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Toxic impacts in mucus of *Halobatrachus didactylus:* modulation of activity by fish's sex, stress and environmental conditions

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Maestrado em Biologia Marinha

Supervisor:
Pedro Miguel Guerreiro Da Costa Guerreiro
Co-supervisor:
Teresa Isabel Mendonça Modesto

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Toxic impacts in mucus of *Halobatrachus didactylus:* modulation of activity by fish's sex, stress and environmental conditions

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Abstract

Halobatrachus didactylus, commonly known as the Lusitanian toadfish, is a benthic sedentary species inhabiting coastal waters, which mucus was recently found to have toxic activity over bacteria and other fish, likely playing a role as an immune and defense mechanism. This study aimed to investigate which conditions, intrinsic or external, may determine the toxicity of the mucus over other fish species, including amount, stress, sex and salinity, while understanding the potential role of the skin and axillary glands in toxin production. Through a series of in vivo and in vitro experiments, the toxicity of the mucus was evaluated using *Sparus aurata* and *Gambusia holbrooki* as test subjects. The experiments showed that the lethal effect of the toxin is dose dependent, either when collected mucus was added to water or when toadfish holding water was used. The results also revealed that male mucus have higher toxicity than female mucus, reflected in higher lethality of test subjects exposed to the same conditions. Haemolytic activity experiments showed differences in toxicity between sexes, with male mucus causing higher hemolytic activity. The toxin is likely hydrophilic as it maintains activity in the toadfish holding water, but environmental salinity was found to influence the toxin's effectiveness: mucus added to seawater was effective while freshwater conditions nullified the mucus's lethality, suggesting that the toxin may require specific environmental conditions or physiological pathways to be active to evoke an effect. While the skin mucus clearly demonstrated toxic properties, no significant toxic effect was detected from the contents of the axillary glands, challenging the hypothesis of their involvement in toxin production. The histological analysis revealed a higher density of skin mucus cells in males compared to females, raising questions about sex-based physiological differences in mucus production and toxicity. This research provides new insights into the toxicological properties of *H. didactylus* and suggests that its mucus plays a dual role in predation defense and microbial protection. However, further biochemical analyses are required to fully understand the molecular composition of the toxin, actual sites and regulatory mechanisms of secretion, and its ecological significance.

Keywords: *Halobatrachus didactylus,* biotoxin, skin, modulation of toxic activity, *Sparus aurata, Gambusia holbrooki,* defense.

Resumo

Este trabalho centra-se sobre as propriedades toxicológicas do muco produzido por *Halobatrachus didactylus* (n.v. xarroco ou peixe-sapo lusitano), uma espécie bentónica e eurihalina pertencente à família Batrachoididae. Os membros desta família estão distribuídos por praticamente todas zonas costeiras temperadas e tropicais do planeta, e muitas das espécies que a compõem produzem substâncias com propriedades tóxicas. O xarroco, que habita as águas costeiras da região nordeste do Oceano Atlântico e algumas zonas do Mar Mediterrâneo, é uma espécie bentónica e eurihalina que vive sob pedras e em fendas, alimentando-se de pequenos peixes, crustáceos, moluscos e poliquetas. Embora consiga deslocar-se pelos estuários ou lagoas, esta espécie é geralmente sedentária, com taxas metabólicas baixas, reproduzindo-se próximo ao fundo do mar, onde os machos territoriais atraem as fêmeas para seus ninhos com sons produzidos pela contração dos músculos sonoros na bexiga natatória; um repertório variado de sons é também utilizado com funções defensivas para afastar outros machos. A espécie apresenta táticas reprodutivas alternativas, com dois tipos de machos: tipo I e tipo II. Os machos do tipo II, geralmente menores, apresentam comportamentos parasitas dos machos tipo I, pois não cortejam as fêmeas nem constroem ninhos, aproveitando-se da sua semelhança com as fêmeas para se infiltrar no ninho do macho tipo I e tentar fertilizar os ovos que ficam aderidos às paredes e tetos dos ninhos.

Apesar dos fortes espinhos na barbatana dorsal, o xarroco, ao contrário de muitas espécies deste grupo, não apresenta espinhos ocos ou outras formas evidentes de inocular substâncias tóxicas. No entanto, estudos recentes mostraram que o muco desta especie é tóxico para várias espécies de peixes, induzindo alterações drásticas no seu comportamento e, inclusivamente a morte. Até ao momento foram avaliadas as propriedades antimicrobianas do muco e foi demonstrado o seu efeito em vários parâmetros hematológicos e na função dos nervos olfativos de outras espécies, mas o papel ecológico da toxicidade do muco de *H. didactylus* é ainda pouco claro.

O objetivo geral desta tese foi investigar as recém-descobertas propriedades tóxicas do muco de *H. didactylus*, com foco nas condições que podem modular a sua secreção e potência, bem como nos locais efetivos de produção. O primeiro objetivo deste estudo foi determinar se o efeito tóxico previamente observado era atribuível ao muco produzido pela pele ou se poderia provir de outros tecidos ou glândulas, já que estudos anteriores haviam apenas utilizado a água na qual foram imersos xarrocos. Para isso, testámos o muco coletado diretamente da pele e as secreções da glândula axilar, comparando os efeitos com aqueles provocados pela água onde estiveram imersos os peixes, conforme os protocolos anteriores, além de analisar a estrutura da

pele e da glândula axilar como possíveis locais de produção e secreção. O segundo objetivo foi investigar a relação entre a potência dos efeitos tóxicos e variáveis intrínsecas, como sexo e/ou níveis de stress nos animais. O muco e a água onde estiveram imersos os peixes ("água de xarroco") de ambos os sexos, antes e após serem submetidos a condições de stress, foram utilizados em diferentes concentrações para avaliar os impactos sobre possíveis espécies-alvo simpátricas (*Sparus aurata* em testes in vivo e em um ensaio de atividade hemolítica in vitro). Finalmente, testou-se a influência do ambiente e das espécies-alvo utilizando duas espécies de peixes eurihalinos, uma de origem marinha (*S. aurata*) e outra originária de água doce (Gambusia holbrooki), aclimatadas e testadas numa gama de salinidades ambientais. A metodologia utilizou abordagens in vivo (testando o muco em indivíduos vivos) e in vitro (testando a hemólise em células sanguíneas). A primeira experiência visou compreender as diferenças, caso existissem, no efeito tóxico do muco entre os sexos e em diferentes condições de stress.

O muco cutâneo de vários individuos foi recolhido na região dorsal, em ambos os lados do corpo do animal, com uma lâmina de vidro e transferido para um tubo de ensaio. Foram agrupadas as amostras de 4-6 individuos. Após a recolha e pesagem, o muco foi dissolvido em água salgada e depois um volume desta solução foi adicionado ao tanques experientais contendo os peixes alvo, previamente aclimatados por 10 minutos. Foram utilizadas duas concentrações: 0,04g/L (dose I) e 0,4g/L (dose II), em 500 mL de água salgada. Após adição da solução de muco, os comportamentos e a ocorrência de mortalidade na espécie alvo foram observados e registados em vídeo durante um período de 25 minutos. Para cada condição (controlo, dose I e dose II) foram preparados 3 tanques, cada um contendo 3 indivíduos da espécie alvo.

Foram efetuados vários grupos de experiências de forma a testar separadamente o efeito do muco de machos e de fêmeas, sem stress ou expostos a stress de manuseamento repetido, num volume de água reduzido. O muco foi adicionado, nas concentrações descritas, a juvenis de dourada, *S. aurata*, como espécie alvo. Os resultados mostram uma diferença entre os impactos causados pelo muco proveniente de machos ou fêmeas, sendo o muco dos machos mais efetivo em causar alterações comportamentais e também mais letal para *S. aurata*. Quanto ao efeito do stress, parece haver uma diminuição da toxicidade quando o muco utilizado provém de animais stressados, mas há que avaliar com alguma atenção esta informação, pois o muco pode ter sido libertado e diluído na água onde os animais permaneceram durante o manuseamento. Numa segunda abordagem foram testados os potenciais efeitos das condições ambientais sobre a toxicidade do muco. Para este efeito foi realizada uma experiência, em condições semelhantes às anteriores, mas em que os peixes alvo foram previamente aclimatados a diferentes salinidades. Foram testadas duas espécies, *S. aurata* a 36ppt, 25ppt, 13 ppt, 6 ppt e 3 ppt, e *Gambusia holbrooki* a 0 ppt, 13 ppt e 25 ppt. Os resultados mostram que a toxicidade em *S. aurata* diminui com a salinidade e que o muco não é tóxico para *G. holbrooki* em água doce, mas que a toxicidade aumenta na salinidade intermédia e é 100% letal a 25 ppt. As razões para este impacto da salinidade sobre a toxicidade do muco não são conhecidas, mas podem dever-se quer à necessidade de determinados compostos no ambiente para ativação da toxina, ou a diferenças na fisiologia entre peixes adaptados a água doce e a água salgada, visto que ambas as espécies mostraram comportamentos semelhantes em resposta à toxina na gama de salinidades utilizada. O terceiro grupo de experiências, em que foi utilizada "água de xarroco" (machos ou femeas) filtrada visou testar se a mortalidade observada em estudos anteriores poderia ser causada pela ação mecânica de grandes partículas de muco sobre a superfície respiratória, e testar ainda o efeito da "água de xarroco" em diferentes concentrações e após diferentes períodos de stress (após 5 ou 30 minutos de manuseamento e exposição ao ar). Foram testadas três diluições: 1:1, 1:5 e 1:50 sendo neste caso, os indivíduos da espécie alvo que adicionados a 500ml desta água. Os resultados confirmaram a diferença de potência entre machos e fêmeas e mostraram que a morte não é causada pelo efeito mecânico do muco. Além disso, a água dos peixes stressados durante mais tempo pareceu ter efeitos mais leves, portanto ser menos tóxica, talvez devido à degradação da toxina ao longo do tempo, mas indicando que esta será libertada no inico do processo de stress e não ser adicionada cumulativamente na água onde estiveram os xarrocos. A última experiência in vivo teve como objetivo investigar se a glândula axilar está envolvida na produção da toxina, tal como se supõe para outras espécies. As secreções desta glândula foram recolhidas com uma zaragatoa e diluídas em água salgada, e o protocolo experimental repetido como descrito para o muco cutâneo. Não foram verificados quaisquer efeitos deste material biológico sobre o comportamento ou a mortalidade em *S. aurata.*

As experiências in vitro consistiram em medir a atividade hemolítica do muco, dosear a quantidade de proteína presente nas amostras de muco recolhido e utilizado nas diferentes experiências, e analisar eventuais diferenças histológicas entre sexos e condições de stress na pele e na glândula axilar. Para a atividade hemolítica, foram recolhidas amostras de sangue de 4 douradas adultas, e após processamento, os erirócitos foram expostos a quatro diluições de muco em solução fisiológica: 1:1, 1:100, 1:1000 e 1:10000. Os resultados mostraram que o muco tem atividade hemolítica, com valores mais elevados registados para o muco dos machos

em comparação com o das fêmeas, especialmente visíveis na diluição 1:100. A concentração de proteínas nas amostras, quer as utilizadas na atividade hemolítica, quer as utilizadas nas experiências in vivo, mostra estar relacionada com quantidade de muco; as amostras de muco obtidas de machos mostraram consistentemente valores mais elevados de proteína para a mesma quantidade de muco. Este resultado parece indicar que a toxina poderá ter uma natureza proteica, corroborado parcialmente pela sua aparente facilidade em ser solúvel em água. Porém, não sabendo a composição exacta da toxina, não podemos assumir categoricamente que maior quantidade de proteínas equivale a maior quantidade de toxina. A análise histológica foi efetuada em três amostras de pele e na glândula axilar. Foram utilizadas diferentes colorações para realçar eventuais diferenças estruturais e de composição química. A partir da reação com a coloração azul de Alcian, foi possível observar que as células do muco contêm substâncias ácidas e que existe uma maior densidade das mesmas na pele dos machos em comparação com as fêmeas. Relativamente às glândulas axilares não há diferença na quantidade de células mucosas mas pode haver diferenças relativamente às células saciformes. Seria necessária uma abordagem histológica mais detalhada para validar possíveis diferenças entre machos e fêmeas e entre animais stressados e não stressados. Em conclusão, este trabalho provou haver uma relação entre a potência da toxina e a dose, diferenças de potência entre machos e fêmeas e a importância das condições ambientais para a sua ação. Este conjunto de informações são úteis para avaliar o potencial significado ecológico da toxina e fornece pistas relativamente à sua natureza e local de produção. No entanto, será necessário efetuar outras abordagens para identificar a composição química da toxina e compreender a sua proveniência, condições de secreção, e os mecanismos de ação noutros peixes.

Palavras-chave: xarroco, biotoxina, muco cutâneo, espécies-alvo, modulação

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Abbreviations

(fill the table and in the end just sort from A to Z)

NSMM	fish receiving mucus from non-stressed male toadfish
SMM	fish receiving mucus from stressed male toadfish
NSFM	fish receiving mucus from non-stressed female toadfish
SFM	fish receiving mucus from stressed female toadfish
SMW5	fish exposed to holding water of male individuals stressed form 5 minutes
SMW30	fish exposed to holding water of male individuals stressed form 30 minutes
SFW5	fish exposed to holding water of female individuals stressed form 5 minutes
SFW30	fish exposed to holding water of female individuals stressed form 5 minutes

Chapter 1 INTRODUCTION

1.1 ANIMAL TOXINS

To survive in the natural world, having evolutionary advantages is essential. Over time, many animals have developed the ability to produce toxins. These substances, when present in biologically relevant quantities, cause dose-dependent pathophysiological injury to living organisms, ultimately reducing their functionality or viability (Gwaltney-Brant, 2017). The level of toxicity and its action, having immediate or delayed effects, ranging from discomfort, pain, transient muscle paralysis, neural impairment or death, are modulated by the nature and dose of exposure. Some highly toxic substances can in fact have no effect if they are administered in small quantities, the same way as some harmless ones can become toxic in high quantities. As Theophrastus of Hohenheim (Paracelsus), the Swiss-German physician and 'Father of Toxicology', said, 'All things are poison and nothing (is) without poison. Only the dose makes a thing not to be poison' (Michaleas et al., 2021).

As previously mentioned, the consequences of the contact with the toxins can vary from small impairment to death (Nelsen et al., 2014). Toxins have evolved for defence or predatory purpose and organisms that produce them can be characterized in two categories. Venomous animals actively deliver their toxins through bites or stings, often using specialized structures. In contrast, poisonous organisms possess toxins that can harm if ingested, inhaled, or absorbed through the skin, but they do not actively deliver the toxin. (Gwaltney-Brant, 2017). The different ways of delivering the toxin highlight the different purposes they can have. Bactericidal functions, protection from predators and more effective predation are some of them (Brodie, 2009).

Toxin characterization

The study of toxins reveals an incredible diversity in their mechanisms of action, reflecting the evolutionary pressures faced by different species. Toxins in animals can be broadly categorized based on their modes of action, which include neurotoxins, hemotoxins, cardiotoxins, and cytotoxins, each targeting specific physiological processes in prey or predators (Karalliedde, 1995). Neurotoxins, such as tetrodotoxin found in pufferfish, block sodium channels, preventing nerve impulse transmission and leading to paralysis. This class of toxins is particularly effective in immobilizing prey quickly (Schiavo et al., 2000). Cardiotoxins, which can be found in some snake venoms, interfere with cardiac muscle function by either causing sustained contraction or disrupting the electrical signals in the heart. This can lead to fatal arrhythmias or cardiac arrest, making them highly effective for defence (Kumar et al., 1997). Hemotoxins, such as those found in viper venoms, disrupt blood coagulation processes, leading to internal bleeding, blood clotting disorders, and tissue damage. (Lu et al., 2005). Cytotoxins, on the other hand, act by disrupting cell membranes, leading to cell lysis and tissue necrosis. These toxins, such as those produced by some sea anemones and scorpions, evolved to cause localized damage, making them useful in both defence and predation (Çelik, 2018).

Toxins can be also classified based on their chemical composition, each category reflecting distinct biological functions and mechanisms of action. Protein toxins are among the most potent, often acting as enzymes that disrupt cellular processes (Parker & Feil, 2005). Peptide toxins, shorter chains of amino acids, similarly interfere with ion channels or cellular receptors, as seen in sea anemones (Honma & Shiomi, 2006). Lipid-based toxins integrate into cell membranes, causing disruption and cell death, a common mechanism in certain marine algae (Fivaz et al., 2000). Glycosylated toxins are proteins with attached carbohydrate groups, which can influence how these toxins bind to cells and elicit their effects (Gerwig et al., 2013). Alkaloid toxins, which are small, nitrogen-containing molecules, interfere with neurotransmitter receptors, producing effects like paralysis or death, typical of some fish venoms and plant-derived compounds (Azzeme & Kamarul Zaman, 2019).

Uses of toxins

Toxins have long been explored as a source of new bioactive molecules for pharmacological applications. However, compared to the venom of terrestrial animals, piscine venoms have received less attention due to the reduced danger they pose to humans and the challenges in obtaining them (Church & Hodgson, 2002). Nonetheless recent study indicates that there could be over 2,000 venomous fish species, with one study estimating the existence of 1200-1600 venomous species within the Siluriformes order alone (Smith & Wheeler, 2006; Wright, 2009). These toxic substances, that are both toxic to fishes or produced by them, are known as “ichthyotoxins” and they can vary in nature and effects (Russell, 1984).

However, the use of toxins within animals is ancient and an example of the “evolutionary arms race”, and the development, acquisition, presence and advertisement of toxins can have many forms. Therefore, it is important to note that fish are not the only group of organisms that produce toxic substances in the marine environment. Nudibranchs, with their vibrant colours signalling their toxic nature, deter predators and thrive in competitive environments even without shell protection. They acquire toxins from their diet, specifically from nocive molecules present in the algae they consume. Over time, they have evolved to synthesize these molecules independently, reducing their reliance on dietary sources (Wright, J. L. 2002). Other organisms, like the blue-ringed octopus and pufferfish, obtain their venom through symbiosis with bacteria (Hwang et al., 1989; Noguchi et al., 1987). The blue-ringed octopus uses tetrodotoxins to capture its prey while the pufferfish use it as a defense. These compounds, which affect neuromuscular junctions and nerve conduction leading to paralysis (Lane, W.R., Sutherland, S., 1967, 1967) are also utilized by other creatures such as cone snails. The latter have evolved a more complex venomous apparatus and use a hollow harpoon-like structure to inject the toxin, paralyzing and killing its prey (Dutertre et al., 2013).

In the life cycle of all organisms, the egg stage is the most vulnerable, facing threats from both predators and pathogens. Research has discovered toxic compounds within or coating the eggs of certain species, which exhibit antimicrobial properties upon further analysis(Benkendorff et al., 2001; Kerwin et al., 2019). In other species, such as the gar fish, these toxins may also serve as a predator deterrent, having proven toxic to other marine organisms (Ostrand et al., 1996).

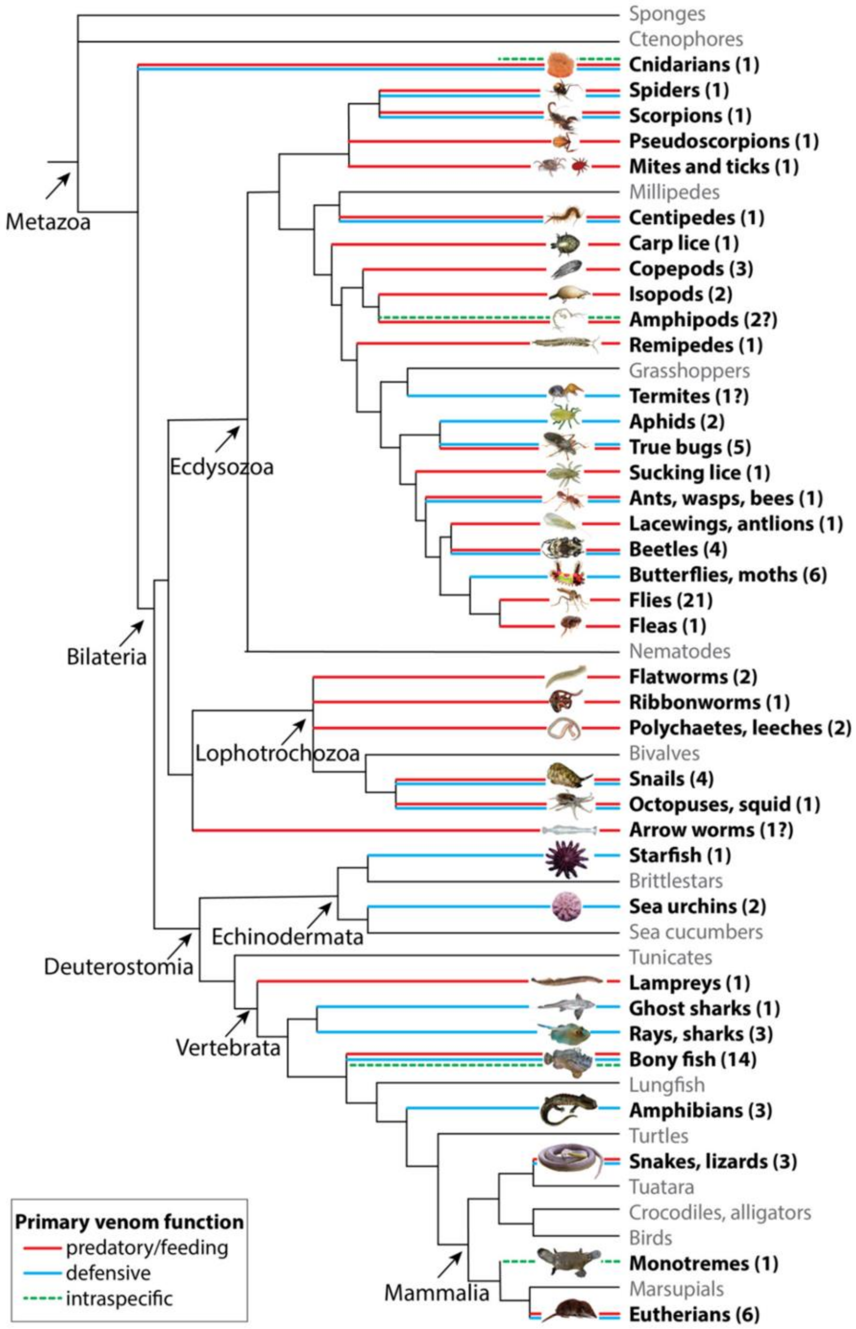

Figure 1.1- Taxonomic diversity and the main primary functions of venom. A pruned and schematic phylogenetic tree of venomous animals form Schendel, (2019) illustrating the frequency with which venoms have evolved within the animal kingdom. Coloured branches highlight venomous lineages, with red branches indicating a predatory/feeding venom function, blue branches indicating a defensive function and dashed green branches indicating a role in intraspecific competition. Taxa for which no direct support of their venomous nature could be found are indicated with a question mark.

Beyond species differences, toxin composition and potency can also vary between sexes. For instance, a study on the Venezuelan scorpion *Tityus nororientalis* revealed that females produce venom with significantly higher toxicity and a different composition compared to males (De

Sousa et al., 2010). Other examples of intersexual differences in venom composition among terrestrial animals have been documented (Escoubas et al., 2002; Furtado et al., 2006; Herzig et al., 2008; Menezes et al., 2006). However, due to previously mentioned reasons, knowledge about this phenomenon in aquatic animals remains limited. A study by Lopes-Ferreira (2016) analysed male and female specimens of *Thalassophryne maculosa*, the Caribbean Cano toadfish, a member of the Batrachoididae family which deliver venom through spines, and showed that male venom has a higher protein concentration and induces greater nociception, while female venom has a higher capacity for necrosis induction and greater proteolytic activity on certain substrates. The differences in venom composition and effects between males and females of the same species can be attributed to several biological and ecological factors that influence venom production and utilization. Research on various venomous species, including spiders and scorpions, has shown that female individuals often produce more potent and complex venoms compared to males. This disparity is thought to be driven by the different ecological roles and physiological needs of each sex. For instance, females, which are typically larger and often responsible for tasks such as protecting offspring or securing food, require a more potent venom to effectively subdue prey and defend against predators. In contrast, males, whose primary role may be to seek mates rather than hunt or defend, often have a less toxic venom (Miller et al., 2016). Biochemical analyses have revealed that female venoms tend to have higher concentrations of toxic proteins and enzymes, such as sphingomyelinases in spiders, which contribute to increased lethality and dermonecrotic effects (de Oliveira et al., 2005). In certain cases, the size difference between sexes can result in varying amounts of venom being injected, which may lead to different effects (Miller et al., 2016).

1.2 SITES OF PRODUCTION AND TOXIC SUBSTANCES IN FISH

Skin

The mucus on fish skin serves as a natural, physical, biochemical, dynamic, and semipermeable barrier, facilitating the exchange of nutrients, water, gases, odorants, hormones, and gametes (Esteban, 2012). This mucus is secreted by specialized goblet cells within the epidermis and is rich in various immune components (Ingram, 1980) The mucus not only prevents pathogen entry by trapping and immobilizing bacteria and other microorganisms, but it also contains substances like lysozyme and proteases that actively degrade and neutralize these potential threats (Cone, 2009; Subramanian et al., 2007). Additionally, the mucus layer is continuously replenished, which helps prevent the stable colonization of pathogens, further protecting the fish from infections (Nagashima et al., 2003; Sanahuja et al., 2023) . A study shows that the mucus properties of the ventral and dorsal skin of the Senegalese sole change in response to stress. The viscosity of the mucus increases to offer mechanical protection, and also the soluble protein concentration and the antioxidant power of the dorsal mucus increase showing the different response to stress on the two sides of the body (Fernández-Alacid et al., 2019)

Some families of fishes have potentiated their chemical defense with the additions of crinotoxins to their mucus. Crinotoxins are unique toxic proteinaceous substances secreted by the skin of various fish species as a form of chemical defense, playing a crucial role in their survival strategies (Satora & Łukasik-Głębocka, 2009). These toxins are typically released into the surrounding water or upon physical damage to the skin, making them distinct from other venoms that are actively injected via specialized apparatuses. Crinotoxins are produced by proteinaceous cells in the epidermis, such as the club cells found in species like moray eels (*Lycodontis nudivomer*), which are responsible for haemolytic and toxic activities that can deter predators or protect against microbial infections (Randall et al., 1981). The evolution of these toxins likely provided significant ecological advantages by enhancing the ability of sedentary or slow-moving fish, such as certain species of catfish, to defend against predators and parasites (Haddad Junior & Lopes-Ferreira, 2023).

There remains an ongoing debate regarding the evolutionary relationship between venom glands and crinotoxins, with researchers still exploring whether these structures share a common origin or evolved independently across different species. Cameron and Endean believe that the modern venom systems may have evolved from the same cells that secreted crinotoxins (Cameron & Endean, 1973) A preliminary study of the catfish *Plotosus lineatus* supports Cameron and Endean’s hypothesis, revealing that the club cells of this species produce a substance similar, and possibly identical, to one found in the venom gland (Shiomi et al., 1988). On the other side, other researchers point out the histochemical and ultrastructural differences between the club cells and the venom gland cells (Whitear et al., 1991). Wherever they might come from, the venomous system has convergently arisen on at least 19 separate occasions across their evolutionary history and has proven essential for many fish species (Harris, 2024).

Venomous glands

In marine fish, venom glands have evolved in various species as specialized structures for defense and competition. These venom glands are often associated with spines or fangs and secrete toxic proteins that can incapacitate predators or competitors. For example, in fangblennies (*Meiacanthus* species), venom glands are closely associated with their enlarged canine fangs, which are used for defense against predators. These glands are located at the base of the fangs and secrete venom into deep grooves along the fangs, facilitating venom delivery during a bite. This venom contains a unique combination of toxins, including phospholipases A2 (PLA2), proenkephalins, and neuropeptide Y, which can induce hypotension, pain relief, and neurotoxic effects. Unlike many venomous fish that use dorsal or opercular spines to inject venom, fangblennies are unique in delivering venom through their bites, which is primarily used as a defensive mechanism rather than for predation (Casewell et al., 2017). Another example is the venom apparatus of the scorpionfish (*Notesthes robusta*), which consists of venom glands located at the base of the dorsal, anal, and ventral fin spines. Each spine is surrounded by an integumentary sheath, which, when puncturing a victim, retracts and exposes the venom glands. These paired venom glands are responsible for delivering venom through ruptured distal ends during envenomation. The venom consists of proteinaceous granules stored in specialized gland cells, which, upon puncture, are released into the wound, causing intense pain and other systemic symptoms in victims. Remarkably, these venom glands can regenerate within six days if damaged, with full regeneration occurring within 13 weeks (Cameron & Endean, 1966). Toadfish species such as T*halassophryne amazonica* also exhibit venom glands located at the base of their dorsal spines, which are responsible for injecting venom during defensive actions. Interestingly, these venom glands differ between species, and in some non-venomous toadfish, the dorsal spines are present but lack associated venom glands (Smith & Wheeler, 2006)

Axillary glands

Apart from the skin cells, dedicated venom glands, draining or injecting venom through specialized spines, usually modified fin rays, are the most evident form of producing and delivering toxic substances in fish, but not necessarily the most abundant; Many species do not have such an evolved venom system, and the venom production is hypothesized to take place in other structures. In the Siluriformes order, which represent the 95% of the venomous freshwater fishes, is hypothesizes that the axillary glands have a role in toxin production (Greven et al., 2006; Kiehl et al., 2006). A study shows that, when subjected to stress, individual of *Corydoras* spp. release substances that have bactericidal properties. Part of these substances were found to be identical to those produced by the axillary glands, proving a role of the latter in stress response. Still, the contribution of axillary gland secretions to the bactericidal properties remains to be proven (Greven et al., 2006; Kiehl et al., 2006). The axillary glands are bilaterally situated close to the surface of the skin in the axil of the pectoral fins, and each has a large lumen opening to the external environment (Vernick & Chapman, 1968). The gland is tubular, non-lobated and covered by a thin capsule of connective tissue. A strong longitudinal muscle is attached to the inner face of each gland (Greven et al., 2006). The external tissue of the gland is highly folded, increasing the internal surface area of the gland. It is composed of mucus cells, epithelial cells and clavate cells (Maina et al., 1998).

Through a series of experiments, Maina (1998) and Wallace (1893), tried to understand the function of the axillary gland in the marine *Opsanus beta*, the Gulf toadfish, another member of the Batrachoididae family. From these studies there is no evidence that the gland has a role in urea excretion, pheromones production toxin production or antimicrobial activity. The actual role of the gland is thus still not clear, especially in marine species, although it has been showed that axillary secretions are discharged, together with other integumental substances, during stressful conditions (Kiehl et al., 2006).

Model species

The Lusitanian toadfish (*Halobatrachus didactylus, Batrachoidiformes, Batrachoididae*) is a benthic euryhaline species that lives in marine coastal areas and occasionally in brackish waters (Costa et al., 2003). It can be found from the Bay of Biscay to Ghana and in the west mediterranean (Cotter et al., 2011). This species lives under rocks and in cervices and feeds on small fishes, crustaceans, molluscs and polychaetae (Cârdenas, 1977; Dos santos et al., 2000). Although its individuals are capable of moving up and down the estuaries or within the lagoons (Pereira et al., 2021) the species is usually sedentary for most of the day and exhibits a stationary behaviour, show low metabolic rates (Molina et al., 2023) and engage in reproduction near the seafloor (Modesto et al., 2024), with territorial males attracting females to their nest using croaking-like sounds (Amorim et al., 2006). This, together with their physical features, contributes to their common name - toadfish. Their capacity to generate sound occurs through the utilization of their swimbladder. This acoustic emission is achieved by the contraction of sonic muscles within the bladder, and it resemble the sound of a toad. Besides their use to attract females, the sounds serve also defensive purposes, as they emit them when guarding their nests to deter other individuals (Amorim et al., 2008; Dos santos et al., 2000). During the reproductive season, from March to August (Palazón-Fernández et al., 2001), the females attach the eggs to the roof of the nest which are then fertilized and guarded by the males (Modesto & Canário, 2003). This species presents an example of "alternative reproductive tactics". There are two types of male toadfish, type I and type II. Type II males, usually smaller, appear to be obligate sexual parasites of type I males. They do not court females or built nest but rather take advantage or their resemblance to females to sneak in the type 1 male's nest and attempt to fertilize the eggs that were laid there by one or more females (Brantley & Bass, 1994).

The Lusitanian toadfish belongs to one of the families with the most known toxic marine fish species: the Batrachoididae family. Composed of a total of 84 species (*CAS - Eschmeyer's Catalog of Fishes - Genera/Species by Family/Subfamily*, 24/07/2024), within this family, there are 4 subfamilies identified: Porichthyinae, Thalassophryninae, Batrachoidinae, and Halophryninae.

All the 11 species belonging to the subfamily Thalassophryninae are known to be venomous and widely studied due to the multitude of human related accidents. The toxin is released involuntarily when pressure is applied to the dorsal spines and cause inflammatory reaction with pain and swelling (Ziegman & Alewood, 2015). However poisonous species are not present only in that subfamily. *Opsanus beta*, belonging to the subfamily Batrachoidinae, is known to secrete a mucus that has neurological effects on other species (Nair et al., 1982). Recent evidence suggests that also *H. didactylus,* also belonging to the subfamily Halophryninae, may produce toxic substances (Gonçalves, 2022). This discovery arose when several fish species (*Sparus aurata*, *Dicentrachus labrax*, *Argyrosomus regius*, and *Gobius paganellus*) died shortly after being in the same container or even just in the same water recirculation as the toadfish (Modesto, Guerreiro, personal communication).

Emerging studies on *H. didactylus* analysed the effects that the toxin has on other species and the chemical composition of the mucus. Gonçalves (2022) found that the water of the container in which a group of toadfish were subjected to crowding stress ("toadfish water") indices changes on several haematological parameters, plasmatic indicators, and behaviour as well as on the olfactory nerve of the target fish, hypothesizing a possible role of the toxin in nest defence. Cunha et al. (2023) assessed the bioactive potential of mucus from *H. didactylus* and showed the antioxidant, antihypertensive, and antimicrobial properties of the mucus.

Chapter 2 OBJECTIVES

The overall goal of this thesis was to address the newly discovered toxic properties of *H. didactylus'* mucus, focusing on the conditions that may modulate its secretion and potency, and the actual sites of production.

The first objective of this study was to ascertain whether the toxic effect previously observed was attributable to the mucus produced by the skin in this species. For this we tested the mucus collected directly from the skin, or the secretions of the axillary gland, and compared the effects with those evoked by fish holding water, as used in previous studies, and analyzed the structure of the skin and axillary gland as possible sites of production and secretion.

The second objective was to investigate the relationship between the potency of the toxic effects and intrinsic variables such as sex and/or stress levels in the animals. Mucus and holding water of fish from either sex, before and after stress, were used, at different concentrations, to evaluate the impacts over potential sympatric target species (*S. aurata) in vivo* and in an *in vitro* hemolytic assay.

Finally, the influence of the environment and of target species were tested using two euryhaline fish species, one from marine origin (*S. aurata*) and another from freshwater origin (*Gambusia holbrooki*) acclimated and tested in a range of salinities.

Chapter 3 MATERIALS AND METHODS

3.1 Animals

3.1.1 - Toadfish

Lusitanian toadfish (*H. didactylus*) were captured by local fisherman in Ria Formosa lagoon (Southern Portugal) using set nets. Fish were transferred to Ramalhete Marine Station where they were placed in seven 600L tanks, in an open flow-through system and kept at natural temperature and photoperiod. Upon acclimation to captivity the fish were transferred to the facilities of the Experimental Laboratory of Aquatic Organisms (LEOA) of university of Algarve/CCMAR at Campus de Gambelas, where they were maintained in 3 250L tanks in a recirculating closed system, with mechanical and biological filters, at 20°C and artificially simulated natural photoperiod. To obtain the mucus necessary for the experiment, 21 toadfish were used. After assessing the sex of each individual by observing the urogenital papilla, fish were weighted and allocated in the 3 tanks: females, males and unidentified. These fish were fed squid three times per week (3% w/w). Male fishes used in experimental conditions averaged 180.7± 40.06 g and had an average length of 24,4 ± 3,31 cm; females averaged 152,2 ± 21,60 g and had an average length of 22.2 ± 2.95 cm.

3.1.2 Target species

Juvenile Gilthead seabream (*S. aurata*) individuals were provided by the Experimental Aquaculture Station in Olhão, from the Portuguese Institute of Sea and Atmosphere (EPPO-IPMA) and transported to the facilities of CCMAR/university of Algarve. Fish were maintained in three 150L tanks connected to a recirculating closed system, with appropriate mechanical and biological filters, at 20C and artificially simulated natural photoperiod. Fish were fed daily with commercial seabream feeds (Aquasoja NneoGold, 2 % w/w) and had an average weight of 1.92 ± 0.229g

This species was chosen due to its the ability to acclimate to a wide range of salinity (Chervinski, 1983) and, since it is a commonly used species in aquaculture, it was easily available and there is extensive knowledge about its physiology (Mhalhel et al., 2023). Juvenile sea bream uses marine lagoons and estuaries as nursing grounds and therefore may occur in sympatry with the toadfish, allowing for ecological considerations. The fish were initially kept at a salinity of 36 ppm. A group was then transferred to another tank, where the salinity was reduced by 4 ppm every two to three days. This gradual adjustment period allowed the fish to acclimate properly, minimizing the risk of mortality due to sudden changes in salinity.

The mosquito-fish *Gambusia holbrooki* is a euryhaline species from freshwater origin, also able to easily acclimate to a wide range of salinities. Mosquito-fish is a commonly used laboratory animal (Chervinski, 1983; Tumlison, 2017), and there is wide knowledge about its biology The individuals were captured in the wild, from local freshwater streams, using electric fishing methods, part of a fish monitoring program at CCMAR. They were transported to the facilities of CCMAR/university of Algarve and maintained in twelve 5L tanks connected to a recirculating closed system, with appropriate mechanical and biological filters, at 20C and artificially-simulated natural photoperiod. Fish were fed daily with commercial freshwater aquarium fish feeds (Aquatic Nature, Tropical Energy S, 2 % w/w) and had an average weight of 0.25 ± 0.058 g. The original salinity of the fish was 0 ppm. Some individuals were moved to another tank and kept at the initial salinity. In the system the salinity was gradually increased by adding saltwater every two to three days, raising the salinity by around 4 points each time. This interval allowed the water to mix thoroughly in the system and to minimize mortality in the process.

This species is considered invasive in Portugal (DL 92/2019) and in many other regions or the world (included in the 100 world's worst invasive species by. *G. holbrooki* was first brough to Portugal as a biological control of mosquitos carrying diseases, and due to its high reproduction rate is now threatening endangered indigenous fish and invertebrate species, and captured individuals must be destroyed by law.

Fish collected in the wild were obtained under the appropriate licenses from the fisheries (DGRM) and nature conservation authorities (ICNF) and fish rearing and handling were conducted under the FELASA type-C license issued by the DGAV (Government of Portugal). Procedures followed the Portuguese legislation and European Union guidelines (DL 113/2013, 2010/63/EU).

Salinity and oxygen were monitored daily using a conductivity meter/salinometer (Lovibond SD325 Con) and an optical oxygen probe/meter (VWR PHenomenal, OX4110H) respectively. Dissolved ammonia was measured weekly using a dedicated commercial kit (Tropic Marin).

3.2 IN VIVO EXPERIMENT

MUCUS EXTRACTION

This procedure aimed at collecting skin mucus to be mixed with seawater and used to expose target fish to different amounts/concentrations of putative toxins.

Toadfish individuals were removed from the holding tank into a bucket. Each fish extraction was processed sequentially to minimize crowding stress. Skin mucus was collected from each fish by first gently drying the body with a cloth, then scraping five times on each dorsal side with a microscope slide at a 45° angle. The mucus of six individuals (males, females or mixed groups, according to the experiments described below) was pooled and weighed, and 5 mL of water (salinity according to the experiments described below) were added to each gram of collected mucus. This mixture was then vortexed briefly and used at different dilution/concentration in the following experiments devised to identify any differences between the impact of mucus from males and females, as well as between stressed and non-stressed fish. After mucus collection the toadfish were returned to their original tanks. Protein levels were measured in this emulsion using the Bradford method (see below).

3.2.2. EXPOSURE EXPERIMENTS

3.2.2.1 EFFECTS OF INTRINSIC CONDITIONS

To establish the possible effect of stress over the amount/potency of the mucus toxins mucus was collected from non-stressed and stressed toadfish using the extraction methodology described above.

Dosage

To evaluate the level and time-course of the impacts of the mucus toxin over target species, a set up to test different concentrations of mucus was designed. This consisted of 9 1L beakers that were used to complete three mucus concentrations, using 3 replicates per concentration, that were set in front of video cameras.

Sea bream juveniles maintained in full-strength seawater were collected from the holding tank and transferred to the 1L glass beakers containing each 500mL of aerated seawater at 20ºC. Three fish were placed in each beaker, so overall, a total of 9 seabream per condition and 27 per experiment were used. Fish were maintained in these conditions for 10 minutes and the video recorded was initiated. At time zero (T0), seawater or the mucus solution were added using a pipette, in amounts to create exposure to 0.00 mg mucus/L (control), 0.04 mg mucus/L (dose I) and 0.40 mg mucus/L (dose II) (Figure 3.1). Aeration was removed shortly after the addition of the mucus solution to ensure mixing and allow clear discrimination of behaviours in video recordings. The maximum time allowed for the exposure was 25 minutes and oxygen levels were measured (VWR PHenomenal, OX4110H) at the beginning and at the end of the experiments to ensure its level was over 75% saturation.

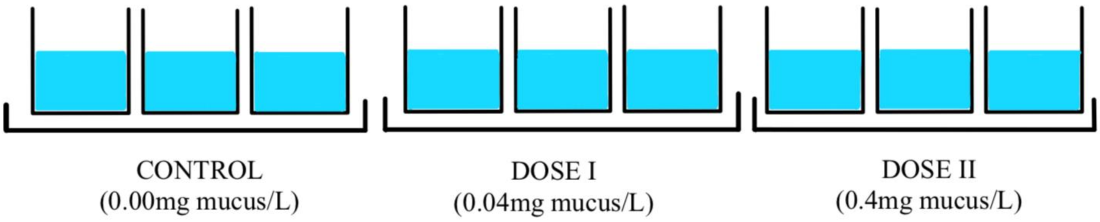

Figure 3.1-Rrepresentative scheme of the disposition of the beaker in the experiment with the different concentrations. In general, 3 seabreams were used per replicate beaker, thus a total of 9 fish per condition.

The swimming behaviour of target fish was followed throughout the duration of the experiments and specific swimming modes, bursts, reduction or cessation of swimming, or of opercular movement, and other observation were evaluated in real-time and then re-assessed by posterior observation and analysis of the video recordings and a basic ethogram was created. Target fish that did not resist to the toxic effects, leading to death were removed and destroyed. Those that survived were reallocated to a different holding tank and not re-used in the experiments.

Gender-related differences in toxic activity

Male and female toadfish have differentiated behaviour and roles in nest-building or egg-caring activities. To test whether fish gender may have an effect on the toxicity levels of skin mucus, the protocols described above were applied using mucus collected from either a pool of male or a pool of female toadfish, separately.

Stress effects in modulating mucus toxic activity

Previous observations indicate that mucus production seems to increase dramatically under stressful conditions such as crowding or air exposure (Guardiola et al., 2014). Moreover, it is likely that a toxin may be released in such circumstance that may be perceived as direct attack or intrusion in the fish territory. Thus, the effects of stress were tested using the same pool of male and female toadfish. At least one week after the mucus collection in minimally stress conditions, the experimental protocol was repeated, but prior to mucus collection, the toadfish were subjected to stress conditions. This was achieved by placing the fish in a bucket with insufficient water to fully cover the animals and handling them every 5 minutes to elevate stress levels. After 30 minutes, the mucus was collected, and the experiment proceeded as per the original protocol.

Experimental groups of target species associated with these experiments will hereafter also be denominated as NSMM - fish receiving mucus from non-stressed male toadfish, SMM - fish receiving mucus from stressed male toadfish, NSFM - fish receiving mucus from non-stressed female toadfish, SFM - fish receiving mucus from stressed female toadfish.

3.2.2.2 EFFECTS OF ENVIRONMENTAL SALINITY

The following set of experiments was designed to test the effects of an environmental variable – water salinity – on the effectiveness of the toadfish mucus toxin. *H. didactylus* is known to be found in river estuaries where it may encounter different salinity gradients and different fish communities. Thus, in this experiment the effect of the toadfish mucus was tested at salinities of 1) 36 ppm, 25 ppm, 13 ppm, 6 ppm, and 3 ppm, using environmental salinity-acclimated *S. aurata* individuals and 2) 25 ppm, 13 ppm and 0 ppm, on environmental salinity-acclimated individuals of *Gambusia holbrooki.*

Target *Sparus aurata*

For this experiment mucus was collected from all 21 toadfish, including males, females and unidentified fish, mixed and aliquoted into five vials, each containing approximately 0.4g of mucus. The mucus was mixed with 10 mL of seawater at 36 ppm and homogenized using a vortex mixer.

Three individuals of *S. aurata* per beaker were used as test subjects. These fish were previously acclimated over two weeks to the environmental salinity to be tested. Five different salinities were selected: 36 ppm, 25 ppm, 13 ppm, 6 ppm, and 3 ppm. Two replicates were used for each salinity and one more is prepared as control (total n/salinity=9). Fish were left in the beaker for 10 minutes and then mucus was added (5ml) to the beakers which contained 500 mL of water at the different salinities, to a final concentration of 0.4g mucus/L. The control beakers received an identical volume of water at 36ppt, the same salinity used for the mucus solution. Aeration was then removed but oxygen was measured at the beginning and end of the exposure. The exposure protocol was conducted as before, behaviour and mortality were observed and video-recorded, and the experiment was terminated after 25 minutes, and all target fish were weighed.

Target Gambusia holbrooki

As for *S. aurata*, mucus was collected from all 21 toadfish, yielding a total of 4.24g from both sexes, mixed and aliquoted into five vials, each containing approximately 0.4g of mucus. The mucus was mixed with 10 mL of seawater at 36 ppm and homogenized using a vortex mixer. As above, for each salinity there were two replicas receiving mucus, and another one used as control, which only received 5ml of seawater. Five individuals of *Gambusia holbrooki* of similar size, previously acclimated to the respective salinity for at least three days were selected and placed in each beaker containing 500 mL of water (total n/salinity=15). After allowing the fish to acclimate for 10 minutes, the oxygen was measured and then 5 mL of water mixed with mucus was added to each beaker, and the behaviour of the fish was observed. After 25 minutes, the recordings were stopped, the oxygen levels in each beaker were measured, and the target fish were weighed.

EXPERIMENTS USING TOADFISH-HOLDING WATER

In this experiment, we aimed to ascertain 1) whether the deleterious impacts observed could result from mechanical actions of large mucus particles, namely over the gills; 2) confirm that toadfish holding-water may have similar impacts as skin mucus emulsions and whether fish condition may translate in differential effects 3) obtain information about the dispersal/nature of the toxin substance in water.

To obtain the “toadfish water” six individuals of the same sex were placed in a single bucket containing 1.5L of water, which was enough to keep the animals only partially submerged, and then were additionally stressed by handling for 5 minutes, in one trial, or for 30 minutes on a second trial. These was done separately for male and for female toadfish. The toadfish were then returned to their holding tanks and the buckets’ water was filtered with a coffee paper-filter to remove large particles of mucus. This filtered toadfish-holding seawater, hereafter named “toadfish water”, was used to expose target fish, in a protocol modified from to those described above. Protein levels were quantified in the filtered water using the Bradford method (see below).

Briefly, 3 specimens of *S. aurata*, reared in full strength seawater, were directly transferred to individual beakers already containing either 300mL of “toadfish water”, 500 mL of seawater containing toadfish water at a 1:5 dilution, 500 mL of seawater containing toadfish water at a 1:50 dilution or 500 mL of seawater, as the control group. Each concentration was tested in duplicate beakers, and the experiment was carried out for 4 conditions: male holding-water, obtained following a stress of 5 or 30 minutes and female holding water obtained following a stress of 5 or 30 minutes. Following the measurement of oxygen levels, the timer was started and the experiment lasted 25 minutes. At the end of the 25 minutes the oxygen was measured again and the fish that succumbed to the exposure were counted and weighted.

Experimental groups of target species associated with these experiments will hereafter be denominated as SMW5 – fish exposed to holding water of males individuals stressed form 5 minutes, SMW30 - fish exposed to holding water of males individuals stressed form 30 minutes, SFW5 - fish exposed to holding water of females individuals stressed form 5 minutes, SFW30 - fish exposed to holding water of females individuals stressed form 5 minutes,

EXPERIMENTS USING AXILLARY GLAND CONTENT

This experiment was conducted to assess whether the axillary glands present in toadfish produce toxic substances that could cause deleterious impacts or even mortality in the target species, similar to the effects observed with the mucus.

Ten male fishes were collected from their holding tank and placed in aerated buckets, where they were stressed for 30 minutes following the methodology described early. Each fish was then dried individually using a cloth and special attention was given to the axillary pore area to prevent mucus contamination. A swab was inserted into the axillary pore and swirled for 10 seconds. The swab was then immersed in 1 mL of seawater in a soft tube and squeezed three times to extract the content. The same swab was used to collect the secretions of the two axillary glands of 5 fish, and all content was pooled in a single vial. Protein levels were measured in this solution using the Bradford method (see below).

Three beakers, each containing 3 seabreams in 500 mL of water, were prepared. After 10 minutes of an acclimation period, two of the beakers received 0.5 mL of the axillary gland solution, while the third served as a control, receiving 0.5 mL of saltwater at a salinity of 36 ppm. The ensuing protocol was as previously described, with the exception that the experiment concluded 60 minutes after addition of the treatment instead of the usual 25 minutes.

BEHAVIOURAL ANALYSIS

During all the experiments, recordings were performed using action cameras (GoPro Hero 4) with the intent of analyzing the behaviour of the target species, with special attention to breaking the surface behaviour, reduction of movement, spasms, and death. It was noted the beginning and end of each behaviour and the number of times it occurred per minute or for the duration of the experiment in each beaker. The sum of the events per minute of all target fish per condition (sum of replicates: e.g 9 – sex/stress and axillary gland experiments; 6/10 – salinity experiments; 6 – holding water experiments) was used as a single replicate to evaluate the chronological occurrence of behaviour. Given that it was not possible to identify individual fish in each beaker, the sum of all events/behaviour per beaker was calculated for the duration of the experiments. The average of replicates per condition was used to compare between treatments.

3.3 IN VITRO EXPERIMENTS

HAEMOLYTIC ACTIVITY

Blood and mucus collection and preparation

We conducted this experiment to evaluate whether the mucus have immediate cytotoxicity. Blood cells were chosen as the target model, and we proceeded to observe the possible haemolytic effect of the mucus.

Approximately 500 µL of blood was taken from 4 individuals of adult *S. aurata (circa 30 cm)*, by puncturing the blood vessels of the caudal peduncle with an heparinized syringe. Blood was transferred to microtubes containing 10ul heparin (1000U/ml in saline solution) and maintained on ice.

The skin mucus was collected from 6 females and 8 males of *H. dydactylus* scraping 5 times on each dorsal side with a microscope slide as previously describe. A total of 1g of mucus was obtained for each sex. The mucus collected was mixed with 1 mL of 0,95% NaCl saline solution and homogenized with the vortex. This emulsion was then centrifuged at 15000 rpm for 15 minutes and the supernatant collected. Protein content was quantified in the supernatant obtained after mucus extraction and homogenization from the pool of either males or females to investigate a potential correlation between protein levels and cytotoxicity. Considering the total amount of extraction, and the preliminary data shown in Gonçalves (2022), 4 dilutions were performed, 1:1, 1:100; 1:1000 and 1:10.000, to be used in the haemolytic assay.

Heamolytic assay

The procedure to determine the hemolytic activity was determined following methodology described from Neely & Campbell (2006) and Al-Rasheed et al (2018). The blood drawn was mixed with the same amount of saline solution and centrifuged at 5000 rpm for 5 min to allow the deposit of red blood cells. This was repeated 4 times, removing the supernatant, and adding new saline solution each time. A solution of 1% red blood cells was prepared by adding 19.800 µL of saline with 200 µL of red blood cells, amounting to a total of 20 mL of solution.

The red blood cell solutions were distributed in a 96 well U-bottom microplate (100ul per well), 24 wells per fish and 100ul of each dilution of mucus extraction was added. For issues related to the limited amount of mucus solution, the lower three concentrations were performed in triplicate while the higher concentration was performed in duplicate, for all 4 seabream samples. For the positive control both saponin (15µg/ml) and distilled water were used, and for the blank a 0.95% saline solution was used.

These mixes in wells were left to incubate for 2 hours. The microplate was then centrifuged at 5000 rpm for 5 min (Heraeus, Bbiofuge Stratos) and the supernatant from each well transferred to a 96-well Flat-bottom microplate and absorbance measured in a microplate spectrophotometer (Thermo Scientific MultiScan Go). The 414nm wavelength was used to quantify the haemolysis, and the absorbances at 620nm used to remove interferences in the reads. Haemolytical activity is calculated as:

Equation 1- equation used to calculate the heamolytic activity

$$\text{Haemolytic Activity (\%)} = (\text{Abs positive control} - \text{Abs blank}) / (\text{Abs sample} - \text{Abs blank}) \times 100$$

Protein quantification

The protein concentration of the supernatant was assessed using the Bradford Method. This method involves a dye-binding assay, where an acidic solution of Coomassie Brilliant Blue undergoes a colour change, shifting its absorbance maximum from 465 to 595. Consequently, this alteration corresponds to varying protein concentrations (Becker et al., 1996). The same method was also employed to determine the protein concentration in the mucus and “toadfish water” samples utilized in the *in vivo* experiments.

HISTOLOGY

Collection of samples

The histological analysis of the skin and axillary gland was conducted to obtain insights into potential differences in tissue composition and structure between sexes and upon various treatments.

The same procedure was followed for two female and two male specimens. One female and one male were selected from a holding tank in the marine station and quickly humanely euthanized by severing the spinal cord. Immediately following this, three skin samples were collected from the right side, while mucus was gathered from the left side using the previously described methodology. The skin samples consisted of two sections from the dorsal region (anterior and posterior, referred to as Sk_d1 and Sk_d2) and one from the ventral area (designated as Sk_v) (Figure 3.2). The axillary gland was extracted from each side of the specimen through an incision above the pectoral fin and the severing of the muscular tissue surrounding the gland.

Another female and another male were placed separately in a smaller bucket and stressed for 45 minutes by chasing it with a small net every 5 minutes. The animals were then sacrificed, and the skin samples and axillary glands were taken as indicated above.

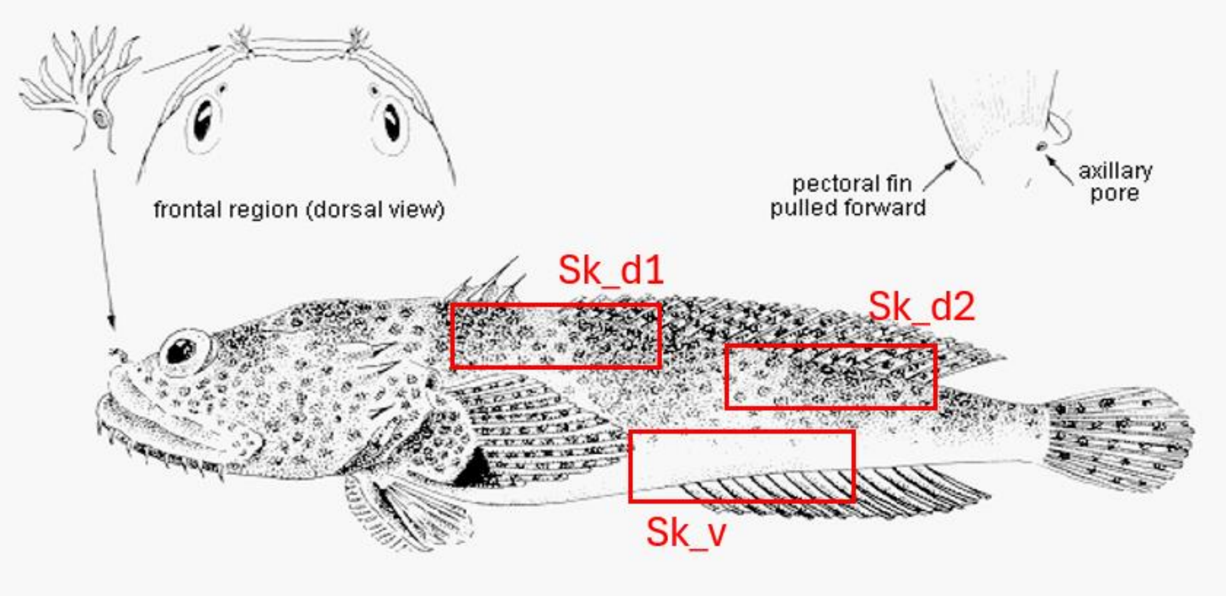

Figure 3.2- Diagram illustrating the specific body locations where skin tissue samples were collected

All samples intended for histological analysis were immersed in 4% paraformaldehyde (PFA) and refrigerated at 4°C. They were allowed to sit for a day to ensure the fixative thoroughly permeated the tissues and then were washed with PBS. To effectively remove the fixative, the samples were washed three times for 15 minutes each with sterile PBS at a pH of 7.4, with agitation during this period. This was followed by a 15-minute wash with sterile water. Subsequently, the samples were stored in 70% ethanol at 4°C.

Histological processing

The samples were trimmed to remove excess tissue, arranged in cassettes, and then placed in the tissue processor (Leica TP1020, Leica®). Over the following 12 hours, the samples underwent a dehydration in an ascending series of ethanol (70%, 95% and 100%), process by being immersed in increasingly concentrated ethanol cleared in xylene, xylene-paraffin (1:1, in volume) and embedded in low melting point paraffin wax Histosec (Merck). Paraffin blocks were prepared in a paraffin console (Miles Scientific) and the blocks formed left at -20°C to harden. Tissues were sliced to a thickness of 5 µm using a microtome (Leica RM2125T, Leica®) with disposable stainless-steel low-profile blades (MX35 Ultra low-profile, Thermo Scientific) and mounted on poly-L-lysine coated glass slide and stored in an oven at 37°C overnight and then 24 hours at room temperature. Transversal slices were made for the axillary glands while longitudinal slices were made for the skin samples.

Staining

Elimination of paraffin (dewax) was achieved by immersing the sample in two xylene baths for 15 minutes each. Following this, the tissue was hydrated, to allow the penetration of the stain, by submerging it in ethanol solutions of decreasing concentrations, specifically 100%, 95%, and 70% for five minutes each. The slides were then immerged for 5 minutes in distilled water to be cleaned. After each coloration, the tissue underwent a dehydration process using the same increasing ethanol concentration as before and was then placed in xylene, which removes residual ethanol and renders the tissue translucent, thereby facilitating its examination under a microscope.

Upon completion of the protocol, a coverslip was affixed to each slide using DPX (Fluka, Sigma) to safeguard the samples.

Different colorations were selected to highlight different structures in the tissues. Hematoxylin-eosin staining is a fundamental histological technique used to identify the morphology of cells and tissues. In this process, the negatively charged nuclei appear purple, while the basic cytoplasm turns pink (Chan, 2014). After undergoing the deparaffination and hydration process, the slides were immersed in Harris hematoxylin solution for five minutes, blued in running tap water, quickly rinsed in distilled water, then immersed in an aqueous eosin Y solution for two minutes, and finally rinsed in distilled water with a few drops of acetic acid.

Masson’s trichrome staining relies upon similar acid dyes to provide a differential visualization of tissue elements; connective tissue is stained green and colloidal and mineralized structures (if present) are stained red. This coloration is based on three stains. The firsts one, Mayer’s haematoxylin stains the nuclei of the cells blue. It is then followed by xylidine ponceau, which stains the cytoplasm and light green that stain the collagen. The protocol followed was the one described by Witten & Hall (2003). Deparaffinized sections were stained for 10 minutes with Mayer`s acid hematoxylin (Sigma-Aldrich), exposed to running tap water for 10 minutes and rinsed in distilled water. Sections were then stained with freshly prepared xylidine ponceau for 2 minutes, rinsed in distilled water, treated for 4 minutes with 1% phosphomolybdic acid, rinsed again and stained with light green for 90 seconds.

Alcian blue pH 2.5 is used to highlight the mucus cells as the dye reacts with anionic groups such as the ones contained in acid muco substances. They are stained blue because of the copper presence in the molecule. The protocol used (modification of Mowry 1963) involved staining the slides in Alcian Blue for 1 hour, followed by rinsing them under running water for 3 minutes and briefly in distilled water. The slides were then immersed in Mayer’s haematoxylin for 5 minutes and rinsed under running water for 15 minutes. The Alcian Blue (pH 2.5) method is used to visualise sulphated and carboxylated acid mucopolysaccharides and sulphated and carboxylated sialomucins (glycoproteins) that stain blue or bluish-purple.

DATA ANALYSIS AND STATISTICS

The obtained data for the haemolytic activity was statistically analysed using a non-parametrical one-way analysis of variance (Kruskal-Wallis), since the data distribution resulted to be not normal according to the Shapiro-Wilk test. The one-way analysis was followed by a post-hoc Dunn’s multiple comparison test. The alpha used was 0.05, so the differences were only considered significant if p-value<0.05. The data about the average total number of events for each behavior are analysed through one-way ANOVAs (alpha = 0.05) followed by a multiple comparison test and t-tests. The results presented in the graphs of behaviour, survival and haemolytic activity represent mean ± standard deviation.

Chapter 4 RESULTS

4.1 MUCUS EXPERIMENTS

Exposure of seabream and mosquitofish to toadfish mucus resulted in deleterious effects that ranges from altered swimming behaviour to lack of responsiveness and eventually to death. Based on the observations in real-time and on video-recordings, a basic ethogram was created to characterize and analyze the onset, frequency, duration, total occurrence and chronology of these different behavioural components and relate them to the dosage and time of exposure. The behaviours identified and their characterization are shown in table 1

Table 1-behaviour identified during the video analysis

BEHAVIOUR	DESCRIPTION
BREAKING THE SURFACE	The fishes repeatedly reach out of the water. Some individuals jump out while others just break the surface with the head/mouth
REDUCED SWIMMING	The swimming of the individuals is drastically reduced; the fishes are most of the time laying at the bottom of the beaker
SPASMS	The fishes perform fast and short movement and then rest again at the bottom of the beaker.
DEATH	An individual is considered dead when laying in the bottom and there is no opercular movement detected for at least 30 seconds

Overall, the occurrence and frequency of these abnormal behaviours, and lethality levels, were significantly depending on the amount of mucus emulsion added to the fish

When examining the results of the experiments conducted with mucus from males stressed or not stressed, a noticeable difference between the two conditions emerges. Figures 4.1 and 4.2 display the survival percentages for each minute of the two experiments.

The first behaviour to appear, usually in the first minute after adding the mucus, is breaking the surface. Depending on the case, after some minutes the fishes present a reduction of swimming followed by spasms. The latter behaviour is present until right before the death of the individuals. Fishes may look deceased at some point but after some seconds they continue to perform fast and short movement that are characterized as spasms. When the opercular movement is not detected anymore for 30 seconds the fish is considered dead.

In the high concentration conditions, the seabream exposed to the mucus of the non-stressed toadfish (NSMM) died within 12 minutes of exposure, with a steep decline in survival rates (showing one or more deaths per minute) between the 7th and 12th minute. In the experiment where mucus was collected from stressed males (SMM), seabream mortality started only at minute 16, but all fishes were dead by the 21st minute, thus within a similar 5-minute interval. Regarding the lower concentration results, mortality occurred only in NSMM group, where the survival rate dropped to 45%, at the end of the experiment, with deaths occurring more gradually over time, with onset at the 18th minute.

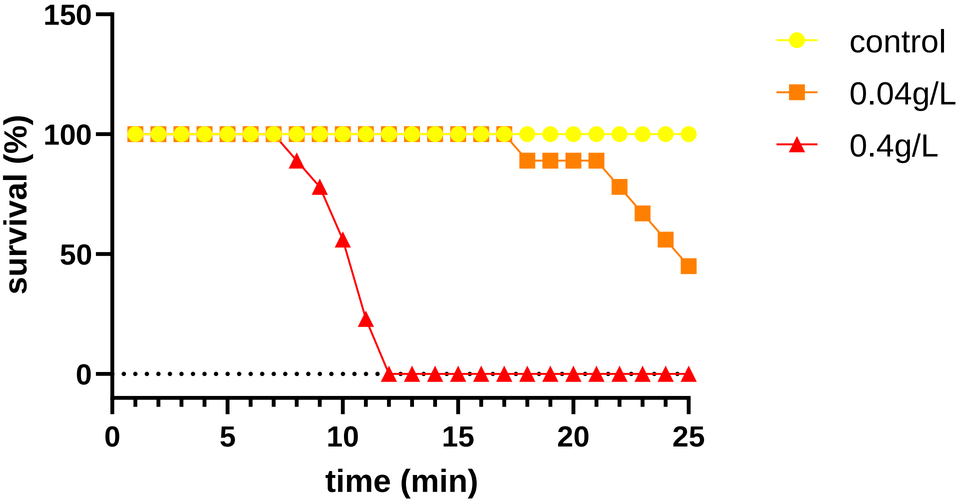

Figure 4.1- Percentage of survival in time for seabream exposed to different concentration of mucus collected from non-stressed toadfish males. Values shown result from the cumulative mortality from n=9 per mucus concentration group. Each condition was tested in triplicate (n=3 fish/replicate)

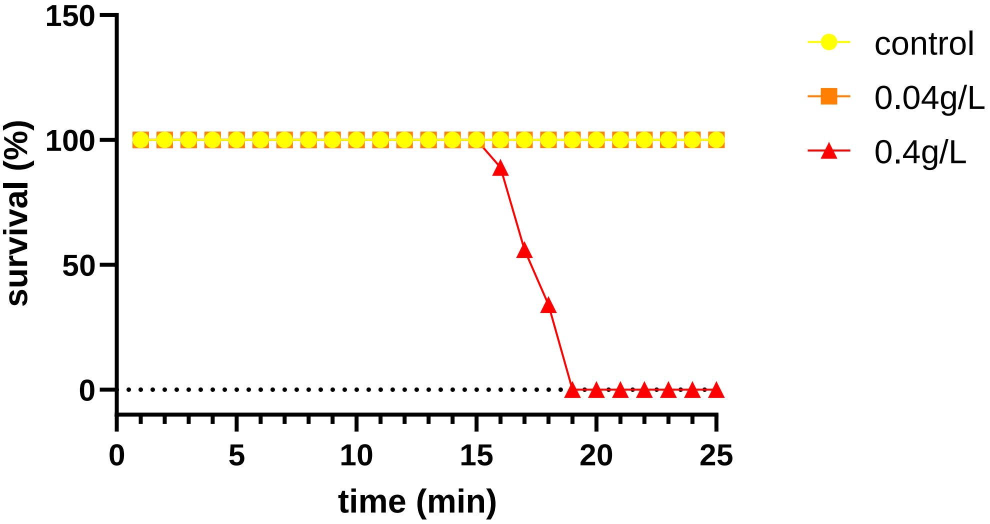

Figure 4.2- Percentage of survival in time for seabream exposed to different concentration of mucus collected from stressed toadfish males. Values shown result from the cumulative mortality from n=9 per mucus concentration group. Each condition was tested in triplicate (n=3 fish/replicate)

The behaviours shown by the seabream exposed to mucus from stressed and non-stressed toadfish males were analysed according to the ethogram in table I and are depicted in Figure 4.3. The occurrence of atypical swimming behaviours was more prominent at the higher mucus concentration, considering both the onset and the number of the events. The "breaking the surface" behaviour (fig. 4.3 a-b) was evident from the beginning of exposure in both non-stressed (NSMM) and stressed groups (SMM), but the number of events was much higher in the NSMM group. The “reduced swimming” and “spasms” behaviours (fig 4.3 c-d-e-f) initiate earlier in the NSMM group than in the SMM group. At the lower concentration the intensity of behaviours was generally lower, and the onset was later for the NSMM group, while fish in the SMM group did not show any of these behaviours throughout the duration of the experiments, similar to what was observed in the control fish, which have not received mucus.

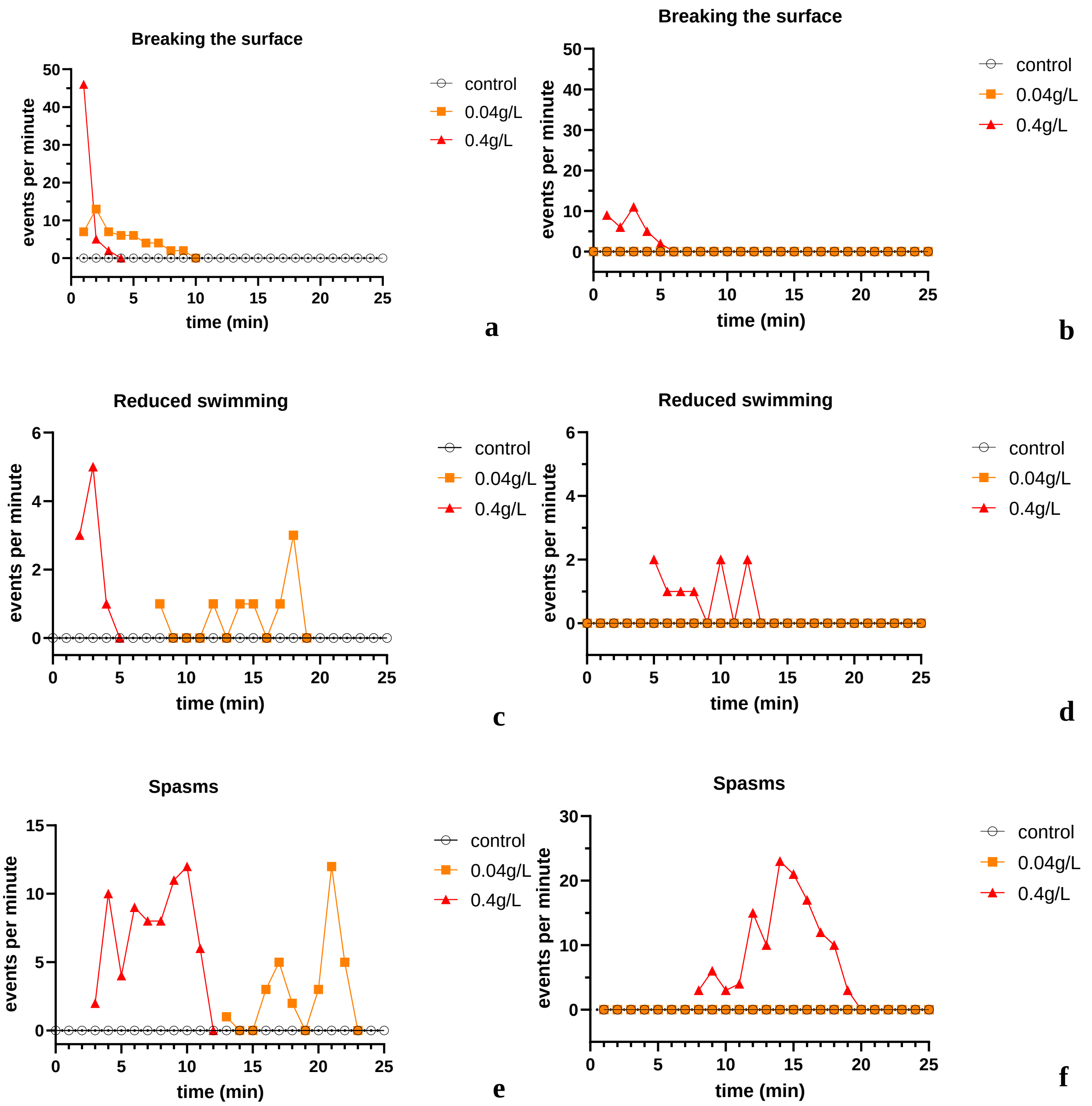

Figure 4.3 – Events per minute of each behaviour performed by seabreams exposed to the mucus of stressed and no-stressed females. values shown results from the sum of events of the three replicas beaker for each concentration. a-c-e) respectively breaking the surface, reduced swimming and spasms behaviours corresponding to non-stressed males ’mucus exposure. b-d-f) respectively breaking the surface, reduced swimming and spasms behaviours corresponding to stressed males’ mucus exposure

Due to the reduced amount of mucus obtained, the experiment with stressed females' mucus was conducted only at the higher concentration. When analysing the results of the experiments with stressed females' mucus (SFM) and non-stressed females' mucus (NSFM) (Figures 4.4 and 4.5), a noticeable difference is observed, similarly to the males. The mucus induced the death of all the test subjects in the NSFM group, with mortality occurring from minute 10 to minute 21 after initial exposure, with 0% survival by the end of the experiment, while the survival rate in the SFM in the other condition only dropped to 89% at the same time-period.

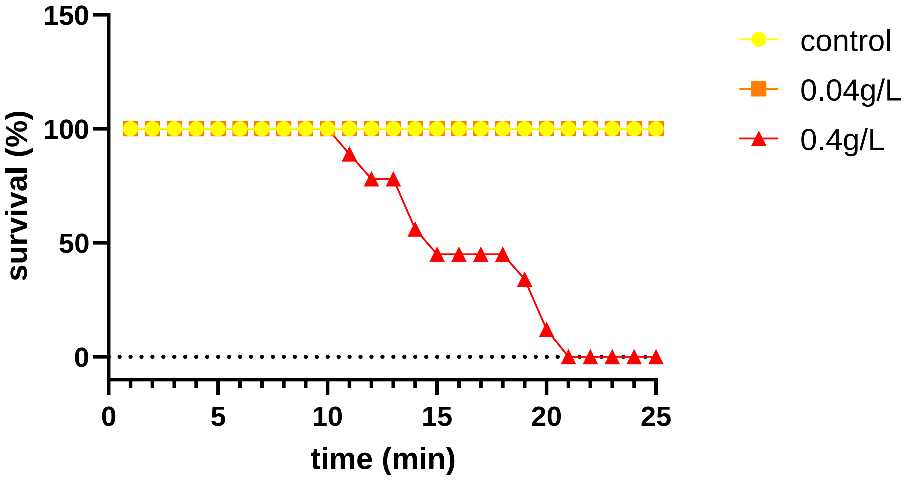

Figure 4.4- Percentage of survival in time for seabream exposed to different concentration of mucus collected from non-stressed toadfish females. Values shown result from the cumulative mortality from n=9 per mucus concentration group. Each condition was tested in triplicate (n=3 fish/replicate)

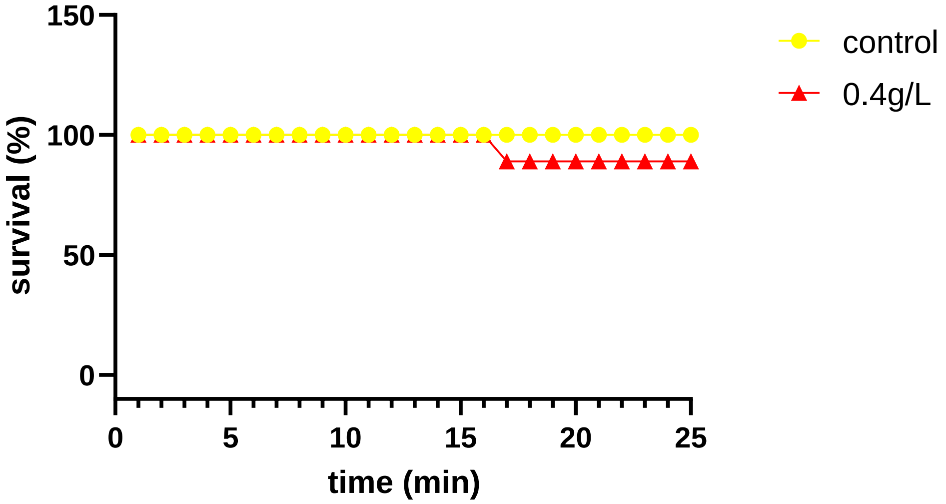

Figure 4.5- Percentage of survival in time for seabream exposed to different concentration of mucus collected from stressed toadfish females. Values shown result from the cumulative mortality from n=9 per mucus concentration group. Each condition was tested in triplicate (n=3 fish/replicate)

When analysing the behaviours, showed in Figure 4.6, the frequency of all of them is much higher for the subject of the NSFM group. Especially when observing the spasms behaviour of the SFM group it’s evident that both the duration in time and the frequency of the behaviour are very reduced.

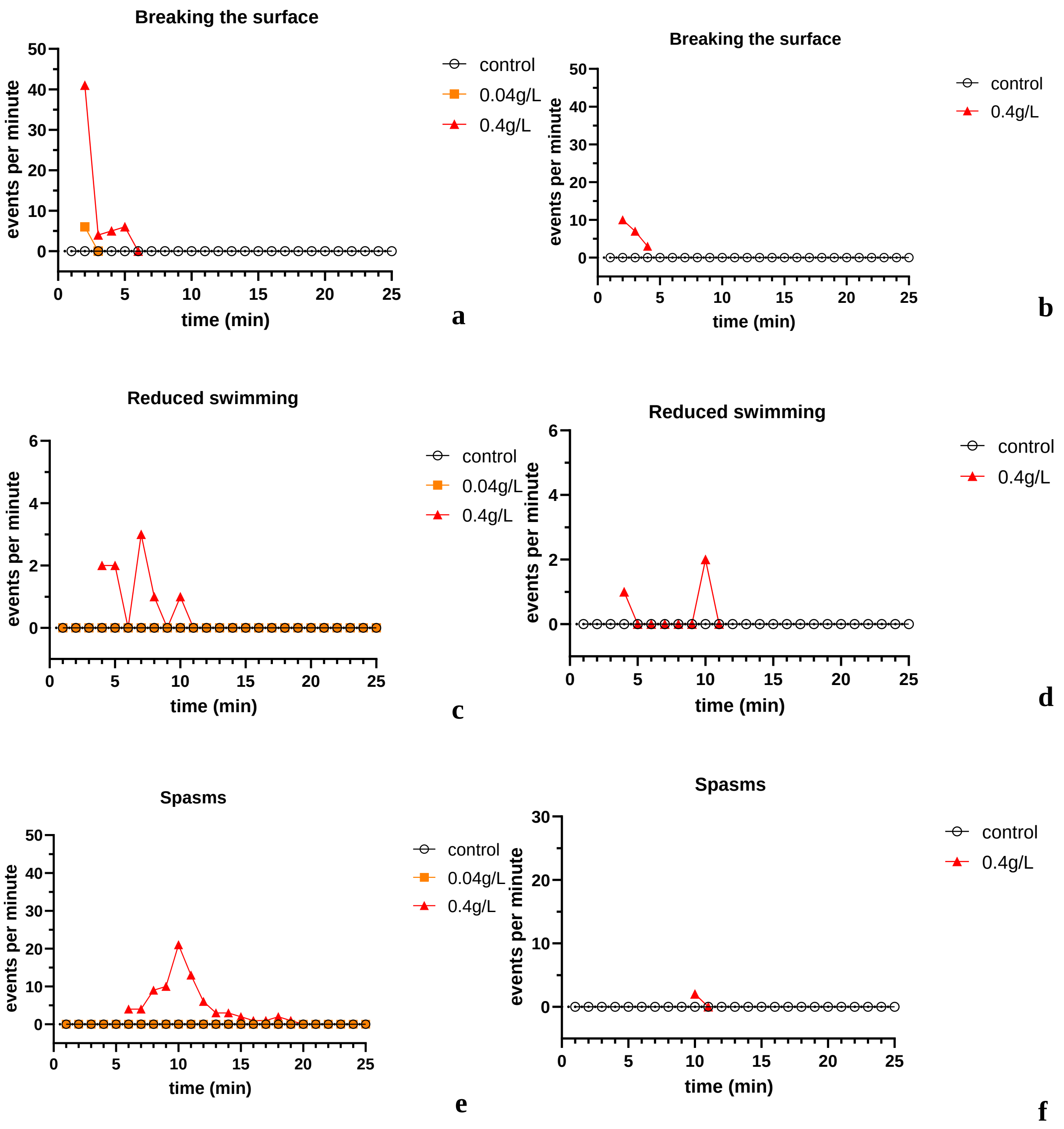

Figure 4.6 - Events per minute of each behaviour performed by seabreams exposed to the mucus of stressed and no-stressed females. values shown results from the sum of events of the three replicas beaker for each concentration. a-c-e) respectively breaking the surface, reduced swimming and spasms behaviours corresponding to non-stressed females 'mucus exposure. b-d-f) respectively breaking the surface, reduced swimming and spasms behaviours corresponding to stressed females' mucus exposure

When comparing the final survival percentages for the NSMM and the NSFM (shown in figures 4.7a and 4.7b) we can notice a difference only at the low concentration. In the NSFM 0 fishes died by the end of the 25 minutes while in the NSMM the survival rate dropped to 45% indicating that only 5 individuals died. For the two SMM and SFM groups the comparison can be done only between the two high concentration. For the SMM group all the individuals died by the end of the 25 minutes, while for the SFM group only one did not survive the exposition to the mucus.

Figure 4.7dc shows the protein concentration of the mucus' samples used in the experiments. Despite the fact that the mucus amount used in all experiments was similar (in weight), and mixed in an identical volume of seawater, the concentration of protein present in the sample used for the NSMM group was higher than the others and both NSMM and NSFM were higher than the corresponding stressed group.

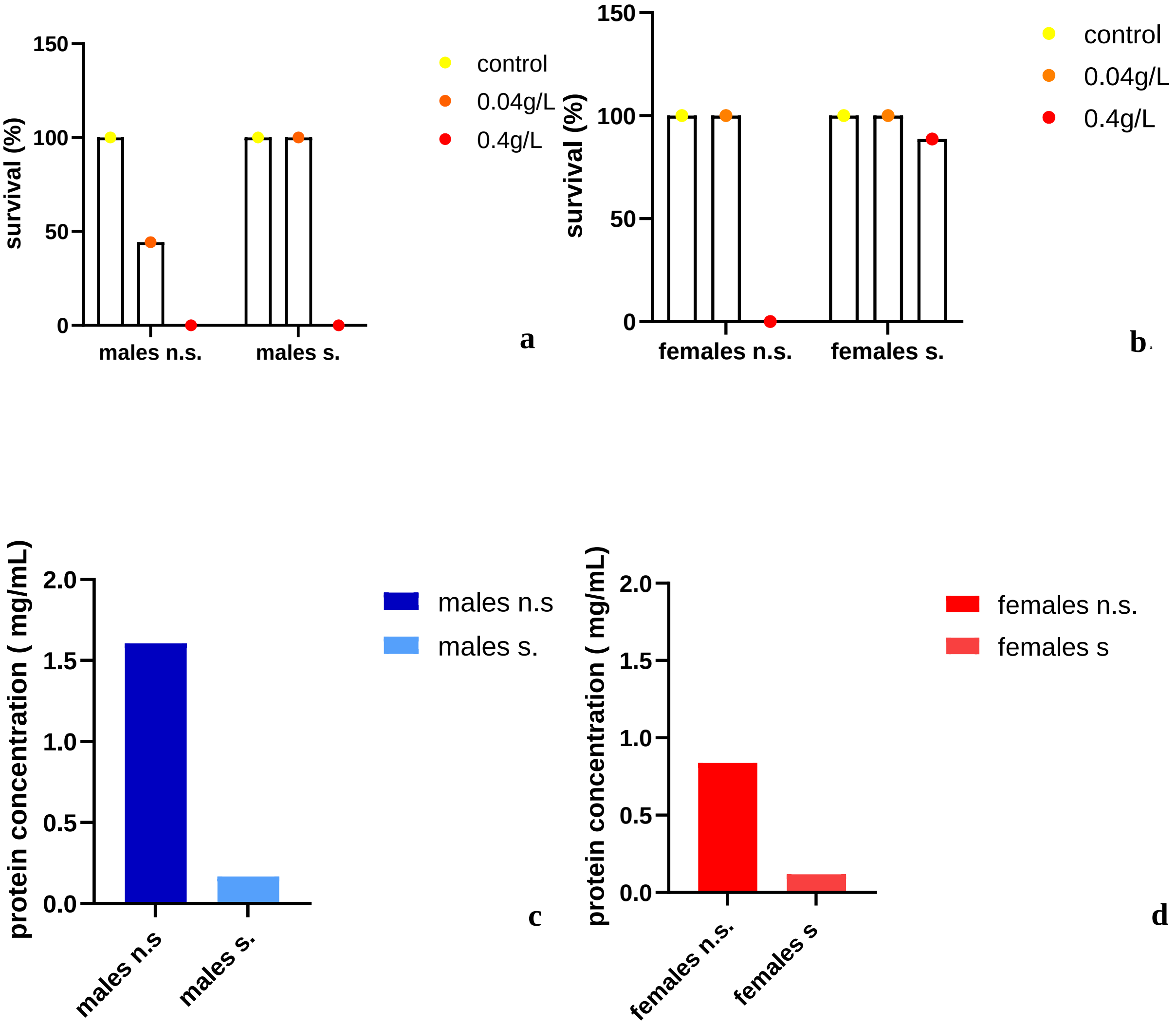

Figure 4.7 – a-b) final percentage of survival in each concentration, respectively stressed and not stressed males and stressed and not stressed females. c-d) protein concentrations' values of samples of the mucus used in the experiments. Respectively not stressed and stressed males and not stressed and stressed females.

Analyzing the average total number of events identified in the three replicates beaker for each concentration (Figure 4.8) we can immediately notice that the lower concentration of the SMM group had no effect on the behaviour of the test subjects. In the two group of the female exposure the same difference is noticeable at the higher concentration, with the average of events of the SFM group, lower than the one of the NSFM group

To statistically analyze the data, a one-way ANOVA followed by a multiple comparison test was performed to analyze the concentrations in each group, and then a non-parametric t-test (Wilcoxon test) was performed to analyze the differences between groups at the same concentrations.

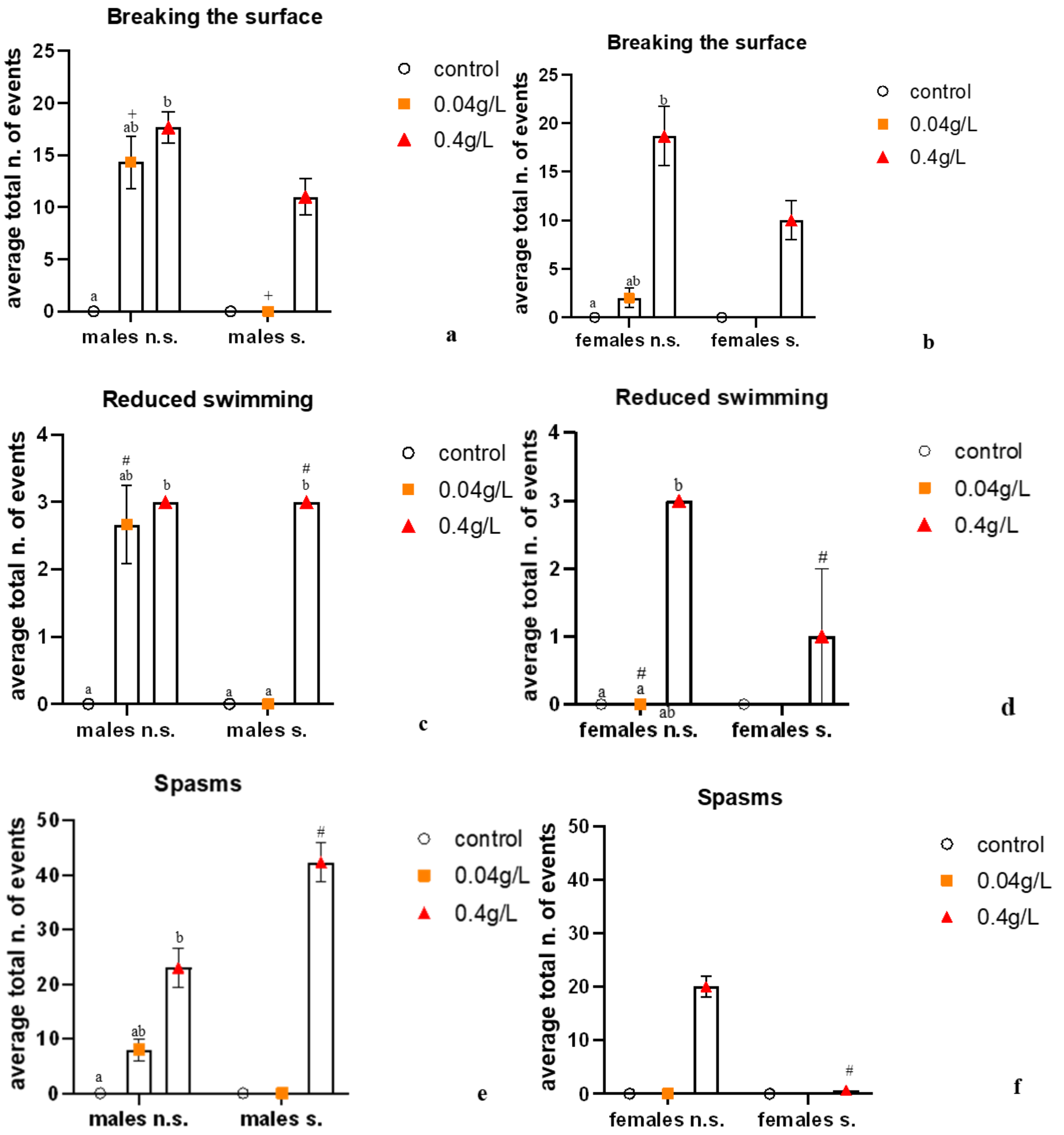

Figure 4.8 - Average total number of events per beaker of each behaviour for the stressed and not stressed males and females' mucus experiments. the results shown come from the mean of the values of three beaker for each concentration. a-c-e) respectively breaking the surface, reduced swimming and spasms behaviours corresponding to stressed and not stressed males' mucus exposure. b-d-f) respectively breaking the surface, reduced swimming and spasms behaviours corresponding to stressed and not stressed females' mucus exposure. Mean ± standard deviation. Different letters on top of the columns indicate statistically significant differences between concentrations, within the same exposure group. the symbol (+) indicates differences between non-stress and stress groups for the same concentration in each sex. The symbol (#) indicate differences between male and female groups for the same concentration for the same stress condition.

4.2 SALINITY EXPERIMENTS

The survival percentages of *S. aurata* at various salinities over time are illustrated in Figure 4.9. At the highest salinity (36 ppm), fish mortality began at 8 minutes, with survival dropping to 0% by the 13th minute. At 25 ppm, the survival rate started to decline at the 16th minute, reaching 0% by the 20th minute. For salinities of 13 and 6 ppm, the survival rate decreased similarly, falling to 67% by the 24th minute. At the lowest salinity of 3 ppm, the lethal effect was delayed, reducing the survival rate to 50% by the end of the experiment. All the control groups showed no lethal effect, so they are represented as an unique line in the graphs.

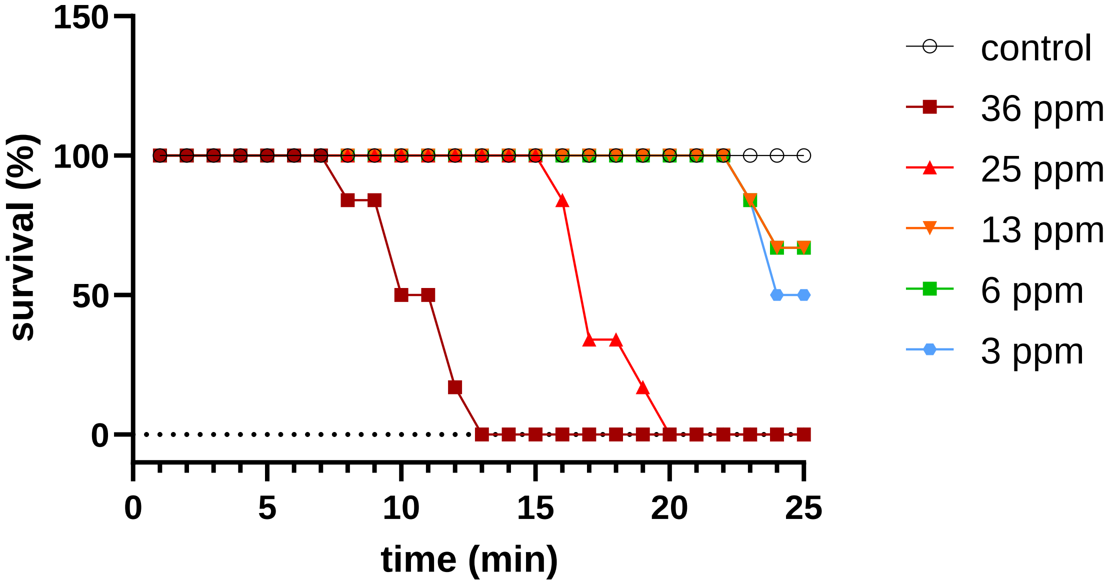

Figure 4.9- Percentage of survival in time for seabream exposed to a pool mucus from males and females at different salinities. Values shown result from the cumulative mortality from n=6 per salinity group. Each condition was tested in duplicate (n=3 fish/replicate)

The survival percentages of *Gambusia holbrooki* at various salinities over time are presented in Figure 4.10. All the control groups, which were not exposed to the mucus, showed no mortality so they are represented in a single line. At a salinity of 25 ppm, the fish began to die after 7 minutes, with all individuals dead by the 20th minute. Similarly, at a salinity of 13 ppm, mortality started at the 6th minute, and all fish were dead by the 20th . In the beaker with 0 ppm salinity, no fish died.

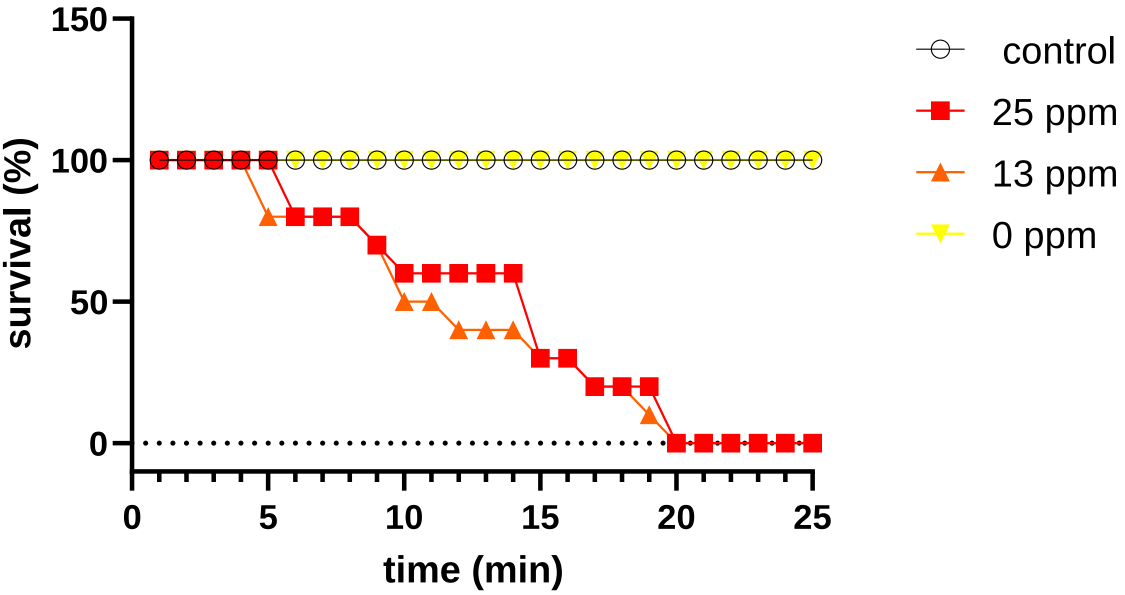

Figure 4.10- Percentage of survival in time for mosquitofishes exposed to a pool mucus from males and females at different salinities. Values shown result from the cumulative mortality from n=10 per salinity group. Each condition was tested in duplicate (n=5 fish/replicate)

When comparing the survival percentages of the two experiments we can see that the survival percentage dropped faster at the highest salinity of 36 ppm. In both experiments the fishes at salinity of 25 ppm were dead by the 20th minute, while the survival rates of the 13 ppm salinity differs in the two experiments, with a 0% survival in the experiment with *G. holbrooki* and a 67% survival in the experiment with *S.aurata.*

When analyzing the behaviour of the test subjects in the two experiments (Figure 4.11), no significant differences were observed between them. In the experiment with *S. aurata*, it doesn't appear to be a clear difference between the salinities. However, in the spasms graph, fish at lower salinities exhibited less frequent spasms compared to those at higher salinities. In the experiment with *G. holbrooki*, the mucus had no effect on the fishes' behaviour at 0 ppm salinity. For the other two salinity levels, the results were quite similar, with no noticeable differences.

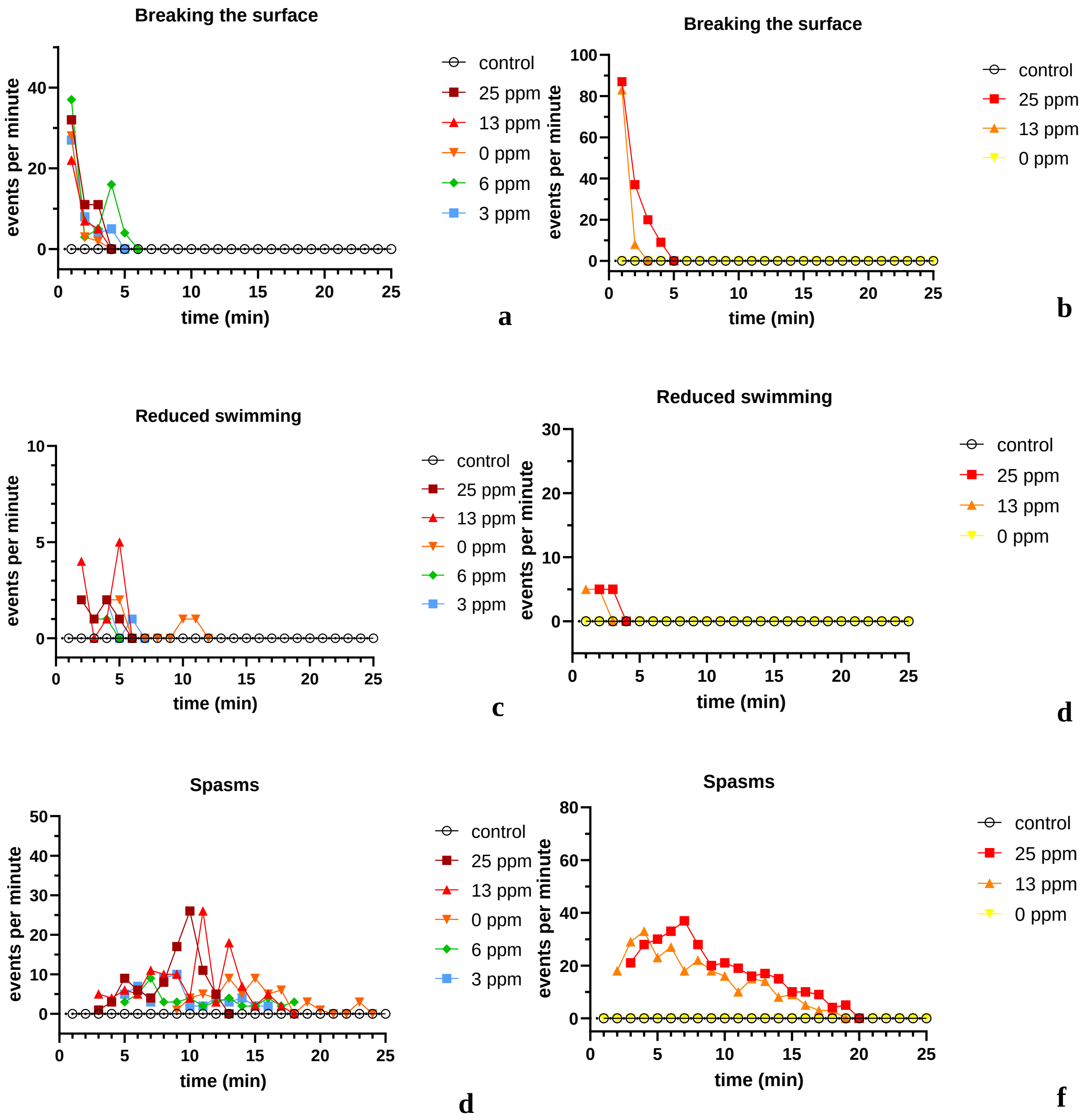

Figure 4.11 - Events per minute of each behaviour performed by seabreams exposed to a pool of males and females mucus at different salinities. values shown results from the sum of events of the two replicates beaker for each concentration. a-c-e) respectively breaking the surface, reduced swimming and spasms behaviours corresponding to individual of *S.aurata* exposed to the mucus at salinities of 36,25,13,6 and 3 ppm. b-d-f) respectively breaking the surface, reduced swimming and spasms behaviours corresponding to individuals of *G. holbrooki* exposed to the mucus at salinities of 25,13 and 0 ppm

When observing the final percentages of survival in the two experiments (Figure 4.12) we can notice a difference in mortality at the salinity of 13 ppm. In the experiment with *G. holbrooki* all the individuals exposed at that salinity died while in the experiment with *S. aurata* less than half perished. This could be due to physiological differences of the two species. In the experiment with the seabreams there doesn't seem to be a correlation between lower salinity and survival.

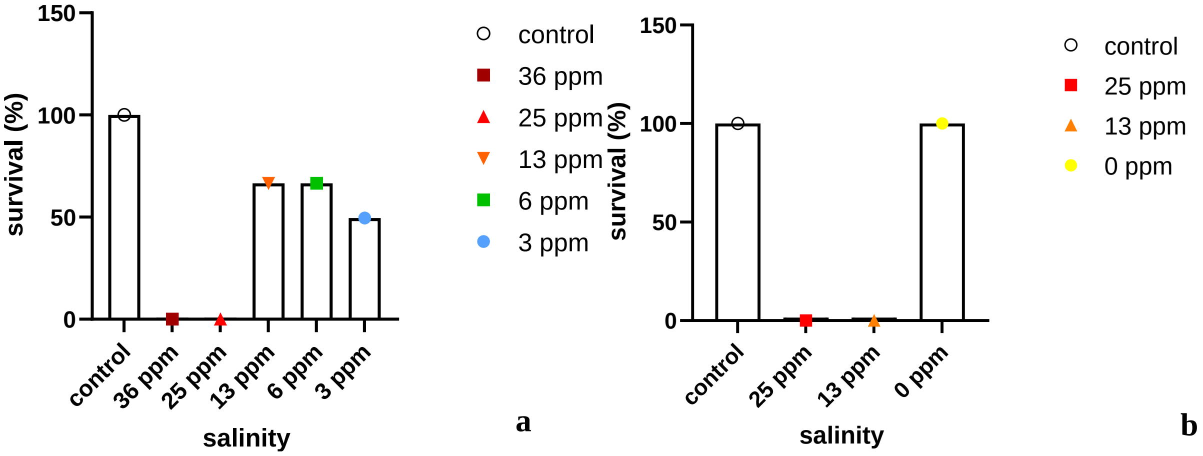

Figure 4.12- percentages of survival at the end of the experiments. Values shown are the mean of the survival percentages of the two replicate beakers of each salinity a) results for the experiment with *S. aurata*. b) results for the experiment with *G. holbrooki*.

When observing the average total number of events per beaker for each behavior (Figure 4.13), in the experiment with *S. aurata* there doesn't seem to be a correlation between salinity and number of events. Regarding the *G. holbrooki* experiment there does seem to be a decrease of number of events when the salinity decreases, especially in the "reduced swimming" and "spasms" behaviours (Figure 4.13d-f). After performing a one way ANOVA to analyse the differences in the two experiments, we know that there are no statistically significant differences between salinities. It is not possible to compare the experiments on the two species because the number of test subjects in each beaker is not the same.

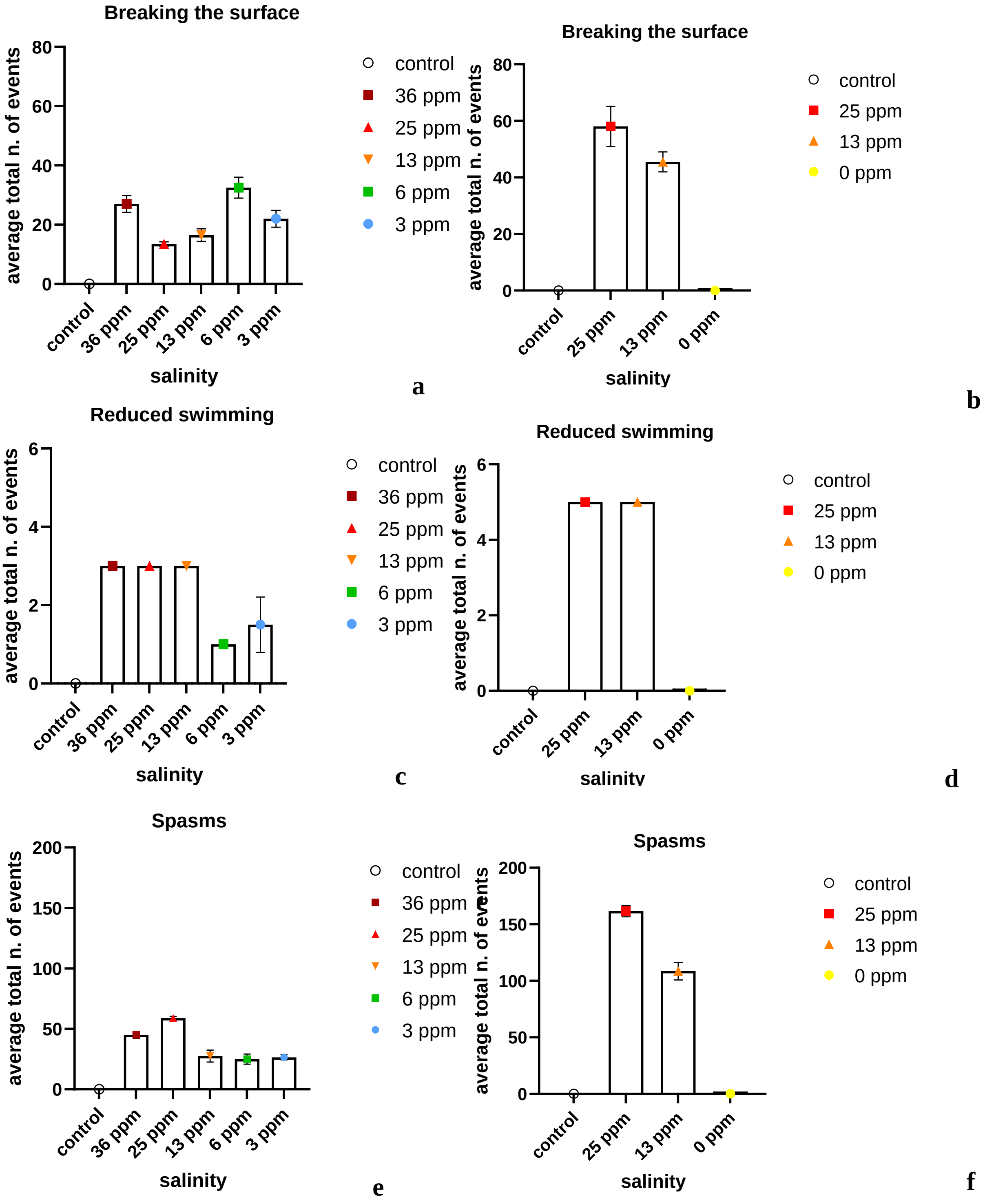

Figure 4.13 - Average total number of events per beaker of each behaviour for mucus experiments in different salinities with *S. aurata* and *G. holbrooki*. The results shown come from the mean of the values of two beaker for each salinity. a-c-e) respectively breaking the surface, reduced swimming and spasms behaviours of the experiment with *S. aurata*. b-d-f) respectively breaking the surface, reduced swimming and spasms behaviours corresponding to *G. holbrooki*. Mean ± standard deviation.

4.3 WATER EXPERIMENTS

Mortality was also observed over time in the fish exposed to holding water from the male toadfish stressed for 5 minutes (SMW5, figure 4.14). In the 1:1 concentration the lethal effect starts after 4 minutes, and the survival percentage quickly decrease until 0 by the 7th minute. In the 1:5 concentration the fishes start to die later, at the 8th minute, but again the percentage quickly decrease to 0 by the 10th minute. In the lower concentration (1:50) more than half of the fishes are dead by the 25th minute. The control fishes did not die through the experiment.

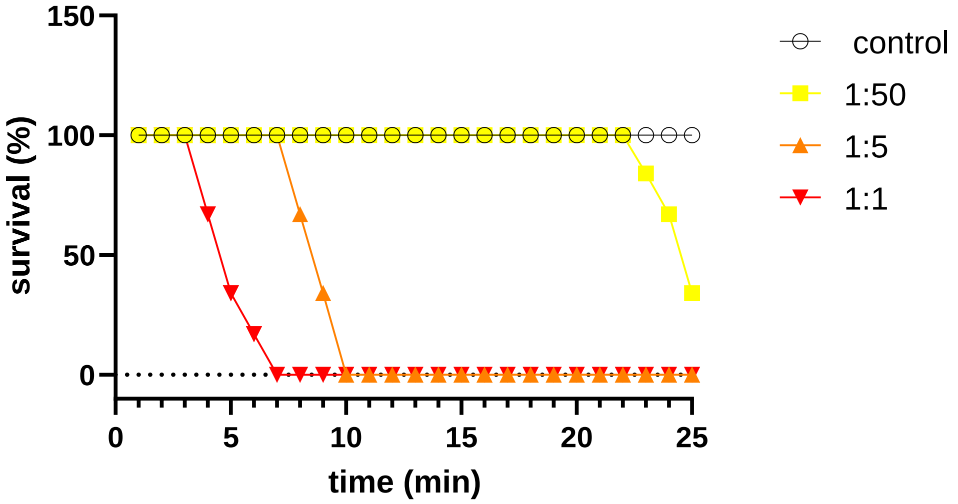

Figure 4.14 - Percentage of survival in time for seabreams exposed to holding water of male individuals stressed for 5 minutes. Values shown result from the cumulative mortality from n=6 per concentration group. Each condition was tested in duplicate (n=3 fish/replicate)

In figure 4.20 Is shown the percentage of survival in time of the group exposed to the 30 minutes stressed males' holding water (SMW30). The "toadfish water" at the higher concentration exhibit fast lethal effect and by the 7th minute all the fishes are dead. In the 1:5 concentration the decrease of survival percentage is less steep, and it reaches 0 at the 13th minute. The lower concentration did not have lethal consequences on the fishes, with a 100% survival at the end of the experiment.

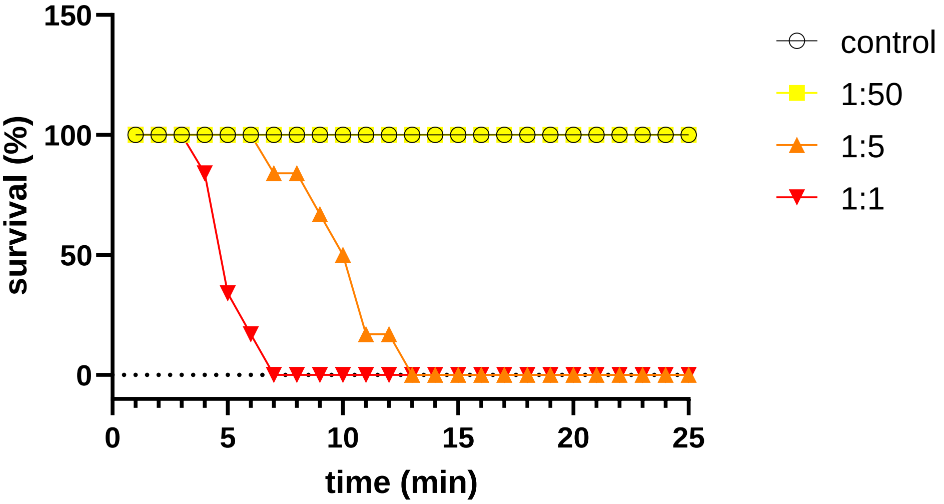

Figure 4.15- Percentage of survival in time for seabreams exposed to holding water of males individuals stressed for 30 minutes. Values shown result from the cumulative mortality from n=6 per concentration group. Each condition was tested in duplicate (n=3 fish/replicate)

In Figure 4.16 are plotted the events for each behaviour for the SMW5 and SMW30. The more evident difference is at the 1:50 concentration where we can see that the SMW30 group exhibit no alteration of the behaviour.

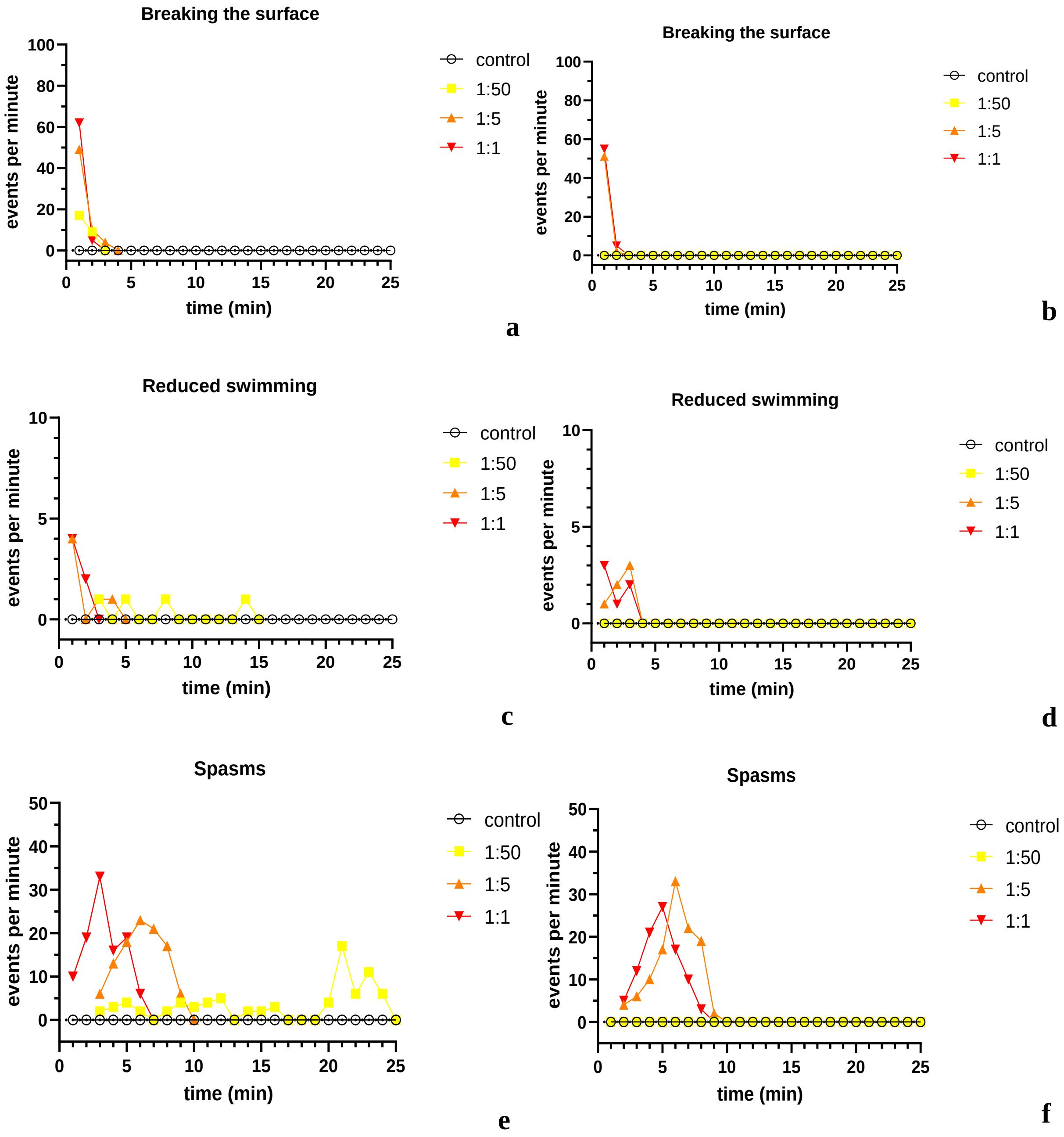

Figure 4.16 - Events per minute of each behaviour performed by seabreams exposed to the holding water of males stressed for 5 and 30 minutes. values shown results from the sum of events of the three replicas beaker for each concentration. a-c-e) respectively breaking the surface, reduced swimming and spasms behaviours corresponding to the exposure to the 5 minutes stressed males' holding water. b-d-f) respectively breaking the surface, reduced swimming and spasms behaviours corresponding to the exposure to the 30 minutes stressed males' holding water.

In Figures 4.17 and 4.18, we observe the survival percentages over time for SFW5 and SFW30, respectively. In both experiments, the 1:50 concentration had no lethal effect on the fish. For the 1:5 concentration in the SFW5, mortality began at the 7th minute, with all fish dead by the 10th minute. At the higher concentration, mortality started at the 3rd minute, with a 0% survival rate by the 7th minute. In the experiment with SFW30, at the higher concentration, fishes began dying at the 6th minute, reaching 0% of survival by the 10th minute. At the 1:50 concentration, the first deaths occurred at the 9th minute, with all fishes dead by the 12th minute. In both experiments, the control fishes did not die

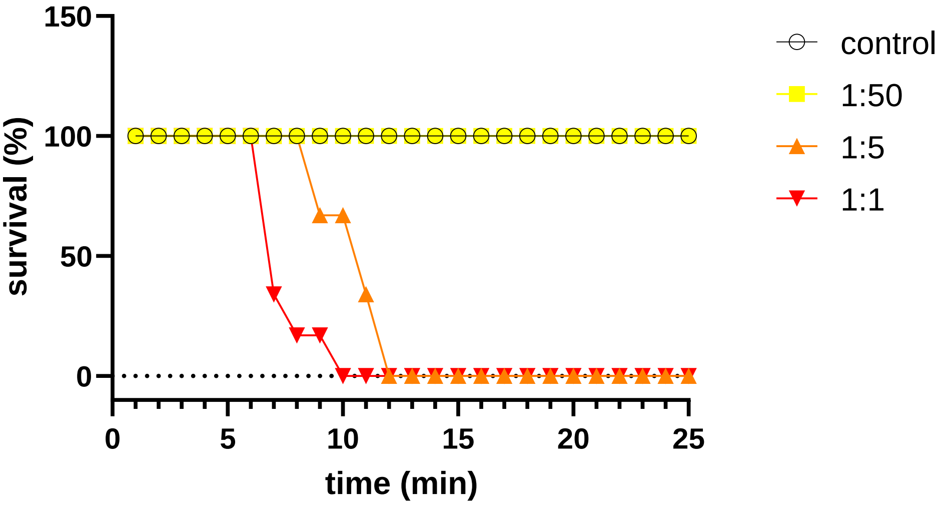

Figure 4.17- Percentage of survival in time for seabreams exposed to holding water of females' individuals stressed for 5 minutes. Values shown result from the cumulative mortality from n=6 per concentration group. Each condition was tested in duplicate (n=3 fish/replicate)

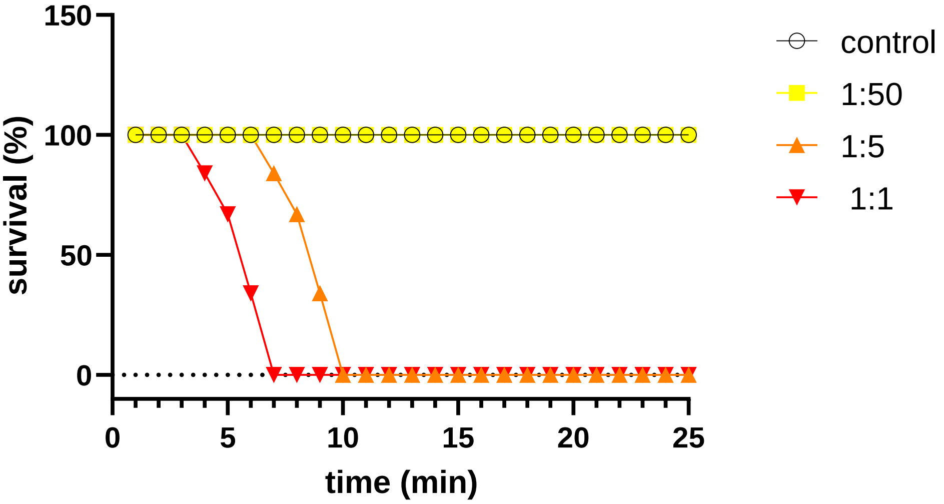

Figure 4.18- Percentage of survival in time for seabreams exposed to holding water of females' individuals stressed for 30 minutes. Values shown result from the cumulative mortality from n=6 per concentration group. Each condition was tested in duplicate (n=3 fish/replicate)

When observing the behaviour plotted in Figure 4.19, we can notice that the SFW5 group exibith no changes in behaviour at the lower concentration. On the other hand the SFW30 group show a slight change of behaviour, causing reduced swimming in some individuals. Regarding the 1:1 concentration there appear to be no difference between the two groups.

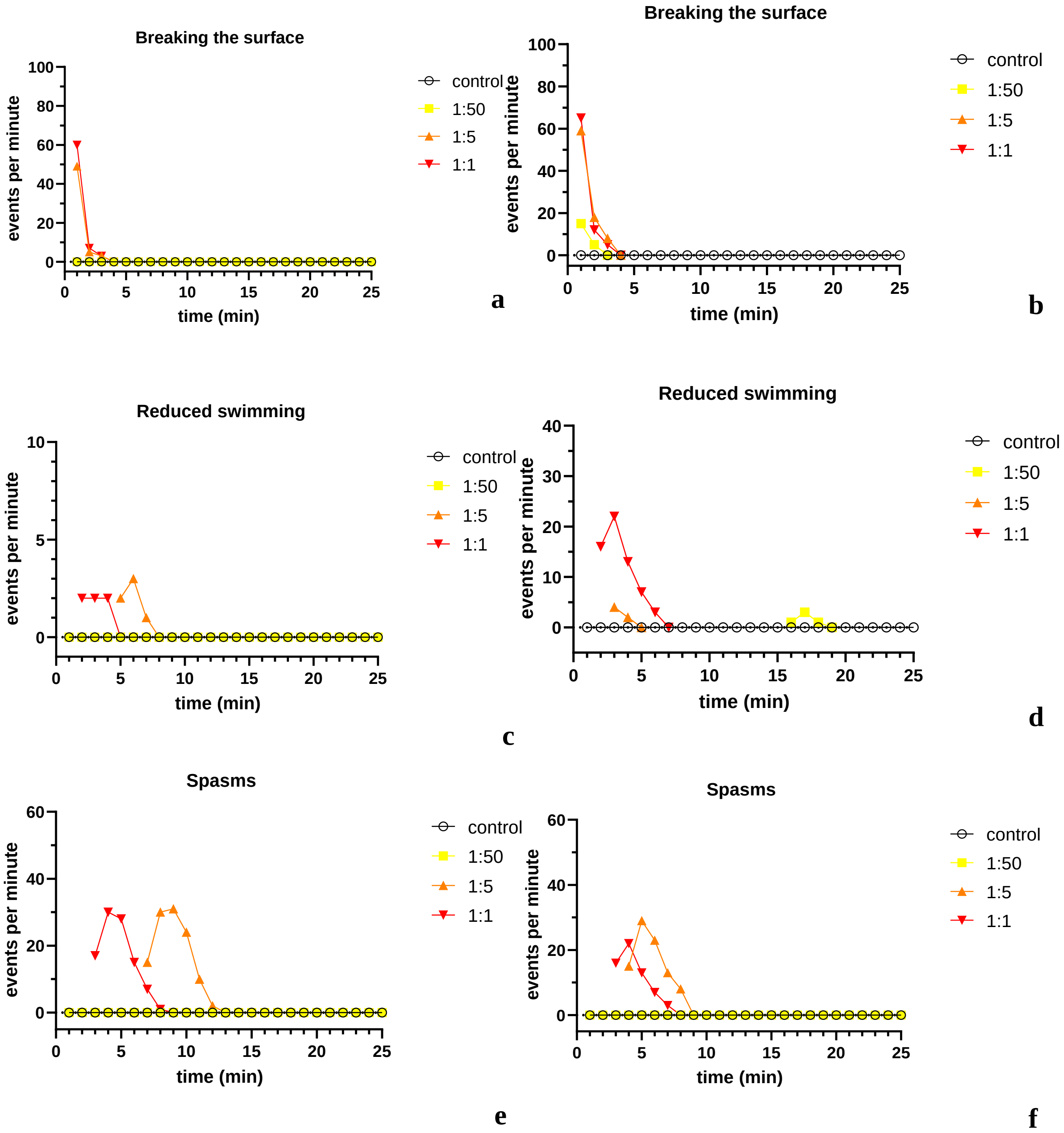

Figure 4.19 - Events per minute of each behaviour performed by seabreams exposed to the holding water of females stressed for 5 and 30 minutes. values shown results from the sum of events of the three replicas beaker for each concentration. a-c-e) respectively breaking the surface, reduced swimming and spasms behaviours corresponding to the exposure to the 5 minutes stressed females' holding water. b-d-f) respectively breaking the surface, reduced swimming and spasms behaviours corresponding to the exposure to the 30 minutes stressed females' holding water.

When observing the final survival rates of the experiments (Figure 4.20) the only difference between males and females is the 1:50 concentration of the SMW5, which is the only one that exhibits a decrease in survival at that concentration. When comparing the protein concentrations with the results of the experiment of the males we can notice a higher value of protein for the water that caused more mortality.

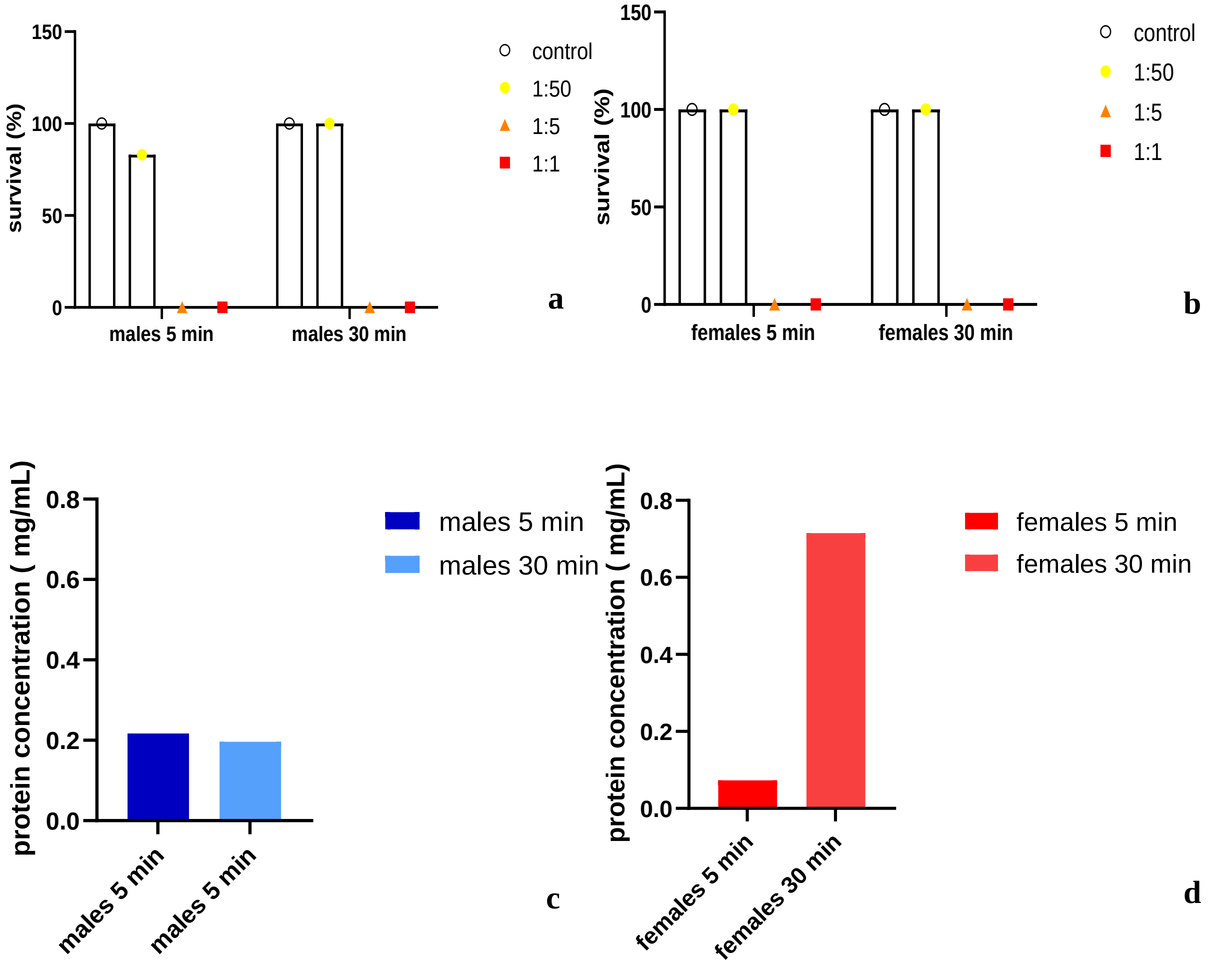

Figure 4.20- a-b) final percentage of survival in each concentration, respectively SMW5, SMW30 and SFW5 and SFW30. c-d) protein concentrations’ values of samples of the mucus used in the experiments.

In the breaking the surface behaviour the males and females exhibited a clear increase in breaking the surface events at the highest concentration (1:1), with this behavior being less present or no present at all in the 1:50 concentration. Regarding the reduced swimming behaviour there doesn’t seem to be a difference between males and females or betweeen the stress periods, at least at the two highest concentrations. At the lowest concentration only the SMW5 and the SFW30 showed an effect. Spasms do not appear at the lowest concentration except fot the SMW5 group. Statystical analysis did not show any statistically significant difference between the conditions or the concentrations.

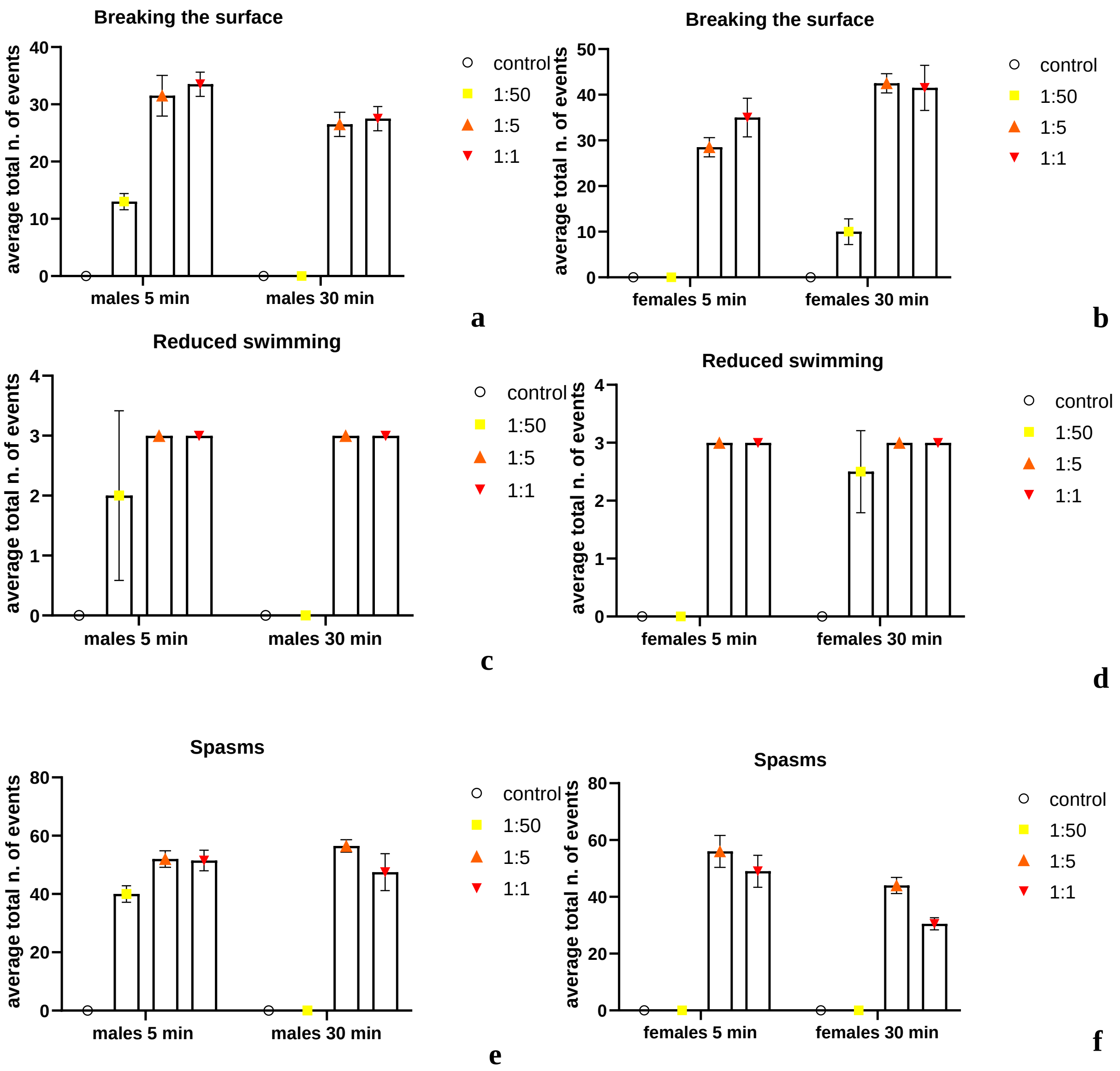

Figure 4.21- Average total number of events per beaker of each behaviour in the holding water experiments. The results shown come from the mean of the values of two beaker for each salinity. a-c-e) respectively breaking the surface, reduced swimming and spasms behaviours of the experiment with holding water of 5 and 30 minutes stressed females. b-d-f) respectively breaking the surface, reduced swimming and spasms behaviours of the experiment with holding water of 5 and 30 minutes stressed males Mean ± standard deviation.

4.4 AXILLARY GLAND CONTENT

By observing the recording of the axillary gland experiment, there doesn’t seem to be an effect on the fishes. No death was recorded at the end of the 25 minutes and analysing the behaviour of the test subject no particular changes occurred. To test the content of the axillary gland a swab is used to collect secretion inside the axillary pore. The swab it then squeezed in a tube with a mL of water and the content is tested in two beakers containing 500mL of saltwater. It’s important to note that maybe the dose might not have been sufficient to cause an effect.

4.5 HAEMOLYTIC ACTIVITY

The percentage of haemolytic activity was calculated assuming that the saponin used caused 100% of plasmolysis which might not be the case. That could explain why we have values of haemolytic activity slightly higher than 100%.

For the female’s mucus the values of heamolytic activity for each concentration are ± 100% for the 1:1 concentration, ± 7% for the 1:100 concentration and ± 4% for both 1:1000 and 1:10000 concentrations (Figure 4.22)

Since the data distribution result as non-normal from the Shapiro-Wilk normality test, a non-parametrical analysis is performed. From the t-tests we know that between the values of haemolytic activity of the 1:1 and the 1:100, 1:1000 and 1:10000 the difference is statistically significant.

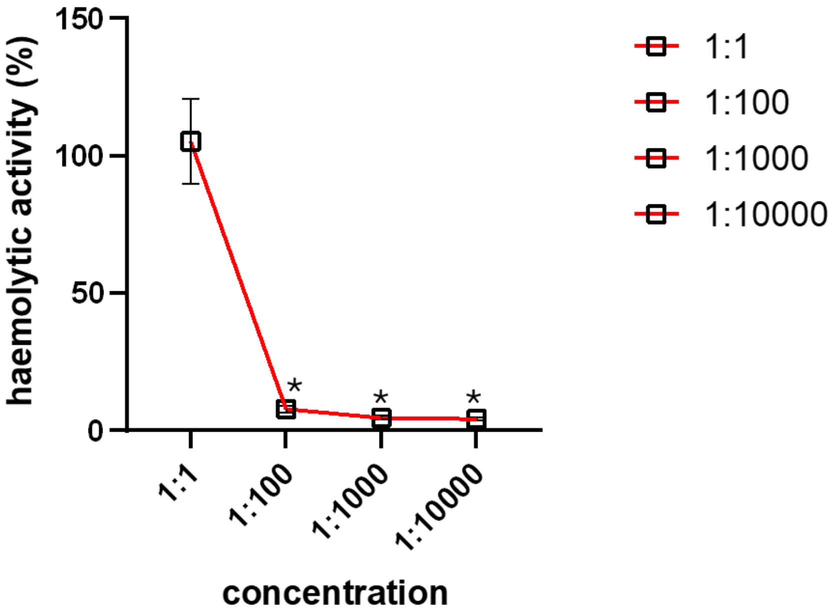

Figure 4.22 - Percentage of haemolytic activity of females’ mucus at different concentrations. Mann-Whitney (p-value > 0,05). The symbol (*) indicates statistical difference from the 1:1 concentration. Mean ± standard deviation

For the males’ mucus the values of heamolytic activity for each concentration are ± 107% for the 1:1 concentration, ± 100% for the 1:100 concentration, and ± 9% for the 1:1000 concentration and ±7 for the 1:10000 concentrations. (Fig. 4.23)

For the male’s heamolytic activity values was performed again a non-parametric test as the distribution of the data is not normal. From the Mann-Whitney test the statistically significant differences are between the value at the concentration 1:1 and at the concentration 1:1000 and 1:10000.

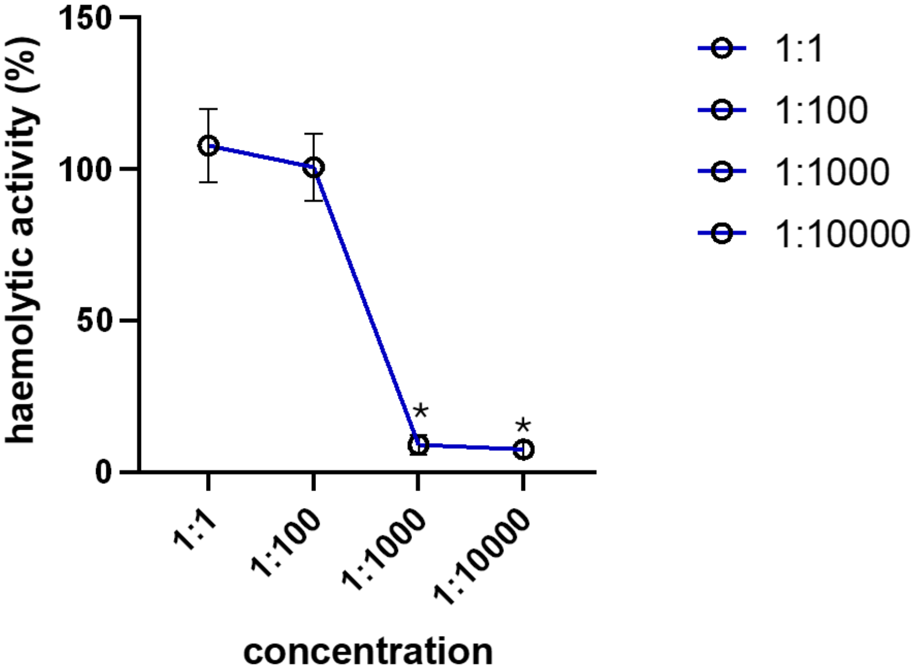

Figure 4.23 - Percentage of haemolytic activity of males' mucus at different concentrations. Mann-Whitney (p-value > 0,05). Mean ± standard deviation. The symbol (*) indicates statistical difference from the 1:1 concentration. Mean ± standard deviation

When comparing the values between males and females it can be noted that the males' mucus has higher values of heamolytic activity compared to the females, especially for the 1:100 concentration (Figure 4.24a). When performing the statistical test (Mann-Whitney) the result is that the only statistically significant difference is between the two 1:100 concentrations.

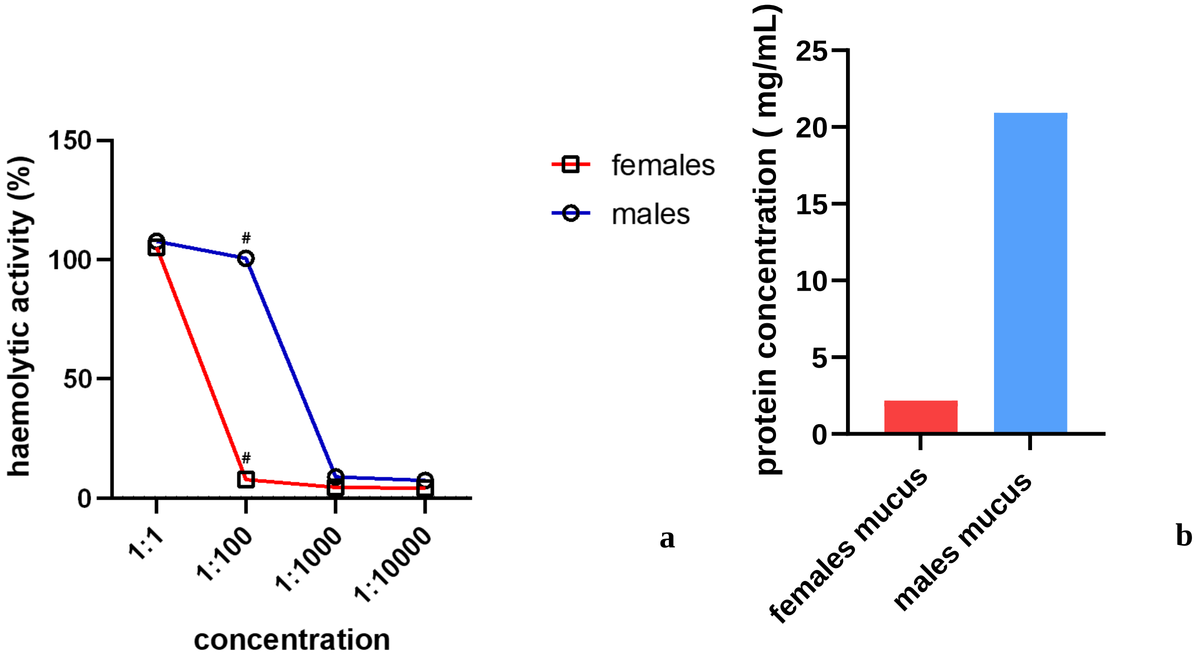

Figure 4.24- a) percentage of haemolytic activity of males and females' mucus at different concentrations. Mann-Whitney (p-value > 0,05). b) protein concentration values for males and females' mucus. Females' mucus was collected from 6 individuals, males' mucus was collected from 8 individuals

In Figure 4.24b is shown the protein concentration in the samples of males and females' mucus used for the haemolytic activity experiments. The females' mucus contained 2.174 mg/mL and the male's mucus contained 20.931 mg/mL.

4.6 HISTOLOGY

Skin

Figure 4.25 shows a cross-section of the skin of *H. didactylus* highlighting its distinct layers. It is possible to identify the basic structural layers found in other teleost: the epidermis, composed of a non-keratinized stratified squamous epithelium, followed by a layer of connective tissue, the dermis, where small scales are embedded in dermal pockets, that do not protrude through the epidermis; below the dermis lies the hypodermis, composed of loose connective tissue supported by a layer of muscle tissue.

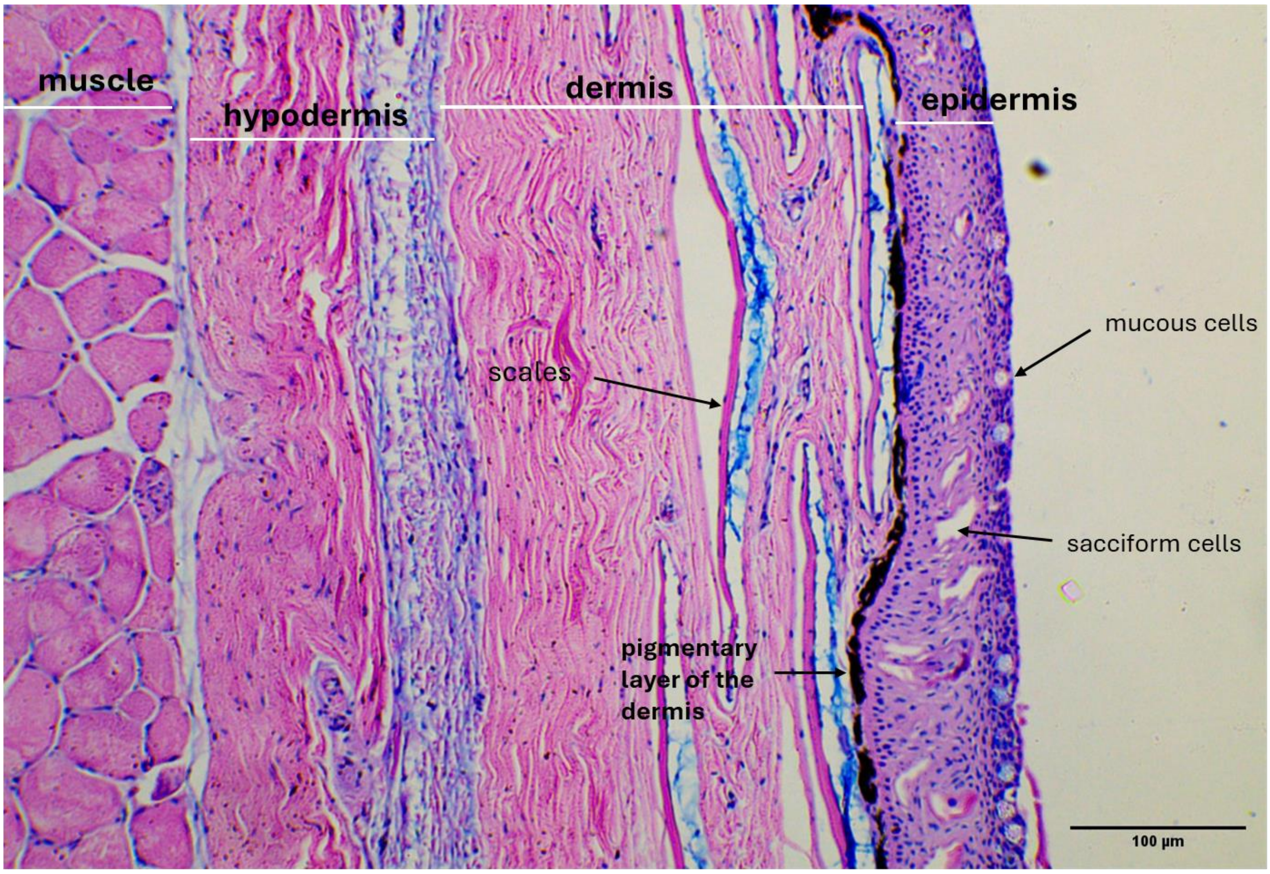

Figure 4.25 – Alcian blue staining of a cross section of Sk_d2 sample of a not stressed female individual of *H. didactylus.* The different layers are shown and labelled. Mucous cells are highlighted as well as sacciform cells, scales and the pigmentary layer of the dermis.

In Figure 4.26, in the outermost layer, numerous mucus cells can be observed, staining positively with Alcian Blue 2.5 and negatively with Hematoxylin/Eosin and Masson's Trichrome. This indicates the presence of acidic components such as sulfated and carboxylated glycoproteins (mucins) (Chan, 2014; Foot, 1933; Layton & Bancroft, 2019; K. S. Suvarna et al., 2018). Immediately below the mucus cell layer, sacciform cells of varying sizes, large secretory cell, are present in abundance both in the male and female skin. These cells are unicellular exocrine glands and occupy most of the thickness of the epidermis. In most of the samples, these cells appear empty or with scarce cytoplasmic content.

There doesn’t seem to be a difference in the skin layers and composition between the different region of the skin (dorsal skin 1, dorsal skin 2, and ventral), or between the two stress conditions. However, it is evident that, in both locations (dorsal and ventral), the number of mucus cells appears to be much greater in males than in females (Figure 4.27).

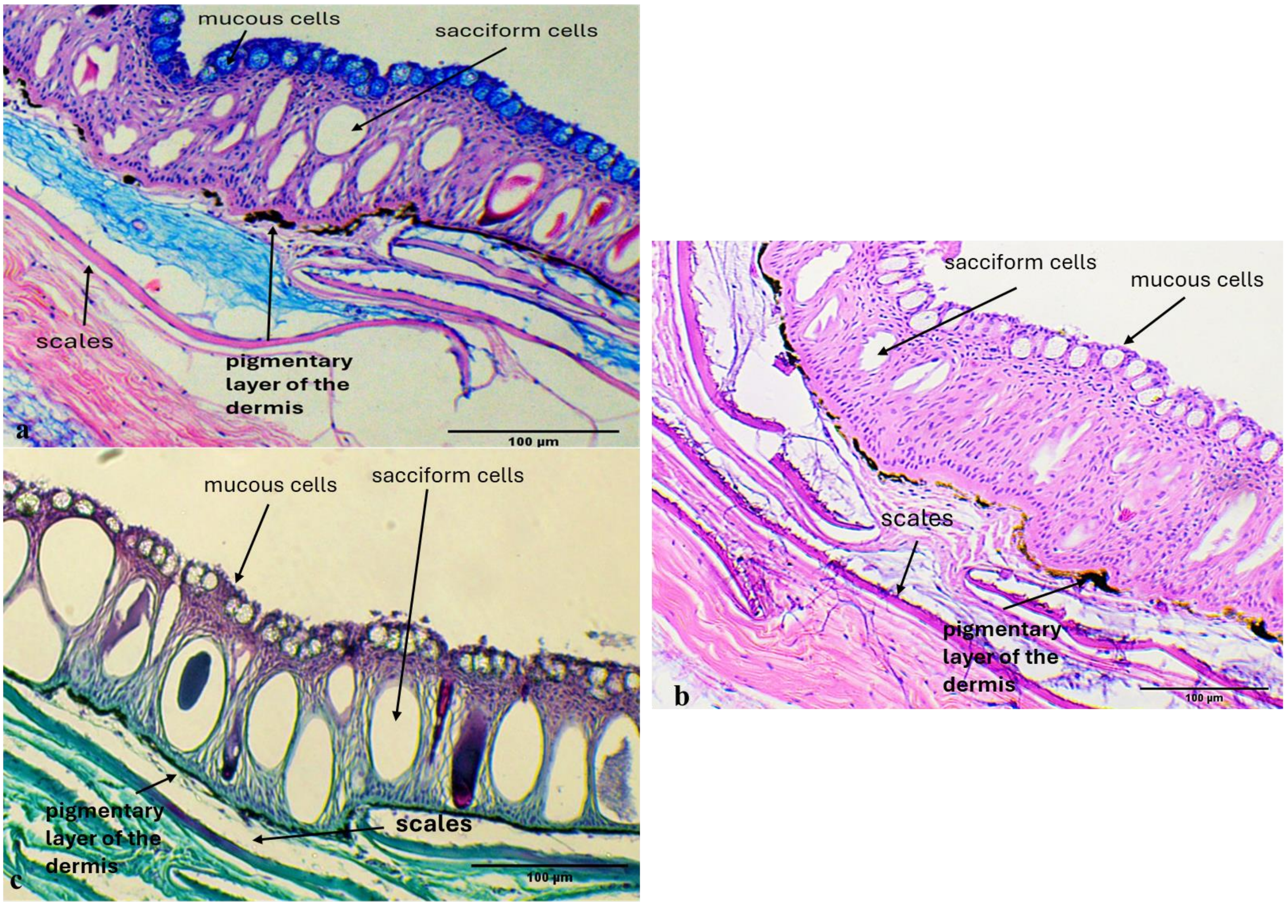

Figure 4.26 - Cross section of Sk_d2 samples of not stressed males of H. didactylus stained with Alcian Blue (a), H&E (b) and Masson’s trichrome (c). Mucous tested positive for Alcian Blue but negative for Masson´s trichrome and H&E staining.

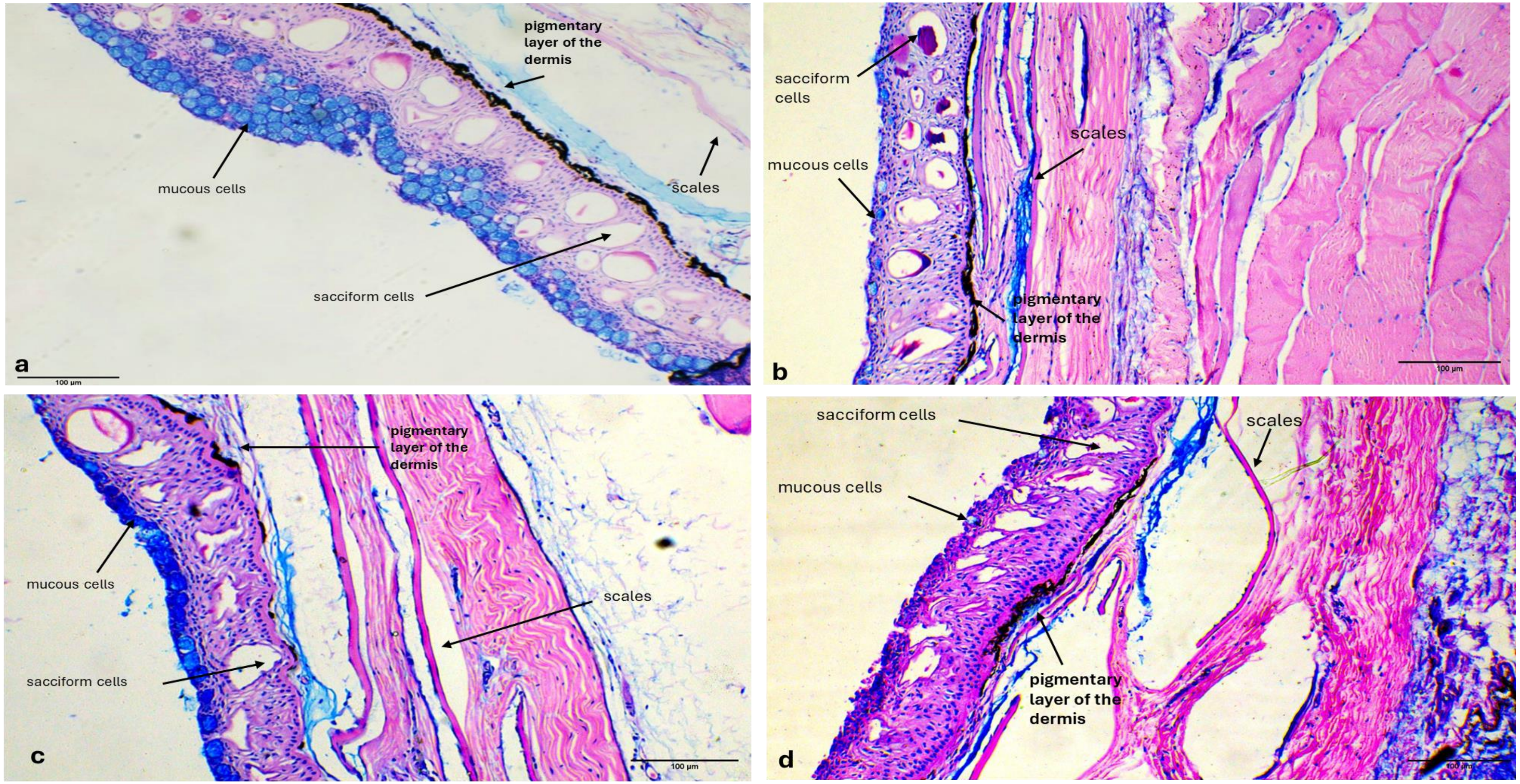

Figure 4.27 - Alcian Blue staining highlighting the difference in number of mucous cells in males and females' skin. a) Sk_d1 sample of a stressed male. b) Sk_d1 sample of a stressed female. c) Sk_v sample of a stressed male. c) Sk_v sample of a stressed female

Axillary glands

Like what has been observed in other species of batrachoidids, *H. didactylus* has a pair of axillary glands located between the body wall and the pectoral fin, which open to the exterior through a pore. These glands have a rosette shape. Histologically, the epithelium is highly folded (Figures 4.28 and 4.29), increasing the internal surface area of the gland in contact with the exterior. No ducts are evident, and the epithelium contains epithelial cells and numerous sacciform cells. In the distal region of the epithelium, numerous mucus cells are observed near the lumen of the gland (Figures 4.28, 4.29 and 4.30), staining intensely with Alcian Blue 2.5 but negatively with Masson's trichrome and Hematoxylin-eosin., indicating the presence of acidic mucins, both carboxylated and sulfated (Figure 4.30) From the analysis of the slides, it's not evident a difference between the stressed and not stressed conditions.

The sacciform cells are abundant and their cytoplasm is acidophilic (H&E staining), but the majority of the cells appear empty when stained with Alcian Blue 2.5, indicating the absence of acidic mucopolysaccharides. Is also noticeable a difference in the number and content of sacciform cells between males and females (Figures 4.28 and 4.29), however, the limited number of samples does not allow us to say that this is a real difference or an artifact that comes from the slide being prepared at different depths within the glands.

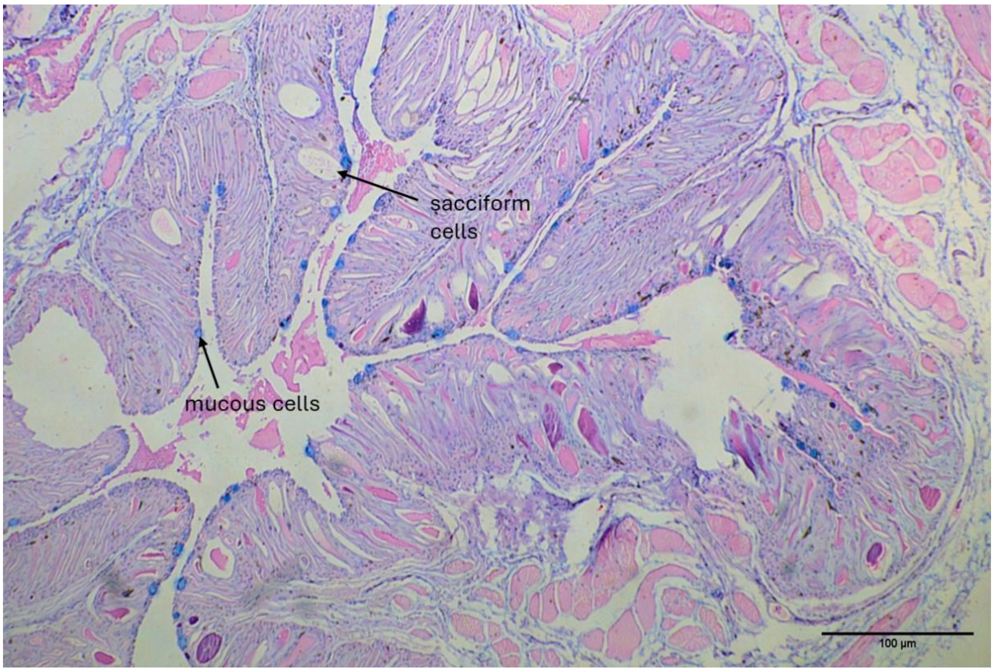

Figure 4.28 - Alcian blue staining of a section of the axillary gland of a stressed male. The coloration highlights the mucous cells (in blue) and the content of some of the sacciform cells

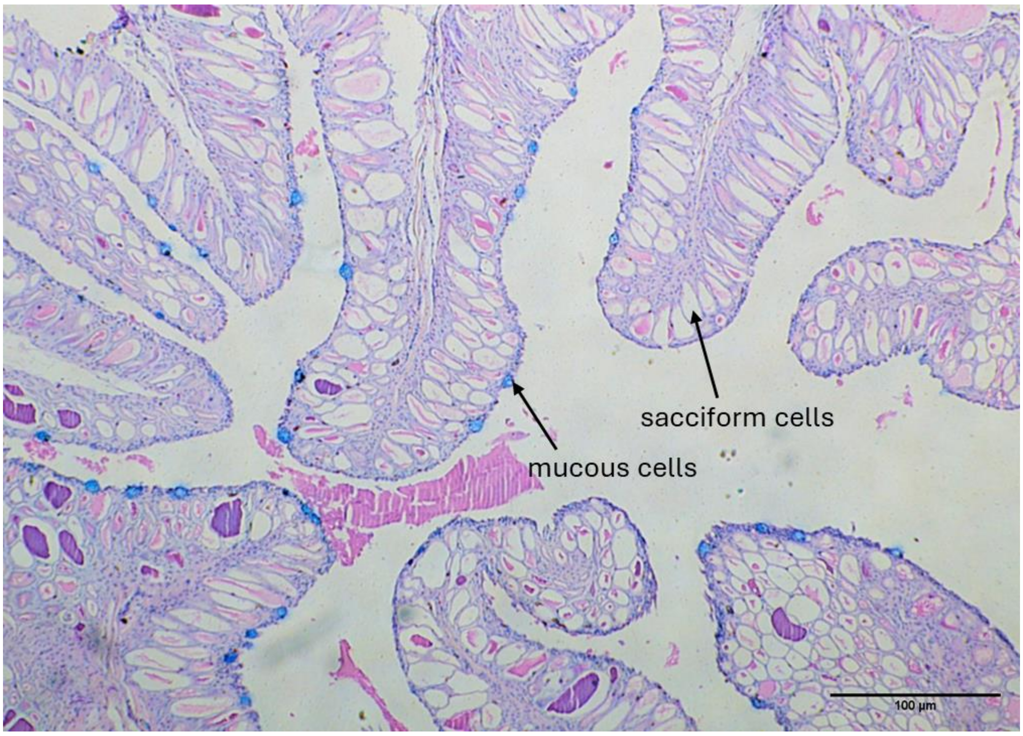

Figure 4.29 - Alcian blue staining of a section of the axillary gland of a stressed female. The coloration highlights the mucous cells (in blue) and the content of some of the sacciform cells.

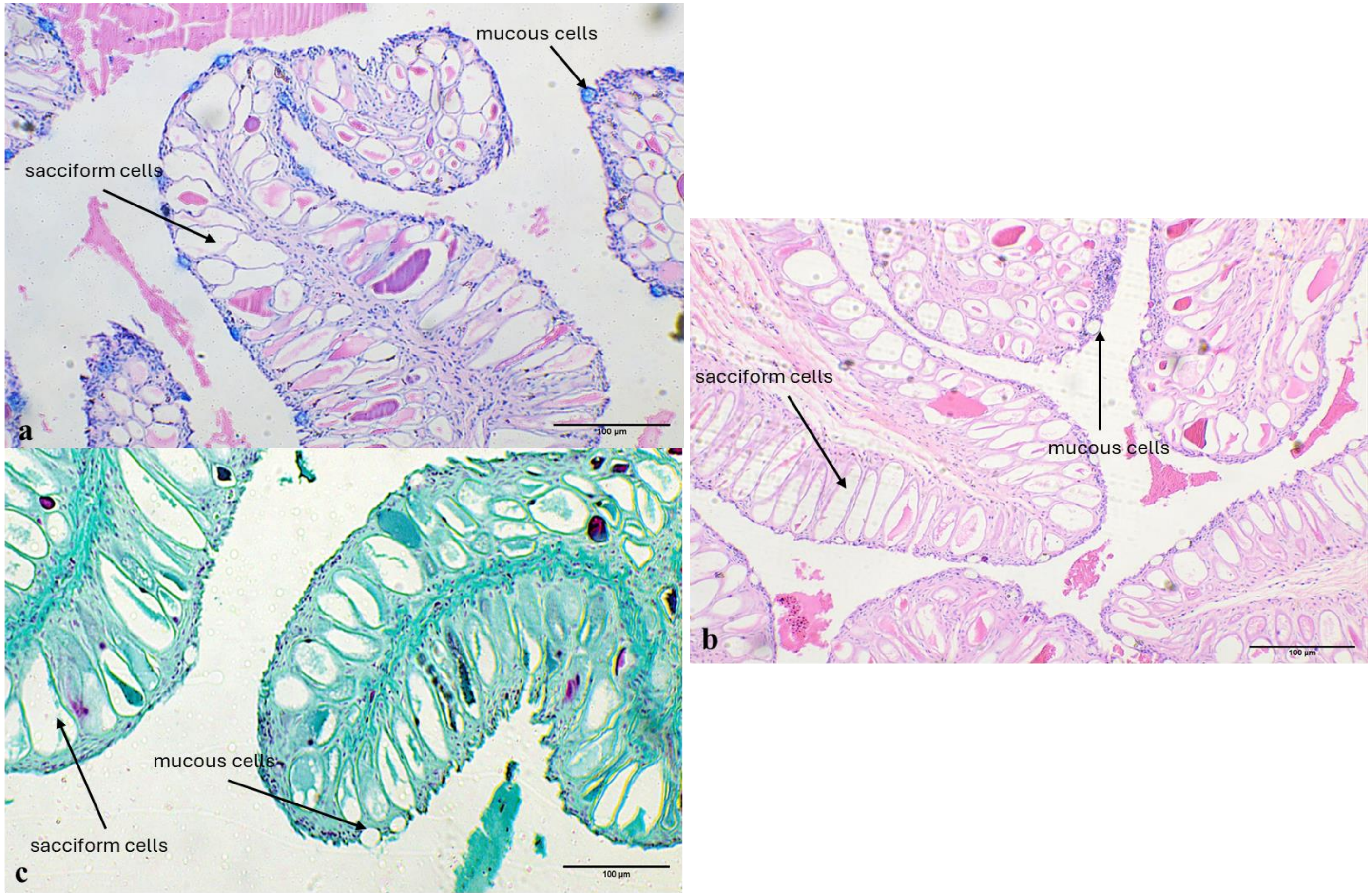

Figure 4.30 - Cross section of the axillary gland sample of not stressed females of H. didactylus stained with Alcian Blue (a), H&E (b) and Masson’s trichrome (c). Mucous tested positive for Alcian Blue but negative for Masson´s trichrome and H&E staining

Chapter 5 DISCUSSION

This study showed that the Lusitanian Toadfish skin mucus contains substances with toxic impacts over other fish species, that range from transient alterations in swimming behaviour to death, the magnitude of the effects being dependent on the dose and duration of the exposure, fish sex and environmental conditions. The nature of the toxin and the mechanisms by which it exerts its effects are yet to be described, but the toxin appears to be readily soluble in water, although its activity decreases in conditions of reduced or no salinity. The toxin also shows direct cytotoxicity over fish red blood cells. Secretions from the axillary gland failed to produce impacts and the main sites of production for this toxin remains unknown.

5.1 Biological and ecological roles of the toxin

When examining the solution containing toadfish mucus on the test subjects, its toxic effects were evident in modifying swimming behavior and by causing yet to be clarified impacts over physiological processes that led to death in many situations. While additional research is needed to identify the exact nature of the toxin(s), potential target and mode of entry, as well as the physiological pathways and processes affected, the *in vivo* experiments clearly demonstrate the lethal potential of the mucus over other fish species. This also begs the question of the biological and ecological roles for this toxin, and how it may have evolved, taking in consideration the species life-history and its phylogeny, radiating aside kin species with poisonous properties or actively using venoms. These experiments also reveal that the toxin’s effectiveness varies with dosage, environmental conditions and depending on the sex of the individual.

In our experiments we used different concentrations of toadfish skin mucus in seawater, that were prepared from an initial emulsion of 1g of skin mucus mixed in 5 mL. The higher dosage of this solution, at 0.4g/L caused 100% mortality in all cases with the exception when mucus used was collected from a pool of stressed female toadfish. A 10x less concentrated dosage was still lethal to over 50% of the target fish tested but, in this case, only when the mucus used was obtained from a pool of non-stressed toadfish. When toadfish holding water was used, we observed a similar effect of concentration – fish exposed to undiluted toadfish holding water were lethally affected, with 100% mortality occurring within 7 minutes after immersion in this media. Total mortality was also observed in a 1:5 dilution, but only after 10-15 minutes, while a 1:50 dilution show no effect over survival, although it still caused abnormal behavior in some target individuals.

The confirmation that the toadfish's mucus contains toxic components seems to highlight a crucial protective and repellent function as it occurs in other species (Reverter et al., 2018). Toxic mucus serves as a defensive barrier, effectively deterring potential predators and preventing harmful organisms from attaching to the fish’s body (Guardiola et al., 2014; He et al., 2023)

However, generally, mucus from non-stressed animals had higher potency than that from stressed ones, both in the experiments with mucus solutions and holding water, which does not support our initial hypothesis that mucus, or at least toxin production, would be trigged or stimulated by threatening condition as a defense response. This supposition was based on the initial observations that other fish sharing confined spaces/volumes with toadfish would be unresponsive or dead within minutes to days depending on the volume. This was replicated by our experiments using mucus solutions or toadfish holding water. The assumption of following the previous premises would be that since the presence or other fish would be a threat or territorial intrusion, or a feeding opportunity, the toadfish would release a toxic substance. Interestingly, in those conditions no obvious predatory actions from toadfish were observed. However, there are also cases, mostly in public aquarium displays, where toadfish shared the tank with other fish species without similar incidents, but these were situation where water volumes were substantial and/or potential targets were large fish.

In the case of the experiments with holding water, where target species were exposed to toadfish holding water, the most evident effects occurred in the groups receiving water from toadfish stressed for 5 minutes, which showed higher frequency and, to some extent, earlier onset, of the abnormal swimming behaviors, and, in some cases, were the only groups showing effects at the lower concentration.

Altogether the data seem to indicate that higher increments in stressful conditions are not linked to increase in toxicity, although they may appear to be associated to increased mucus production. It is therefore possible that the toadfish produce the toxin constantly or were already stimulated in our "low- stress" or "non-stressed" conditions and stored toxin was released at this point. It is also interesting that a longer duration of stress did not seem to maintain a constant release of the toxin, and that the one present in the holding water may appear to have lost potency over this period of time.

It is also interesting to note that from the present experiments, and from preliminary evidence in experimental work from Gonçalves (2022), male mucus appears to be more potent than female mucus. This was emphasized not only by the percentage of target individuals where it evoked lethal effects, but also by the time it took for those lethal effects to occur, which was longer in the case of the fish exposed to female mucus. The same pattern was observed for the fish that showed abnormal behaviors – both the intensity and the onset of those behaviors were lower and later in the case of female mucus. Knowing the ecology of the species and that the toxin is effective only at determined concentrations, we can hypothesize a role in shelter or nest defence. Being the shelter a relatively small and closed environment it is possible that the concentration of the toxic mucus inside of it could be enough to cause some disturbances to possible predators. The nest will be here considered as a shelter occupied only by territorial males where females lay eggs. The higher toxicity of the males' mucus supports the hypothesis that the toxin may be relevant to protect the sitting male, or the eggs, from predators, eventually used as a repellent or cloaking defense mechanism. A similar use of toxins has been described for two species of coral-dwelling gobies, which evolved skin toxins. When a predator captures one of these fishes, most of the time it expels it quickly and alive, giving them a higher chance of surviving a predator attack. Also when there are other non-toxic species, the predator tend to hunt them over the gobies (Gratzer et al., 2015). It can also be hypothesised, given the antimicrobial properties of fish mucus (Sanahuja et al., 2023) and specifically from toadfish (Fernandez Cunha et al., 2023), that the toxins in the mucus can be used to protect the eggs in the nest from being colonised and destroyed by harmful bacteria or other pathogens. As previously mentioned, some species do equip their eggs with toxins (Benkendorff et al., 2001; Kerwin et al., 2019) although there is no evidence of such phenomenon in *H. dydactylus.* Regardless, the evolution a toxin as a deterrent against pathogens and predators could be just linked to the specie's stationary lifestyle (Dos santos et al., 2000; Haddad Junior & Lopes-Ferreira, 2023). This difference between males and females individuals, as already seen in other species (Miller et al., 2016), could also depend on the size of the animal, with the females being smaller than the males, and so maybe producing less toxic compounds, resulting in less efficacy when the toxin in too diluted. The difference in feeding ecology and behaviour is known to influence the venom composition between sexes in some animals (de Oliveira et al., 2005; Miller et al., 2016), and should so be taken into account for further studies on this species.

5.2 Nature and site of production of the toxin

The nature of the toxin is still elusive, but our experiments provide some information that can be used to characterize some of its properties or the requirements for its activity. This appears to be a potent and fast actin toxin, especially at high concentrations in reduced volumes of water. Using both a solution prepared from a mucus/seawater emulsion and toadfish-holding water indicates that there is a hydrophilic component for the toxin, as it dilutes into seawater. This is consistent with the existence of bioactive peptides in mucus as characterized by Fernandez Cunha et al (2023), which are polar substances and were fractioned using saline solutions. However, the activity of these peptides was tested only in bacterial cultures and not in other fish. Moreover, the medium used to convey the mucus to the bacterial cultures appear to have been distilled water. In the present study we determined the amounts of protein used in the mucus solutions previous to addition to the exposure beakers, and in toadfish-holding water. Overall, the relative amounts of protein in each condition seem to correlate well with the amplitude of the effects observed, both in onset and frequency of affected behaviors and mortality. Protein levels were higher in mucus retrieved from males when compared to those from female's samples, despite the same amount/concentration of mucus per sample. Also, in the *in vitro* experiments the haemolytic activity is higher for the males, whose mucus had a higher protein concentration than the females. Thus, it is likely that the toxic activity observed is caused by a substance of a peptide/proteic nature.

In the study of toxins it is well-documented that many toxins are of proteic nature, with a direct correlation often observed between the amount of protein and the toxicity exhibited (Chen et al., 2018; Karlsson, 1979). Proteins such as neurotoxins and hemotoxins, which disrupt cellular function through enzyme activity or receptor binding, are prime examples where higher protein concentrations frequently correspond to increased toxic effects (Osipov & Utkin, 2023). This relationship between protein content and toxicity has been explored in various marine organisms, and understanding the specific composition of these proteins can provide crucial insights into their potency (Grim et al., 2011).

In our experiments the toxin showed much lower activity when used with hypoosmotic media, especially below the fish isotonic conditions. It may be then that specific conditions only present in seawater (such as a threshold concentration of specific ions existing down to limited dilutions) are required to activate or maintain activity of the toxin. This deserves further research, possibly with artificial seawater, as it may be crucial for the biological and ecological role of this substance.

In addition to its apparent higher potency in seawater, we also observed a decline in activity with time, and previous preliminary testes carried out by Gonçalves and collaborators during his study (Gonçalves, 2022) also shown an important loss in activity in frozen holding water when compared with freshly used holding-water.

Considering the experiments carried out with skin mucus vs those where secretions of the axillary gland was used, it becomes apparent that only the skin mucus evokes altered behavior and mortality. Although the discussion could be made about the amounts delivered from each tissue, it is clear that the skin mucus does contain toxic activity, shown in the scraped mucus experiments, and if any toxic substance was produced by the axillary gland, which could be a component of the holding water, it would have been released before extraction with the swab.

The structure of the different regions of the skin and of the axillary glands was analyzed through different staining to better observe all their components.

The epidermis of *H. didactylus* includes mucous cells, and sacciform cells, in abundance, each playing distinct roles (Palazón-Fernández & Sarasquete Reiriz, 2015)

Sacciform cells and mucous cells play critical roles in the integumentary systems of fish, aiding in defense and environmental interaction. Sacciform cells, as identified in species like brown trout and Arctic char, secrete proteinaceous substances thought to be defensive, protecting the fish from parasites and possibly predators. These cells typically contain a large vacuole filled with eosinophilic secretion, released through holocrine mechanisms. Sacciform cells proliferate in response to parasitic infestations, indicating their role in immune defense (Pickering & Fletcher, 1987) . Sacciform cells in fish skin have been associated with the secretion of alarm substances, which act as a defense mechanism. These alarm substances, used for example from the species in the superorder Ostariophysi, are released when the skin is damaged, triggering a fright response in nearby fish. This fright reaction helps alert other fish to potential threats or predators. The alarm substance secreted by sacciform cells plays a key role in this chemical signaling process, helping to reduce predation risk by warning conspecifics of danger (Zaccone et al., 1990)

Meanwhile, mucous cells (also called goblet cells) are widespread across fish species, producing mucus that forms a protective layer over the skin. This mucus not only aids in lubricating the skin but also serves as a barrier against pathogens and environmental irritants (Elliott, 2011).

By analyzing the histology of the skin, we observed that male toadfish possess a higher density of mucus cells in the skin compared to females. This finding raises interesting questions about potential physiological differences between the sexes, particularly since we know that the toxin is present in the mucus and that the female’s is less toxic. However, it is important to note that while the toxin is contained in the mucus, we do not know where it is produced. Therefore, the difference in the number of mucus cells may not directly correlate with the variation in toxicity observed between males and females. In the histological analysis we conducted in this study, we were unable to find a clear difference in the quantities of sacciform cells between males and females, as we found for mucous cells. However, such a difference might exist and could be related to their role in producing toxic substances in the epidermis. A more systematic histological analysis aimed at quantifying sacciform cells would be necessary.

From the histological analysis of sacciform cells and mucus cells using various staining techniques, such as Hematoxylin & Eosin (H&E), Alcian Blue, and Masson's Trichrome, we can deduce the composition of the secretions. For instance, Alcian Blue highlight acidic mucopolysaccharides (Layton & Bancroft, 2019), while Masson’s Trichrome can differentiate between collagen, muscle, and other protein-rich tissues (Foot, 1933), and H&E provides basic insights into the cellular structures (Chan, 2014). This allow us to know that mucous cells are filled with acidic components like sulphated and carboxylated glycoproteins. However, while the staining patterns can suggest the general composition of the secretions, they do not directly indicate whether these secretions are toxic.

No differences in the skin composition and in the axillary glands between the stressed and non-stressed individuals emerged, as well as no differences in the different regions of the skin.

Therefore, while histology is useful for characterizing the types of substances produced by these cells, further analytical methods are required to confirm whether these secretions have toxic properties and so determine whether the production of toxins is influenced by the amount of mucus cells.

In our study of the Lusitanian toadfish (*H. didactylus*), we tried to understand whether the axillary glands have a role in the production of the toxin. We used the experimental design outlined by Maina (1998), who investigated the axillary gland's function in *Opsanus beta,* making some changes. Our adaptation of this experiment involved using saltwater to test the content of the glands, based on our prior findings that the suspected toxin would not be effective in freshwater. This shift in environmental conditions was crucial, as the ionic composition of saltwater might support the proper function of toxins that would otherwise be inactive in freshwater environments. Despite this adjustment, our results still indicate the absence of detectable toxic activity in the axillary gland secretions, raising questions about the gland’s function in the Lusitanian toadfish. It’s important to consider also that the high dilution used in this experiment, due to the small amount of secretion collected from the pore, could have influenced the results of the experiment. Further biochemical analyses of these secretions are necessary to fully understand the role of these glands, if any, in the ecology and behaviour of this specie.

5.3 Impacts and environmental modulation of effect

The observed behavioural effects of the toadfish toxin on the test subjects provide valuable insights into the nature of the toxin. The fishes’ responses, such as breaking the surface, occasionally jumping out of the water, may indicate an impairment in oxygen transport, preventing the efficient transfer of oxygen from the water to the blood. This behavior is typically observed in fish experiencing hypoxic conditions (Zhu et al., 2013); however, in this study, oxygen levels remained above 75% throughout the experiments. Another possible explanation for this behavior is that the fish are attempting to escape the perceived threat. This type of response is common in some species when they are trying to avoid capture by a predator (Soares & Bierman, 2013). The spasmodic behavior observed could be attributed to neurotoxins such as tetrodotoxin (TTX) or other sodium channel blockers, which disrupt normal nerve function and muscle control. Tetrodotoxin, commonly found in pufferfish, is known to interfere with nerve signalling, leading to muscle paralysis and spasms, which can result in erratic behaviors prior to death (Noguchi et al., 1987).

The results of these experiments show that the toxin causes a pronounced haemolytic activity, particularly at high concentrations. This aligns with the findings of Gonçalves (2022), which also observed significant alterations in the haematological parameters in response to the toadfish-derived toxin, which was found to cause haemolysis and plasmolysis in the red blood cells of *S. aurata*. In that study, where plasma indicators were analysed in seabream exposed to toadfish-holding water, the author also reported changes in red blood cell shape and alteration in the cell membrane compatible with osmotic imbalance, and increased plasma osmolality. In addition, the same fish showed increased levels of calcium, magnesium, sodium, potassium, chloride and phosphate, paralleled by reduced activity of the branchial gill Na+/K+-ATP, the pump responsible for the gradients required for proper ion exchange at the gills. Interestingly there was also significantly higher levels of plasma ammonia and reduced blood pH, which are known to evoke neural impairment and altered behavior, (Ruyet et al., 1995) and osmotic and respiratory compromise (Perry et al., 2023) . Ammonia and pH were not measured in blood of target fish nor in the water but, as in the present case, the effects observed by Gonçalves (2022) were more prominent in target fish exposed to holding water from male toadfish.

The observation that the toxins produced by the toadfish (*H. dydactylus)* exhibit no effect in freshwater environments raises questions about the action mechanisms of the toxin and its dependency on environmental conditions.

One possible explanation for this phenomenon could be related to the differences in water intake behaviours between freshwater and saltwater fish. Living in aquatic environments, whether in saltwater or freshwater, requires adaptations to maintain constant body fluid osmolarity. Teleost fishes keep their extracellular bodily fluid osmolarity around 300 mosmol kg^{-1}1 (Kültz, 2015). Freshwater osmolarity ranges from 1 to 10 mol kg^{-1}, while saltwater osmolarity can reach up to 1000 mol kg^{-1}, depending on salinity. Unlike marine fish, freshwater fish do not actively drink water, relying instead on osmosis to balance their internal fluids (Greenwell et al., 2003; Perry, 1997; Takei et al., 2014). If the toadfish toxin requires ingestion and subsequent action within the gastrointestinal tract to have an effect, the lack of water intake in freshwater fish could prevent the toxin from reaching its site of action, and so rendering it ineffective.

Another potential explanation lies in the ionic composition of the water and how fish deal with the ionic gradients. Marine environments are rich in ions such as sodium, chloride, and calcium, which might play a crucial role in the activation or efficacy of the toadfish toxin. As suggested above, in freshwater, where these ions are present in much lower concentrations, the toxin may not be able to function properly, either due to a failure in activating its toxic components or because its potential molecular targets in freshwater fish do not respond in the same way without the necessary ionic co-factors or are not even present. For example, ion transporters/channels that are required for chloride excretion occur abundantly in the epithelia of saltwater fish but are practically absent in freshwater fish (Kovac & Goss, 2024; Kültz, 2015). Similarly, a toxin that acts by blocking channels that must counter a steep gradient may have less impact when that gradient is lower. These hypothesis align with the known dependency of many marine toxins on specific ionic conditions for their activity (Anderson et al., 1988; Park & Miller, 1992), suggesting that the toadfish toxin's mechanism might be closely tied to the salinity and ionic composition of its environment. Our experiment also showed that even if the mucus is initially mixed with saltwater and then put in freshwater, it still have no toxic effect, so it is not needed an “activation” but rather a continuous environmental condition. Salinity is not the first nor the only environmental factor influencing the efficacy of a toxin. Another example are the venoms of the family Trachinidae and of the Scorpionfish; these venom are made of protein that are thermolabile and lose their effect when exposed to high temperatures (Haddad et al., 2003; Paolino et al., 2023).

Experiment on the isolated intestinal tract of the test subject as well as experiments involving freshwater fish species under controlled ionic conditions, namely using modifications of artificial seawater, could help clarify these hypotheses. Also, further experiments involving production of “toadfish water” with toadfish acclimated at different salinities would be important to better understanding the toxin and its ecological role.

Chapter 6 CONCLUSIONS AND PERSPECTIVES

The present study successfully demonstrated some toxicological properties of the mucus secreted by *Halobatrachus didactylus*, confirming its detrimental effects on other fish species, specifically *S. aurata* and *Gambusia holbrooki*. The results revealed a strong correlation between mucus toxicity and environmental factors, including salinity, as well as other factors such as sex and stress levels of the toadfish. Notably, the toxicity of the mucus was greater in males, particularly under non-stressed conditions, aligning with previous observations.

Moreover, the studies revealed that the effectiveness of the toxin is dose-dependent, showing significant toxic effects only at higher concentrations. At lower doses, the toxin had a limited or null impact. This evidence suggests that individuals of *H. didactylus* may use the toxin to defend their nests, and the eggs inside them, from predators or pathogens, being the nest a small environment in which the toxin can be accumulated. This theory might also account for the increased toxicity of the males' mucus, given that, ecologically, they are responsible for guarding the nest and the eggs.

The findings also suggest that salinity plays a crucial role in modulating the effects of the toxin. When tested at various salinity levels, the toxin demonstrated differential effectiveness. The study showed that lower salinities (e.g., 0 ppm, 6 ppm, 3 ppm) resulted in reduced or null toxicity to some fish species, while at higher salinities (e.g., 25 ppm and 36 ppm, 13 ppm), the toxic effects were more pronounced. This suggest that salinity might influence the stability and potency of the toxin. Additionally, this introduce the possibility that different species may react differently to the toxin depending on their natural habitat and the salinity levels they are accustomed to.

Histological analysis revealed differences in the number of mucous cells between males and females, with males exhibiting a greater number of these cells. Despite this, the higher number of mucous cells does not necessarily correlate with the observed increase in toxicity.

The function of the axillary glands remains unclear, as no conclusive evidence of toxic substance production from these glands was observed. While this challenges initial hypotheses, further investigations focusing on specific conditions or stress responses of the axillary glands may still yield important discoveries.

The results of this research can be used as a base layer for future studies. One critical step is to further investigate the production site and the molecular composition of the toxin to identify its active components. Advanced biochemical analysis, such as proteomics, could uncover the exact molecules responsible for the observed effects, enhancing our understanding of the biological function and potential applications of the toxin. Additionally, understanding the pathways through which environmental conditions, such as salinity, modulate the toxicity will be needed. This could involve testing the toxin in various controlled environmental conditions, as well as experimenting to obtain a better understanding of the mode of entry of the toxin(s).

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Annexes