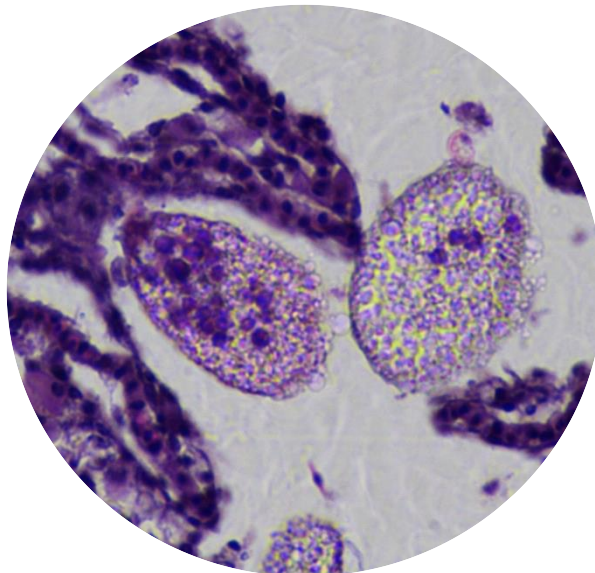


University of Algarve
Faculty of Sciences and Technology



Utilizing hydrophilic extracts of *Amyloodinium ocellatum* (Brown, 1931) in the development of new marine fish therapies

António Pedro da Fonseca Jorge Machado Ribeiro

Nº 68839

2022

University of Algarve
Faculty of Sciences and Technology

Utilizing hydrophilic extracts of *Amyloodinium ocellatum* (Brown, 1931) in the development of new marine fish therapies

António Pedro da Fonseca Jorge Machado Ribeiro

Nº68839

Dissertation to the obtention of the master's degree in Aquaculture and Fisheries

Project supervised by:

Doctor Florbela Soares (IPMA-EPPO)

Prof. Doctor Ana Grenha (FCT-UALG)

2022

**Utilizing hydrophilic extracts of *Amyloodinium ocellatum* (Brown, 1931)
in the development of new marine fish therapies**

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.

Copyright© António Pedro da Fonseca Jorge Machado Ribeiro, N° 68839 Faculdade de Ciência e Tecnologias - Universidade do Algarve

A Universidade do Algarve tem o direito, perpétuo e sem limites geográficos, de arquivar e publicitar este trabalho através de exemplares impressos reproduzidos em papel ou em forma digital, ou por qualquer outro meio conhecido ou que venha a ser inventado, de o divulgar através de repositórios científicos e de admitir a sua cópia e distribuição com objetivos educacionais ou de investigação, não comerciais, desde que seja dado crédito ao autor e editor.



Vista aérea da Estação Piloto de Piscicultura de Olhão do IPMA onde foi realizado este trabalho.

Este trabalho foi financiado e realizado na Estação Piloto de Piscicultura de Olhão (EPPA) do IPMA utilizando corvinas nascidas nesta Estação e as infraestruturas e meios disponíveis para o cultivo, amostragens biológicas e análises laboratoriais. Ao IPMA reserva-se o direito de utilizar toda a informação nela contida para efeitos de publicações, relatórios da entidade financiadora e divulgação ao público em geral.

Acknowledgements

I would like to express my gratitude to Dr Florbela Soares of the National Institute for the Sea and Atmosphere's (IPMA) Aquaculture Research Centre for all her patience, motivation, scientific formation, and support during the writing of my thesis. I also would like to express my gratitude to Professor Ana Grenha of the University of Algarve's CCMAR - Centre of Marine Sciences, for the possibilities, scientific training, and assistance she offered for this thesis. I also thank the financial support for this thesis from the projects SAUDE&AQUA (MAR-02.05.01-FEAMP-0009) and DIVERSIAQUA II (MAR2020-P02M01- 0656P).

I also want to thank all the EPPO-IPMA staff Tetyana Urshulyak, Ravi Luna, Rita Viegas, Carolina Ribeiro, Beatriz Cordeiro, Sara Sousa, Marisa Barata, Isa Dias, Margarida Gamboa and Raquel Quintã for all the support, companionship and friendship throughout these months; To Conceição Batista for all the support, friendship and scientific formation provided on this project; To Dr Pedro Pousão from EPPO-IPMA, for the inestimable contribution by providing facilities and fish for my thesis; And especially to Rui Sousa, Eloise De Sá, Diogo Esteves and Marcelo Livramento, for the scientific formation, support, friendship and all the great conversations about science, pathology, parasites and bacteria.

My heartfelt gratitude goes to my parents and brother for making all of this possible and for their unending support. To all of my friends in Lisbon for their encouragement and friendship, especially Diogo Fernandes, who listened to all of my complaints.

Resumo

Espera-se que a população global atinja os 9 mil milhões em 2050, desafiando o sector alimentar mundial a tornar-se mais produtivo, eficiente e sustentável para garantir a segurança alimentar às gerações futuras. Os oceanos, em todo o mundo, são sobre-explorados e a atividade de pesca é muitas vezes insuficiente para satisfazer a procura, pelo que a aquacultura representa uma solução para o problema. Um dos principais desafios ao desenvolvimento da aquacultura é proliferação de doenças que afetam os indivíduos em cultivo. Na região mediterrânica, no Sul da Europa, amyloodiniosis representa um grande risco para a aquacultura semi-intensiva e intensiva, afetando a maioria das explorações piscícolas em todos os sistemas de produção e causando elevada mortalidade. Embora existam métodos preventivos e desinfetantes, a maioria tem um custo elevado, é pouco prática de aplicar em sistemas semi-intensivos com tanques de terra e altamente prejudicial para o ambiente, tornando a inovação de novas terapias, tratamentos e vacinas de especial importância. Esta tese centra-se no desenvolvimento de uma possível vacina para o robalo (*Dicentrarchus labrax*), utilizando extratos hidrofílicos obtidos a partir do dinoflagelado *Amyloodinium ocellatum*. Os extratos foram injetados intraperitonealmente em robalos de 139.8 g de peso médio e as respostas imune, de *stress* e hematológica foram analisadas após 7, 14, 21 e 28 dias da vacinação. Vinte e oito dias após a vacinação procedeu-se ao desafio com duas concentrações de *A. ocellatum* (35 e 10 dinosporos/mL), sobre o grupo controlo e o grupo vacinado. Para os parâmetros hematológicos, foi registada uma disparidade de resultados nos valores da hemoglobina (Hgb), hematócrito (Htc), lactato e pH, apresentando o grupo vacinado valores mais elevados de Hgb e Htc e valores mais baixos de lactato e pH no sangue. O grupo vacinado, também, apresentou um maior número de monócitos e um menor número de trombócitos. A nível histológico, não foram observadas diferenças no fígado e nas brânquias entre os dois grupos. Quanto aos resultados do desafio, conclui-se que, com a concentração utilizada, não existiu diferença significativa entre os peixes imunizados e o grupo de controlo.

Palavras-chave: Aquacultura, patologia, *Dicentrarchus labrax*, *Amyloodinium ocellatum*, resposta imune, onicação, acinação

Abstract

The global population is expected to reach 9 billion before 2050, and the world food-producing sector must become more productive, efficient, and sustainable to provide food security for future generations. Fisheries worldwide are overfished, and stocks are often incapable of satisfying the demand, aquaculture represents a solution to the problem. However, the development of aquaculture is constrained by different causes, diseases being one of the most significant. In the Mediterranean area, Southern Europe, *amyloodiniosis* represents a key bottleneck for semi-intensive aquaculture production, affecting most fish farms across all production systems and causing high mortalities at precipitous rates. Although treatments exist, most are either expensive, unpractical, or highly harmful to the environment, making the innovation of new therapies, treatments, and vaccines of paramount importance. This thesis focuses on the development of a possible vaccine for seabass (*Dicentrarchus labrax*), using hydrophilic extracts obtained from the dinoflagellate (*Amyloodinium ocellatum*). The Extracts were injected intraperitoneally in seabasses with an average weight of 128.9 g and the immune, stress and haematological responses were evaluated at 7-, 14-, 21-, and 28-days post-injection. After 28 days the challenge started, where both the control and the vaccinated group were injected with two concentrations of *A. ocellatum* (35 and 10 dinospores/mL). For the haematological parameters, it was registered a difference in the values of haemoglobin (Hgb), haematocrit (Htc), lactate and pH, where the vaccinated fish showed higher values in Hgb and Htc and lower values in lactate and pH. Vaccinated fish also presented a higher number of monocytes and a lower number of thrombocytes. At the histological level, no significant differences were noted in the gills and liver. As for the results from the challenge, it was concluded that there were no differences between the vaccinated fish and the control with the selected concentration.

Keywords: *Aquaculture, pathology, Dicentrarchus labrax, Amyloodinium ocellatum, Immune Response, Sonication, Vaccination*

Index of Contents

1. Objectives and Motivation	1
2. General Outlook on Aquaculture	2
2.1 Impacts of Diseases in Aquaculture.....	2
3. Characterization of the Parasite	3
3.1 Taxonomy	3
3.2 Biological Cycle and Morphology.....	4
3.3 Diagnostics and Pathology.....	6
3.4 Treatments, Prevention, Control, and Prophylaxis	8
4. Stress Indicators	10
5. Immune Response.....	13
5.1 Innate Immune Response.....	13
5.2 Acquired Immune Response.....	14
6. Methodology.....	17
6.1 <i>Amyloodinium ocellatum</i> Infection, Incubation and Collection	18
6.2 <i>Amyloodinium ocellatum</i> Sonication and Extract Obtention.....	19
6.3. Target Species and Culture Conditions.....	20
6.4. Vaccination and Challenge	20
6.5 Sampling	22
6.6 Analysis of Haematological Parameters	23
6.6.1 Blood Collection	23
6.6.2 Haematocrit.....	24
6.6.3 Haemoglobin.....	24
6.7 Analysis of Stress Parameters	25
6.8 Analysis of Histological Parameters.....	26

6.9 Statistical Analysis.....	27
7. Results.....	28
7.1 Biometric Data, Condition Index and Mortality	29
7.2 Haematological Data.....	30
7.2.1 Haemoglobin.....	30
7.2.2 Haematocrit.....	31
7.2.3 Haematological Cell Counting and Identification	32
7.2.4 K ⁺	35
7.2.5 Ca ⁺	35
7.3. Haematological Stress Parameters	36
7.3.1 Glucose	36
7.3.2 Lactate.....	37
7.3.3 pH.....	38
7.4 Histology.....	39
7.4.1 Gills.....	39
7.4.2 Liver.....	41
7.5 Challenge with <i>Amyloodinium ocellatum</i> , After Seabass Vaccination.....	43
7.5.1. Challenge With 35 Dinospores/mL - Survival and Infection Level	43
7.5.2. Challenge With 10 Dinospores/mL - Survival and Infection Level	45
8. Discussion	47
9. Conclusions and Considerations	51
10. Bibliography	52

List of Figures

FIGURE 3.1- <i>AMYLOODINIUM OCELLATUM</i> LIFE CYCLE PHASES (AMP. 200X AND 400X).....	5
FIGURE 3.2 AND 3.3- <i>AMYLOODINIUM OCELLATUM</i> ATTACHED AND DETACHED FROM GILLS OF SEABASS (<i>DICENTRARCHUS LABRAX</i>), RESPECTIVELY (40X AND 100X AMP., IPMA).....	6
FIGURE 6.4 - THE FIGURE DESCRIBES THE SYSTEM USED TO CHALLENGE <i>DICENTRARCHUS LABRAX</i> WITH TWO CONCENTRATIONS (35 AND 10 DINOSPORES PER ML) OF <i>AMYLOODINIUM OCELLATUM</i> 28 DAYS-POST THE ADMINISTRATION OF A VACCINE.	21
FIGURE 6.5- BRANCHIAL REMOVAL (1° ARCH) OF <i>DICENTRARCHUS LABRAX</i>	22
FIGURE 6.6 - BLOOD COLLECTION OF <i>DICENTRARCHUS LABRAX</i> THROUGH THE PUNCTION OF THE CAUDAL VEIN.	23
FIGURE 6.7 - TRANSFORMATION PROCESS WITH IMAGEJ OF A HISTOLOGICAL SLIDE OF LIVER TISSUE OF <i>DICENTRARCHUS LABRAX</i> (AMP. 400X). WITH IMAGEJ. A- ORIGINAL IMAGE; B, C- RGB STACKING AND PROCESSING.	27
FIGURE 7.8 - FULTON’S CONDITION INDEX (MG.MM ⁻³) OF <i>DICENTRARCHUS LABRAX</i> IN THE CONTROL AND VACCINATED TANK DURING THE 28 DAYS AFTER INJECTING THE HYDROPHILIC EXTRACT OF <i>AMYLOODINIUM OCELLATUM</i> . VALUES MARKED WITH A DIFFERENT LETTER DIFFER SIGNIFICANTLY FROM EACH OTHER (N= 6; P < 0.05).....	30
FIGURE 7.9 - CONCENTRATION OF HGB (G/DL) IN <i>DICENTRARCHUS LABRAX</i> IN THE CONTROL AND VACCINATED GROUP. VALUES MARKED WITH DIFFERENT LETTERS DIFFER SIGNIFICANTLY FROM EACH OTHER (N= 6; P < 0.05).....	31
FIGURE 7.10 - HAEMATOCRIT OF <i>DICENTRARCHUS LABRAX</i> IN THE CONTROL AND VACCINATED TANK DURING THE TRIAL. VALUES MARKED WITH DIFFERENT LETTERS DIFFER SIGNIFICANTLY FROM EACH OTHER (N= 6; P < 0.05)	32
FIGURE 7.11 - BLOOD CELL IDENTIFICATION OF <i>DICENTRARCHUS LABRAX</i> . (M - MONOCYTES; T- THROMBOCYTES; L- LYMPHOCYTES; G- GRANULOCYTES; E- ERYTHROCYTES). (A, B) – 1500X AMP.; (C, D) – 1000X AMP.	33
FIGURE 7.12 - LEUCOCYTES IN THE BLOOD OF <i>DICENTRARCHUS LABRAX</i> AT 7, 21 AND 28 DAYS POST THE INJECTION OF THE HYDROPHILIC EXTRACT OF <i>AMYLOODINIUM OCELLATUM</i>	34
FIGURE 7.13 - CONCENTRATION OF K ⁺ IN <i>DICENTRARCHUS LABRAX</i> IN THE CONTROL AND VACCINATED TANK. VALUES MARKED WITH A DIFFERENT LETTER DIFFER SIGNIFICANTLY FROM EACH OTHER (N= 6; P < 0.05).	35
FIGURE 7.14 - CONCENTRATION OF CA ⁺ IN <i>DICENTRARCHUS LABRAX</i> IN THE CONTROL AND VACCINATED TANK. VALUES MARKED WITH DIFFERENT LETTERS DIFFER SIGNIFICANTLY FROM EACH OTHER (N= 6; P < 0.05).....	36
FIGURE 7.15 - CONCENTRATION OF GLUCOSE IN <i>DICENTRARCHUS LABRAX</i> IN THE CONTROL AND VACCINATED TANK. VALUES MARKED WITH DIFFERENT LETTERS DIFFER SIGNIFICANTLY FROM EACH OTHER (N= 6; P < 0.05).....	37
FIGURE 7.16 - CONCENTRATION OF LACTATE IN <i>DICENTRARCHUS LABRAX</i> IN THE CONTROL AND VACCINATED TANK. VALUES MARKED WITH A DIFFERENT LETTER DIFFER SIGNIFICANTLY FROM EACH OTHER (N= 6; P < 0.05).....	37
FIGURE 7.17 - pH VALUES OF <i>DICENTRARCHUS LABRAX</i> IN THE CONTROL AND VACCINATED GROUP AT 14 AND 28 DAYS AFTER THE ADMINISTRATION OF THE HYDROPHILIC EXTRACT. VALUES MARKED WITH A DIFFERENT LETTER DIFFER SIGNIFICANTLY FROM EACH OTHER (N= 6; P < 0.05).....	38

FIGURE 7.18 - IMAGES OF HISTOLOGICAL SECTIONS OF GILLS OF <i>DICENTRARCHUS LABRAX</i> STAINED WITH HAEMATOXYLIN-EOSIN (H&E) DYE. (A, B) SHOW THE NORMAL APPEARANCE OF HEALTHY GILLS OF THE VACCINATED TANK (A) AND CONTROL TANK (B) AT 14 DAYS POST- INJECTION OF THE HYDROPHILIC EXTRACT OF <i>AMYLOODINIUM OCELLATUM</i> AT 400X AND 400X AMP. RESPECTIVELY. WHILE (C, D, E, F) SHOW TISSUE INFECTED BY <i>AMYLOODINIUM OCELLATUM</i> OF BOTH THE CONTROL TANK (C, D) AND THE VACCINATED TANK (E, F) AT 400X, 1000X, 400X, 100X AMP. RESPECTIVELY. LF - LAMELLAR FUSION; AO - <i>AMYLOODINIUM OCELLATUM</i> ; HP - LAMELLAR HYPERPLASIA	40
FIGURE 7.19 - PERCENTAGE OF LIPID RETENTION IN HEPATOCYTES OF <i>DICENTRARCHUS LABRAX</i> DURING THE PERIOD AFTER THE INJECTION OF A HYDROPHILIC EXTRACT OF <i>AMYLOODINIUM OCELLATUM</i> IN THE VACCINATED GROUP AND PBS IN THE CONTROL GROUP. VALUES MARKED WITH A DIFFERENT LETTER DIFFER SIGNIFICANTLY FROM EACH OTHER (N= 6; P < 0.05)	41
FIGURE 7.20 - REPRESENTATIVE PHOTOS OF HISTOLOGICAL FEATURES OF LIVER OF <i>DICENTRARCHUS LABRAX</i> , WHERE PHOTOS A, C AND E BELONG TO A GROUP INJECTED WITH A HYDROPHILIC EXTRACT OF <i>AMYLOODINIUM OCELLATUM</i> AND B, D AND F BELONG TO A GROUP INJECTED WITH PBS. A, B. LIVER WITH HYPERAEMIA AND HIGH HEPATOCYTES VACUOLIZATION; C, D. LIVER WITH PRONOUNCED HEPATOCYTE VACUOLIZATION., E, F. LIVER WITH NECROTIC FOCL	42
FIGURE 7.21 - TOMONTS AND TROPHONTS COLLECTED FROM THE 2 FIRST BRANCHIAL ARCHES OF EACH <i>DICENTRARCHUS LABRAX</i> THAT DIED IN THE CHALLENGE WITH 35 DINOSPORES / mL OF <i>AMYLOODINIUM OCELLATUM</i> 28 DAYS AFTER THE INJECTION OF THE HYDROPHILIC EXTRACT IN THE VACCINATED GROUP AND PBS IN THE CONTROL GROUP.	44
FIGURE 7.22 - SURVIVAL IN THE CHALLENGE OF <i>DICENTRARCHUS LABRAX</i> WITH 35 DINOSPORES / mL OF <i>AMYLOODINIUM OCELLATUM</i> 28 DAYS AFTER THE VACCINATION WITH THE HYDROPHILIC EXTRACT (VACCINATED GROUP) AND PBS IN THE CONTROL GROUP. THE Y-AXIS REPRESENTS THE PERCENTAGE OF ALIVE FISH IN THE CHALLENGE, WHERE N= 9 AND THE X-AXIS THE TIME IN HOURS.	44
FIGURE 7.23 - MORTALITIES IN THE CHALLENGE OF <i>DICENTRARCHUS LABRAX</i> WITH 10 DINOSPORES/mL OF <i>AMYLOODINIUM OCELLATUM</i> 28 DAYS AFTER THE INJECTION OF THE HYDROPHILIC EXTRACT (VACCINATED GROUP) AND PBS IN THE CONTROL GROUP. THE Y-AXIS REPRESENTS THE PERCENTAGE OF ALIVE FISH IN THE CHALLENGE, WHERE N = 9 AND THE X-AXIS IS THE TIME IN HOURS.....	45
FIGURE 7.24 - TOMONTS AND TROPHONTS COLLECTED FROM THE 2 FIRST BRANCHIAL ARCHES OF EACH MORTALITY OF <i>DICENTRARCHUS LABRAX</i> IN THE CHALLENGE WITH 10 DINOSPORES / mL OF <i>AMYLOODINIUM OCELLATUM</i> 28 DAYS AFTER THE INJECTION OF THE HYDROPHILIC EXTRACT IN THE VACCINATED GROUP AND PBS IN THE CONTROL GROUP.	46

List of Tables

TABLE 7.1 BIOMETRIC DATA FOR <i>DICENTRARCHUS LABRAX</i> DURING THIS TRIAL (N=6), WHERE T IS TIME IN DAYS. VALUES MARKED WITH A DIFFERENT LETTER DIFFER SIGNIFICANTLY FROM EACH OTHER (N= 6; P < 0.05).....	29
TABLE 7.2- LEUCOCYTES IN THE BLOOD OF <i>DICENTRARCHUS LABRAX</i> DURING THE TIME BETWEEN VACCINATION AND THE FIRST CHALLENGE. HTC AND HGB VALUES WERE TAKEN USING THE EPOC® BLOOD ANALYSIS SYSTEM. VALUES MARKED WITH DIFFERENT LETTERS DIFFER SIGNIFICANTLY (N= 6; P < 0.05) IN EACH HAEMATOLOGICAL PARAMETER.	34

Abbreviations

Amp. - Ampliation

AMPPs - Antimicrobial polypeptides

E – Erythrocytes

ELISA - Enzyme-linked immunosorbent assay

G - Granulocytes

Hgb - Haemoglobin

HLPs - Histone-like proteins

Htc - Haematocrit

IC – Condition index

Ig - Immunoglobulin

L - Lymphocytes

M - Monocytes

MHC - Major histocompatibility complex molecules

PBS - Phosphate-buffered saline

RBC - Red blood cells

T - Thrombocytes

TCR - T-cell antigen receptor

UV – Ultraviolet

1. Objectives and Motivation

The main objective of this thesis is the development of a preventive therapy for the seabasses *Dicentrarchus labrax* based on the hydrophilic extracts of sonicated *Amyloodinium ocellatum* tomonts. The preventive therapy will be based on vaccination, explicitly, sonicated tomonts of *A. ocellatum* will be fractionated into hydrophilic and lipophilic fractions and the hydrophilic fraction will be injected intraperitoneally. Then the immune response and health of the fish, for the chosen concentration, will be evaluated through various parameters determining if this approach was successful.

A. ocellatum is an agent responsible for the occurrence of Amyloodiniosis in marine fish produced in aquaculture. Due to the life cycle of *A. ocellatum*, with an enlisting phase, and the pollutant characteristics of the more common treatments, the complete eradication of this parasite is still a severe challenge. The discovery of new methods that allow the treatment of infected animals by chemically destroying the initials stages of the parasite; eliminating the tomonts (encapsulated state, a cyst that develops after the trophont leaves the fish) in the tanks or by helping the organism build a resistance to the pathogen, has a significant value an industry that annually suffers significant losses in damages due to *A. ocellatum*.

2. General Outlook on Aquaculture

Humans have relied on fish as a primary source of animal protein in the past and will continue to do so in the future. Food production (e.g., growing of crops, breeding of domestic animals) has and will continue to compete with other human activities (e.g., transportation, housing, industry) for the limited usable/inhabitable land as the population approaches 8 billion in around 20 years. Many species of marine fish provide significant health components, such as polyunsaturated fatty acids, in addition to being a cheaper animal protein (e.g., Omega 3). However, the capture-fishery is either stagnant or declining, as natural fish stocks have been considerably reduced in many regions of the world as a result of overfishing, indiscriminate fishing, and/or the destruction of spawning grounds. Many harmful discharges (e.g., organophosphates, heavy metals) into aquatic habitats, particularly from industry, have been shown to impair fish survival and reproduction. Because of excessive amounts of accumulated pollution in some locations, fish are no longer fit for human consumption, and no new fishing grounds have been located. Aquaculture continues to be the fastest-growing food-producing sector, according to the Food and Agriculture Organization, with an annual growth rate of roughly 10%. If not for illness outbreaks, it would be far higher (Woo et al., 2002). Many countries, particularly those with significant numbers of rivers and lakes and/or lengthy coastlines, have established intense cage culture of freshwater and marine fishes (e.g., China, Chile, Norway). Disease outbreaks, on the other hand, are becoming more common as intensive fish culture facilitates the transmission of parasites amongst caged fish and the acquisition of pathogens from wild fish attracted to the uneaten food in cages (Woo et al., 2002).

2.1 Impacts of Diseases in Aquaculture

Diseases are one of the most important constraints in aquaculture, especially in intensive fish farming, representing severe annual costs to producers (Murray and Peeler, 2005). The open design of many aquaculture systems allows the transmission of infectious pathogens, where they find ideal conditions to cause a disease outbreak and complicates the implementation of host defence mechanisms (Balcázar et al., 2006; Mladineo et al., 2010). Global warming tends to

increase the frequency of outbreaks and increase the ecological range of the diseases (Diez and Perlado, 2014; FAO, 2015a, b). Parasitic diseases caused by obligate or opportunistic eukaryotic pathogens have a major impact on global finfish and shellfish aquaculture, and in many regions, they represent a key constraint to the production, sustainability, and economic viability of aquaculture facilities (Shinn et al., 2014).

Amyloodiniosis, often known as 'marine velvet,' is a parasite illness that poses a severe danger to semi-intensive and intensive aquaculture in Southern Europe and the Mediterranean region. It is regarded as one of the most serious barriers to the production of various warm-water aquaculture species (Noga et al., 1996; Menezes et al., 2000). *Amyloodinium* is known to infect over a hundred species of fish. It is one of the few fish parasites that can infest both elasmobranchs and teleosts, and most fish that dwell within its ecological range are susceptible to infestation (Lawler et al., 1980). In brackish and marine warm water aquaculture facilities, it can cause substantial morbidity and death, as well as a moderate-to-intense tissue reaction in numerous commercially important shrimp species (Aravindan, Chaganti & Aravindan 2007). It is frequently regarded as the most serious parasite infection affecting marine fish (Paperna et al., 1981). Outbreaks can happen very quickly, with 100 percent death in just a few days. When freshwater fish are raised in saltwater waters, they are prone to infection (Lawler et al., 1980; Noga and Bower, 1987; Kuperman and Matey, 1999). Tilapia growers in the Salton Sea, California, USA, lost as much as 77 million dollars owing to an *Amyloodinium* infestation in 1997-1998. (Kuperman & Matey, 1999).

3. Characterization of the Parasite

3.1 Taxonomy

A. ocellatum is a dinoflagellate that parasitizes fish (Becker et al., 1977; Lawler et al., 1980; Lauckner et al., 1984; Noga et al., 1996). A study made by Murray et al., (2005a), using rRNA (ribosomal RNA), indicates that *A. ocellatum* is closely related to *Pfiesteria piscicida*. Saldarriaga et al., (2004) and Ki et al., (2012), puts *A. ocellatum* in the *Blastodiniales* order, but phenotypical characteristics put it near the order *Peridinales*, which agrees with Landsberg et al., (1994), Steidinger et al., (1996) and Fensome et al., (1999). Some studies have suggested that there may

be more than one species or strain of *Amyloodinium*, however none is registered at the moment (Lom and Lawler, 1973, Lawler et al., 1980, Noga et al, 1991).

3.2 Biological Cycle and Morphology

A. ocellatum is a dinoflagellate with a three-stage life cycle: trophont (parasitic state); tomont (encapsulated state, a cyst that develops after the trophont leaves the fish); and dinospores (free-living state, discharged from the tomont) with a length of 6.1 ± 0.8 microns and a width of 11.7 ± 0.5 microns (Landsberg et al., 1994). The actively feeding parasitic trophont attaches to fish gills and skin, the reproductive encysted tomont is introduced into sediments, and the free-swimming infective dinospores form after the tomonts divide (Kuperman and Matey, 1999; Woo et al., 2007). (Figure 3.1). At 25 degrees Celsius, each tomont can produce up to 256 dinospores in three days, each of which can infect a new host and produce a trophont.

The trophonts of *A. ocellatum* adhered to the fins, eyes, or mouth cavity of fish are brownish or yellow spheric, ovoid, or piriform structures with a size range of $49 \times 26 \mu\text{m}$ to $120 \times 79 \mu\text{m}$ (Cruz-Lacierda et al., 2004; Kuperman e Matey, 1999). (Kuperman e Matey, 1999). In the cytoplasm, a huge spheric nucleus with numerous chromatophores, starch granules, and digesting vacuoles can be seen in a central location. Externally, trophonts have a pseudo-chitinous capsule, a stomatopod, a structure specialized in nutrition absorption, and a short stalk with multiple rhizoids that allow them to pierce and connect to hosts in the polar zone (Zambrano et al., 2001).

In the final phase of the nutrient absorption process, the rhizoids and stomatopod are absorbed through an opening in the capsule that, when closed, creates a cyst or a tomont (Zambrano et al., 2001; Noga and Levy, 2006). Before the tomonts final division, the cell's shape shifts from ovoid to elongated, and its cytoplasm darkens, compresses, and suffers strangling. The tomites (daughter cells of the tomont) divide through synchronized binary fission every 9-10 hours inside the tomont. The tomont always has 2 n tomites of identical size in each division state.

The initial size of the tomont when it detaches from the host determines its reproductive potential (division number before sporulation), however the incubation conditions are critical for achieving maximum reproductive capability (Paperna et al., 1984). Each tomite differentiates and

converts into two dinospores that are ejected from the capsule after the last division, a process known as "sporulation." The dinospores occasionally escape from the tomite while still attached, and the final separation takes place outside of the "mother" cell (Paperna et al., 1984).

The dinospores are the active state of the parasite and assume a classic morphological shape of a dinoflagellate with two flagella. Usually, each tomont creates between 64 and 256 dinospores (Menezes et al., 2000; Paperna et al., 1984).

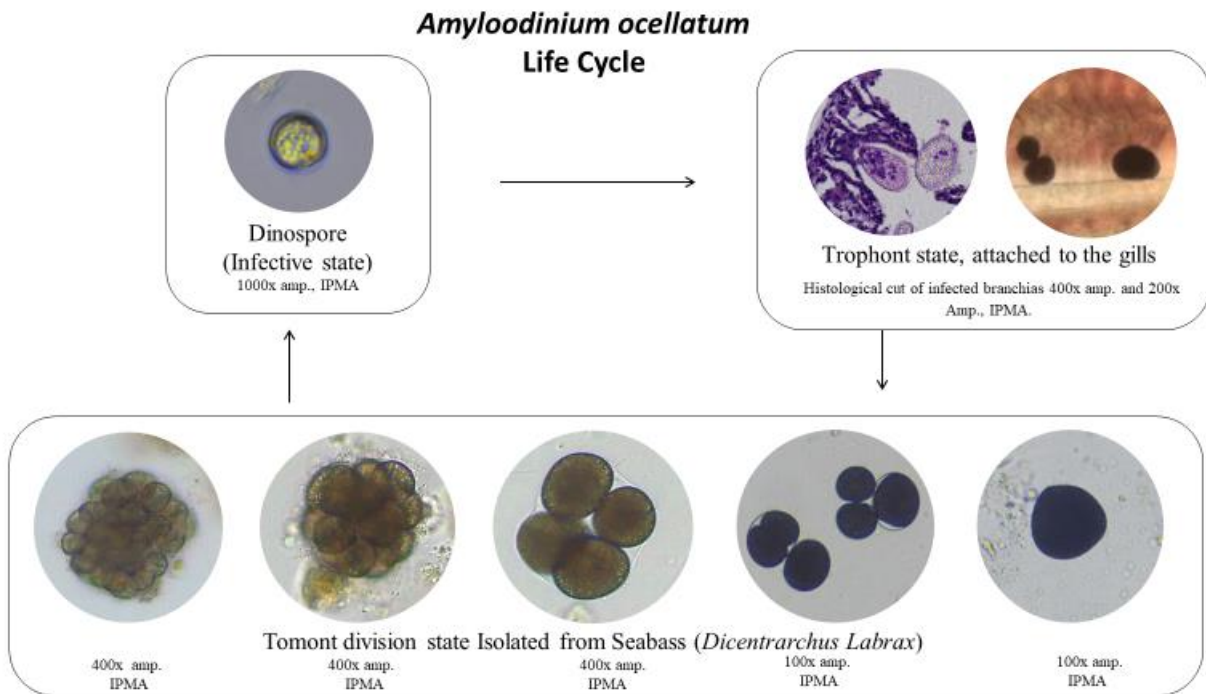


Figure 3.1- *Amyloodinium ocellatum* life cycle phases (amp. 200x and 400x)

3.3 Diagnostics and Pathology

The symptomatology is characterized by changes in fish behaviour, with slow jerky movements, swimming at the water surface and decreased appetite, a condition generally

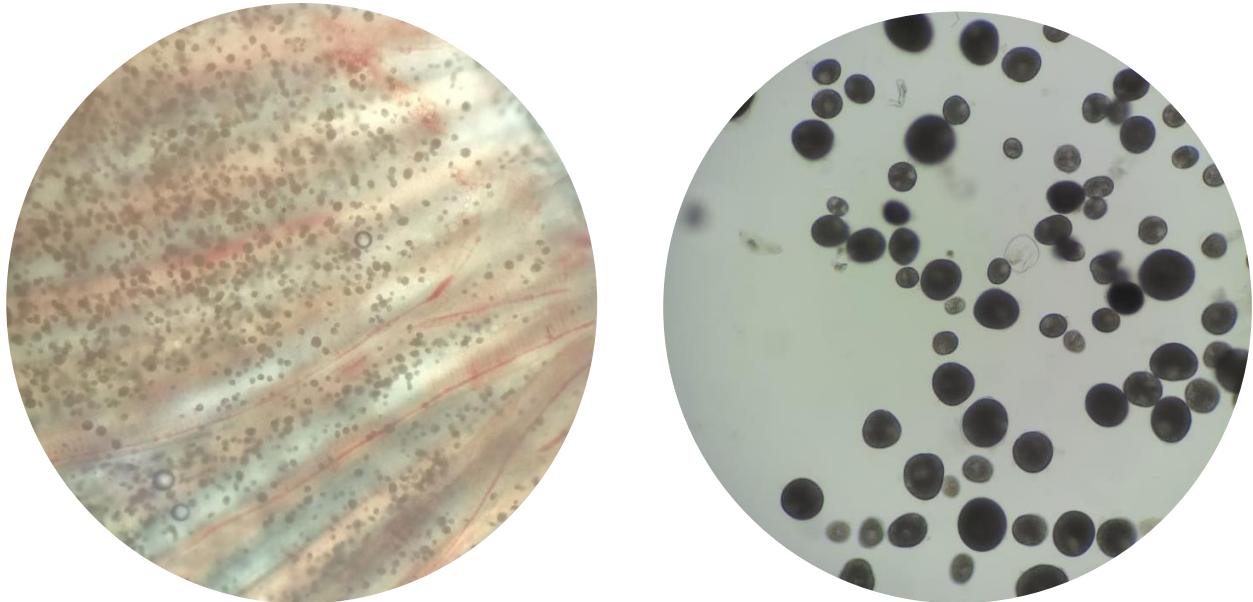


Figure 3.2 and 3.3- *Amyloodinium ocellatum* attached and detached from gills of seabass (*Dicentrarchus labrax*), respectively (40x and 100x amp., IPMA)

associated with most skin and gill infections (Soares et al., 2011; Menezes et al., 2000). If the primary site of infection is the skin, infected fish sometimes develop a white or brown colouration (“velvet”) or cloudy appearance, which is most visible when viewed with indirect lighting such as a flashlight (Levy et al., 2007). Such fish may display signs of “flashing” or rubbing on tank walls, the substrate, or other structures in their environment. The method of diagnostic usually involves using a fresh extract of either skin or gill of recently dead or moribund animals and sequent observation of the sample in a microscope (Menezes et al., 2000).

In some cases, when the “velvet” is not present, direct identification of the parasite may be possible. For example, if the skin infection is large enough and the fish's coloration is dark, it may be possible to identify the parasite by applying diluted lugol directly to the affected area, as it reacts with amid, which is produced by the trophonts (Noga et al., 2012). Bower et al., 1987

discovered that if infected fishes are transferred to a receiver with distilled water, the tomonts will gather in the bottom in roughly 15 minutes, allowing them to be observed.

Serum antibodies detected by ELISA or dinospores agglutination assay may be produced by fish that have recovered from an *Amyloodinium* infestation or have been experimentally exposed to parasite antigen (Smith et al., 1992; Cobb et al., 1998 a,b; Cecchini et al., 2001). Because increased antibody titers have been related to resistance, such assays could be beneficial for both monitoring and identifying subclinical infestations in vulnerable groups (Cobb et al., 1998 a, b). Examining it under a microscope to assess its presence in the water column in fish farms is impractical because one stage of its life cycle is free (dinospores). In this regard, the PCR-based method for detecting dinospores (free phase) in water is a sensitive and specific method for detecting a small number of individuals (Levy et al., 2007). As a result, it can be utilized in an aquaculture environmental monitoring program, as effective disease control requires the ability to detect an infestation in its early stages and execute effective control measures (Soares et al., 2011).

Anoxia (associated with significant gill hyperplasia, inflammation, haemorrhage, and necrosis) is usually blamed for fish deaths, which can happen in as little as 12 hours in heavy infestations (Lawler et al., 1980; Noga et al., 2012). Mild infestations, on the other hand, have a high mortality rate, which Noga et al., (2012) attributes to osmoregulatory dysfunction and subsequent microbial infections as a result of extensive epithelium damage.

Paperna et al., (1980) also observed that, in infections of *A. ocellatum* in juveniles and reproducers of seabream and seabass, the infection focus was on the gills, while in the larval stage the pathogen is mainly found in the skin.

3.4 Treatments, Prevention, Control, and Prophylaxis

As *A. ocellatum* is a virulent parasite, outbreaks must be treated as soon as possible to prevent excessive mortality rates (Ramos e Oliveira, 2001). Efforts to remove *A. ocellatum* at various stages of its life cycle have shown some results using various techniques and drugs. However, because *A. ocellatum* tolerates a wide range of temperature and salinity, modifying physical and chemical parameters for disease management is extremely difficult. Temperatures below 15 °C and lower salinities can inhibit parasite growth (Paperna et al., 1984). Lowering the temperature to 15 °C stops the disease development, while decreasing salinity slows but does not prevent infestations (Barbaro and Francescon, 1985), unless fish are placed in freshwater. Most, but not all, trophonts are dislodged by a brief freshwater bath lasting up to 5 minutes (Kingsford et al., 1975; Lawler et al., 1977b). These measures, however, are ineffective in cropping systems that use earthen ponds (tanks of large dimensions, 0.4 to 1 hectare and larger) and offshore systems, because most pathogen-affected species cannot tolerate wide variations in salinity and temperature, which is the reality in most southern European aquaculture. According to a recent study by Pereira et al. (2011), the water quality maintained within production ponds with a defined fish stock density and a good water renewal rate helps avoid and control *A. ocellatum* infestation in seabream. Chemotherapy is effective against the free-living state (dinospores), but the parasitic and encapsulated states of trophont and tomont are difficult to remove or treat (Lawler, 1980; Paperna et al., 1984). (Soares et al., 2011). The most commonly used treatment for managing and removing this parasite is copper in the form of copper sulphate, a chemical widely used in agriculture but that can have serious environmental consequences if used irresponsibly (Soares et al., 2012).

Because the cell wall prevents the entry of copper ions, the process of encapsulation and division normally happens until sporulation after dislodging the trophonts of the host tissues. Dinospores are exposed to the cytotoxic impact of copper during sporulation, interrupting the parasite's life cycle (Paperna et al., 1984). Tomonts can withstand copper concentrations ten times higher than those that are poisonous to dinospores (Paperna et al., 1984). According to Canosa et al., (2006), copper treatments should last 10-14 days to control the disease, at a concentration of 0.12-0.2 mg. L⁻¹. However, Vaz et al., (2010) by exposing bream juveniles (*Diplodus sargus*) to 0.2 mg. L⁻¹, 0.5 mg. L⁻¹ and 1 mg. L⁻¹ for 23 days and 0.25 mg. L⁻¹ and 0.5 mg. L⁻¹ for 60 days

observed evidence of toxic effect during exposure to copper sulphate, with an increase in the concentration of copper in the liver accompanied by morphological changes in fish. Because free copper is unstable in water, levels should be monitored to ensure that they remain at the correct concentration. It should be noted that at high water temperatures (25 to 30 °C), the infections and, consequently, the copper sulphate treatment may linger for more than a month, which, when combined with the high cost of the product, may imperil the commercial sustainability of a fish farm (Soares et al., 2011). Treatments with formaldehyde at concentrations of 100-200 mg/L for 6 to 9 hours, on the other hand, act on the detached trophont stage. The most effective medical approach has found to be repeated treatments, frequently followed by the removal of the fish to an uncontaminated tank. For example, treating juvenile bullseye puffers (*Sphoeroides annulatus*) in saltwater with 51 mg/L formalin for 1 hour or 4 mg/L formalin for 7 hours reduced *A. ocellatum* load on the skin and gills considerably. After 15 days, reinfestation occurred, but it was managed by continuing the treatment (Fajer-vila et al., 2003). Hydrogen peroxide at 75-150 mg/L was successful in removing trophonts from Pacific threadfin (*Polydactylus sexfilis*) (Montgomery-Brock et al., 2001). All treatments must be maintained for an extended period of time in order to be effective, albeit rigorous management is still essential by testing the fish for probable reinfestation. Many different medications, including chloroquine diphosphate, have been used to treat this parasite, but the outcomes have been mixed. In vitro tests using N-methylglucamine lasalocid indicated that it inhibited dinospore emergence at dosages as low as 0.01 mg/L. Treatment of infestations on red drum fry for 24 h with 0.10 mg/L reduced gill infestation intensity by 80 % and none of the fish treated with 1 mg/L had signs of infestation (Oestmann and Lewis, 1996).

Oestmann et al., (1995) presented a biocontrol technique for dinospores control based on the discovery that larval brine shrimp (*Artemia salina*) readily feast on dinospores. In an in vitro study with a ratio of 8 nauplii/mL and 10,000 dinospores/mL (1 nauplius/1250 dinospores), all dinospores were removed after 8 hours. Adding nauplii before the fish experiment also reduced, but did not eliminate, the infestation. Soares et al. (2012) also indicated that a greater investment in biological treatments may result in a higher quality final product and lower waste management costs. There are also new plant-based research studies (Harikrishnan et al., 2011).

Another approach that is yielding results is immunological stimulation of fishes (Noga et al., 2001; Ewart & Tsoi 2004; Ullal et al., 2008; Noga et al., 2009; Corrales et al., 2010; Noga et al., 2011), with a focus on H2B histones and other antimicrobial peptides (AMP) found in most reared species. The introduction of live yeast from *Debaryomyces hansenii*, for example, has increased the resistance of juvenile leopard grouper *Mycteroperca rosacea* to *A. ocellatum* (Reyes-Becerril et al., 2008). There is additional research being done to develop a vaccine for this parasite. The grouper *Epinephelus coioides* was inoculated with a modest dosage of *Cryptocaryon irritans* theronts and demonstrated acquired protective immunity (Yambot and Song, 2006), indicating the promise of *A. ocellatum* immunization. In terms of risk prevention, the introduction of dinospores in aquaculture systems can be prevented by treating the intake water with ultraviolet (UV), ozone and chlorine (Lawler et al., 1977; Noga et al., 2012). This method, however, is only applicable in closed systems. In the case of Portuguese aquaculture production, which is mainly done in open systems in tanks ranging from 0.4 to 1 hectare, UV water treatment is unfeasible. Furthermore, UV does not disinfect the tomonts-containing pond silt. In open systems, prevention is only possible by closely monitoring the parasite's presence on fish during optimum development phases and avoiding stressful situations for the fish. When fish are stressed, the parasite multiplies rapidly and kills a large number of them. Routine hygiene protocols, such as the removal of dead fish from culture tanks, must be followed (Soares et al., 2011)

4. Stress Indicators

Marine animals including fish are very sensitive to stressors in nature and artificial aquaculture conditions (Iwama et al., 2006). Some aquaculture studies describe that chronic and acute stress is associated with many aspects regarding biochemical or physical perturbations and have physiological effects on the organisms (Kubilay and Ulukoy, 2002; Vijayan et al., 2009).

There are different definitions of stress. Cannon (1935) defined for the first time the "homeostasis" theory. Selye et al., (1950) proposed the notion of non-specific physiological response to stressors, in which he classified all stressor reactions into two categories: special stressor reactions and general stressor reactions with no prior focus on the stressor. Later, McCarty

et al., (1996) claimed that stress is a non-specificity biological reaction or a lack of specificity response of animals to any stimuli in emergencies.

The origin of stressors can be divided into:

1. Biological stressors due to parasites (Landsberg et al., 1998), and high organism density.
2. Chemical stressors are created by spilling contaminants into the water, abiotic environmental stressors (Van Weerd and Komen, 1998), nitrogen, and faeces (Askarian and Kousha, 2006).
3. Physical stressors such as capture (Bolasina et al., 2011), heat fluctuations, crowding or density (McCormick et al., 1998; Bolasina et al., 2011), bad water quality (Santos et al., 2010), water-soluble gases, light, water pressure, feed intake versus FCR, and noisiness (Askarian and Kousha, 2006). Input water quality monitoring is critical for preventing physical stressors such as periodic decreases in dissolved oxygen or increases in CO₂ (Person-Le Ruyet et al., 2002).

Numerous research on stress and biological interactions in marine creatures have been conducted. Most studies state that cortisol and glucose are the most prevalent stress indicators (Einarsdóttir and Nilssen, 1996; Einarsdóttir et al., 2000; Olsen et al., 2008; Fernandes de Castilho et al., 2008; Martinez-Porchas et al., 2009) that can affect fish physiological activities such as increasing cardiac output and gill vascularity along with glycogenolysis with activation of "brain-sympathetic-chromaffin cell axis" (). (Nolan et al., 1999; Olsen et al., 2008).

Activation of the hypothalamus-pituitary gland-gonad axis (Carragher et al., 1989; Pottinger and Mosuwe, 1994) with the release of catecholamines (Einarsdóttir and Nilssen, 1996) such as epinephrine, adrenaline (Martinez-Porchas et al., 2009), norepinephrine (Askarian and Kousha, 2006), glucocorticoid steroid hormones (Vijayan et al., 2009) and corticosteroid hormones within the bloodstream (Weirich et al., 1997; Martinez-Porchas et al., 2009) have been the focus of different studies.

The activation of the stress system is defined by many levels of responses whenever stress occurs:

1. *Alarm*, primarily with an increase in plasma cortisol and catecholamines via neuro-physiological axis that is called the hypothalamic-pituitary-interrenal (HPI) axis (Vijayan et al., 2009).
2. *Resistance*, secondary to an increase in plasma metabolites (such as glucose and lactate) and a decrease in tissue reserves (i.e., glycogen).
3. *Exhaustion*, with reductions in several physiological and immunological processes (growth, reproduction, and others) (Wedemeyer et al., 1990; McCarty et al., 1996; Wendelaar et al., 1997; Askarian and Kousha, 2006).

The critical activities of animals, including fish, are dependent on the intake of oxygen from the environment. Fish have mechanisms for dealing with oxygen demands and adapting to changes in the environment in terms of supply (Martemyanov et al., 2013; Martemyanov et al., 2015). Elevated sodium levels in erythrocytes are found during fish adaptation to oxygen shortage, which occurs due to a decrease in sodium solubility in water when water temperature rises (Martemyanov et al., 2009). The levels of catecholamines in blood increase significantly during the initial stage of stress (Mazeaud et al., 1977; Mazeaud and Mazeaud, 1981), increasing the oxygen needs (Klyashtorin and Smirnov, 1981; Aardt and Booyesen, 2004; Fu et al., 2007).

There is a hypothesis that the functions of Na^+ / H^+ counter-transport enhance in stress conditions, due to catecholamines that increase the oxygen-carrying capacity of fish erythrocytes. This could be measured with a different analysis, like pH and osmolarity (Thomas and Perry, 1992; Wendelaar Bonga, 1997; Perry and Bernier, 1999).

5. Immune Response

“Teleost fish possess both innate and adaptive immune responses, the latter is the key component of the immune system that provides protection following vaccination.” (Adam et al., 2021).

5.1 Innate Immune Response

Innate responses are rapidly mounted reactions to invading organisms and include a complex network of molecules and cells that operate to kill and/or inactivate the assumed pathogen. The leucocytes involved in innate responses are mononuclear phagocytes, polymorphonuclear leucocytes, and natural-killer cells. Molecules involved in innate responses are antibacterial peptides, lysozyme, transferrin, complement, reactive oxygen intermediates (ROI), cytokines, chemokines, and Toll-like receptors (TLR) (Zaccone et al., 2017)

Some fish species, such as killifish (*Fundulus grandis*), American eel (*Anguilla rostrata*), and molly (*Poecilia latipinna*), are inherently immune to *A. ocellatum* parasitism. Resistant species are typically those that create thick mucus or can endure low oxygen levels, probably as a result of the parasite's higher capacity to withstand an attack or feeding on gill tissue (Lawler et al., 1977). In host-parasite interactions, host factors must play a crucial role. Fish parasite oodiniids only feed on or within the skin or gill epithelial tissues. Thus, host-parasite interactions (i.e., host recognition, defensive systems responsible for defending against these infections, and so on) are found in mucus or on/in epithelial cells and extracellular fluid of the epithelium. *In vitro* data suggests that serum can have strong anti-*Amyloodinium* activity (Landsberg et al., 1992). *Amyloodinium* is also highly sensitive to natural, host-produced antibiotics, known as antimicrobial polypeptides (AMPPs). Histone-like proteins (HLPs), a form of AMPP, are found in large amounts in the skin and gills of hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) and other fish (Noga et al., 2001). The amounts of HLPs that were fatal to *Amyloodinium* were well within the range that these compounds are found in fish tissues, implying that they play an essential role in protecting fish against this parasite. Surprisingly, HLPs are very toxic to trophonts but have no effect on dinospores (Noga et al., 2001). Recent research has also revealed that another class of antimicrobial polypeptides, piscidins, are highly toxic to both dinospores and trophonts

(Colorni et al., 2008). Piscidins are present in high concentrations in epithelial tissues including skin and gills (Noga et al., 2009). They are widespread in higher teleost's, especially perciform fish (Silphaduang et al., 2006; Noga et al., 2011) and, thus, this defence may play an important role in resistance to amyloodiniosis. In terms of upregulated defence's, recent data shows that a natural AMPP response in fish, consisting of a suite of AMPPs, some if not all of which are derived from the β -chain of the respiratory protein haemoglobin (Hb β), can be strongly upregulated *in vivo* to levels that are lethal to important pathogens (Ullal et al., 2008), including *Amyloodinium* (Ullal and Noga, 2010; Noga et al., 2011). These AMPPs originate not in the blood but rather in the skin and gill epithelium, the target tissue of *Amyloodinium*. Most importantly, the Hb AMPP concentrations expressed *in vivo* appear to be well within the antiparasitic concentrations measured *in vitro* (Ullal et al., 2008). These data suggest that mechanisms exist to increase at least some AMPP responses to highly lethal levels *in vivo*, thus providing the opportunity to use this as a direct protective tool. In this regard, feeding leopard grouper (*Mycteroperca rosacea*) a diet having the live probiotic yeast *Debaryomyces hansenii* resulted in significantly greater resistance to experimental challenge with *Amyloodinium*, compared with fish feed the same diet without the probiotic. Probiotic-feed fish recovering from the infection also had higher serum antibody levels (Becerril et al., 2008). However, red drum (*Sciaenops ocellatus*) fed a diet containing brewer's yeast and nucleotides, either alone or in combination, did not affect resistance to amyloodiniosis (Li et al., 2005).

5.2 Acquired Immune Response

In jawed vertebrates, lymphocytes (T cells and B cells), a type of white blood cell (leucocyte) with specialized receptors on their surface to detect foreign substances, mediate adaptive immune responses. Despite the fact that the repertoire of these receptors is extensive, each cell has only one specificity. When these receptors come into touch with their ligand (or antigen), the cells multiply and clonally expand, resulting in a larger population capable of fighting off microbial intruders. Furthermore, some of the cells survive for a long time as memory cells, allowing a second encounter with the same antigen to elicit a faster, more specific, and better immune response, resulting in protection. This is the premise of vaccination: prime the animals with an innocuous antigen that can trigger this immunological memory so that when they come

into contact with the true virus, their responses will protect them. Teleost fish have lymphocyte-like cells circulating in their blood and immune-competent tissues such the thymus, kidney, spleen, gills, and gut for a long time. Recently, molecular evidence has emerged confirming that these cells are, in fact, lymphocytes (Adams et al., 2016). They are tiny and have little cytoplasm while at rest compared to other leucocytes. T cells and B cells are the two main types, and they differ in a number of ways. T cells are produced in the thymus and then move to numerous tissue sites to initiate responses. B cells, on the other hand, appear to be made at different sites in different vertebrate groups, with the kidney appearing to be the most important site in teleost fish, similar to bone marrow in mammals, though they are first seen in developing zebrafish in between the dorsal aorta and posterior cardinal vein. Although the genetic mechanism underlying receptor variation is similar, antigen receptors in T and B cells are composed of independent genes and have different shapes. The T-cell antigen receptor (TCR) on most T-cells requires antigen presentation in the form of processed peptides from the original protein provided by major histocompatibility complex (MHC) molecules. B cells, on the other hand, may directly recognize and adhere to soluble antigens via their B-cell antigen receptor (BCR). Furthermore, B cells can produce a soluble form of this receptor that is released as an antibody or immunoglobulin (Ig) that can either act directly against the pathogen (neutralizing antibody) or aid in the internalization of a pathogen by phagocytes (opsonising antibody) (Adams et al., 2016).

There are currently 24 commercially marketed vaccinations for viral and bacterial aquaculture illnesses. However, there is just one commercial parasite vaccine available: Providean Aquatec Sea Lice, manufactured by Tecnovax S.A. Argentina. (Shivam et al., 2021). Recent research has uncovered critical defence systems that could be utilized to specifically boost parasite protection. Anecdotal observations suggested that fish recovered from amyloodiniosis were resistant to reinfestation in early research (Lawler et al., 1977b, 1980; Paperna et al., 1980). Smith et al. (1993) then shown in cell culture that serum from fish inoculated with dinospores could agglutinate living dinospores and kill *Amyloodinium*. Immunized fish also produced an antibody response that could be detected using an ELISA (enzyme-linked immunosorbent assay). This ELISA revealed anti-*Amyloodinium* serum antibody in blue tilapia (*Oreochromis aureus*) inoculated with the DC-1 isolate and in sea bream immunized with a Red Sea *Amyloodinium* isolate using 85-1 of DC-1 isolate *A. ocellatum* dinospores as the capture antigen (Smith et al., 1993). (Noga et al., 1992). An anti-*Amyloodinium* serum antibody was also found in hybrid striped

bass recovered from a spontaneous amyloodiniosis outbreak on a North Carolina fish farm using the same ELISA (Smith et al., 1994). This research indicates that fish may mount a strong antibody response to both artificial and natural stressors, and that there was significant cross-reactivity between these three *Amyloodinium* isolates. The latter backs up molecular evidence suggesting *Amyloodinium* is a fairly homogeneous taxon (Levy et al., 2007). Cecchini et al. (2001) discovered anti-*Amyloodinium* antibody in cultured European sea bass (*Dicentrarchus labrax*) that had recovered from an amyloodiniosis outbreak using tomont antigen. Following experimental trials, *Amyloodinium*-infested fish can build protective resistance, according to subsequent research. After a month of weekly sub-lethal *Amyloodinium* exposures, tomato clownfish (*Amphiprion frenatus*) developed strong immunity to infection. This defence was long-lasting (at least 6 months) and appeared to be directed towards the trophont (Cobb et al., 1998a). Antibody response, as determined by ELISA, was related with protection. In Western blots, the reactivity of immune fish serum to dinospores and trophont antigens revealed the existence of both common and stage-specific antigens (Cobb et al., 1998b). In an indirect fluorescent antibody test, the immune serum also responded with trophonts and dinospores. It was proposed that immunity may potentially be passively conveyed to naive fish (Cobb et al., 1998b). Because protection remained long after serum antibody was undetectable via ELISA, it was suggested that local antibody was more essential than serum antibody in protection. Probiotic-fed leopard grouper (*Mycteroperca rosacea*) which were more resistant to *Amyloodinium* challenge than fish fed a control diet, also had higher convalescent serum antibody titres than previously challenged fish not fed the probiotic (Reyes-Becerril et al., 2008).

Recent research with the European sea bass (*Dicentrarchus labrax*) has confirmed that, while an immediate local immune response to *A. ocellatum* parasite infection exists, three out of four genes coding for molecules related to adaptive immunity (Mhc I, Mhc II, and IgM) did not show upregulation, indicating that an acquired immune response is unlikely. Even though, IgM also has an important role in adaptive immune response, the lower level, or no expression of IgM in infected samples indicated that the parasitic toxins might negatively influence the systemic specific immune response (like production of IgM) (Covello et al., 2009) or that the disease reached its onset very rapidly and the individuals did not have enough time to activate a specific humoral response.

6. Methodology

6.1 *Amyloodinium ocellatum* Infection, Incubation and Collection

For *A. ocellatum* infection and incubation it was chosen the method described by Moreira et al., (2018), namely, a 600 L infection tank with closed recirculation seawater system, temperature between 20-25 °C, artificial aeration (100% dissolved oxygen) and 12 h light photoperiod was used to incubate the parasite *A. ocellatum* and obtain a maximal amount of tomonts. The initial inoculum of *A. ocellatum* tomonts was provided by the Aquaculture Research Centre, National Institute for the Ocean and Atmosphere, Olhão, Portugal (EPPO-IPMA). Several gilthead seabreams weighing 50 to 150 g were exposed to the parasite to increase the dinospores infective population of the tank and to obtain more *A. ocellatum* tomonts for future trials.

For the tomont collection, an adaptation of the method described by Paperna et al., (1984b) was used. When the fish manifest jerky movements (a clinical sign of *A. ocellatum* infection), the infested fish were bathed in freshwater for 1 to 2 min and passed the bathwater sequentially through a 500 µm screen (to take the bigger debris), a 100 µm screen and finally a 60 µm screen. Filtered tomonts, with a diameter between 120 and 90 µm, were placed into a petri dish in clean seawater and counted. Finally, tomonts were preserved in sterilized seawater at -80 °C until further use.

To calculate the total amount of tomonts collected, the container where the tomonts were stored was agitated for 2 min and 1 mL of the solution was removed into a Sedgewick rafter counting cell in triplicate and the total amount of tomonts were counted using a microscope with an amplification of 100x.

6.2 *Amyloodinium ocellatum* Sonication and Extract Obtention

To extract the hydrophilic components of *A. ocellatum* the method described by Moreira et al., (2019) was applied. The collected tomonts (300 000 tomonts) were resuspended in a 5 mL eppendorf on ice, containing 300 μ L of NaCl (0,85 %) and 700 μ L of acetone (99,5 %). Subsequently, the solution was sonicated on ice (QSonica, Q125 Sonicator with standard probe) with pulses every 10 seconds (80 % amplitude) until all tomonts were properly broken down.

After sonication, the solution was centrifuged at 3000g for 10min at 4 °C, and the supernatant (1 mL) was removed into a second eppendorf (2 mL). A volume of 500 μ L of acetone (99,5 %) at 4 °C was added to the initial eppendorf containing the pellet, agitated, and centrifuged again. The resulting supernatant was again removed and added to the second eppendorf. To evaporate the acetone, an eppendorf heat block (VWR Digital Heat block) at 42 °C was used for 10 h, until all acetone evaporated. The latter step must be performed in a hotte

After evaporating the acetone, 500 μ L of bi-distilled water and 500 μ L of diethyl ether (C_2H_5)₂O were added to the remaining solution, agitated, and then rested to let the fractions separate. Then, the diethyl ether (or lipophilic fraction) was removed to another eppendorf (2 mL) and another portion of 500 μ L of diethyl ether was added to the initial eppendorf. The solution was once again allowed to fractionate and after separation the fraction containing diethyl ether was removed. The remaining solution comprises the hydrophilic fraction and is the one used in this trial. The fraction containing diethyl ether or lipophilic fraction was stored for future trials.

6.3. Target Species and Culture Conditions

The target species for this study is seabass. The species is both well-known and studied, making it an ideal candidate. Furthermore, its economic value in the Mediterranean aquaculture and susceptibility to *A. ocellatum*, make its inclusion in this study of particular interest.

Seabasses were reared following the protocol used in EPPO-IPMA. The fish that were used in the vaccination trial (n = 84) have a mean weight of 139.8 g and were maintained in 9000 L tanks (density of 140 g/36 L) with a 12 h light:12 h dark photoperiod and water temperature between 20 and 25 °C. The fish were fed (Sparus[®], 3 mm marine-EPPO) 2 times a day, until satiation.

6.4. Vaccination and Challenge

Fish (n = 84) were immersed in a container with 200 ppm of 2-phenoxyethanol. After the fish were sufficiently anaesthetized, their total length and weight were registered with a ruler and a balance, respectively. At the same time, fish (n = 42) were injected intraperitoneally with 0.5 mL of the diluted hydrophilic extract (1: 300 in PBS or 1000 of the sonicated and processed tomonts per 0.5 mL), obtained from the sonicated tomont of *A. ocellatum* and the control (n = 42), with 0.5 mL of PBS. Fish were maintained in separate tanks of 1500 L under the same conditions they were reared in until the challenge trial at 28 days post-vaccination.

During this time, mortalities were recorded daily and fish from the vaccinated (n = 6) and control (n = 6) groups were sampled weekly (0, 7, 14, 21, 28 days post-infection). Each fish was measured and weighed, haemoglobin and hematocrit levels analyzed, liver and branchial tissue removed for histological analysis, the liver, branchial tissue, head-kidney, and spleen removed for gene analysis. Additionally, on days 14 and 28 an EPOC BGEM test card and card reader were used for Hgb, Htc, pH, K⁺, Ca⁺, glucose and lactate analysis.

The challenge with *A. ocellatum* was performed 28 days post-injection (according to commercial vaccines for other pathogenic agents, Li and Woo., (1995), and previous *A. ocellatum* immunization trials on *Oreochromis aureus* and the clownfish *Amphiprion frenatu*, (Smith et al., 1992; Cobb et al., 1998a). On the 28-day, 32 fish (16 vaccinated and 16 control) were divided into 12 tanks, 3 tanks per triplicate, with the same oxygen concentration (6 mg/L), temperature (25 °C), day-night cycle (12 h day: 12 h night), volume (70 L) and exposed to different concentrations of *A. ocellatum* dinospores, respectively 35 dinospores/mL (2.45×10^6 dinospores per tank) and 10 dinospores/mL (7×10^5 dinospores per tank). Figure 6.4 describes the distribution of fish in the challenge.

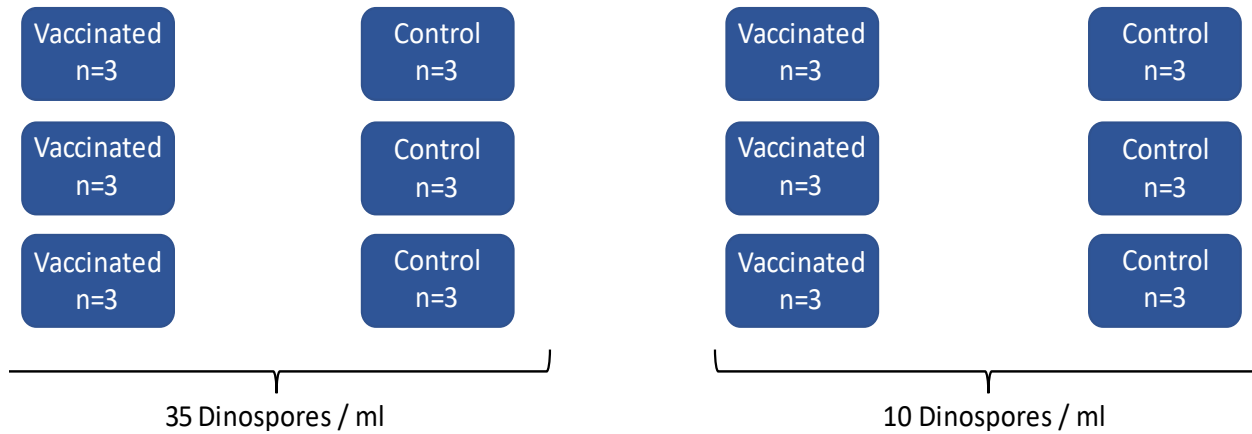


Figure 6.4 - The figure describes the system used to challenge *Dicentrarchus labrax* with two concentrations (35 and 10 dinospores per mL) of *Amyloodinium ocellatum* 28 days-post the administration of a vaccine.

The tomons for the challenge were collected with the same method used for the extract, described in section 6.2 of this thesis. To determine tomont fertility (number of infectious dinospores released per tomont) tomont concentration was determined using a Sedgewick Rafter cell counter and a sample (200 μ L) was allowed to incubate *in vitro* using a 96-well microplate for 72 h in triplicate, the dinospores deemed un-infectious (no mobility) were allowed to settle at the bottom of the plate and counted; then 10 μ L of lugol solution (I₃K) was added to each well, mixed

and allowed to settle for 10 min. Next, the total amount of dinospores in the bottom of the plate was counted and the infectious fraction calculated.

Fish mortalities and tomont/trophont quantity present in the first 2 branchial arches were visually assessed under a microscope. If the amount was deemed uncountable (>1000 trophonts and tomonts) the branchial tissue was washed and the total amount of tomonts was determined using the method described in section 6.1 of this thesis.

6.5 Sampling

After vaccination, fish were sampled weekly (0, 7, 14, 21, 28 days post-vaccination). All the animals were collected (n = 6 from the vaccination tank plus n = 6 from control) and inserted in a vessel containing anaesthetic (2-phenoxyethanol, Sigma, 300 ppm) to euthanize the animals. Once euthanized, the animals were weighed, and their standard length measured.

During this time, blood samples were taken for haemoglobin, hematocrit and blood cell counting and identification. Furthermore, one-third of the liver and the first branchial arch (additionally to the gills removed from regular sampling the gills from mortalities in the challenge were also removed for the same purpose) were fixed in formalin (CH₂O) at 10% (pH 7.2) for 48 h. Subsequently, the samples were changed to 70% ethanol until they were placed in histological cassettes. The other two-thirds of the liver, as well as the head-kidney, spleen and the second branchial arch, were removed and immediately immersed in liquid N₂ and stored at -80 °C until RNA extraction and liver enzyme analysis were performed.



Figure 6.5- Branchial removal (1^o arch) of *Dicentrarchus labrax*.

Additionally, on days 14 and 28 an EPOC bgem test card and card reader were used for Hgb, Htc, pH, K⁺, Ca⁺, glucose and lactate analysis.

6.6 Analysis of Haematological Parameters

6.6.1 Blood Collection

Blood collection followed a laboratory safety protocol that prevented contamination risks (Figure 6.6).



Figure 6.6 - Blood collection of *Dicentrarchus labrax* through the puncture of the caudal vein.

Blood collection was performed through a puncture in the Seabass caudal vein. The fish were previously anaesthetized with 300 ppm of 2-phenoxyethanol, and heparinized needles and syringes were used for the collection. The collected blood was stored in heparinized eppendorf tubes (2 mL) containing 10 μ L of heparin to prevent clotting before further analysis. The blood was used at the site to fill the capillary tubes used for haematocrit analysis, for the blood smear and for the analysis of haemoglobin.

6.6.2 Haematocrit

Hematocrit (Htc) was analyzed at the sampling site shortly after blood collection. The volume of Htc or packed cell volume is a technique used to assess the number of red blood cells or erythrocytes and leukocytes in each volume of blood. The result is given as a percentage of globular volume. The capillary tube was initially filled with 3/4 of blood and placed in the hematocrit rotor of the Hettich eba-21 centrifuge. Centrifugation occurred at 10,000 rpm for 5 min. The Htc value was determined by measuring the total length of Htc, the length of the zone of the red blood cells, using a ruler.

6.6.3 Haemoglobin

Haemoglobin (Hgb) is an iron-containing protein also known as metalloprotein. It is present in erythrocytes and allows the transport of oxygen throughout the circulatory system. In fish, haemoglobin has a great variability of functions and is related to both physiological needs and environmental availability of oxygen. Haemoglobin in ectothermic animals, such as fish, has demonstrated the ability to adapt to large variations of oxygen (Smarra et al., 2000).

Hgb concentration was determined using the Drabkin method (Moore et al. 1981). For the quantitative determination of Hgb, it was used heparinized blood and a working solution previously prepared with distilled water and Drabkin's reagent. The Hgb reacts, forming a compound – cyanmethemoglobin. The Hgb samples were centrifuged to remove dispersed nuclear material and incubated for 3 min at room temperature, their absorbance was subsequently determined in a microplate reader (Thermo Scientific™ Multiskan™ GO) at 540 nm against the blank which only contains 2.5 mL of working solution.

6.6.4 Counting and Identification of Blood Cells

To perform the blood smear, a drop of blood was placed on a slide. Another blade was placed in contact with the first and with an inclination of about 45°. The blood was allowed to spread by capillary action and the blood was distended with a quick and uniform movement, keeping the two blades in contact. The slides were air-dried and identified. Staining started after the slides were dry and using Giemsa solution. The smear was placed in a container of Giemsa solution for 1 minute. The slide was immersed in Phosphate-buffered saline (PBS) solution (7.4 pH) for 5 min, washed to remove excess dye and allowed to air dry before preparation.

This technique allows the observation of blood cells. For each slide, five random fields were selected, and counting was performed by observing the slides in an optical microscope, Nikon eclipse Ci and photographed on a Nikon DS-L3, under immersion oil at a magnification of 1000x.

6.7 Analysis of Stress Parameters

6.7.1 Glucose, Lactate, pH, K⁺ and Ca⁺

The determination of glucose, lactate, pH, K⁺ and Ca⁺ in fish blood was performed using the epoc[®] blood analysis system (Epocal Inc., Ottawa, Ontario, Canada). Immediately after the collection of blood, the needle is inserted into the apparel and a reading is taken which can be later downloaded into a computer or read on screen.

6.7.2 Fulton's Condition Index

Fulton's K condition index is considered a good indicator of the general welfare of the fish (Lambert & Dutil, 1997) and is directly linked with food (Ferron & Leggett, 1994). This morphometric index assumes that heavier fish for a given length is in better condition. It was calculated with the following formula:

$$K \text{ (mg. mm}^{-3}\text{)} = (W/TL^3) \times 100,$$

where W is the weight (mg) and TL is the total length (mm).

6.8 Analysis of Histological Parameters

The biological material (gills and liver) collected for histology were initially stored in 10% buffered formalin (pH 7.2) for 48 h. This fixation, in addition to preventing the destruction of cells by their enzymes or by bacteria, also aims to harden the tissues, making them more resistant to the subsequent steps of the histological technique. Subsequently, the formalin was removed, and the tissues were preserved in 70% ethanol until they were placed in histological cassettes.

After the impregnation of the tissues was carried out, the tissues were treated with molten paraffin (58-60 °C) and allowed to solidify. Afterwards, the block was sliced into sections 5 μm thick in a Leica model 5M 2000 R slide microtome. These sections were placed in a water bath (20 °C) and placed on glass slides with a solution of albumin and glycerine. Drying was carried out in an oven at 58 °C for at least 30 minutes.

To perform the histological examination, the sections were stained with Haematoxylin-Eosin (H&E) dye, as described in Martoja and Martoja, (1967).

6.8.1 Hepatocyte Measuring

For this step, five random fields were selected from each slide and, using FIJI-ImageJ, stained liver tissue was quantified, and the percentage of fat content calculated.

The process involves three steps:

1. Convert the image to grayscale
2. Segment (isolate) the red-stained collagen using thresholding
3. Measure the thresholded area

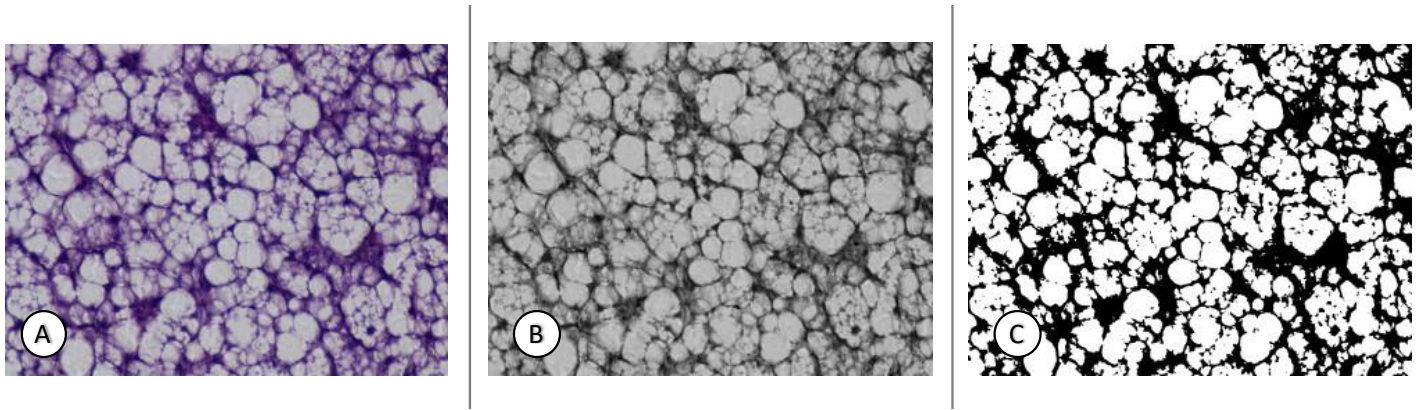


Figure 6.7 - Transformation process with ImageJ of a histological slide of liver tissue of *Dicentrarchus labrax* (amp. 400x). with ImageJ. A- Original Image; B, C- RGB stacking and processing.

6.9 Statistical Analysis

All results were expressed as means \pm standard deviation. Haematological and immunological parameters were analysed using the F-Test analysis to assess the equality of variances (F-test). One-Way ANOVA analysis of variance and the t-test for two samples assuming equality of variance (t-test: two samples assuming equal variance) were also used.

Differences were considered statistically significant when $P \leq 0.05$. All data were analysed using the Microsoft Office Excel 2010[®] program.

7. Results

7.1 Biometric Data, Condition Index and Mortality

The mean weight of seabass individuals was 141.4 ± 22.5 g, at the start of the experiment on day 0. On day 7 control showed superior growth with 147.7 ± 16.2 g over the vaccinated 139.2 ± 15.8 ; at day 14 control showed the same pattern with 155.9 ± 7.8 g over 149.90 ± 14.99 g; on day 21 the trend continued with 160.2 ± 19.1 g over 146.9 ± 33.4 g. However, on day 28 the tendency was inverted, the group of vaccinated fish showed a mean weight of 172.0 ± 29.3 g while the control showed 148.4 ± 18.2 g.

Over the experimental period, fish showed a high variation in growth rate. Nevertheless, the differences were not statistically significant ($p < 0.05$), except for the vaccinated group from T7 to T28.

Table 7.1 Evolution of biometric data of *Dicentrarchus labrax* during the trial ($n = 6$). T is time in days. Values marked with a different letter differ significantly from each other ($n = 6$; $p < 0.05$)

Time (days)	Weight (g)		Length (cm)	
	Control	Vaccinated	Control	Vaccinated
T0	141.4 ± 22.5^{ab}	141.4 ± 22.5^{ab}	22.6 ± 0.8^a	22.6 ± 0.8^a
T7	147.7 ± 16.2^{ab}	139.2 ± 15.8^a	23.0 ± 1.0^a	22.6 ± 0.9^a
T14	155.9 ± 7.8^{ab}	149.9 ± 15.0^{ab}	24.4 ± 0.4^a	23.7 ± 0.6^a
T21	160.2 ± 19.1^{ab}	146.9 ± 33.8^{ab}	23.8 ± 1.3^a	23.8 ± 1.6^a
T28	148.4 ± 18.2^{ab}	172.0 ± 29.3^b	23.4 ± 0.8^a	24.0 ± 1.4^a

The fish condition index (Figure 7.8) did not reveal significant differences in the health status of fish, being within the expected values of good physical health. However, on day 21 the control group was significantly higher than the vaccinated ($p < 0.05$).

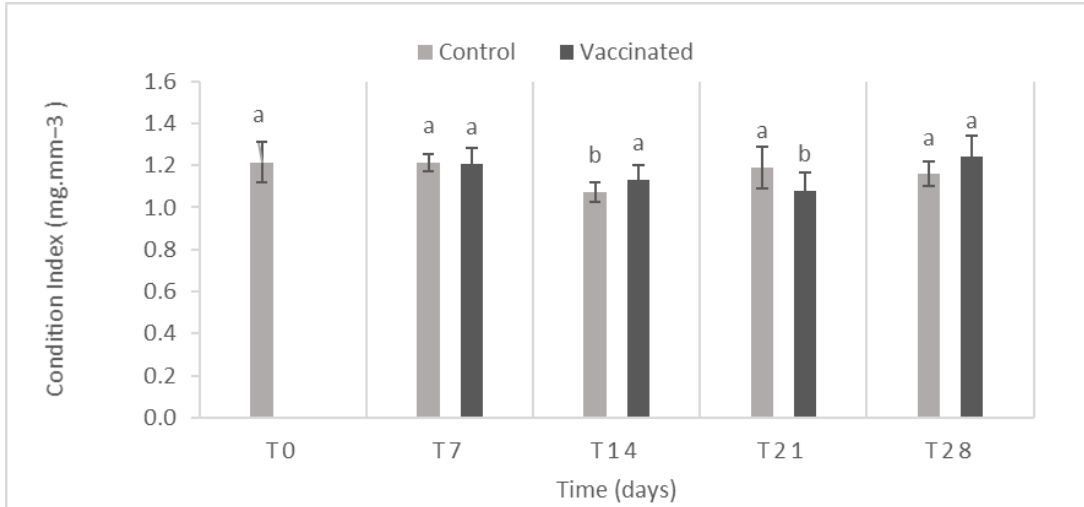


Figure 7.8 - Fulton's condition index (mg.mm⁻³) of *Dicentrarchus labrax* in the control and vaccinated tank during the 28 days after injecting the hydrophilic extract of *Amyloodinium ocellatum*. Values marked with a different letter differ significantly from each other (n = 6; $p < 0.05$).

No mortalities were recorded during the period between seabass vaccination with the hydrophilic extract and the day where they were subjected to the challenge with *A. ocellatum*.

7.2 Haematological Data

7.2.1 Haemoglobin

It was possible to observe through Figure 7.9 that there were no significant differences between the control and vaccinated tanks at both time points.

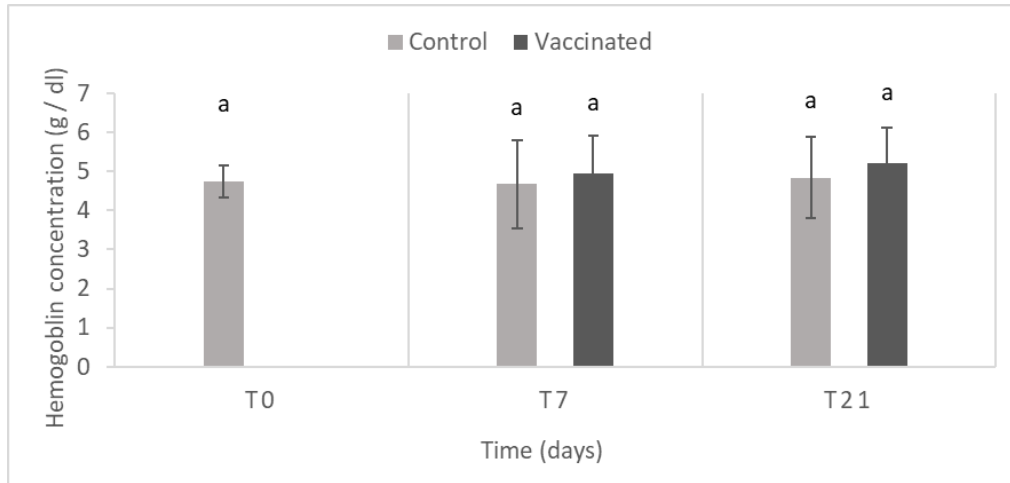


Figure 7.9 - Concentration of Hgb (g/dL) in *Dicentrarchus labrax* in the control and vaccinated group. Values marked with different letters differ significantly from each other (n = 6; p < 0.05)

7.2.2 Haematocrit

The results obtained by analyzing the percentage of erythrocytes in the total blood collected by individuals over time (Figure 7.10) revealed that the fish of the vaccinated group had a higher value than the control group at both time points (p < 0.05).

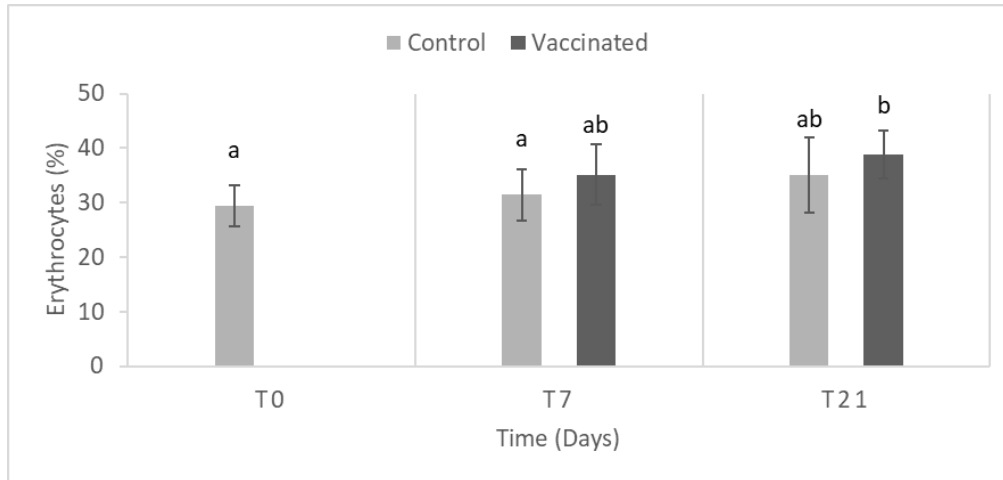


Figure 7.10 - Haematocrit of *Dicentrarchus labrax* in the control and vaccinated tank during the trial. Values marked with different letters differ significantly from each other (n= 6; p < 0.05)

7.2.3 Haematological Cell Counting and Identification

The identified leukocytes were monocytes (M), whose main function is the defense of foreign bodies (bacteria, viruses, parasites, among others). Thrombocytes (T), which have as main function the promotion of blood coagulation. Granulocytes (G) that consist of neutrophils, basophils, and eosinophilic leukocytes. Neutrophils are the first to act in case of infection and perform the function of defense through phagocytosis, basophils, react in cases of immediate hypersensitivities, such as allergic reactions and are cells that usually act in the presence of parasites, destroying the tissues of the same. Lymphocytes (L) are responsible for the identification and production of antibodies to fight antigens (foreign bodies).

Figure 7.11 shows the evolution of the leukocytes during the time between vaccination and the challenge. values show little difference between control and vaccinated groups, however, both

M and T values show differences from the time point day 7 until the final day 28, although not of statistical significance.

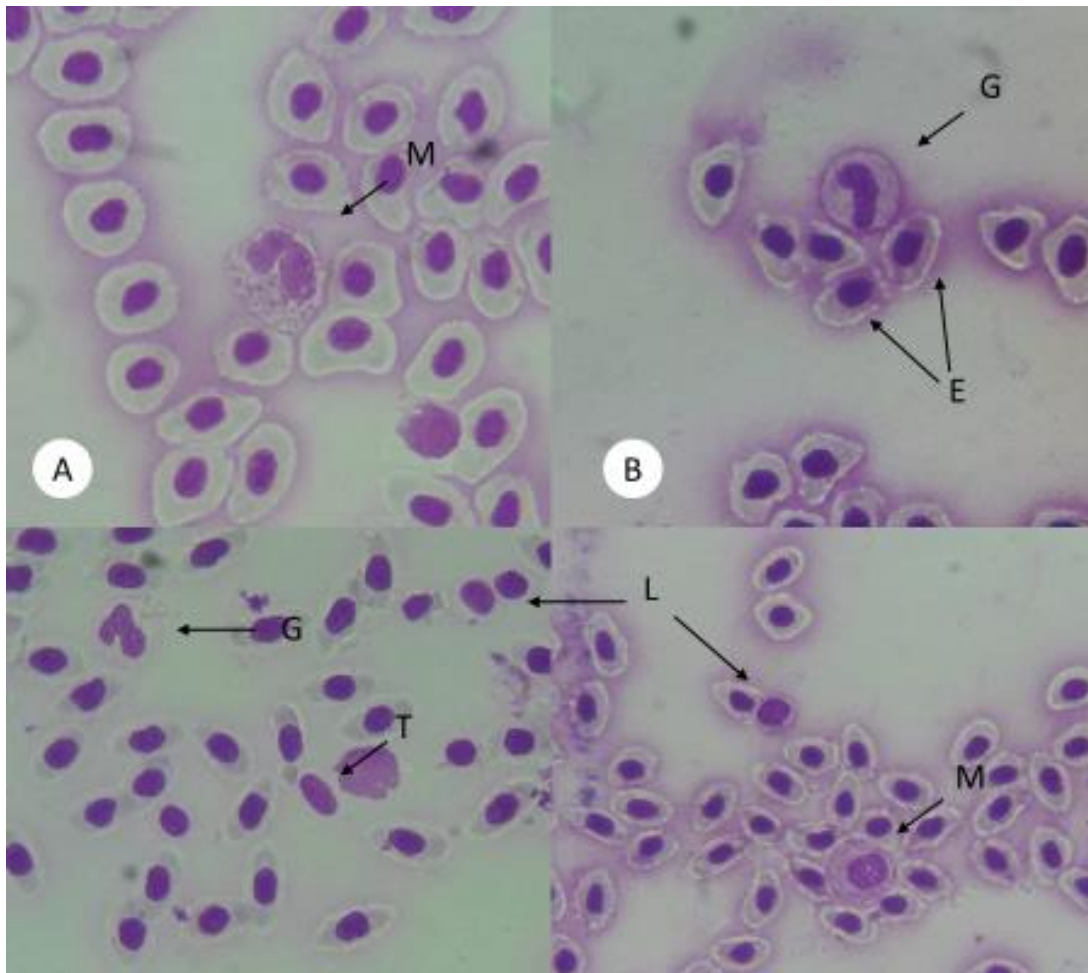


Figure 7.11 - Blood cell identification of *Dicentrarchus labrax*. (M - Monocytes; T- Thrombocytes; L- Lymphocytes; G- Granulocytes; E- Erythrocytes). (A, B) – 1500x amp.; (C, D) – 1000x Amp.

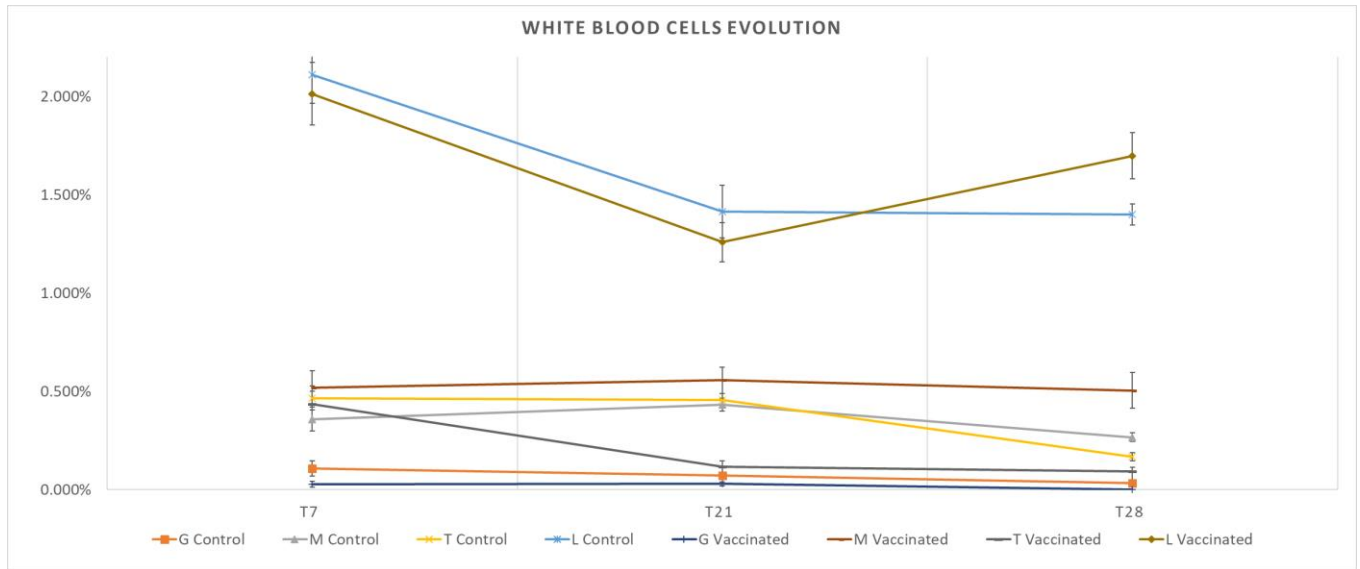


Figure 7.12 - Leucocytes in the blood of *Dicentrarchus labrax* at 7, 21 and 28 days post the injection of the hydrophilic extract of *Amyloodinium ocellatum*.

Table 7.2- Leucocytes in the blood of *Dicentrarchus labrax* during the time between vaccination and the first challenge. Values are mean \pm standard deviation. Htc and Hgb values were taken using the epoc[®] blood analysis system. Values marked with different letters differ significantly ($n = 6$; $p < 0.05$) in each haematological parameter.

Cell type	Treatment	T0	T7	T14	T21	T28
WHITE BLOOD CELLS (%)	control	1.65 \pm 0.81% ^a	2.96 \pm 0.90% ^b	N/A	2.37 \pm 1.22% ^{bc}	1.89 \pm 0.33% ^c
	vaccinated	1.65 \pm 0.81% ^a	3.03 \pm 1.12% ^b	N/A	1.98 \pm 1.01% ^{bc}	2.30 \pm 1.03% ^{bc}
GRANULOCYTES (%)	control	0.00 \pm 0.00% ^a	0.09 \pm 0.14% ^a	N/A	0.07 \pm 0.08% ^a	0.03 \pm 0.08% ^a
	vaccinated	0.00 \pm 0.00% ^a	0.03 \pm 0.07% ^a	N/A	0.06 \pm 0.14% ^a	0.00 \pm 0.00% ^a
MONOCYTES (%)	control	0.08 \pm 0.10% ^a	0.37 \pm 0.32% ^{ab}	N/A	0.43 \pm 0.20% ^b	0.29 \pm 0.18% ^{ab}
	vaccinated	0.08 \pm 0.10% ^a	0.55 \pm 0.51% ^{ab}	N/A	0.50 \pm 0.31% ^b	0.48 \pm 0.54% ^b
THROMBOCYTES (%)	control	0.14 \pm 0.19% ^{ab}	0.38 \pm 0.49% ^{ab}	N/A	0.44 \pm 0.49% ^{ab}	0.17 \pm 0.16% ^{ab}
	vaccinated	0.14 \pm 0.19% ^{ab}	0.43 \pm 0.32% ^b	N/A	0.13 \pm 0.25% ^{ab}	0.09 \pm 0.14% ^b
LYMPHOCYTES (%)	control	1.42 \pm 0.72% ^{ab}	2.12 \pm 0.70% ^b	N/A	1.43 \pm 0.78% ^{ab}	1.40 \pm 0.30% ^b
	vaccinated	1.42 \pm 0.72% ^{ab}	2.02 \pm 0.69% ^b	N/A	1.29 \pm 0.94% ^{ab}	1.74 \pm 0.80% ^{ab}
HAEMATOCRIT (%)	control	N/A	N/A	26.00 \pm 3.43% ^a	N/A	25.67 \pm 3.502% ^a
	vaccinated	N/A	N/A	28.17 \pm 3.43% ^a	N/A	26.83 \pm 1.329% ^a
HAEMOGLOBIN (g/dl)	control	N/A	N/A	8.82 \pm 1.27 ^a	N/A	8.70 \pm 1.16 ^a
	vaccinated	N/A	N/A	9.57 \pm 1.27 ^a	N/A	9.18 \pm 0.52 ^a

7.2.4 K⁺

The results obtained in the K⁺ analysis (Figure 7.13) show that there were no significant differences between the control and vaccinated tanks at both time points.

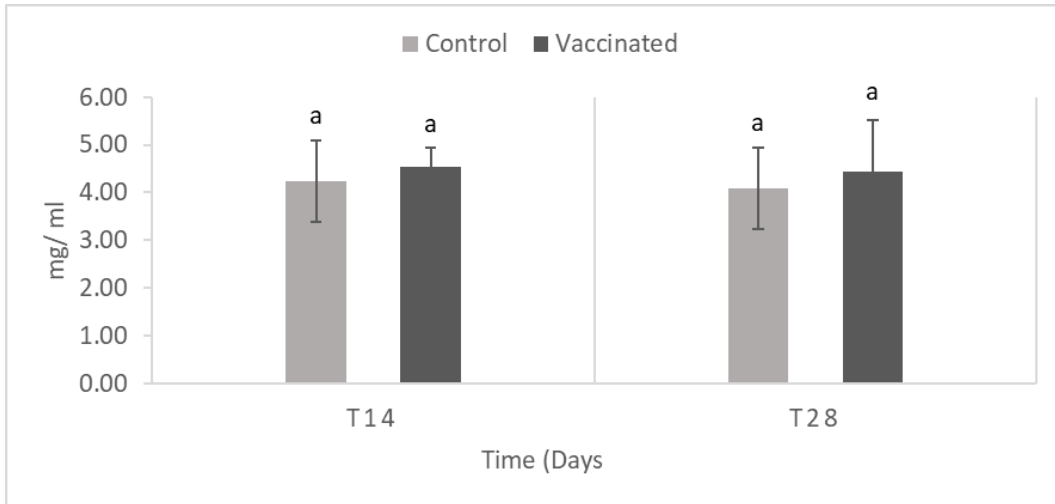


Figure 7.13 - Concentration of K⁺ in *Dicentrarchus labrax* in the control and vaccinated tank. Values marked with a different letter differ significantly from each other (n= 6; p < 0.05).

7.2.5 Ca⁺

The results obtained in the Ca⁺ analysis (Figure 7.14) show a significant decrease of Ca⁺ in the vaccinated group between the first and the second sampling. However, there was no significant difference (p < 0.05) between the vaccinated and control group.

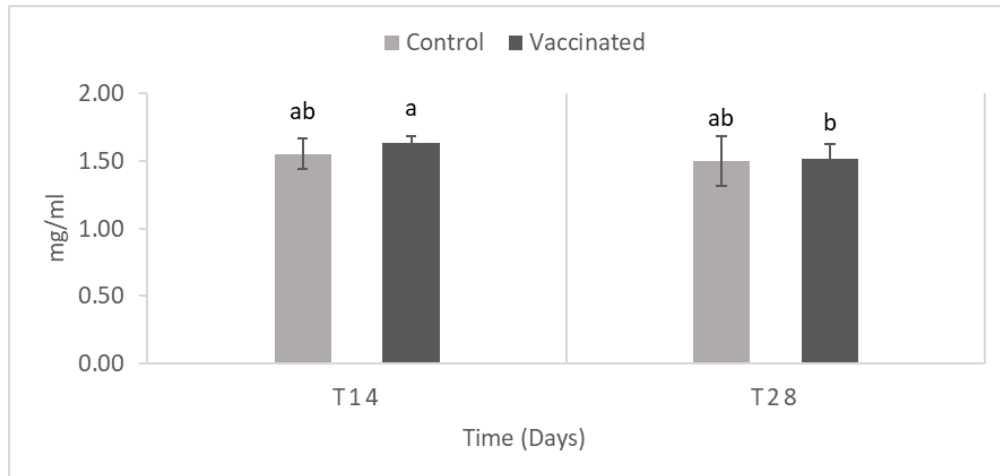


Figure 7.14 - Concentration of Ca⁺ in *Dicentrarchus labrax* in the control and vaccinated tank. Values marked with different letters differ significantly from each other (n= 6; p < 0.05)

7.3. Haematological Stress Parameters

7.3.1 Glucose

The results obtained in the glucose analysis in fish blood (7. 15) show that in the time point 14 days, the vaccinated group had a higher value than the control group. However, on day 28 both groups showed similar values only differentiating by 3 mg/mL.

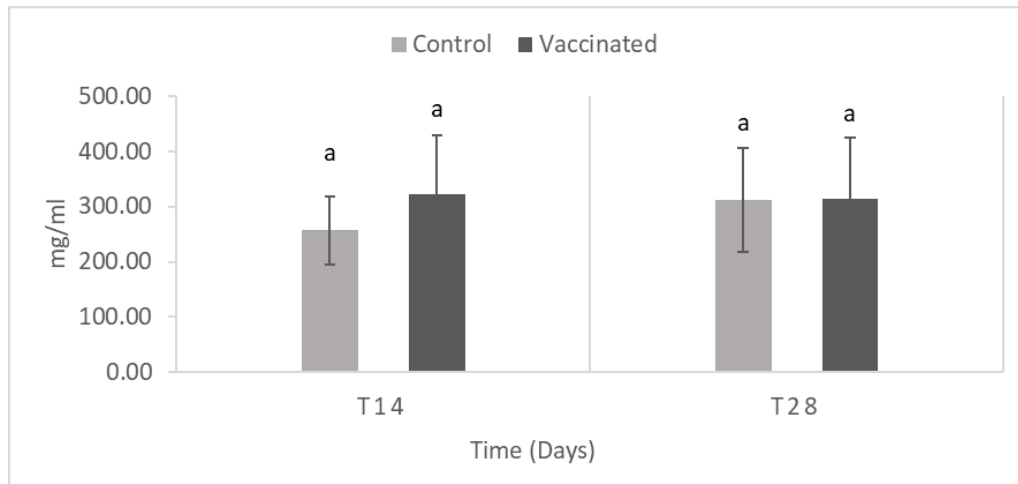


Figure 7.15 - Concentration of glucose in the blood of *Dicentrarchus labrax* in the control and vaccinated tank. Values marked with different letters differ significantly from each other (n= 6; p < 0.05)

7.3.2 Lactate

The results obtained in the lactate analysis in fish blood (Figure 7.16) show there were no significant differences between the control and vaccinated group (p < 0.05).

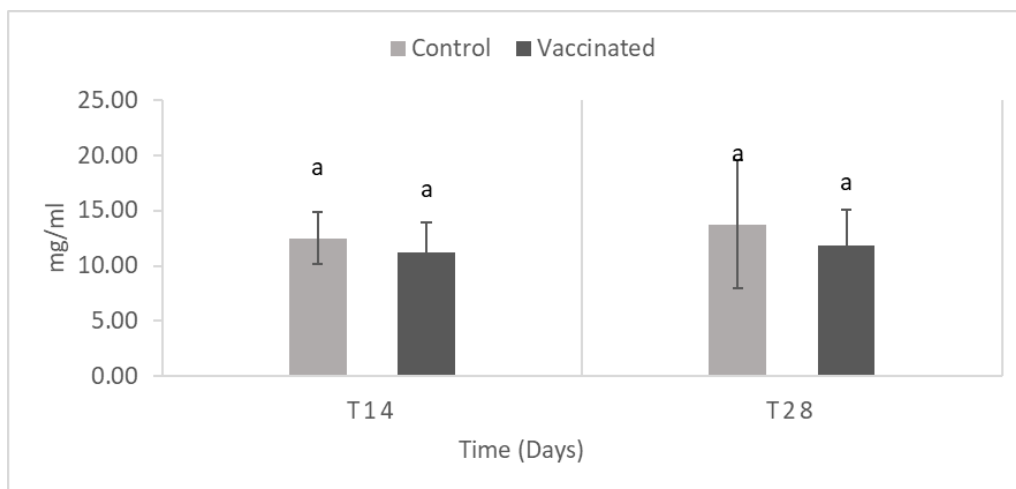


Figure 7.16 - Concentration of lactate in the blood of *Dicentrarchus labrax* in the control and vaccinated tank. Values marked with a different letter differ significantly from each other (n = 6; p < 0.05)

7.3.3 pH

The results obtained in the blood pH analysis (Figure 7.17) show the control group had higher pH values throughout the experiment and at T28 was significantly different from the values of the vaccinated group.

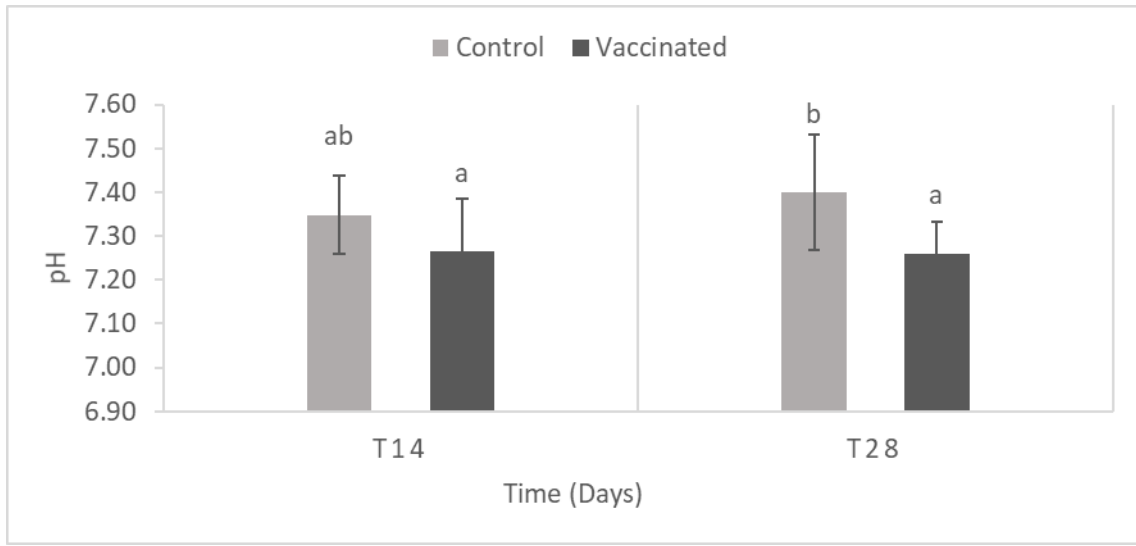


Figure 7.17 - Blood pH values of *Dicentrarchus labrax* in the control and vaccinated group at 14 and 28 days after the administration of the hydrophilic extract. Values marked with a different letter differ significantly from each other (n = 6; p < 0.05)

7.4 Histology

The histological parameters analyzed were organ status and tissue differences. Gills and fish liver from the control tank and infection tank were analyzed visually using a microscope. The fish from both the control tank and the vaccinated tank did not show significant differences or marked changes in tissue level over time in both gills and liver.

7.4.1 Gills

There was no detectable change or effect in fish sampled from both treatments as is demonstrated in figure 7.18, both during the period between vaccination and the challenge or from the fish that died during the challenge due to infection by *A. ocellatum*.

While the samples removed before the challenge show healthy tissue, those removed from the fish that died during the challenge, both vaccinated and control, show lamellar hyperplasia and fusion and detachment of the respiratory epithelium.

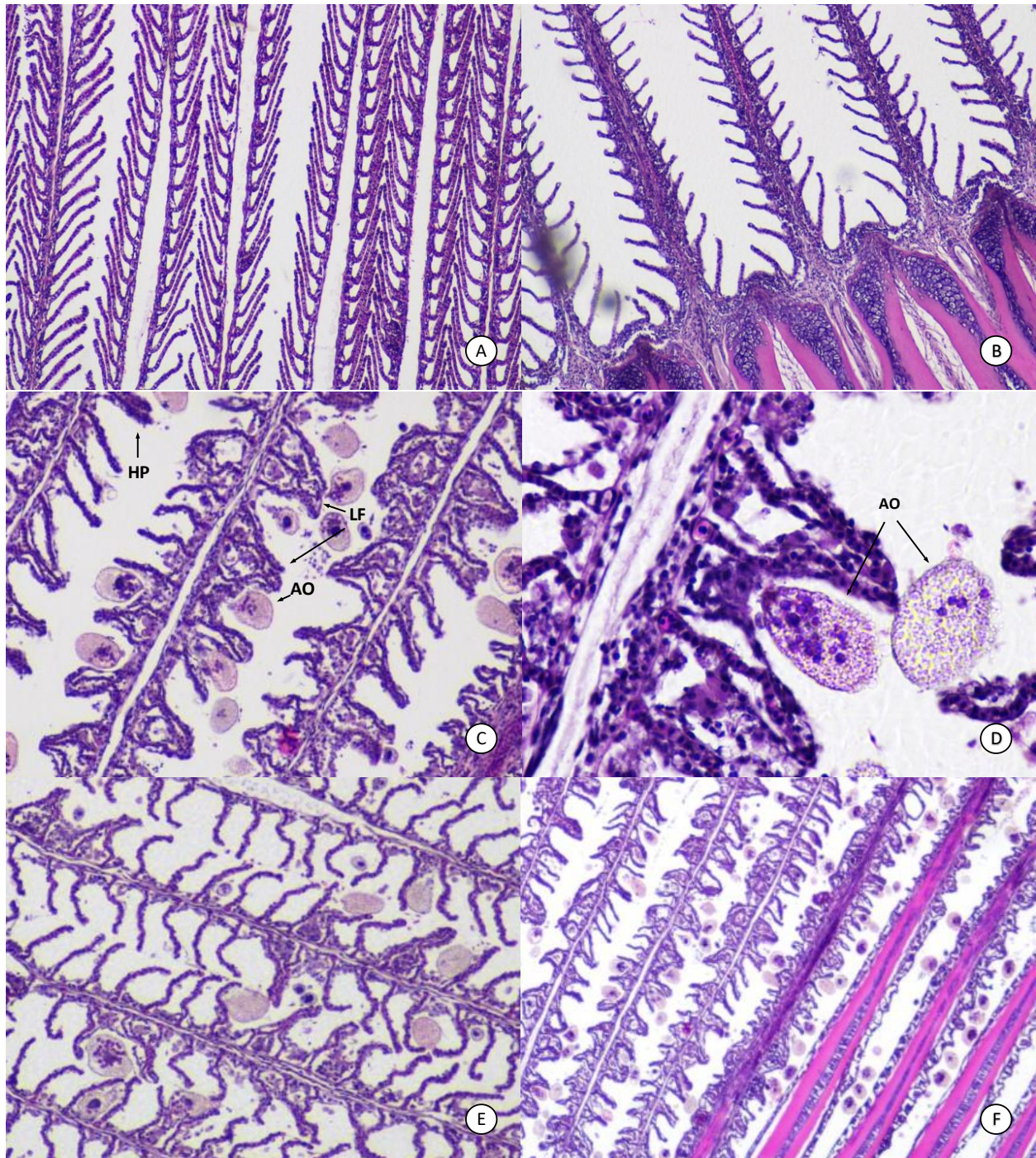


Figure 7.18 - Images of histological sections of Gills of *Dicentrarchus labrax* stained with Haematoxylin-Eosin (H&E) dye. (A, B) Show the normal appearance of healthy gills of the vaccinated tank (A) and control tank (B) at 14 days post- injection of the hydrophilic extract of *Amyloodinium ocellatum* at 400x and 400x amp. respectively. While (C, D, E, F) Show tissue infected by *Amyloodinium ocellatum* of both the control tank (C, D) and the vaccinated tank (E, F) at 400x, 1000x, 400x, 100x amp. respectively. **LF**- Lamellar fusion; **AO**- *Amyloodinium ocellatum*; **HP**- Lamellar hyperplasia

7.4.2 Liver

There weren't any notable changes in lipid content between the vaccinated group and the control as shown on figure 7.19.

However, some fish presented slight hyperaemia and high vacuolization (Fig. 7.20. A, B) or very high vacuolization (Fig. 7.20 C, D) that caused hypertrophy of hepatocytes. In more affected livers necrotic foci were also observed (Fig. 7.20 E, F). However, no significative difference between vaccinated and control was detected.

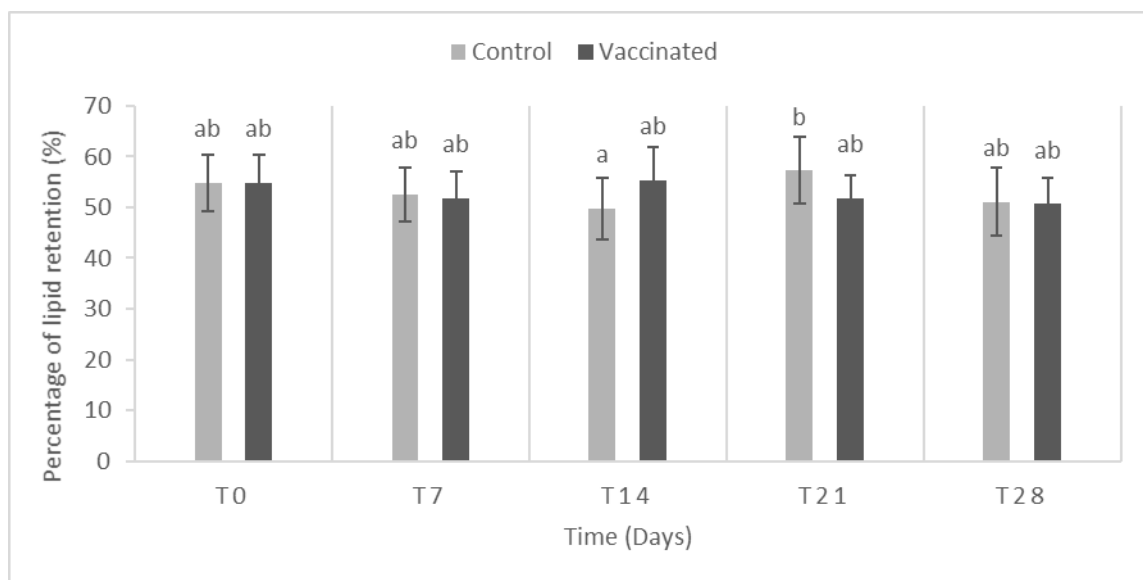


Figure 7.19 - percentage of lipid retention in hepatocytes of *Dicentrarchus labrax* during the period after the injection of a hydrophilic extract of *Amyloodinium ocellatum* in the vaccinated group and PBS in the control group. Values marked with a different letter differ significantly from each other (n = 6; p < 0.05)

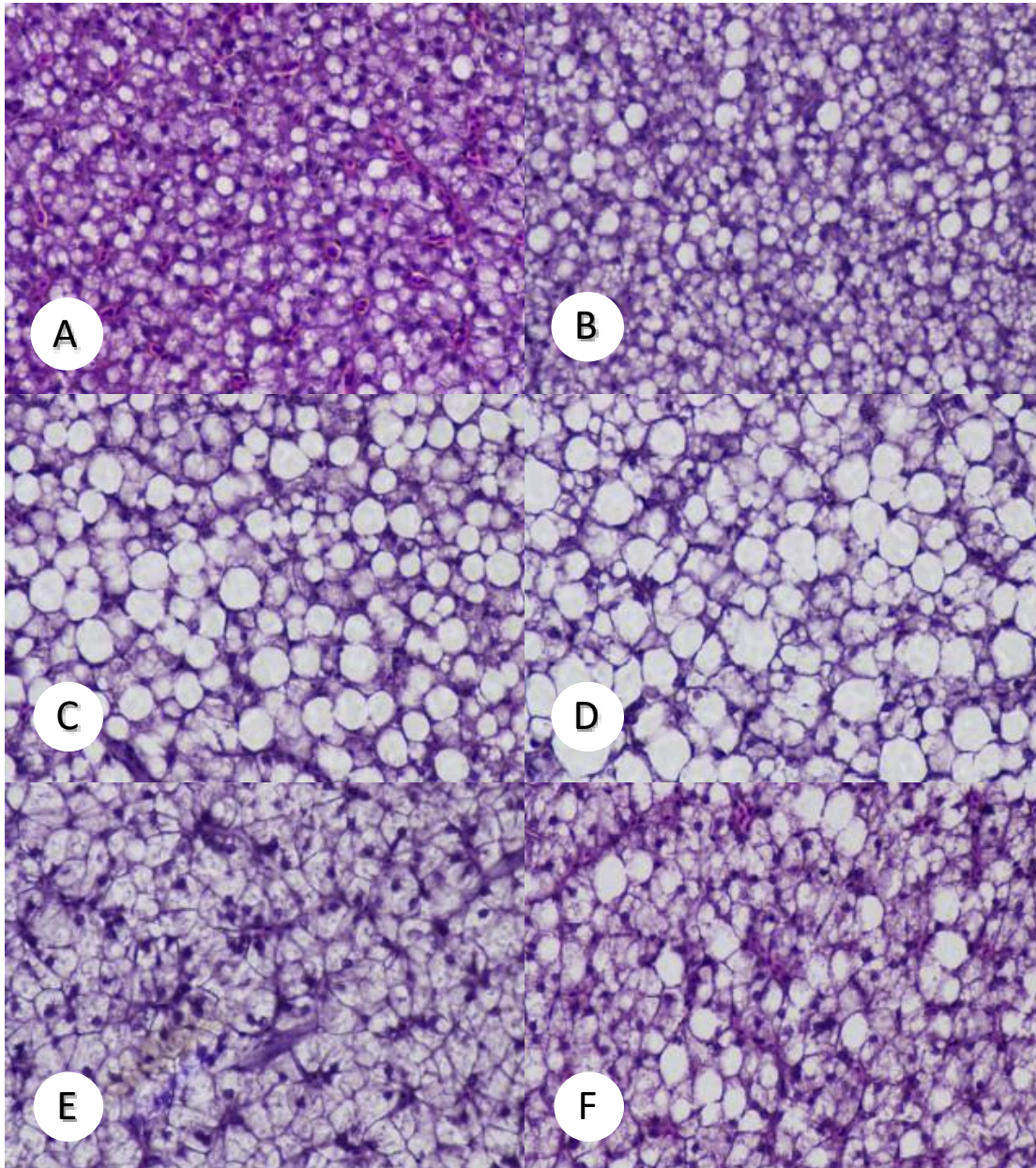


Figure 7.20 - Representative photos of histological features of liver of *Dicentrarchus labrax*, where photos **A**, **C** and **E** belong to a group injected with a hydrophilic extract of *Amyloodinium ocellatum* and **B**, **D** and **F** belong to a group injected with PBS. **A**, **B**. liver with hyperaemia and high hepatocytes vacuolization; **C**, **D**. liver with pronounced hepatocyte vacuolization., **E**, **F**. Liver with necrotic foci.

7.5 Challenge with *Amyloodinium ocellatum*, After Seabass Vaccination

Two concentrations of *A. ocellatum* were chosen for this step, a higher concentration to test for a sudden onset and a lower one to test for a prolonged response.

7.5.1. Challenge With 35 Dinospores/mL - Survival and Infection Level

During the challenge performed with a concentration of 35 dinospores per mL, fish first manifested symptoms of infection 48 h after the start of the challenge. At 82 h both groups had mortalities and in less than 48 h after the first mortality, all fish had died. However, while the control at 105 h showed 100% mortalities, the vaccinated group as demonstrated in figure 7.21 lasted an additional 28 h, surviving until the 129 h since the start of the trial.

For the extracted tomons, (from the first 2 branchial arches of each mortality) the vaccinated group showed a greater spread of the mortalities throughout the trial, while in the control group the mortalities occurred more abruptly having only two time-points with data about the collected tomons as demonstrated in figures 7.21 and 7.22. The vaccinated group showed greater fluctuation in the extracted quantity of tomons, varying on average from 10 000 to 60 000 as shown in figure 7.22, while the control only had a variation in the average of roughly 1000 tomons (28471 and 27310 tomons).

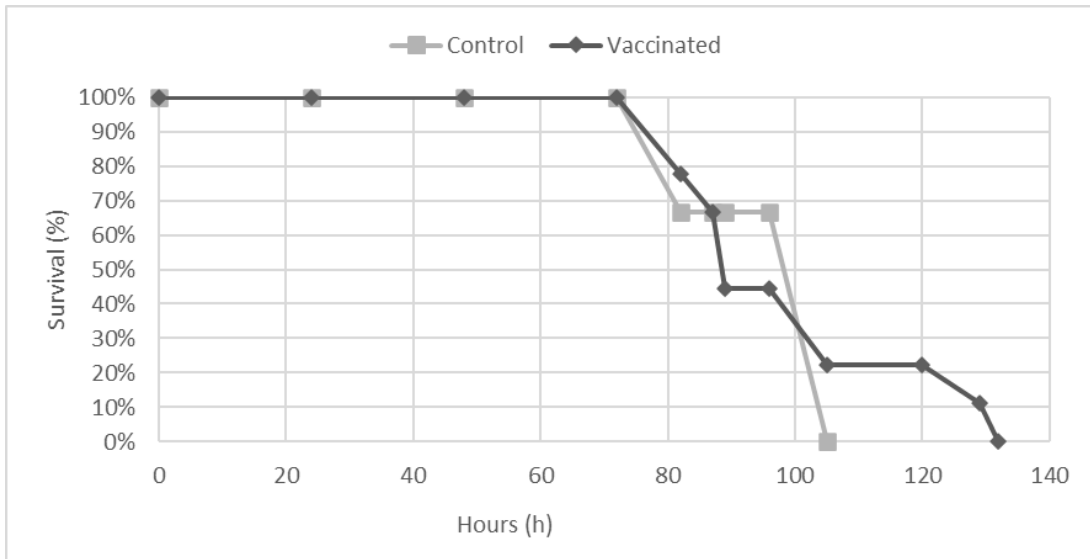


Figure 7.22 - Survival in the challenge of *Dicentrarchus labrax* with 35 dinospores / mL of *Amyloodinium ocellatum* 28 days after the vaccination with the hydrophilic extract (vaccinated group) and PBS in the control group. The Y-axis represents the percentage of alive fish in the challenge, where n = 9 and the X-axis the time in hours.

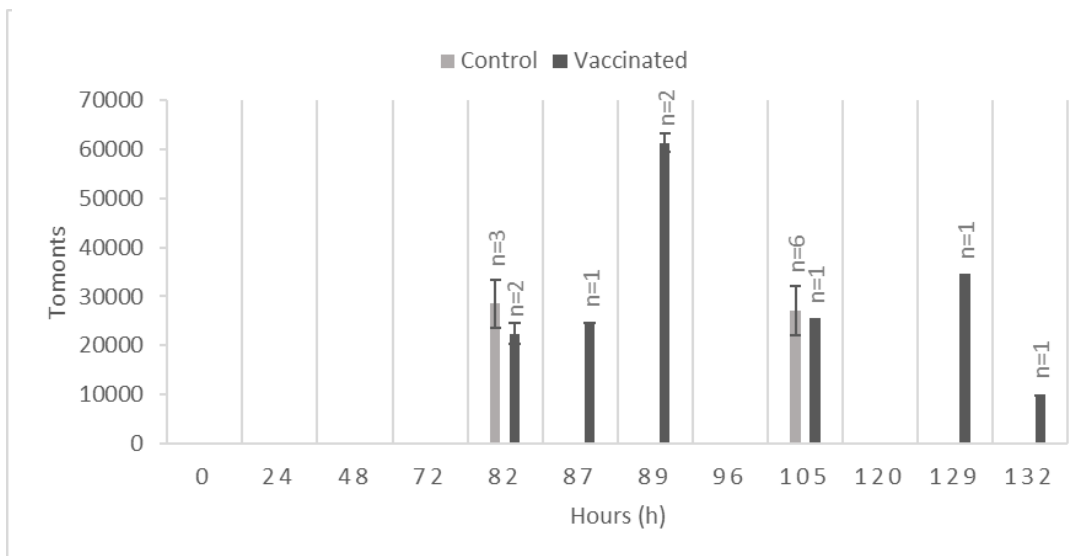


Figure 7.21 - Tomonts and trophonts collected from the 2 first branchial arches of each *Dicentrarchus labrax* that died in the challenge with 35 dinospores / mL of *Amyloodinium ocellatum* 28 days after the injection of the hydrophilic extract in the vaccinated group and PBS in the control group.

7.5.2. Challenge With 10 Dinospores/mL - Survival and Infection Level

During the challenge performed with a concentration of dinospores of 10 dinospores per mL, fish started showing symptoms of infection after 96 h since the start of the challenge, and the vaccinated group had its first mortality at 168 h, while the control group only had its first mortality at 192 h. At the same time, as the control group had its first mortality at 192 h, merely 24 h after the vaccinated group had its first mortality, the rest of the population of the vaccinated group decreased sharply rapidly to 100% mortality. In the interim, the control group showed acute symptoms and reached 100% mortality at 216 h, as shown in figure 7.23.

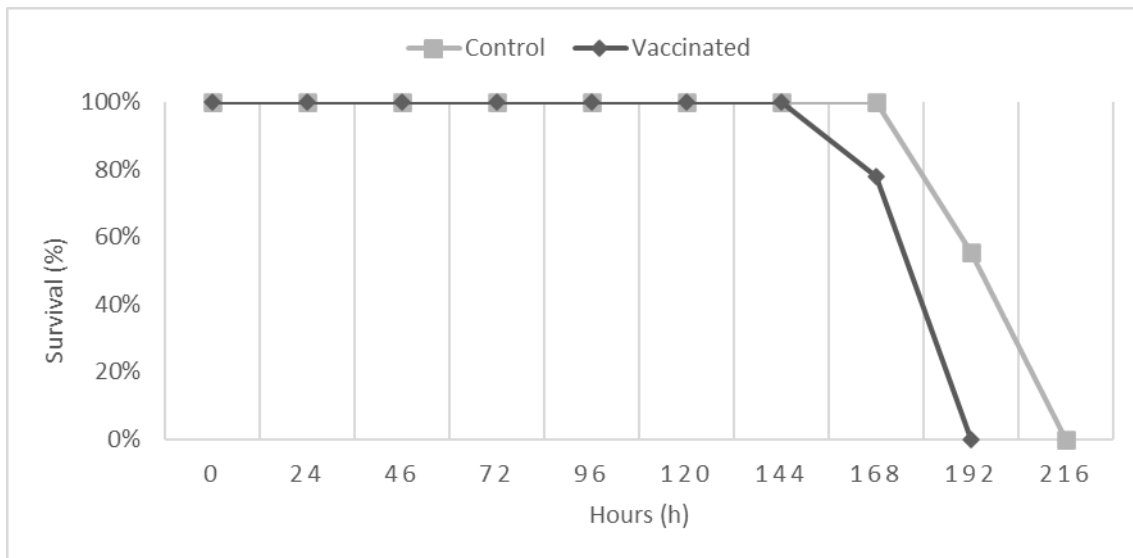


Figure 7.23 - Mortalities in the challenge of *Dicentrarchus labrax* with 10 dinospores/mL of *Amyloodinium ocellatum* 28 days after the injection of the hydrophilic extract (vaccinated group) and PBS in the control group. The Y-axis represents the percentage of alive fish in the challenge, where n = 9 and the X-axis is the time in hours

For the extracted tomons (from the first 2 branchial arches of each mortality), both groups suffered mortalities very rapidly from when they first recorded mortality (in less than 24 h of the

first mortality of each respective group, both groups showed 100% mortality), which concentrated the data to only two data points for each group. For the values of the collected tomonts, the vaccinated group showed a rapid increase in the collected amount from an average of 2750 tomonts to 30 210 tomonts, while the control showed the opposite, a rapid decrease in the collected quantity, from an average of 25 000 to 10 476 tomonts as shown in figure 7.24.

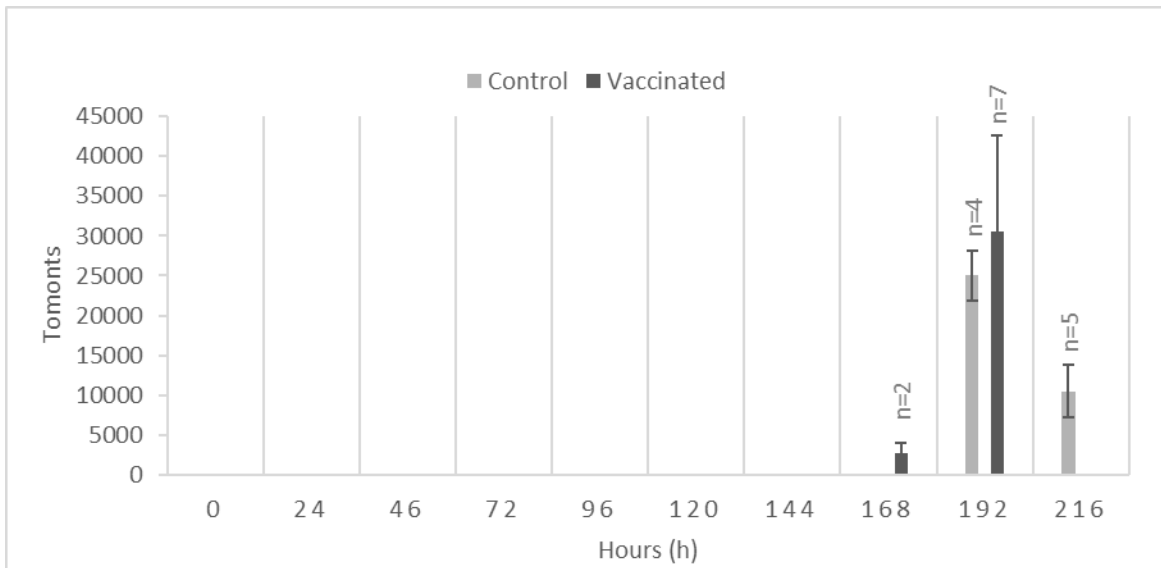


Figure 7.244 - Tomonts and Trophonts collected from the 2 first branchial arches of each mortality of *Dicentrarchus labrax* in the challenge with 10 dinospores / mL of *Amyloodinium ocellatum* 28 days after the injection of the hydrophilic extract in the vaccinated group and PBS in the control group.

8. Discussion

A. ocellatum is commonly identified as a parasite of marine fish with a strong negative impact on aquaculture. This study, set out to determine the impacts of injecting its hydrophilic extract on the immune system of the seabass, *Dicentrarchus labrax*, and assess its viability as a base to develop future vaccines against *A. ocellatum*.

The condition index permits the assessment of general wellbeing and growth of individuals. It can be affected by numerous independent factors that impact appetite, digestion, and growth mechanics. In this study, the condition index was used to understand if the vaccination with hydrophobic extract of *A. ocellatum* had any significant impact on the growth of the seabass. Over the trial, fish showed irregular and nonlinear growth rates between weekly measurements, at times even showing negative values, for example, on the 28th day in the control group and at day 7 in the vaccinated group (Table 1). However, the condition index allows having a closer idea of what might be happening. Throughout the 28 days of the trial, the vaccinated group showed a progressive decrease from T0, becoming significantly different on the 21st day of the trial, and recovering to the initial values on the 28th day. On the other hand, the control group had more stable values except for the 14th day, where they were significantly lower than the initial values. It is theorized that the difference in results between the vaccinated and control group of the trial might be due to inherent reactions to the injection and vaccination. Such a process can cause pain, restrict normal gut function, and cause energy-demanding immune responses. A greater immune response from the vaccinated group, would draw more energy from resources dedicated to the growth of the fish. In turn, the response from the control group could be due to the stress caused by the injection process, which would have a sharp response and then, no further alterations. Sørum and Damsgaard, (2004), had similar results in a trial with Atlantic salmon (*Salmo salar*), where vaccinated fish showed an acute reduction in appetite that was progressively alleviated until the 21-28th day after treatment.

Haematological indices are important parameters for the evaluation of fish physiological status. Their changes depend on fish species, age, the cycle of the sexual maturity of spawners, diseases, and environment (Luskova et al., 1997; Golovina & Trombicky, 1989; Golovina et al., 1996; Zhiteneva et al., 1989). Like in warm-blooded animals, changes in the blood parameters of fish, caused by injuries or infections of some tissues or organs, can be used to determine and

confirm the dysfunction or injuries of the latter (organs or tissues). Haematocrit measures the concentration of red blood cells in whole blood and low values are indicative of an anaemic condition, indicating that fish may not be eating or that they may have osmoregulatory problems (Fazio et al., 2013). Altered haematocrit problems can also be indicative of a stressed state (Casillas and Smith, 1997; Urbina et al., 2012), where values generally increase in a stressed situation. It has been suggested that a slightly hypoxic situation could be created by muscular exertion and higher oxygen demand during stress (Casillas and Smith, 1977) and that such a situation can result in an increase in haematocrit levels by as much as 10-30%. Al-Hassan et al, (1993) also suggests the existence of a positive correlation between Htc and Hgb, and Htc and body weight/length up to a certain point.

In this study, the haematocrit values climbed from their initial value of $29.4 \pm 5.51\%$ to $38.83 \pm 4.41\%$ at day 21, while the control group maintained constant values throughout the trial. However, while there was a slight difference in Hgb values, with the vaccinated group showing higher values than the control group, it cannot be considered statistically significant. Thanga Viji et al., (2013) noticed an increase in the level of red blood cells (RBC) compared to the control groups in *Carassius auratus* vaccinated against *Aeromonas hydrophila*. Likewise, Silva et al., (2009), noticed an increase on day 21, in RBC in fish vaccinated with a polyvalent bacterin vaccine against *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Enterococcus durans* both through oral vaccination and intraperitoneal vaccination.

Regarding leucocytes, the total amount of identified leucocytes didn't differ significantly throughout the 28 days of the trial, except for the 28th day, where the control decreased from $2.96 \pm 0.90\%$ on day 7 to $1.89 \pm 0.33\%$. However, neither of these values were significantly different to any of the registered values on the vaccinated group. Granulocytes and monocytes remained constant throughout the trial, with neither group showing any changes.

Thrombocytes are the main responsible agent for haemostasis. Additionally, they play an important role in the host defence mechanisms, demonstrated by their action in coagulation and inflammatory processes, and the phagocytic activity in infectious processes (Tavares-Dias et al., 2003). Martins et al., (2011) noticed an increase in the number of thrombocytes 20 days after vaccination of *Ictalurus punctatus* with live theronts of *Ichthyophthirius multifiliis*. However, Silva et al., 2009 noticed no significant change in a trial with *Oreochromis niloticus* where they

tested multiple avenues of vaccination against *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Enterococcus durans*.

In this study, thrombocyte values differed in the vaccinated group, where it suffered a decrease from $0.43 \pm 0.32\%$ on the 7th day to $0.09 \pm 0.14\%$ on the 28th day. This thrombopenia could be possibly caused by damages to the liver, however, the histological analysis of the liver didn't show any damage that might be responsible for this variation. This might indicate a possible undetected infection or inflammation where the thrombocytes would be migrating.

Similarly, to the total leucocyte amount, the observed lymphocytes maintained similar values for the vaccinated group but decreased in the control from $2.12 \pm 0.70\%$ on day 7 to $1.40 \pm 0.30\%$ on the 28th day.

Blood pH was significantly lower on the 28th day after injection in the vaccinated group, 7.26 ± 0.07 than the control group 7.4 ± 0.13 . Fromm et al., (1980), suggests that a decrease in blood pH might be linked to an increase in stress. This is contrary to the results obtained from glucose and lactate analysis, which were stable throughout the trial.

Ca⁺ and K⁺ values were stable throughout the trial, which according to Blazer et al., (1992) is within normal values for vaccination trials.

Regarding challenges, both the vaccinated group and the control group reached 100% mortality after a certain time interval. In the challenge with an initial higher concentration of dinospores (35 dinospores/mL), the vaccinated group had a steady decline throughout the challenge, reaching 100% mortality at 130 h, while the control group had a quicker onset where it suffered 3 mortalities at 82 h and then the remaining 6 at 105 h. This difference in mortality rate might be due to a cascade in the decline of water quality, where the death of one individual might decrease the water quality enough that another mortality would occur and so on, particularly since the six individuals that died at 105 h were found in the morning. In addition to these factors, it is also believed that the period where most of these deaths occurred coincided with a peak number of dinospores in the water since *A. ocellatum* has a life cycle of approximately 100 h, which would have increased the damage to the gills and lead to a quicker death by hypoxia.

Regarding the challenge with a lower concentration of dinospores (10 dinospores/mL), as expected, the length of the trial was extended. It is believed that with a lower concentration of dinospores the fish would have a longer time to react and the difference between groups would be

more pronounced than in the first challenge, allowing a more thorough analysis of any difference potentially existing between vaccinated and control groups. However, while the length of the trial was indeed greater, the response between the two groups was very similar both in the time of the mortalities and in the rate of the occurrences. This result may possibly be due to the chosen concentration of the extract, that was perhaps, too conservative, or due to the fraction of the sonicated tomonts used, the hydrophilic fraction. Concluding that while the hydrophilic fraction might not be as harmful to the injected fish (Moreira et al., 2018), it might require a greater concentration or injected volume to initiate a perceptible immunological response.

9. Conclusions and Considerations

In summary, fish injected with 0.5 ml of the hydrophilic extract of sonicated tomonts of *A. ocellatum* (1000 tomonts/0.5 mL) suffered a prolonged decrease in the condition index, while the control group suffered a similar pattern but shorter. Behaviour that was attributed to the stress caused by the manipulation of the injection process and of an immunological response. Immunized fish also showed an increase in haematocrit values throughout the 28 days previous to challenge and as well as a decrease in pH values, which might indicate an increase in stress compared to the control group. Importantly, no abnormalities were observed at a histological level in both gills and liver compared to the control, implying that the administered dosage does not provoke damages at this level.

Regarding parameters directly related to the immunological response, neither leukocyte nor data from the challenges provided data that would indicate a significant immunization against *A. ocellatum*. This is possibly a consequence of using an extract with insufficient concentration to generate a strong and adequate immune response.

10. Bibliography

- Adams, A., & Subasinghe, R. (2021). Fish Vaccines. In Veterinary Vaccines. <https://doi.org/10.1002/9781119506287.ch9>
- Aravindan, N., Chaganti, K., & Aravindan, S. (2007). 'Protozoan parasites in commercially important shrimp species from northeast coast of Andhra Pradesh, India', Journal of Experimental Zoology, 10 (1): 9-20.
- Askarian, F., & Kousha, A. (2009). The influence of photoperiod in farming Beluga sturgeon (*Huso huso*): Evaluation by growth and health parameters in serum. Journal of Fisheries and Aquatic Science, 4(1). <https://doi.org/10.3923/jfas.2009.41.49>
- Balcázar, J. L., Blas, I. de, Ruiz-Zarzuola, I., Cunningham, D., Vendrell, D., & Múzquiz, J. L. (2006). The role of probiotics in aquaculture. In Veterinary Microbiology (Vol. 114, Issues 3–4). <https://doi.org/10.1016/j.vetmic.2006.01.009>
- Barbaro, A., Francescon, A. (1985). 'Parassitosi da *Amyloodinium ocellatum* (Dinophyceae) su larve di *Sparus aurata* allevate in un impianto di riproduzione artificiale'. Oebalia, 11: 745–752.
- Becker, C.D. (1977). 'Flagellate parasites of fishes'. In: Krier, J.P. (ed.) Parasitic Protozoa. Academic Press, New York, pp. 357–416.
- Bakhom A., El-Bermawi M., Srour M., Goda M., and Hebalah M. (2015). Some biological aspects of European seabass *Dicentrarchus labrax* in Egyptian Mediterranean water and aquaculture, Egypt. Mediterranean Aquaculture Journal, 7(1). <https://doi.org/10.21608/maj.2015.4630>

- Benfey, T. J., & Biron, M. (2000). Acute stress response in triploid rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). *Aquaculture*, 184(1–2), 167–176.
[https://doi.org/10.1016/s0044-8486\(99\)00314-2](https://doi.org/10.1016/s0044-8486(99)00314-2)
- Bolasina, S. N. (2011). Stress response of juvenile flounder (*Paralichthys orbignyanus*, Valenciennes 1839), to acute and chronic stressors. *Aquaculture*, 313(1–4).
<https://doi.org/10.1016/j.aquaculture.2011.01.011>
- Bower, C. E., Turner, D. T., & Biever, R. C. (1987). A standardized method of propagating the marine fish parasite, *Amyloodinium ocellatum*. *The Journal of Parasitology*, 73(1).
<https://doi.org/10.2307/3282348>
- Brune, D. E. (1997). Striped Bass and Other Morone Culture. *Developments in Aquaculture and Fisheries Science*, 30.
- Byadgi, O., Beraldo, P., Volpatti, D., Massimo, M., Bulfon, C., & Galeotti, M. (2019). Expression of infection-related immune response in European sea bass (*Dicentrarchus labrax*) during a natural outbreak from a unique dinoflagellate *Amyloodinium ocellatum*. *Fish and Shellfish Immunology*, 84. <https://doi.org/10.1016/j.fsi.2018.09.069>
- Carragher, J. F., Sumpter, J. P., Pottinger, T. G., & Pickering, A. D. (1989). The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* L. and *Salmo gairdneri* Richardson. *General and Comparative Endocrinology*, 76(2).
[https://doi.org/10.1016/0016-6480\(89\)90163-9](https://doi.org/10.1016/0016-6480(89)90163-9)
- Cannon, W.B. (1935). 'Stresses and strains of hemostasis', *The American Journal of the Medical Sciences*, 189: 1-14.
- Canosa, B. (2006) Test of effects caused by chemical action of CuSO₄ (widely used fungicide-algicide). Tese de mestrado em aquaculture. Universidade do Algarve. 55

- Casillas, E., & Smith, L. S. (1977). Effect of stress on blood coagulation and haematology in rainbow trout (*Salmo gairdneri*). *Journal of Fish Biology*, 10(5). <https://doi.org/10.1111/j.1095-8649.1977.tb04081.x>
- Cecchini, S., Saroglia, M., Terova, G., & Albanesi, F. (2001). Detection of antibody response against *Amyloodinium ocellatum* (Brown, 1931) in serum of naturally infected European sea bass by an enzyme-linked immunosorbent assay (ELISA). *Bulletin of the European Association of Fish Pathologists*, 21(3).
- Change, C., & Communities, R. (2015). Coping with climate change – the roles of genetic resources for food and agriculture. In FAO.
- Cobb, C. S., Levy, M. G., & Noga, E. J. (1997). V5 2:45 Development of immunity by the tomato Clownfish (amphiprion frenatus) to the dinoflagellate parasite *Amyloodinium ocellatum* as evidenced by in vivo and in vitro analysis. *Developmental & Comparative Immunology*, 21(2). [https://doi.org/10.1016/s0145-305x\(97\)88790-3](https://doi.org/10.1016/s0145-305x(97)88790-3)
- Cobb, C. S., Levy, M. G., & Noga, E. J. (1998a). Acquired immunity to amyloodiniosis is associated with an antibody response. *Diseases of Aquatic Organisms*, 34(2). <https://doi.org/10.3354/dao034125>
- Cobb, C. S., Levy, M. G., & Noga, E. J. (1998b). Development of immunity by the tomato clownfish amphiprion frenatus to the dinoflagellate parasite *Amyloodinium ocellatum*. *Journal of Aquatic Animal Health*, 10(3). [https://doi.org/10.1577/1548-8667\(1998\)010<0259:DOIBTT>2.0.CO;2](https://doi.org/10.1577/1548-8667(1998)010<0259:DOIBTT>2.0.CO;2)
- Colorni, A. (1989). 'Pathology of marine warmwater finfish in Israel: problems and research', *Advances in Tropical Aquaculture, AQUACOP, IFREMER, Actes de Colloque 9*: 133
- Colorni, A. (1994). Hyperparasitism of *Amyloodinium ocellatum* (Dinoflagellida: Oodinidae) on *Neobenedenia melleni* (Monogenea: Capsalidae). *Diseases of Aquatic Organisms*, 19(2). <https://doi.org/10.3354/dao019157>

- Colorni, A., Ullal, A., Heinisch, G., & Noga, E. J. (2008). Activity of the antimicrobial polypeptide piscidin 2 against fish ectoparasites. *Journal of Fish Diseases*, 31(6). <https://doi.org/10.1111/j.1365-2761.2008.00922.x>
- Covello, J. M., Bird, S., Morrison, R. N., Battaglione, S. C., Secombes, C. J., & Nowak, B. F. (2009). Cloning and expression analysis of three striped trumpeter (*Latris lineata*) pro-inflammatory cytokines, TNF- α , IL-1 β and IL-8, in response to infection by the ectoparasitic, *Chondracanthus goldsmidi*. *Fish and Shellfish Immunology*, 26(5). <https://doi.org/10.1016/j.fsi.2009.03.012>
- Cristina García Díez, C. & Remiro Perlado, J. P. (2014). Impactos del Cambio Climático sobre la Acuicultura en España. Oficina Española de Cambio Climático, Ministerio de Agricultura, Alimentación y Medio Ambiente. Madrid, 38.
- Cruz-Lacierda, E. R., Maeno, Y., Pineda, A. J. T., & Matey, V. E. (2004). Mass mortality of hatchery-reared milkfish (*Chanos chanos*) and mangrove red snapper (*Lutjanus argentimaculatus*) caused by *Amyloodinium ocellatum* (Dinoflagellida). *Aquaculture*, 236(1–4). <https://doi.org/10.1016/j.aquaculture.2004.02.012>
- Dange, R. (1992) Fish red blood cells. in W. Hoar and D.J. Randall (eds.). *Fish physiology* vol. XIIB. Academic press inc. London, 1-54
- Edward J. Noga. (2010). *Fish Disease: Diagnosis and Treatment*, Second Edition. Wiley-Blackwell.
- Edward, K. (1975). *Treatment of Exotic Marine Fish Diseases* (First ed.). Palmetto Publishing, 90 (2).
- Einarsdóttir, I. E., Nilssen, K. J., & Iversen, M. (2000). Effects of rearing stress on Atlantic salmon (*Salmo salar* L.) antibody response to a non-pathogenic antigen. *Aquaculture Research*, 31(12). <https://doi.org/10.1046/j.1365-2109.2000.00506.x>
- Ewart, K. V., & Tsoi, S. C. (2004). Innate Immune Recognition of Pathogens in Teleost Fish. https://doi.org/10.1142/9789812565709_0003

- Fajer-Ávila E. J., La Parra, I. A. de, Aguilar-Zarate, G., Contreras-Arce, R., Zaldívar-Ramírez, J., & Betancourt-Lozano, M. (2003). Toxicity of formalin to bullseye puffer fish (*Sphoeroides annulatus* Jenyns, 1843) and its effectiveness to control ectoparasites. *Aquaculture*, 223(1–4). [https://doi.org/10.1016/S0044-8486\(03\)00166-2](https://doi.org/10.1016/S0044-8486(03)00166-2)
- Fazio, F., Marafioti, S., Arfuso, F., Piccione, G., & Faggio, C. (2013). Comparative study of the biochemical and haematological parameters of four wild Tyrrhenian fish species. *Veterinari Medicina*, 58(11). <https://doi.org/10.17221/7139-VETMED>
- Fensome, R. A., Saldarriaga, J. F., & Taylor, F. J. R. “Max.” (1999a). Dinoflagellate phylogeny revisited: reconciling morphological and molecular based phylogenies. *Grana*, 38(2). <https://doi.org/10.1080/713786919>
- Fensome, R. A., Saldarriaga, J. F., & Taylor, M. F. J. R. (1999b). Dinoflagellate phylogeny revisited: Reconciling morphological and molecular based phylogenies. *Grana*, 38(2–3). <https://doi.org/10.1080/00173139908559216>
- Fernandes-de-Castilho, M., Pottinger, T. G., & Volpato, G. L. (2008). Chronic social stress in rainbow trout: Does it promote physiological habituation? *General and Comparative Endocrinology*, 155(1). <https://doi.org/10.1016/j.ygcen.2007.04.008>
- Ferron, A., & Leggett, W. C. (1994). An Appraisal of Condition Measures for Marine Fish Larvae. *Advances in Marine Biology*, 30(C). [https://doi.org/10.1016/S0065-2881\(08\)60064-4](https://doi.org/10.1016/S0065-2881(08)60064-4)
- Fish Defenses. (2019). In *Fish Defenses*. <https://doi.org/10.1201/9780429187285>
- Food Outlook – Biannual Report on Global Food Markets. (2021). In *Food Outlook – Biannual Report on Global Food Markets*. <https://doi.org/10.4060/cb7491en>
- Francis-Floyd, R., & Floyd, M. R. (2011). *Amyloodinium ocellatum*, an Important Parasite of Cultured Marine Fish. SRAC Publication No. 4705, 4705.
- Fromm, P. O. (1980). A review of some physiological and toxicological responses of freshwater fish to acid stress. In *Environmental Biology of Fishes* (Vol. 5, Issue 1). <https://doi.org/10.1007/BF00000954>

- Fu, S. J., Cao, Z. D., & Peng, J. L. (2007). The effect of acute stress on post-stress oxygen consumption rate in southern catfish, *Silurus meridionalis* Chen. *International Journal of Zoological Research*, 3(2). <https://doi.org/10.3923/ijzr.2007.101.106>
- Harikrishnan, R., Balasundaram, C., & Heo, M. S. (2011). Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish. In *Aquaculture* (Vol. 317, Issues 1–4). <https://doi.org/10.1016/j.aquaculture.2011.03.039>
- Iwama, G. K. (1998). 'Stress in Fish'. *Annals of the New York Academy of Sciences*, 851: 304–310.
- Jørgensen, J. B. (2014). The Innate Immune Response in Fish. In *Fish Vaccination* (Vol. 9780470674550). <https://doi.org/10.1002/9781118806913.ch8>
- Ki, J. S. (2012). Hypervariable regions (V1-V9) of the dinoflagellate 18S rRNA using a large dataset for marker considerations. *Journal of Applied Phycology*, 24(5). <https://doi.org/10.1007/s10811-011-9730-z>
- Klyashtorin, L.B., Smirnov B.P. (1981), 'Possibilities for Increase of Metabolism Activity in Fishes Affected by Stress and during Their Growth', *Biologicheskije Nauki*, 5: 58–61.
- Kumar, P. R., Nazar, A. K. A., Jayakumar, R., Tamilmani, G., Sakthivel, M., Kalidas, C., Balamurugan, V., Sirajudeen, S., Thiagu, R., Gopakumar, G., Regional, M., & Marine, C. (2015). *Amyloodinium ocellatum* infestation in the broodstock of silver pompano *Trachinotus blochii* (Lacepede, 1801) and its therapeutic control. *Indian Journal of Fisheries*, 62(1).
- Kuperman, B. I., & Matey, V. E. (1999). Massive infestation by *Amyloodinium ocellatum* (Dinoflagellida) of fish in a highly saline lake, Salton Sea, California, USA. *Diseases of Aquatic Organisms*, 39(1). <https://doi.org/10.3354/dao039065>
- Lambert, Y., & Dutil, J. D. (1997). Can simple condition indices be used to monitor and quantify seasonal changes in the energy reserves of atlantic cod (*Gadus morhua*)? *Canadian Journal of Fisheries and Aquatic Sciences*, 54(SUPPL.). <https://doi.org/10.1139/cjfas-54-s1-104>

- Landsberg, J. H., Blakesley, B. A., Reese, R. O., Mcrae, G., & Forstchen, P. R. (1998). Parasites of fish as indicators of environmental stress. *Environmental Monitoring and Assessment*, 51(1–2). <https://doi.org/10.1023/A:1005991420265>
- Landsberg, J., Steldinger, K., Blakesley, B., & Zondervan, R. (1994). Scanning electron microscope study of dinospores of *Amyloodinium* of. *ocellatum*, a pathogenic dinoflagellate parasite of marine fish, and comments on its relationship to the Peridinales. *Diseases of Aquatic Organisms*, 20. <https://doi.org/10.3354/dao020023>
- Lawler, A. R. (1977). Dinoflagellate (*Amyloodinium*) infestation of pompano. In: C. J. Sindermann (ed), *Disease Diagnosis and Control in North American Marine Aquaculture. Developments in Aquaculture and Fisheries Science*. Elsevier Scientific Publishing Company, Amsterdam, Oxford, and New York. 257-264.
- Lawler, A. R. (1980). Studies on *Amyloodinium ocellatum* (Dinoflagellata) in Mississippi Sound: Natural and Experimental Hosts. *Gulf Research Reports*, 6. <https://doi.org/10.18785/grr.0604.08>
- Levy, M. G., Poore, M. F., Colorni, A., Noga, E. J., Vandersea, M. W., & Litaker, R. W. (2007). A highly specific PCR assay for detecting the fish ectoparasite *Amyloodinium ocellatum*. *Diseases of Aquatic Organisms*, 73(3). <https://doi.org/10.3354/dao073219>
- Li, C. H., & Chen, J. (2013). Molecular cloning, characterization and expression analysis of a novel wap65-1 gene from *Plecoglossus altivelis*. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, 165(2). <https://doi.org/10.1016/j.cbpb.2013.03.014>
- Li, C. H., Chen, J., Shi, Y. H., & Lu, X. J. (2011). Use of suppressive subtractive hybridization to identify differentially expressed genes in ayu (*Plecoglossus altivelis*) associated with *Listonella anguillarum* infection. *Fish and Shellfish Immunology*, 31(3). <https://doi.org/10.1016/j.fsi.2011.06.011>
- Lom, J., Lawler A.R. (1973). An ultrastructural study on the mode of attachment in dinoflagellates invading gills of Cyprinodontidae, *Protistologica*, 9: 293-309

- Martemyanov, V. I. (2009). The regularities of changes in the sodium ion levels in fish erythrocytes during adaptation to a temperature. *Biology Bulletin*, 36(4). <https://doi.org/10.1134/S1062359009040153>
- Martemyanov, V. I. (2013). Patterns of changes in sodium content in plasma and erythrocytes of freshwater fish at stress. *Journal of Ichthyology*, 53(3). <https://doi.org/10.1134/S0032945213020094>
- Martemyanov, V. I. (2015). Stress reaction in freshwater fish in response to extreme impacts and during the reproduction period. *Journal of Coastal Life Medicine*. 3. 169-177. 10.12980/JCLM.3.201514J97.
- Martínez-Porchas, M., Rafael Martínez-Córdova, L., & Ramos-Enriquez, R. (2009). Cortisol and Glucose: Reliable indicators of fish stress? In *Pan-American Journal of Aquatic Sciences* (Vol. 4, Issue 2).
- Martins, M. L., Xu, D. H., Shoemaker, C. A., & Klesius, P. H. (2011). Temperature effects on immune response and hematological parameters of channel catfish *Ictalurus punctatus* vaccinated with live theronts of *Ichthyophthirius multifiliis*. *Fish and Shellfish Immunology*, 31(6). <https://doi.org/10.1016/j.fsi.2011.07.015>
- Martoja, R. & Martoja-Pierson, M. (1967). *Initiation aux techniques de l'histologie animale*. Paris-Masson
- Mazeaud, M. M., Mazeaud, F., & Donaldson, E. M. (1977). Primary and Secondary Effects of Stress in Fish: Some New Data with a General Review. *Transactions of the American Fisheries Society*, 106(3). [https://doi.org/10.1577/1548-8659\(1977\)106<201:paseos>2.0.co;2](https://doi.org/10.1577/1548-8659(1977)106<201:paseos>2.0.co;2)
- Mazeaud, M.M., Mazeaud F. (1981). 'Adrenergic Responses to Stress in Fish', In *Stress and Fish*, Pickering, A.D.L. (Ed), Academic Press Academic: 50–75
- McCarty, R., E.L. Sabban, R. Kvetnansky. (1996). 'Stress: Molecular genetic and neurobiological advances', *Harvard Academic*, Amsterdam, Vol. 1-2, 1002pp.

- McCormick, S. D., Shrimpton, J. M., Carey, J. B., O’Dea, M. F., Sloan, K. E., Moriyama, S., & Björnsson, B. T. (1998). Repeated acute stress reduces growth rate of Atlantic salmon parr and alters plasma levels of growth hormone, insulin-like growth factor I and cortisol. *Aquaculture*, 168(1–4). [https://doi.org/10.1016/S0044-8486\(98\)00351-2](https://doi.org/10.1016/S0044-8486(98)00351-2)
- Menezes, J. (2000) Parasitoses. In: Manual sobre Doenças de Peixes Ósseos. Publicações Avulsas do IPIMAR. 3: 75 - 114.
- Mladineo, I., Petrić, M., Šegvić, T., & Dobričić, N. (2010). Scarcity of parasite assemblages in the Adriatic-reared European sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*). *Veterinary Parasitology*, 174(1–2). <https://doi.org/10.1016/j.vetpar.2010.08.015>
- Montgomery-Brock, D. R., Sylvester, J. Y., Tamaru, C. S., Brock, J. A. (2000) Hydrogen peroxide treatment for *Amyloodinium* sp. on mullet (*Mugil cephalus*) fry. *Aqua Tips*, 70 Regional Notes, Center for Tropical and Subtropical Aquaculture, Waimanalo, HI. 11(4):4-6.
- Montgomery-Brock, D., Sato, V. T., Brock, J. A., & Tamaru, C. S. (2001). The application of hydrogen peroxide as a treatment for the ectoparasite *Amyloodinium ocellatum* (Brown 1931) on the Pacific Threadfin polydactylus sexfilis. *Journal of the World Aquaculture Society*, 32(2). <https://doi.org/10.1111/j.1749-7345.2001.tb01103.x>
- Moore, G. L., Ledford, M. E., & Merydith, A. (1981). A micromodification of the Drabkin hemoglobin assay for measuring plasma hemoglobin in the range of 5 to 2000 mg/dl. *Biochemical Medicine*, 26(2). [https://doi.org/10.1016/0006-2944\(81\)90043-0](https://doi.org/10.1016/0006-2944(81)90043-0)
- Moreira, M., Herrera, M., Pousão-Ferreira, P., Triano, J. I. N., & Soares, F. (2018). Stress effects of amyloodiniosis in gilthead sea bream *Sparus aurata*. *Diseases of Aquatic Organisms*, 127(3). <https://doi.org/10.3354/dao03199>
- Murray, A. G., & Peeler, E. J. (2005). A framework for understanding the potential for emerging diseases in aquaculture. *Preventive Veterinary Medicine*, 67(2-3 SPEC. ISS.). <https://doi.org/10.1016/j.prevetmed.2004.10.012>

- Murray, S., Flø Jørgensen, M., Ho, S. Y. W., Patterson, D. J., & Jermiin, L. S. (2005). Improving the analysis of dinoflagellate phylogeny based on rDNA. *Protist*, 156(3). <https://doi.org/10.1016/j.protis.2005.05.003>
- Nilssen, K. J. (1996). Stress responses of Atlantic salmon (*Salmo salar* L.) elicited by water level reduction in rearing tanks. *Fish Physiology and Biochemistry*, 15(5). <https://doi.org/10.1007/bf01875582>
- Noga, E. J., & Bower, C. E. (1987). Propagation of the marine dinoflagellate *Amyloodinium ocellatum* under germ-free conditions. *The Journal of Parasitology*, 73(5). <https://doi.org/10.2307/3282512>
- Noga, E. J., Silphaduang, U., Park, N. G., Seo, J. K., Stephenson, J., & Kozłowicz, S. (2009). Piscidin 4, a novel member of the piscidin family of antimicrobial peptides. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, 152(4). <https://doi.org/10.1016/j.cbpb.2008.12.018>
- Noga, E. J., Smith, S. A., & Landsberg, J. H. (1991). Amyloodiniosis in cultured hybrid striped bass (*morone saxatilis* x *m. chrysops*) in north carolina. *Journal of Aquatic Animal Health*, 3(4). [https://doi.org/10.1577/1548-8667\(1991\)003<0294:AICHSB>2.3.CO;2](https://doi.org/10.1577/1548-8667(1991)003<0294:AICHSB>2.3.CO;2)
- Noga, E. J., Ullal, A. J., Corrales, J., & Fernandes, J. M. O. (2011). Application of antimicrobial polypeptide host defenses to aquaculture: Exploitation of downregulation and upregulation responses. *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics*, 6(1). <https://doi.org/10.1016/j.cbd.2010.06.001>
- Nolan, D. T., Op't Veld, R. L. J. M., Balm, P., & Wendelaar Bonga, S. E. (1999). Ambient salinity modulates the response of the tilapia, *Oreochromis mossambicus* (Peters), to net confinement. *Aquaculture*, 177(1–4). [https://doi.org/10.1016/S0044-8486\(99\)00093-9](https://doi.org/10.1016/S0044-8486(99)00093-9)
- Oestmann, D. J., & Lewis, D. H. (1995). A method for producing microbe-free *Amyloodinium ocellatum* (Brown) with Percoll®. *Veterinary Parasitology*, 59(2). [https://doi.org/10.1016/0304-4017\(94\)00746-Y](https://doi.org/10.1016/0304-4017(94)00746-Y)

- Oestmann, D. J., & Lewis, D. H. (1996). Improved cell culture propagation of *Amyloodinium ocellatum*. *Diseases of Aquatic Organisms*, 24(3). <https://doi.org/10.3354/dao024173>
- Olsen, R. E., Sundell, K., Ringø, E., Myklebust, R., Hemre, G. I., Hansen, T., & Karlsen, Ø. (2008). The acute stress response in fed and food deprived Atlantic cod, *Gadus morhua* L. *Aquaculture*, 280(1–4). <https://doi.org/10.1016/j.aquaculture.2008.05.006>
- Paperna, I. (1980). *Amyloodinium ocellatum* (Brown, 1931) (Dinoflagellida) infestations in cultured marine fish at Eilat, Red Sea: epizootiology and pathology. *Journal of Fish Diseases*, 3(5). <https://doi.org/10.1111/j.1365-2761.1980.tb00421.x>
- Paperna, I. (1984a). Chemical control of *Amyloodinium ocellatum* (Brown 1931) (Dinoflagellida) infections: In vitro tests and treatment trials with infected fishes. *Aquaculture*, 38(1). [https://doi.org/10.1016/0044-8486\(84\)90133-9](https://doi.org/10.1016/0044-8486(84)90133-9)
- Paperna, I. (1984b). Reproduction cycle and tolerance to temperature and salinity of *Amyloodinium ocellatum* (Brown, 1931) (Dinoflagellida). *Annales de Parasitologie Humaine et Comparée*, 59(1). <https://doi.org/10.1051/parasite/1984591007>
- Paperna, I., Colorni, A., Ross, B., & Colorni, B. (1981). Diseases of marine fish cultured in Eilat Mariculture Project based at the Gulf of Aqaba, Red Sea. *Shefayim (Israel)*, 6.
- Pereira, J. C., Abrantes, I., Martins, I., Barata, J., Frias, P., & Pereira, I. (2011). Ecological and morphological features of *Amyloodinium ocellatum* occurrences in cultivated gilthead seabream *Sparus aurata* L.; A case study. *Aquaculture*, 310(3–4). <https://doi.org/10.1016/j.aquaculture.2010.11.011>
- Perry, S. F., & Bernier, N. J. (1999). The acute humoral adrenergic stress response in fish: Facts and fiction. *Aquaculture*, 177(1–4). [https://doi.org/10.1016/S0044-8486\(99\)00092-7](https://doi.org/10.1016/S0044-8486(99)00092-7)
- Person-Le Ruyet, J., Pichavant, K., Vacher, C., le Bayon, N., Sévère, A., & Boeuf, G. (2002). Effects of O₂ supersaturation on metabolism and growth in juvenile turbot (*Scophthalmus maximus* l.). *Aquaculture*, 205(3–4). [https://doi.org/10.1016/S0044-8486\(01\)00689-5](https://doi.org/10.1016/S0044-8486(01)00689-5)

- Pottinger, T. G., & Mosuwe, E. (1994). The corticosteroidogenic response of brown and rainbow trout alevins and fry to environmental stress during a 'critical period'. *General and Comparative Endocrinology*, 95(3). <https://doi.org/10.1006/gcen.1994.1133>
- Pouder, D. B., Curtis, E. W., & Yanong, R. P. (2019). *Common Freshwater Fish Parasites Pictorial Guide: Flagellates*. EDIS, 2005(9). <https://doi.org/10.32473/edis-fa109-2005>
- Ramos, P. e Oliveira, J.M. (2001) Amiloodinose em pregado, *Psetta maxima* (L.) *Revista Portuguesa de Ciências Veterinárias*. 96: 201-205
- Reyes-Becerril, M., Tovar-Ramírez, D., Ascencio-Valle, F., Civera-Cerecedo, R., Gracia-López, V., & Barbosa-Solomieu, V. (2008). Effects of dietary live yeast *Debaryomyces hansenii* on the immune and antioxidant system in juvenile leopard grouper *Mycteroperca rosacea* exposed to stress. *Aquaculture*, 280(1–4). <https://doi.org/10.1016/j.aquaculture.2008.03.056>
- Saldarriaga, J. F., Taylor, F. J. R., Cavalier-Smith, T., Menden-Deuer, S., & Keeling, P. J. (2004). Molecular data and the evolutionary history of dinoflagellates. *European Journal of Protistology*, 40(1). <https://doi.org/10.1016/j.ejop.2003.11.003>
- Santos, G. A., Schrama, J. W., Mamauag, R. E. P., Rombout, J. H. W. M., & Verreth, J. A. J. (2010). Chronic stress impairs performance, energy metabolism and welfare indicators in European seabass (*Dicentrarchus labrax*): The combined effects of fish crowding and water quality deterioration. *Aquaculture*, 299(1–4). <https://doi.org/10.1016/j.aquaculture.2009.11.018>
- Selye, H. (1950). Stress and the general adaptation syndrome. *British Medical Journal*, 1(4667). <https://doi.org/10.1136/bmj.1.4667.1383>
- Shinn, A. P., Pratoomyot, J., Bron, J. E., Paladini, G., Brooker, E. E., & Brooker, A. J. (2015). Economic costs of protistan and metazoan parasites to global mariculture. In *Parasitology* (Vol. 142, Issue 1). <https://doi.org/10.1017/S0031182014001437>
- Silva, B. C., Martins, M. L., Jatobá, A., Neto, C. C. B., Vieira, F. N., Pereira, G. v., Jerônimo, G. T., Seiffert, W. Q., & Mouriño, J. L. P. (2009). Hematological and immunological

- responses of Nile tilapia after polyvalent vaccine administration by different routes. *Pesquisa Veterinaria Brasileira*, 29(11). <https://doi.org/10.1590/s0100-736x2009001100002>
- Smarra, A. L. S., de Azevedo, W. F., Fadel, V., Delatorre, P., Dellamano, M., Colombo, M. F., & Bonilla-Rodriguez, G. O. (2000). Purification, crystallization and preliminary X-ray analysis of haemoglobin I from the armoured catfish *Liposarcus anisitsi*. *Acta Crystallographica Section D: Biological Crystallography*, 56(4). <https://doi.org/10.1107/S0907444900001724>
- Smith, R. W., Wood, C. M., Cash, P., Diao, L., & Pärt, P. (2005). Apolipoprotein AI could be a significant determinant of epithelial integrity in rainbow trout gill cell cultures: A study in functional proteomics. *Biochimica et Biophysica Acta - Proteins and Proteomics*, 1749(1). <https://doi.org/10.1016/j.bbapap.2005.02.009>
- Smith, S. A., Levy, M. G., & Noga, E. J. (1992). Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of antibody to the parasitic dinoflagellate *Amyloodinium ocellatum* in *Oreochromis aureus*. *Veterinary Parasitology*, 42(1–2). [https://doi.org/10.1016/0304-4017\(92\)90110-U](https://doi.org/10.1016/0304-4017(92)90110-U)
- Smith, S. A., Levy, M. G., & Noga, E. J. (1994). Detection of anti-*Amyloodinium ocellatum* antibody from cultured hybrid striped bass (*morone saxatilis* × *m. chrysops*) during an epizootic of amyloodiniosis. *Journal of Aquatic Animal Health*, 6(1). [https://doi.org/10.1577/1548-8667\(1994\)006<0079:CDOAAO>2.3.CO;2](https://doi.org/10.1577/1548-8667(1994)006<0079:CDOAAO>2.3.CO;2)
- Smith, S., Noga, E., Levy, M., & Gerig, T. (1993). Effect of serum from tilapia *Oreochromis aureus* immunized with the dinospore *Amyloodinium ocellatum* on the motility infectivity and growth of the parasite in cell culture. *Diseases of Aquatic Organisms*, 15. <https://doi.org/10.3354/dao015073>
- Soares, F., Leitão, A., Moreira, M., Teixeira De Sousa, J., Almeida, A. C., Barata, M., Feist, S. W., Pousão-Ferreira, P., & Ribeiro, L. (2012). Sarcoma in the thymus of juvenile meagre *Argyrosomus regius* reared in an intensive system. *Diseases of Aquatic Organisms*, 102(2). <https://doi.org/10.3354/dao02545>

- Soares, F., Quental Ferreira, H., Cunha, E., Pousão-Ferreira, P. (2012a) Occurrence of *Amyloodinium ocellatum* in aquaculture fish production. *Aquaculture Europe*. 36(4): 13-16.
- Soares, F., Quental-Ferreira, H., Moreira, M., Cunha, E., Ribeiro, L., & Pousão-Ferreira, P. (2012). First report of *Amyloodinium ocellatum* in farmed meagre (*Argyrosomus regius*). *Bulletin of the European Association of Fish Pathologists*, 32(1).
- Sørum, U., & Damsgård, B. (2004). Effects of anaesthetisation and vaccination on feed intake and growth in Atlantic salmon (*Salmo salar* L.). *Aquaculture*, 232(1–4). [https://doi.org/10.1016/S0044-8486\(03\)00529-5](https://doi.org/10.1016/S0044-8486(03)00529-5)
- Steidinger, K. A., Burkholder, J. M., Glasgow, H. B., Hobbs, C. W., Garrett, J. K., Truby, E. W., Noga, E. J., & Smith, S. A. (1996). *Pfiesteria piscicida* gen. et sp. nov. (Pfiesteriaceae fam. nov.), a new toxic dinoflagellate with a complex life cycle and behavior. *Journal of Phycology*, 32(1). <https://doi.org/10.1111/j.0022-3646.1996.00157.x>
- Tavares-Dias M. (2003). Variáveis hematológicas de teleósteos brasileiros de importância zootécnica. Tese de Doutorado em Aqüicultura, Centro de Aqüicultura, Universidade Estadual Paulista, Jaboticabal, 209.
- Thomas, S., & Perry, S. F. (1994). Influence of initial respiratory status on the short-and long-term activity of the trout red blood cell β -adrenergic Na^+/H^+ exchanger. *Journal of Comparative Physiology B*, 164(5). <https://doi.org/10.1007/BF00302554>
- Ullal, A. J., Wayne Litaker, R., & Noga, E. J. (2008). Antimicrobial peptides derived from hemoglobin are expressed in epithelium of channel catfish (*Ictalurus punctatus*, Rafinesque). *Developmental and Comparative Immunology*, 32(11). <https://doi.org/10.1016/j.dci.2008.04.005>
- Urbina, M. A., & Glover, C. N. (2012). Should I stay or should I go?: Physiological, metabolic and biochemical consequences of voluntary emersion upon aquatic hypoxia in the scaleless fish *Galaxias maculatus*. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, 182(8). <https://doi.org/10.1007/s00360-012-0678-3>

- Van Aardt, W. J., & Booyesen, J. (2004). Water hardness and the effects of Cd on oxygen consumption, plasma chlorides and bioaccumulation in *Tilapia sparrmanii*. *Water SA*, 30(1). <https://doi.org/10.4314/wsa.v30i1.5027>
- Van Weerd, J. H., & Komen, J. (1998). The effects of chronic stress on growth in fish: A critical appraisal. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, 120(1). [https://doi.org/10.1016/S1095-6433\(98\)10017-X](https://doi.org/10.1016/S1095-6433(98)10017-X)
- Vaz, C. L. O. (2010) Efeitos do cobre no sargo (*Diplodus sargus*, Linnaeus 1758): implicações quer a nível fisiológico, quer de crescimento. Dissertação de mestrado em Engenharia Zootécnica – Produção animal apresentada à Faculdade de Medicina Veterinária e ao Instituto Superior de Agronomia da Universidade técnica de Lisboa, 119(1).
- Vijayan, M. M., Aluru, N., & Leatherland, J. F. (2010). Stress response and the role of cortisol. In *Fish Diseases and Disorders (Vol. 2)*. <https://doi.org/10.1079/9781845935535.0182>
- Viji, V. T., Deepa, K., Velmurugan, S., Donio, M. B. S., Jenifer, J. A., Babu, M. M., & Citarasu, T. (2013). Vaccination strategies to protect goldfish *Carassius auratus* against *Aeromonas hydrophila* infection. *Diseases of Aquatic Organisms*, 104(1). <https://doi.org/10.3354/dao02581>
- Wedemeyer, G.A., Barton, B.A., McLeay, D.J. (1990). Stress and acclimation, C.B. Shreck, P.B. Moyle (eds), *Methods for fish biology*. American Fisheries Society, U.S.A.: 451- 477.
- Wendelaar Bonga, S. E. (1997). The stress response in fish. In *Physiological Reviews (Vol. 77, Issue 3)*. <https://doi.org/10.1152/physrev.1997.77.3.591>
- Woo, P. T. K. (2007). Protective immunity in fish against protozoan diseases. *Parassitologia*, 49(3).
- Woo, P. T. K., & Bruno, D. W. (2014). Diseases and disorders of finfish in cage culture: Second edition. In *Diseases and Disorders of Finfish in Cage Culture: Second Edition*.

Yambot, A. v., & Song, Y. L. (2006). Immunization of grouper, *Epinephelus coioides*, confers protection against a protozoan parasite, *Cryptocaryon irritans*. *Aquaculture*, 260(1–4). <https://doi.org/10.1016/j.aquaculture.2006.05.055>

Zambrano, J.L.F., Dezón, D.E., González, C.R., & Fermín, E.G. (2001) Ciclo de vida de *Amyloodinium ocellatum* (Brown, 1931) (Dinoflagellata: Oodinidae). *Boletín Oceanográfico*, 83-89.