João Pedro dos Santos Gonçalves

Interspecific physiological and behavioral effects of potential biotoxins from *Halobatrachus didactylus*

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2022

João Gonçalves

Interspecific physiological and behavioral effects of potential biotoxins from *Halobatrachus didactylus*

2022

João Pedro dos Santos Gonçalves

Interspecific physiological and behavioral effects of potential biotoxins from *Halobatrachus didactylus*

Masters in Marine Biology

Supervisors:

Pedro M. Guerreiro

Centre of Marine Sciences (CCMAR-Algarve)

Teresa Modesto

Universidade do Algarve (UALg)

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2022

Interspecific physiological and behavioural effects of potential biotoxins from *Halobatrachus didactylus*

Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída,

Acknowledgements

First, I would like to thank both of my supervisors, prof. Pedro Guerreiro and prof. Teresa Modesto, for giving me the opportunity to develop this work. Furthermore, I thank them both not only for the continuous support and guidance, but also for their availability and time dedicated to helping me to learn more and being able to complete this work.

A very special thanks to Alexandra Alves, who spent countless hours helping me through this chapter of the masters, which without her help was sure to be impossible. I would also like to thank her for all her support and teachings, which were crucial for the understanding and development of both the practical and theoretical parts of the thesis.

I would also like to extend my gratitude to Dr. Peter Hubbard, since without his help the electrophysiology chapters of this work could not be possible.

I would like to extend my gratitude to Dra. Zélia Velez, for all her help and availability, with the performance of the HPLC procedure.

I would also like to thank my family for their support, especially my parents, who have helped me accomplish my goals for the last 22 years and will surely continuo to do so. Their continuous support and help were a great motivation for the completion of this master degree.

Resumo

A capacidade para produzir toxinas encontra-se num grande número de espécies, que apresentam diferentes mecanismos de produção e de libertação destas substâncias. As toxinas produzidas por estes organismos desempenham principalmente duas funções: proteção contra os predadores e captura de presas. Os peixes com esta capacidade podem ser divididos em três classes conforme os mecanismos de produção e de libertação da toxina: peixes em que a toxina se encontra nos tecidos corporais, peixes em que a toxina é libertada através de espinhas e peixes cuja secreção cutânea contém a toxina. A família Batrachoididae é muito estudada devido ao facto de incluir várias espécies tóxicas. Esta família é composta por quatro subfamílias: Thalassophryninae, Porichthyinae, Batrachoidinae, e Halophryninae. Destas, aquela que tem merecido mais atenção é a subfamília Thalassophryninae, devido aos seus membros terem a capacidade de produzir toxinas que causam efeitos severos. No entanto, em espécies que pertencem tanto à subfamília Porichthyinae como à Batrachoidinae também foi observada a produção de toxinas, apesar dos mecanismos de produção serem diferentes. Estudos recentes indicam que a espécie *Halobatrachus didactylus*, que pertence à subfamília Halophryninae, também poderá ser capaz de produzir substâncias tóxicas e que a água que contém estas substâncias tem um efeito severo em várias espécies de peixes, como a corvina *Argyrosomus regius*, levando à sua morte em poucos minutos. *H. didactylus*, comummente chamada de xarroco lusitano, é uma espécie bentónica que pode ser encontrada enterrada em sedimentos ou escondida em fendas de rochas. O limite norte da sua distribuição ocorre no sudoeste da Península Ibérica, e o sul no Golfo da Guiné. O xarroco lusitano tem sido usado como modelo experimental em variadas áreas de investigação (ciclo reprodutivo, produção de sons, estudos hematológicos, estudos ecológicos, etc). O objetivo desta tese consiste em estudar os efeitos que esta toxina, ainda não identificada e caracterizada, poderá provocar em indivíduos da espécie *Sparus aurata*. Foi realizado um conjunto de experiências *in vivo* que consistiram na exposição crónica e aguda de indivíduos da espécie *S. aurata* a diferentes diluições da água de contenção de xarrocos (designada como "água de xarroco"). As experiências *in vitro* consistem em estudar a atividade hemolítica da toxina, através da exposição de glóbulos vermelhos de *S. aurata* a muco colhido de indivíduos da espécie *H. didactylus*. Adicionalmente, e assumindo a hipótese de que os compostos libertados para a água pelos xarrocos possam exercer algum tipo de ação neurotóxica nas espécies alvo, foi testado o

efeito da água de xarroco na sensibilidade do nervo olfativo (Olfactory Nerve Recording – ONR). A obtenção da "água de xarroco" foi realizada colocando vários indivíduos num contentor num rácio de 0,5kg de biomassa por cada litro de água. Após a adição da água, os peixes foram manipulados a cada 5 minutos durante 30 minutos de modo a induzir stress. Duas exposições crónicas foram realizadas, correspondendo à utilização de duas diluições da "água de xarroco": 1:1000 e 1:100. Estas exposições tiveram durações de dezassete e cinco dias respetivamente. Durante a realização deste trabalho, surgiram evidências que indicam a existência de diferenças nos efeitos da água de xarrocos machos e de xarrocos fêmeas nos indivíduos da espécie *S. aurata*. Assim, as exposições agudas foram realizadas com "água de xarroco" fêmeas e machos em separado, numa concentração de 1:1. Estas exposições tiveram uma duração máxima de uma hora. Após a realização de ambas as exposições agudas, uma terceira foi realizada, com uma diluição de 1:30 de "água de xarroco" de machos e teve uma duração de vinte e quatro horas. Depois do término das exposições, os indivíduos utilizados como alvos, foram sacrificados e amostras de sangue e das brânquias foram coletadas. Os parâmetros hematológicos medidos foram: hematócrito, concentração de hemoglobina, número de glóbulos vermelhos e os índices associados (volume celular médio, hemoglobina celular média, concentração de hemoglobina celular média), a presença de oxi, deoxi, e metahemoglobina, hemólise, plasmólise e morfologia dos glóbulos vermelhos, pH do sangue, e osmolalidade. Os indicadores plasmáticos medidos foram: glucose, lactato, fósforo, magnésio, cloro, cálcio, sódio, potássio, LDH, e amónia. Adicionalmente, nas exposições agudas, a atividade total das ATPases e a atividade da Na K-ATPase também foram observadas. A análise da atividade hemolítica foi feita utilizando diferentes concentrações de extrato aquoso de muco de *H. didactylus*, que consistia numa mistura de muco com igual quantidade de solução salina. O extrato foi então incubado juntamente com eritrócitos de *S. aurata,* e após a incubação, a absorbância foi medida de modo a indicar a quantidade de glóbulos vermelhos que tinham sido destruídos, o que quando comparado com um controlo positivo, permite calcular a atividade hemolítica. Foram também realizados registos em vídeo do comportamento em situação de exposição aguda. Os parâmetros registados foram perda de equilíbrio, a ocorrência de espasmos e imobilização total dos indivíduos. Os registos eletrofisiológicos de nervo olfativo, foram realizados expondo o epitélio olfativo de indivíduos da espécie alvo a uma série de estímulos (cisteína, serina, leucina, glutamato, arginina, e bílis de *S. aurata*). Após a exposição aos estímulos, o epitélio olfativo é então exposto a concentrações cada vez

maiores de “água de xarroco” e de seguida avaliada novamente a resposta aos estímulos. Esta abordagem permite avaliar as possíveis alterações na sensibilidade olfativa devido à exposição a “água de xarroco”. Os resultados demonstram que a toxina produzida por *H. didactylus*, não aparenta ter efeitos nos indicadores fisiológicos medidos, quando utilizada em baixas concentrações (utilizadas nas exposições crónicas e na exposição de 1:30). Porém, a exposição a “água de xarroco” sem diluição (exposição aguda), especificamente se for produzida pelos machos, provoca a morte dos animais alvo em alguns minutos. Estas exposições agudas, provocam alterações significativas nas espécies alvo, não só nos parâmetros hematológicos (hematócrito, pH do sangue, osmolalidade e morfologia dos glóbulos vermelhos) como nos indicadores plasmáticos (glucose, lactato, fósforo, magnésio, cloro, cálcio, sódio, potássio e amónia). Em relação á atividade hemolítica, tanto a “água de xarroco” fêmea como a de macho, apresentam capacidade de hemolisar 100% do glóbulos vermelhos presentes na solução, mesmo quando presente em concentrações baixas. Os registos no nervo olfativo apontam para um efeito a nível do sistema neuronal tendo a “água de xarroco” a capacidade de inibir a função olfativa.

Os resultados apresentados neste trabalho, mostram claramente que a espécie *H. didactylus* é capaz de produzir substâncias tóxicas que são libertadas para a água, possivelmente através do muco, e que induzem alterações fisiológicas e neurotoxicidade, levando à morte dos indivíduos da espécie *S. aurata,* apesar de que o efeito se fazer sentir apenas em concentrações elevadas. Dado o facto de que, os efeitos mais severos serem encontrados na “água de xarroco” machos, e de nesta espécie serem os machos que protegem os ninhos, é possível que a função primária desta toxina esteja associada aos cuidados parentais, no sentido de ser um mecanismo utilizado para repelir organismos que se aproximem dos ninhos e das posturas. Este trabalho consistiu numa primeira abordagem aos possíveis efeitos da toxina produzida pelo xarroco lusitano, que poderá servir como base para futuros estudos, com o foco de identificar e caracterizar quimicamente esta toxina, identificar os mecanismos que estão por detrás da sua produção e libertação, e compreender a sua função biológica e/ou ecológica.

Palavras-Chave: *Halobatrachus didactylus*, , Batrachoididae, toxina, *Sparus aurata*

Abstract

Several fish species have shown the ability to produce biotoxins, that are used as a means of protection against predators or to subdue prey. The Batrachoididae family of fish, commonly referred to as toadfishes, presents several species that have been described to produce toxic compounds. This family can be divided into four subfamilies, Thalassophryninae, Porichthyinae, Batrachoidinae, and Halophryninae. The subfamily Thalassophryninae is the most studied, due to the species belonging to this subfamily, being able to produce strong venoms. Batrachoididae members belonging to the Porichthyinae and Batrachoidinae, have also been described to be able to produce toxic compounds. Preliminary experiments have shown that the species *Halobatrachus didactylus*, belonging to the Halophryninae subfamily, is also able to produce toxic substances, and the water containing these substances has a strong impact on various fish species. The objective of this thesis is to evaluate the toxic effects of this uncharacterized biotoxin, in the fish species *Sparus aurata*. To evaluate the toxic effects, several *in vivo* and *in vitro* experiments were performed. The *in vivo* experiments consist of performing chronic and acute exposures of the target fish, using different concentrations of toxin, with the intent of analysing the effects on several haematological parameters, plasmatic indicators, and behaviour. *In vitro* experiments consisted, of studying the destructive effect of the toxin on the erythrocytes and analysing the effects on the function of the olfactory nerve of the target fish. The obtained results indicate that the *H. didactylus* toxin causes an effect on the previously mentioned parameters, when present in high concentrations, especially if it is produced by male specimens. This indicates that the toxin may be used to repel organisms during nest defence. Follow-up studies should focus on the characterization, and production of the toxin,

Keywords: *Halobatrachus didactylus*, Batrachoididae, Toxins, *Sparus aurata*

Index

List of Figures

List of tables

1. Introduction

In the animal kingdom, several organisms have developed the ability to produce toxins that increase their chances of survival (Gwaltney-Brant et al., 2018). The production of toxic compounds can occur constantly, under stress, or in the presence of different physical or chemical stimuli. These organisms can be separated into two main categories: poisonous and venomous. These categories are differentiated by the way that the toxins are released. Poisonous organisms produce and accumulate the toxins in the tissues, resulting in the exposure of other organisms that have enter in physical contact, with the toxin. Venomous organisms produce toxins that are delivered to the target by wounding it with either fangs, stinger or spines (Gwaltney-Brant, 2017).

The contact with these toxins can lead to temporary illness or death. These toxins can be used as a means of defence against predators or can be used as a tool to catch prey. An example of toxins used for defensive purposes, is the poison frogs, native to Central and South America, that belong to the family Dendrobatidae. These aposematic frogs (which present bright warning coloration to inform potential predators of their poisonous nature) accumulate lipophilic alkaloids, more specifically Batrachotoxins, on their skin, that have originated from dietary sources (Caldwell, 1996; Summers and Clough, 2001). When a predator comes into contact with this toxin, it causes the voltage dependant sodium channel of the nerves and muscles to stay open, which leads to an influx of sodium that depolarizes the membrane, blocking their function (Daly et al., 1999; Maan and Cummings, 2012). On the other hand, an example of toxins used to catch prey, are the venomous snakes. Several species belonging to the suborder Serpentes, are able to produce toxins that can be used to kill prey, and to defend themselves (Timmerman, 1995). These toxins also help with the digestion of the prey. When a venomous snake encounters a prey, bites it, and injects venom into the prey through hollow or grooved fangs (Reinert, et al., 1984; Sherbrooke, 2008). The venom of several snake species contains neurotoxins and basic polypeptides, that can cause pathophysiological effects including paralysis and respiratory failure (Lee, 1972).

It is not only on land that it is possible to find poisonous or venomous organisms. There are many aquatic organisms with the ability to produce or accumulate toxins. These organisms can be both vertebrates and invertebrates, but even several species of phytoplankton. As on land, these toxins can be used for defence or hunting prey. For example, some cephalopod species of the genus *Hapalochlaena* commonly known as the

blue ringed octopus, that has a wide distribution over the Pacific Ocean and Indian Ocean. These are venomous species that possess tetrodotoxin that is accumulated from tetrodotoxin producing bacteria (Hwang et al., 1989). The blue ringed octopus uses this toxin to kill its prey, since it affects neuromuscular junctions and nerve conduction, causing paralysis (Lane and Sutherland, 1967). One example of toxic marine vertebrates are several species belonging to the family Tetraodontidae commonly referred to as pufferfish. Besides its ability to expand its size when threatened, the pufferfish also possess tetrodotoxin, that it is passed into its predators, after the pufferfish has been eaten. In the case of the pufferfish, the tetrodotoxin is obtained through a dietary source, and is accumulated mainly in the digestive tract (Noguchi et al., 2006a). After being exposed to the toxin, the predators, will most likely die, since it can cause paralysis (Oliveira et al., 2003). Other examples of well-known organisms due to their potent toxin are the stonefishes, and the cone snails. Despite one being a vertebrate and the other an invertebrate, both are venomous. Stonefish belong to the family Synanceja, and it delivers its toxin, through the dorsal spines. This toxin can cause several severe effects, such as extreme pain, swelling, and tissue death, sometimes even culminating in death (Khoo, 2002). The cone snail belonging to the Conidae family, uses a specialized tool in the shape of a hollow harpoon, present in the radula, to deliver its toxin to the prey. When a prey is injected with the toxin, it will enter the circulatory system, where it can interact with nerves, ultimately causing paralysis (Dutertre et al., 2013).

Besides using toxins to hunt prey, or defending themselves, organisms can use these toxins to protect their eggs. Since in the marine environment there are many potentially pathogenic microorganisms, some species of invertebrates and vertebrates are able to use toxins to protect their eggs from microbial infection. This is achieved, because some of the chemical compounds in the toxins have antibiotic properties, which ensures the survival of the eggs (Benkendorff et al., 2001). Several species, including the previously mentioned blue ringed octopus and pufferfish, alongside with some frog and newt species, present this behaviour (Hwang et al., 1989).

Toxic or venomous fish species

Toxic fishes can be divided into three general classes, based on their mechanisms for venom production and release. One group is fishes with toxic material found within the body tissues and is composed mainly of basic low molecular weight nitrogenous

compounds, like tetrodotoxin and ciguatoxin. Another group is fishes where the toxic material is delivered by a stinging apparatus and is composed mainly of labile toxic proteins. The last group is composed by fish whose skin secretion contain toxic components called Ichthyocrinotoxins (Primor and Zlotkin, 1975). These components have anti-parasitic effects and protect the fish from being eaten by larger fish. Since the piscine venoms have evolved from Ichthyocrinotoxins, these components are also physiologically relevant (Ziegman and Alewood, 2015). These toxins are surfactants, and present the ability to disrupt biological membranes, hemolysis and icthyotoxicity to small fish. Ichthyocrinotoxins can be grouped into four categories: polypeptide surfactants, followed by steroid glycosides, cationic surfactants, and nonionic surfactants. Ichthyocrinotoxins function as a repellent, and therefore these components must be rapidly dispersed, water-soluble substances that act on the mouth or gills and have a noxious taste or tissue destructive action, that affects the predator before it has time to bite or swallow the fish. Therefore, neurotoxins are not practical since they do not act fast enough on the predator (Abdul-Haqq and Shier, 1991). The evolutionary ecology and adaptive consequences of developing venoms in fish was recently reviewed by Harris and Jenner (2019), including possible advantages and energetic trade-offs. They indicate that venom systems are thought to have convergently evolved 19 times with more than 2900 species utilising venom as a form of defence or for predation/competition and produced a phylogenetic tree of fish families highlighting all know venomous families (figure 1.1). The authors also thoroughly described the many structures and their locations, and forms used to deliver venom, which include spines, barbs, and teeth/fangs, among other.

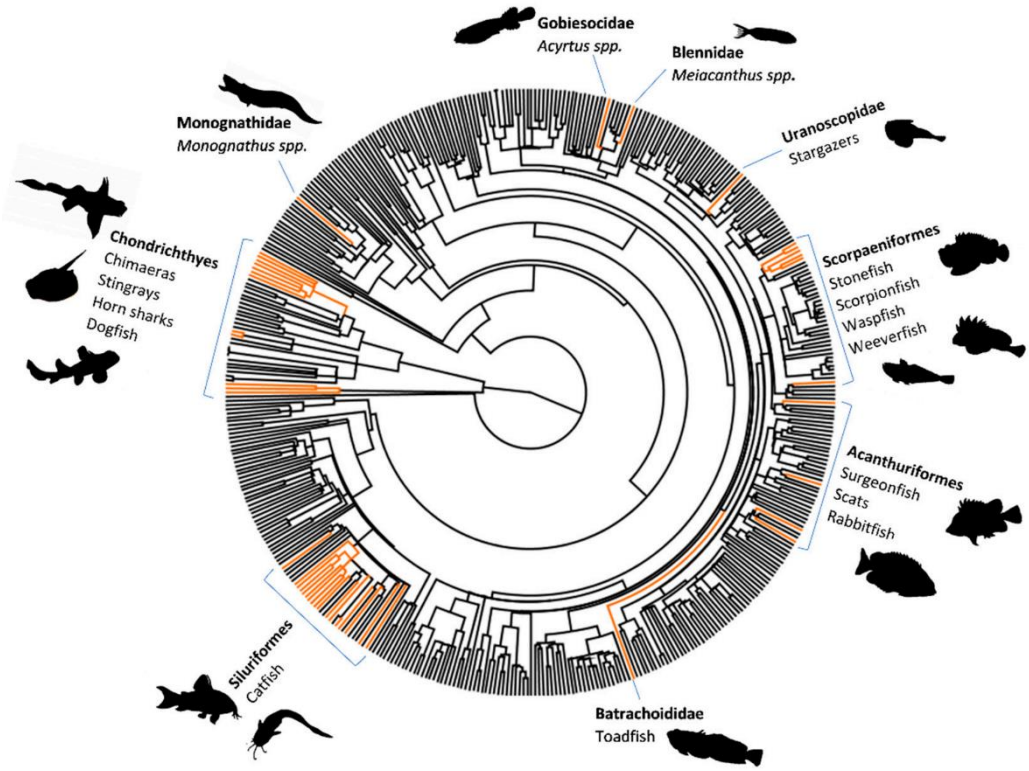

Figure 1.1 – Phylogenetic tree of fish families, with all the known venomous families highlighted in orange, adapted from Harris and Jenner, 2019.

One family of fish that possess several toxic species is the Batrachoididae. This family is composed by a total of 84 species (CAS - Eschmeyer's Catalog of Fishes, 2021) distributed across the Atlantic, Indian and Pacific Oceans, inhabiting both shallow inshore areas and deep waters. These organisms are bottom dwelling, ambush predators that feed on invertebrates, mainly molluscs and crustaceans, and small fish. This family of fish presents a broad and flattened head with barbels and fleshy flaps, and a wide mouth. Two dorsal fins, the first consists of sharp, strong spikes, and the second consists of soft rays. The pelvic fins are inserted before the pectoral fins and consist of one strong spike and soft rays. Usually presents a brown coloration with black spots. Their body is either naked or covered with small cycloid scales (Fishbase, 2021). The species of this family demonstrate a sedentary behaviour, and benthic spawning (Félix et al., 2016; Sousa et al., 2018). These organisms are commonly referred to as toadfish, not only due to their appearance, but also due to their ability to produce sound using their swim bladder. The sound is produced, by contracting the sonic muscles in the bladder, and the frequency of the sound matches the rate of contraction. The produced sounds are used for mating, the males produce sound to attract females to their nests, and are also used in an aggressive manor, which are emitted during nest defence (Fine et al., 2001; Mann, 2006; Rice and

Bass, 2009). Phylogenetic studies divided this family into two major clades, corresponding to the New World and Old World species. This family can then be divided into 4 subfamilies: Porichthyinae, Thalassophryninae, Batrachoidinae, and Halophryninae. Species of the Porichthyinae are present in the western Atlantic and eastern Pacific and are characterized by the presence of 2 dorsal fin spines, and photophores or canine teeth. Thalassophryninae presents a similar distribution as Porichthyinae, being present in the western Atlantic and eastern Pacific. This subfamily is also characterized by the existence of 2 dorsal fin spines and a venomous apparatus, comprised of hollow spines connected to venom glands. The subfamily Batrachoidinae corresponds to the species present in the New World, that possess 3 dorsal fin spines, and do not possess neither photophores, canine teeth or a venom apparatus. Halophryninae corresponds to the Old World species, present in the eastern Atlantic, Indic ocean, and Pacific ocean. Like the Batrachoidinae, this subfamily is characterized by 3 dorsal fin spines, and a lack of photophores, canine teeth and a venomous apparatus (Greenfield et al., 2008).

The subfamily Thalassophryninae contains only venomous species, being comprised of 11 species of toadfish (CAS - Eschmeyer's Catalog of Fishes, 2021) distributed across the continental shelves of the eastern Pacific and western Atlantic. This subfamily is well known, and studied since, individuals belonging to this subfamily are responsible for a large number of severe accidents involving humans. The organisms of these subfamily differ from other species of the Batrachoididae family because they possess hollow dorsal and opercular spines that are connected to venom glands, that are located at the base of the spines (Collette, 1966; Greenfield et al., 2008). These organisms do not possess the musculature associated with their venomous apparatus, so the venom is released involuntarily. The release occurs when there is a mechanical pressure applied to the apparatus, which forces the venom to be expelled through the hollow spines. Since the venom apparatus of these fish is relatively primitive, consisting of involuntary discharges of the venom, they serve for defence purposes (Ziegman and Alewood, 2015). Overall, the effect of these toxins, can ultimately cause necrosis (Facó et al., 2003; Sosa-Rosales et al., 2005).

Although, Thalassophryninae is the more famous of the Batrachoididae subfamilies, due to their toxicity, there are other toadfish species belonging to the other subfamilies that can also present the ability to produce toxic compounds. An example is the *Opsanus tau*

that belongs to the subfamily Batrachoidinae. This species can produce and releases into the water a mucous that can affect the central nervous system of some fish species (Nair et al., 1982). Other example, is the species *Porichthys porosissimus*, belonging to the subfamily Porichthyinae. Despite belonging to a different subfamily, this species, has some similarities with the species of Thalassophryninae. Both use their spines to release the toxin, however, while Thalassophryninae has hollow spines connected to venom glands, *Porichthys porosissimus* has grooved spines with glandular tissue inside. The toxin present in this species, can cause intense pain, and inflammatory response on the victim (Lopes-Ferreira et al., 2014).

Evaluation of venom composition and potency in fish

Compared to research done on terrestrial animal venoms, little is known of the composition and possible effects of aquatic animal venoms. This is mainly due to the fact that fish venom is difficult to obtain, and work with since they are highly labile and sensitive to heat, pH, freezing and thawing, and lyophilisation. Also, venom samples often contain fish mucus, which causes problems when trying to separate and identify the venom components. Nevertheless, there has been some research done on the toxin of several fish species belonging to different families. (Baumann et al., 2014; Carrijo et al., 2005). When it comes to the collection of the venom, the techniques applied depend on the mechanisms for venom production and release, of the species being study. If the venom is present on the body tissues, then several organs like the liver, muscle, skin, and gonads are removed. The tissues are then extracted using an acid compound and centrifuged. The supernatant contains the venom (Landsberg et al., 2006; Noguchi et al., 2006b). In the case that the fish possess a stinging apparatus, the venom can be obtained by removing and homogenizing the spines (Kiriake et al., 2013; Kiriake et al., 2017), by removing and homogenizing the tissue that contains the venom (Chhatwal and Dreyer, 1992), or by collecting the venom directly from the tip of the spines (Sosa-Rosales et al., 2005; Lopes-Ferreira et al., 1998). In the first two cases, the extraction of the material is followed by the centrifugation of the extract, with the supernatant containing the venom. When it comes to obtaining the Ichthyocrinotoxins, it is possible to gather venom samples by scraping the skin of the fish, diluting it with saline, homogenizing, and centrifuging the sample to collect the supernatant (Manivasagan et al., 2009; Ramos et al., 2012). Or it possible to place the fish in a container with water and agitate it so the mucus is released into the water (Gratzer et al., 2015). After removing the water and filtering the extract it

is possible to obtain a sample of the venom (Nair et al., 1982). In some cases it is even possible to obtain the venom by applying a gentle pressure over the glandular area which releases the toxic secretion allowing it to be collected by a pipette (Primor and Zlotkin, 1975). During the analysis of the venom samples, the contents are separated and analysed using electrophoresis (usually SDS-PAGE), different types of chromatography (reverse-phase, gel filtration), and mass spectrometry, sometimes associated to the chromatography in a Liquid Chromatography-Mass Spectrometry (LC-MS) (Baumann et al., 2014; Kiriake et al 2013; Noguchi et al., 2006b; Ramos et al., 2012). To evaluate the effects of these venoms, it is common to calculate LD50, and to study the edema-forming, nociceptive, necrotizing activity, leukocyte influx and haemorrhagic and vascular permeability by injecting laboratory mice with diluted samples of the venom, and when necessary, sacrificing the mice to do histological studies (Baumann et al., 2014; Sosa-Rosales et al., 2005; Lopes-Ferreira et al., 2014; Kiriake et al., 2013). The application of the venom also allows to observe alterations on the microcirculatory network using intravital microscopy (Conceição et al., 2009; Ramos et al., 2012). It is also possible to study the haemolytic activity, usually by using animal erythrocytes mixed with diluted samples of the venom. After incubation the absorbance of the solution is measured to observe the effect of the poison (Manivasagan et al., 2009; Primor and Zlotkin, 1975). The possible antimicrobial effect of the venom can be studied using fish pathogens or other microorganisms, like *Escherichia coli.* These studies are done by adding a diluted sample of the venom to the culture with later incubation. Finally, the effect that the venom had on the culture can be observed by measuring the absorbance of the solution (Maina et al., 1998; Ramos et al., 2012). Other studies have focused on the behavioural effect that the venom can have. These studies can focus on the effect that the venom might have on the respiration on other fish (Maina et al., 1998; Nair et al., 1982), or they can focus on the behaviour that predators might demonstrate in the presence of the venom (Abdul-Haqq and Shier, 1991; Gratzer et al., 2015).

Model species and previous rationale of this work

The work described in this report, focuses on the study of the Lusitanian toad-fish, *Halobatrachus didactylus*. This is a species belonging to the family Batrachoididae, and subfamily Halophryninae (CAS - Eschmeyer's Catalog of Fishes, 2021). This toadfish inhabits coastal areas and brackish water environments. It is a benthic species, that it is often found buried in soft sediments or concealed in rock crevices. It is present from the

south-western Iberian Peninsula to the Gulf of Guinea, however, there have been observed digressive specimens, above the northern limit (Costa et al., 2003). Several aspects of the biology, physiology and ecology of this species are well studied, since these organisms are used as laboratory animals, for different types of experimental studies. (Amorim and Vasconcelos, 2008; Félix et al., 2016; Modesto and Canário, 2003; Modesto et al.; 2015; Palazón-Fernández et al., 2001).

Figure 1.2 – Specimens of *Halobatrachus didactylus.*

Recently discovered evidence shows that *H. didactylus* may also be able to produce some toxic components. This evidence emerged, when several fish species (*Sparus aurata*, *Dicentrachus labrax*, *Argyrosomus regius*, and *Gobius paganellus*) that were present in the same water circulation system as the toadfish, died within a short period of time. Not only that, but fish that were placed in the same water container in which the toadfish were previously present ended up dying in a short period of time also. Based on this information an experiment was done with *Argyrosomus regius,* in which these individuals were exposed to different dilutions of water of the container in which a group of toadfish were subjected to crowding stress (here on referred to as "toadfish water"). In this experiment, the behaviour of the exposed individuals was observed, and some plasmatic indicators were measured. The effect that the "toadfish water" may cause on the olfactory nerve was observed with an olfactory nerve recording (Guerreiro, Modesto and co-authors, unpublished). The results from this experiment indicate that *H. didactylus* is

capable of releasing a toxin into the water, since, during the exposure, the *Argyrosomus regius* individuals died within minutes, and there was an inhibition of the olfactory nerve in the presence of "toadfish water". Therefore, it would be interesting to follow up on these results with a fish species that during a stage of their life cycle inhabit the same area has *H. didactylus*, to not only confirm the previously obtained results, but to also perform a more complete analysis of all the possible effects that the toxin can have.

2. Objectives

Since the nature, source and mechanisms of release of this toxin to the water (gills, urine, glands,…) is yet unknown, the studies of this thesis will use the toadfish containment water to evaluate its effect on other species. Later, future projects, should address the route through which the toxin is released, as well as attempt the chemical identification of the substance(s) involved.

Thus, the main objective of this work was to evaluate the physiological effects of the toxic substance released by the toadfish on the target species, the gilthead sea bream, *Sparus aurata* to develop a suite of bioassays.

To achieve this goal the next steps were be taken:

1. Obtain "toadfish water" samples using pools of several individuals;
2. Perform acute and chronic exposures to "toadfish water";
3. Characterize the behaviour of the target species when exposed to "toadfish water", using time to Loss of Equilibrium (LOE) as an indicator of toxin action;
4. Determine changes in various physiological parameters (haematological parameters, plasmatic indicators, neurological effects) of the target species during acute and chronic exposures.

3. Materials and methods

The preliminary results indicate that the exposure to the putative toxin of *H. didactylus* causes several physiological effects (including alterations of plasma parameters and the inhibition of the olfactory nerve), and ultimately leading to the death of the exposed organisms. Therefore, the aim of the work described in this report, is to better identify these effects by performing several *in vivo* and *in vitro* experiments.

1. *Animals*

For the purpose of this experiments two species of fish were used: the Lusitanian toad-fish, *H. didactylus* (used for the production of "toadfish water"), and the Gilthead sea bream, *S. aurata* (used to identify the effects of the toxin). The toadfish were captured by local fisherman and were then transferred to Ramalhete research station where they were placed in seven 250L tanks that were connected to an open system and kept at natural temperature and photoperiod. These fish were fed squid three times per week and have an average weight of 238g. The sea bream were provided by the Portuguese Institute of Sea and Atmosphere (IPMA) and they were maintained in three 500L tanks connected to a recirculating closed system. They were fed fish feed daily and had an average weight of 117g and a length of 18cm. Temperature in the holding systems, both for the toadfish and sea bream ranged between 20-25ºC throughout the duration of the experimental work. Ammonia concentrations were in water were measured at least twice a week and were always blow 0.6 mg/L.

2. *Production of the "toadfish water"*

To perform both the chronic and acute exposures, it is necessary to first obtain the water in which the toxin is found. The first batch of "toadfish water" was produced using 5 individuals placed in 2 L which in total corresponded to 0,5kg/L. These fish were then stressed by manipulation 5 min intervals-for 30 minutes. The water was then collected and transferred to glass bottles. The next batches of water corresponded to larger volumes of water, 7L and 40L (20L of male "toadfish water" and 20L of female "toadfish water"). All of these batches of water were obtained with the same fish mass to water volume ratio (0,5kg/L), to try to maintain the same toxin concentration across all batches. These larger amounts of water were produced in separate buckets, each one with a number of fish to match the 0,5kg/L. These individuals were stressed for 30 minutes, the water produced in all of the buckets was then mixed together and transferred to glass bottles.

3. *Experimental design*

To evaluate the effect that the toxin may have on the short and long term, two types of exposures were performed, the chronic and acute exposures. The chronic exposures were done to observe the effects on fish physiology that take longer to be expressed and can be the result of cumulative exposure, such as changes in some blood parameters and

plasmatic indicators, while the acute exposures were done to test larger dosage and observe more drastic effects such as immediate behaviour. Both acute and chronic exposures were carried out at natural temperature, similar to that of the holding tanks.

Chronic exposures

To perform the chronic exposures, 10 sea bream individuals were equally divided between two 100L tanks each connected to a mechanical/biological filter, constituting separate closed system, one serving as control and the other as experimental treatment. Two rounds of treatments were utilized corresponding to the addition of "toadfish water" to a final concentration of: 1:1000, and 1:100, in which 100ml, and 1L were added daily to the system, respectively. Each of the exposures had a different duration, the first being 17 days, the next being 5 days. After the exposure, sea bream individuals were anaesthetised with 2-phenoxyethanol, and blood samples were collected by puncturing the blood vessels of the caudal region with heparinised syringes and transferred to Eppendorf tubes containing heparin, to prevent the formation of blood clots. After the blood samples were collected the fish were measured and weighted and sacrificed by spinal transection. Gill samples were collected by cutting the second branchial arch and placed in Eppendorf tubes containing SEI (sucrose-EDTA-imidazole) buffer, to later measure Na/K-ATPase activity. The blood samples were later used in the laboratory to study several haematological parameters (haematocrit, haemoglobin concentration, number of red blood cells and associated indexes, presence of oxy, deoxy, and methaemoglobin), and plasmatic indicators (glucose, lactate, phosphorous, magnesium, chloride, calcium, sodium, potassium, LDH, and ammonia).

Acute exposures

The acute exposures were performed once with pure "toadfish water" produced by either males and females separately, since evidence of possible differentiated effect related to fish sex emerged during the activities. To accommodate for the available amount of water, two 10L tanks were filled with "toadfish water" (either male or female) and then 2 sea bream individuals were placed in each. A similar approach was used for the control fish, for which the 10L tanks were filled with unspoiled seawater. The exposures lasted for a maximum period of 1h, unless there was an observed effect on activity or vital conditions. The fish were removed before the 1h mark if the organisms in the tank were immobile

with no opercular movement. After these organisms were removed, 2 more sea bream individuals were placed in the tank. This means that a total of 8 individuals were used for each of the treatments (male and female "toadfish water") and in the control (unspoiled seawater).

During the exposures, recordings of the behaviour and direct observations were performed. Once again, blood samples were collected, and their pH was measured. The fish were then measured, weighted and then sacrificed. Afterwards, samples of the gills were collected, to measure Na/K-ATPases. The blood samples were then later used to analyse haematological parameters (haematocrit, blood pH, osmolality, haemolysis, morphology of red blood cells, presence of oxy, deoxy, and methaemoglobin) and plasmatic indicators (glucose, lactate, phosphorous, magnesium, chloride, calcium, sodium, potassium, and ammonia). During the acute exposure samples of the "toadfish water" were collected at different times, to later analyse the ammonia concentrations.

An additional exposure was done using only diluted male "toadfish water", since this water caused the rapid death of the exposed organisms. This exposure consists of adding "toadfish water" to a final concentration of 1:30 by adding 3,33L in 100L containing 7 *S. aurata* individuals. This exposure had a duration of only one day, after which the fish were sacrificed and blood samples, and gill samples were collected. Due to the small duration of the exposure and the fact that it was done with male "toadfish water" the obtained results were compared to those from the acute exposures.

4. *Haematological Parameters*

The blood samples collected after the chronic and acute exposures were then used to evaluate several haematological parameters, which allows a better understanding of the possible effects that the putative toxin can have. When the blood sample was collected, a drop of blood was placed on a microscope slide, to produce a smear, that was later used to analyse changes in cell morphology (percentage of plasmolysis). The smears were used to analyse the effect of the 1:30 exposure and al of the acute exposure. The haematological parameters measured were: concentration of haemoglobin, number of both red and white blood cells, the changes in the concentration of oxy, deoxy, and methaemoglobin, and haemolysis.

4.1. *Haematocrit*

Before, proceeding with the count of blood cells, the haematocrit (Hct) was evaluated to determine the volume percentage of erythrocytes in blood. This was done by transferring part of the blood sample to a haematocrit tube by capillarity. The tubes were then centrifuged at 10000 rpm. The haematocrit is the percentage of red blood cells over the total volume of red blood cells, white blood cells and plasma. In practice this was calculated by measuring the distance that the red blood cells occupy and compare it against the distance occupied by the plasma in a dynamic scale.

4.2. *Haemoglobin (Hb)*

The concentration of haemoglobin (Hb) was calculated by using the Drabkin method. This is a colorimetric method in which, haemoglobin is oxidized into methaemoglobin and later to cyanomethaemoglobin by potassium ferricyanide and potassium cyanide, respectively. After the reaction occurs, the absorbance of the solution is measured in a spectrophotometer at 540nm. Haemoglobin concentration is proportional to the colour produced by the reactions, the more intense is the colour produced, the higher the haemoglobin concentration (van Kampen et al.,1961). This was determined using a SPIN react kit (1001230) following the manufacturer's instructions.

4.3. *Blood Cell counts*

To do the red blood cell direct counts (RBC), or erythrocyte counts, the blood samples were diluted using a physiological solution for teleosts, with a ratio of 1:100 and later 1:200. After, the sample was placed on a Neubauer counting chamber using a pipette. The chamber was then observed on a microscope, and the blood cells were counted. The red blood cells are counted on 5 of the secondary squares present in the central area of the slide. The results of the counting were presented in nº red blood cells/mm^3 and was given by the formula:

$$RBC\ (nº\ erythrocytes/mm^3) = \left(\frac{400 \times erythrocytes \times\ dilution\ factor}{nº\ terciary\ squares\ counted}\right)\ /0{,}1;$$

After calculating the number of erythrocytes, these results were used to calculate erythrocytes indices that describe the morphology and properties of the red blood cells, such as, the mean cell volume (MCV) of erythrocytes, the mean cell haemoglobin (MCH), and the mean cell haemoglobin concentration (MCHC) using the following equations:

$$MCV = \left(\frac{Hct \times 10}{RBC\ (\times 10^{12} L)}\right);\qquad MCH = \left(\frac{Hb \times 10}{RBC\ (\times 10^{12} L)}\right);\qquad MCHC = \left(\frac{Hb \times 100}{Hct}\right).$$

4.4. *Oxy, Deoxy, and Methaemoglobin*

The haemoglobin present in the blood is responsible for the transport of oxygen. When the blood reaches the oxygen exchange sites, in this case the gills, oxygen is bound to the haemoglobin, and this is called oxyhaemoglobin (HbO_2). When the oxygen is transferred to the body tissues, oxyhaemoglobin turns to deoxyhaemoglobin (HbH). When exposed to certain chemicals, the iron present in the haemoglobin can change its ionic state from Fe^{2+} to Fe^{3+} which is incapable of oxygen biding (Cooling, 2014). This is called methaemoglobin (MHb). To identify and measure the presence of MHb, the blood samples were haemolysed by mixing them with distilled water in a dilution of 1:20. Afterwards, the haemolysed samples were centrifuged for 5 minutes at 3000 rpm. The absorbance of the supernatant was then measured in a spectrophotometer between 450 and 700nm. As it can be seen in figure 3.1 (adapted from Nguyen et al., 2016) the presence of oxyhaemoglobin is indicated by two absorption peaks between 500 and 550nm and 550 and 600nm, the presence of deoxyhaemoglobin is indicated by a single peak between 550 and 600nm, and the presence of methaemoglobin is indicated by an irregular slope and plateau across a set of absorbances.

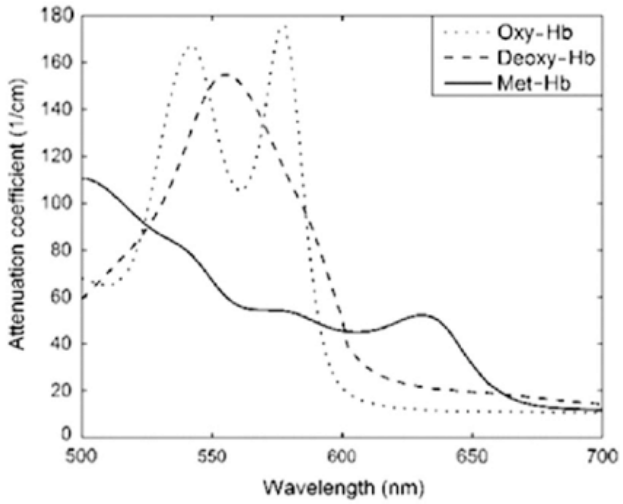

Figure 3.1 – Absorbance spectrum of oxy, deoxy, and methaemoglobin, adapted from Nguyen et al., 2016.

4.5. *Haemolysis*

After the blood analysis was performed, the blood samples were centrifuged at 10000 rpm for 5 minutes to obtain the plasma. After the plasma was obtained, 100µl were

transferred to a 96-well u-bottom microplate. An absorbance spectrum was executed with a wavelength interval ranging between 350 and 700nm, in which peaks of absorbance were observed at 414, 540 and 575nm, with the highest being 414nm. This wavelength was used to quantify the percentage of haemolysis, since a study performed, in which carp erythrocytes were used, demonstrated an increase of the assay sensibility for lysed erythrocytes when compared to 540nm. (Eschbach et al., 2001). Additionally, the absorbance at 620nm was used as a reference wavelength to remove non-specific interferences in the reads.

4.6. *Analysis of the Blood Smears*

The blood smears produced, were stained using the Hemacolor staining set (Hemacolor® Rapid staining of blood smear, Sigma-Aldrich), which uses 4 reagents to give colour to the smears. After staining, the smears were mounted with DPX. The smears were then observed on a microscope and pictures were taken of 3 fields of each smear. This procedure was done only to the blood smears produced in the chronic exposure of 1:30 and on the acute exposure. These pictures were later analysed using ImageJ software. The analysis consisted of counting both the total number of red blood cells in the field and the number of plasmolyzed cells, to calculate the plasmolysis percentage. Then 30 red blood cells in each field were measure for the longer and shorter diameter. With these measurements it is possible to calculate the area of the cell.

Figure 3.2 – Examples of regular red blood cells (left) and plasmolyzed red blood cells (right).

5. *Plasmatic Indicators*

The study of the haematological parameters also allows for a simultaneous study of the stress indicators and other plasmatic indicators. The plasma used for these analysis was obtained by centrifuging the blood samples, which separates the plasma from the other blood components.

5.1. *Stress indicators*

5.1.1. *Glucose*

Glucose was measure using a kit (Spinreatc Ref. 41013) that is based on an enzymatic reaction in which the glucose present in the samples is oxidised by the glucose oxidase into gluconic acid and hydrogen peroxide. The hydrogen peroxide is then transformed into quinone and water by peroxidase. This final reaction produces colour, whose intensity is proportional to the concentration of glucose (Trinder, 1969).

5.1.2. *Lactate*

The lactate was also be measured using a kit (Spinreact Ref. 1001330) that is also based on an enzymatic reaction, in which lactate is oxidized into pyruvate and hydrogen peroxide by lactate oxidase. The hydrogen peroxide is then converted into quinone and water by peroxidase. Once again, this reaction produces a colour, that will be proportional to the concentration of lactate in the sample (Burtis et al., 1999).

5.2. *Electrolytes*

The electrolytes that are going to be measured are phosphorous, magnesium, chloride, calcium, sodium, and potassium. The phosphorous, magnesium, chloride, and calcium, like the glucose and lactate were measured using kits. The sodium and potassium were measured with flame photometry.

5.2.1. *Phosphorus*

The inorganic phosphorous present in the samples reacts with the ammonium molybdate present in the kit (Spinreact Ref.1001155) when in an acid medium to form a phosphomolybdate complex with a yellow colour. The intensity of this colour is proportional to the concentration of phosphorous of the samples (Daly and Ertingshausen, 1972).

5.2.2. *Magnesium*

The magnesium kit (Spinreact Ref. 1001285) uses Magon sulfonate in alkaline solution to react with the magnesium in the sample to form a coloured complex, whose intensity is proportional to the concentration of magnesium in the sample (Burtis et al., 1999).

5.2.3. *Chloride*

When the chloride in the sample reacts with the mercuric thiocyanate present in the kit (Spinreact Ref. 1001360), leads to the displacement of thiocyanate and subsequent formation of a red ferric thiocyanate complex. The intensity of the colour produced is proportional to the concentration of chloride (Schoenfeld and Lewellan, 1964).

5.2.4. *Calcium*

When in an alkaline medium, the calcium present in the samples reacts with the o-Cresophthalein present in the kit reagent (Spinreact Ref. 1001061), which leads to a formation of a colour complex, whose intensity is proportional to the concentration of calcium (Burtis et al., 1999).

5.2.5. *Sodium and Potassium*

The process of measuring both the sodium and potassium present in the samples, consists in diluting the samples in water to 1:50. The solution is then atomized into a gas burner, with the vapor being ignited once it reaches the flame. The intensity of the light produced by this reaction is then measured. This value is then compared with a previously prepared calibration curve, that was made with solutions containing known concentrations of both sodium and potassium (Berry et al., 1946).

5.3. *Lactate Dehydrogenase (LDH)*

LDH is an enzyme that catalyses the reduction of pyruvate by NADH present in the kit (Spinreact Ref. 41222), leading to the formation of L-lactate and NAD^+. Since this is a kinetic reaction, the rate at which the concentration of NADPH decreases is proportional to the concentration of LDH (Burtis et al., 1999).

5.4. *Ammonia*

The ammonia concentration was measured not only in the plasma, but also in the water of the chronic and acute exposures. The ammonia kit (Spinreact Ref. 1001410) is based on an enzymatic reaction, in which the ammonia, in the presence of glutamate dehydrogenase (GLDH), combines with α-ketoglutarate and NADPH, releasing glutamate and NADP+. As this reaction occurs, the absorbance at 340nm suffers a decrease that is proportional to the concentration of ammonia (Mondzac et al.,1965).

6. *Haemolytic Activity*

Characterization of the haemolytic activity was performed by incubating blood samples of *S. aurata* with two mucus extractions from toadfish skin, one corresponding to the male and the other to the female. The mucus was collected by scraping the sides of the fish with a microscope slide and transferring the collected mucus present in the skin to 15ml falcon tubes. This experiment was performed with an aqueous extract of the mucus. This extract was prepared by mixing equal parts of mucus with 0,95% NaCl saline solution. The extraction was homogenized and later centrifuged at 15000rpm for 15min, and the supernatant was collected. Afterwards, the protein concentration of the supernatant was measured with the Bradford Method. The Bradford Method consists of a dye-binding assay, in which an acidic solution of Coomassie Brilliant Blue changes its absorbance maximum from 465 to 595, and therefore, there is a colour change as a response to different protein concentrations (Becker et al., 1996). Based on the lowest protein concentration indicated by the Braford Method, 5 serial dilutions were prepared, 1:1, 1:10, 1:100, 1:1000, 1:10000, by mixing the aqueous extract with saline solution.

The blood used in this procedure was collected using the previously mentioned procedure, from 3 *S. aurata* individuals that were not under experimental conditions. In this experiment only the red blood cells were used, therefore, the blood was pooled in 50ml falcon tubes, and mixed with double the amount of 0,95% NaCl saline solution, and was centrifuged at 5000 rpm for 5 minutes, to deposit the red blood cells at the bottom of the tube. Afterwards the supernatant was discarded, and more saline solution was added. This procedure was repeated several times to clean the red blood cells. When the cells were clean, a 1% red blood cell solution was prepared by mixing 1ml of red blood cells with 99ml of saline.

To observe the haemolytic activity, both the aqueous extract dilutions and red blood cell solution were transferred to a 96-well v-bottom microplates. Each dilution was done in triplicate, and in each well there was 100µl of the dilution and 100µl of the red blood cell solution. For the positive control it was used both saponin (15µg/ml) and distilled water, and for the blank it was used the 0,95% saline solution. Then the was incubated at room temperature for 2h. Afterwards, the plate was centrifuged, and the supernatant was transferred to a 96-well u-bottom microplate (Al-Rasheed et al 2018; Neely and Campbell, 2006). Like, the procedure for haemolysis, the 414nm wavelength was used to

quantify the haemolysis, and the absorbances at 620nm were once again used to remove interferences in the reads.

7. *Na K-ATPase activity*

The gills of teleost fish are of key importance to the relation of the fish with the surrounding environment. Therefore, the branchial Na K–ATPase enzymatic system found in the epithelial cells of the gills is of extreme importance for osmoregulation since it is responsible for active transport of electrolytes across the gills (Ay et al., 1999). The Na K–ATPase transports Na^+ ions out of cells and k^+ into the cell, duo to the energy provided by the hydrolysis of one molecule of ATP. Due to the importance of this enzymatic system to the health of the fish it is very important to observe if the toadfish toxin present in the water may have an effect not only on the activity of the Na K–ATPase., but on the total activity of the ATPase.

The procedure to measure the activity of the Na K–ATPase is based on three enzymatic reactions: the first in which ATP is phosphorylated into ADP with the release of inorganic phosphate; the second in which the ADP is transformed into ATP with the formation of pyruvate, in the presence of phosphoenolpyruvate with the help of the pyruvate kinase; and the third in which, in the presence of LDH, the pyruvate is reduced to lactate by NADH which is oxidized in to NAD^+ (Nørby, 1988).

To measure the Na K–ATPase activity of the gill samples of the acute exposure these had to firstly be homogenized with a cell disruptor, and later centrifuged at 5000g for 10min, and the supernatant is collected. Part of supernatant was transferred into a microplate with the remaining being used to measure the protein concentration, in the presence and absence of ouabain which is an inhibitor of the Na^+/k^+ to observe the pump to prevent the process to occur on other ATPases on the cell. The different components for the reactions were added later to the microplates as a mixture. The linear rate of NADH disappearance was measured at 340nm for 10min, with these values the Na K–ATPase activity was calculated as the difference between the difference in ATP hydrolysis in the presence and absence of the inhibitor (McCormick,1993).

8. *Olfactory Nerve Recording (ONR)*

A possible effect that the toadfish toxin may have on the target species could be at a neurotoxic level. In fact, previous observations have shown that the *H. didactylus* toxin causes inhibition of the responses of the olfactory nerve. *S. aurata* individuals were anaesthetised by immersion in seawater containing a mixture of 1000 mgL^{-1} of MS222

buffered with 2000 mgL^{-1} of $NaHCO_3$, and were immobilized by and intramuscular injection of gallamine triethiodide. The fish was then placed on a holder and maintained with a continuous supply of aerated seawater containing 50 mgL^{-1} of MS222 flowing over the gills. To allow the performance of this recording, a surgery must be performed to expose the olfactory nerve. This was done by removing the bone of the skull between the snout and clearing the connective tissue and fat. The recording was done by placing two tungsten electrodes that are inserted into one of the olfactory nerves and a ground electrode placed down the body of the fish. These electrodes are connected to an a.c. preamplifier. The signal produced was then filtered, integrated, and later digitized. The target stimulant substances were automatically delivered to the olfactory epithelium by a glass tube held in position in the inhalant olfactory opening, connected to a gravity-fed tubing system, connected to a computer that controls the time and flow of the release. The tubing is also connected to a glass container with a control stimulant, and this system allows for the switching between the control and the target substance, which allows to better identify the response (Hubbard et al., 2000).

To evaluate if the system is calibrated and the fish is in good condition to allow the performance of the recording, first it was performed a control test using L-cysteine 10^{-3} M in triplicate as the positive control and seawater as the negative control. If the produced data for these tests is correct, then it is possible to proceed with the analysis of the effect of the "toadfish water". The exposure of the fish to the "toadfish water" was done by exposing the olfactory epithelium, during one minute, to consequently smaller dilution of the water, 1:1000, 1:100, 1:30, and 1:10. These dilutions were produced by mixing "toadfish water" with seawater. To observe the effect that these dilutions have, the fish were exposed to an array of different amino acids, such as an L-cysteine curve corresponding to the concentrations $10^{-3,}$ $10^{-4,}$ $10^{-5,}$ 10^{-6} M, L-leucine 10^{-4} M, L-glutamic acid 10^{-4} M, L-arginine 10^{-4} M, and L-serine 10^{-4} M, and sea bream bile in a dilution of 1:5000 since it is a very potent stimulant. The response to these stimulants were measured before applying any "toadfish water" to serve as a control, and they were measured once again after every dilution has been applied. To ensure that there is no de-calibration of the system, and the fish is still in good condition, the response to the negative control (seawater) was measured after each dilution. Finally, after testing all the dilutions, a wash-out of the olfactory epithelium is performed by letting seawater flow through it for 30 min, after which the response to the different stimulants was measured once more to observe if there was a recovery of the olfactory nerve.

9. *High Performance Liquid Chromatography (HPLC)*

Samples of "toadfish water" and mucus from both the males and females were fractionated using a preparative liquid chromatograph system (Shimadzu, LC-20AP, Izasa Scientific, Portugal) consisting of a quaternary pump, a degassing device, an auto-sampling injector, an automatic sample collector, a column oven and a diode array detector scanning from 190 to 900 nm. The column was a Phenomenex Luna C18 column (25 cm, 4.6 mm, 5 mm). Samples were run with a linear gradient of water and acetonitrile (10–100%) over 30 min and a flow rate of 1.0 ml min-1. Spectral data were collected and analysed with the software "Labsolutions, Postrun system, Shimadzu".

10. *Behavioural Analysis*

During the acute exposures direct observations and recordings were performed with the intent of analysing the behaviour the sea bream in the presence of "toadfish water", with special attention to spasms, loss of equilibrium (LOE) and immobility. When immobility was observed, the experiment was ended, however if no immobility was observed the experiment would last for 1 hour. Due to limitations of the availability of the "toadfish water" the acute exposures had to be done in 10L tanks, which were not suitable to maintain the seabream individuals for long periods of time. Therefore, these individuals were only placed in these tanks at the time of the experiment, thus there wasn't a suitable period for acclimation.

11. *Statistical analysis*

The obtained data was statistically analysed using one-way analysis of variance (ANOVA), followed by a post-hoc Tukey test, to observe the differences between the means of each group. The alpha used was 0,05, so the differences were only considered significant if p-value<0,05. In the case of the haematocrit values of the chronic exposures, due to errors present in the obtained values for the 1:1000 exposure, these were not taken in consideration and therefore the statistical analysis consisted of performing a t-test to observe differences between the control and the treatment of the 1:100 exposure. In the case that there are significant differences, bars or cells that are represented by different letters, indicate the presence of a significant difference. The results present in both the graphs and tables represent the mean ± standard deviation.

4. Results

Chronic Exposures

1. *Haematological parameters*

1.1. *Haematocrit*

The haematocrit values obtained during the chronic exposures are present in figure 4.1. Due to some problems during the laboratory procedure the values for the 1:1000 exposure were incorrect and therefore are not part of the final results used for the analysis of the effect of the toadfish toxin. The difference between the control and exposure of the 1:100 treatment was not statistically significant.

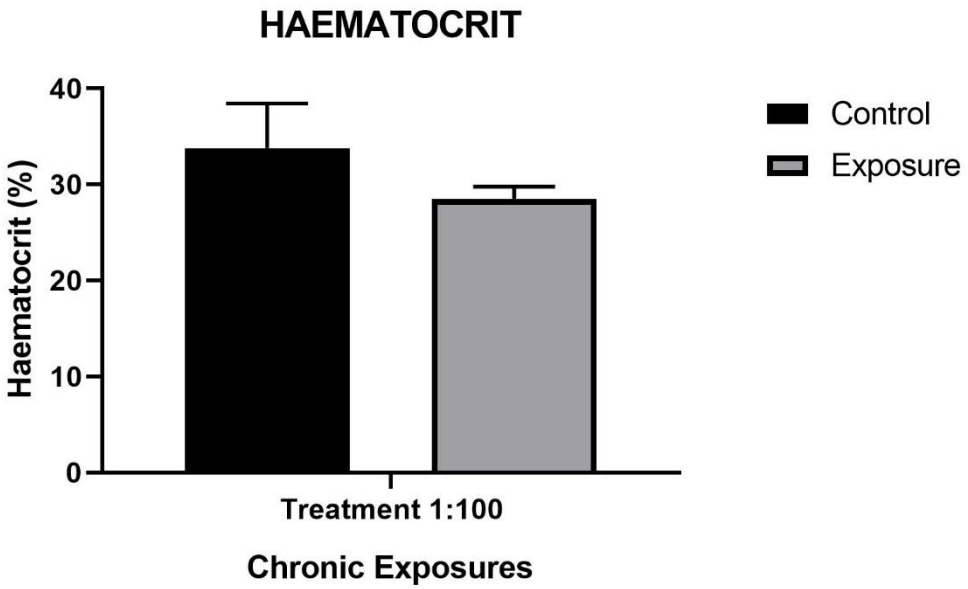

Figure 4.1 – Obtained haematocrit values for the chronic treatment of 1:100. N=4-5. T-test (p-value > 0,05), between control and treatment. Mean ± Standard deviation.

1.2. *Haemoglobin (Hb)*

After performing the Drabkin method, the haemoglobin concentration was obtained. Despite the small increase from the 1:1000 to the 1:100 treatment, there were no significant differences.

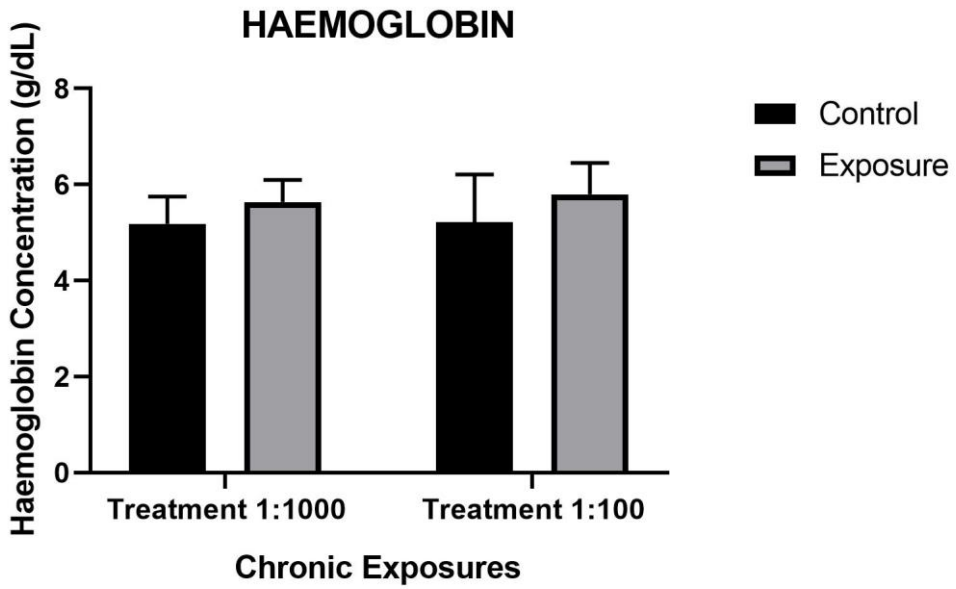

Figure 4.2 – Obtained haemoglobin concentration for the chronic treatments of 1:1000 and 1:100. N=4-5. One-way ANOVA (p-value > 0,05). Mean ± Standard deviation.

1.3. *Blood Cell counts*

The obtained blood cell counts are shown in figures 4.3, red blood cells. The increase in concentration of "toadfish water" did not seem to cause an effect on the number of red blood cells, since there was no significant difference.

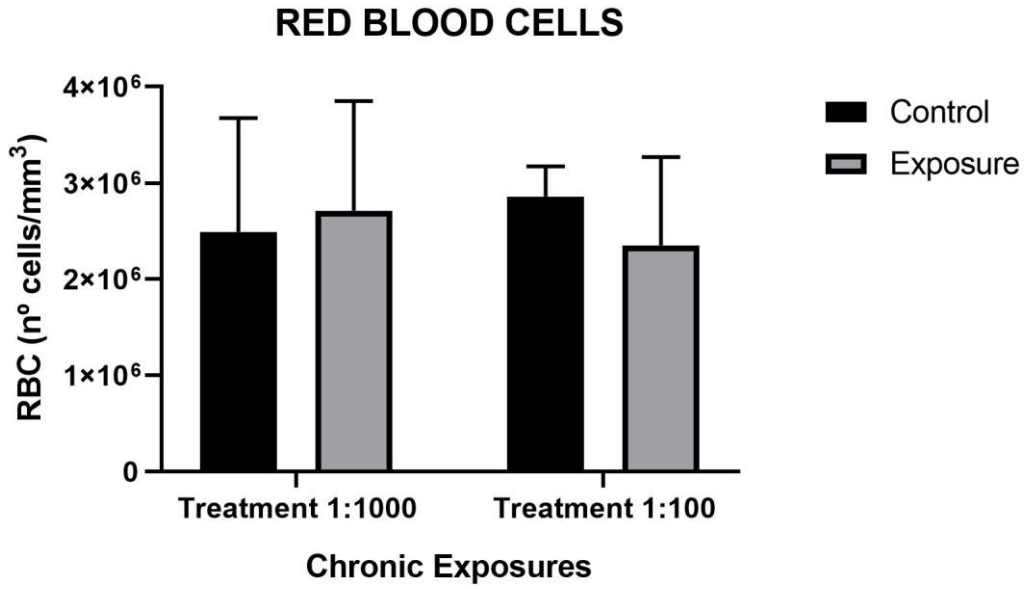

Figure 4.3 – Obtained red blood cell counts for the chronic treatments of 1:1000 and 1:100. N=4-5. One-way ANOVA (p-value > 0,05). Mean ± Standard deviation.

Based on the haemoglobin, haematocrit, and red blood cells, the mean cell volume, mean cell haemoglobin, and mean cell haemoglobin concentration were calculated for the

chronic exposures and are presented on table 4.1. Since the haematocrit values of the 1:1000 treatment weren't considered in these results, the mean cell volume and mean cell haemoglobin concentration were not possible to calculate for this treatment.

In relation to the mean cell volume, there was an increase from the control to the 1:100 treatment, however this increase was not statistically significant.

Because the mean cell haemoglobin was calculated based on the haemoglobin and red blood cell counts, it presented a higher value on the 1:100 treatment, since it was in this treatment that the haemoglobin was also highest, and the number of red blood cells was smaller. Despite there being a difference between the treatments, these were not significant. The differences between the controls and their treatments were also not significant.

When it comes to the mean cell haemoglobin concentration, the difference between the treatment and the control of the 1:100 exposure was significant.

Table 4.1 – Haematological parameters obtained from the chronic exposures of the 1:1000 and 1:100 treatment. N=4-5. One-way ANOVA. Mean ± Standard deviation.

	1:1000 treatment		1:100 treatment	
	Control	Treatment	Control	Treatment
Haematocrit (%)			33,80 ± 4,66	28,50 ± 1,29
Haemoglobin (g/dL)	5,19 ± 0,57	5,64 ± 0,46	5,22 ± 0,99	5,80 ± 0,65
RBC (nº cell/mm^3)	$2,492\times10^6$ ± 1182717	$2,71\times10^6$ ± 1143175	$2,8575\times10^6$ ± 314152	$2,3525\times10^6$ ± 916311
MCV (fL)			110,01 ± 20,32	135,91 ± 50,53
MCH (pg/cell)	19,23 ± 4,93	17,31 ± 5,86	17,12 ± 4,49	28,34 ± 12,81
MCHC (g/dL)			15,43 ± 1,73[a]	20,71 ± 2,06[b]

2. *Plasmatic indicators*

2.1. *Stress indicators*

2.1.1. *Glucose*

The glucose measurements of the chronic exposures show that there were no evident alterations that occur when the concentration of "toadfish water" increased. There was a slight decrease from the 1:1000 treatment to the 1:100 treatment, however there were no significant differences.

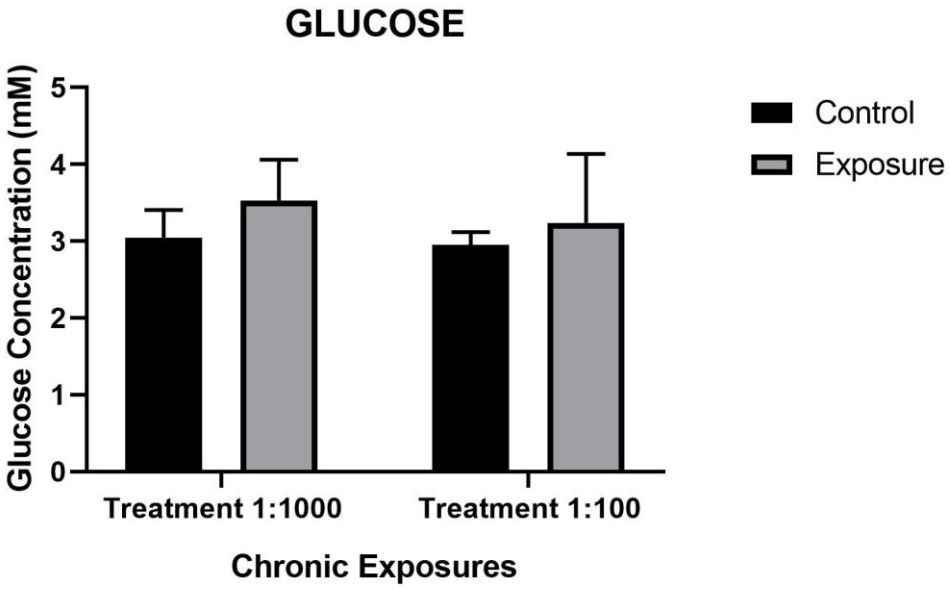

Figure 4.4 – Obtained glucose concentration for the chronic treatments of 1:1000 and 1:100. N=4-5. One-way ANOVA (p-value > 0,05). Mean ± Standard deviation.

2.1.2. *Lactate*

Like the glucose concentration, the lactate concentration did not present large alterations as the "toadfish water" concentration increased, however there was a decrease in the controls of the treatment. Despite the changes in the control, the statistical significance of the lactate concentration, was the same as the glucose concentration, that is, there were no statistically significant differences.

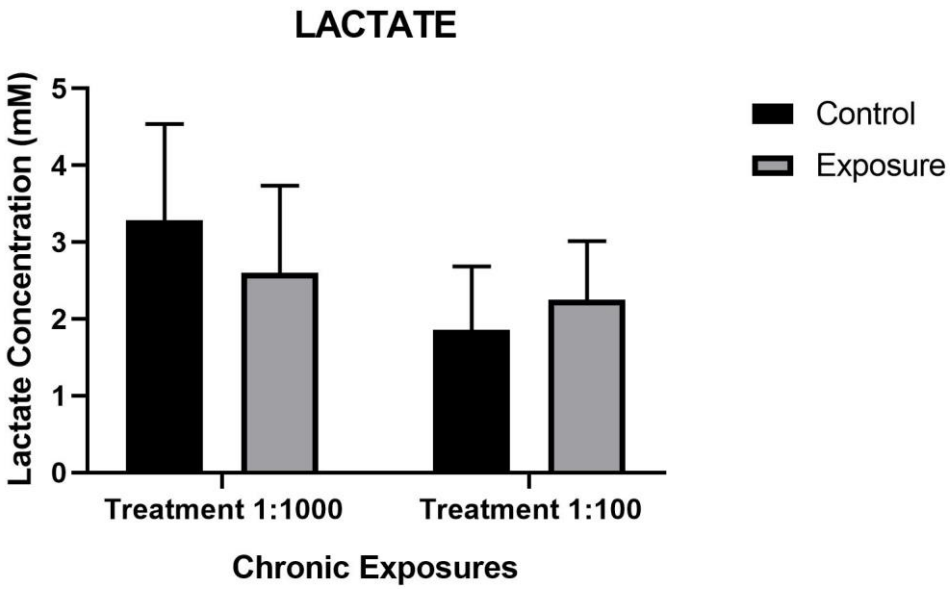

Figure 4.5 – Obtained lactate concentration for the chronic treatments of 1:1000 and 1:100. N=5. One-way ANOVA (p-value > 0,05). Mean ± Standard deviation.

2.2. *Electrolytes*

2.2.1. *Phosphorus*

The phosphorous concentration increased not only from the chronic exposure of 1:1000 to the 1:100 but also in their respective controls. The significant differences were present between the 1:1000 control and the 1:100 treatment, and between the 1:1000 treatment and the 1:100 treatment.

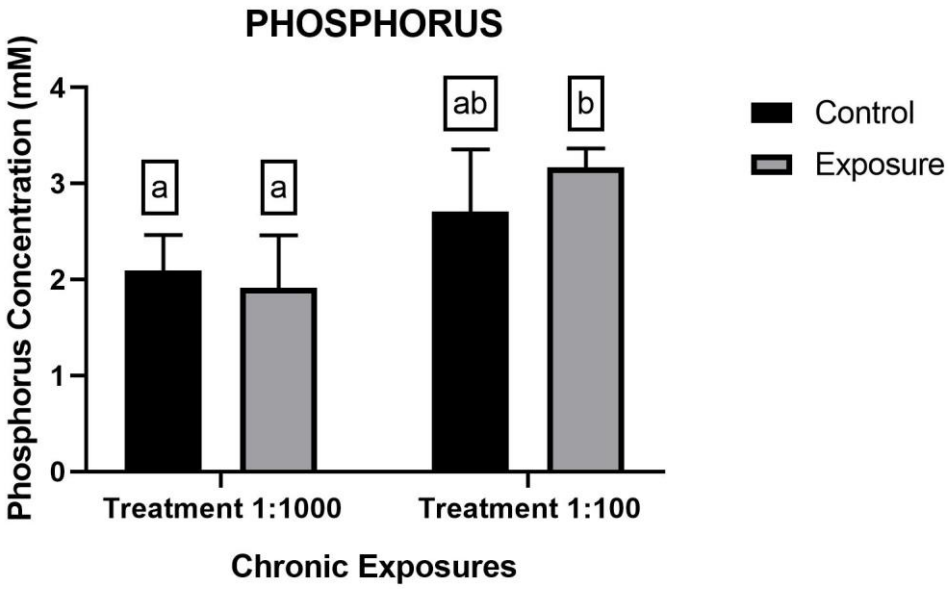

Figure 4.6 – Obtained phosphorus concentration for the chronic treatments of 1:1000 and 1:100. N=5. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

2.2.2. *Magnesium*

The obtained magnesium concentrations from chronic exposures presented a very slight increase, that was not significant, as the concentration of the "toadfish water" increased. The magnesium concentration did not seem to present any significant differences

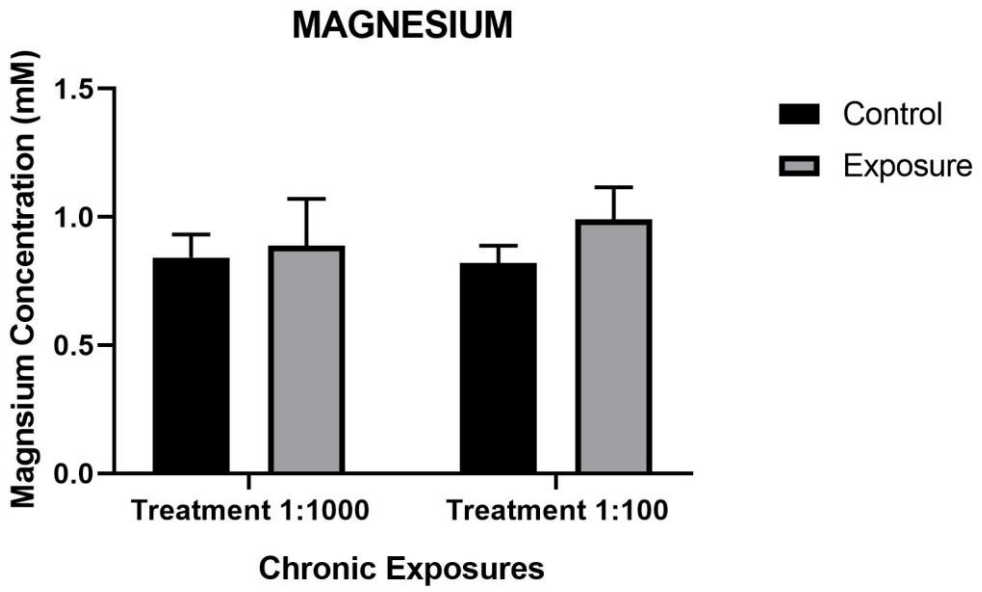

Figure 4.7 – Obtained magnesium concentration for the chronic treatments of 1:1000 and 1:100. N=5. One-way ANOVA (p-value > 0,05). Mean ± Standard deviation.

2.2.3. *Chloride*

The chloride concentration decreased as the concentration of "toadfish water" increased, being higher in the 1:1000 and lower in the 1:100 treatment. The significant differences were between the two controls, between 1:1000 control and the 1:100 treatment, and between the two treatments.

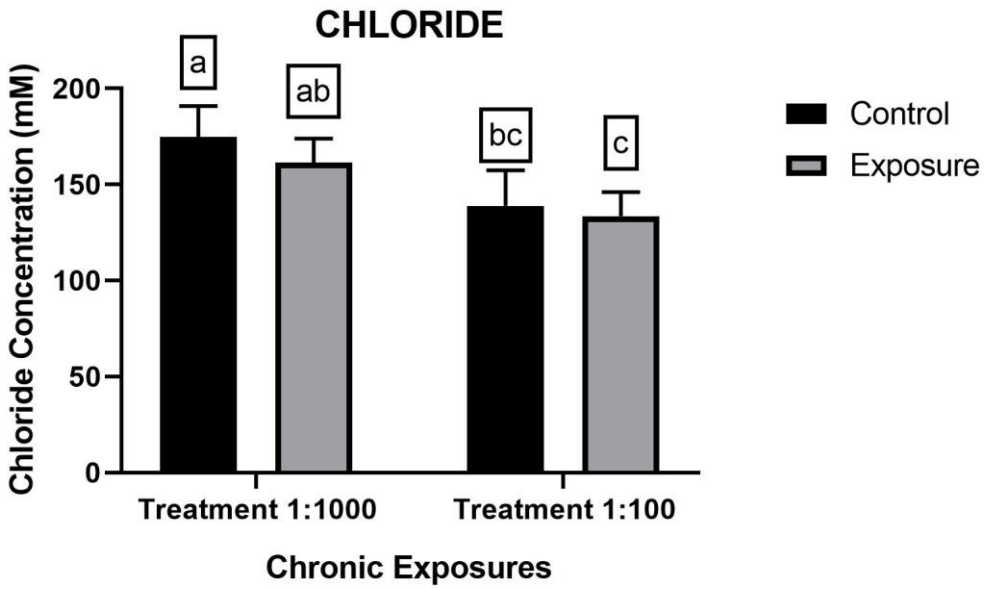

Figure 4.8 – Obtained chloride concentration for the chronic treatments of 1:1000 and 1:100. N=4-5. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

2.2.4. *Calcium*

The calcium concentration did not seem to suffer an effect when the "toadfish water" concentration increased. In the case of the calcium concentration there were no significant differences.

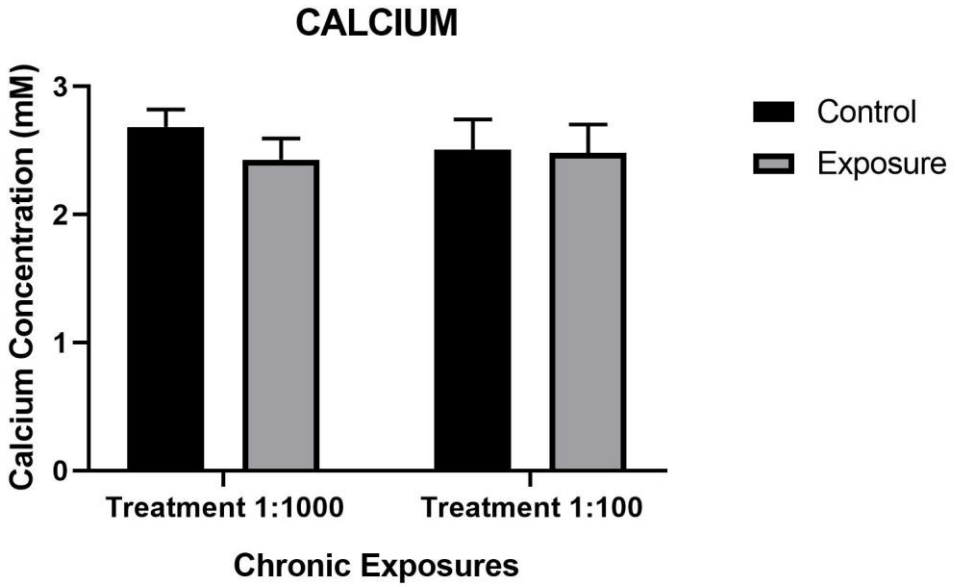

Figure 4.9 – Obtained calcium concentration for the chronic treatments of 1:1000 and 1:100. N=5. One-way ANOVA (p-value > 0,05). Mean ± Standard deviation.

2.2.5. *Sodium and Potassium*

In the case of the chronic exposures, the sodium and the potassium followed opposite trends. The sodium decreased as the "toadfish water" concentration increased, while the potassium concentration increased with the increase of the "toadfish water" concentration. The sodium concentration presented significant differences between the two controls, between the 1:1000 control and the 1:100 treatment, and between the 1:1000 treatment and the 1:100 control. The potassium concentration did not present any significant difference.

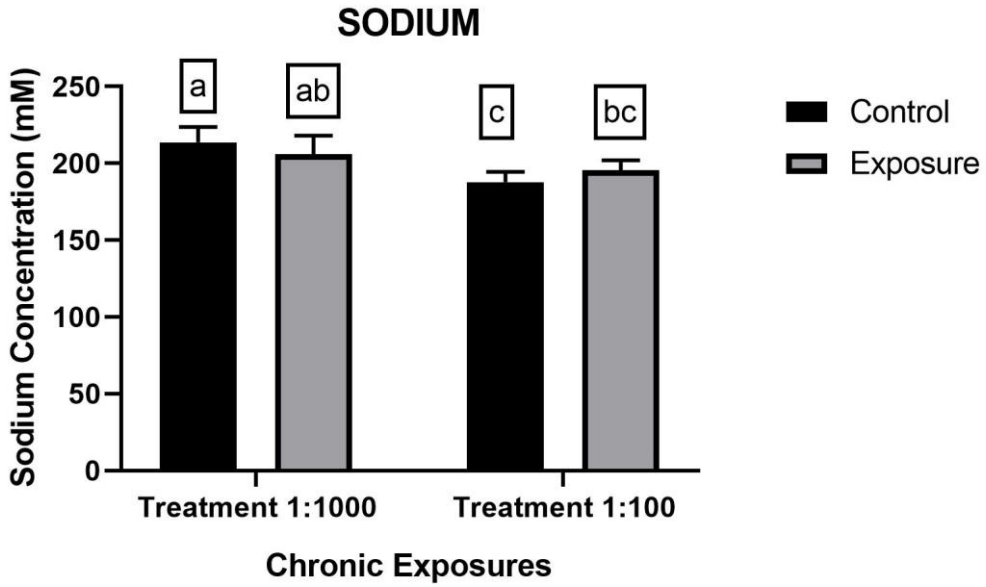

Figure 4.10 – Obtained sodium concentration for the chronic treatments of 1:1000 and 1:100. N=4-5. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

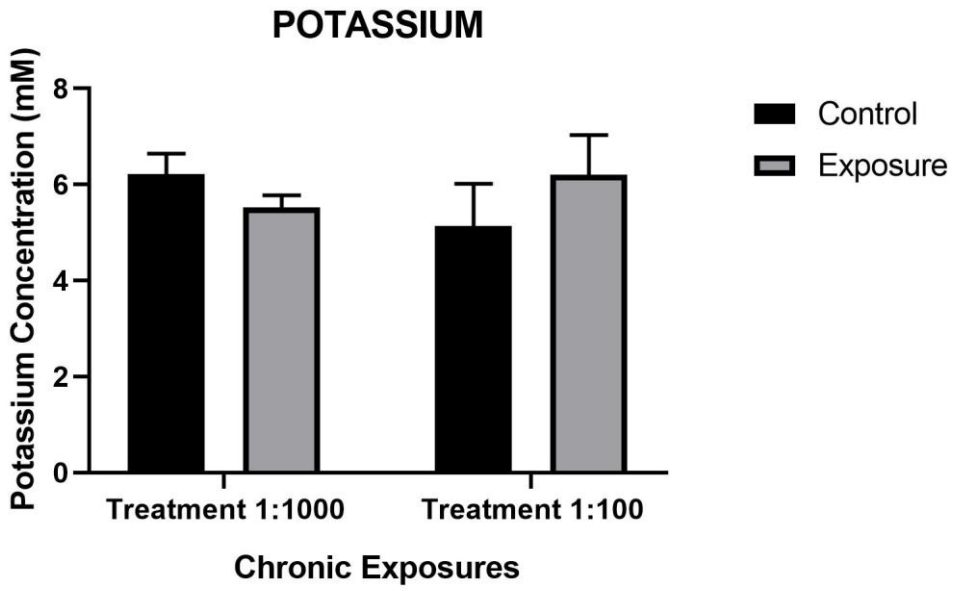

Figure 4.11 – Obtained potassium concentration for the chronic treatments of 1:1000 and 1:100. N=4-5. One-way ANOVA (p-value > 0,05). Mean ± Standard deviation.

2.3. *Lactate dehydrogenase (LDH)*

The LDH concentration decreased as the "toadfish water" concentration increased, however there were no significant differences.

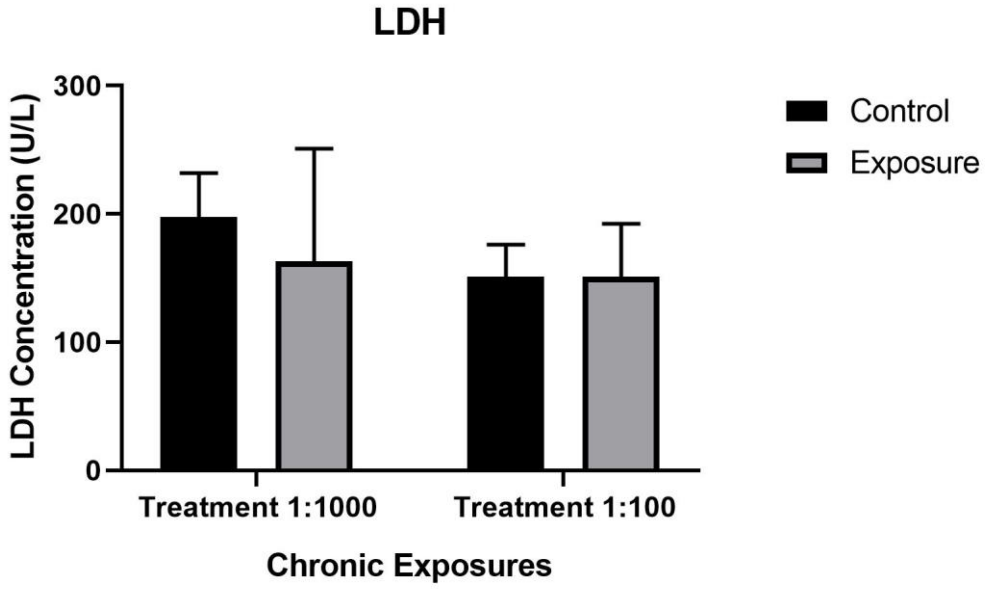

Figure 4.12 – Obtained LDH concentration for the chronic treatments of 1:1000 and 1:100. N=4. One-way ANOVA (p-value > 0,05). Mean ± Standard deviation.

2.4. *Ammonia*

The ammonia concentration suffered an increase from the 1:1000 to the 1:100 treatment. However, there were no significant differences.

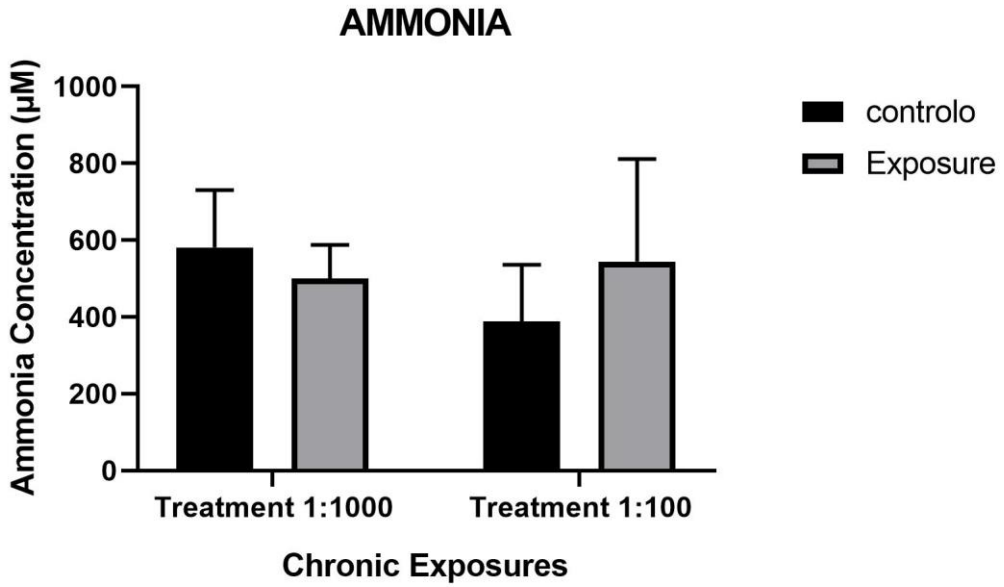

Figure 4.13 – Obtained Ammonia concentration for the chronic treatments of 1:1000 and 1:100. N=4-5. One-way ANOVA (p-value > 0,05). Mean ± Standard deviation.

Acute Exposures

1. *Haematological Parameters*

1.1. *Haematocrit*

In relation to the haematocrit values of the acute exposures there was a clear decreasing trend from the control to the female and to the male "toadfish water" treatments. However, when the concentration of the male "toadfish" water was decreased to 1:30, there was an increase of the haematocrit. Despite the clear changes, only the difference between the control and male "toadfish water" was significant.

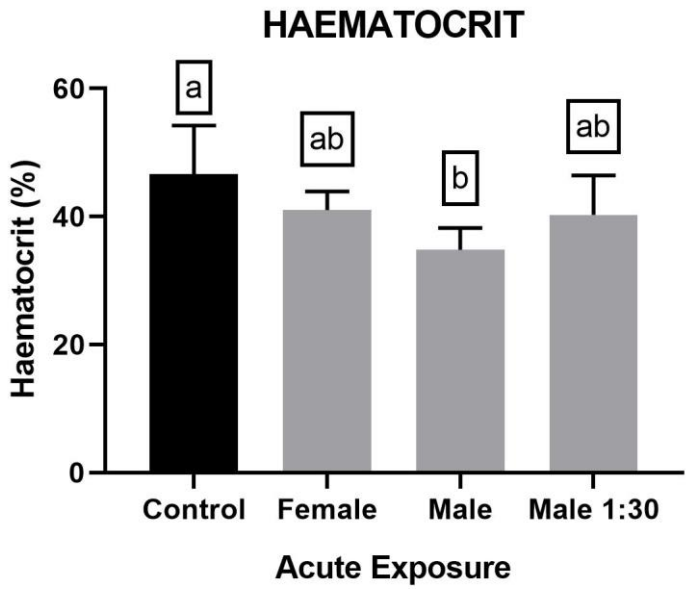

Figure 4.14 – Obtained haematocrit values for the acute exposures. N=7-12. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

1.2. *Blood PH and Osmolality*

For the acute exposures, instead of the haemoglobin, and blood cell counts, the blood pH and osmolality were measured. When it comes to the blood pH the male treatment presented the smallest value obtained, while both the female and 1:30 did not seem to have a large difference from the control. The significant differences were present between the control and female, between female and 1:30, and between the male and all the other treatments.

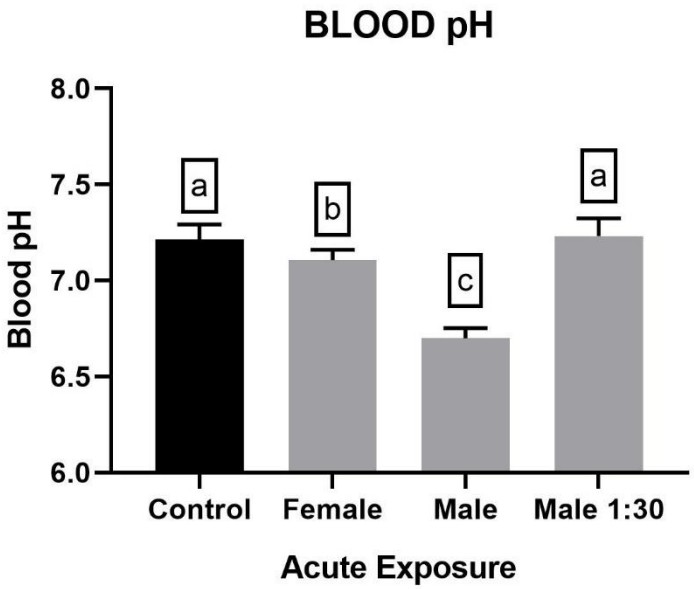

Figure 4.15 – Obtained blood pH values for the acute exposures. N=7-12. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

In relations to osmolality, the highest value was present on the male treatment, and the smallest was present on the 1:30 treatment. The significant differences were between the control and male, and between the 1:30 and both the female and male treatments.

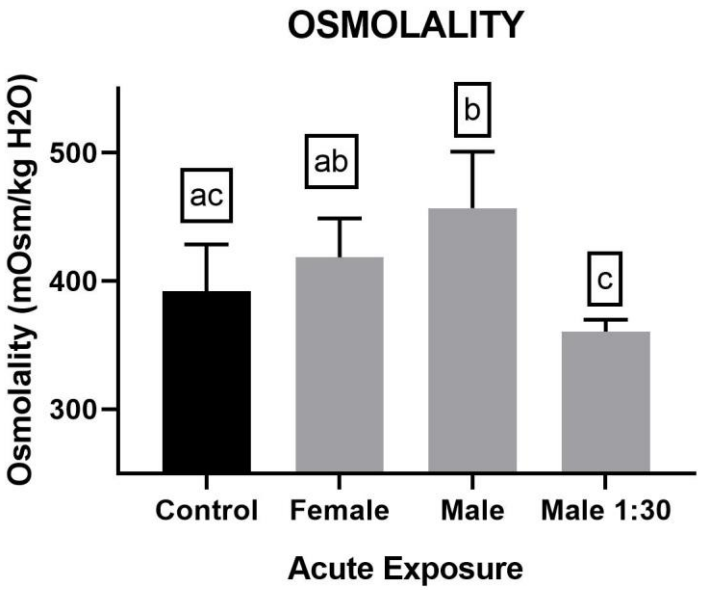

Figure 4.16 – Obtained osmolality values for the acute exposures. N=7-8. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

1.3. *Haemolysis*

The haemolysis percentage that was obtained during the acute exposures are represented on figure 4.17, as a fold change in relation to the control. There was a clear increase of the haemolytic activity not only from the control to both the treatments, and from the female water treatment to the male water treatment. Despite the clear differences present in the figure, these were not statistically significant.

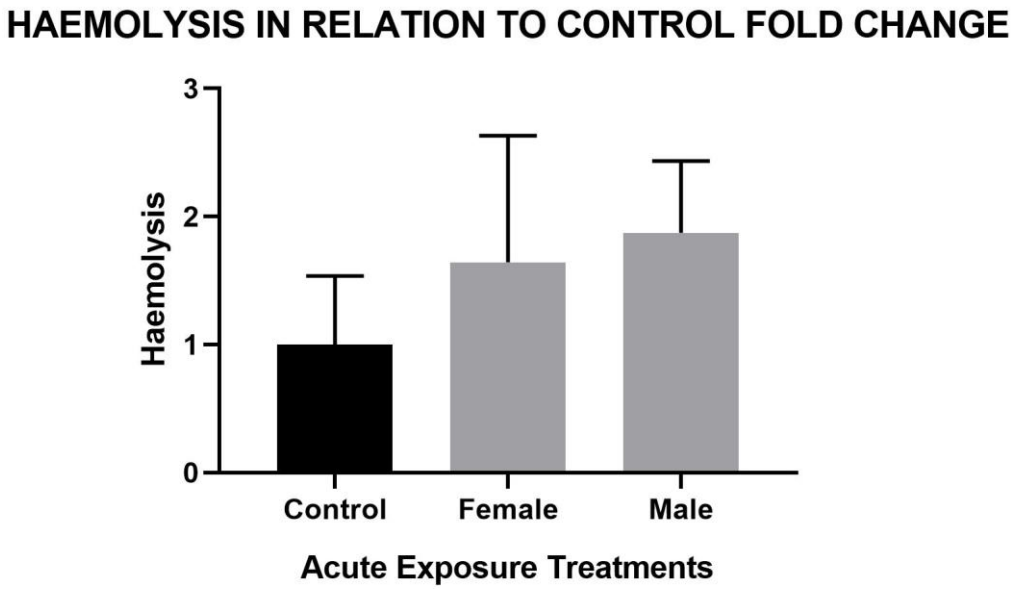

Figure 4.17 – Obtained haemolysis values for the acute exposures. N=8. One-way ANOVA (p-value > 0,05). Mean ± Standard deviation.

1.4. *Analysis of blood smears*

The obtained measurements from the smear analysis are present in table 4.2.

Table 4.2 – Results from the analysis of the blood smears of *S. aurata*. N=21-24 Fields. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

	Control	1:30	Female	Male
Long diameter of RBC (µm)	1,98 ± 0,16[a]	2,00 ± 0,16[a]	1,91 ± 0,18[b]	1,92 ± 0,19[b]
Short diameter of RBC (µm)	1,28 ± 0,12[a]	1,24 ± 0,12[b]	1,27 ± 0,15[a]	1,24 ± 0,15[b]
Area of RBC (μm^2)	1,98 ± 0,25[a]	1,94 ± 0,25[ab]	1,91 ± 0,32[bc]	1,87 ± 0,30[c]
Plasmolysis percentage (%)	36,17 ± 6,48	54,25 ± 9,45[a]	56,36 ± 12,54[a]	87,67 ± 5,38
Diameter ratio	1,56 ± 0,19[a]	1,63 ± 0,20	1,53 ± 0,20	1,57 ± 0,23[a]

As it is possible to observe the male "toadfish water" caused the largest cell plasmolysis, which in turn lead to a decrease in cell area. It is also possible to observe that despite the fact that the1:30 exposure was done with male "toadfish water" it presented similar results to that of the female water acute exposure, in terms of both area and plasmolysis percentage, with these differences not being statistically significant. In relation to the diameter ratio, it suffered slight changes between the treatments, being highest on the 1:30 treatment and lowest on the female treatment. The differences between the groups were all significant with the exception between the control and male treatment.

2. *Plasmatic indicators*

2.1. *Stress indicators*

2.1.1. *Glucose*

In terms of the acute exposures, the glucose concentration increased in both the female and 1:30 treatment and decreased in the male treatment. Despite the glucose concentration varying between the treatments, the only difference that was significant is between the female and male treatments.

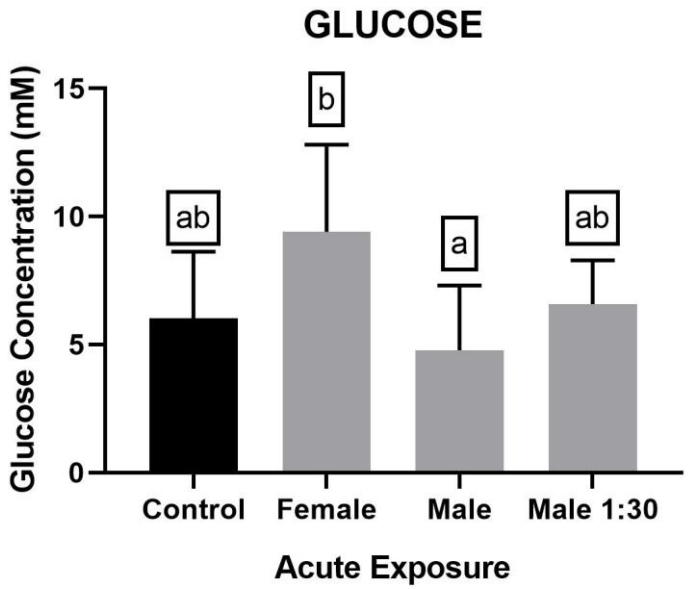

Figure 4.18 – Obtained glucose concentration for the acute exposures. N=7-12. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

2.1.2. *Lactate*

When it comes to the acute exposures, the lactate concentrations behaved like the opposite of the glucose concentration, with the male treatment presenting the highest value. Also, the glucose concentration presented only one difference between the groups that was

significant, however, the lactate presents four significant differences. These were between the control and female and male, and between the 1:30 and the female and male treatments.

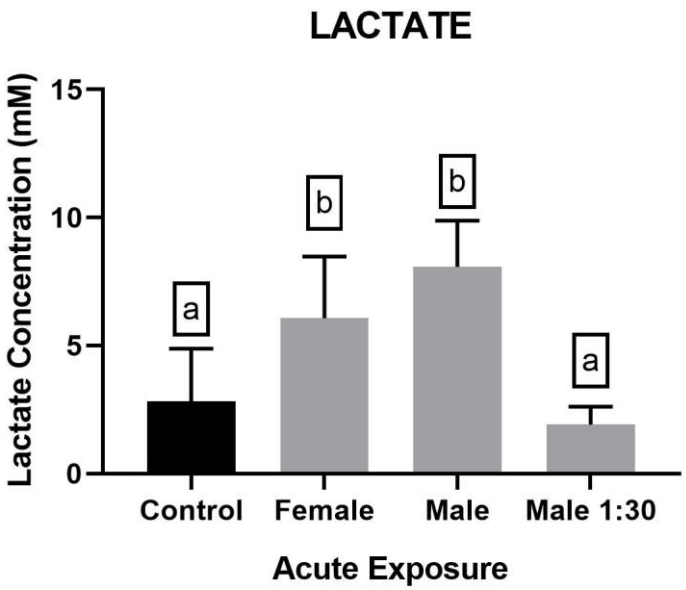

Figure 4.19 – Obtained lactate concentration for the acute exposures. N=7-11. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

2.2. *Electrolytes*

2.2.1. *Phosphorus*

The phosphorous concentration of the acute exposures followed the same trend as the lactate concentration, there was an increase from the control to the female and to the male treatments, while there was a decrease to the 1:30 treatment. The significant differences present in these results were between the male and all the other treatments.

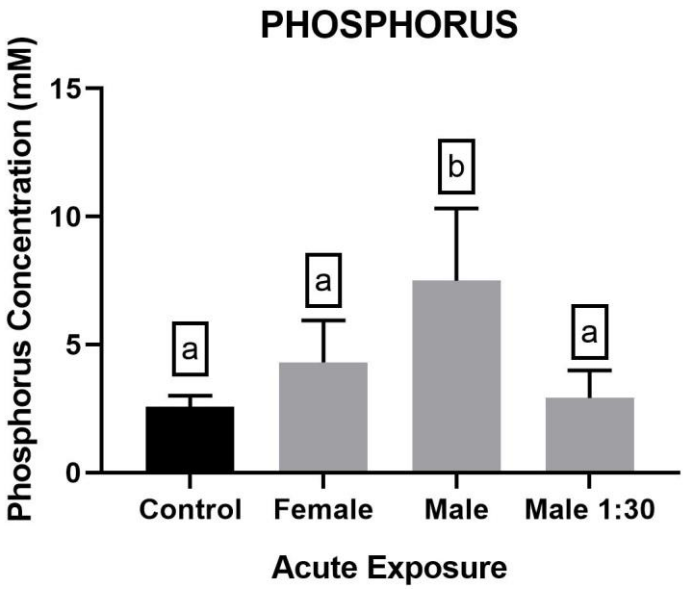

Figure 4.20 – Obtained phosphorus concentration for the acute exposures. N=7-8. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

2.2.2. *Magnesium*

Once again, in the acute exposures, the male treatment presents highest concentration. The female treatment did not seem to have much of an effect since there was not much of a change from the control. Like the concentration of phosphorous and lactate, the 1:30 treatment presented the lowest value. Like the phosphorous concentration, the significant differences were between the male and the other treatments.

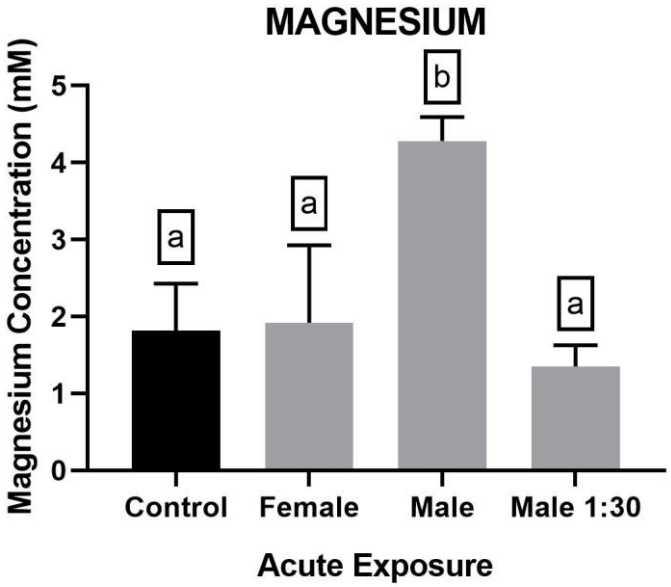

Figure 4.21 – Obtained magnesium concentration for the acute exposures. N=6-8. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

2.2.3. *Chloride*

The chloride concentration of the acute exposures followed the same trend as the previous electrolytes, that is, highest on the male, and lowest on the 1:30 treatment, and the female treatment suffered very little change from the control. The significant differences present in the results were between the control and the male treatment, the 1:30 and both the female and male treatments.

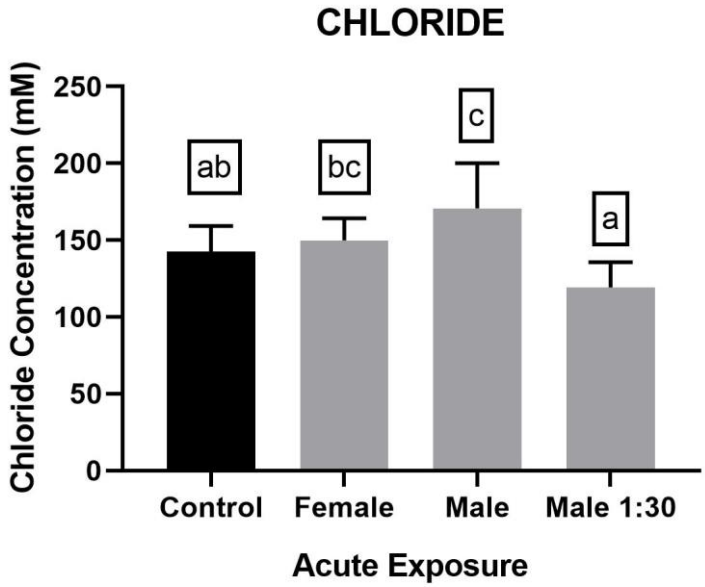

Figure 4.22 – Obtained chloride concentration for the acute exposures. N=7-8. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

2.2.4. *Calcium*

The calcium concentration of the acute exposures, presented the highest value on the male treatment, however in this case the 1:30 exposure presented the second highest value. The significant differences present in the calcium concentration, was between the male and all the other treatments.

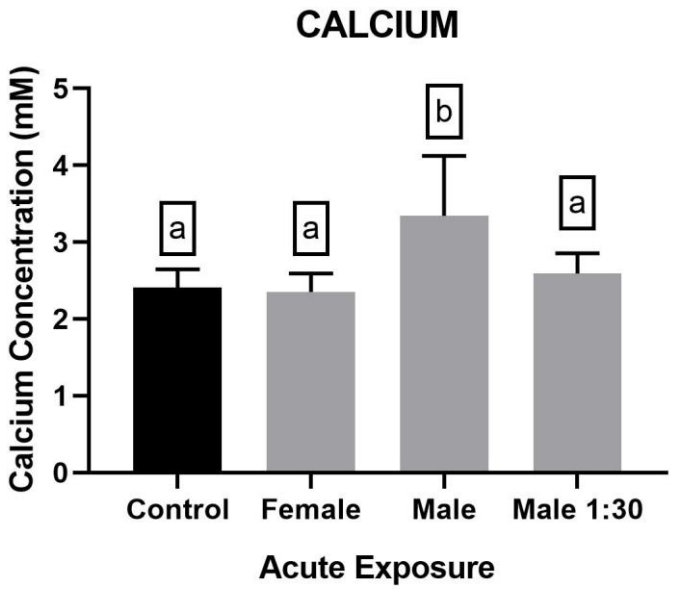

Figure 4.23 – Obtained calcium concentration for the acute exposures. N=7-8. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

2.2.5. *Sodium and Potassium*

In the acute exposures, the sodium and the potassium followed the same trend, presenting higher values on the male, followed by the female, and the lowest values on the 1:30 treatment. The significant differences of the sodium concentration were present between the control and both the male and 1:30 treatments, and between the 1:30 and both the female and male treatments. The potassium concentration presented significant differences only between the control and male treatments, and between male and 1:30 treatments.

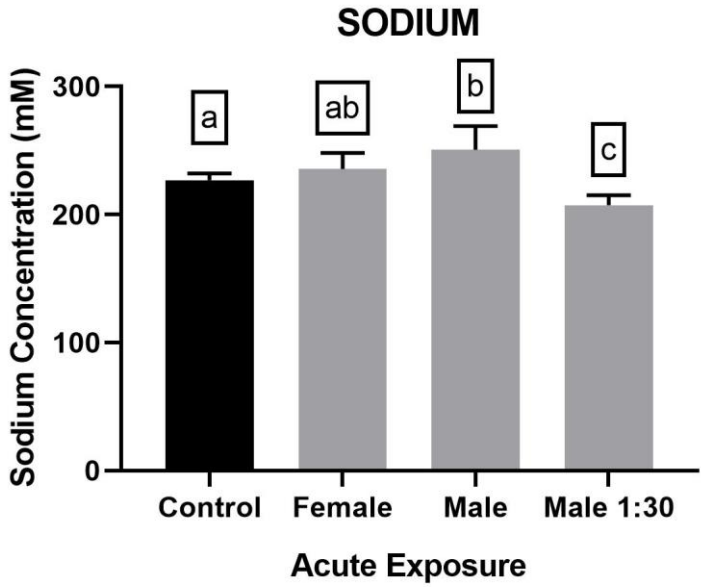

Figure 4.24 – Obtained sodium concentration for the acute exposures. N=6-8. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

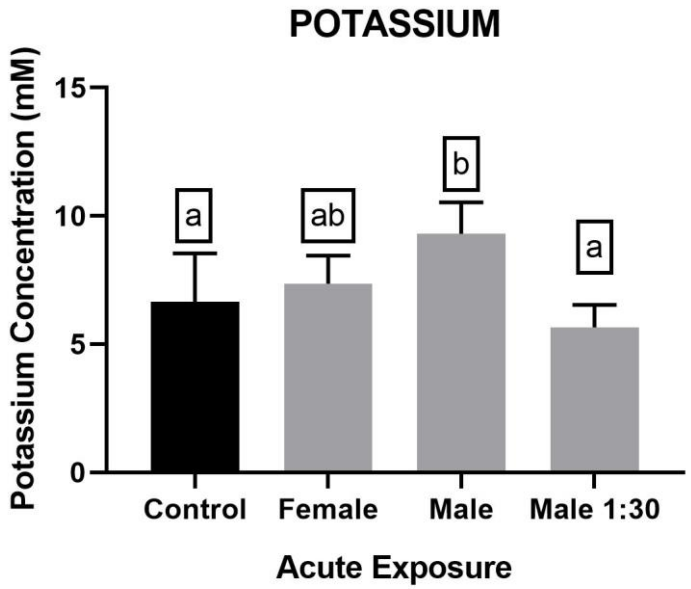

Figure 4.25 – Obtained potassium concentration for the acute exposures. N=6-8. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

2.3. *Ammonia*

During the acute exposures, the ammonia concentration increased from the control to the female and male treatments but decreased in the 1:30 treatment. The significant differences were present between both the female and male treatments and the control, between the female and male treatments, and between both the female and male and the 1:30 treatment.

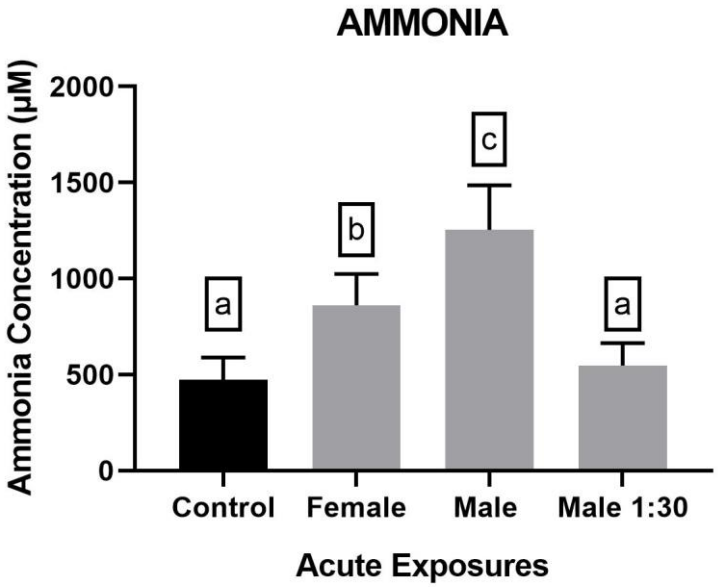

Figure 4.26 – Obtained Ammonia concentration for the acute exposures. N=6-8. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

It is worth point out that before starting the acute exposures, samples of the water were collected, to allow the measurement of the ammonia concentration in the water. Both the water used for the control, and the female and male "toadfish waters" had an ammonia concentration lower than 70 µM.

3. *Na K-ATPase activity*

The resultant total activity and Na K-ATPase activity, after exposure to "toadfish water" are presented on figures 4.27 and 4.28, respectively. When it comes to the total activity, the female treatment was very similar with the control. The only clear difference was with the male treatment, which was statistically significant, with both the control and with the female treatment. The Na K-ATPase activity presented clearer differences between both the treatments and the control. The statistical differences were the same as the total activity.

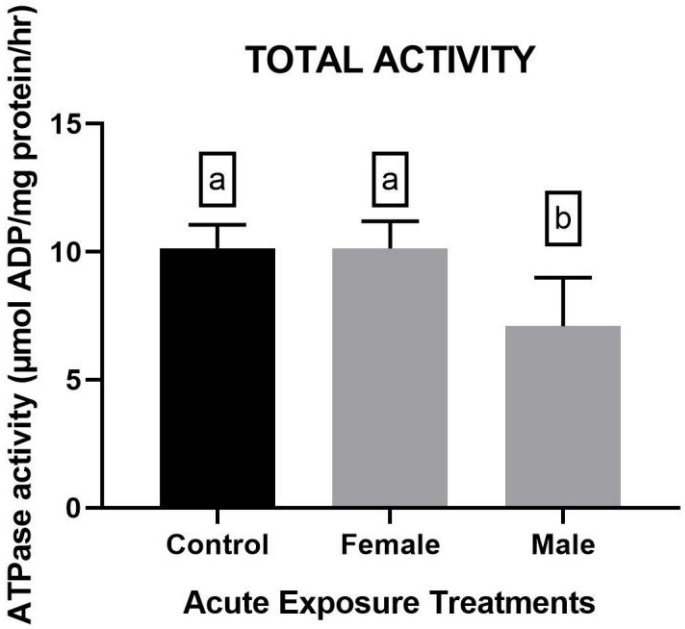

Figure 4.27 –Total activity of ATPase of seabream when exposed to the acute exposures. N=8. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

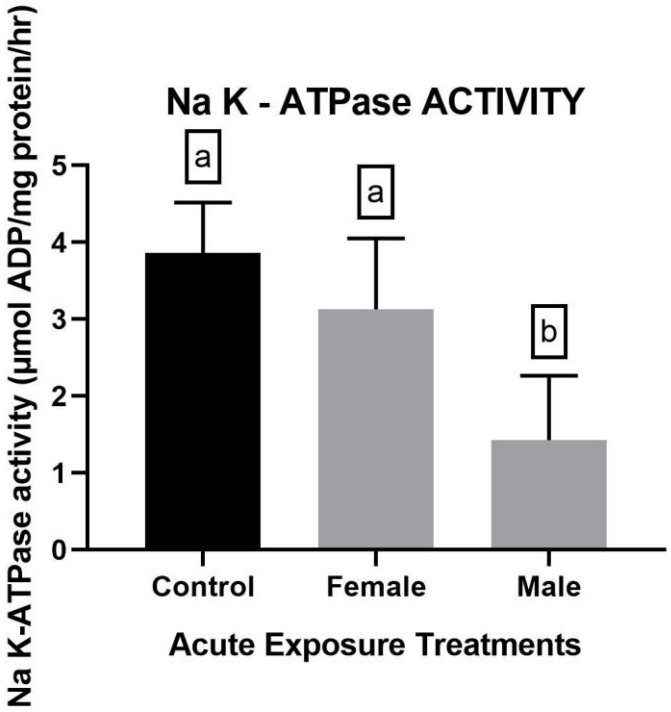

Figure 4.28 – Na K-ATPase activity of seabream when exposed to the acute exposures. N=8. One-way ANOVA (p-value < 0,05). Mean ± Standard deviation.

4. *Behavioural Analysis*

The observed behaviours that were observed during the behavioural analysis is described on table 4.3.

Table 4.3 – Observed behaviours of *S. aurata* during the acute exposures.

	Initial Phase	Exposure
Control	When the sea bream individuals were first placed on the new tanks used for the experiment, they immediately demonstrated erratic behaviours, like fast movements in and outside the water.	After the first minute, the individuals placed in the tanks tended to calm down, stopping the erratic behaviours. Until the end of the exposure the individuals tended to swim calmly or stay stationary.
Female	Due to the stress of being placed in a new tank, the individuals exposed to female "toadfish water" also demonstrated fast movements in and outside the water.	Like the control exposure, after the first minute the individuals used for the female "toadfish water" treatment calmed down and from here on they swam calmly or stayed stationary.
Male	Like the control and female treatments, when the sea bream used for the male "toadfish water" treatment were placed in a new tank, they presented the same erratic behaviours.	After the first minute the individuals used for the male "toadfish water" treatment calmed down, however around two minutes of exposure, the individuals started to demonstrated spasms, loss of equilibrium, and eventually immobility with no apparent opercular movements. The average time of occurrence of these behaviours is present on figure 4.29.

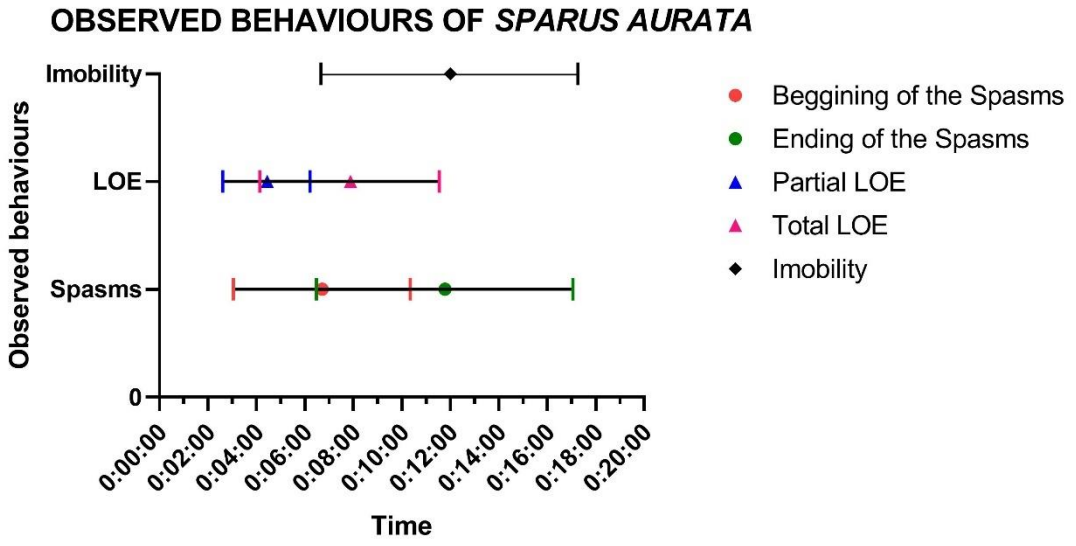

Figure 4.29 – Average time of occurrence of the observed behaviours of *S. aurata* when exposed to male "toadfish water". Mean ± Standard deviation.

5. *Oxy, deoxy, methaemoglobin*

As it can be seen on figures 4.30 to 4.36, during the several chronic and acute exposures, the formation of deoxyhaemoglobin and methaemoglobin did not occur. Nevertheless, it was possible to compare the obtained spectrums by observing the differences between the two peaks and the valley presented in the graphs. In this case to allow the study of the 1:30 and male spectrums, these were compared to the other two controls of the chronic exposures. According to the statistical analysis, when it comes to the first peak (between 500 and 550nm) the only statistically significant differences were between the 1:100 control, 1:100, male treatments and the 1:30 treatment. The second peak (between 550 and 600nm) presented the same significant differences. In relation to the valley (between 550and 600nm) the significant differences were once again equal to the first peak. The average values for these three variables were present in table 4.4.

Table 4.4 – Average values for the two peaks and valley of the haemoglobin spectrums.

	First Peak	Second Peak	Valley
Control 1:1000	1,60 ± 0,20$^{\underline{abc}}$	1,76 ± 0,20$^{\underline{abc}}$	0,67 ± 0,10$^{\underline{abc}}$
1:1000	1,60 ± 0,23$^{\underline{abc}}$	1,77 ± 0,24$^{\underline{abc}}$	0,65 ± 0,10$^{\underline{abc}}$
Control 1:100	1,34 ± 0,38$^{\underline{ab}}$	1,47 ± 0,41$^{\underline{ab}}$	0,55 ± 0,15$^{\underline{ab}}$
1:100	1,26 ± 0,54$^{\underline{ab}}$	1,41 ± 0,60$^{\underline{ab}}$	0,50 ± 0,22$^{\underline{ab}}$
1:30	2,02 ± 0,16$^{\underline{ac}}$	2,17 ± 0,14$^{\underline{ac}}$	0,84 ± 0,08$^{\underline{ac}}$
Male	1,41 ± 0,13$^{\underline{ab}}$	1,52 ± 0,13$^{\underline{ab}}$	0,59 ± 0,05$^{\underline{ab}}$

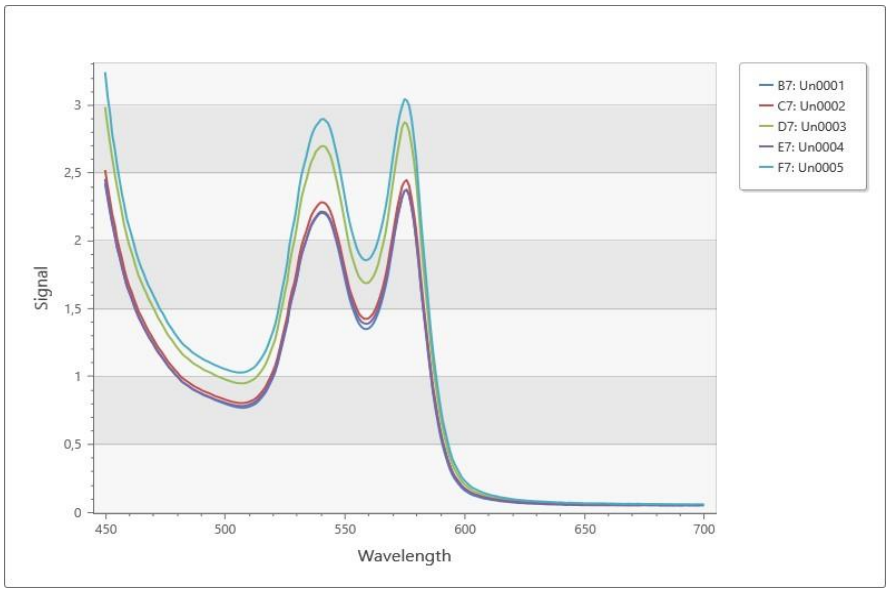

Figure 4.30 – Haemoglobin spectrum of the control of the 1:1000 treatment of the chronic exposures.

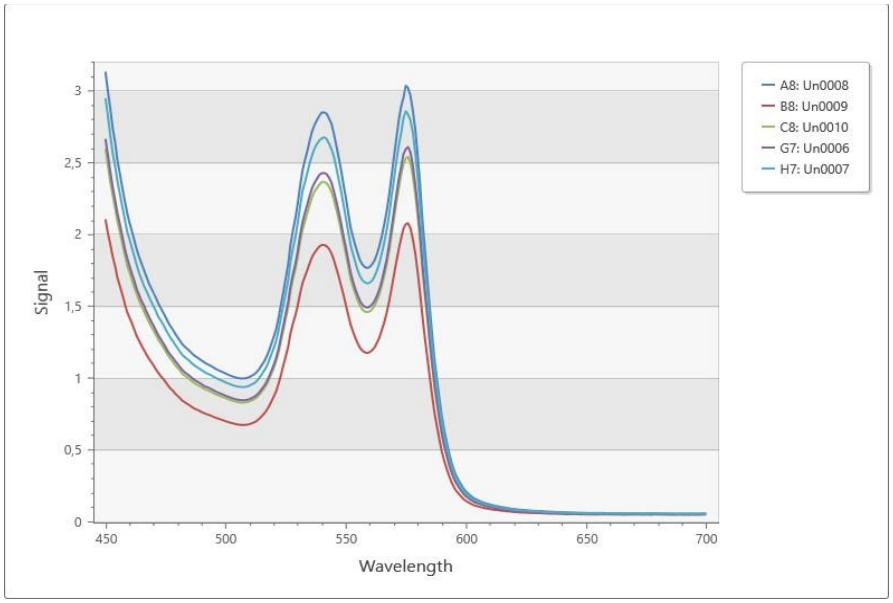

Figure 4.31 – Haemoglobin spectrum of the 1:1000 treatment of the chronic exposures.

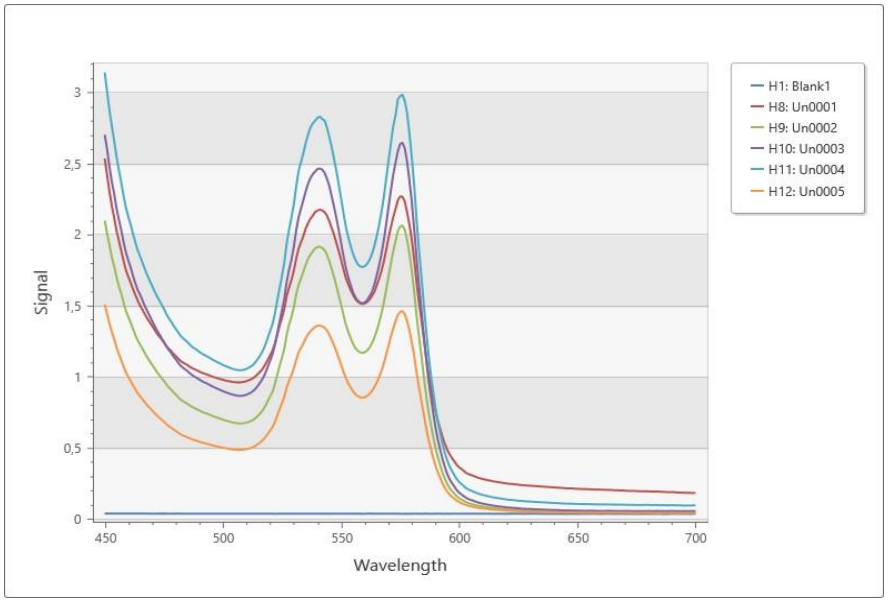

Figure 4.32 – Haemoglobin spectrum of the control of the 1:100 treatment of the chronic exposures.

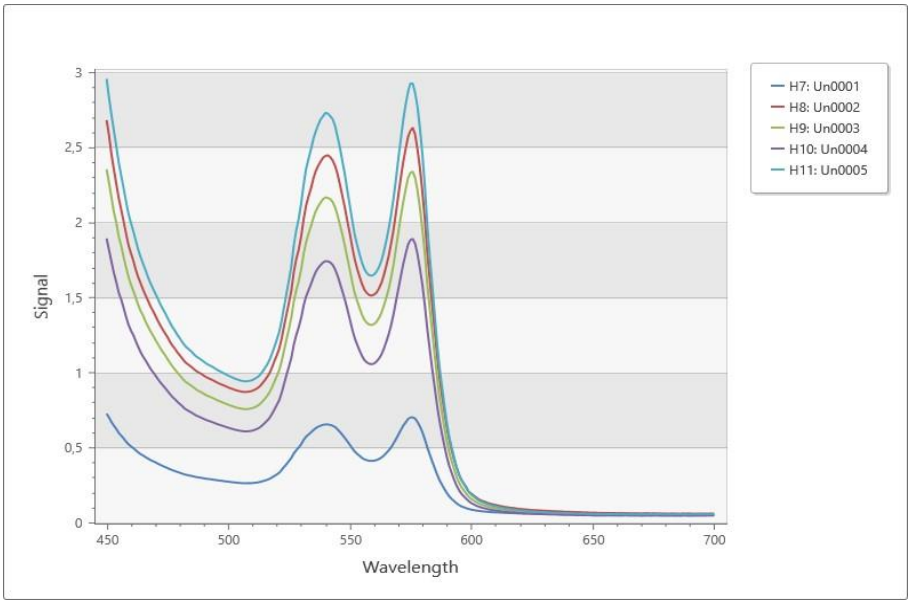

Figure 4.33 – Haemoglobin spectrum of the 1:100 treatment of the chronic exposures.

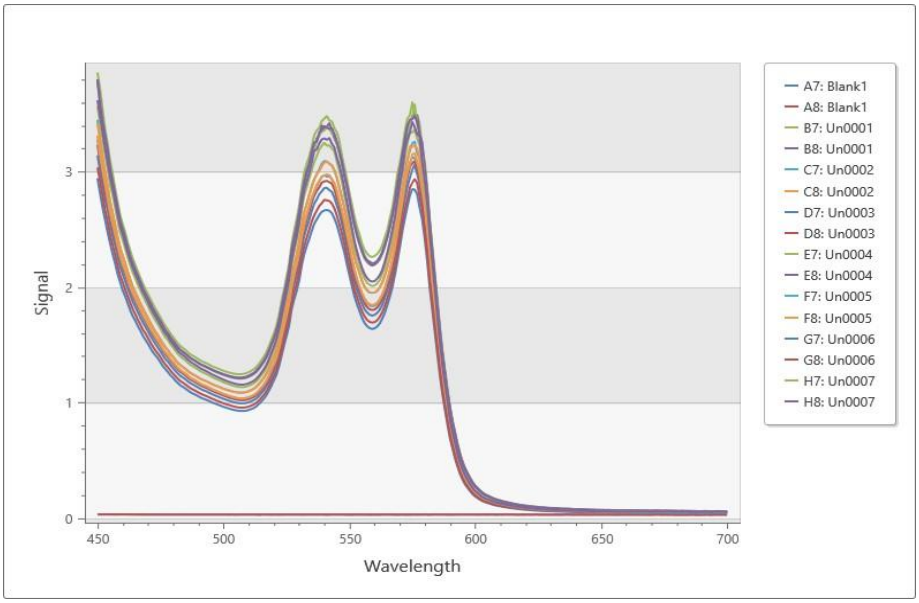

Figure 4.34 – Haemoglobin spectrum of the 1:30 treatment of the transition exposure.

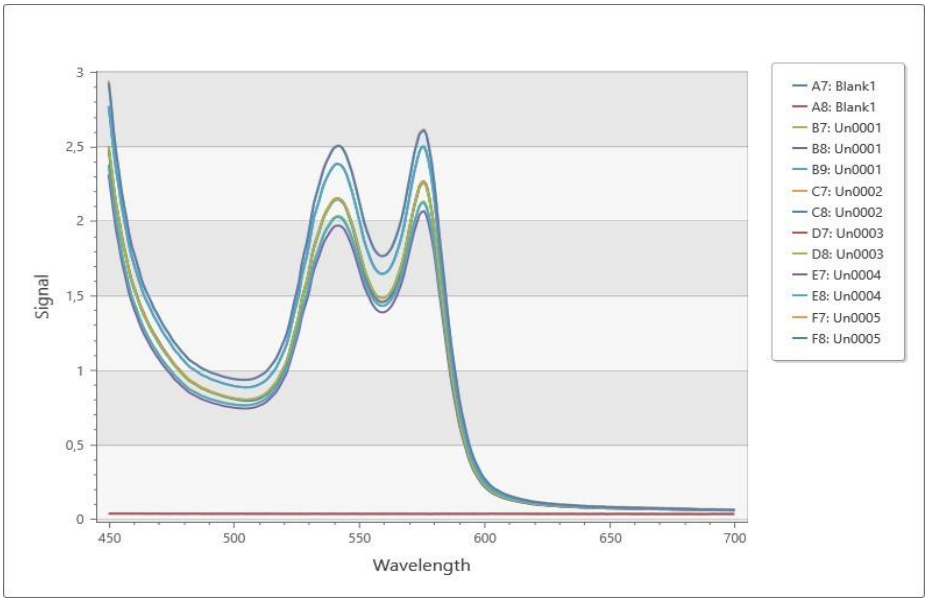

Figure 4.35 – Haemoglobin spectrum of the male "toadfish water" of the acute exposures.

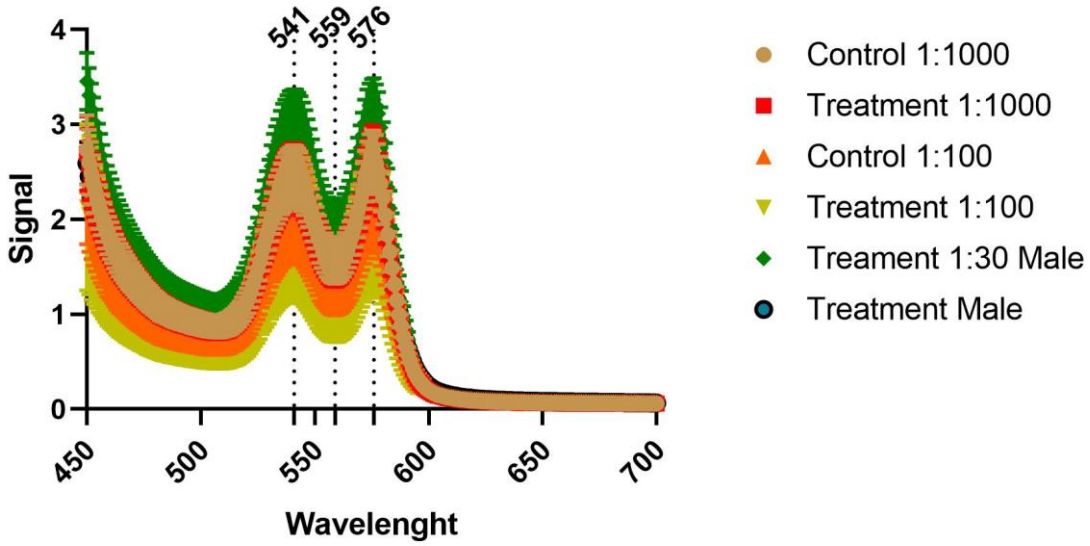

Figure 4.36 – Haemoglobin spectrum of all the treatments.

1. *Haemolytic activity*

The haemolytic activity of the toadfish toxin on sea bream blood is presented on figure 4.37. As previously mentioned, the dilutions used on this experiment were prepared based on the lowest protein concentration measured by the Bradford method. In this case that concentration corresponded to 9mg/ml, and therefore, the dilutions used, 1:1, 1:10, 1:100, 1:1000, 1:10000, correspond to 9,00; $9{,}00\times10^{-01}$; $9{,}00\times10^{-02}$, $9{,}00\times10^{-03}$, $9{,}00\times10^{-04}$, respectively. Both the Female and Male extract cause haemolytic activity as the protein concentration increases., with the main statistical difference being in the $9{,}00\times10^{-03}$. The protein concentration that causes 50% haemolysis (HA_{50}) was 6.8×10^{-03} mg/mL for the males and $7{,}6\times10^{-03}$ mg/mL for the females (these values were calculated using the AAT Bioquest ED50 Calculator).

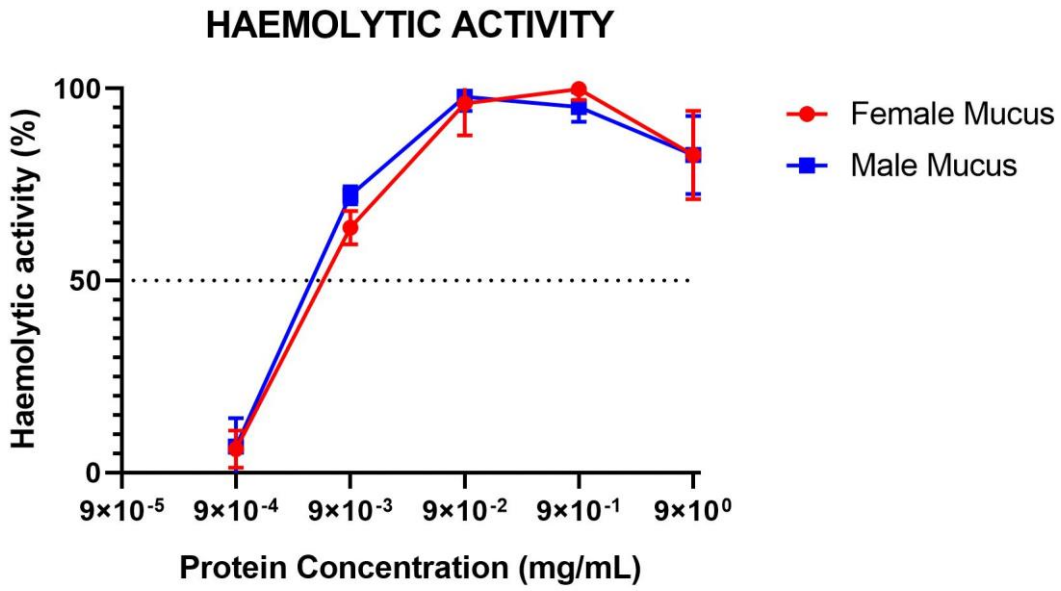

Figure 4.37 – Haemolytic activity of the toadfish toxin on the blood of sea bream.

2. *Olfactory Nerve Recording*

The effects of the "toadfish water" and both the female and male "toadfish water" over the olfactory nerve are present on figures 4.38, 4.39 and 4.40.

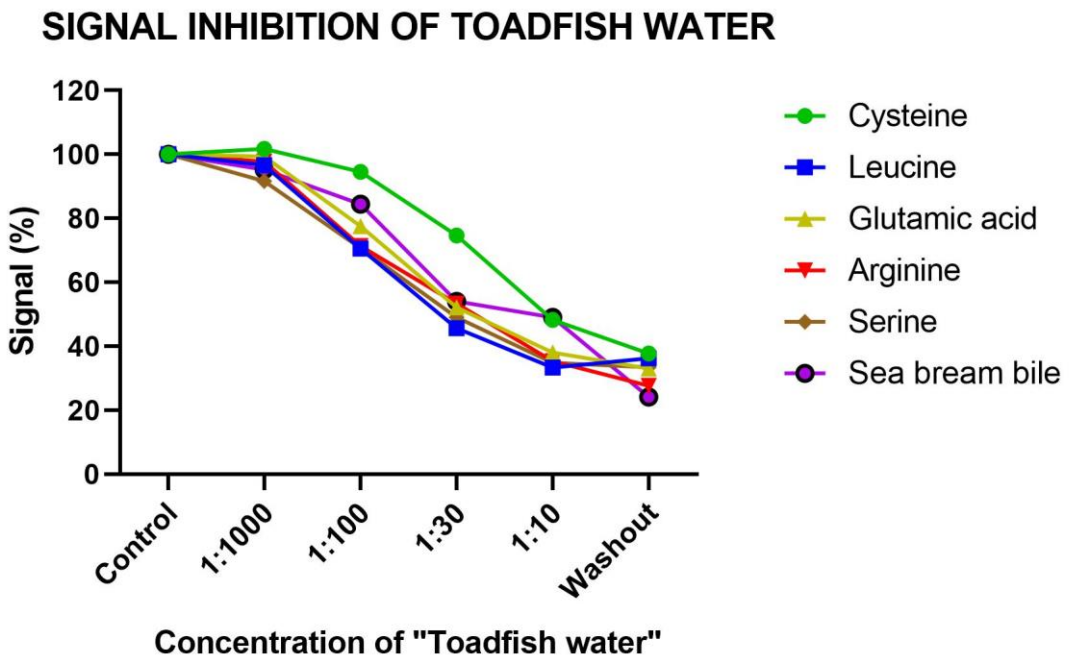

Figure 4.38 - Effect of the Toadfish toxin on the olfactory nerve of sea bream upon exposure to different dilutions of the "toadfish water" (produced with a mixture of both males and females). N=10.

It was possible to observe that overall, the exposure to the toxin leads to decrease of the signal to all the stimuli, which confirms that exposure to the "toadfish water" affects the function of the olfactory nerve of the exposed organisms.

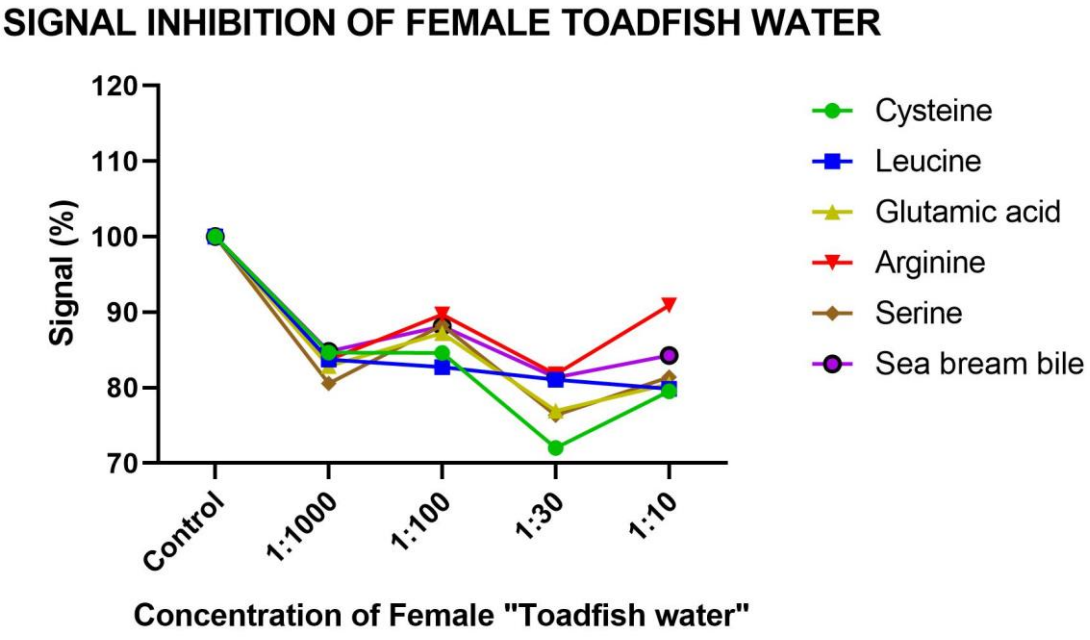

Figure 4.39 - Effect of the female "toadfish water" on the olfactory nerve of sea bream upon exposure to different dilutions of "toadfish water" (produced with females). N=4

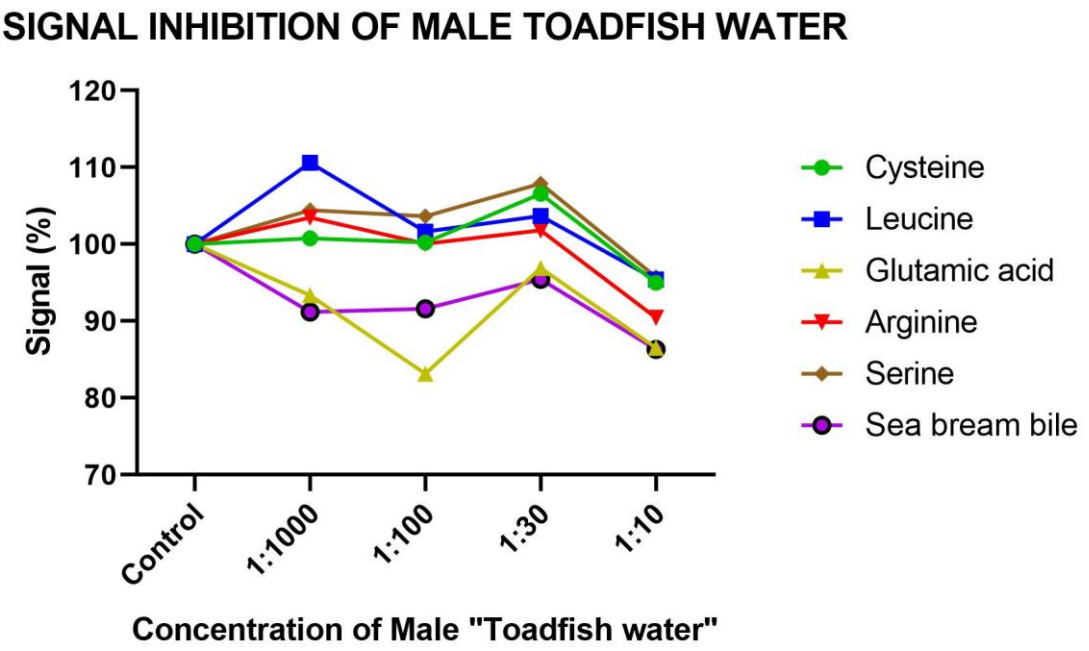

Figure 4.40 - Effect of the male "toadfish water" on the olfactory nerve of sea bream upon exposure to different dilutions of "toadfish water" (produced with males). N=4

When it comes to the female and male "toadfish water" it was also possible to see that in both cases there was also a decrease of the signal produced for the different stimuli, although it was in a smaller, and more irregular way than the regular "toadfish water". Another thing that was observed was the fact that the inhibitions produced by the female and male "toadfish water" did not reach the same level as the regular "toadfish water".

3. *High Performance Liquid Chromatography (HPLC)*

After performing the HPLC, chromatograms of the "toadfish water" are presented on figures 4,41 and 4,42. The chromatograms of the mucus were obtained and are presented on figures 4.43 and 4.44.

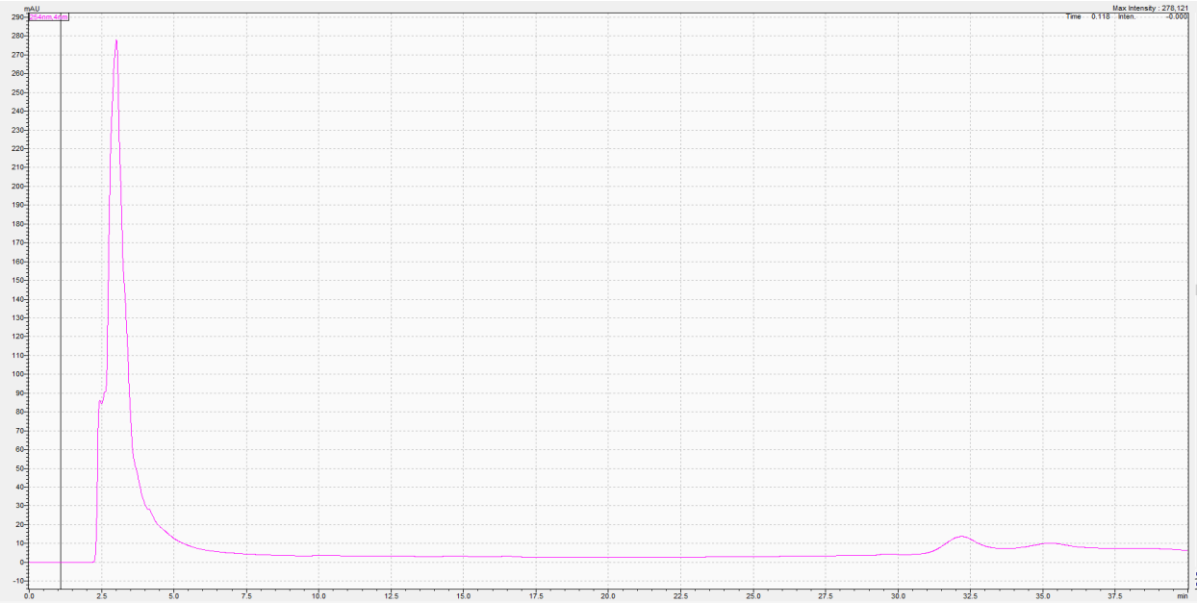

Figure 4.41 – Chromatogram o the female toadfish mucus.

Figure 4.42 – Chromatogram of the male toadfish mucus.

When comparing the obtained chromatograms of both the female and male mucus, it was possible to observe differences in the area and the retention time of the peaks. In the mucus of the female there were two initial peaks at times 2.4 min (area=829587) and 3.0 min (area=11708716). At the end of the HPLC run, there were two more peaks at time 32.2 min (area=593452) and 35.2 min (area= 233196). In the mucus collected from males there were three initial peaks at time 2.42 min (area=2220420), 2.6 min (area=1852454) and 2.9 min (area=8362301). At the end, two more peaks were observed at times 32.0 min (area=768580) and 35.5 min (area=694705).

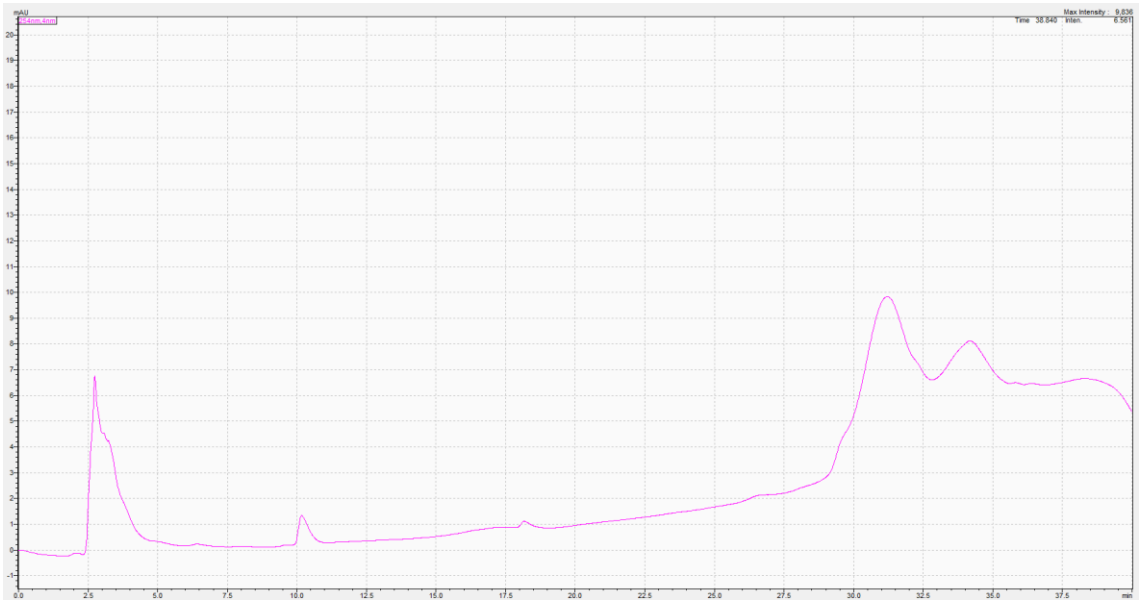

Figure 4.43 – Chromatogram of the female “toadfish water”.

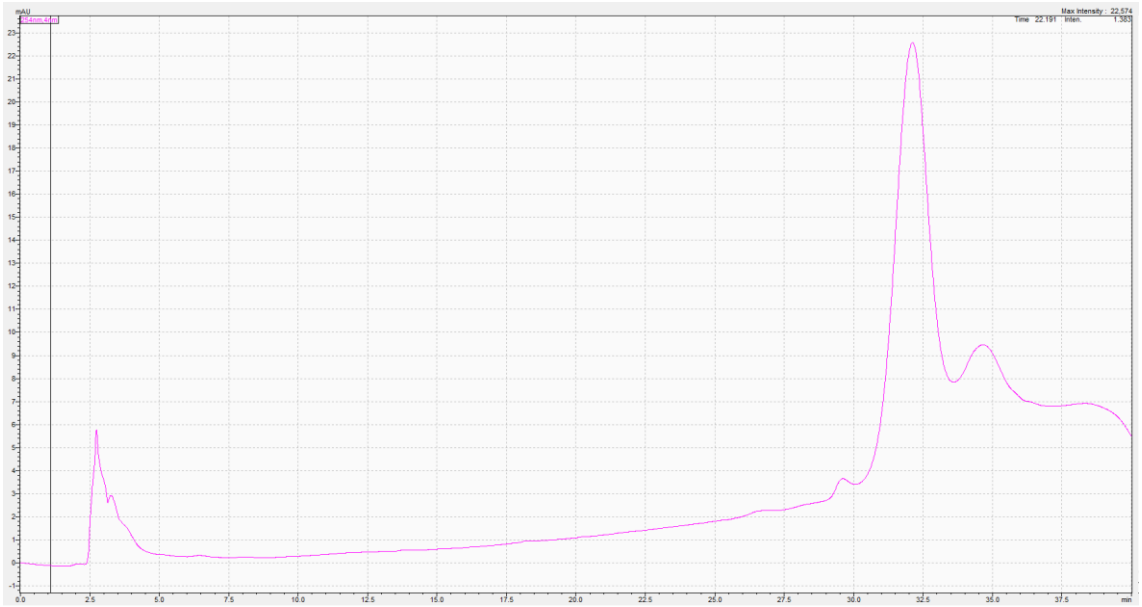

Figure 4.44 – Chromatogram of the male “toadfish water”.

Like the mucus, the “toadfish water” of males and females also showed some differences in the area and retention time of the peaks. The most evident differences were the presence of a peak at retention time 10.2 min in females. The area of the later peak at retention time 32.1 min was three times bigger in the males (area=1545338) than in the females (area=502862).

5. Discussion

To evaluate the effect of the putative toxin produced by the toadfish species *H. didactylus*, different experiments *in vivo* and *in vitro* were performed, with the objective of analysing a large array of parameters, to understand how the toxin acts upon the exposed individuals.

The *in vivo* experiments were divided based on the concentration of "toadfish water" in which the toxin is present. Initially, small concentrations were tested, which allowed for longer experiment durations, and therefore were considered chronic exposures to the toadfish toxin. Afterwards, pure female and male "toadfish water" were tested in high concentrations, which lead to shorter experiment durations, and were considered acute exposures. One additional exposure was performed, with an intermediate concentration to serve as a transitional exposure between the chronic and the acute exposures.

Impact of chronic "toadfish water" exposure on sea bream physiology

Haematological impacts

During the chronic exposures, several haematological parameters, like haematocrit, haemoglobin, red blood cell counts and related indices, were observed. When it comes to the results obtained for the haematological parameters, these present few significant differences, which indicates that the increase of the "toadfish water" concentration from 1:1000 to 1:100 used in the chronic exposure, of up to seventeen and five days, respectively, doesn't seem to affect the haematological parameters. Published haematocrit values in sea bream varied from close to 25% (Carbonara et al., 2019) to upwards of 50% (Fazio et al., 2012), which is the range in which the haematocrit values obtained during this experiment are inserted. Similarly, the haemoglobin range observed in previous studies seems to be between 7,5 g/dL (Fazio et al., 2012) to 12 g/dL (Carbonara et al., 2019), which once again is the range in which the obtained results are present. Red blood cell counts seem to fall between around 2×10^6 cells/mm^3 (Carbonara et al., 2019) to around 3,5 cells/mm^3 (Fazio et al.,2012). In an experiment performed by Fazio et al., 2015 the obtained values for MCV, MCH, and MCHC varied in a range between 92,00 to 168 fL, 12,63 to 36,11 pg, and 13,33 to 28,90%, with the average values being 149,30 ± 17,79 fL, 33,40 ± 4,17 pg, and 22,61 ± 3,33%, respectively. Like the previously mentioned parameters, the obtained values for these indices in this experiment fall within the ranges considered to be normal .These differences can most likely be

justified due to the great variability of these indicators since they can be affected by several parameters, related to the individuals (like sex, size and diet), related to the sampling procedures, and environmental condition (like salinity, temperature, nitrate, and dissolved oxygen) (Michail et al., 2022).

Osmoregulation parameters

Plasma electrolytes are usually kept within tight limits as some may have profound effects on cell physiology, impacting the renal, cardiovascular and neurological functions. Values outside these limits often indicate inability of the system to maintain homeostasis due to abnormal function of one or several organs, namely liver and kidney, caused by disease or toxic exposures (Greenwell et al., 2003). We did not observe any obvious changes in locomotory or respiratory behaviour between the control and the treatment fish, at either concentration of "toadfish water" and overall plasma electrolytes do not indicate reduced osmoregulatory function.

The phosphorous concentration presents an increase as the "toadfish water" increases, however, so does the control, and since the difference between the 1:100 control and treatment is not significant, it is safe to assume that the "toadfish water" did not cause an effect. In fact, phosphorus concentrations seem to fall within a range between 3,23 to 4, 59 mM (Peres et al., 2012), which is slightly high but still considered to be normal, so even though the 1:1000 exposure presents smaller values, there is no difference between the control and treatment. The calcium concentration presents a decrease from the control to the treatment in the 1:1000 exposure, however, the treatment values do not differ from those of the 1:100 exposure. Therefore, it doesn't reflect an effect of the "toadfish water". Additionally, the obtained values fall within the 3,3 to 4,5 mM range, which, like phosphorus, are slightly elevated but still considered to be normal (Peres et al., 2012). Like the phosphorus, the magnesium concentration also presents an increase from the 1:1000 to the 1:100 exposures, although not significant. In this case, the control does not suffer any alterations, nevertheless, this increase of the magnesium concentration is relatively small and, doesn't appear to be biologically significant, since normal magnesium concentrations can be within 0,87 and 1,69 mM (Peres et al., 2012). When it comes to the potassium concentration although there is a decrease from the control on the 1:1000 to the 1:100 exposures, the concentrations obtained from the treatments aren't significantly different. Therefore, despite the control and the treatment from the 1:1000 exposure being different, the "toadfish water" does not appear to have an effect, especially

since the values considered to be normal can fall between 3,00 (Gallardo et al., 2003) to 10,2 mM (Guardiola et al., 2018). Potassium levels in plasma are relatively low when compared to their concentration within blood cells. The results are therefore in agreement with a low haemolysis rate, which would be a possible effect of some toxins, which cause blood cell bursts and the consequent release of potassium into the plasma (Mirghaed et al., 2017).

The extracellular fluid is mostly composed by water and sodium and chloride ions, which account for about 90% of the impact on plasma osmolality (Field et al., 1943). Large changes in these ions are likely to cause important uncontrolled exchange of water across cells membranes with deleterious effects and their presence in the plasma is one of the factors controlling renal function and renal regulation (Greenwell et al., 2003). The chloride concentration suffers a decrease from the 1:1000 to the 1:100 exposures, but so does the respective control, and therefore there doesn't appear to be an effect of the "toadfish water" on the chloride concentration, and normal chloride concentration values seem to be on average close to 150 mM (Laiz-Carrión et al., 2005; Miguel Mancera et al., 2002). Despite there being a slight variation from the 1:1000 exposure to the 1:100, it happens both on the treatment and on the control and therefore, the sodium concentration does not seem to be affected by the "toadfish water". On average the normal sodium values seem to be around 180 mM (Laiz-Carrión et al., 2005; Miguel Mancera et al., 2002) which is close to the obtained values. This likely contributed to the maintenance of normal osmolality, close to 300-350 mosmol/Kg (Wang and Kültz, 2017), which does not pose an extra load on the energy requirement to maintain osmoregulation.

Energy and metabolism related indicators

Alongside the study of the haematological parameters, several plasmatic indicators were also observed, like glucose, lactate, electrolytes, ammonia, and LDH. According to the statistical analysis, neither the glucose nor lactate suffer significant changes when the concentration of "toadfish water" increases, despite the concentration of both being slightly smaller on the 1:100 exposure. However, the concentration decreases also occurs in the control, with the controls from the 1:100 exposure presenting smaller values than those of the 1:1000 exposure. In 2012, Peres et al, obtained glucose values in the range of 3,27 to 10, 88 mM with an average of 6,01 mM (Peres et al., 2012). Although the obtained values for the glucose on the chronic exposures are close to the bottom end of this range, there doesn't seem to be an effect on the glucose concentration due to chronic

exposure to the “toadfish water”. The lactate concentration can vary from around 1mM (Arends et al., 1999) to close to 6,15 mM (Fazio et al., 2015). This parameter is often an indicator of metabolic changes that uses increased anaerobic cell respiration, and increased values are considered a sign of cell stress when the animal is exceeding its normal aerobic scope. Once again, the obtained results for the chronic exposures fall within the range that can be considered normal, thus suggesting no impact of the fish metabolic pathways. This seem to be confirmed by the values of plasma lactate-dehydrogenase, usually used as an indicator of increased glucose and lactate metabolism, and eventually tissue damage (Agrahari et al., 2007). The LDH concentration of the treatments suffers a decrease from the 1:1000 to the 1:100 exposure, but once again so do the controls. Since there are no significant differences between the treatments and their respective control, there doesn’t appear to be an effect caused by the “toadfish water”, and the possible range that can be considered normal values is between 129 and 1528 U/L, with an average of 618,5 U/L (Peres et al., 2012). Ammonia is the major product of protein and amino acid break down, and thus elevated values in circulation are relevant as indicators of increased metabolism, either as an alternative source of energy or as the result of tissue damage. On the other hand ammonia is highly toxic for cells and has important neurological effects, disturbing cognition, behaviour and ultimately impairing the connection between neuronal signalling and muscular action, leading to death (Ip and Chew, 2010). Thus, ammonia poisoning must be checked either as a result of its direct toxicity due to elevated concentration in water or if any toxic substance eventually present in “toadfish water” interferes with the normal ammonia excretion pathways, leading to accumulation and internal toxicity. However, the ammonia concentration, presents a decrease from the 1:1000 control to the 1:100 control, while the treatment concentration slightly increases. Nevertheless, the differences between the treatments and the 1:100 control and treatment are not significant, which indicates that there is no effect caused by the “toadfish water”, plus on average the considered normal values are around 643 μM (Oliva-Teles et al., 2006).

In conclusion, the analysis of several blood and plasma indicators do not show any effect of exposure to “toadfish water” that could be compatible with the striking results observed in previous assays and that spurred this experimental work. However, this chronic exposure approach differed from those assays in the concentration of toadfish water used. Thus, these low concentrations, of up to 1:100 of “toadfish water” were not enough to

clearly modify the physiology, even for a prolonged period. However one cannot exclude that the fact that these fish were allocated in closed system fitted with filters may have reduced the potency of the eventual substances in toadfish water, although one can also consider that such substances could have also deleterious impacts on the biological filters, depending on their nature.

Acute effects of male and female "toadfish water"

Haematological parameters and haemolytical action of "toadfish water"

When it comes to the acute exposures, the only haematological parameters measured were the haematocrit, blood pH, osmolality, haemolysis, and the effect on the shape of the red blood cells. The haematocrit values obtained for acute exposures, present a clear decreasing trend from the control to the treatments using "toadfish water", with the male treatment being the lowest. This may be due to the fact the male treatment not only presents the largest haemolysis, but also the largest plasmolysis percentage, leading not only to a fewer number of red blood cells, but also a decrease in the cell area, and therefore a smaller haematocrit value. However, the acute exposures, present overall larger haematocrit values than the chronic exposure, possibly due to red blood cell swelling (Hrubec and smith, 2010), that can be caused not only due to the stress caused by the acute exposure design, since the fish had no time to acclimatize to the tanks, and these were relatively small tanks compared to the size of the fish used, but also due to the presence of higher concentrations of "toadfish water", with the exception of the 1:30 exposure since this was done on a larger tank. Osmolality and almost all electrolyte levels were also elevated which may indicate loss of water from plasma, thus contributing to an increased haematocrit. Nevertheless, the obtained haematocrit values, still seem to be within the previously mentioned range. When observing the blood smears, the main result that can be observed is the difference in cell plasmolysis. The male treatment presents a plasmolysis percentage of close to 90%, which in turn leads to alterations in the morphology of the cell. These alterations can be explained by the increase of the concentration of plasma electrolytes, which forces the water to diffuse out of the blood cells.

The treatment had no impact on haemoglobin profiles. Besides the previously mentioned haematological parameters, the haemoglobin spectrum was also observed, for both the chronic treatments and the acute male and 1:30 treatments. When the obtained results are

compared to figure 3.1, it is possible to observe that there was no transformation of the haemoglobin, being always present in the oxyhaemoglobin form, with consistency of peaks at the same wavelengths, but at variable concentrations, given by absorbance values at peak wavelength. The changes obtained in the height of the peaks or valleys are related to variations of haemoglobin concentration between the individuals used for the exposure, and overall, do not show average differences between test groups. This indicates that neither in low nor high concentrations, the putative toadfish toxin does not appear to have an effect on the form and quantity of haemoglobin.

When it comes to the haemolytic capability of the toadfish toxin it is possible to observe that both the "toadfish water", in which the toxin is believed to be present, and the toadfish mucus are both able to induce haemolysis in the *S. aurata* red blood cells. As it can be seen in figures 4.17 and 4.37 both the female and male "toadfish water" present a similar ability to cause haemolysis, although in figure 4.17 there is a slight difference, with higher haemolysis in the male treatment, while in figure 4.37 this capability does not seem to be that different. These differences can be explained by the fact that the procedures used to obtain these values were different. The haemolysis present in figure 4.17 was obtained by measuring the plasma collected after the acute exposures were performed, while the haemolytic activity, in figure 4.37, was measured by directly exposing the *S. aurata* blood to *H. didactylus* mucus. One of the more known causes of haemolysis is the presence of phospholipase A_2 in the venom of a few animals, however, this component is not found in fish venom (Ziegman and Alewood, 2015). A great number of studies performed on several fish species the toxic factor responsible for haemolysis revealed a great diversity of toxins, which seem to vary among the fish species, with each one having its own acting mechanism, with the severity of the effect being dependent on the target species. Nevertheless, the type of toxin studied in this report seems to be more related to the ichthyocrinotoxins, which contain different components than the regular fish toxins (Ziegman and Alewood, 2015). The more well-known ichthyocrinotoxins can be grouped into polypeptide surfactants, steroid glycosides, cationic surfactants, and nonionic surfactants (Abdul-Haqq and Shier, 1991). However, without knowing the composition of the *H. didactylus* mucus, which appears to be where the toxin might be found, it is difficult to identify to which category it might belong, or even which is the haemolytic mechanisms associated. Preliminary data from the High Performance Liquid Chromatography (HPLC) shows the presence of relevant differences between the

composition profiles of the mucus retrieved from male and female toadfish, either in quantity or quality of specific, yet unidentified, substances, that can be involved, and responsible for, the variability of the impacts mentioned above.

Osmoregulatory impacts

Normal osmolality values seem to be around 370 mOsm/kg (Barton et al., 2005; Vargas-Chacoff et al., 2020). The obtained values from the acute exposures seem to be higher than this value, with the exception of the control and the 1:30 exposure which appear to be close. Since osmolality is related to the concentration of electrolytes, especially sodium and chloride, the increase in osmolarity can be explained by the overall increase of the concentration of all electrolytes measured in fish exposed to male "toadfish water", which may be due to the accumulation of ions released from cells and tissues, or by loss of water from plasma into the tissue or the environment.

When it comes to the phosphorous concentration, the only treatment that is out of the normal range is the male treatment, which suffers an increase of phosphorus concentration. This can be explained by the fact that fish erythrocytes contain high concentrations of phosphorus, that when haemolysis occurs, can be released into the plasma (Mirghaed et al., 2017). It is not only the phosphorus concentration that might change due to haemolysis. The chloride and potassium concentrations can also suffer the same trend as phosphorus, since erythrocytes also present higher concentrations of both chloride and potassium, which, again, when haemolysis occurs are released into the plasma (Mirghaed et al., 2017). It is also in the male treatment that the concentration of both chloride and potassium are at a maximum, with the chloride concentration being higher than 150 mM, but the potassium concentration being within the normal interval. With the exception of the 1:30 treatment, every other treatment has a much higher sodium concentration than 180 mM. A possible explanation for the increase of sodium, which can also justify the increase of chloride can be the fact after physical exercise, water moves out of the plasma into the cell, due to a shift of the osmotic gradient, due to the lactate accumulation in the cells (Wood et al.,1983). This seems to happen in all the acute treatments because when the fish are placed in tanks used for the acute exposure, they tend to react abruptly since they did not have time to acclimate to these tanks. Overall, the magnesium concentration seems to be above the normal range, however, the great difference is observed mainly in the male treatment. The calcium concentration presents a similar trend, except, in this case, all the treatments, except the male have lower

concentrations than the normal range, however, the male still presents a maximum, when compared to the other treatments. This can possibly be explained by an increase of gill permeability during the stress caused by the exposure to male "toadfish water" which allows for an influx of both magnesium and calcium since seawater concentrations of both magnesium and calcium are higher than those of the plasma. Although the concentration of magnesium is higher than that of calcium, and therefore, the increase of magnesium in plasma is much more pronounced than the increase of calcium (Redding and Schreck, 1983).

Energy balance and metabolism and pH levels

The glucose concentration of the acute exposures appears to be within the previously normal range of values, however, there is a decrease in the male "treatment". When it comes to the lactate concentration, although there is an increase in both the female and male treatments, only the male treatment is significantly elevated out of the previously mentioned range. The decrease of glucose and increase of lactate can be once again explained by the increase of physical activity during the male treatment. During this exposure fish presented increased locomotory activity, and showed what appeared to be uncontrolled movements, spasms, which could result from involuntary muscle contractions that may have led to increased anaerobic respiration, and therefore exhaustion of glucose, and increased production and use of lactic acid, converted into lactate in solution. Such behaviours lasted for 5-15 min in average, as indicated on figure 4.29 describing the behavioural analysis, and were only observed in the fish exposed to male water, which confirms the differences in composition of the released substance seen in the mucus chromatograms.

The ammonia concentration obtained during the acute exposures, shows an increase in both the female and male treatments, while both the control and the 1:30 treatment, do not seem to be much different from the normal value, in comparison to the other treatments. Although the increase in ammonia concentration in the female treatment could be explained by the fact that the concentration of ammonia already present in the female "toadfish water", was the highest in all the treatments, and it was shown that *S. aurata* can accumulate ammonia in plasma, as environmental ammonia concentration also increases (Le Ruyet et al., 1998). However, the fish in the male treatment show an even greater and significant ammonia peak, which can be explained by the fact that during physical exercise, which happened in this treatment, ammonia can be produced from the

catabolism of amine products (including amino acids with energetic properties) due to the deamination of adenosine monophosphate present in the skeletal muscles (Ip and Chew, 2010). This may also indicate that the channels used to excrete ammonia are impaired in these fish, which may correlate with the increased ion concentration, namely sodium, as both ions, NH4 and Na, can be exchanged by the membrane transporter and channel complex.

Constant pH is crucial for the correct functioning of cells, impacting the integrity of membranes, transmembrane exchange of substances and buffering enzymatic reactions. Correct pH is also important for neurological function and changes in plasma pH that impact the pH of the cerebrospinal fluid have large impacts on behaviour and cognitive ability (Fromm, 1980). For this reason animals developed mechanism to counterbalance acidosis, which can usually come from increased metabolism or impaired renal function (Wright et al., 2014). The blood pH of *S. aurata* individuals to varies from around 7,5 (Papoutsoglou et al., 1999) to around 7,8 (Chambeyron and Zohar, 1990), depending on several factors. Although the obtained values all seem to be slightly below the normal range, the greatest difference is present, when the fish is exposed to the male "toadfish water". A possible explanation for this effect is the fact that, when a fish increases its physical activity, which is the case of the individuals exposed to the male "toadfish water", it will result in the accumulation of lactic acid in the blood, with the effect on the pH being related to the intensity of the physical activity (Fromm, 1980). Another possible explanation is related to the findings of Burton (1996), in which, while comparing the blood pH of several teleost species, with their respective plasma sodium concentration, found that there is a negative correlation between the two factors, that is, the higher the concentration of sodium the lower the blood pH (Burton, 1996). Perhaps it is then possible to assume that the increase of plasma sodium concentration in the male "toadfish water" treatment can lead to a decrease of blood pH. This is explained by the fact that the excretion of H+ from renal and gill cells can be done by an apical transporter that exchanges sodium (that enter the organism) by H+ (that exits). In a Na+ loaded organism there is no gradient for the Na+ to enter and thus H+ are also retained inside, decreasing pH. This same transporter can also aid in the transport of ammonia (exchange with Na+) and its impairment can explain the accumulation of ammonia, which in its ionic form, NH4, can lose a H+, to become the more neutral NH3, thus releasing a H+ to the extracellular fluid, contributing to lower pH. The pH in water was not measured and thus

the possibility of an acidic environment that would either create a flux of H+ into the fish (Fromm, 1980) or reduce the gradient for the excretion of H+ cannot be tested.

Branchial ATPase activity

Many toxicants exert their detrimental effect by blocking membrane channels and transporter or by reducing or impairing the activity of crucial enzymes (Evans, 1987). When comparing figures 4.27 and 4.28, it is possible to observe that the decrease of both the total ATPase and Na K-ATPase activity, seems to follow the same pattern, especially in the male treatment. The total activity decreases 0,0046 µmol ADP/mg protein/hr from the control to the female treatment, and 3,0516 µmol ADP/mg protein/hr from the control to the male treatment. Many functions of the gills and in other organs depend on a myriad of ATPases. If the negative effects on gills are a reflex of other organisms, it is conceivable that the substances in "toadfish water" are shutting down the organism, so to say. The same is true for the Na K-ATPase, which are widely spread and one of the most important active transport pump. The Na K- ATPase decreases 0,7312 µmol ADP/mg protein/hr from the control to the female treatment, and 2,4345 µmol ADP/mg protein/hr from the control to the male treatment. This may indicate that the decrease in the total activity of the ATPase might be due to the decrease of the Na K-ATPase. The inhibition of the Na K-ATPase activity may be due to the toadfish toxin disrupting the energy producing pathways, by the direct interaction of the toxin with the Na K-ATPase itself, or by the toadfish toxin causing lesions on the gills (Agrahari and Gopal, 2008). Although during the sampling procedures, the gills of the treatments did not appear to be wounded. Since the Na-K ATPase pump is one of the main ways to eliminate both sodium and chloride, by releasing them to the environment (Vargas-Chacoff et al., 2020), the decrease in activity shown in these results can also be a reason for the increase of both sodium and chloride concentrations, additionally many of the other ions, ammonia and pH, which balance results from secondary pathways for which NaK-ATPase creates the necessary gradients.

Impacts on fish behaviour

When it comes to the behavioural analysis, the only difference noted was in the male treatment, neither the control nor the female treatment presented any irregular behaviour, or LOE during the exposures. Many of the parameters measured and discussed above can contribute to the altered behaviour but also be a consequence of some of these altered

muscular activities. Some reasons that can possibly justify the alterations of behaviour and LOE in the male treatment are the decrease in blood pH, and possibly the increase in ammonia concentration. When the blood pH of the male treatment decreases, the haemoglobin affinity to bind to oxygen also decreases, which means that the ability of oxygen transport in the blood from the respiratory surfaces to the tissues is lowered (Fromm, 1980). The increase in ammonia concentration can be responsible for the occurrence of irregular behaviours and LOE, since both ammonia and pH can have an effect on a large array of cellular processes, it can affect the balance of ions in the fish, but most severely it can interfere with the nervous system, due to its capability to replace K^+ in ion transporters, which can alter the electrochemical gradients of the nervous system, which can result in the ceasing of activity (Eddy, 2005; Ip and Chew, 2010). The same is true for the reduction of ATpase activity, which may have important neutral and muscular impacts, as well as those in organs with the responsibility of detoxification such as the liver and the kidneys. During the exposure procedure, the timing of occurrence of the irregular behaviours was delayed for the second batch of fish used, that is, the last four fish took longer to display any of the irregular behaviours. This may indicate that the toxin present in the "toadfish water" may be consumed by the fish who are exposed, decreasing its effectiveness. The site(s) of entry or action of the putative toxin are unknown, but it is clear that the fish can sense it in the water through the olfactory system, although there is no clue about how this information is integrated or if it leads to aresponse, as the experimental paradigm did not allow for a typical fight or flight response.

Olfactory Nerve Recordings

When it comes to the recording of the olfactory nerve, it is possible to observe that the "toadfish water" causes an effect over the olfactory nerve response to different odorants. According to the literature, fish are olfactory sensitive to small and relatively polar molecules, such as amino acids, bile acids, steroids, amines, nucleotides, and prostaglandins (Olivares and Schmachtenberg, 2019). Olfactory sensitivity to amino acids and nucleotides appears to be related to the detection and location of food, sensitivity to bile acids and steroids is often associated with intra and interspecific communication. In this study, amino acids with different properties were tested, as well as bile fluid, with an inhibition effect on olfactory sensitivity to all stimuli tested. So, these results suggest that the effect of the "toadfish water" may be a general processes,

not being specific to the stimulus tested. Thus, it is possible that this is due to inhibition of receptor proteins or signal transduction pathways involved in olfaction. Future studies may clarify the inhibition mechanisms involved.

Additionally, as it can be seen on figure 4.38, even after performing a washout, there wasn't a recovery of the response signal. The inhibition of the signal can be related to the deterioration of not only the receptor cells present in olfactory epithelium, but also of the epithelium itself. However, the washout only lasted for 30 min, so perhaps this wasn´t enough time for the recovery, since it may take several hours for the recovery of olfactory response (Kasumyan, 2004). In the case that the inhibition effect is irreversible, it may be due to the toxin affecting the olfactory neuron by affecting the cellular surface proteins, the membrane structure, or even the cell organelles. These effects can ultimately lead to the destruction of the cell (Sandahl et al., 2006).

When comparing the signal inhibition of the regular "toadfish water" with the female and male "toadfish water" it is clear that the inhibitions produced by the female and male "toadfish water" do not reach the same level as the regular "toadfish water". Instead of reaching a signal of close to 20%, in the female, the lowest signal is close to 70% and in the male is 80%. Although these decreases in the female and male "toadfish water" aren't regular, since there are also signal increases, even in the highest concentrations of "toadfish water". These results suggest that there is either an accumulative effect when both these types of water are mixed, amplifying the effect, or perhaps the female and male "toadfish waters" contain different components that need to be mixed together to be able to produce any severe effect. It is worth mentioning that during the performance of the olfactory nerve recordings, it was noticed that there is a large variability between the individuals used for the experiment. Some presented large and clear response signals to the stimuli, while others presented smaller response signals. Some presented a clear signal decrease when exposed to the toadfish water, while others didn't seem to suffer a large effect.

Final Considerations

Overall, the results presented in this work, clearly show that the toadfish species *H. didactylus* is capable of producing toxic components, that have an effect not only on the previously tested species *Argyrosomus regius* but also on the *S. aurata*. These toxic substances are released into the water (possibly through the muccus, but other forms of

release should be considered), and induce physiological changes and neurotoxicity, leading to the death of individuals, although the effect is felt only at high concnetrations. When present on low concentrations, it does not appear to have an effect, neither on the haematological parameters nor on the plasmatic indicators that were measured. Results also showed that the toxin produced by the females differs from that produced by the males. Differences in the effects on the exposed individuals to the female and male waters can be related to the fact that, both the mucus and the water apparently have different chemical compositions as revealed by analytical chemistry approach (HPLC chromatograms). Although the male "toadfish water" caused the more severe effects, when its concentration is reduced to 1:30, it ceases to have an effect. This indicates that the toxin only has an effect when present in very large concentrations. Despite the changes on the haematological parameters, the main effects are noticed not only on the plasmatic indicators but also on the behaviour of the exposed organisms. It is, also interesting that another species of the family Batrachoididae, seems to have a similar toxin that ultimately causes death on the exposed individuals. In 1982, Nair et al., performed an experiment using the secretion of the species *Opsanus tau*, in which silverfish individuals (*Menidia menidia)* were exposed to the skin secretion of *O. tau.* After 15 min the silverfish started gasping and after 27 min the silverfish died (Nair et al., 1982). Perhaps this type of toxin may even be common among several species of this family.

Considering the fact that the male individuals of *H. didactylus* seem to produce a toxin with more severe effects, it is possible that the main function of this toxin could be related to nest protection since, it is the males that display parental care and protect the nest (Santos et al., 2000; Vasconcelos et al., 2010). Although, possibly, the purpose of the toxin is simply to repel any organisms that may come close, since, in the natural environment, when fish come into contact with any adverse conditions, they will move away, possibly not staying enough time in the area, for the toxin to cause the more severe effect, like LOE, immobilization and death.

The obtained results serve as a first approach, to the study of the *H. didactylus* toxin, upon which new studies should continue to be performed. With the obtained results, the following studies should perhaps focus mainly on the identification of the toxin and its composition using different analytical chemical techniques, which would not only grant a better insight into the mechanisms behind the effects presented here but possibly help identify any other effects that the toxin may have. The mechanism, and factors (size,

weight, sex, stress, among others) behind the production of the toxin should also be investigated since understanding how and when the toxin is produced can lead to the identification of its purpose. Alongside the process of the toxin identification, additional studies should be performed on both the toxin produced both the female and males, with the intent of truly identifying their differences and functions.

6. References

AAT Bioquest, Inc. (2022, September 7). *Quest Graph™ ED50 Calculator*. AAT Bioquest. https://www.aatbio.com/tools/ed50-calculator.

Abdul-Haqq, A.J., Shier, W.T., 1991. Icthyocrinotoxins and Their Potential Use as Shark Repellents. Journal of Toxicology: Toxin Reviews 10, 289–320. https://doi.org/10.3109/15569549109053859

Agrahari, S., Gopal, K., 2008. Inhibition of Na+–K+-ATPase in different tissues of freshwater fish Channa punctatus (Bloch) exposed to monocrotophos. Pesticide Biochemistry and Physiology 92, 57–60. https://doi.org/10.1016/j.pestbp.2008.06.003

Agrahari, S., Pandey, K.C., Gopal, K., 2007. Biochemical alteration induced by monocrotophos in the blood plasma of fish, Channa punctatus (Bloch). Pesticide Biochemistry and Physiology 88, 268–272. https://doi.org/10.1016/j.pestbp.2007.01.001

Al-Rasheed, A., Handool, K.O., Garba, B., Noordin, M.M., Bejo, S.K., Kamal, F.M., Daud, H.H.M., 2018. Crude extracts of epidermal mucus and epidermis of climbing perch Anabas testudineus and its antibacterial and hemolytic activities. The Egyptian Journal of Aquatic Research 44, 125–129. https://doi.org/10.1016/j.ejar.2018.06.002

Amorim, M.C.P., Vasconcelos, R.O., 2008. Variability in the mating calls of the Lusitanian toadfish Halobatrachus didactylus: cues for potential individual recognition. Journal of Fish Biology 73, 1267–1283. https://doi.org/10.1111/j.1095-8649.2008.01974.x

Arends, R.J., Mancera, J.M., Muñoz, J.L., Wendelaar Bonga, S.E., Flik, G., 1999. The stress response of the gilthead sea bream (Sparus aurata L.) to air exposure and confinement. J Endocrinol 163, 149–157. https://doi.org/10.1677/joe.0.1630149

Ay, Ö., Kalay, M., Tamer, L., Canli, M., 1999. Copper and Lead Accumulation in Tissues of a Freshwater Fish Tilapia zillii and Its Effects on the Branchial Na,K-ATPase Activity. Bulletin of environmental contamination and toxicology 62, 160–8. https://doi.org/10.1007/s001289900855

Barton, B.A., Ribas, L., Acerete, L., Tort, L., 2005. Effects of chronic confinement on physiological responses of juvenile gilthead sea bream, Sparus aurata L., to acute handling. Aquaculture Research 36, 172–179. https://doi.org/10.1111/j.1365-2109.2004.01202.x

Baumann, K., Casewell, N.R., Ali, S.A., Jackson, T.N.W., Vetter, I., Dobson, J.S., Cutmore, S.C., Nouwens, A., Lavergne, V., Fry, B.G., 2014. A ray of venom: Combined proteomic and transcriptomic investigation of fish venom composition using barb tissue from the blue-spotted stingray (Neotrygon kuhlii). Journal of Proteomics 109, 188–198. https://doi.org/10.1016/j.jprot.2014.06.004

Becker, J.M., Caldwell, G.A., Zachgo, E.A., 1996. Exercise 13 - Protein Assays, in:

Becker, J.M., Caldwell, G.A., Zachgo, E.A. (Eds.), Biotechnology (Second Edition). Academic Press, San Diego, pp. 119–124. https://doi.org/10.1016/B978-012084562-0/50069-2

Benkendorff, K., Davis, A.R., Bremner, J.B., 2001. Chemical Defense in the Egg Masses of Benthic Invertebrates: An Assessment of Antibacterial Activity in 39 Mollusks and 4 Polychaetes. Journal of Invertebrate Pathology 78, 109–118. https://doi.org/10.1006/jipa.2001.5047

Berry, J.W., Chappell, D.G., Barnes, R.B., 1946. Improved Method of Flame Photometry. Ind. Eng. Chem. Anal. Ed. 18, 19–24. https://doi.org/10.1021/i560149a005

Burtis, C.A., Ashwood, E.R., Tietz, N.W., 1999. Tietz textbook of clinical chemistry. W.B. Saunders, Philadelphia.

Burton, R.F., 1996. The dependence of normal arterial blood pH on sodium concentration in teleost fish. Comparative Biochemistry and Physiology Part A: Physiology 114, 111–116. https://doi.org/10.1016/0300-9629(95)02102-7

Caldwell, J.P., 1996. The evolution of myrmecophagy and its correlates in poison frogs (Family Dendrobatidae). Journal of Zoology 240, 75–101. https://doi.org/10.1111/j.1469-7998.1996.tb05487.x

Carbonara, P., Alfonso, S., Zupa, W., Manfrin, A., Fiocchi, E., Pretto, T., Spedicato, M.T., Lembo, G., 2019. Behavioral and physiological responses to stocking density in sea bream (Sparus aurata): Do coping styles matter? Physiol Behav 212, 112698. https://doi.org/10.1016/j.physbeh.2019.112698

Carrijo, L.C., Andrich, F., de Lima, M.E., Cordeiro, M.N., Richardson, M., Figueiredo, S.G., 2005. Biological properties of the venom from the scorpionfish (Scorpaena plumieri) and purification of a gelatinolytic protease. Toxicon 45, 843–850. https://doi.org/10.1016/j.toxicon.2005.01.021

CAS - Eschmeyer's Catalog of Fishes - Genera/Species by Family/Subfamily [WWW Document], n.d. URL https://researcharchive.calacademy.org/research/ichthyology/catalog/SpeciesByFamily.asp#Batrachoididae (accessed 10.16.21).

Chambeyron, F., Zohar, Y., 1990. A diluent for sperm cryopreservation of gilthead seabream, Sparus aurata. Aquaculture 90, 345–352. https://doi.org/10.1016/0044-8486(90)90258-O

Chhatwal, I., Dreyer, F., 1992. Biological properties of a crude venom extract from the greater weever fish Trachinus draco. Toxicon 30, 77–85. https://doi.org/10.1016/0041-0101(92)90503-W

Collette, B.B., 1966. A Review of the Venomous Toadfishes, Subfamily Thalassophryninae. Copeia 1966, 846–864. https://doi.org/10.2307/1441412

Conceição, K., Santos, J.M., Bruni, F.M., Klitzke, C.F., Marques, E.E., Borges, M.H., Melo, R.L., Fernandez, J.H., Lopes-Ferreira, M., 2009. Characterization of a new bioactive peptide from Potamotrygon gr. orbignyi freshwater stingray venom. Peptides 30, 2191–2199. https://doi.org/10.1016/j.peptides.2009.08.004

Cooling, L., 2014. The RBC as a Physiological Object, in: McManus, L.M., Mitchell, R.N. (Eds.), Pathobiology of Human Disease. Academic Press, San Diego, pp. 3049–3067. https://doi.org/10.1016/B978-0-12-386456-7.06202-X

Costa, J.L., Almeida, P.R. de, Costa, M.J., 2003. A morphometric and meristic investigation of Lusitanian toadfish *Halobatrachus didactylus* (Bloch and Schneider, 1801): evidence of population fragmentation on Portuguese coast. Scientia Marina 67, 219–231. https://doi.org/10.3989/scimar.2003.67n2219

Daly, J.A., Ertingshausen, G., 1972. Direct method for determining inorganic phosphate

in serum with the “CentrifiChem.” Clin Chem 18, 263–265.

Daly, J.W., Martin Garraffo, H., Spande, T.F., 1999. Chapter One - Alkaloids from Amphibian Skins, in: Pelletier, S.W. (Ed.), Alkaloids: Chemical and Biological Perspectives. Pergamon, pp. 1–161. https://doi.org/10.1016/S0735-8210(99)80024-7

Dutertre, S., Jin, A., Kaas, Q., Jones, A., Alewood, P.F., Lewis, R.J., 2013. Deep venomics reveals the mechanism for expanded peptide diversity in cone snail venom. Mol Cell Proteomics 12, 312–329. https://doi.org/10.1074/mcp.M112.021469

Eddy, F.B., 2005. Ammonia in estuaries and effects on fish. Journal of Fish Biology 67, 1495–1513. https://doi.org/10.1111/j.1095-8649.2005.00930.x

Eschbach, E., Scharsack, J.P., John, U., Medlin, L.K., 2001. Improved erythrocyte lysis assay in microtitre plates for sensitive detection and efficient measurement of haemolytic compounds from ichthyotoxic algae. J Appl Toxicol 21, 513–519. https://doi.org/10.1002/jat.797

Evans, D.H., 1987. The fish gill: site of action and model for toxic effects of environmental pollutants. Environmental Health Perspectives 71, 47–58. https://doi.org/10.1289/ehp.877147

Facó, P.E.G., Havt, A., Barbosa, P.S.F., Nobre, A.C.L., Bezerra, G.P., Menezes, D.B., Fonteles, M.C., Lopes-Ferreira, M., Monteiro, H.S.A., 2003. Effects of Thalassophryne nattereri fish venom in isolated perfused rat kidney. Toxicon 42, 509–514. https://doi.org/10.1016/S0041-0101(03)00229-0

Field, J.B., Elvehjem, C.A., Juday, C., 1943. A STUDY OF THE BLOOD CONSTITUENTS OF CARP AND TROUT. Journal of Biological Chemistry 148, 261–269. https://doi.org/10.1016/S0021-9258(18)72280-1

Fishbase - FAMILY Details for Batrachoididae - Toadfishes [WWW Document], n.d.

URL https://www.fishbase.de/Summary/FamilySummary.php?ID=189 (accessed 10.16.21).

Fazio, F., Filiciotto, F., Marafioti, S., Di Stefano, V., Assenza, A., Placenti, F., Buscaino, G., Piccione, G., Mazzola, S., 2012. Automatic analysis to assess haematological parameters in farmed gilthead sea bream (Sparus aurata Linnaeus, 1758). Marine and Freshwater Behaviour and Physiology 45, 63–73. https://doi.org/10.1080/10236244.2012.677559

Fazio, F., Saoca, C., Casella, S., Fortino, G., Piccione, G., 2015. Relationship between blood parameters and biometric indices of Sparus aurata and Dicentrarcus labrax cultured in onshore tanks. Marine and Freshwater Behaviour and Physiology 48, 289–296. https://doi.org/10.1080/10236244.2015.1041239

Félix, P.M., Amorim, M.C.P., Pereira, T.J., Fonseca, P.J., Sousa-Santos, C., Costa, J.L., 2016. Feeding ecology and life-history strategy of nesting males in a fish with long parental care, Lusitanian toadfish (Halobatrachus didactylus, Batrachoididae). Journal of the Marine Biological Association of the United Kingdom 96, 657–665. https://doi.org/10.1017/S0025315415001022

Fine, M.L., Malloy, K.L., King, C., Mitchell, S.L., Cameron, T.M., 2001. Movement and sound generation by the toadfish swimbladder. J Comp Physiol A 187, 371–379. https://doi.org/10.1007/s003590100209

Fromm, P.O., 1980. A review of some physiological and toxicological responses of freshwater fish to acid stress. Environ Biol Fish 5, 79–93. https://doi.org/10.1007/BF00000954

Gallardo, M.Á., Sala-Rabanal, M., Ibarz, A., Padrós, F., Blasco, J., Fernández-Borràs, J., Sánchez, J., 2003. Functional alterations associated with “winter syndrome” in gilthead sea bream (Sparus aurata). Aquaculture 223, 15–27. https://doi.org/10.1016/S0044-8486(03)00164-9

Gratzer, B., Millesi, E., Walzl, M., Herler, J., 2015. Skin toxins in coral-associated Gobiodon species (Teleostei: Gobiidae) affect predator preference and prey survival. Marine Ecology 36, 67–76. https://doi.org/10.1111/maec.12117

Greenfield, D., Winterbottom, R., Collette, B., 2008. Review of the toadfish genera (Teleostei: Batrachoididae). Proc Calif Acad Sci 59, 665–710.

Greenwell, M.G., Sherrill, J., Clayton, L.A., 2003. Osmoregulation in fish: Mechanisms and clinical implications. Veterinary Clinics: Exotic Animal Practice 6, 169–189. https://doi.org/10.1016/S1094-9194(02)00021-X

Guardiola, F.A., Bahi, A., Esteban, M.A., 2018. Effects of dietary administration of fenugreek seeds on metabolic parameters and immune status of gilthead seabream (Sparus aurata L.). Fish & Shellfish Immunology 74, 372–379. https://doi.org/10.1016/j.fsi.2018.01.010

Gwaltney-Brant, S.M., 2017. Chapter 49 - Zootoxins, in: Gupta, R.C. (Ed.), Reproductive and Developmental Toxicology (Second Edition). Academic Press, pp. 963–972. https://doi.org/10.1016/B978-0-12-804239-7.00049-4

Gwaltney-Brant, S.M., Dunayer, E., Youssef, H., 2018. Chapter 58 - Terrestrial Zootoxins, in: Gupta, R.C. (Ed.), Veterinary Toxicology (Third Edition). Academic Press, pp. 781–801. https://doi.org/10.1016/B978-0-12-811410-0.00058-1

Harris, R.J., Jenner, R.A., 2019. Evolutionary Ecology of Fish Venom: Adaptations and Consequences of Evolving a Venom System. Toxins (Basel) 11, 60. https://doi.org/10.3390/toxins11020060

Hrubec, T.C., Smith, S.A., 2010. Chapter 126 – Hematology of Fishes, in: Weiss, D.J., Wardrop K.J., (Ed.), Schalm's Veterinary hematology (Sixth edition). Wiley-Blackwell, pp. 994-1003.

Hubbard, P.C., Barata, E.N., Canario, A.V., 2000. Olfactory sensitivity to changes in environmental [Ca(2+)] in the marine teleost Sparus aurata. Journal of Experimental Biology 203, 3821–3829. https://doi.org/10.1242/jeb.203.24.3821

Hwang, D.F., Arakawa, O., Saito, T., Noguchi, T., Simidu, U., Tsukamoto, K., Shida, Y., Hashimoto, K., 1989. Tetrodotoxin-producing bacteria from the blue-ringed octopus Octopus maculosus. Mar. Biol. 100, 327–332. https://doi.org/10.1007/BF00391147

Ip, A., Chew, S., 2010. Ammonia Production, Excretion, Toxicity, and Defense in Fish: A Review. Frontiers in Physiology 1.

Kasumyan, A., 2004. The Olfactory System in Fish: Structure, Function, and Role in Behavior. Journal of Ichthyology 44, S180–S223.

Khoo, H.E., 2002. Bioactive proteins from stonefish venom. Clinical and Experimental Pharmacology and Physiology 29, 802–806. https://doi.org/10.1046/j.1440-1681.2002.03727.x

Kiriake, A., Ishizaki, S., Nagashima, Y., Shiomi, K., 2017. Occurrence of a stonefish toxin-like toxin in the venom of the rabbitfish Siganus fuscescens. Toxicon 140, 139–146. https://doi.org/10.1016/j.toxicon.2017.10.015

Kiriake, A., Suzuki, Y., Nagashima, Y., Shiomi, K., 2013. Proteinaceous toxins from three species of scorpaeniform fish (lionfish Pterois lunulata, devil stinger Inimicus japonicus and waspfish Hypodytes rubripinnis): Close similarity in properties and primary structures to stonefish toxins. Toxicon 70, 184–193. https://doi.org/10.1016/j.toxicon.2013.04.021

Laiz-Carrión, R., Sangiao-Alvarellos, S., Guzmán, J.M., Martín del Río, M.P., Soengas, J.L., Mancera, J.M., 2005. Growth performance of gilthead sea bream Sparus aurata in different osmotic conditions: Implications for osmoregulation and energy metabolism.

Aquaculture 250, 849–861. https://doi.org/10.1016/j.aquaculture.2005.05.021

Landsberg, J.H., Hall, S., Johannessen, J.N., White, K.D., Conrad, S.M., Abbott, J.P., Flewelling, L.J., Richardson, R.W., Dickey, R.W., Jester, E.L.E., Etheridge, S.M., Deeds, J.R., Van, D.F.M., Leighfield, T.A., Zou, Y., Beaudry, C.G., Benner, R.A., Rogers, P.L., Scott, P.S., Kawabata, K., Wolny, J.L., Steidinger, K.A., 2006. Saxitoxin Puffer Fish Poisoning in the United States, with the First Report of Pyrodinium bahamense as the Putative Toxin Source. Environmental Health Perspectives 114, 1502–1507. https://doi.org/10.1289/ehp.8998

Lane, W.R., Sutherland, S., 1967. The ringed octopus bite: a unique medical emergency. Medical Journal of Australia 2, 475–6.

Le Ruyet, J.P., Boeuf, G., Infante, J.Z., Helgason, S., Le Roux, A., 1998. Short-Term Physiological Changes in Turbot and Seabream Juveniles Exposed to Exogenous Ammonia. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 119, 511–518. https://doi.org/10.1016/S1095-6433(97)00458-3

Lee, C.Y., 1972. Chemistry and Pharmacology of Polypeptide Toxins in Snake Venoms. Annual Review of Pharmacology 12, 265–286. https://doi.org/10.1146/annurev.pa.12.040172.001405

Lopes-Ferreira, M., Barbaro, K.C., Cardoso, D.F., Moura-Da-Silva, A.M., Mota, I., 1998. Thalassophryne nattereri fish venom: biological and biochemical characterization and serum neutralization of its toxic activities. Toxicon 36, 405–410. https://doi.org/10.1016/S0041-0101(97)00115-3

Lopes-Ferreira, M., Ramos, A., Martins, I., Lima, C., Conceição, K., Junior, V., 2014. Clinical manifestations and experimental studies on the spine extract of the toadfish Porichthys porosissimus. Toxicon : official journal of the International Society on Toxinology 86. https://doi.org/10.1016/j.toxicon.2014.04.014

Maan, M.E., Cummings, M.E., 2012. Poison Frog Colors Are Honest Signals of Toxicity, Particularly for Bird Predators. The American Naturalist 179, E1–E14. https://doi.org/10.1086/663197

Maina, J.N., Wood, C.M., Hogstrand, C., Hopkins, T.E., Luo, Y.-H., Gibbs, P.D.L., Walsh, P.J., 1998. Structure and Function of the Axillary Organ of the Gulf Toadfish, Opsanus beta (Goode and Bean). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 119, 17–26. https://doi.org/10.1016/S1095-6433(97)00410-8

Manivasagan, P., Annamalai, N., Ashokkumar, S., Sampathkumar, P., 2009. Studies on the proteinaceous gel secretion from the skin of the catfish, Arius maculatus (Thunberg, 1792). African Journal of Biotechnology 8. https://doi.org/10.4314/ajb.v8i24.68807

Mann, D.A., 2006. Fish Communication, in: Brown, K. (Ed.), Encyclopedia of Language & Linguistics (Second Edition). Elsevier, Oxford, pp. 489–493. https://doi.org/10.1016/B0-08-044854-2/00832-4

McCormick, S.D., 1993. Methods for Nonlethal Gill Biopsy and Measurement of Na+, K+-ATPase Activity. Can. J. Fish. Aquat. Sci. 50, 656–658. https://doi.org/10.1139/f93-075

Michail, G., Berillis, P., Nakas, C., Henry, M., Mente, E., 2022. Haematology reference values for Dicentrarchus labrax and Sparus aurata: A systematic review and meta-analysis. Journal of Fish Diseases 45. https://doi.org/10.1111/jfd.13680

Miguel Mancera, J., Laiz Carrión, R., del Pilar Martín del Río, M., 2002. Osmoregulatory action of PRL, GH, and cortisol in the gilthead seabream (Sparus aurata L.). General and Comparative Endocrinology 129, 95–103. https://doi.org/10.1016/S0016-6480(02)00522-1

Mirghaed, A.T., Ghelichpour, M., Hoseini, S.M., Amini, K., 2017. Hemolysis

interference in measuring fish plasma biochemical indicators. Fish Physiol Biochem 43, 1143–1151. https://doi.org/10.1007/s10695-017-0359-y

Modesto, T., Canário, A.V.M., 2003. Morphometric changes and sex steroid levels during the annual reproductive cycle of the Lusitanian toadfish, Halobatrachus didactylus. General and Comparative Endocrinology 131, 220–231. https://doi.org/10.1016/S0016-6480(03)00027-3

Modesto, T., Freitas, A.M.M.S., Canario, A.V.M., 2015. Steroidogenesis by testis and accessory glands of the Lusitanian toadfish, Halobatrachus didactylus, during reproductive season. General and Comparative Endocrinology 223, 120–128. https://doi.org/10.1016/j.ygcen.2015.09.026

Mondzac, A., Ehrlich, G.E., Seegmiller, J.E., 1965. An enzymatic determination of ammonia in biological fluids. J Lab Clin Med 66, 526–531.

Nair, M.S., Leong, I., Nayar, M.S., 1982. Ichthyotoxins from the oyster toadfish, Opsanus tau (Linnaeus). Toxicon 20, 933–935. https://doi.org/10.1016/0041-0101(82)90082-4

Neely, T., Campbell, L., 2006. A modified assay to determine hemolytic toxin variability among Karenia clones isolated from the Gulf of Mexico. Harmful Algae 5, 592–598. https://doi.org/10.1016/j.hal.2005.11.006

Nguyen, T., Nguyen Phan, K., Lee, J.-B., Kim, J., 2016. Met-myoglobin formation, accumulation, degradation, and myoglobin oxygenation monitoring based on multiwavelength attenuance measurement in porcine meat. Journal of biomedical optics 21, 57002. https://doi.org/10.1117/1.JBO.21.5.057002

Noguchi, T., Arakawa, O., Takatani, T., 2006a. Toxicity of pufferfish Takifugu rubripes cultured in netcages at sea or aquaria on land. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, Papers presented at the TODAI International Symposium on Functional Genomics of Pufferfish Recent Advances and Perspective, The

University of Tokyo, Yayoi Auditorium, Tokyo, Japan, 3rd –6th November 2004 1, 153–157. https://doi.org/10.1016/j.cbd.2005.11.003

Noguchi, T., Arakawa, O., Takatani, T., 2006b. TTX accumulation in pufferfish. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, Papers presented at the TODAI International Symposium on Functional Genomics of Pufferfish Recent Advances and Perspective, The University of Tokyo, Yayoi Auditorium, Tokyo, Japan, 3rd –6th November 2004 1, 145–152. https://doi.org/10.1016/j.cbd.2005.10.006

Nørby, J.G., 1988. [11] Coupled assay of Na+,K+-ATPase activity, in: Methods in Enzymology, Biomembranes Part P: ATP-Driven Pumps and Related Transport: The Na,K-Pump. Academic Press, pp. 116–119. https://doi.org/10.1016/0076-6879(88)56014-7

Olivares, J., Schmachtenberg, O., 2019. An update on anatomy and function of the teleost olfactory system. PeerJ 7, e7808. https://doi.org/10.7717/peerj.7808

Oliva-Teles, A., Guedes, M.J., Vachot, C., Kaushik, S.J., 2006. The effect of nucleic acids on growth, ureagenesis and nitrogen excretion of gilthead sea bream Sparus aurata juveniles. Aquaculture 253, 608–617. https://doi.org/10.1016/j.aquaculture.2005.09.010

Oliveira, J.S., Pires Junior, O.R., Morales, R. a. V., Bloch Junior, C., Schwartz, C.A., Freitas, J.C., 2003. Toxicity of puffer fish: two species (Lagocephalus laevigatus, linaeus 1766 and Sphoeroides spengleri, Bloch 1785) from the Southeastern Brazilian coast. J. Venom. Anim. Toxins incl. Trop. Dis 9, 76–88. https://doi.org/10.1590/S1678-91992003000100005

Palazón-Fernández, J.L., Arias, A.M., Sarasquete, C., 2001. Aspects of the reproductive biology of the toadfish, *Halobatrachus didactylus* (Schneider, 1801) (Pisces: Batrachoididae). Scientia Marina 65, 131–138. https://doi.org/10.3989/scimar.2001.65n2131

Papoutsoglou, S.E., Miliou, H., Chadio, S., Karakatsouli, N., Zarkada, A., 1999. Studies on stress responses and recovery from removal in gilthead sea bream Sparus aurata (L.) using recirculated seawater system. Aquacultural Engineering 21, 19–32. https://doi.org/10.1016/S0144-8609(99)00021-7

Peres, H., Santos, S., Oliva-Teles, A., 2012. Selected plasma biochemistry parameters in gilthead seabream (Sparus aurata) juveniles. Journal of Applied Ichthyology 29, 630.

Primor, N., Zlotkin, E., 1975. On the ichthyotoxic and hemolytic action of the skin secretion of the flatfish Pardachirus marmoratus (Soleidae). Toxicon 13, 227–231. https://doi.org/10.1016/0041-0101(75)90128-2

Ramos, A.D., Conceição, K., Silva, P.I., Richardson, M., Lima, C., Lopes-Ferreira, M., 2012. Specialization of the sting venom and skin mucus of Cathorops spixii reveals functional diversification of the toxins. Toxicon 59, 651–665. https://doi.org/10.1016/j.toxicon.2012.02.002

Redding, J.M., Schreck, C.B., 1983. Influence of Ambient Salinity on Osmoregulation and Cortisol Concentration in Yearling Coho Salmon during Stress. Transactions of the American Fisheries Society 112, 800–807. https://doi.org/10.1577/1548-8659(1983)112<800:IOASOO>2.0.CO;2

Reinert, H.K., Cundall, D., Bushar, L.M., 1984. Foraging Behavior of the Timber Rattlesnake, Crotalus horridus. Copeia 1984, 976–981. https://doi.org/10.2307/1445342

Rice, A.N., Bass, A.H., 2009. Novel vocal repertoire and paired swimbladders of the three-spined toadfish, Batrachomoeus trispinosus: insights into the diversity of the Batrachoididae. Journal of Experimental Biology 212, 1377–1391. https://doi.org/10.1242/jeb.028506

Sandahl, J.F., Miyasaka, G., Koide, N., Ueda, H., 2006. Olfactory inhibition and recovery in chum salmon (Oncorhynchus keta) following copper exposure. Can. J. Fish. Aquat.

Sci. 63, 1840–1847. https://doi.org/10.1139/f06-074

Santos, M.E. dos, Modesto, T., Matos, R., Grober, M.S., Oliveira, R.F., Canario, A.V.M., 2000. Sound production by the Lusitanian toadfish, Halobatrachus didactylus. Bioacoustics 309–321.

Schoenfeld, R.G., Lewellan, C.J., 1964. A Colorimetric Method for Determination of Serum Chloride. Clinical Chemistry 10, 533–539. https://doi.org/10.1093/clinchem/10.6.533

Sherbrooke, W.C., 2008. Antipredator Responses by Texas Horned Lizards to Two Snake Taxa with Different Foraging and Subjugation Strategies. hpet 42, 145–152. https://doi.org/10.1670/07-072R1.1

Sosa-Rosales, J.I., Piran-Soares, A.A., Farsky, S.H.P., Takehara, H.A., Lima, C., Lopes-Ferreira, M., 2005. Important biological activities induced by Thalassophryne maculosa fish venom. Toxicon 45, 155–161. https://doi.org/10.1016/j.toxicon.2004.10.003

Sousa, R.P.C. de, Oliveira-Filho, A.B., Vallinoto, M., Cioffi, M. de B., Molina, W., de Oliveira, E.H., Silva-Oliveira, G.C., 2018. Cytogenetics description in Batrachoides surinamensis, (Batrachoididae: Batrachoidiformes): What does the estuary have to say? Estuarine, Coastal and Shelf Science 213, 253–259. https://doi.org/10.1016/j.ecss.2018.08.008

Summers, K., Clough, M.E., 2001. The evolution of coloration and toxicity in the poison frog family (Dendrobatidae). PNAS 98, 6227–6232. https://doi.org/10.1073/pnas.101134898

Timmerman,W.W. 1995. Home range, habitat use, and behavior ofthe eastern diamondback rattlesnake (Crotalus adamanteus) on the Ordway Preserve. Bulletin Florida Museum Natural History Vol 38, Pt I(5):127-158.

Trinder, P., 1969. Determination of Glucose in Blood Using Glucose Oxidase with an Alternative Oxygen Acceptor. Ann Clin Biochem 6, 24–27. https://doi.org/10.1177/000456326900600108

van Kampen, E.J., Zijlstra, W.G., 1961. Standardization of hemoglobinometry II. The hemiglobincyanide method. Clinica Chimica Acta 6, 538–544. https://doi.org/10.1016/0009-8981(61)90145-0

Vargas-Chacoff, L., Arjona, F.J., Ruiz-Jarabo, I., García-Lopez, A., Flik, G., Mancera, J.M., 2020. Water temperature affects osmoregulatory responses in gilthead sea bream (Sparus aurata L.). Journal of Thermal Biology 88, 102526. https://doi.org/10.1016/j.jtherbio.2020.102526

Vasconcelos, R.O., Simões, J.M., Almada, V.C., Fonseca, P.J., Amorim, M.C.P., 2010. Vocal Behavior During Territorial Intrusions in the Lusitanian Toadfish: Boatwhistles Also Function as Territorial 'Keep-Out' Signals. Ethology 116, 155–165. https://doi.org/10.1111/j.1439-0310.2009.01722.x

Wang, X., Kültz, D., 2017. Osmolality/salinity-responsive enhancers (OSREs) control induction of osmoprotective genes in euryhaline fish. Proceedings of the National Academy of Sciences 114, E2729–E2738. https://doi.org/10.1073/pnas.1614712114

Wood, C.M., Turner, J.D., Graham, M.S., 1983. Why do fish die after severe exercise? Journal of Fish Biology 22, 189–201. https://doi.org/10.1111/j.1095-8649.1983.tb04739.x

Wright, P.A., Wood, C.M., Wilson, J.M., 2014. Rh versus pH: the role of Rhesus glycoproteins in renal ammonia excretion during metabolic acidosis in a freshwater teleost fish. Journal of Experimental Biology 217, 2855–2865. https://doi.org/10.1242/jeb.098640

Ziegman, R., Alewood, P., 2015. Bioactive Components in Fish Venoms. Toxins 7,

1497–1531. https://doi.org/10.3390/toxins7051497