.3	6.1
	Amino acid and protein metabolism										
126	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial	*Larimichthys crocea*	A0A0F8CCJ1	Bckdha	309	1.3E-25	17	5	17	42.0	6.8
151	Eukaryotic translation elongation factor 1 beta 2	*Xiphophorus maculatus*	M3ZV25	Ef1b2	74	4.2E-02	3	2	13	24.9	4.6
152	Proteasome subunit alpha type	*Oreochromis mossambicus*	Q3ZLC8	Psa	76	2.9E-02	6	2	25	22.8	9.1
164	DDB1- and CUL4-associated factor 11 isoform X1 (fragment)	*Xiphophorus maculatus*	UPI000C6EA71E	Dcaf11	35.8	7.00E-03					

(a) Spot number in reference 2-DE gel (Figure 3)

(b) Protein name, organism and UniProt/UniParc ID of the first hit returned by Mascot search. Spots 164 and 170 gave scores under threshold but fragmented peptides derived from them were used to a BLAST against non-redundant protein sequences database (nr) of *Actinopterygii*. The first hits reported were included.

(c) Protein symbol according to ZFIN Zebrafish Nomenclature Conventions.

(d) MOWSE score based on MS data. Protein scores greater than 76 are significant ($p<0.05$).

(e) The number of times we would expect to obtain an equal or higher score by chance.

(f) PM: Number of non-redundant matching peptides. PF: Number of fragmented peptides matching the protein. SC: % of sequence coverage.

Table 2 - Proteins identified by 2-DE and combined PMF + MS/MS in the skin mucus of *S. dumerili* parasitized by *N. girellae*

SN[a]	Protein[b]	Organism[b]	UniProt/UniParc ID[b]	Symbol[c]	Score[d]	Expect[e]	PM[f]	PF[f]	SC %[f]	Mass (kDa)	p*I*
	Structural proteins										
216	Beta-actin	*Tetraodon nigroviridis*	Q4SMI4	Actb	334	4.10E-28	18	5	43	42.9	5.6
217	Beta-actin	*Monopterus albus*	Q7SZL6	Actb	586	2.6E-53	22	8	58	42.1	5.3
219	Capping protein (actin filament), gelsolin-like b	*Xiphophorus maculatus*	M4AMP9	Capg	149	1.3E-09	10	3	20	38.5	5.2
220	Capping protein (actin filament), gelsolin-like b	*Oreochromis niloticus*	I3KRY6	Capg	137	2.0E-08	12	3	27	38.9	5.4
250	Destrin	*Seriola dumerili*	A0A3B4T7V6	Dstn	78	3.5E-02	13		41	22.6	6.0
307	Cofilin-2-like	*Seriola lalandi dorsalis*	A0A3B4WDY5	Cfl2	126	5.9E-07	18		57	22.7	6.0
221	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	403	5.1E-35	22	4	35	54.1	5.6
223	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	402	6.50E-35	21	4	33	54.1	5.6
232	Keratin 4	*Astyanax mexicanus*	W5K1N1	Krt4	324	4.1E-27	13	4	20	54.1	5.6
239	Keratin 4	*Oncorhynchus mykiss*	A0A060XRR9	Krt4	301	8.1E-25	14	4	19	55.3	5.5
241	Keratin 4	*Oncorhynchus mykiss*	A0A060XRR9	Krt4	372	6.5E-32	14	5	17	55.3	5.5
249	Keratin 4	*Oncorhynchus mykiss*	A0A060XRR9	Krt4	418	1.6E-36	15	6	20	55.3	5.5
242	Keratin 5	*Astyanax mexicanus*	W5LD34	Krt5	224	4.1E-17	16	3	29	53.4	4.9
237	Keratin 5	*Astyanax mexicanus*	W5LD34	Krt5	143	5.1E-09	13	2	27	53.4	4.9
230	Keratin 91	*Astyanax mexicanus*	W5K0S9	Krt91	230	1.0E-17	15	3	21	49.1	5.6
231	Keratin 91	*Astyanax mexicanus*	W5K0S9	Krt91	275	3.2E-22	16	3	28	49.1	5.6
233	Keratin 91	*Astyanax mexicanus*	W5K0S9	Krt91	264	4.1E-21	14	3	21	49.1	5.6
234	Keratin 91	*Astyanax mexicanus*	W5K0S9	Krt91	256	2.6E-20	14	3	21	49.1	5.6
235	Keratin 91	*Astyanax mexicanus*	W5K0S9	Krt91	164	4.1E-11	15	3	25	49.1	5.6
236	Keratin 91	*Astyanax mexicanus*	W5K0S9	Krt91	254	4.1E-20	13	3	17	49.1	5.6
240	Keratin 91	*Astyanax mexicanus*	W5K0S9	Krt91	265	4.1E-21	14	3	18	49.1	5.6
247	Keratin 91	*Danio rerio*	Q6DHB6	Krt91	207	2.0E-15	10	3	18	50.0	5.3
252	Keratin 91	*Danio rerio*	Q6DHB6	Krt91	106	2.6E-05	9	3	18	50.0	5.3
243	Keratin 97	*Oreochromis niloticus*	I3JR11	Krt97	90	1.0E-03	10	2	18	48.2	5.3
238	keratin, type I cytoskeletal 13-like	*Oreochromis niloticus*	I3JS45	Krt	107	2.0E-05	8	2	16	52.6	5.3
244	keratin, type I cytoskeletal 13-like	*Oreochromis niloticus*	I3JS45	Krt	91	7.4E-04	8	3	12	52.6	5.3
227	Keratin type II (Fragment)	*Epinephelus coioides*	F6KMG4	Krt	189	1.3E-13	10	3	34	26.6	7.8
228	Keratin type II (Fragment)	*Epinephelus coioides*	F6KMG4	Krt	335	3.2E-28	11	3	39	26.6	7.8
248	Keratin type II (Fragment)	*Epinephelus coioides*	F6KMG4	Krt	340	1.0E-28	14	4	44	26.6	7.8
	Stress response										
201	Heat shock pHeat shock cognate 70 kDa	*Xiphophorus maculatus*	M3ZHB7	Hsp7c	325	3.2E-27	20	6	31	71.2	5.2
213	Protein disulfide-isomerase	*Dicentrarchus labrax*	U3LRB6	Pdi	201	8.1E-15	9	5	14	56.3	5.4
303	Glucose-regulated protein 78kDa	*Seriola lalandi dorsalis*	A0A3B4X3W4	Grp78	233	1.2E-17	30	5	42	72.4	5.0
205	Warm temperature acclimation protein (hemopexin)	*Dicentrarchus labrax*	D5A7I0	Wap65	121	8.1E-07	7	3	16	49.7	5.5
206	Warm temperature acclimation protein (hemopexin)	*Seriola dumerili*	A0A3B4U472	Wap65	91	1.8E-03	15		35	49.3	5.7
207	Warm temperature acclimation protein (hemopexin)	*Dicentrarchus labrax*	A1YTM9	Wap65	213	5.1E-16	10	4	17	49.7	5.4
208	Warm temperature acclimation protein (hemopexin)	*Seriola dumerili*	A0A3B4U472	Wap65	154	9.3E-10	23		44	49.3	5.7
209	Warm temperature acclimation protein (hemopexin)	*Seriola dumerili*	A0A3B4U472	Wap65	156	5.9E-10	24		46	49.3	5.7
210	Warm temperature acclimation protein (hemopexin)	*Seriola dumerili*	A0A3B4U472	Wap65	179	3.0E-12	26		51	49.3	5.7
211	Warm temperature acclimation protein (hemopexin)	*Seriola dumerili*	A0A3B4U472	Wap65	153	1.2E-09	24		45	49.3	5.7
222	Cathepsin D	*Oplegnathus fasciatus*	F8WPA8	Catd	113	1.0E-05	4	3	13	43.3	5.8
245	DELTA-stichotoxin-Hcr4a-like	*Seriola dumerili*	A0A3B4TN03	Shtx	81	1.8E-02	12		55	20.8	5.8

246	DELTA-stichotoxin-Hcr4a-like	*Seriola dumerili*	A0A3B4TN03	Shtx	91	1.9E-03	14		64	20.8	5.8
	Metabolism										
	Iron metabolism										
202	Serotransferrin	*Epinephelus coioides*	G9I0G6	Trfe	312	6.5E-26	11	3	12	75.9	5.7
203	Serotransferrin	*Epinephelus coioides*	G9I0G6	Trfe	236	2.6E-18	11	4	12	75.9	5.7
204	Serotransferrin	*Epinephelus coioides*	G9I0G6	Trfe	259	1.3E-20	11	3	12	75.9	5.7
212	Serotransferrin	*Epinephelus coioides*	G9I0G6	Trfe	200	1.0E-14	10	3	10	75.9	5.7
218	Transferrin (Fragment)	*Epinephelus coioides*	B9V308	Trfe	180	1.0E-12	7	3	18	33.9	5.9
	Glycolysis and central metabolism										
214	Enolase 1a, (alpha)	*Xiphophorus maculatus*	M3ZNX0	Enoa	237	2.0E-18	10	5	25	47.3	6.4
215	Alpha-enolase	*Larimichthys crocea*	A0A0F8CJR2	Enoa	358	1.6E-30	18	5	38	47.3	6.2
304	Alpha-enolase isoform X1	*Seriola dumerili*	A0A3B4TYP6	Enoa	77	4.7E-02	14	3	29	47.3	6.1
224	Pyruvate dehydrogenase (lipoamide) beta	*Oreochromis niloticus*	I3IV89	Pdhb	76	2.9E-02	6	2	20	39.9	5.9
	Nucleotide metabolism										
225	Cytosolic 5'-nucleotidase 1A-like isoform X6	*Seriola dumerili*	UPI000BBF09A1	Nt5c3a	78	3.6E-02	11		40	33.3	6.1
226	Cytosolic 5'-nucleotidase 1A-like isoform X6	*Seriola dumerili*	UPI000BBF09A1	Nt5c3a	112	1.5E-05	19		45	33.3	6.1
306	Cytosolic 5'-nucleotidase 1A-like isoform X6	*Seriola dumerili*	UPI000BBF09A1	Nt5c3a	115	7.6E-06	20		45	33.3	6.1
	Amino acid metabolism										
305	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial	*Seriola dumerili*	A0A3B4WQF4	Bckdha	157	4.70E-10	23	5	39	51.1	6.9

(a) Spot number in reference 2-DE gel (Figure 3).
(b) Protein name, organism and UniProt/UniParc ID of the first hit returned by Mascot search.
(c) Protein symbol according to ZFIN Zebrafish Nomenclature Conventions.
(d) MOWSE score based on MS data. Protein scores greater than 76 are significant ($p<0.05$).
(e) The number of times we would expect to obtain an equal or higher score by chance.
(f) PM: Number of non-redundant matching peptides. PF: Number of fragmented peptides matching the protein. SC: % of sequence coverage.

Table 3. Immune system process-related proteins (GO:0002376) found in the skin mucus samples of *S. dumerili* by gel-free LC-MS/MS

Sequence ID[(a)]	Protein name[(a)]	Sample[(b)]
A0A3B4VG93	cell wall integrity and stress response component 4-like	NP
A0A3B4TCY4	Homer scaffold protein 2	NP
A0A3B4T6N4	interferon-induced protein with tetratricopeptide repeats 1-like	NP
A0A3B4V5N9	Myotrophin	NP
A0A3B4VAY3	NACHT, LRR and PYD domains-containing protein 12-like	NP
A0A3B4UXT2	NACHT, LRR and PYD domains-containing protein 12-like isoform X1	NP
A0A3B4URJ5	NLRC4_HD2 domain-containing protein	NP
A0A3B4T4C1	Platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit	NP
A0A3B4UZ11	SAM domain and HD domain 1	NP
A0A3B4T7B0	SBDS ribosome maturation factor	NP
A0A3B4V008	SCY domain-containing protein	NP
A0A3B4U8Y6	Signal transducer and activator of transcription	NP
A0A3B4UTG8	Synuclein alpha	NP
A0A3B4TZF2	TAP binding protein (tapasin), tandem duplicate 2	NP
A0A3B4TWD5	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	NP
A0A3B4UAR5	40S ribosomal protein S24	NP, P
A0A3B4V3M0	40S ribosomal protein S27	NP, P
A0A3B4TY16	40S ribosomal protein S7	NP, P
A0A3B4VGQ5	60S ribosomal protein L27	NP, P
A0A3B4TWX6	actin-related protein 2-like	NP, P
A0A3B4U0P6	Adenylate kinase 2, mitochondrial (AK 2) (EC 2.7.4.3)	NP, P
A0A3B4T8N7	Aminopeptidase (EC 3.4.11.-)	NP, P
A0A3B4V3H8	AP-2 complex subunit alpha	NP, P
A0A3B4V303	Apolipoprotein H	NP, P
A0A3B4VIL6	apoptosis-associated speck-like protein containing a CARD isoform X3	NP, P
A0A3B4V2V2	Beta-2-microglobulin	NP, P
A0A3B4THR8	complement C3-like	NP, P
A0A3B4TZX0	complement C3-like	NP, P
A0A3B4T9V8	Complement component 6	NP, P
A0A3B4TAI6	Complement component 9	NP, P
A0A3B4TJ19	complement factor B-like	NP, P
A0A3B4VF14	complement factor H	NP, P
A0A3B4VQW2	complement factor H-related protein 1-like, partial	NP, P
A0A3B4VFP0	Copine I	NP, P
A0A3B4TKU2	Copine III	NP, P
A0A3B4TGN3	Dihydrolipoamide S-succinyltransferase	NP, P
A0A3B4U544	Dyskeratosis congenita 1, dyskerin	NP, P
A0A3B4V789	Epoxide hydrolase 2, cytoplasmic	NP, P
A0A3B4T535	fucolectin-4	NP, P
A0A3B4VEV3	Guanine nucleotide binding protein (G protein), beta polypeptide 1b	NP, P

A0A3B4VFD6	Heat shock protein 9	NP, P
A0A3B4TGS0	Heat shock protein 90, alpha (cytosolic), class B member 1	NP, P
A0A3B4T8T8	Ig-like domain-containing protein	NP, P
A0A3B4U2L6	Ig-like domain-containing protein	NP, P
A0A3B4VP40	immunoglobulin light chain precursor	NP, P
A0A3B4U4P8	Interleukin enhancer binding factor 2	NP, P
A0A3B4V2E6	Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	NP, P
A0A3B4UUE3	Methionine aminopeptidase 2 (MAP 2) (MetAP 2) (EC 3.4.11.18)	NP, P
A0A3B4T3W5	N-myc-interactor-like	NP, P
A0A3B4VB63	Nucleosome assembly protein 1-like 4a	NP, P
A0A3B4T6N7	Osteoclast stimulating factor 1	NP, P
A0A3B4U0N0	Peroxiredoxin 1	NP, P
A0A3B4T203	Potassium inwardly-rectifying channel, subfamily J, member 11, like	NP, P
A0A3B4VA55	Proteasome 26S subunit, non-ATPase 13	NP, P
A0A3B4VH49	Proteasome subunit beta (EC 3.4.25.1)	NP, P
A0A3B4VQC6	Proteasome subunit beta (EC 3.4.25.1)	NP, P
A0A3B4TPB4	Pyrin domain-containing protein	NP, P
A0A3B4TZ30	Ribosomal protein L11	NP, P
A0A3B4ULM2	Ribosomal protein L35	NP, P
A0A3B4TP28	Ribosomal protein L35a	NP, P
A0A3B4VPG5	Ribosomal protein S14	NP, P
A0A3B4VEJ4	Ribosomal protein S15a	NP, P
A0A3B4U2T8	Ribosomal protein S19	NP, P
A0A3B4TIT1	Ribosomal protein S3	NP, P
A0A3B4UFG7	Ribosomal protein, large, P1	NP, P
A0A3B4U4F9	S_100 domain-containing protein	NP, P
A0A3B4U6S9	Serine peptidase inhibitor, Kunitz type 1 a	NP, P
A0A3B4T2U3	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	NP, P
A0A3B4T847	Sorting nexin	NP, P
A0A3B4UC06	Splicing factor 3a, subunit 3	NP, P
A0A3B4TBI0	SUMO1 activating enzyme subunit 1	NP, P
A0A3B4TMU9	tolloid-like protein 1	NP, P
A0A3B4U5L7	Tropomyosin 4a	NP, P
A0A3B4UL57	tropomyosin alpha-3 chain-like isoform X2	NP, P
A0A3B4V127	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	NP, P
A0A3B4UV44	Actin-related protein 2/3 complex subunit	P
A0A3B4UCJ3	bactericidal permeability-increasing protein-like	P
A0A3B4TA33	Complement component 7b	P
A0A3B4VEZ1	complement factor H	P
A0A3B4VIA0	complement factor H	P
A0A3B4U2G6	Family with sequence similarity 49 member Bb	P
A0A3B4TL02	Ig-like domain-containing protein	P
A0A3B4UH18	immunoglobulin light chain	P
A0A3B4U7K2	Matrix metallopeptidase 9	P
A0A3B4US08	Nucleophosmin 1a	P
A0A3B4TAH0	Proteasome 26S subunit, non-ATPase 4b	P
A0A3B4TE66	Ribosomal protein L22-like 1	P

A0A3B4U618	Tropomyosin 4	P
A0A3B4U5X8	tropomyosin alpha-1 chain isoform X1	P

[(a)] Protein ID and protein name in *UniProt* database.
[(b)] Detection in non-parasitized (NP) and/or parasitized (P) experimental condition.

Table 4. Protease activity-related proteins found in the skin mucus samples of *S. dumerili* by gel-free LC-MS/MS

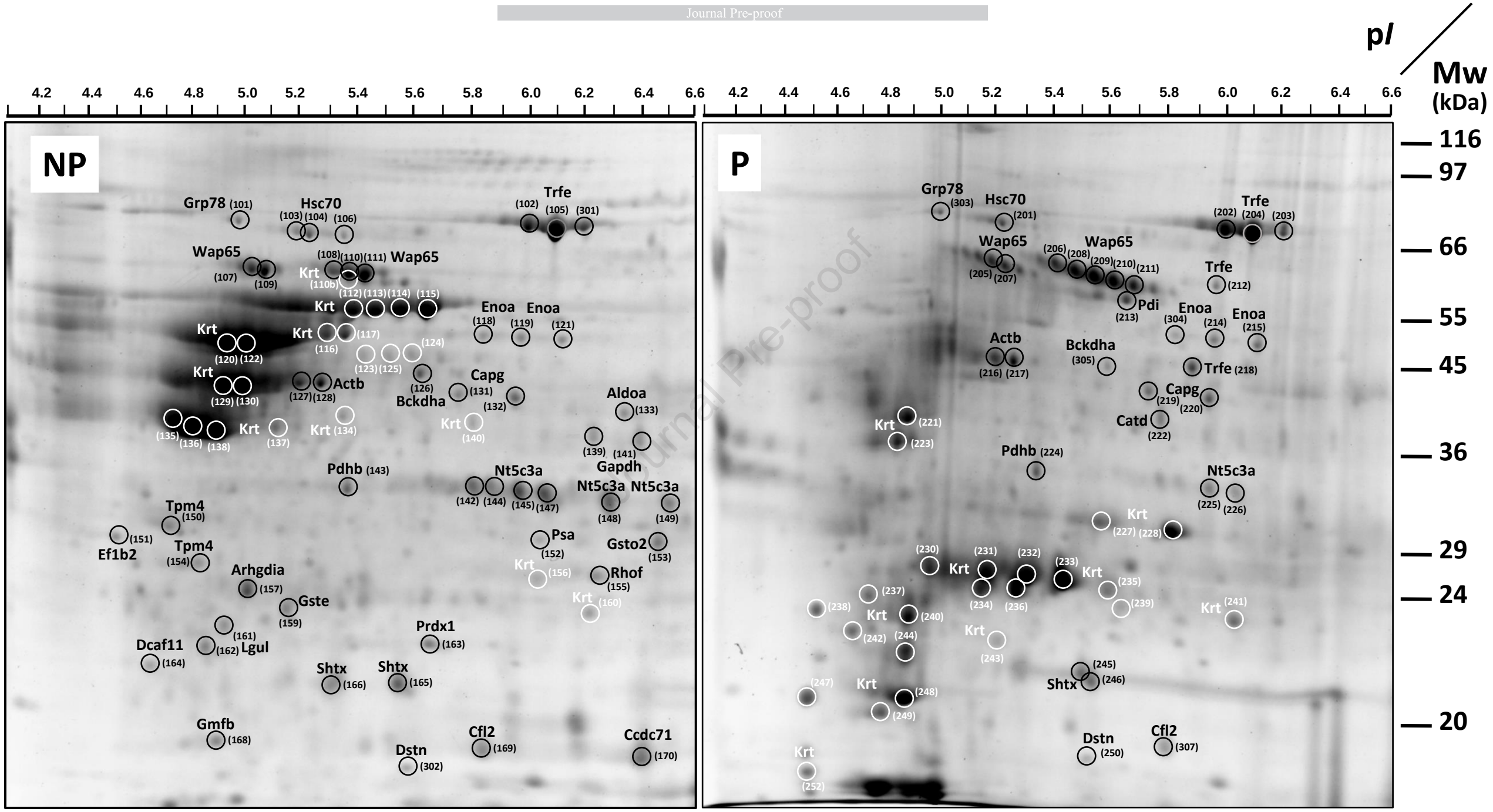
Sequence ID[(a)]	Protein name[(a)]	Sample[(b)]
A0A3B4UIT0	alpha-2-macroglobulin-like isoform X1	NP
A0A3B4UZ31	CAAX prenyl protease (EC 3.4.24.84)	NP
A0A3B4TG30	Calpain 5a	NP
A0A3B4UKC4	calpain-1 catalytic subunit-like	NP
A0A3B4V640	Carboxypeptidase (EC 3.4.16.-)	NP
A0A3B4V8Q2	Caspase 3, apoptosis-related cysteine peptidase a	NP
A0A3B4VFE7	DNA-damage inducible protein 2	NP
A0A3B4ULR2	inter-alpha-trypsin inhibitor heavy chain H3-like isoform X3	NP
A0A3B4V3V7	Metalloendopeptidase (EC 3.4.24.-)	NP
A0A3B4T5Z8	Papilin a, proteoglycan-like sulfated glycoprotein	NP
A0A3B4T2V3	Papilin b, proteoglycan-like sulfated glycoprotein	NP
A0A3B4V2Q5	Peptidase A1 domain-containing protein	NP
A0A3B4UQ15	Peptidase S1 domain-containing protein	NP
A0A3B4T4C1	Platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit	NP
A0A3B4VLM4	Proteasome subunit beta (EC 3.4.25.1)	NP
A0A3B4TG57	Signal peptidase complex catalytic subunit SEC11 (EC 3.4.21.89)	NP
A0A3B4TT41	Signal peptidase complex subunit 3 (EC 3.4.-.-)	NP
A0A3B4UTG8	Synuclein alpha	NP
A0A3B4UQR7	Ubiquitin specific peptidase 7 (herpes virus-associated)	NP
A0A3B4V2N1	Ubiquitin thioesterase (EC 3.4.19.12)	NP
A0A3B4TLB9	26S proteasome non-ATPase regulatory subunit 1	NP, P
A0A3B4U3P2	26S proteasome non-ATPase regulatory subunit 2	NP, P
A0A3B4USR2	Acylaminoacyl-peptide hydrolase	NP, P
A0A3B4T226	Alpha-1-microglobulin/bikunin precursor	NP, P
A0A3B4U4M0	Alpha-2-HS-glycoprotein 2	NP, P
A0A3B4VID4	alpha-2-macroglobulin-like	NP, P
A0A3B4VID7	alpha-2-macroglobulin-like isoform X2	NP, P
A0A3B4T7Y7	Aminopeptidase (EC 3.4.11.-)	NP, P
A0A3B4T8N7	Aminopeptidase (EC 3.4.11.-)	NP, P
A0A3B4TG61	Aminopeptidase like 1	NP, P
A0A3B4TWK0	Aspartyl aminopeptidase	NP, P
A0A3B4VNC0	ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92)	NP, P
A0A3B4UFH9	Bleomycin hydrolase (EC 3.4.22.40)	NP, P
A0A3B4V4C6	Calpain 12	NP, P
A0A3B4VGM9	Calpain 2, (m/II) large subunit b	NP, P
A0A3B4T7P8	Calpain 2, (m/II) large subunit, like	NP, P
A0A3B4VGI5	Calpain 2, (m/II) large subunit, like	NP, P
A0A3B4U4V2	Calpain 6	NP, P
A0A3B4T6A0	Calpain 9	NP, P
A0A3B4U1F6	Calpain-1	NP, P
A0A3B4UJA4	Calpain-1 catalytic subunit	NP, P
A0A3B4T7B5	calpain-2 catalytic subunit-like	NP, P
A0A3B4TD23	Calpain-9-like	NP, P
A0A3B4VKU9	calpastatin isoform X16	NP, P

A0A3B4UJ69	Carnosine dipeptidase 2	NP, P
A0A3B4UPB8	Caspase 3	NP, P
A0A3B4T8J4	Caspase 6, apoptosis-related cysteine peptidase a	NP, P
A0A3B4V6B3	Caspase a	NP, P
A0A3B4T680	Cathepsin Ba	NP, P
A0A3B4T5H6	Cathepsin C	NP, P
A0A3B4V9M3	Cathepsin D	NP, P
A0A3B4U9I5	Cathepsin H	NP, P
A0A3B4T9F3	Cathepsin S, ortholog2, tandem duplicate 1	NP, P
A0A3B4TFM1	Cathepsin Z	NP, P
A0A3B4TB78	Coagulation factor II (thrombin)	NP, P
A0A3B4THR8	complement C3-like	NP, P
A0A3B4TZX0	complement C3-like	NP, P
A0A3B4V6S2	complement C5	NP, P
A0A3B4TJ19	complement factor B-like	NP, P
A0A3B4VDW1	complement factor I-like	NP, P
A0A3B4USI9	Cystatin 14a, tandem duplicate 1	NP, P
A0A3B4URQ5	Cystatin domain-containing protein	NP, P
A0A3B4VDQ2	Dipeptidyl peptidase 3 (EC 3.4.14.4) (Dipeptidyl aminopeptidase III) (Dipeptidyl peptidase III)	NP, P
A0A3B4U157	dipeptidyl peptidase 9-like	NP, P
A0A3B4VH63	Fetuin B	NP, P
A0A3B4VJC1	Fetuin B	NP, P
A0A3B4VI55	Hyaluronan binding protein 2	NP, P
A0A3B4TIJ4	hyaluronan-binding protein 2-like	NP, P
A0A3B4USE1	Inter-alpha-trypsin inhibitor heavy chain 2	NP, P
A0A3B4VHV2	inter-alpha-trypsin inhibitor heavy chain H3-like	NP, P
A0A3B4UVK5	kininogen-1-like	NP, P
A0A3B4V6R5	Latexin	NP, P
A0A3B4UG29	Metalloendopeptidase (EC 3.4.24.-)	NP, P
A0A3B4UUE3	Methionine aminopeptidase 2 (MAP 2) (MetAP 2) (EC 3.4.11.18) (Initiation factor 2-associated 67 kDa glycoprotein) (Peptidase M) (p67) (p67eIF2)	NP, P
A0A3B4TUR3	Microseminoprotein, beta-like	NP, P
A0A3B4T767	Peptidase S1 domain-containing protein	NP, P
A0A3B4VNC7	Peptidase S1 domain-containing protein	NP, P
A0A3B4THQ6	Plasminogen	NP, P
A0A3B4VFI1	prolyl endopeptidase-like	NP, P
A0A3B4T516	Proteasome 26S subunit, ATPase 6	NP, P
A0A3B4VF73	Proteasome 26S subunit, non-ATPase 14	NP, P
A0A3B4UT73	Proteasome 26S subunit, non-ATPase 3	NP, P
A0A3B4USC0	Proteasome 26S subunit, non-ATPase 7	NP, P
A0A3B4VF98	Proteasome 26S subunit, non-ATPase 8	NP, P
A0A3B4VCN7	Proteasome activator subunit 1	NP, P
A0A3B4UAA1	Proteasome activator subunit 2	NP, P
A0A3B4T2Z5	Proteasome subunit alpha type (EC 3.4.25.1)	NP, P
A0A3B4T4S6	Proteasome subunit alpha type (EC 3.4.25.1)	NP, P
A0A3B4T6U4	Proteasome subunit alpha type (EC 3.4.25.1)	NP, P
A0A3B4T8E3	Proteasome subunit alpha type (EC 3.4.25.1)	NP, P

A0A3B4TSQ2	Proteasome subunit alpha type (EC 3.4.25.1)	NP, P
A0A3B4U7H2	Proteasome subunit alpha type (EC 3.4.25.1)	NP, P
A0A3B4UCU8	Proteasome subunit alpha type (EC 3.4.25.1)	NP, P
A0A3B4VDF2	Proteasome subunit alpha type (EC 3.4.25.1)	NP, P
A0A3B4T404	Proteasome subunit beta (EC 3.4.25.1)	NP, P
A0A3B4T8E5	Proteasome subunit beta (EC 3.4.25.1)	NP, P
A0A3B4TIT3	Proteasome subunit beta (EC 3.4.25.1)	NP, P
A0A3B4VGJ0	Proteasome subunit beta (EC 3.4.25.1)	NP, P
A0A3B4VH49	Proteasome subunit beta (EC 3.4.25.1)	NP, P
A0A3B4VQC6	Proteasome subunit beta (EC 3.4.25.1)	NP, P
A0A3B4TDQ1	Putative aminopeptidase W07G4.4	NP, P
A0A3B4TPB4	Pyrin domain-containing protein	NP, P
A0A3B4U6S9	Serine peptidase inhibitor, Kunitz type 1 a	NP, P
A0A3B4UV59	Serine peptidase inhibitor, Kunitz type 1 b	NP, P
A0A3B4TLT8	SERPIN domain-containing protein	NP, P
A0A3B4VGP7	Serpin family B member 1	NP, P
A0A3B4T2U3	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	NP, P
A0A3B4UJ25	Suppressor of tumorigenicity 14 protein homolog	NP, P
A0A3B4VEX2	Thimet oligopeptidase 1	NP, P
A0A3B4TMU9	tolloid-like protein 1	NP, P
A0A3B4TXL6	Ubiquitin carboxyl-terminal hydrolase (EC 3.4.19.12)	NP, P
A0A3B4U0N5	Ubiquitin carboxyl-terminal hydrolase (EC 3.4.19.12)	NP, P
A0A3B4UKX2	Ubiquitin carboxyl-terminal hydrolase (EC 3.4.19.12)	NP, P
A0A3B4UWX5	Ubiquitin carboxyl-terminal hydrolase (EC 3.4.19.12)	NP, P
A0A3B4V076	Ubiquitin carboxyl-terminal hydrolase (EC 3.4.19.12)	NP, P
A0A3B4V8C5	Ubiquitin carboxyl-terminal hydrolase (EC 3.4.19.12)	NP, P
A0A3B4VAW7	Ubiquitin carboxyl-terminal hydrolase (EC 3.4.19.12)	NP, P
A0A3B4U5T6	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble	NP, P
A0A3B4TQ46	ADAM metallopeptidase domain 28	P
A0A3B4T9E1	Cathepsin K	P
A0A3B4VAZ1	cathepsin L1	P
A0A3B4TCH1	Coagulation factor VIIi	P
A0A3B4UWA1	Cystatin domain-containing protein	P
A0A3B4V967	Insulin-degrading enzyme	P
A0A3B4U717	Legumain	P
A0A3B4U7K2	Matrix metallopeptidase 9	P
A0A3B4THA8	Peptidase S1 domain-containing protein	P
A0A3B4TKS5	Peptidase S1 domain-containing protein	P
A0A3B4UNL2	Peptidase S1 domain-containing protein	P
A0A3B4VDW8	Peptidase S1 domain-containing protein	P
A0A3B4VJ61	Peptidase S1 domain-containing protein	P
A0A3B4T3P6	Prolyl endopeptidase	P
A0A3B4U778	Proteasome 26S subunit, non-ATPase 11b	P
A0A3B4TAH0	Proteasome 26S subunit, non-ATPase 4b	P
A0A3B4UBH6	Proteasome subunit beta (EC 3.4.25.1)	P
A0A3B4UKV3	Proteasome subunit beta (EC 3.4.25.1)	P
A0A3B4V8I4	Proteasome subunit beta (EC 3.4.25.1)	P
A0A3B4UV22	Serpin peptidase inhibitor, clade C (antithrombin), member 1	P
A0A3B4UCS5	TIMP metallopeptidase inhibitor 2b	P

A0A3B4TQX4	Tripeptidyl peptidase 2	P
A0A3B4VBW1	ZnMc domain-containing protein	P

[(a)] Protein ID and protein name in *UniProt* database.
[(b)] Detection in non-parasitized (NP) and/or parasitized (P) experimental condition.



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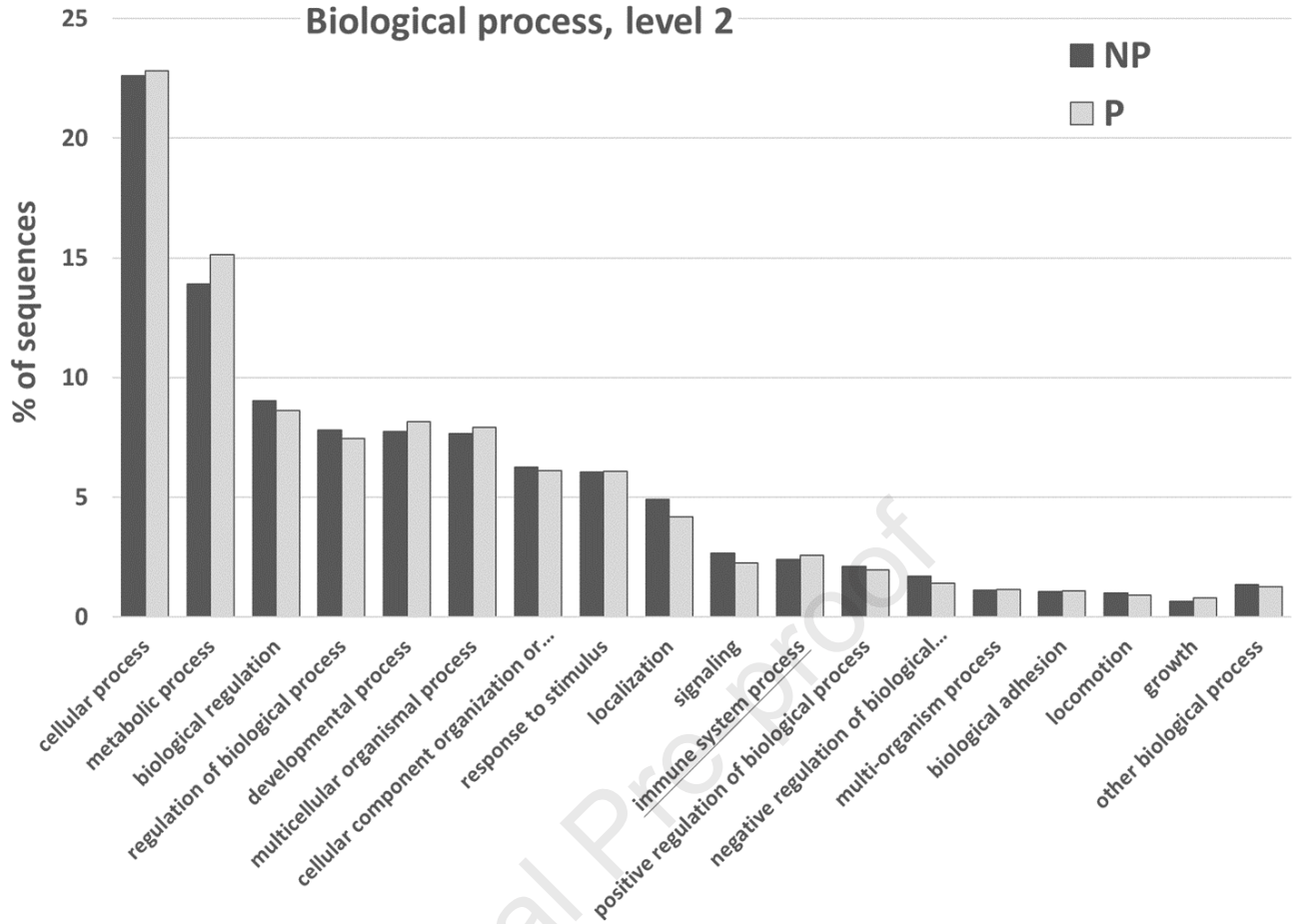

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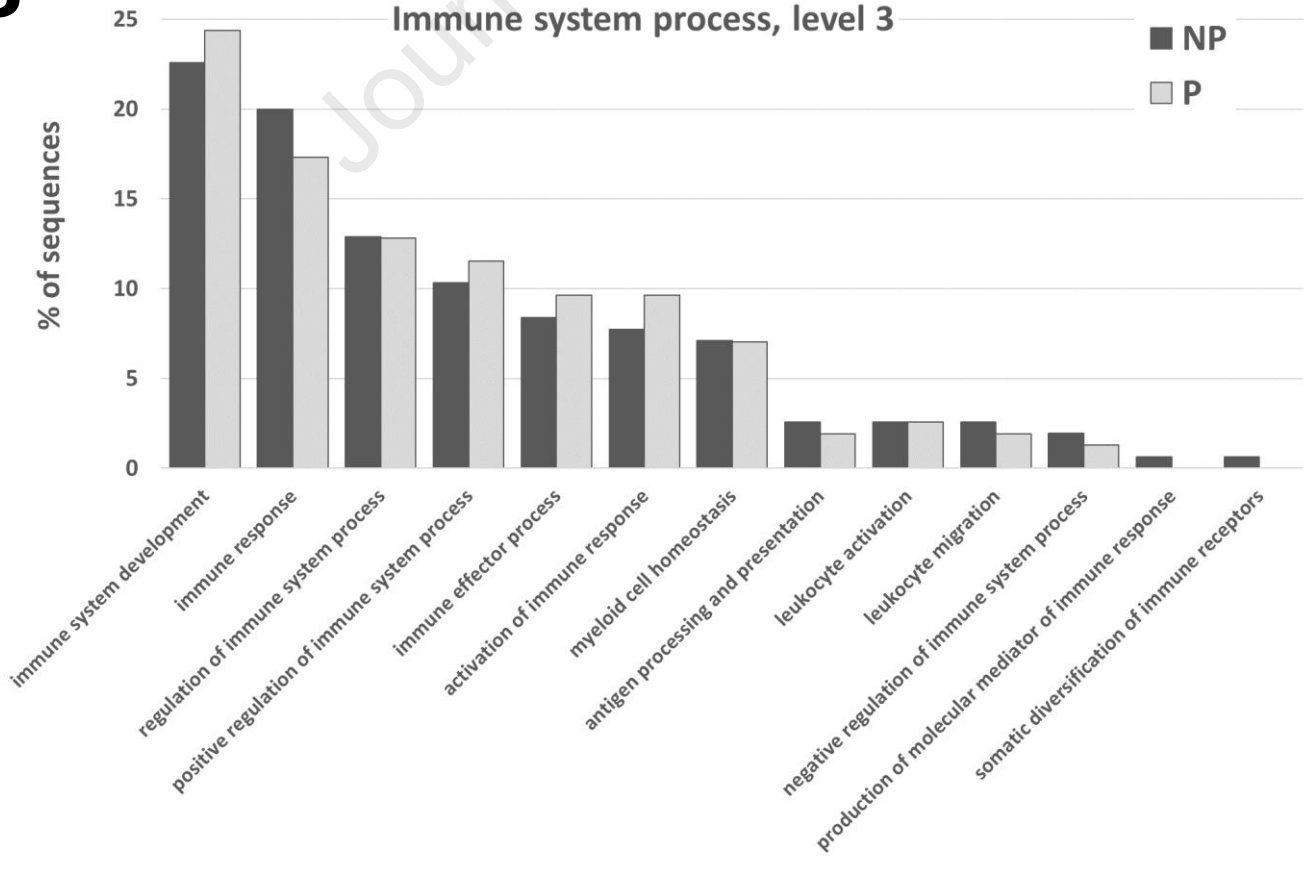

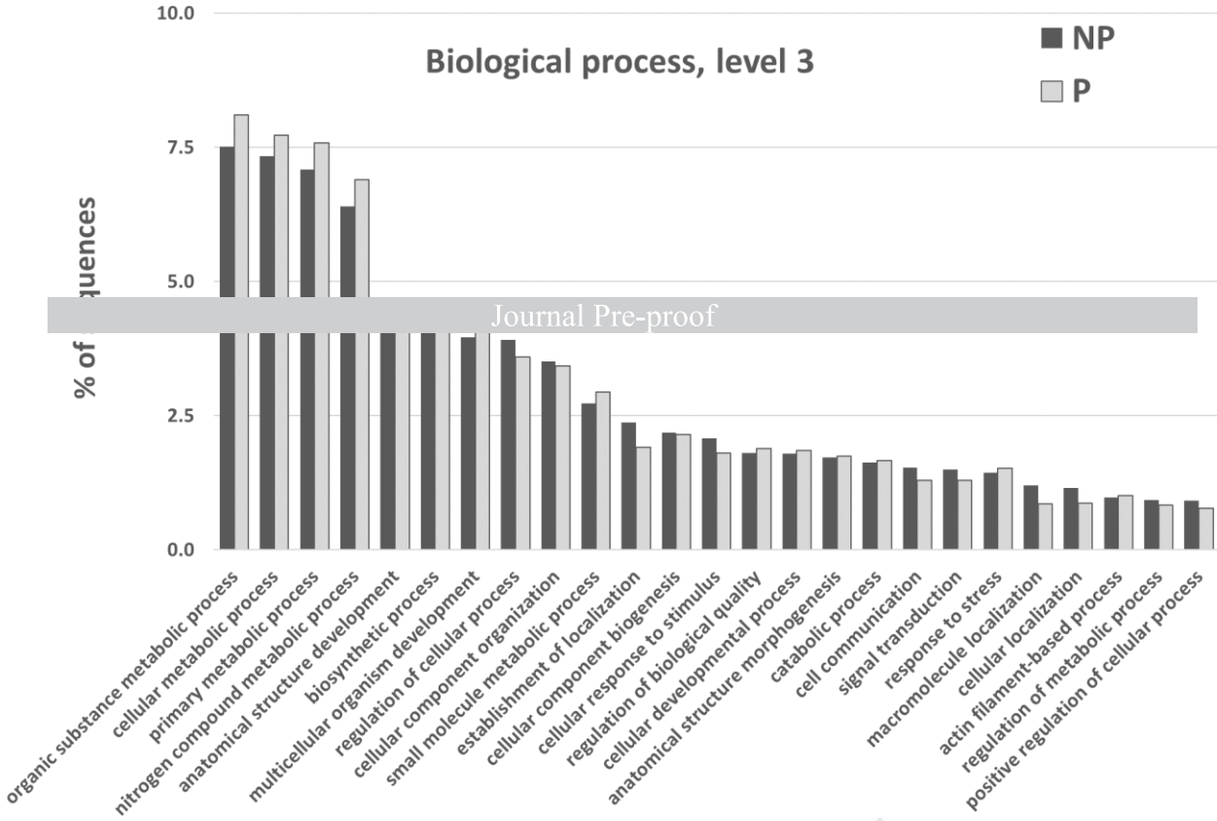

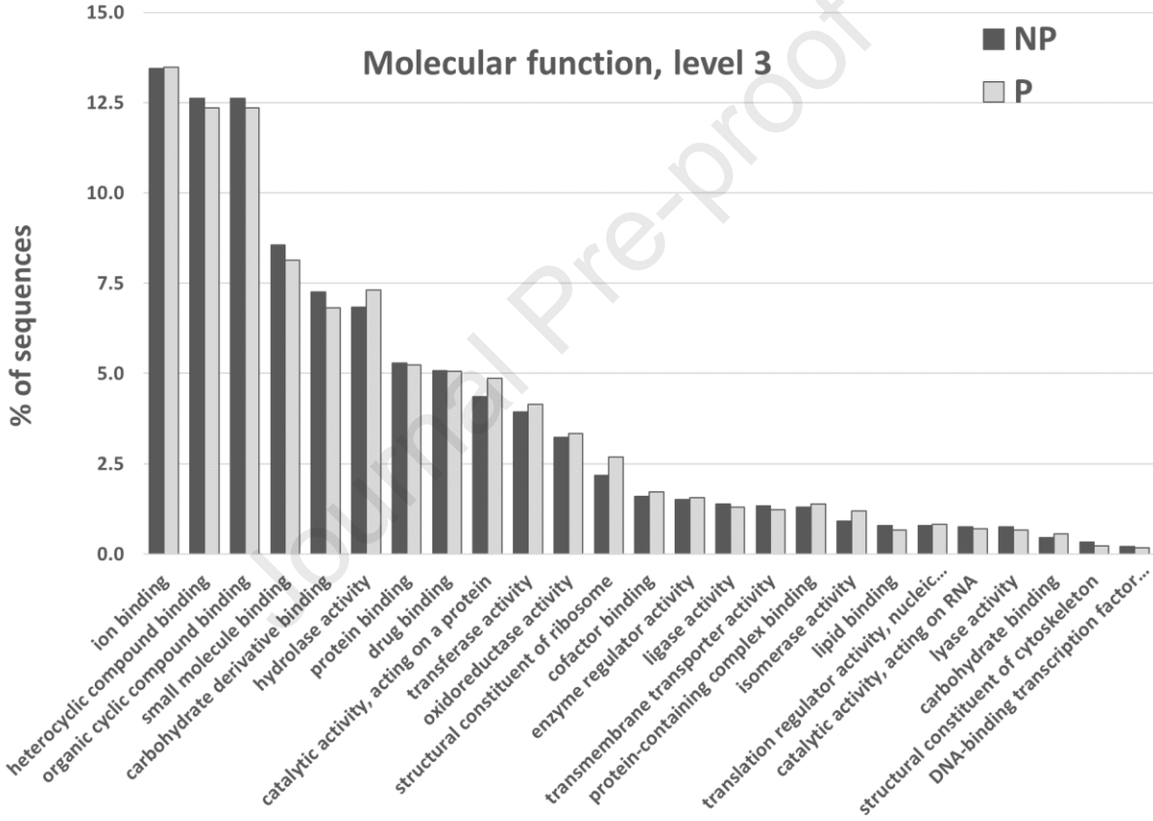

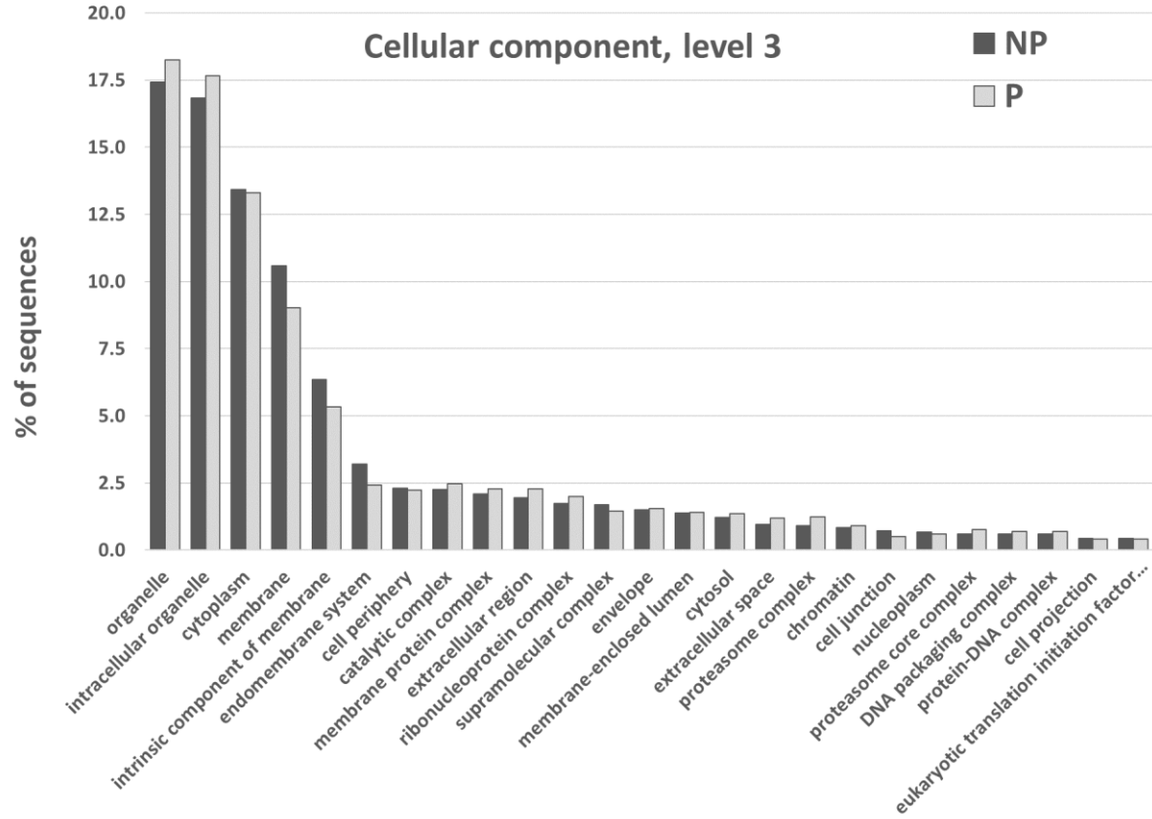

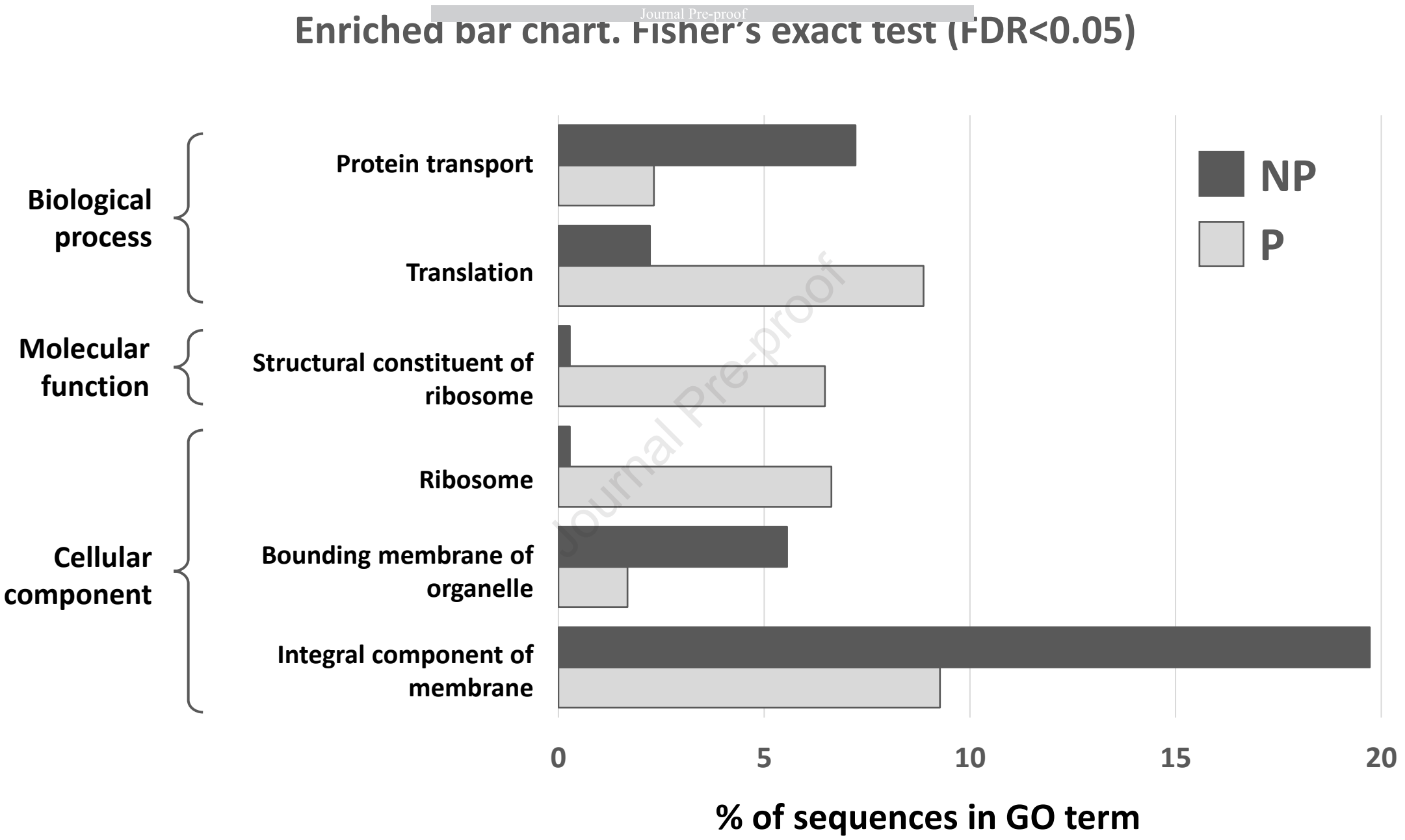

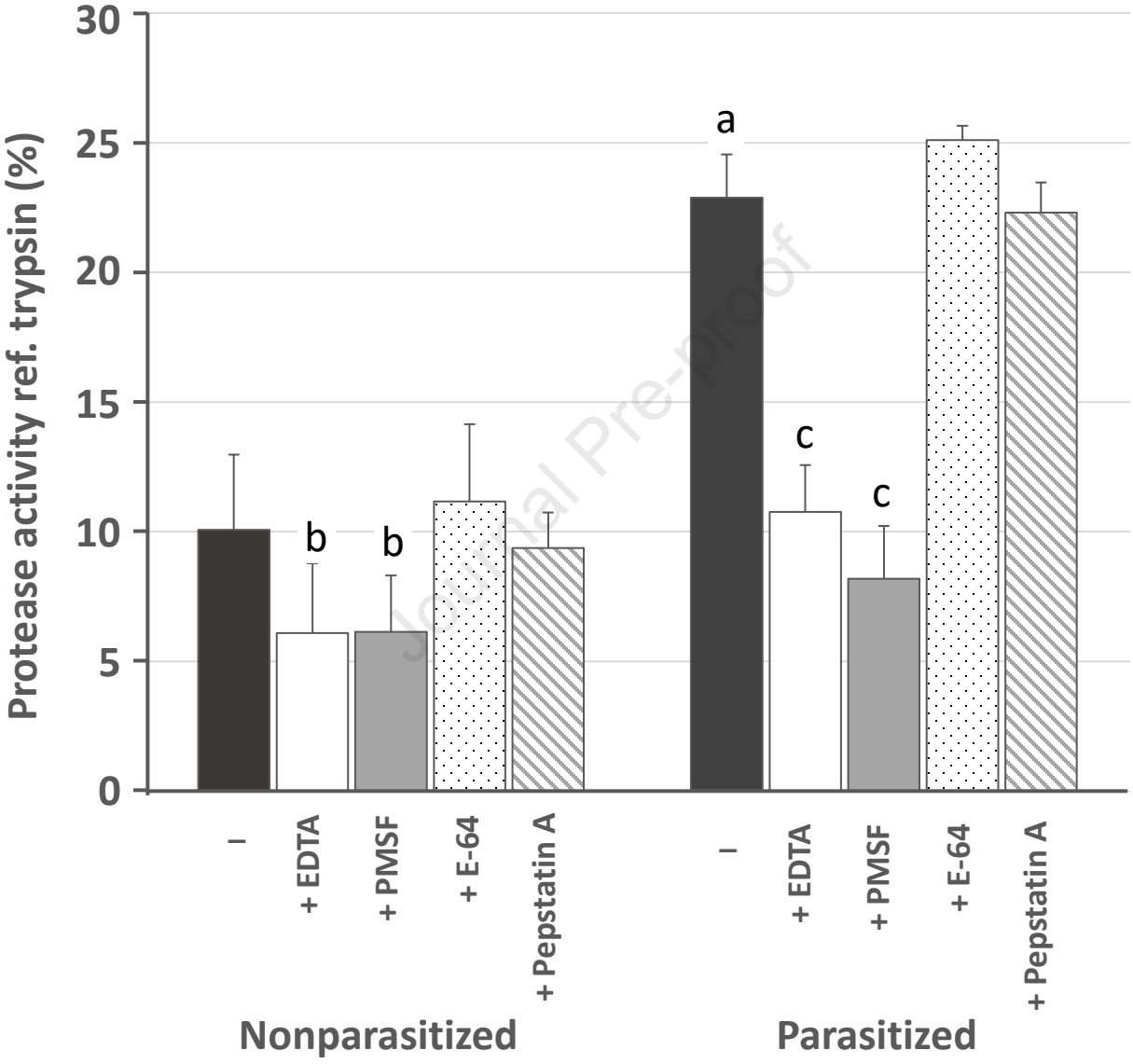

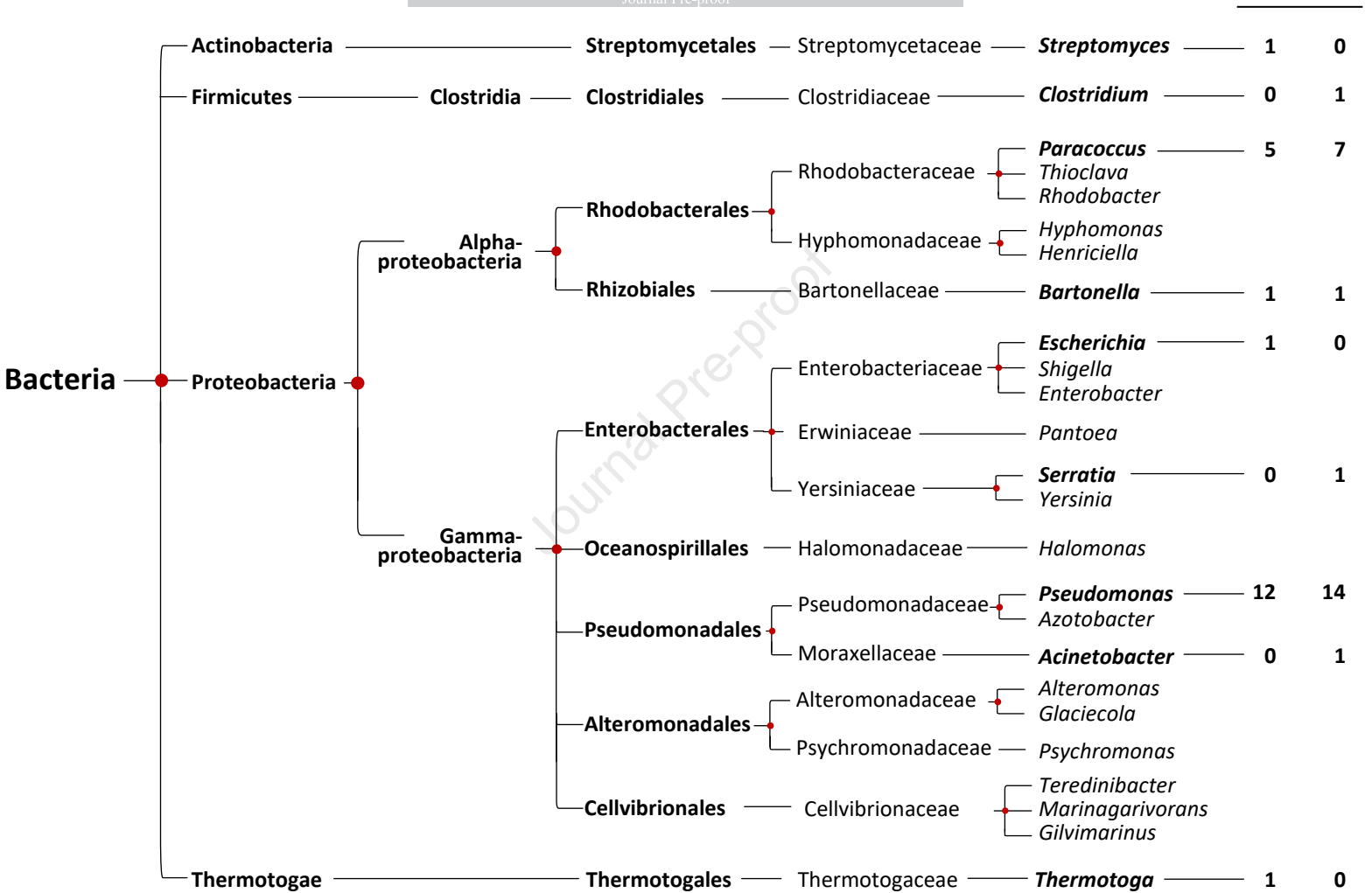

Highlights

- The skin mucus proteome of *Seriola dumerili* was analyzed for the first time.
- The effect of *Neobenedenia girellae* on the proteome, proteases, and the microbiota was assessed.
- Ribosomal proteins were overrepresented in the skin mucus of parasitized fish.
- 2-DE proteomics reveals that specifically keratins were cleaved in parasitized fish.
- The mucus of infected fish showed high metal-dependent protease and serine protease activities.