

Research Article

Cite this article: Borges Osório J, Gosch Berton G, Pereira LM, Potriquet J, Cândido RRF, Mulvenna J, Jones MK, Graeff-Teixeira C, Morassutti AL (2025) Infection with *Angiostrongylus cantonensis* reveals up- and down-regulation of the protein profile in the mucus of infected slugs. *Parasitology*, 1–4. <https://doi.org/10.1017/S0031182025000435>

Received: 17 November 2024

Revised: 4 March 2025

Accepted: 11 March 2025





Keywords:

biomarkers; host–parasite relationship; mollusc infection; non-invasive diagnosis; proteomics

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Infection with *Angiostrongylus cantonensis* reveals up- and down-regulation of the protein profile in the mucus of infected slugs

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Abstract

Host–parasite adaptation is crucial for evolutionary success of a parasite. The nematode *Angiostrongylus cantonensis* has a heterogenic life cycle involving molluscs as intermediate hosts and rats as definitive hosts. Several mollusc species are susceptible hosts of *A. cantonensis*, allowing the development of first-stage larvae (L1) into third-stage larvae (L3). Changes in the metabolism of infected molluscs have been demonstrated, disturbing regular routes and inducing host defence mechanisms. This study aimed to identify changes in the proteomic content of *Phyllocaulis* spp. mucus during *A. cantonensis* infection. Proteins were extracted from the mucus samples of infected and non-infected slugs and identified using liquid chromatography–tandem mass spectrometry. We found 26 up-regulated and 15 down-regulated proteins in infected slugs compared to non-infected slugs. Protein profiles are promising markers of parasite infection, and a better understanding of proteomic profiles during infection may help inform *in vivo* studies and promote new tools for the non-invasive identification of infected hosts.

Introduction

Understanding the interactions between parasites and their hosts is fundamental to elucidating the mechanisms of disease transmission and developing control strategies. The nematode *Angiostrongylus cantonensis*, the causative agent of eosinophilic meningitis in humans, has a complex life cycle involving molluscs as intermediate hosts and rats as definitive hosts (Wang et al., 2012). Various slug and snail species can serve as intermediate hosts, supporting the development of first-stage larvae into infective third-stage larvae (Caldeira et al., 2007; Chan et al., 2015).

Molluscs from the Veronicellidae family, such as *Phyllocaulis* spp., are known to be susceptible to *A. cantonensis* and are utilized for life cycle maintenance in laboratory settings (Graeff-Teixeira et al., 1993; Morassutti et al., 2014). Molluscs secrete complex mucus rich in glycoproteins, enzymes and antimicrobial peptides, which play roles in locomotion, defence and environmental adaptation (Qvarnstrom et al., 2007). The biological potential of molluscan mucus has been explored for therapeutic and cosmetic applications because of its antimicrobial and anti-inflammatory properties (Tsvetanova et al., 2020; Rizzi et al., 2021).

Infection with *A. cantonensis* can modulate the immune response and metabolic pathways of molluscs, leading to alterations in protein expression and oxidative stress responses (Mendes et al., 2020). Investigating these proteomic changes could reveal biomarkers for infection and enhance our understanding of host–parasite dynamics.

This study aimed to analyse the proteomic alterations in the mucus of *Phyllocaulis* spp. experimentally infected with *A. cantonensis*, to identify potential biomarkers for infection and to contribute to the development of non-invasive diagnostic tools.

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Materials and methods

Collection of molluscs and maintenance of the parasite lifecycle

Phyllocaulis spp. slugs were collected in Porto Alegre, Brazil, and reared in laboratory conditions at Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS). The slugs were maintained in plastic containers with garden soil and fed fresh vegetables *ad libitum*.

Phyllocaulis spp. infection and collection of mucus

Ten slugs were divided into 2 groups: a control group (CG) and an infected group (IG). IG was exposed to approximately 6,000 first-stage larvae of *A. cantonensis* from the Vila Fátima isolate (Cognato et al., 2013). After a 30-day infection period, mucus samples were collected by gently scraping the slug bodies with a moistened swab. The samples were stored at -80°C until analysis. Infection was confirmed by artificial digestion of slug tissues and microscopic examination of the larvae.

Proteomic experiment

Protein extraction from mucus samples was performed using a modified filter-aided sample preparation method (Potriquet et al., 2017). Proteins were reduced, alkylated and digested with trypsin. The peptides were purified and concentrated using ZipTip C18 pipette tips.

Liquid chromatography–tandem mass spectrometry was conducted using a Shimadzu Prominence Nano High-Performance Liquid Chromatography coupled with a TripleTOF 5600+ mass spectrometer (AB SCIEX). Data acquisition was performed using Information Dependent Acquisition, and data analysis was performed using Analyst 2.0 software.

Protein identification was accomplished by searching spectral data with 2 independent software programmes: X! Tandem and ProteinPilot. Our workflow utilized X! Tandem to search gastropod protein sequences from GenBank and *Angiostrongylus* transcriptomic data, comparing experimental mass spectra with theoretical spectra generated from known protein sequences. A minimum of 2 unique peptides, each containing at least eight amino acids, was required to confidently confirm a protein's identity (Nakayasu et al., 2021). In parallel, ProteinPilot employed curated reference databases from UniProtKB and SwissProt, adding a further validation layer. This dual approach enhanced the robustness of protein identifications by cross-validating outputs from distinct data sources.

Quantification was performed using Sequential Window Acquisition of All Theoretical Fragment Ion Spectra (SWATH), enabling precise and reproducible measurements of protein abundance across samples (Messner et al., 2021). SWATH captures ion fragments in sequential mass windows, ensuring a comprehensive and unbiased acquisition of fragmentation data, even in complex mixtures. Stringent statistical thresholds were applied to validate our findings: only proteins with a false discovery rate of $\leq 2\%$ and a *P*-value of ≤ 0.05 were considered significant. These rigorous filters ensure that only high-confidence, statistically robust protein identifications are reported.

Results

A total of 103 proteins were identified in the mucus samples of infected and non-infected *Phyllocaulis* sp. slugs. In the IG,

five proteins matched the *Angiostrongylus* transcriptomic data: actin, ATP synthase subunit alpha, ATP synthase subunit beta, AAA family ATPase CDC48 subfamily and the ribosomal protein S11 domain. An additional 41 proteins were matched to the *Biomphalaria glabrata* reference sequences.

Our study demonstrated that *A. cantonensis* infection induces significant proteomic alterations in the mucus of *Phyllocaulis* sp. slugs. Similar proteomic modifications have also been observed in another mollusc, *B. glabrata*, supporting the notion of conserved host-response mechanisms across molluscan species (Mendes et al., 2020). Among the proteins highlighted in our study, an F-BAR (Fes/CIP4 Homology–Bin/Amphiphysin/Rvs) domain-containing protein was particularly notable. Using NCBI (National Center for Biotechnology Information) BLASTp (*E*-value threshold of 10^{-20}), we identified homologous sequences bearing the F-BAR domain in multiple *B. glabrata* proteins, suggesting the presence of this domain in the *B. glabrata* proteome. F-BAR proteins have been implicated in active cellular responses, including membrane remodelling and reparative processes (Itoh et al., 2005; Frost et al., 2009), hinting at a possible mechanism by which these gastropod hosts adapt to *A. cantonensis* infection.

Key up-regulated proteins included glycolytic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as well as proteins involved in immune responses, such as von Willebrand factor type A and epidermal growth factor (EGF)-like calcium-binding domain proteins. Down-regulated proteins include histone H4, indicating potential changes in gene expression. Additionally, 2 proteins, the low-complexity protein and globin, were found to be both up- and down-regulated simultaneously (Figure 1). This observation may indicate the presence of distinct isoforms. We recommend further molecular sequence analyses of these proteins to clarify these findings.

Discussion

Our study demonstrated that *A. cantonensis* infection induced significant proteomic alterations in the mucus of *Phyllocaulis* sp. slugs. The up-regulation of the F-BAR domain-containing protein suggests an active cellular response involving membrane remodelling and repair processes (Itoh et al., 2005; Frost et al., 2009). Similar findings in *B. glabrata* infected with *A. cantonensis* support the notion of conserved immune response mechanisms across mollusc species (Mendes et al., 2020).

The down-regulation of histone H4 indicates possible epigenetic modifications that affect gene expression in response to infection (Kornberg and Lorch, 1999; Saha et al., 2006). This aligns with previous observations in *B. glabrata*, suggesting that histone modifications may play a role in molluscan defence against parasites.

The up-regulation of glycolytic enzymes, such as PEPCK and GAPDH, reflects metabolic adjustments to meet increased energy demands during infection (Hanson and Garber, 1972; Sirover, 1999). Enhanced glycolysis may provide the necessary ATP for immune response and tissue repair.

Identifying proteins such as von Willebrand factor type A and EGF-like calcium-binding domain proteins underscores their potential as biomarkers for *A. cantonensis* infection. These proteins are involved in coagulation and cell signalling pathways, which are critical for immune functions (Ober and Litington, 2001; Ruggeri, 2003).

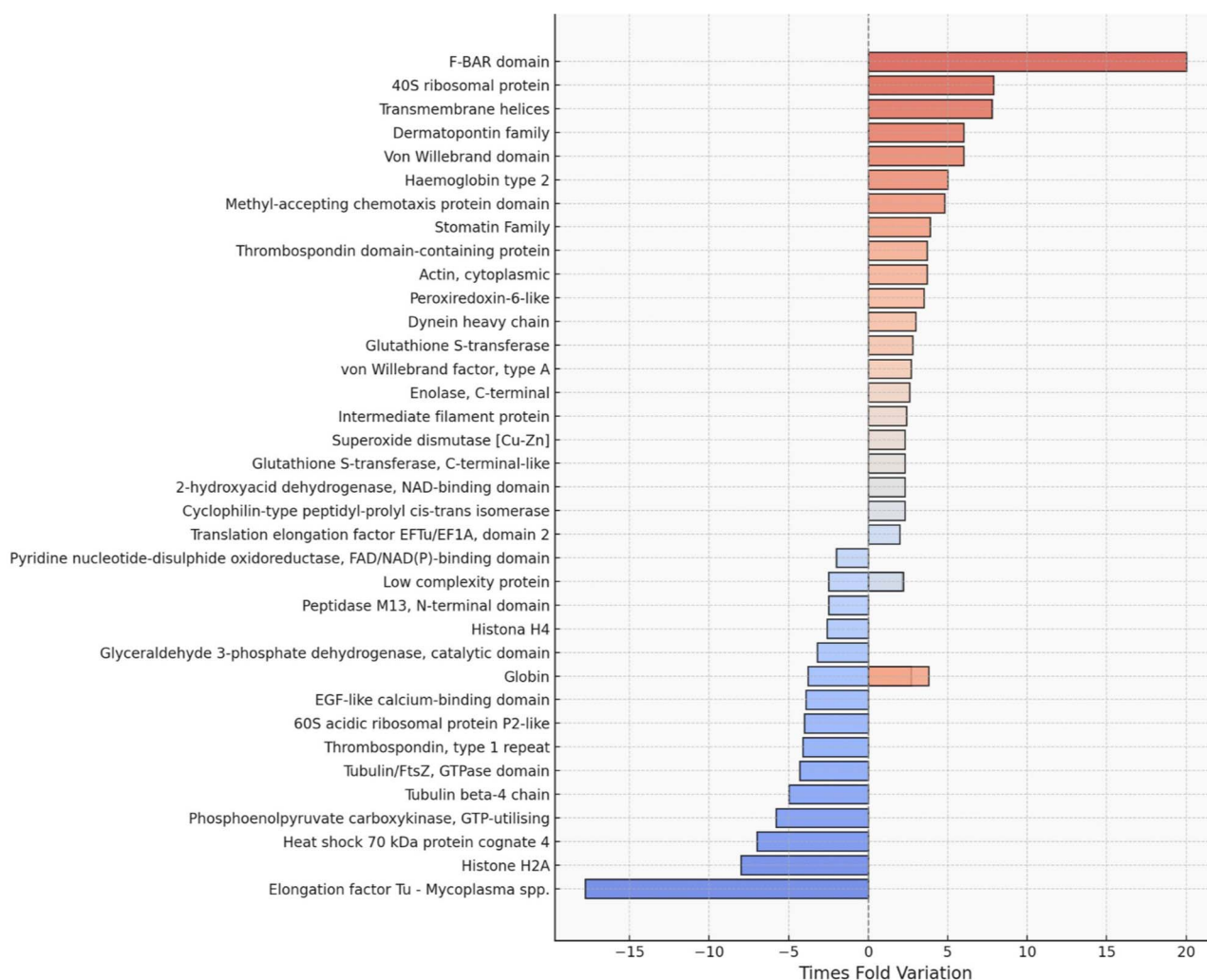


Figure 1. Protein expression in the mucus of *Phyllocaulis* spp. slugs infected with *Angiostrongylus cantonensis*.

The significant down-regulation of elongation factor Tu from *Mycoplasma* sp. suggests a functional interplay between helminth infection and the mollusc microbiota. *Mycoplasma* sp. has been detected in the microbiota of both *A. cantonensis*-infected and non-infected molluscs (Osório et al., 2020). This observation may be explained by immune-mediated processes that influence bacterial adhesion (Jonák, 2007).

Detecting *Angiostrongylus*-derived proteins in slug mucus, such as actin and ATP synthase subunits, may result from larval degradation or immune responses, leading to larval death (Bonetti and Graeff-Teixeira, 1998; Lange et al., 2017). Thus, these proteins may serve as direct indicators of infection.

The presence of lipopolysaccharide-binding proteins, such as BgLBP/BPI1 and LBP/BPI1.2, exclusively in infected mucus samples, indicates an activated innate immune response against gram-negative bacteria (Bingle and Craven, 2004; Baron et al., 2013). This may reflect a broader immune activation in response to parasitic infections.

The proteomic changes observed in the mucus of *Phyllocaulis* sp. infected with *A. cantonensis* highlight potential biomarkers for infection and provide insights into host immune and metabolic responses. The identification of up-regulated proteins involved in

membrane remodelling, energy metabolism and immune function suggests active host defence mechanisms.

Future studies should focus on validating these biomarkers in larger populations and diverse mollusc species, studying possible changes among species from the same genus. Investigating the molecular pathways underlying these proteomic shifts could enhance our understanding of host–parasite interactions and support the development of non-invasive diagnostic tools for monitoring *A. cantonensis* transmission.

Acknowledgements. We thank the High-Performance Computing Laboratory (Laboratório de Alto Desempenho, LAD/PUCRS) for providing access to computational resources for the high-throughput sequence analyses.

Financial support. This work was supported by the Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico do Brasil (CNPq, grant number 401904/2013-0) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior 'Edital 32/2010'. C.G.-T. received a CNPq PQ 1D fellowship (grant number 307005/2014-3).

Competing interests. The authors declare none.

Ethical standards. Experiments were conducted following ethical guidelines approved by the Animal Ethics Committee of the PUCRS (Brazil; registration number 14/00399) and the Animal Ethics Committee of the QIMR Berghofer Medical Research Institute (Australia; project number P1468).

Use of artificial intelligence tools. During the preparation of this work, the authors used artificial intelligence tools in order to review the manuscript and improve grammar and clarity. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

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