NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

MARTA DA CONCEIÇÃO RAMALHO GATO

BSc in Biochemistry

EXPRESSION AND CHARACTERISATION OF CYSTEINE-RICH SECRETORY PROTEINS IN A MARINE GASTROPOD

MASTER IN BIOCHEMISTRY

NOVA University Lisbon

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"Nada é impossível"

ACKNOWLEDGMENTS

"Aqueles que passam por nós, não vão sós, não nos deixam sós.
Deixam um pouco de si, levam um pouco de nós."
Antoine de Saint-Exupéry

Se há algo que aprendi nesta vida, é agradecer porque nada disto seria possível sem a força, carinho, apoio e dedicação de algumas pessoas que de uma forma ou de outra são responsáveis por estas minhas conquistas. Assim, gostaria de agradecer:

Aos meus orientadores, o Professor Pedro Costa e o Professor Mário Diniz. Primeiramente, obrigada por terem aceitado fazer este projeto comigo, por terem-me recebido de braços abertos. Professor Pedro, obrigada por todo o apoio, paciência, disponibilidade e preocupação em me proporcionar as condições para que eu fizesse o meu percurso académico "normal". Obrigada também pelo constante incentivo e motivação em não duvidar das minhas capacidades. Professor Mário, também obrigado por tudo.

À equipa do SeaTox, nomeadamente à Cátia, Mariaelena, Ana, Carla e Carolina. Não poderia pedir melhor equipa. Obrigada por toda a ajuda, apoio e orientação que me deram ao longo deste ano. Contribuíram tanto para que eu crescesse profissionalmente, aprendi muito com cada uma de vocês. Obrigada também por todos os momentos divertidos, gargalhadas e convívio!

À Sónia Campos, Telma Luís e Inês Cabral, as "prendas-amigas" que o SeaTox me deu. Nada disto seria a mesma coisa sem vocês. Obrigada!

À intérprete Rita Valente, por ter sido a minha "ponte de comunicação" muitas vezes ao longo destes dois anos.

Aos meus colegas da Faculdade, especialmente à Ana Carvalho. O meu "pilar" do mestrado. Um obrigado não chegará para agradecer tudo o que fizeste por mim.

À Catalina Cepeleaga, a minha principal companheira durante esta etapa da minha vida académica e certamente vai ser para a vida. Obrigada pela amizade, por me fazeres a rir de tudo e de nada, por me dares a mão, por seres a rede quando parece faltar o chão, por tudo o que de bom trazes à minha vida!

À Joana Veríssimo, uma das melhores pessoas que já conheci. Obrigada por fazeres parte da minha vida, por seres a pessoa fantástica que se coloca sempre no lugar dos outros, por tudo!

Aos meus amigos de sempre, a minha família de coração, a minha âncora, a minha casa, o meu porto de abrigo. Um obrigado especial à Daniela Alvarinho. Obrigada por teres-me feito sorrir naqueles dias em que achava que já não era capaz, por teres-me feito a acreditar em mim quando duvidava de tudo, por teres-me mostrado que era capaz e que ia dar tudo certo.

À Rita Botas, por fazer parte da minha vida há tantos anos e por ser uma pessoa fantástica que está sempre ao meu lado, mesmo à distância, a apoiar-me, a mostrar-me que sou capaz de tudo. Obrigada por cada palavra-amiga, cada força e cada abraço-casa que me dás sempre.

À Jéssica e à Nádia, simplesmente por existirem na minha vida. Por iluminarem a minha vida, por ouvirem sempre os meus medos e dúvidas, por não me deixarem desistir de acreditar, por me fazerem a acreditar que não há nenhum obstáculo nesta vida que não consiga ultrapassar. Por tudo.

Por fim mas não menos importante, aos meus pais, ao meu irmão e à minha Família, com F maiúscula. Por serem o meu pilar, por estarem sempre lá para mim, a vibrar as vitórias como fossem as suas, a chorar nas derrotas como fossem as suas. Obrigada pela força que me dão para lutar por tudo o que ambiciono, obrigada por me ajudarem a subir as montanhas da vida, por tudo! Um obrigado especial para ti, mãe. Sei que não foi fácil para ti quando descobriste que era surda e quando te disseram que provavelmente iria ficar muito “limitada” no futuro mas, ainda assim, não deixaste que isto te abatesse, que isto me definisse. Nunca desististe de mim. Aprendeste a LGP logo quando eu tinha alguns meses de vida só para conseguires comunicar comigo, sempre lutaste pelos meus direitos, para que eu fizesse a vida normalmente, sempre acreditaste em mim, sempre estiveste do meu lado, para me aplaudir pelas minhas conquistas, para tudo! Não chegam as palavras. Obrigada.

Mesmo que a palavra "obrigado" signifique tanto não expressará por inteiro o quanto estou grata pelo que fizeram por mim, por vos ter cruzado na minha vida. Obrigada!

ABSTRACT

Cysteine-rich secretory proteins (CRISPs) are highly bioactive natural products, bearing potential biotechnological and biomedical applications, from anti-oxidants to biocides. They can be found in a wide range of organisms, with emphasis on venom-secreting animals. Still, little is known about CRISPs in marine invertebrates despite their immense biodiversity and relevance for the bioprospecting for novel natural products. These proteins play important roles in many organs, from digestion to toxins for preying and defence against predators and competitors. Even though venoms, such as those secreted by marine cone snails, contain some of the best-studied cysteine-rich toxins, and indeed already giving rise to approved painkiller drugs, most marine invertebrate venoms remain uncharacterized, as well as their individual components. *Nucella lapillus* (L. 1758) is a common gastropod in W Europe that is suspected to secrete immobiliser toxins related to cysteine-rich proteins. The present work aimed at comparing the secretion, presence and bioreactivity of cysteine-rich proteins between foot, salivary gland, digestive gland and gonad. Histological results demonstrate that the salivary gland is structurally compatible with the secretion of enzymes and toxins. The salivary gland still contains the highest amount and diversity of proteins, with the largest fraction within 20-40 kDa, which is compatible with the proteins under study. In turn, isolating proteins from digestive gland and gonads was problematic, in large part due to the presence of peptidases. However, it was in these organs that the highest content of thiols was found. *Ex-vivo* bioassays with mussel gills revealed the bioreactivity of protein extracts from the salivary gland and foot, their secretions caused some morphological changes, such as increased haemocytes and apoptosis. These results indicate that these two organs are a promising target for bio-prospecting.

Keywords: Marine biotechnology, Mollusc, Bioprospecting, Bioactives, Proteome

RESUMO

As proteínas secretoras ricas em cisteína (CRISPs) são produtos naturais altamente bioativos, com potenciais aplicações biotecnológicas e biomédicas, de antioxidantes a biocidas. Podem ser encontradas em diversos organismos, com ênfase nos animais secretores de veneno. Ainda assim, pouco se sabe sobre CRISPs em invertebrados marinhos, apesar da sua grande biodiversidade e relevância para a bioprospecção de novos produtos naturais. Essas proteínas desempenham papéis importantes em muitos órgãos, desde a digestão até toxinas para ataque e defesa contra predadores e competidores. Embora os venenos, como os secretados por caracóis marinhos, contenham algumas das toxinas ricas em cisteína mais estudadas e, na verdade, já deram origem a medicamentos analgésicos aprovados, a maioria dos venenos de invertebrados marinhos permanece descaracterizada, assim como os seus componentes individuais. *Nucella lapillus* (L. 1758) é um gastrópode comum no oeste da Europa, suspeito de secretar toxinas imobilizadoras relacionadas com as proteínas ricas em cisteína. O presente trabalho teve como objetivo comparar a secreção, presença e bioreatividade de proteínas ricas em cisteína entre pé, glândula salivar, glândula digestiva e gônada. Os resultados histológicos demonstram que a glândula salivar é estruturalmente compatível com a secreção de enzimas e toxinas. A glândula salivar contém ainda a maior quantidade e diversidade de proteínas, com a maior fração entre 20-40 kDa, o que é compatível com as proteínas em estudo. Por outro lado, isolar proteínas da glândula digestiva e gônadas revelou-se problemático, em grande parte devido à presença de peptidases. No entanto, foram nestes órgãos que se encontraram o maior teor de tióis. Os bioensaios *ex-vivo* com a brânquia dos mexilhões revelaram a bioreatividade de extratos proteicos da glândula salivar e do pé, as suas secreções causaram algumas alterações morfológicas, como o aumento de hemócitos e de apoptoses. Esses resultados indicam que esses dois órgãos são um alvo promissor para bioprospecção.

Palavras-chave: Biotecnologia marinha, Molusco, Bioprospecção, Bioativos, Proteoma

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LIST OF ABBREVIATIONS

Ac-DEVD-pNA	Acetyl-Asp-Glu-Val-Asp p-nitroanilide
ADG	Adipogranular cell
APS	Ammonium persulfate
CA	California
CNS	Neutral nervous system
CRISP	Cysteine-rich secretory protein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPA	3,4-dihydroxyphenylalanine
DPX	Dibutylphthalate Polystyrene Xylene
DTNB	Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)]
DTT	Ditiotreitol
EDTA	Ethylenediamine tetraacetic acid
GSH	Glutathione
GST	Glutathione S-transferase
H&E	Haematoxylin and eosin staining
HMPA	High melting point agarose
LMPA	Low melting point agarose
MA	Massachusetts
MQ	Milli-Q water
MO	Missouri
MT	Metallothionein
MW	Molecular weight
PBS	Phosphate buffered saline
pNA	p-nitroanilide
RER	Rough endoplasmic reticulum
RyR	Ryanodine receptors
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SH	Sulfhydryl group
TAE	Tris-Acetic Acid-EDTA
TBT	Tributyltin
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
UK	United Kingdom
USA	United States of America
VC	Vesicular cell
VT	Vermont

1. INTRODUCTION

1.1. Marine biotechnology

Having emerged in the 1960s to 1970s, marine biotechnology, a major part of "blue biotechnology", is based on the same scientific and technological principles as other fields of biotechnology (Rotter et al., 2020). The differentiating factor is the origin of processed bioproducts. In this case, biotechnology makes use of biological resources from the marine environment, applying the latest biological knowledge and techniques to develop or modify products and processes to meet various societal challenges, from food safety to new therapies (Rotter et al., 2020; Vieira et al., 2020).

The marine environment represents more than 70% of the planet's surface and over 90% of the biosphere. It is the global habitat with the greatest biological and biochemical diversity, which results from the most ancient radiation of life and from adaptation to the most diverse ecosystems on Earth, from estuaries and mangroves to deep-sea vents (Brennecke et al., 2018; Rotter et al., 2020). In the last few decades, the oceans' biodiversity has been responsible for the growing interest on the discovery of novel marine biopolymers and bioactives with potential applications in several areas, including biomedicine and drug discovery (Brennecke et al., 2018). Marine organisms have an infinity of bioactive properties, whose structure and chemical characteristics generally do not exist in terrestrial animals and these compounds can be divided into various structural types, including peptides, polysaccharides, sterols, alkaloids and unsaturated fatty acids (Barreca et al., 2020; Lu et al., 2021). This is due to the fact that organisms compete for survival in inhospitable environments, with extreme and differentiated conditions of pressure, temperature, pH, lighting, salinity and oxygenation, in addition to nutrient limitation and space limitation/competition, and as a result, develop different mechanisms of adaptation, defence and capture, mechanisms that are unnecessary in terrestrial animals (Nigam et al., 2019; Rotter et al., 2020; Ameen et al., 2020; Lu et al., 2021). These mechanisms include the production of complex molecules with interesting biological activities such as antioxidant, antimicrobial, antiproliferative, anticoagulant (Rotter et al., 2020; Ameen et al., 2020). It is nowadays acknowledged that marine organisms can provide an increasing number important therapeutic products, industrial and environmental applications and analytical tools.

1.2. Venoms, toxins and their potentialities

Animal venoms are among the most complex secretions known (Gorson et al., 2015). Venoms can be defined as a complex mixture of proteins, peptides and other bioactive molecules, often referred to as toxins, secreted by a specialized gland in an animal and delivered to another animal in response to predatory or defensive stimuli (King, 2011; Zhang, 2015). The targets of these toxins mainly include sodium, calcium and potassium ion channels and can interrupt functions or even affect, for example, the neuromuscular, cardiovascular and immune systems (King, 2011; Zhang, 2015; Minutti-Zanella et al., 2021; Gonçalves & Costa, 2021). As a result, animal toxins have high specificity and potency against molecular receptors and metabolic pathways, making them high-value products (see for instance King, 2011; Verdes et al., 2016; Gonçalves & Costa, 2021, and references therein).

One of the predominant examples that revolutionized the world of blue biotechnology was ziconotide (ω-conotoxin, also known as ω-MVIIA), the first drug approved by the Food and Drug Administration for the treatment of severe and chronic pain (Webster, 2015; Nigam et al., 2019; Rotter et al., 2020). It is a synthetic neuroactive peptide developed by the marine snail *Conus magus* to incapacitate its prey. The drug acts as an antagonist of the N-type calcium channels that control neurotransmission, causing decreased neurotransmitter release and inhibiting the transmission of pain signals to the CNS, that is, resulting in pain relief (Webster, 2015; Deer et al., 2018).

Although venoms have assumed great importance and brought highly significant contributions to society, most of these venoms remain uncharacterized or unidentified for several reasons: they have numerous little-known organisms with strange physiology and some of them are small, difficult to acquire and maintain in laboratory conditions and, despite technological advances, analytical techniques for characterizing the components of venoms are still limited (King, 2011; Rodrigo & Costa, 2019; Rotter et al., 2020).

1.3. Venoms and cysteines

Most venoms, namely cone snails, scorpions and spiders, are characterized by the presence of proteins and peptides rich in cysteines that provide them with stability and resistance to proteases, in other words, most venoms are aimed at their prey and, therefore, the venom components must be stable enough to reach their molecular targets before being degraded or excreted (Fry et al., 2009; King, 2011; Verdes et al., 2016). In some cases, the presence of proteins with multiple cysteine-repeating motifs in animal venom glands is a chemical signature for toxin secretion (Gonçalves & Costa, 2020). In addition, cysteine-rich peptides and proteins also play important roles in metabolic and physiological pathways, acting as inhibitors of enzymes, hormones, growth factors, ion channel modulators and host defence molecules and can be found in all organisms (Lavergne et al., 2012; D'Ambrosio et al., 2021).

The stability and high nucleophilicity of cysteines and their small size that can range from 12 - 13 in snails to 40 - 80 residues in scorpions and snakes make highly reactive venoms, with biotechnological potential (Norton & Olivera, 2006; Lavergne et al., 2012; Poole, 2015; Verdes et al., 2016; Dos Santos et al., 2020; D'Ambrosio et al., 2021).

1.3.1. Cysteine-rich secretory proteins (CRISPs)

Cysteine-rich secretory proteins (CRISPs) are a family of highly conserved cysteine-rich proteins with a molecular mass of 20 - 30 kDa and are commonly found in venoms, mainly snake (Yamazaki & Morita, 2004; Estrella et al., 2010; Verdes et al., 2016; Tadokoro et al., 2020). Still, a variety of proteins have been found in other organisms, including marine ones like *Conus textile* (Milne et al., 2003), exhibiting diverse biological functions. However, evidence on the functions and molecular targets of these proteins is scarce, especially in marine invertebrates. Few of these proteins have been functionally characterized, most studies have suggested that they function as ion channel antagonists (Lodovicho et al., 2017; Tadokoro et al., 2020; Deka et al., 2020). Furthermore, CRISPs in venoms can interact with ryanodine receptors (RyR), inhibiting the liberation of Ca^{2+} ions, while others have the ability to block Ca^{2+} and/or K^{+} activity, causing smooth muscle paralysis (Roberts et al., 2007; Estrella et al., 2010). It has also been shown that CRISPs also promote inflammatory responses, being involved in complement activation, mast cell degranulation, leukocyte recruitment and cytokine release (Tadokoro et al., 2020; Deka et al., 2020). Proteins can still be found in the male reproductive system, mainly in mammals, being expressed in practically all stages of development and maturation of gametes, where they interfere with the acrosome development and function of sperm, sperm motility, fertilization and protection of the immune system but the functions are still far from being fully understood (Koppers et al., 2010; Weigel Muñoz et al., 2019; Björkgren & Sipilä, 2019; Gaikwad et al., 2019, 2020; Arévalo et al., 2020).

1.3.2. Metallothioneins (MTs)

Metallothioneins (MTs) are small metalloproteins of low molecular weight (6 - 7 kDa) with a high content of cysteine residues that allow them to bind to specific metals such as mercury, copper, silver, cadmium and zinc and have been reported in marine invertebrates, including bivalve and gastropods molluscs such as *Nucella lapillus* (Leung & Furness, 2001; Leung et al., 2005; Mao et al., 2012). Given their metal-binding capacity, they play an important role in several physiological functions that include metal detoxification, scavenging free radicals, metabolic regulation and shield against heavy metals (Mao et al., 2012; Samuel et al., 2021). Many organisms, including mammals and marine invertebrates, have been seen to synthesize MTs, under excess metals, as a defence mechanism and, for this reason, MTs concentrations in organisms are used as biomarkers for monitoring exposure to heavy metals (Amiard et al., 2006; Mao et al., 2012).

1.3.3. Glutathione S-transferases (GSTs) and glutathione (GSHs)

Glutathione S-transferases (GSTs), whose molecular mass can vary between 23 to 28 kDa, are a group of enzymes that play fundamental roles in cellular detoxification, endogenous and exogenous compounds, catalysing the conjugation of reduced glutathione (GSH) with these compounds (Blanchette et al., 2007; Saranya Revathy et al., 2012; Park et al., 2020). Glutathione (GSH) is a tripeptide composed of glycine, glutamic acid and cysteine and in its reduced form, a glutathione has a free thiol group (Monostori et al., 2009). Their concentrations are indicators of cellular functionality and, in view of this, GSH have been used as oxidative stress biomarkers in several studies (Blanchette et al., 2007; Monostori et al., 2009). Currently, information on marine GST is very limited, but even so there are studies that have detected GSTs in marine invertebrates, such as *Mytilus edulis* by Fitzpatrick & Sheehan (1993) and *Mytilus galloprovincialis* by Hoarau et al. (2006).

1.4. The potential of marine invertebrates in bioprospecting

There is growing interest in the bioactivity of marine invertebrate phyla, specifically molluscs . Molluscs belong to the second largest phylum of animals on Earth (Mollusca) and have become the focus of many studies due to their enormous diversity, not only in terms of richness, but also as they encompass a wide variety of morphologies, ecological niches, lifestyles, and trophic niches compared to many other invertebrate phyla (Benkendorff, 2010; Cuezzo et al., 2020). The richness of bioactive compounds is mainly due to the fact that most marine invertebrates are soft-bodied, sessile and slow-moving, which makes them vulnerable to predators and pathogens, requiring alternative defence strategies, such as the production of potent venoms (Proksch et al., 2002; Norton & Olivera, 2006; Benkendorff, 2010; Pyron & Brown, 2015).

Mollusca is composed of eight distinct classes: Gastropoda, Bivalvia, Scaphopoda, Cephalopoda, Polyplacophora, Monoplacophora, Caudofoveata and Solenogastres (Benkendorff, 2010). Of these classes, gastropods and bivalves are the most investigated molluscs and represent around 98% of the total molluscs (Avila, 2006; Benkendorff, 2010; Cuezzo et al., 2020). Gastropods constitute the largest and most diverse class of the phylum and include whelks, slugs, and snails (Benkendorff, 2010; Haszprunar & Wanninger, 2012). Predation and defence are habits that require morphological and physiological adaptations, and, in the case of gastropods, most have developed specialized glands or other anatomical characteristics that secrete chemical substances to dominate their prey (Modica & Holford, 2010; Ponte & Modica, 2017). Adaptations include specific venom glands in Conoidea and primary and/or accessory salivary glands in other groups of gastropods that do not have a venom gland (Modica & Holford, 2010). There are several publications that demonstrate that toxins can be secreted in these glands, associating the salivary gland with the venom gland (e.g., Andrews, 1991 and Power et al., 2002). The possession of accessory and acinous tubular salivary glands is a plesiomorphic feature of

neogastropods, seen in some members of three superfamilies, Muricoidea, Cancellarioidea and Conoidea (Ponder, 1973).

The most studied gastropods are the *Conus*, which is characterized by the presence of a specialized venom gland (Modica & Holford, 2010; Dutertre et al., 2015; Ponte & Modica, 2017; Rajaian Pushpabai et al., 2021). The species secrete cysteine-rich neurotoxins, known as conopeptides or conotoxins, which are used to immobilize or paralyze prey as well as for defence. The main targets are the ion channels, G protein-coupled receptors and transporters in the nervous system (Terlau & Olivera, 2004; Gao et al., 2017). Due to their high specificity and affinity for targets, neurotoxins have been a valuable source for the discovery of new drugs. The discovery of ziconotide (described above), ω-conotoxin isolated from *Conus magus*, is a predominant example.

In addition to Conoidea, other families of gastropods, such as Muricidae and Tonnoidea, have also developed specialized compounds that also have potential in biotechnological applications but still need to be explored (Modica & Holford, 2010; Turner et al., 2018). The Muricidae family comprises a diverse group of predatory snails, with more than 2 000 species found on all continents and, although they have not yet been chemically analysed in clinical trials, Muricidae produce a set of brominated indoles with anti-inflammatory and anti-cancer properties, as well as choline esters with muscle relaxant and analgesic effect (Benkendorff et al., 2015).

1.4.1. Nucella lapillus L.1758

Nucella lapillus (L. 1858) is a gastropod mollusc and a predator of bivalves, especially mussels, that is common on the rocky coasts of Europe and the north-western Atlantic coast. The species has been widely employed for monitoring tributyltin (TBT) contamination, a compound used in antifouling paints, which causes imposex in this species, which is a condition in which TBT causes dysregulations in the endocrine system of marine organisms, developing male sex characteristics in female gastropods, which can lead to sterility and death (Schøyen et al., 2018).

Evidence was found to indicate that *Nucella* secretes unidentified sulfhydryl-rich neurotoxins through salivary glands with a paralyzing effect on musculature to its target *Mytilus sp.* (Martoja, 1971; Andrews, 1991). Furthermore, preliminary studies confirmed the presence of cysteine-rich proteins in the digestive gland and in the peribuccal mass which includes the salivary glands (D'Ambrosio et al., 2021). The species has a similar anatomy to *Conus*, both contain a muscular layer surrounded by glandular cells, which helps in the expulsion of secretory vesicles, but the food mechanisms are different, *Nucella* feeds by inserting the siphon between the mussel valves while *Conus* injects venoms through a radular tooth similar to a harpoon to hunt prey (Andrews, 1991; West et al., 1996; Norton & Olivera, 2006).

1.5. Objectives

There has been a growing interest in bioactive compounds from marine organisms for the development of new drugs and CRISPs have unique properties to be the main targets. They can be easily found in snake venoms but the same cannot be said with marine invertebrates. Despite having already detected it in some marine invertebrates, information about the proteins is scarce. Therefore, the main focus of this thesis is to localise and analyse the distribution of cysteine-rich secretory proteins in the dogwhelk, *Nucella lapillus*, a potential target species for the bioprospecting of novel bioactives, as it is suspected to secrete venomous saliva. Thus, the main objectives of this work can be summarized in the following topics:

- Identify and characterise the main secretory organs in the species and associate morphoanatomy with the potential secretion of cysteine-rich proteins.

- Provide a comparative overview of the proteome of the main secretory organs of the species and attempt to identify fractions containing CRISPs and analyse their expression.

- Analyse the toxicity and bioreactivity of crude protein extracts from main glandular organ and associate effect with the potential presence of CRISPs.

2. MATERIALS AND METHODS

2.1. Organism collection and rearing

Adult dogwhelk *Nucella lapillus* were collected manually in October 2020 and March and April 2021 during the low ride, from rocky intertidal areas at Costa da Caparica, western Portugal (38°38'44.9"N 9°14'37.0"W). A total of 150 animals were collected. The animals ranged between 17 and 22 mm total length, with aperture length between 12 and 16 mm and weighed around 1.5 g, including the shell.

The organisms were then transported alive to the laboratory and acclimatised in an ≈ 7 L capacity aquarium fitted in a closed circulation seawater mesocosm system with artificial seawater, constant aeration and water filtration. Rearing parameters were constrained as temperature = 17 ± 1 ºC; salinity = 30 % and 16 light : 8 dark photoperiod. The mesocosm environment consisted of natural pebbles with mussel clumps collected from Costa da Caparica. Mussels were used as a food source for *Nucella* and being fed daily with *Spirulina*.

Figure 2.1. *Nucella lapillus* in its natural environment. A) *Nucella lapillus* can be found in the rocky areas of Costa da Caparica beach. B) *Nucella* is usually penetrated together with mussels which are their food source. Inset: detail of *Nucella lapillus*.

2.2. Histological analysis of glands

Nucella lapillus were fixated by injecting Davidson's fixative (9 - 10 % v/v formalin, 10 % v/v glacial acetic acid and 30 % ethanol) through a pre-made perforations in the apex, spiral zone and below the operculum, to maximize preservation of the internal structure. After fixation for 15 - 20 minutes, the shell and operculum were gently removed and the organs were then placed in histo-cassettes, where they were washed in distilled water, dehydrated in a progressive series of ethanol (70, 95 and 100 %), infiltrated in xylenes and embedded in paraffin, through Shandon Pathcentre tissue processor (Labexchange, Burladingen, Germany) (Table 2.1).

Table 2.1. Paraffin-embedding procedure performed on the enclosed tissue processor. The samples start by washing with distilled water, then they are dehydrated with a progressive series of ethanol (70, 95 and 100%) and infiltrated in xylene. In the end, they are embedded in paraffin overnight.

Step	Duration
Fixation	
Davidson's	24 h total
Washing	
Distilled water	4 x 15 min
Dehydration	
70% ethanol	1 × 30 min
95% ethanol	2 × 15 min
Absolute ethanol	3 × 30 min
Intermediate infiltration	
Xylenes	3 × 15 min
Impregnation	
Molten paraffin	Overnight

Paraffin-embedded samples were sectioned at 5 µm thickness, using a RM2035 model rotary microtome (Leica Microsystems, Wetzlar, Germany). At least two slides per sample were obtained, each containing two rows of sections. The slides were later deparaffinated in xylene, rehydrated in a regressive series of ethanol (100, 95 and 70%), washed in distilled water and stained with Haematoxylin and Eosin (H&E), to enhance structural detail. Haematoxylin highlights acidic structures such as nuclei,

rough endoplasmic reticulum (RER) and acidic mucopolysaccharides (all of which are termed basophilic structures). In turn, alcoholic Eosin Y stains more alkaline (acidophilic) substances such as cytoskeleton and muscle fibres. The procedure follows Costa (2017) (Table 2.2).

Table 2.2. Haematoxylin and eosin staining protocol (Costa, 2017). The samples go through several processes: deparaffination with xylene, rehydration with a progressive series of ethanol and distilled water, staining with haematoxylin and eosin, dehydration again with ethanol and ending up by clearing with xylene.

Step	Duration
Deparaffination	
Xylenes	2 × 30 s
Rehydration	
Absolute ethanol	2 × 30 s
95% ethanol	2 × 30 s
70% ethanol	1 × 30 s
Brief rinse in distilled water	
Distilled water	1 × 6 min
Staining	
Harris's haematoxylin	1 × 2 min
Differentiate in tap water	2 × 2 min
Brief rinse in distilled water	
70% ethanol	1 × 3 min
Alcoholic Eosin Y	1 × 3 min
Dehydration	
70% ethanol	1 × 30 s
95% ethanol	2 × 30 s
Absolute ethanol	2 × 30 s
Clearing	
Xylenes	1 × 30 s

Slides were mounted with Dibutylphthalate Polystyrene Xylene (DPX) resin (DBH, Poole, UK) and analysed with a DM2500 optical microscope equipped with a DFC 480 digital camera (all from Leica Microsystems, Wetzlar, Germany). Images were processed using ImageJ and GIMP (2.10.24). In absence of reference studies for *N. lapillus*, identification of histological structures is based on other gastropod studies as proxy, namely Lobo-da-Cunha (2002), Bravo Portela et al. (2012), Vasconcelos et al. (2012), Arrighetti et al. (2015), Rodrigo & Costa (2017), Costa (2017), Li et al. (2018), Abdel Gawad et al. (2018), Landro et al. (2019) and Dennis et al. (2021).

2.3. Preparation of crude protein extracts

Total protein was extracted from the four main glandular organs: foot, salivary gland, digestive gland and gonad (Figure 2.2). Organs were separately homogenized with a pestle in lysis buffer, consisting of 0,05 M Tris-HCl pH 7, 200 mM Ditiotreitol (DTT) and Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged for 5 minutes, 12 000 rpm, at 4 ºC. The obtained supernatants were used to determine the total protein, using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

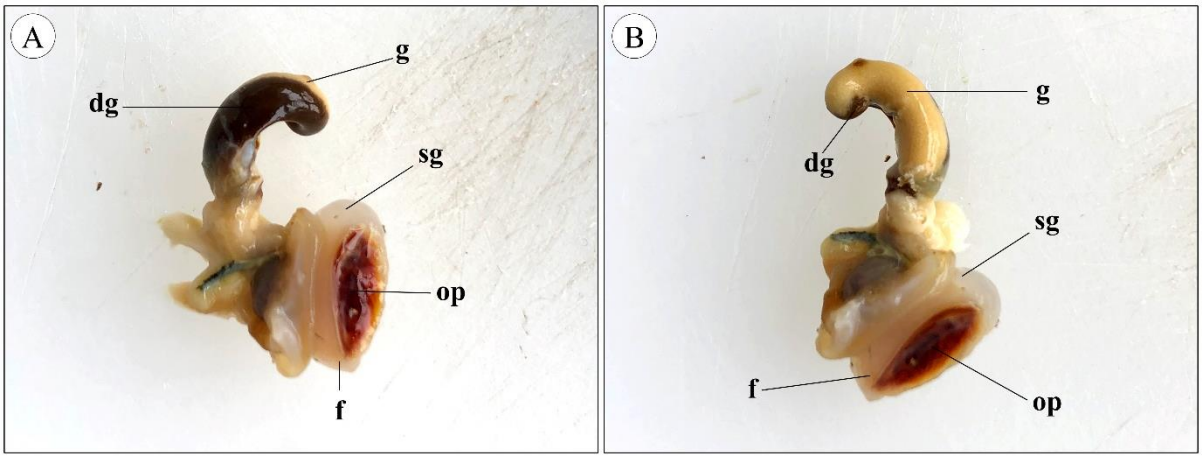

Figure 2.2. Anatomy of *Nucella lapillus*. The main organs are foot (f), salivary gland (sg), digestive gland (dg) and gonad (g). Next to the foot is the operculum (op) which acts as a protective layer and is removed during extraction.

Crude protein extracts were fractionated and concentrated using Amicon Ultra Centrifugal Filters for volumes up to 4 mL (Merck, Darmstadt, Germany). Different filter pore sizes were selected to divide the protein extracts in two fractions based on molecular weight (10 - 100 and > 100 kDa). Previously washed with phosphate buffered saline (PBS), the filters 100 were filled with protein extracts and also PBS (1:1), in order not to obstruct the filters, and centrifuged at 4 000 rpm until the extract was filtered, but without leaving the filter completely dry. The first part of the filtration was terminated by washing the filters 10 with PBS and the filtrates were transferred to filters 100, where the same process was repeated. Concentrates were been stored -81 ºC for future analyses.

2.4. Protein gel electrophoresis

Protein crude and fractionated extracts were resolved in Sodium Dodecyl Sulphate-Polyacrylamide Gel Electroforesis (SDS-PAGE), performed in an omniPAGE Mini Vertical Protein Electrophoresis system (Cleaver Scientific Ltd, Rugby, UK), using the discontinuous system developed by Laemmli (1970). In brief, aliquots of protein solutions were mixed with sample buffer (0.5 M Tris-HCl with 10%

SDS) in 1:1 ratio and boiled for 2 min in a water bath. After cooling and spin-down, samples were loaded into a gel consisting of 12% m/v acrylamide/bis-acrylamide (9% for the fraction > 100 kDa) resolving gel and 6% stacking gel (Table 2.3). Tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) were used as catalysts for polymerisation. Lanes were loaded with the same amount of protein for permit comparison between fractions and extracts from the different organs.

Table 2.3. Formulations of SDS-PAGE gels. The 9% and 12% resolving gels were prepared for samples from the fraction > 100 kDa and for unfractionated samples and from the fraction < 100, respectively.

Reagent	Resolving gel		Stacking gel
	9 % acrylamide (for the fraction > 100 kDa)	12 % acrylamide	6 % acrylamide
H_2O MQ	3.3 mL	2.55 mL	2.025 mL
Acrylamide	2.25 mL	3 mL	0.75 mL
Resolving buffer (1.5 M Tris HCl pH 8.8)	1.875 mL	1.875 mL	-
Stacking buffer (0.5 M Tris HCl pH 6.8)	-	-	0.938 mL
SDS 10%	0.075 µL	75 µL	37.5 µL
APS 10%	37.5 µL	37.5 µL	18.75 µL
TEMED	3.75 µL	3.75 µL	3.75 µL

Electrophoresis was run at 90 V for 30 minutes, followed by another run at 110 V for 45 minutes. The resulting gel was stained for overnight with Coomassie Brilliant Blue R250, with gentle agitation. Protein staining was developed by destaining in a solution containing 10 % v/v glacial acetic acid and 30 % v/v absolute methanol. Gel images were acquired using a the GelDoc EZ Imaging System (Bio-Rad, Hercules, CA, USA) and processed using imageJ (Schneider et al., 2012).

2.5. Quantification of protein thiols - total SH and free SH

The quantification of thiols was done according to the protocol of determination of sulfhydryl groups through Ellman's reagent developed by Sedlak & Lindsay (1968). Briefly, acetylcysteine was using to prepare standards varying from 31 to 1000 µM by successive dilutions. For determination of total sulfhydryls group (SH), a reaction mix solution was prepared by adding 20 µL of protein extracts, 75 µL of dilution buffer (30 mM Tris-HCl, 3 mM EDTA pH 8.2), 25 µL of DTNB reagent and 400 µL of methanol and centrifuged at 3000 × g for 5 min at room temperature. Then, on a 96 well-plate, 90 µL

of supernatants were added to each well. In this assay, DTNB reacts with the thiol group of the extracts to form 2-nitro-5-thiobenzoic acid (TNB^{2-}), the coloured product that absorbs light at 412 nm.

Free SH was obtained by precipitation of 50 µL of sample with 50 µL of 10% (w/v) trichloroacetic acid (TCA), to separate protein from SH groups and then was centrifuged for 15 min, 1 500 rpm and room temperature. 50 µL of supernatants were then placed to a 96-well-plate, where were also added 200 µL Tris-HCl 262 mM EDTA 13 mM pH 8.9 and 20 µL of DTNB reagent. Finally, the optical absorbance extinction was read at 412 nm on a Synergy HTX model microplate reader (BioTek, Winooski, VT, USA). The subtraction of free SH from the total fraction allowed the quantification of the bound SH fraction.

2.6. Toxicity and bioreactivity testing

2.6.1. Ex-vivo bioassays

Ex-vivo bioassays using *Mytilus edulis* gills as biological model were performed to analyse the toxicity and bioreactivity of protein extracts from the salivary gland and foot, the first selected as main (toxin) secretory organ, the second as reference for comparison. The gills were exposed for 10 min in the cold to protein extracts from two organs, diluted to four different total protein concentrations: 0.25; 0.1; 0.025 and 0.01 mg/mL, plus control (PBS only). As first step, the valves of mussels were gently separated so as not to compromise the integrity of the gills and flooded with PBS to avoid desiccation of tissue. One valve was used as a test sample and the other as its respective control, being exposed to 1ml of diluted protein extract and PBS, respectively. Ten min later, exposure was terminated by washing each valve with PBS. The gills were then carefully excised and divided for DNA damage assessment, histopathology, histopathology and caspase 3 activity to investigate type of cell death (Figure 2.3). Assays were performed with six biological replicates (n = 6).

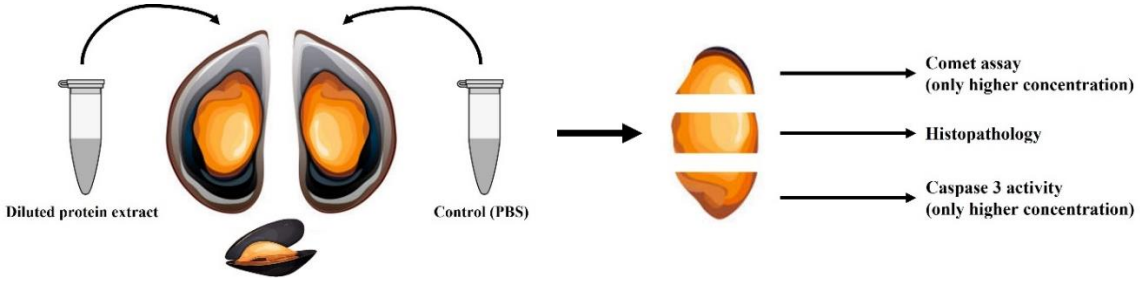

Figure 2.3. Illustration of excision and division of the mussel gill tissue for the several biomarkers analyses. After exposure to diluted protein extracts and controls, the mussel gills are dissected into three parts for the respective analyses.

2.6.2. Comet assay

Molecular damage to DNA was assessed by the alkaline single-cell gel electrophoresis ("Comet") assay, originally developed by Singh et al. (1988) and adapted by Martins & Costa (2020). In short, tissues were crushed with a pestle in PBS and centrifuged for 2 min at 1500 rpm. 20 µL of supernatant was incorporated into 180µL of molten (36 - 40 ºC) low melting point agarose (LMPA) prepared in PBS and then two drops of 80 µL of the mixture were placed on slides pre-coated with high melting point agarose (HMPA) prepared in TAE (Tris - Acetic Acid - EDTA) buffer and allowed to dry for at least 48h. Slides were then covered with a coverslip. After agarose solidification for 15 min at 4 ºC, the coverslips have been rigorously removed and the slides were immersed in cold (4 ºC) lysis buffer (0.45 M NaCl, 40 mM EDTA and 5 mM Tris, pH 10), to which were previously added 10 % v/v DMSO e 1 % v/v Triton X-100, during 1 h and in dark. Afterwards, the slides were placed in cold electrophoresis buffer (0.2 mM EDTA and 10 M NaOH) for 40 min to allow DNA unwinding and increase the expression of alkaline labile sites (Martins & Costa, 2020). Forty min after, electrophoresis was run during 30 min in dark, at 25 V. In the end, slides were neutralized in Tris-HCl (pH 7.5) for 15 min and then dried with methanol for the same time, in cold, and archived dry until analyses.

Before analysis, slides were rehydrated in cold MQ water for 30 min, stained with GreenSafe Premium (Nzytech, Lisbon, Portugal) for 10 min in dark and examined with a DM 2500 LED microscope adapted for epifluorescence with an EL 6000-light source (all from Leica Microsystems, Wetzlar, Germany). Approximately 100 comets per slide were analysed using CometScore. The percentage of DNA in the tail was recorded as metric for DNA damage.

2.6.3. Histopathology

Mussel gills samples were prepared for histology as described previously. In brief, the dissected tissues were fixed in Davidson's fixative solution for 24h. Before placing in the histo-cassettes, the samples were immersed in MQ water for 4 × 15 min. Then, the samples were washed in distilled water, dehydrated and embedded in molten paraffin, as described in section *2.2. Histological analysis of glands*. The 5 µm thickness sections were stained with the haematoxylin and eosin staining protocol, as detailed on the Table 2.2. The final images were taken through DM2500 optical microscope (Leica Microsystems, Wetzlar, Germany). A preliminary histopathological analysis was performed on the internal structure of the gills, searching for morphological and cellular alterations, such as haemocyte infiltration, vacuolation, lipofuscin aggregates e fibrosis (Costa et al., 2013).

2.6.4. Caspase 3 activity

The activity of caspase 3 (effector caspase involved in intrinsic apoptosis) was evaluated using the Caspase 3 Assay Kit Colorimetric (Sigma-Aldrich, St. Louis, MO, USA) designed for human/mammalian tissue, as described by the manufacturer. The method is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) catalysed by caspase 3, producing the pNA. The p-nitroaniline (pNA) standards were prepared from pNA stock solution to obtain the concentrations of the range of 10 to 200 µM, diluting the pNA in 1 × Assay Buffer. Then, 100 µL of each concentration was added to each well of the 96 well-plate. On the other 96 well-plate, the samples (that were previously lysed with a pestle in 1 × Assay Buffer) or caspase 3 positive control 5 µg/mL, 1 × Assay Buffer and caspase 3 inhibitor 200 mM were added in the appropriate wells as indicated in Table 2.4. The reaction was started by adding 10 µL of caspase 3 substrate 2 mM to each well and mixed gently by shaking. Then, the plate was covered with aluminium foil and incubated for four different times in an attempt to optimise the protocol for mussel tissue: first, at room temperature for 90 min; second, at room temperature for another 90 min; third, another 30 min at 37 °C and finally, overnight at room temperature.

Table 2.4. Preparation of reaction mix for determination of caspase 3 activity. For each well, a 100 µL total reaction mix was prepared containing: cell lysate/caspase 3, 1 × buffer assay, caspase 3 inhibitor (for control + inhibitor) and caspase 3 substrate.

	Cell lysate	**Caspase 3 5 µg/mL**	**1 × Assay Buffer**	**Caspase 3 inhibitor Ac-DEVD-CHO 200 µM**	**Caspase 3 substrate Ac-DEVD-pNA 2 mM**
Reagent blank	-	-	90 µL	-	10 µL
Non-induced cells (control)	5 µL	-	85 µL	-	10 µL
Induced (samples)	5 µL	-	85 µL	-	10 µL
Caspase 3 positive control	-	5 µL	85 µL	-	10 µL
Caspase 3 positive control + inhibitor	-	5 µL	75 µL	10 µL	10 µL

The absorbance was measured at 405 nm, using a Multiskan Sky model microplate reader (Thermo Scientific, Waltham, MA, EUA). With the pNA standards, a calibration curve was made, and the caspase 3 activity was determined, using the following equation,

$$Capase\ 3\ activity\ (\mu mol\ pNA/min/\ mL) = \frac{\mu mol\ pNA \times d}{\varepsilon^{mM} \times t \times v} \quad [2.1]$$

where $\mu mol\ pNA$ is the value obtained from the calibration curve, ε^{mM} extinction coefficient (for pNA, ε^{mM} = 10.5), v volume of samples in mL, d dilution factor and t reaction time in min.

2.7. Statistical analysis

Statistical analyses were conducted using R 4.0 (Ihaka & Gentleman, 1996), where the data were analysed by the non-parametric Kruskal-Wallis ANOVA-by Ranks H, followed by the Dunn's test for multiple comparisons. Adjustment of p-values was done through the Benjamini-Hochberg method. The significance level was set at $p < 0.05$.

3. RESULTS

3.1. Microanatomy of the glandular organs

3.1.1. Foot

Histological analysis of the foot (Figure 3.1) revealed that the organ consists of muscle fibres that occupy practically the entire space of the foot, being spread in different directions (inset of Figure 3.1.A). These, when subjected to haematoxylin & eosin stains, are stained pink. With the 400x magnification, it was also possible to verify that the organ is covered by two main layers (Figure 3.1.B): inside this coating, there is an epithelial layer that acts as a protective layer and contains multiples of mucocytes that are oval and colourless when subjected to H&E stains and next to the epithelium, there is a brush border, composed of microvilli. It is also possible to observe the groves on the foot.

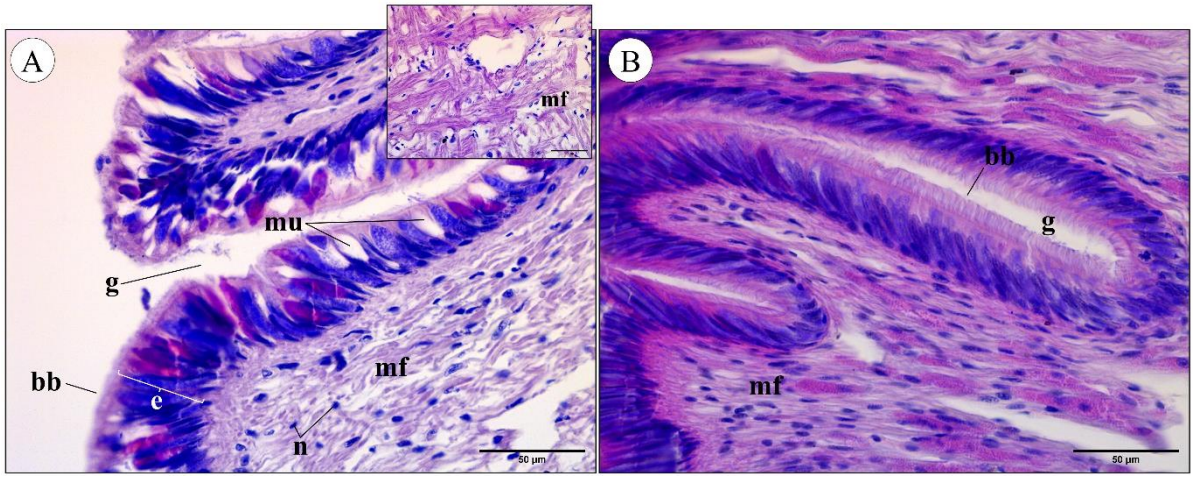

Figure 3.1. Histological representation of the foot (H&E). The foot consists mainly of muscle fibres (mf) with dispersed nucleus (n) and has two layers: epithelium (e) and brush border (bb). The first contains multiples of mucocytes (mu). The foot yet has grooves (g). Inset: detail of muscles fibres (mf) scatter in different directions. Scale bars: 50 µm.

3.1.2. Salivary gland

Right after the foot, comes the peribucal glandular mass, which includes the salivary glands. Histological studies were revealed little information about the salivary gland, showing only the presence of three types of cells that make up the glandular epithelium: vacuolar cells, granular cells and mucocytes (Figure 3.2.A). Vacuolated cells were the most abundant cells and were identified by the presence of vacuoles that manifested colourless / transparent when stained with H&E. As for granular cells, which can also be called basophilic cells, they were recognized by the blue colour of H&E stains and most had the shape of a chalice. Mucocytes, known as "goblet" cells, have a shape similar to granular cells, but these cells are relatively larger and are very frankly coloured. The salivary gland can be found in the digestive system, being connected to the oral cavity by a duct (Figure 3.2.B). The duct is characterized by the presence of three main layers: microvilli, epithelium and muscle fibres.

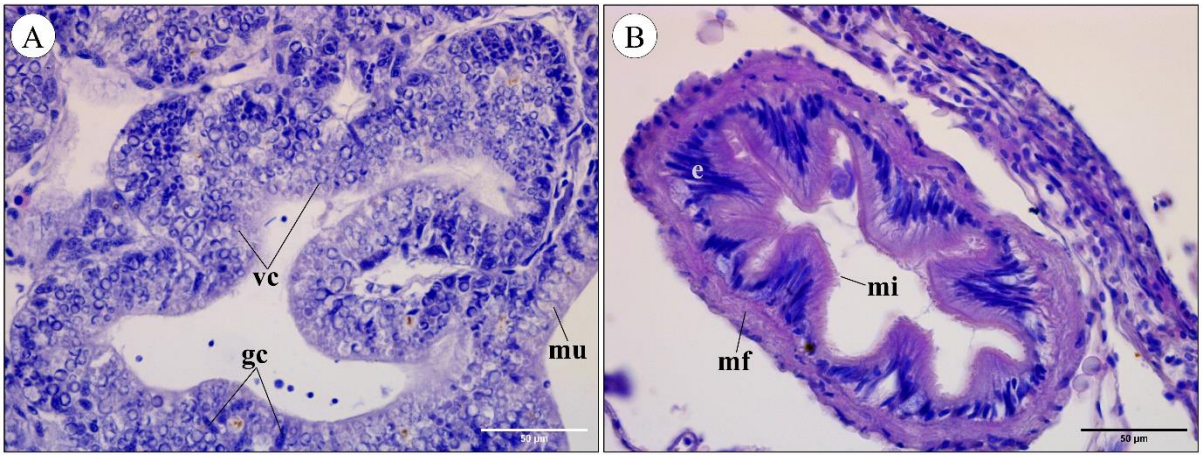

Figure 3.2. Histological representation of the salivary gland (A) and the duct (B) (H&E). Two types of cells are present in the salivary gland: vacuolar cells (vc), granular cells (gc) and mucocytes (mu). The salivary gland is connected to the digestive system by a duct that is covered by epithelium (e), muscle fibres (mf) and microvilli (mi). Scale bars: 50 µm.

3.1.3. Digestive gland

The digestive gland (Figure 3.3) histologically showed the typical structure of the molluscan digestive gland, characterized by multiples of tubules, also called digestive diverticula. The tubules consist of columnar structures made up of monolayered epithelium (bound to a thin basal membrane) and were interconnected by connective tissue, also known as intertubular tissue. Around the tubules, two main types of cells were seen: basophilic cells and digestive cells with numerous digestive vacuoles. Stained blue due to haematoxylin, it is evident that basophilic cells are the most abundant cells in the digestive tubules. As for digestive cells, these are found at the ends of the tubule and are pink thanks to eosin.

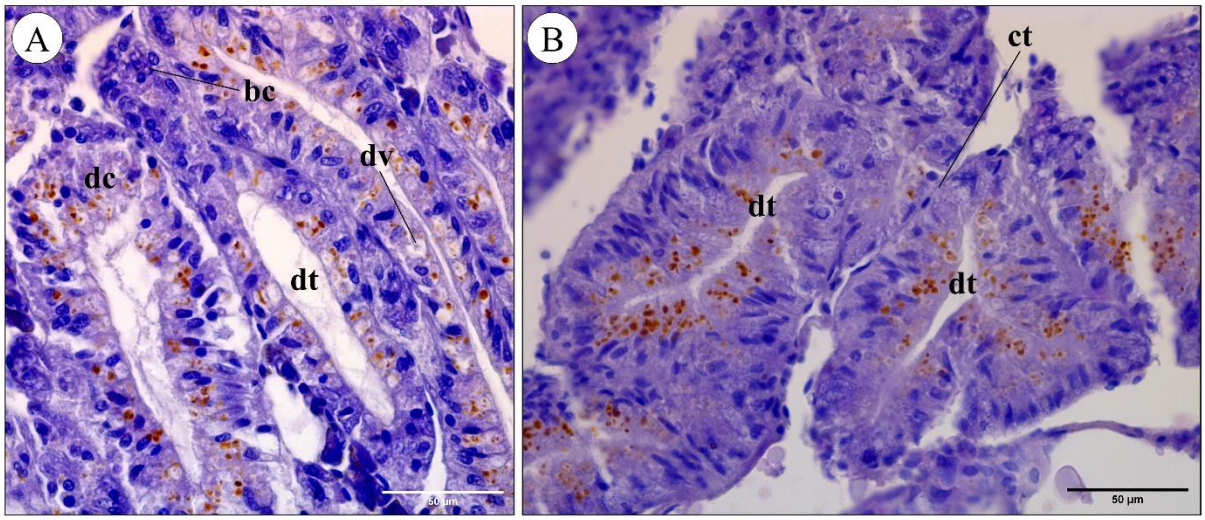

Figure 3.3. Histological representation of the digestive gland (H&E). The digestive gland is composed of multiple tubules, also known as digestive diverticula (dt), connected by connective tissue (ct) and are surrounded by basophilic cells (bc) and digestive cells (dc) with digestive vacuoles (dv). Scale bars: 50 µm.

3.1.4. Gonad

Histological analysis of the gonads revealed that the animals of both sexes were maturing, being possible to visualize different stages of germ cells within the same gonad. Cells proliferate mitotically in follicles (female) / seminiferous tubules (male). In the female gonad (Figure 3.4.A), germ cells give rise to oogonia that line the follicles. Oogonium differentiate into oocytes. Maturing oocytes are identified by the presence of intracytoplasmic bright eosinophilic vitelline granules. In the other hand, the male gonad (Figure 3.4.B) is full of sperm with different shapes, and they are totally basophilic. Spermatogonium form seminiferous tubules containing spermatocytes, spermatids and spermatozoa.

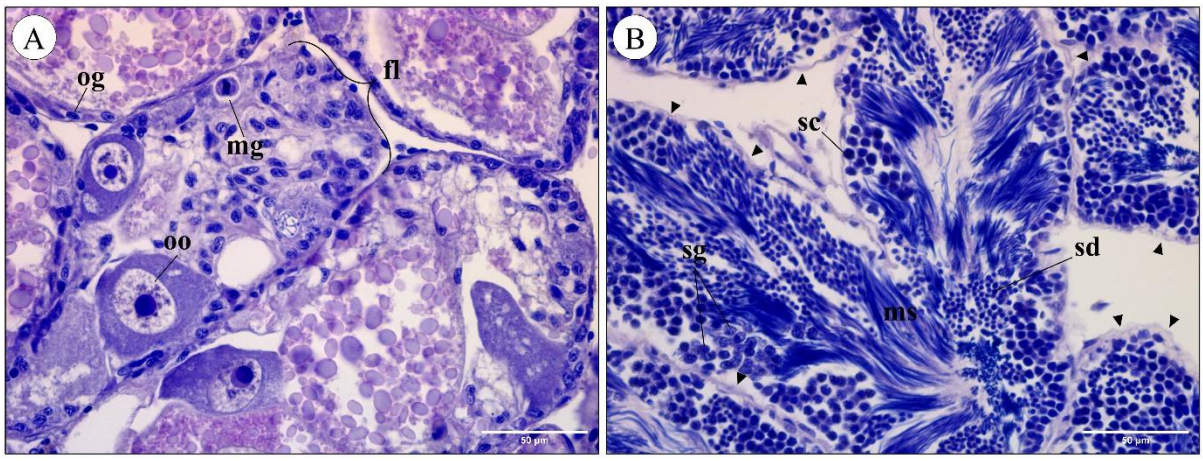

Figure 3.4. Histological representation of the female (A) and male (B) gonad (H&E). A) Each follicle (fl) houses mitosing oogonium (mg), maturing oocyte (oo) and oogonium (og) in which mitosis can be observed. B) In the male, several stages of sperm in the seminiferous tubules (arrowheads) are evident: spermatogonium (sg), spermatocyte (sc), spermatid (sd) and mature spermatozoa (ms). Scale bars: 50 µm.

3.2. Organ proteomes

Two electrophoresis were performed in order to have a better comparative visualization of the proteomes of the main organs: first with the four non-fractionated organs (Figure 3.5) and second with the fractionated salivary gland and foot (Figure 3.6). In the first electrophoresis, the salivary gland (band 3 in Figure 3.5) presented the greatest amount and diversity of proteins, with similarities to the foot proteome. The highest protein expression of both organs in the range of 35 to 45 kDa, more specifically in the 37 and 42 kDa. In relation to the digestive gland (band 2 in the Figure 3.5), this organ had less defined bar, without giving an understanding of its molecular weight. The same applied with the gonad (band 1 in the Figure 3.5).

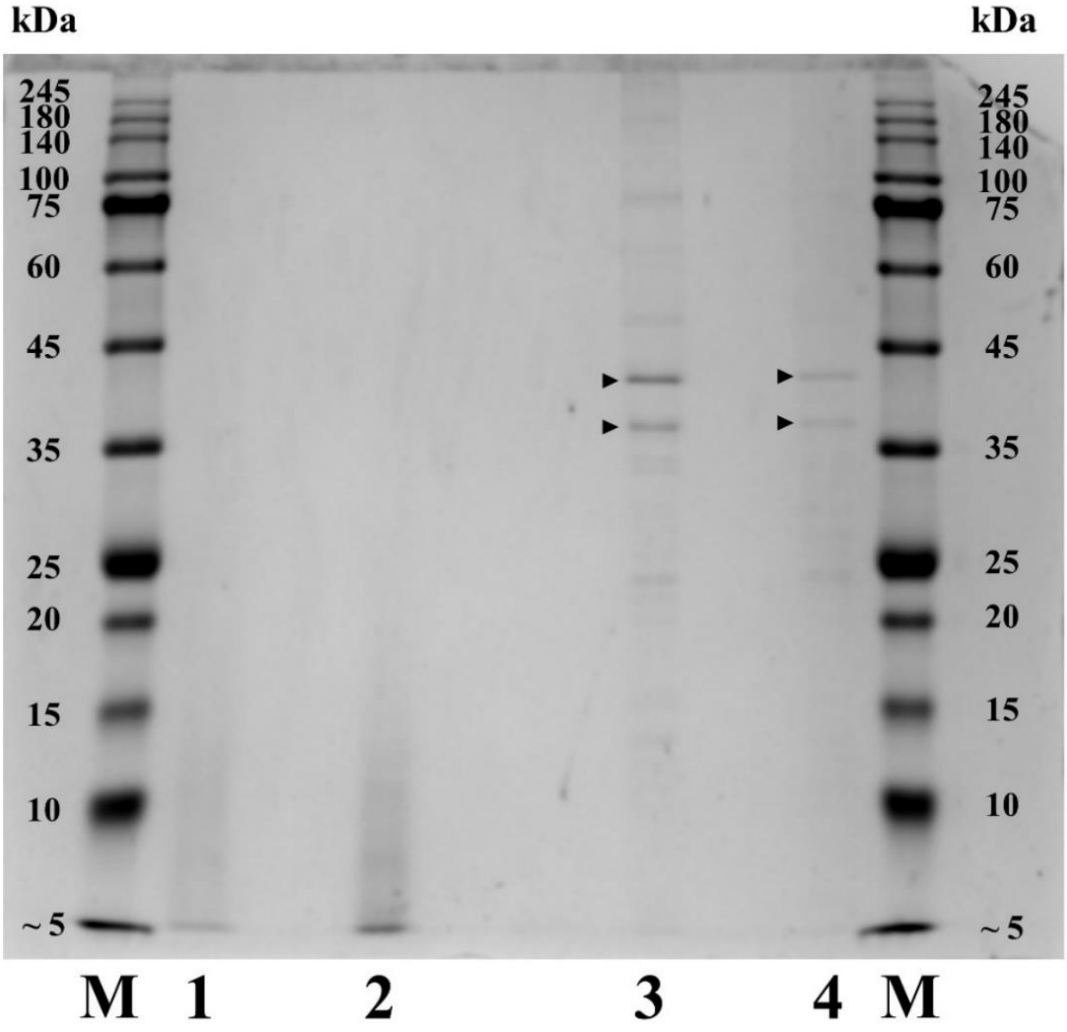

Figure 3.5. Gel resulting from SDS-PAGE of the 4 organs (foot, salivary gland, digestive gland and gonad). The electrophoresis was performed with the 12 % resolving gel. Bands 1, 2, 3 and 4 correspond, respectively, to the gonad, digestive gland, salivary gland and foot of *N. lapillus*. The first and last rows of bands refer to the market "M" NZYColour Protein Marker I (NZYTech, Lisbon, Portugal).

As the isolation of proteins from the digestive gland and gonads revealed more problematic, it focused only on the salivary gland and foot. There was a need to fractionate protein extracts to reduce protein complexity and, consequently, improve the comparative view of protein integrity. Samples were, as far as possible, divided into two fractions (10 – 100 and > 100 kDa) and again loaded with the same quantity. The results (Figure 3.6) confirmed once again that the proteome of the salivary gland and the foot have some resemblances, but they also showed some differences between the organs, especially in the fraction > 100 kDa (Figure 3.6.B). This same fraction has the best visualization of the diversity and expression of proteins between the two extracts, with the greatest prominence in the molecular mass around 42 kDa for both organs. The salivary gland has a greater variety of proteins than the foot, with the bands most expressed in molecular weights of approximately 36, 80 and 85 kDa. In the 10 - 100 fraction (Figure 3.6.A), the salivary gland proteome is very identical to the foot, both extracts have intense bands with a molecular mass around 22 and 36 kDa.

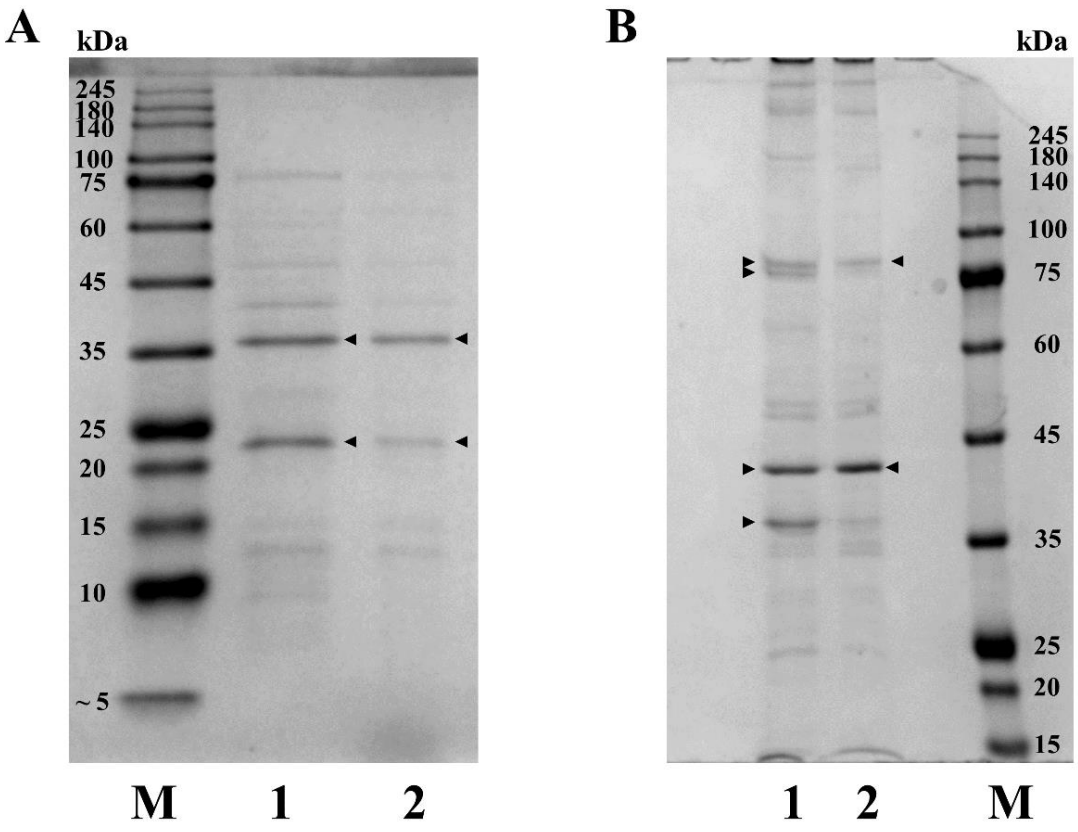

Figure 3.6. Gels resulting from SDS-PAGE of the two fractions > 10 kDa (A) and > 100 kDa (B). The fractions > 10 and > 100 kDa were executed with the 12 and 9 % resolving gel, respectively. A) Fraction > 10 kDa: bands 1 and 2 correspond to the foot and salivary gland, respectively. B) Fraction >100 kDa: Bands 1 and 2 correspond to the salivary gland and foot, respectively. The M stands for NZYcolour Protein Marker I (NZYTech, Lisbon, Portugal).

3.3. Thiols distribution

The results from concentration of thiols are present in Figures 3.7 and 3.8. Figure 3.7 shows the number of nanomoles of thiols existing in each mg of each target organ (foot, salivary gland, digestive gland and gonad) and it is noticeable that the organs produced distant concentrations of thiols. The highest concentration of total thiols was found in the reproductive organ (688.94 nmol/mg), while the salivary gland had the lowest (90.76 nmol/mg). In all organs, except the salivary gland, of the total thiols found in this organ, approximately 98.7% were strongly bound to proteins (Figure 3.7.C). The salivary gland has about 1.5 % free thiols.

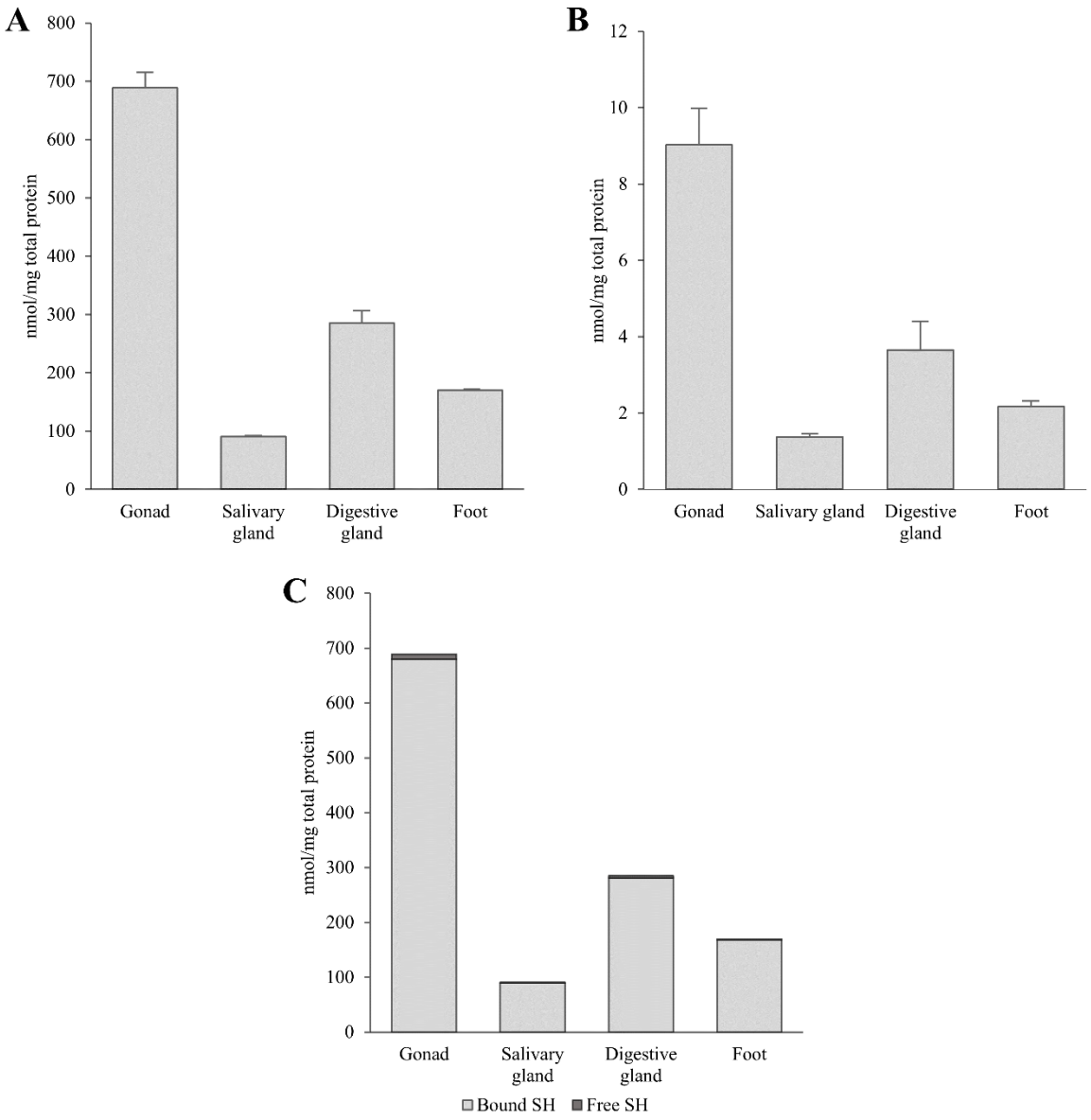

Figure 3.7. Concentration of thiols found in 4 different organs of *Nucella Lapillus*: gonad, salivary gland, digestive gland and foot. A) Concentration of total thiols. B) Concentration of free thiols. The samples were precipitated with 25 µL of TCA, to separate the thiols from the proteins. C) Final results of total thiols, bound thiols + free thiols.

For the fractionated protein extracts (Figure 3.8), the salivary gland was the organ that obtained the highest concentration of total thiols, with emphasis on the range from 10 to 100 kDa (235.32 nmol/mg) which was found almost quadruple thionic substances than the fraction > 100 kDa (Figure 3.8.A). However, it is in the fraction > 100 that the highest number of nanomoles of free thiols found in each mg of total protein is found. In turn, the foot has the highest concentration in the fraction > 10 (173.48 nmol/mg), nearly three times as many thiols as the higher fraction, yet the free thiols found were equivalent for both fractions (Figure 3.8.B). It was also noteworthy that the greater than 100 fraction of two organs contains similar total thiols values.

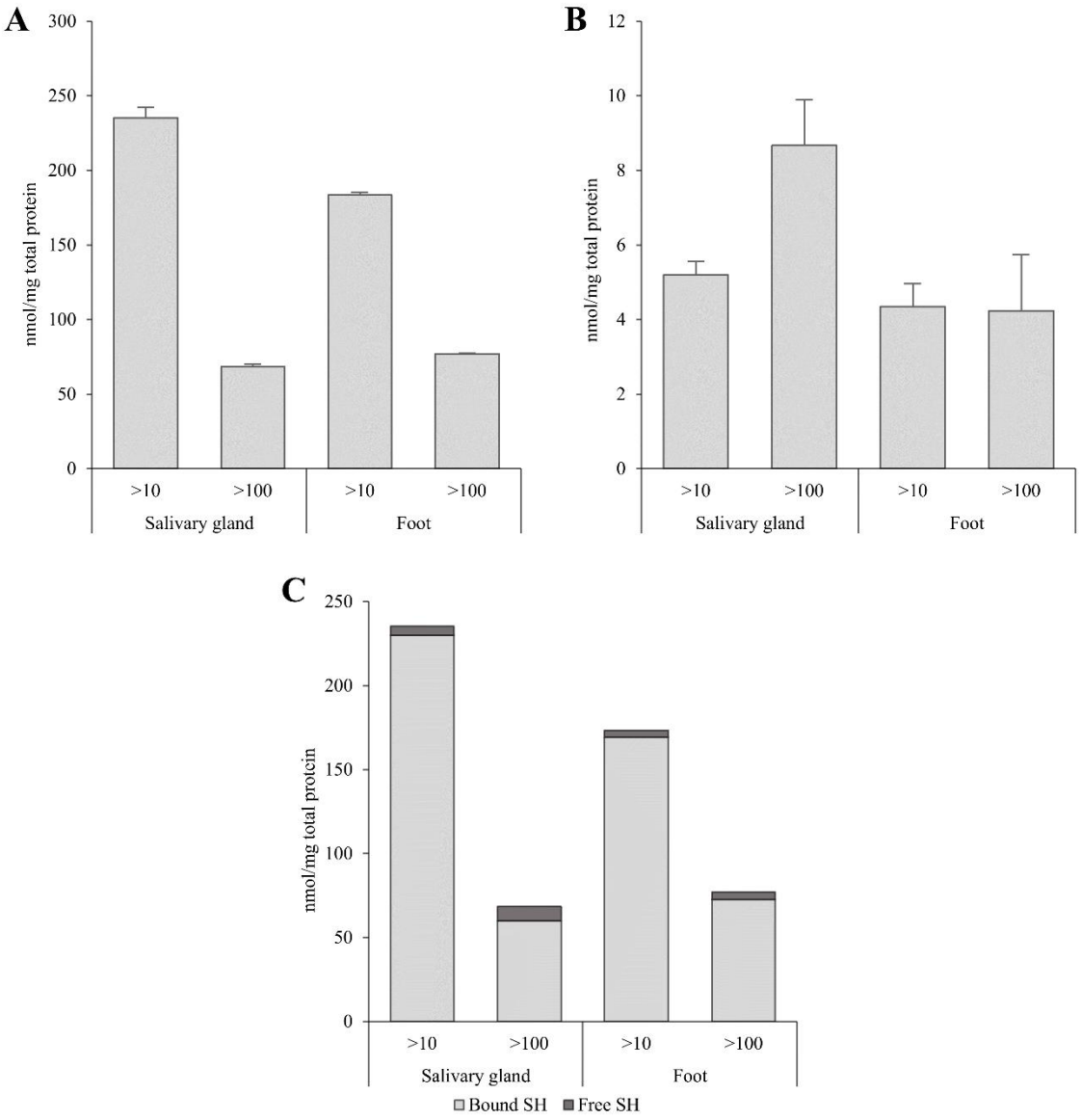

Figure 3.8. Concentration of thiols found in the two different fractions of salivary gland and foot: > 10 and > 100 kDa. The results are expressed as nmol of thiols per mg total protein (mean + standard deviation). A) Concentration of total thiols. B) Concentration of free thiols. C) Final results of total thiols, bound thiols + free thiols.

3.4. Bioreactivity and toxicity of protein extracts

3.4.1. Comet assay

About 150 comets were analysed per sample to quantify the amount of fragmented DNA from mussels exposed to the highest concentration (0.25 mg/mL). The results obtained are shown in Figures 3.9 and 3.10. The comets are grouped into 5 classes, depending on their morphology and degree of damage to the comet's tail: [0-20[, [20-40[, [40-60[, [60-80[e [80-100[% DNA in tail (Figure 3.9). The tail represents the level of DNA damage and the increase in length and intensity of fluorescence from the comet tail is proportional to DNA degradation (in the form of breaks and relaxed coils).

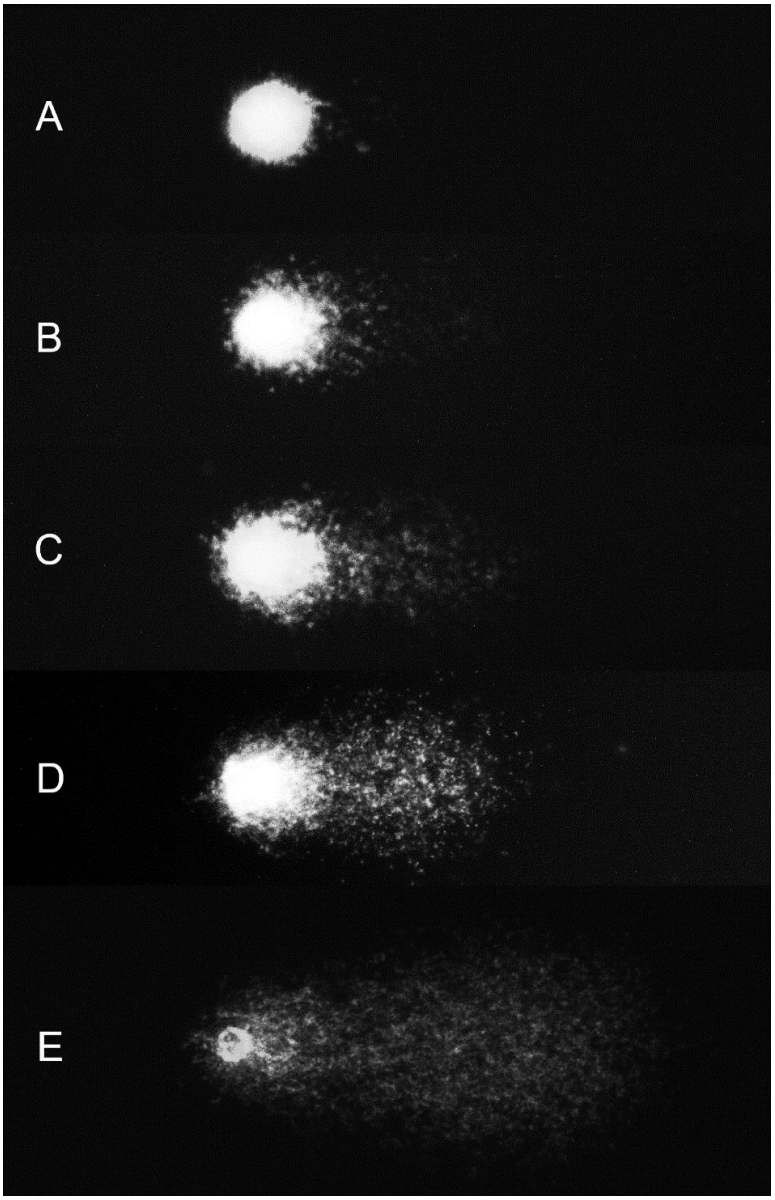

Figure 3.9. Scheme of comet classes based on % DNA in Tail in *Nucella lapillus*. A) [0-20[, where the tail is reduced, meaning most of the DNA is intact. B) [20-40[. C) [40-60[. D) [60-80[. E) [80-100[, where the amount of degraded DNA is the highest.

For an overview of the level of damage presented in each condition, the average value of DNA present in the tail of the comet was determined (Figure 3.10). Thus, it was found that all exposed conditions had about 50% of the DNA in the tail, that is, half of the mussel DNA of all conditions was harmed by the exposure. The gills treated with the salivary gland extract achieved a maximum average of 54.1% tail DNA, followed by an average of 45.2% tail DNA recorded on the exposed gills with the foot extract. The control obtained an average of 53.9% of DNA in the tail.

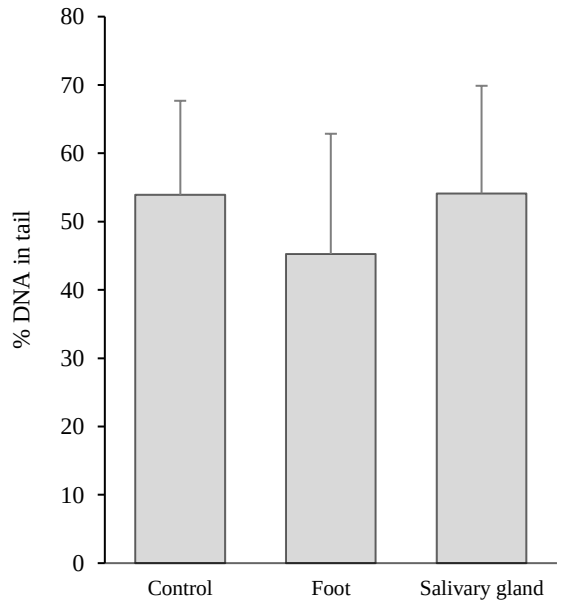

Figure 3.10. % DNA in Tail from the gills of mussels exposed to the most concentrated extract from the foot and salivary gland of *Nucella lapillus*. The values are represented as mean + standard deviation.

The Kruskal-Wallis test was applied to check the difference between conditions, a p-value < 0.05 was considered significant and in this analysis, no statistically significant differences were detected (Kruskal-Wallis test: chi-squared = 1.0643, df = 2, p-value = 0.59).

3.4.2. Histopathology

The histopathological results showed that the branchial tissue treated with *Nucella lapillus* extract does not present serious alterations, regardless of dilution and extract. Most of the gills of *Mytilus sp.* presented a typical bivalve structure, with well-defined lamellae formed by a layer of epithelial cells and branched from the filaments which, in turn, are joined by ciliary plates. But still, they presented some small morphological alterations. The main histopathological changes recorded in the target organisms are summarized in Table 3.1.

Table 3.1. List of the cellular and morphological alterations observed in the gills of mussels exposed to 0.25, 0.1, 0.025 and 0.01 mg/mL of salivary gland (SG) and foot (F). Legend: ++ Fully present; + Present; ± Moderately present; - Absent

Cellular and morphological changes	Exposure							
	0.25 mg/mL		0.1 mg/mL		0.025 mg/mL		0.01 mg/mL	
	SG	F	SG	F	SG	F	SG	F
Presence of haemocytes	++	++	++	++	+	+	±	+
Haemolytic infiltration	-	-	-	±	+	±	-	-
Apoptotic cells	++	++	++	++	++	+	+	+
Edematous (fluid-retaining)	++	++	++	++	++	++	++	+
Lipofuscin aggregates	-	±	-	±	±	+	+	±
Vacuolation	-	±	-	-	±	-	-	-
Epithelial detachment	-	-	-	-	+	±	-	-
Epithelial thickening	+	+	-	-	-	-	-	-

Analysing the semi-quantitative data presented in Table 3.1, it is possible to verify that haemocytes, apoptotic cells and edematous are the most frequent lesions in all exposed individuals. Comparing with controls (Figure 3.11), it is noteworthy that there was a large increase in haemocytes that are in haemolymph vessels at practically all concentrations, being most notable at the highest concentration (Figure 3.12). As for apoptotic cells, these were also found in all exposures and were morphologically identified by the highly and densely basophilic cytoplasm, that is, they had a high affinity for haematoxylin, showing blue when stained. The other most recurrent change was edematous (fluid-retaining) that were extremely present in all concentrations and is characterized by abnormal accumulation of fluids, presenting colourless when stained with H&E. The lipofuscin (which are known as the "brown cells") aggregates were another alteration that is moderately present, especially in the lower concentrations of the two extracts.

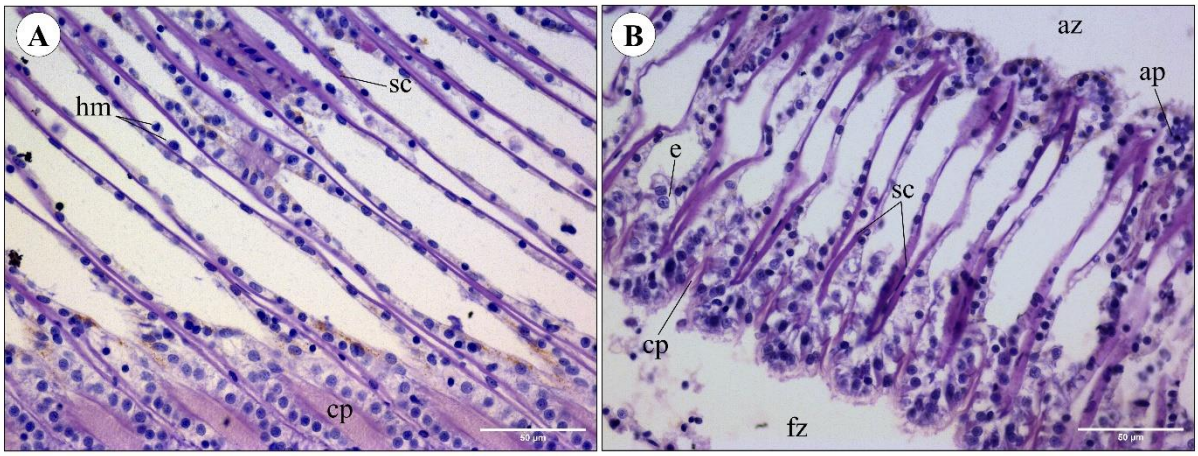

Figure 3.11. Representative histological gill section of *Mytilus sp*. treated with PBS only (controls). The gills have a lamellar structure, formed by filaments that are joined by ciliary plates (cp) and supported by supporting cartilage (sc). It has two zones: abfrontal zone (az) and frontal zone (fz). Some apoptotic cells (ap) and edematous (e) were observed but not relevant. Scale bars: 50 µm.

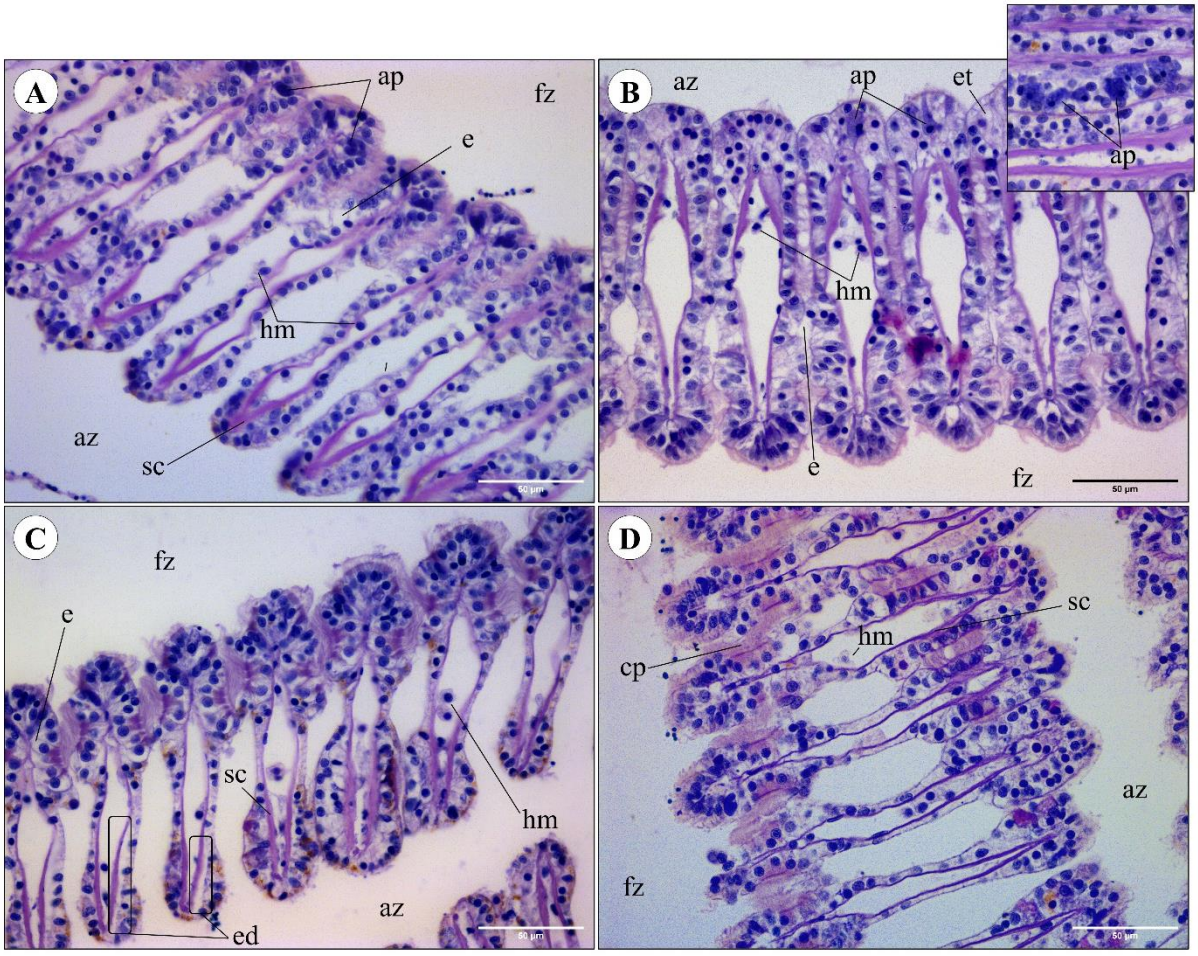

Figure 3.12. Representative histological gill section of *Mytilus sp.* with 0.25 and 0.025 mg/mL extract from the *Nucella lapillus* salivary gland (A and C, respectively) and foot (B and D, respectively) (H&E). Even with the morphological alterations, the gills maintain a lamellar structure, with the filaments interconnected by ciliary plates (cp) and supported by supporting cartilage (sc). The presence of haemocytes (hm), apoptotic cells

(ap) and edematous (e) were the most observed alterations. Epithelial detachment (ed) was also observed, which consists of the separation of epithelial cells from the supporting cartilage (sc), caused by edematous (e). Inset: detail of apoptosis (ap). Fz: frontal zone, az: abfrontal zone. Scale bars: 50 µm.

In contrast, haemolytic infiltration, vacuolation, epithelial detachment and epithelial thickening stood out as the least present alterations but were found in some individuals of different concentrations (Figure 3.13). Haemolytic infiltrations were only seen in mussels exposed to intermediate concentrations (0.1 and 0.025 mg/mL) and they were surrounded by densely packed haemocytes, which can be easily confused with apoptotic cells. Epithelial detachment is a consequence of edematous which, due to the accumulated fluid, caused the epithelium to lose its "link" with the supporting cartilage, leaving only an empty space between them. Epithelial thickening was only observed at 0.25 mg/mL of both extracts, they are characterized by multiple overlapping epithelial layers. Finally, vacuolation stood out as the least observed change, being only detected in individuals of 0.25 mg/mL and 0.025 mg/mL of foot and salivary gland extracts, respectively.

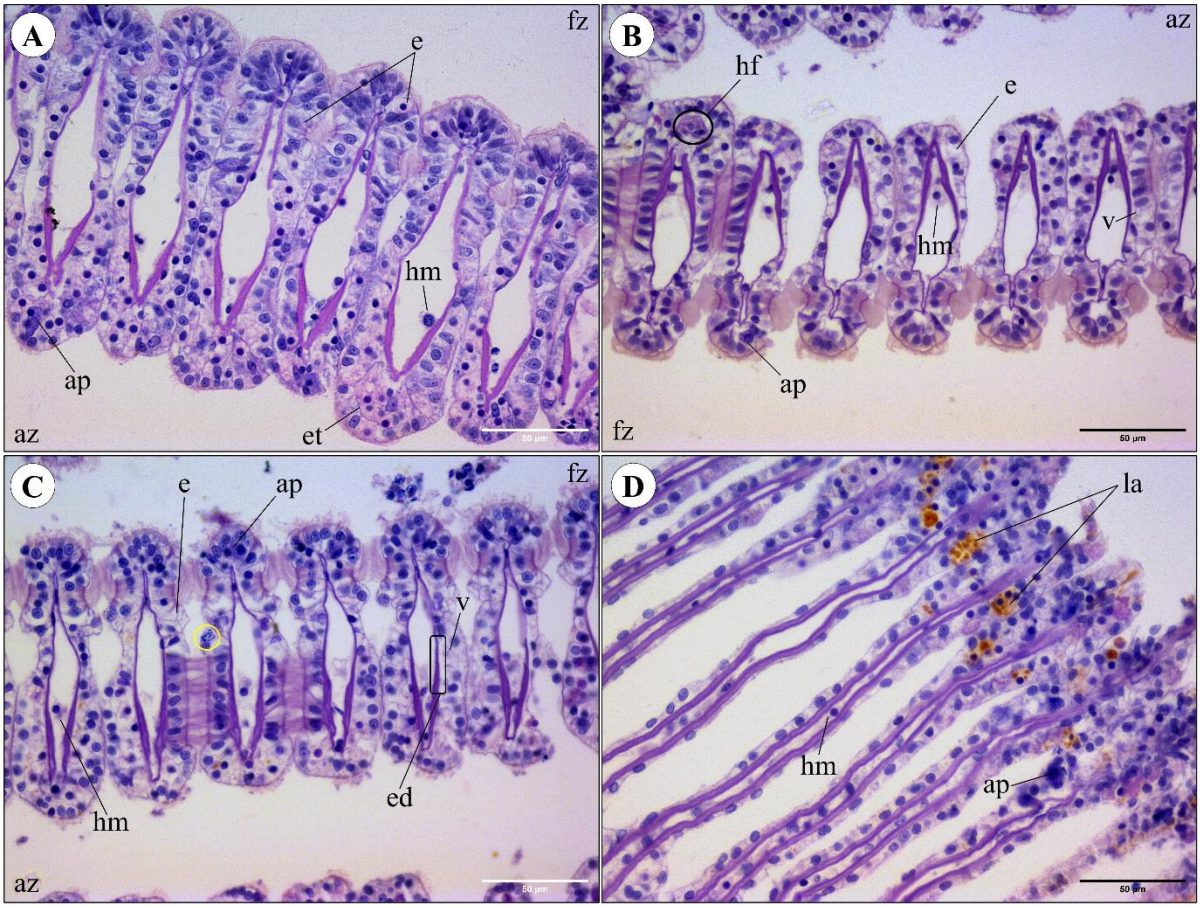

Figure 3.13. Representative histological of observed histopathological alterations in the gill of *Mytilus sp.* exposed to foot (A) and salivary gland (B, C and D) (H&E). A) Gill structure exposed to the most concentrated extract of the foot. B) and C) Gill treated with treated with 0.025 mg/ml extract of the salivary gland. D) Gill exposed to the lower concentrated extract of the salivary gland. All morphological and cellular changes observed:

presence of haemocytes (hm), apoptotic cells (ap), haemolytic infiltration (hi), lipofuscin aggregates (la), vacuolation (v), edematous/fluid-retaining (e) , epithelial detachment (ed) and epithelial thickening (et). Mitosis (yellow circle) was also observed. Scale bars: 50 µm.

3.4.3. Caspase 3 activity

The results of caspase 3 activity determined in mussels exposed to the highest concentration of foot and salivary gland extract are shown in Figure 3.14, as the mean + standard deviation. There was no significant difference between the exposures of extracts from the salivary gland and the foot but still, 1320 min showed a large trend, that is, the greatest difference between the extracts, compared to the other times. However, 90 min was the time in which all extracts registered the highest caspase 3 activities, with the salivary gland breaking the maximum record of 0.000145 µmol pNA/min/mg and, right behind, the foot extract that reached 0.00138 µmol pNA/min/mg. Analysing the other incubation times, it is possible to see that, in general, the activity decreased over time. But there was a slight increase in activity in mussels exposed to the foot extract, from 180 to 225 min which includes 30 minutes of incubation at 37°C, rising about 0.0004 µmol pNA/min/mg.

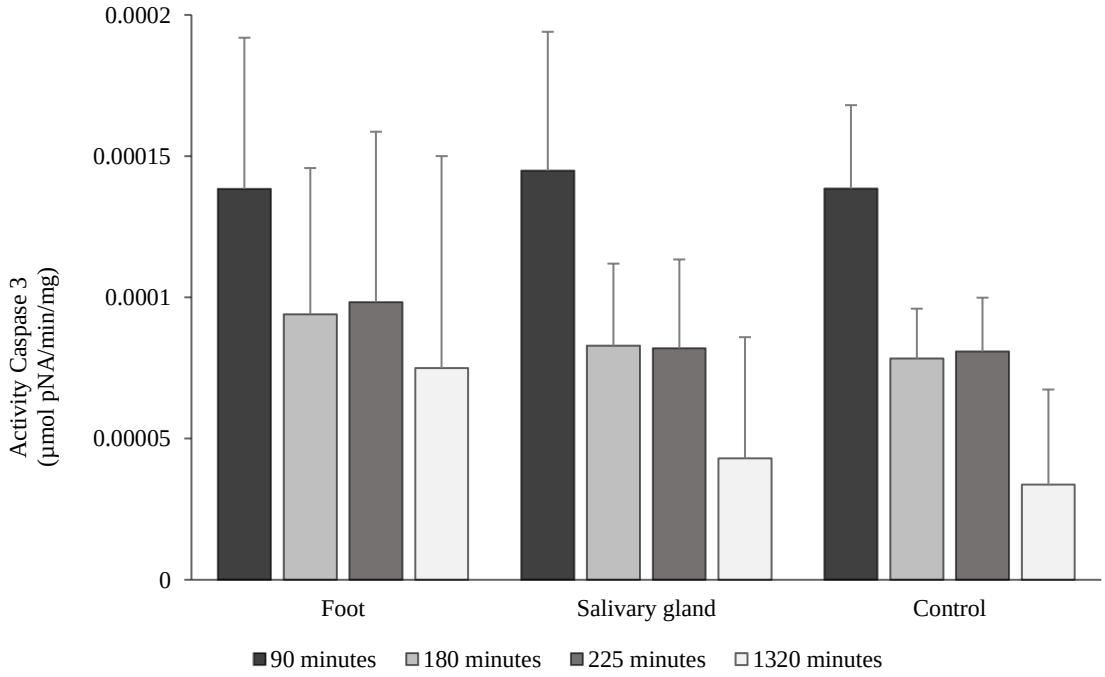

Figure 3.14. Caspase 3 activity in mussels exposed to 0.25 mg/mL of extract from the foot and salivary gland of *Nucella lapillus*. The experiment was performed with four different incubation times: 90, 180, 225 and 1320 min.

The statistical analysis performed by Kruskal-Wallis showed that there was no significant difference ($p > 0.05$) in the incubation times.

4.
DISCUSSION

The current work demonstrated that the different glandular organs of the gastropod *Nucella lapillus* have different microanatomical characteristics, as well as distinct histological signatures. In addition, the results also showed levels of thiolic proteins and peptides which seemingly reflects their different roles in the physiology, behaviour and ecology of this species of intertidal gastropod. Most importantly, these findings also suggest distinct interest for potential biotechnological research on this common species long suspected to secrete toxins and other bioactives as part of its predatory behaviour and defence. The salivary gland and the digestive gland had multiples of secretory cells and ducts, so we can say that these two organs have a complex glandular structure. The salivary gland still showed the greatest diversity and expression of proteins, with some similarities to the foot proteome. On the other hand, the digestive gland and the gonad presented blurred bands, not implying the expression or the diversity of proteins, however, as for thiols, it was in these organs that they found the highest concentrations of thiols. As the isolation of proteins from the digestive gland and the gonads became the most complicated challenge, it ended up only analysing the toxicity and bioreactivity of the remaining organs, salivary gland and foot.

4.1. Foot

The foot is mainly characterized by dense musculature that is used mainly for locomotion. However, their histological analysis also revealed the presence of the dense mucus layer, produced by epithelial cells, that coat the foot. That is, the foot also secretes mucus, which seems to be important to protect the foot during locomotion and also to help adhere to the rock surface. Given that marine gastropods of the intertidal creep over a variety of types of substrates (especially rocky) and that the foot is usually one of the main entry sites for pathogens, secreted mucus is also used for microbial defence and tissue hydration (Zhong et al., 2013; McDermott et al., 2021). In addition, the foot is also characterized by the abundance of microvilli on the brush border that is likely used to distribute mucus to lubricate the organ during movement. Histological and ultrastructural studies on invertebrate foot are

practically scarce, however, those that exist reinforce the importance of mucus for animals and, moreover, they reported that they secrete different types of mucus with different functions, depending on how it is stimulated, such as for example, tidal activity cycles, which can happen to *Nucella*, as it inhabits rocky areas exposed to tidal cycles (Bravo Portela et al., 2012; Noothuan et al., 2021; McDermott et al., 2021). Different studies, such as Ireland & Faulkner (1978), Kubota et al. (1985) and Dolashka et al. (2011), already demonstrated that the secreted mucus has several biological activities such as antimicrobial, antioxidant and antitumor. In addition to mucus, the foot can also be involved in the secretion of cysteine-rich proteins, as it obtained a considerable number of thiols, even more than the salivary gland. As the foot is involved in lubrication and adhesion to the surface, the organ secretes essential protein-based "adhesives" to ensure that animals stick firmly to surfaces, and cysteine appears to be one of the amino acids that play vital roles in the process of adhesion (Almeida et al., 2019; Li et al., 2021; Zeng et al., 2021). Several publications (see for instance, Yu et al., 2011a, b) detected high content of thiols in this organ of marine invertebrates, namely in mussels, which showed to be important for the high addition to surfaces, especially in humid, turbulent and salty environments, controlling the redox reaction of the catechol groups of 3,4-dihydroxyphenylalanine (DOPA) that are responsible for adhesion. This indicates that this is likely to happen in other molluscs such as *Nucella*.

It was noticeable that the proteome of the foot obtained a proteome that was very similar to that of the salivary gland, presenting common and pronounced bands in the range of 35 to 42 kDa. It must be noted that, in *Nucella lapillus* as in other molluscs, the foot is not well differentiated from the salivary glands and other organs, as the coelom is a much-reduced cavity. In view of this, both organs bear dense and tangled muscle fibres, which made their individualization very difficult. This fact may explain the identical results between the two proteomes. However, it could also have been due to the overshadow caused by the common proteins, that is, the common proteins that unite the two organs may have "masked" the specific proteins that differentiate the two organs, preventing their visibility in the gels. For this reason, there was a need to fractionate samples from the salivary gland and foot, to prevent overshadowing of less-represented proteins by highly abundant proteins such as actin and other cytoskeletal elements, reducing the complexity of proteins between the two organs. It is important to point out that Amicon filters are not always efficient, they may have selectivity problems, that is, they may not separate the contents by molecular weight and, consequently, they may have very similar filtrate profiles. According to Johnsen et al. (2016), Amicon filters are inadequate for separating proteins into fractions. Still, protein fractionation has been advanced. The results showed once again the similarities between the proteomes of the salivary gland and the foot, with emphasis on the range of 20 to 45 kDa, however, the salivary gland showed bands that differ from the foot.

Finally, *ex-vivo* bioassays results revealed a differential bioreactivity of foot protein extracts. Their secretions induced an increase in the presence of haemocytes and apoptotic cells that appear to be cellular immune responses in molluscs, being more evident at the highest concentration. Even so, damage

was also observed, namely lipofuscin aggregates, at lower concentrations. In addition, the protein extracts from the foot also showed the ability to damage the DNA, having destroyed about 50% of the DNA of the exposed mussels. As a large increase in apoptosis was histologically observed, caspase 3 activity was analysed to determine the intrinsic apoptosis pathway and the results were insufficient to draw conclusions. It was possible to detect some pNA at all exposures, but caspase 3 activity was too low. One of the justifications could be linked to the possibility that the procedure does not work for this type of organization.

4.2. Gonad

As the foot, the gonad also has some secretory cells. The gonads have different stages of development of maturation in which the morphology of the germ cells varies within the gonad, depending on the stage of development of maturation and, therefore, the histological determination of the stage of maturation is based on evolution and arrangement of germ cells (Costa, 2017). Therefore, before analysing the histological results of the reproductive organ, it is important to take into account the developmental stages of maturation, there are classifications of gonadal development that were made for specific organisms, however, so far, there is no literature regarding the classification of *N. lapillus*. Still, the maturation phases are very similar in oviparous aquatic animals, on which *Nucella lapillus* is part, and therefore can be classified into six phases: immature, early ripening (early maturation), ripening (mature), spawning and postspawning (spent) (Costa, 2017). Taking this into account, it was possible to observe that the organisms were maturing, that is, they were still immature. At this stage, the gonads are morphologically differentiated, with different stages of germ cells scattered in the follicles and tubules and surrounded by numerous adipogranular cells. According to Costa (2017), the gonads are formed by mesodermal-originated cells and, for example, in bivalves, the gonads originate in the mantle's adipogranular tissue. The mantle can be used as a place for the production and maturation of gametes, as well as storage of reserves, being composed of several types of storage cells, such as vesicular cells (VC) and adipogranular cells (ADG) (Lobo-da-Cunha et al., 2006; Eckelbarger & Hodgson, 2021). Although there are some theories about their involvement in reproduction, the functions of adipogranular cells have not been fully clarified, particularly in gastropods. Even so, already described in several species of bivalves, such as *Mytilus edulis*, adipogranular cells were identified as storage sites for proteins, lipids and glycogen and showed high metabolic activity during gametogenesis, that is, stored compounds are used for the formation of reproductive organs and gamete proliferation (e.g., Pipe, 1987; Peek et al., 1989). Biochemical studies in gastropods are limited, but in bivalves, the cellular and biochemical compositions of the mand tissue vary seasonally, depending on the reproductive cycle, that is, the availability of stored reserves varies in the time and duration of gametogenesis, being lower during the gamete production (Ruiz et al., 1992; Mathieu & Lubet, 1993; Ojea et al., 2004). Seasonal metabolic activities in molluscs are also influenced by food availability (Ojea et al., 2004). There are publications

that demonstrate the important connection of the gonad with the digestive gland, which apparently plays a secondary role as a storage site for essential nutrients for gamete development (Vélez-Arellano et al., 2016; Eckelbarger & Hodgson, 2021). Many studies have associated the increase in the size of the digestive gland with the reduction of the gonad, proposing that the material can be transported from the digestive gland to the gonad and this transfer was observed in different groups of molluscs, but the pathways and mechanisms are still poorly understood (Gabbot, 1975; Najmudeen, 2007). Thus, it is likely that the connection with the digestive gland has influenced the results of the gels in the reproductive organ, with all blurred bands, such as those in the digestive gland. In bivalves, glycogen appears to be the main source of energy, yet proteins can also be used as an energy source in cases of nutritional stress and energy imbalance or during gonad maturation (Gabbott & Bayne, 1973; Ruiz et al., 1992; Mathieu & Lubet, 1993; Liu et al., 2008). In this present work, a high content of thiol proteins was detected, which may indicate that most of the stored proteins contain cysteine, but there are no studies that could support this theory. As the value of thiols was very significant compared to other organs, these proteins may have some importance for the organ. There are many articles that report the presence and importance of CRISPs in the male reproductive system, particularly in fertilization, but most of these studies refer to mammals, there is almost nothing about marine invertebrates, which makes the analysis difficult. In mammals, although the mechanisms are far from being fully understood, the proteins have been associated with epididymal proteins and are involved in sperm-egg fusion through interaction with complementary sites in the egg, where they "help" the sperm to fertilize an egg, binding it with the plasma membrane of the egg (Cohen et al., 2011; Da Ros et al., 2015; Weigel Muñoz et al., 2019).

4.3. Digestive gland

The digestive gland is also a compound gland, presenting a tubular structure typical of the digestive gland of molluscs, formed by multiple tubules (also called digestive diverticula) with numerous basophilic and digestive cells. Digestive cells are involved in intracellular digestion, more specifically, in the absorption and digestion of nutrients and, on the other hand, basophilic cells appear to be digestive enzyme secreting cells (Zaldibar et al., 2007; Zarai et al., 2011). Thus, the structure reflects that the digestive gland is a glandular organ with important functions in digestion. In addition, different digestive enzymes secreted by the digestive gland have already been detected, such as peptidases, acid phosphatase, glycosidases and esterases, which reinforce their involvement in digestion (Foster et al. 1999; Taïeb, 2001). This function can justify the blurry bar obtained in the gel, making it impossible to draw conclusions about the expression and diversity of proteins and, for this reason, the isolation of proteins from this organ proved to be problematic, as it was not possible to fractionate the proteins. However, it was possible to find a high content of cysteine-rich substances in this organ, most of which are strongly linked to other proteins. This evidence shows that the digestive gland may have the ability to secrete

cysteine-rich proteins and there may be some connection to its role in detoxification. To explain better, the digestive gland of molluscum is analogous to the liver of vertebrates, being a multifunction organ that accumulates digestive roles with storage and detoxification and, therefore, can contain digestive enzymes just as well as detoxification agents such as glutathione and metallothioneins (for instance), which, when combined, are responsible for the relatively high content of thiols (D'Ambrosio et al., 2021). This assertion can be corroborated by different publications (e.g., Ireland, 1979; Leung & Furness, 1999) in which they detected high concentrations of different heavy metals, such as cadmium, zinc and copper in these same glands of *Nucella lapillus*. To finalise, exposure to metals can cause a change in the cellular proportions of the digestive tubules, increasing the volume of basophilic cells in relation to digestive cells, which seems to be evident in the histological results of the salivary gland (Soto & Marigómez, 1997a, b; Soto et al., 2002). It is for these reasons that the digestive gland has been a target organ in toxicological studies.

4.4. Salivary gland

In similarity to the digestive gland, the salivary glands of *N. lapillus* also contain a complex gland structure, having three types of morphologically distinct secretory cells that form the glandular epithelium: vacuolated cells, granular cells and mucocytes. There has been some controversy regarding the number, function and secretion of secretory cell types existing in the salivary glands, which can be highly variable between species. The number of morphologically distinct secretory cell types observed in the glands has ranged from one to seventeen (e.g., Serrano et al., 1996; Moura et al., 2004; Lobo-da-Cunha & Calado, 2007). Each cell type must secrete different types of proteins and peptides. According to available data, vacuolated cells may have acidic polysaccharides and small amounts of protein in secretory vacuoles, and granular cells appear to be protein-secreting cells that are used to produce granules (Lobo-da-Cunha, 2002; Lobo-da-Cunha & Calado, 2007; Lobo-da-Cunha et al., 2009; Abdel Gawad et al., 2018). However, secretions are not yet fully understood and further studies are needed. Despite this, salivary gland secretions in gastropods have been associated with several physiological functions, such as lubrication, food intake, enzyme production and prey capture (Andrews, 1991).

The salivary gland is connected to the digestive system by a duct that is lined by epithelial cells with several folds of the basement membrane and still contains an abundance of microvilli, suggesting the existence of an absorption capacity by the epithelium. Another important feature observed in *Nucella* is the presence of muscle fibres around the duct. Thus, it is likely that saliva is driven by peristaltic contractions of muscle fibres, which reinforces the involvement of the salivary gland in pre-digestion, more specifically, lubrication and agglutination of food and secretion of digestive enzymes. This evidence can be corroborated by the activities of digestive enzymes detected in the salivary glands of different gastropods, such as amylase, protease and maltase (e.g., Charrier & Rouland, 1992; Moura, 2004). Information about the presence of digestive enzymes present in the salivary glands of molluscs

is reduced, but when compared, the activities are considered significant (Moura, 2004). In view of this, the salivary gland has a complex gland structure that is compatible with the secretion of enzymes and toxins. Different toxins in salivary secretion have been reported in different species of gastropods (e.g., Shiomi et al., 1994; Andrews et al., 1999; Power et al., 2002). Thus, they would be expected to have the greatest diversity and expression of proteins on SDS-PAGE. Instead, they showed very similar results with the foot proteome, showing common and pronounced bands in the 35 to 42 kDa range, which are compatible with cysteine-rich proteins, such as CRISPs or GSTs that can range from 20 - 30 kDa and 23 - 28 kDa, respectively. These results can be explained by the problems mentioned above (problems of individualization and/or overshadow of proteins). After protein fractionation, the salivary gland showed greater diversity than the foot, namely in the upper fraction of 100. In this fraction, the salivary gland obtained more pronounced bands than the foot, with specific proteins being more expressed in the 36 and 80 kDa. In general, the three gels performed (unfractionated and fractions 10 - 100 and > 100) showed the highest expression particularly in the range of 20 to 45 kDa, which is compatible with cysteine-rich proteins. This is further supported by the existence of thiols in this organ. It should be noted that the 36 kDa protein also appeared in the other fraction, unlike the other bands that only appear in a fraction, which may mean that this protein in question has a certain importance for the organ and could be CRISP, whose MW varies between 20 and 30 kDa. Even so, of the four non-fractionated organs, the salivary gland is not the main secretor organ of cysteine-rich proteins, as it was in this organ that the lowest thiol concentration was found, contrary to expectations. However, the proteins from the salivary gland were fractionated to reduce their complexity and improve thiol quantification and positioning within the proteome. The fractionation results provide further evidence for the differential proteome complexity hypothesis, with the salivary gland presenting a higher concentration of thiols than the foot, namely in the fraction from 10 to 100 kDa. Regarding the type of thiols, it should be noted that the fraction > 100 it has the widest distribution of free thiols, accounting for about 13% of the total thiols while the other organs contain a maximum of 5.5%.

The existence of thiols in the salivary gland can also be supported by other different studies that showed the secretion of cysteine-rich proteins in *Nucella lapillus*, such as Andrews (1991) or D'Ambrosio et al. (2021). First, the species under study has effectively two pairs of salivary glands, one acinous and one tubular which is known as the accessory gland and their structural differences reflect their functions (Andrews, 1991). The acinar glands are covered by a thin layer of connective tissue with muscle fibres and are probably responsible for the synthesis and secretion of protein materials, being composed of two types of secretory cells: one secretes glycoprotein and the other produces acidic mucopolysaccharides with a small amount of proteins (Andrews, 1991; Lobo-da-Cunha, 2019; D'Ambrosio et al., 2021). On the other hand, the accessory tubular glands have a similar structure to the Conoidea glands, being characterized by the presence of muscular tubular walls and the presence of epithelial and subepithelial secretory cells, which secrete, respectively, tryptophan-rich proteins and a cysteine-rich glycoprotein (Andrews et al., 1991; Ball et al., 1997). Andrews (1991) and West et al. (1994) associated

the tubular salivary gland as a possible venom gland, highlighting the presence and secretion of a compound in the salivary glands of *Nucella* that was identified as serotonin, with the ability to induce cardiac arrest and muscle paralysis in the prey, the mussels. They also detected another unidentified compound, a glycoprotein with multiple disulphide bonds, similar to conotoxin and may be responsible for part of the gland's bioreactivity (Andrews et al., 1991; Andrews, 1991). Furthermore, preliminary studies carried out by D'Ambrosio et al. (2021) confirmed the presence of cysteine-rich proteins in the perioral mass, which includes the salivary glands.

Lastly, the salivary gland protein extract showed a differential bioreactivity, as did the foot. It was noted that the morphological changes observed in the exposed mussels were identical for both conditions (salivary gland and foot), but this can be explained by the fact that the fraction above 100 was used (the fraction that showed the greatest diversity and difference between the two organs) and both extracts obtained a very similar total thiol concentration value, which shows more that the foot and the salivary gland can secrete cytotoxic agents rich in cysteines. The detection of DNA damage after exposure to the extracts revealed a slightly higher percentage in mussels exposed to the salivary gland than the foot, which can be explained by their functions in secretion. Like in the foot, caspase 3 activity in tissues exposed to salivary gland extract was also analysed and compared to the results of the foot, these tissues showed a greater tendency to decrease activity after 180 minutes. This observation may be related to the secretory cells that make up the salivary glands. These results indicate that salivary glands may be a promising target for the extraction and characterization of cysteine-rich protein toxins.

5.
CONCLUSION

The present work revealed the presence of cysteine-rich proteins in each of the four glandular organs identified (foot, salivary gland, digestive gland and gonad) of *Nucella lapillus*, with the gonad and digestive gland having the highest concentrations. A probable reason is their important roles in fertilization and detoxification, respectively. Even with the reduced concentration, the salivary gland, whose structure is compatible with the secretion of toxins, and the foot revealed the greatest quantity and diversity, with some similarities between their proteomes and the more pronounced bands residing in the range from 20 to 45 kDa, which are compatible with cysteine-rich proteins such as cysteine-rich secretory proteins (CRISPs) and glutathione (GST). In turn, the digestive gland and the gonad revealed poorly defined bands, due to the presence of peptidases, making their detection and isolation more complicated.

Toxicity and bioreactivity tests revealed similar results for the two organs (salivary gland and foot), probably because they were integrated in the same mass. The results showed that proteins from the salivary gland and the foot affected the gill structure of mussels, namely the epithelial cells. In addition, they also caused an increase in haemocytes and apoptotic cells, which shows that the proteins bear cysteine residues and have noticeable toxicity and bioreactivity even at low concentrations. That said, we can conclude that both the salivary gland and the foot are promising targets for bioprospecting of new marine bioactives for biotechnological purposes. Nonetheless, more studies are needed, with emphasis on the optimisation of protein extraction, fractionation (e.g., by HPLC and LC) and identification (namely through tandem mass spectrometry). For the purpose, the gonad and the digestive gland should be prioritised since these are the organs where the highest concentrations of thiolic substances are found. Most importantly, the current work demonstrated that marine invertebrates that are common in temperate waters, such as *Nucella*, are a promising source of bioreactives, with implications for ocean-based biotechnology and the awareness for the need to safeguard marine biodiversity.

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APPENDIX

Appendix 1. R Script for the bioinformatics analysis of the comet assay and caspase 3 activity data.

```
library(gplots)
library(dunn.test)

##Data import to R
filename <- "C:\\Users\\Marta Gato\\Desktop\\RStudio\\CSV\\Data_Biomarkers.csv"
Data <- read.table(filename, sep=";",header = TRUE)
summary(Data)
rownames(Data)<-Data[,1]
Data<-Data[,-1]

##Multiple comparisons (non-parametric, alpha = 0.05)
for(i in 5:ncol(Data)){
     ##Test
     print(colnames(Data[i]))
     print(dunn.test(Data[,i],g=Data$Organ, method = "bh"))
```

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