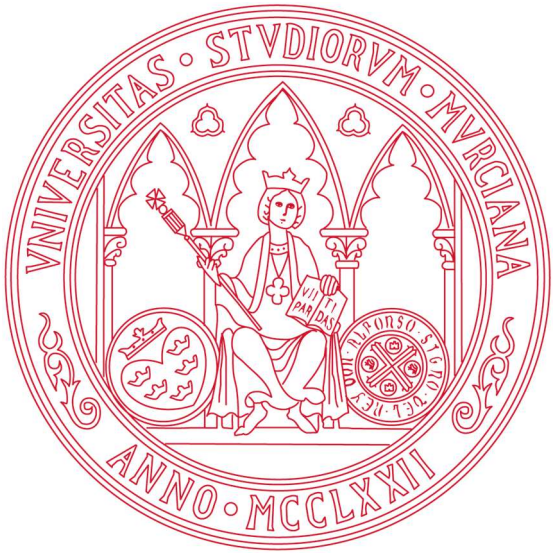

UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Clove essential oil nanoencapsulated in β-cyclodextrins improves the welfare at slaughter in different farm fish species: parameters of stress and innate immune response

El aceite esencial de clavo nanoencapsulado en β-ciclodextrinas mejora el bienestar en el sacrificio en diferentes especies de peces: parámetros de estrés y respuesta inmune innata.

Dña. Amanda Esperanza López Cánovas

2020

"The individual has always had to struggle to keep from being overwhelmed by the tribe. If you try it, you will be lonely often, and sometimes frightened. But no price is too high to pay for the privilege of owning yourself"

Friedrich Wilhelm Nietzsche

A mis padres.

A mi familia.

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Publications

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López-Cánovas, A.E., García-Ayala, A., López-Gómez, A., Fernandes, J.M.O & Galindo-Villegas., J., 2019. Effect of nanoencapsulated clove-oil anaesthesia in the physiological response and immune status of Nile tilapia. *Fish & Shellfish Immunology*. 91, 449.

López-Cánovas, A.E., García-Ayala, A., López-Gómez, A., Cabas, I., & Chaves-Pozo, E., 2019. Nanoencapsulation of essential oils improves its anesthetics characteristics in farmed gilthead seabream (*Sparus aurata* L.) by modulating stress parameters and antimicrobial activities. *Aquaculture* (in revision).

López-Cánovas, A.E., García-Ayala, A., López-Gómez, A., Cabas, I., & Chaves-Pozo, E., 2019. Incorporations of a new anesthesic method in Aquaculture to farmed European seabass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) (in preparation).

López-Cánovas, A.E., García-Ayala, A., López-Gómez, A., Fernandes, J.M.O., & Galindo-Villegas, J., 2019. Behavioral differences between Atlantic salmon (*Salmo salar*) and Nile tilapia (*Oreochromis niloticus*) after being stunned with nanoencapsulated clove essential oil in β-Cyclodextrins (in preparation).

Other publications/collaborations

Navarro-Segura, L., Ros-Chumillas, M., **López-Cánovas, A.E**., García-Ayala, A., & López-Gómez, A., 2019. Nanoencapsulated essential oils embedded in ice improve the quality and shelf life of fresh whole seabream stored on ice. *Heliyon* 5, e01804.

Works submitted to conferences or congress

López-Gómez, A., Ros-Chumillas, M., **López-Cánovas, A.E**., García-Ayala, A., Maté, J., & Soto-Jover, S., 2016. MAP packaging with essential oils vapour increases the shelf-life of refrigerated seabream fillets. CYTEF. VIII Congreso Ibérico/VI Congreso Iberoamericano de las Ciencias y Técnicas del Frío (Coimbra-Portugal).

López-Cánovas, A.E., Mulero-Méndez, V., Ros-Chumillas, M., García-Ayala, A., & López-Gómez, A., 2016. Liquid ice with an anaesthetic additive improves animal welfare in farmed seabream during stunning and slaughtering. CYTEF. VIII Congreso Ibérico/VI Congreso Iberoamericano de las Ciencias y Técnicas del Frío (Coimbra-Portugal).

López-Cánovas, A.E., Mulero-Méndez, V., Ros-Chumillas, M., García-Ayala, A., & López-Gómez, A., 2016. Improves animal welfare in farmed seabream during stunning and slaughtering through liquid ice with an anaesthetic additive. II Jornadas Doctorales de la Universidad de Murcia (Escuela Internacional de Doctorado de la Universidad de Murcia, EIDUM).

López-Cánovas, A.E., García-Ayala, A., Cabas, I., Mulero-Méndez, V., & López-Gómez, A., 2017. Mejora del bienestar de la dorada (*Sparus aurata* L.) cultivada en la industria acuícola durante el momento del sacrificio a través de la incorporación de un aditivo nanoencapsulado. III Jornadas Doctorales de la Universidad de Murcia (Escuela Internacional de Doctorado de la Universidad de Murcia, EIDUM).

López-Cánovas, A.E., Cabas, I., Mulero-Méndez, V., García-Ayala, G., & López-Gómez, A., 2017. Clove oil encapsulated β-cyclodextrins improves animal welfare in farmed fish during stunning and slaughtering. Aquaculture Europe 17 (Dubrovnik, Croacia).

López-Cánovas, A.E., Ros-Chumillas, M., Navarro-Segura, L., López-Gómez, A., Cabas, I., & García-Ayala, A., 2017. Nanoencapsulated clove essential oil used in stunning during slaughtering operation improves the quality and shelf-life of farmed gilthead seabream (*Sparus aurata* L.) and decreases fish wastes in the urban consumption chain. CONAMA Local Valencia (Valencia, España).

López-Cánovas, A.E., García-Ayala, A., López-Gómez, A., Cabas, I., & Chaves-Pozo, E., 2018. Comparación del uso de dos anestesiantes nanoencapsulados para la mejora del bienestar animal de la dorada (*Sparus aurata* L.) de acuicultura durante el momento del sacrificio. IV Jornadas Doctorales de la Universidad de Murcia (Escuela Internacional de Doctorado de la Universidad de Murcia, EIDUM).

López-Cánovas, A.E., Ros-Chumillas, M., Navarro-Segura, L., López-Gómez, A., Cabas, I., & García-Ayala, A., 2018. La mejora de la calidad y vida útil de la dorada (*Sparus aurata* L.) y lubina europea (*Dicentrarchus labrax*), tras el uso del aceite esencial de clavo nanoencapsulado en el momento de aturdido y sacrificio, disminuye el número de desechos de peces en la cadena de consumo humano. III Congreso de Biodiversidad y Conservación de la Naturaleza (Universidad de Murcia, España).

López-Cánovas, A.E., Cabas, I., Ros-Chumillas, M., López-Gómez, A., & García-Ayala, A., 2018. Efectos del aceite esencial de clavo encapsulado e incorporado al hielo en el proceso de aturdido previo al sacrificio en dorada (*Sparus aurata* L) y lubina europea (*Dicentrarchus labrax*) cultivadas. I Congreso Internacional Jls del Mar (Cádiz, España).

López-Cánovas, A.E., Cabas, I., Ros-Chumillas, M., Navarro Segura, L., López-Gómez, A., Chaves-Pozo, E., & García-Ayala, A 2019. Efecto del aceite de clavo encapsulado en β-ciclodextrinas sobre los niveles de glucosa y lactato y actividades humorales en el plasma de dorada (*Sparus aurata* L.) y lubina europea (*Dicentrarchus labrax* L.) durante el sacrificio. XVII Congreso Nacional de Acuicultura (CNA) 2019 (Cartagena, España).

López-Cánovas, A.E., García-Ayala, A., López-Gómez, A., Fernandes, J.M.O., & Galindo-Villegas, J., 2019. Effect of nanoencapsulated clove-oil anaesthesia in the physiological response and immune status of Nile tilapia. 3rd *Conference* of the International Society of *Fish & Shellfish Immunology (*Las Palmas de Gran Canaria, España).

Lopez-Canovas, A.E., Lopez-Gomez, A., Ros-Chumillas, M., Cabas, I., & García-Ayala, A., 2019. Crushed ice including nanoencapsulated clove essential oil improves animal welfare in farmed sea bass during stunning and slaughtering. ICR 2019 (Montreal, Quebec, Canadá).

.

Honours & awards

López-Cánovas, A.E., Cabas, I., Ros-Chumillas, M., Navarro-Segura, L., López-Gómez, A., García-Ayala, A, Mulero-Méndez, V.F. *XVI National Aquaculture Research Award (JACUMAR 2017) granted by the Ministry of Agriculture and Fisheries, Food and Environment*. "El aturdido y sacrificio de peces de acuicultura con aceite esencial de clavo nano-encapsulado embebido en cristales de hielo disminuye el sufrimiento animal y mejora la calidad y la vida útil del pescado refrigerado".

Index

Abbreviation index

act-β	Beta-actin gene
ACTH	Adrenocroticotropin
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ASC	Apoptosis associated speck-like protein containing a C-terminal CARD
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
bm	Body mass
Ca^{+2}	Calcium ion
CARD	Caspase activating recruiting domain
CD	Cyclodextrin
cDNA	Complementary DNA
CEO	Clove essential oil
CEO+β-CD	Clove essential oil nannoencapsulated in beta-cyclodextrin
CI	Crushed ice
CICR	Calcium induced calcium release
CRH	Corticotrophin releasing hormone
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
DHPR	Dihydropyridine receptor
ECC	Excitation-contraction coupling
ELISA	Enzyme linked immunosorbent assay

EU	Eugenol
EU+β-CD	Eugenol nanoencapsulated in β-cyclodextrin.
FAO	Food and agriculture organisation
FDA	Food and drug administration
FW	Fresh water
GALT	Gut associated lymph tissue
GIALT	Gill associated lymph tissue
GRAS	Generally recognized as safe
H_2O_2	Hydrogen peroxide
HBSS	Hanks buffer
HCO_3	Bicarbonate
Hsps	Heat-shock or stress proteins
Hsp70	Heat-shock protein 70
HPI	Hypothalamic-pituitary-interrenal
iL-1β	Interleukin 1 beta
LHD	Lactate dehydrogenase
LI	Liquid ice
Mg^{+2}	Magnesium ion
mmHg	Millimetre of mercury
MS-222	Tricaine methane-sulfonate
NALT	Nose associated lymph tissue
NaOH	Sodium hydroxide
NCCs	Natural cytotoxic cells
NK	Natural killer cells
NEF	Nucleotide exchange factor
OD	Optical density

PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
pCO_2	partial pressure of carbon dioxide
PCR	Conventional polymerase chain reaction
PHD	Pyruvate dehydrogenase
pO_2	Partial pressure of oxygen
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rps18	Ribosomal protein S18 gene
RyR3	Ryanodine receptor 3
SALT	Skin associated lymph tissue
SERCA	Sarco/endoplasmic reticulum ATPase
SR	Sarcoplasmic reticulum
SW	Seawater
SYBR green	Asymmetrical cyanine dye used as a nucleic acid stain
TCA	Trichloroacetic acid
TCO_2	Total carbon dioxide
TCR	T-cell antigen receptor
tgf-β	Transforming growth factor beta
TMB	Tetramethylbenzidine
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TT	Transverse tubule

Figures index

Introduction

Material and methods

Results

Tables index

Introduction

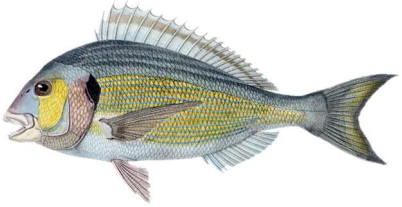
Material and methods

Results

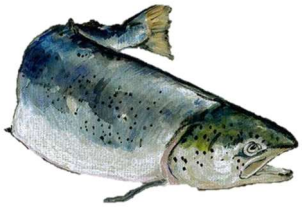
Table of fish species

Common Name
Scientific name

Gilthead seabream
Sparus aurata Linnaeus, 1758

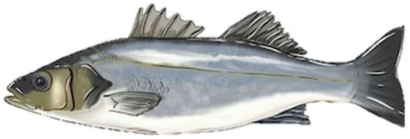


Atlantic salmon
Salmo salar Linnaeus, 1758



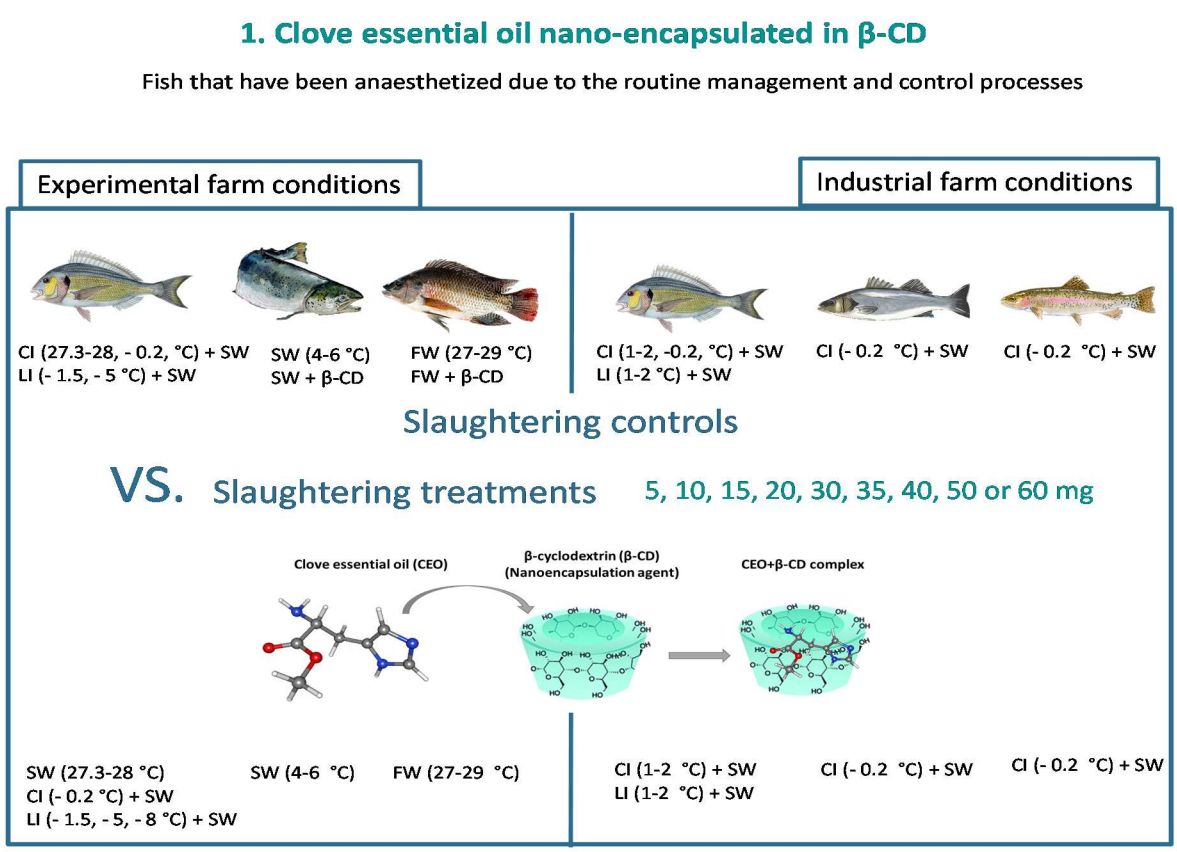
Nile tilapia
Oreochromis niloticus Linnaeus, 1758

European sea bass
Dicentrarchus labrax Linnaeus, 1758



Rainbow trout
Oncorhynchus mykiss Walbaum, 1792

Graphical abstract



2. Innovative management of gilthead seabream under industrial farm conditions

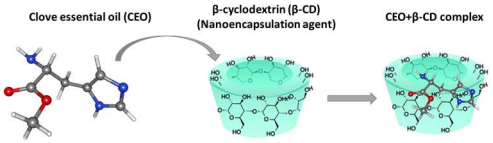
Fish that have been monitored by cameras during rearing and had never been exposed to anaesthesia until sampling

Industrial farm conditions

Slaughtering controls

CI (1-2 °C) + SW

VS. Slaughtering treatments 20 mg



CI (1-2 °C) + SW

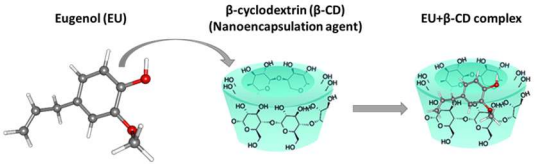
3. Eugenol nanoencapsulated in β-CD in gilthead seabream under experimental farm conditions

Experimental farm conditions

Slaughtering controls

CI (1-2 °C) + SW

VS. Slaughtering treatments 20 mg



CI (1-2 °C) + SW

2.

3.

1. Anaesthesia-induction stages
- Loss of equilibrium
- Loss of reflex activity

2. Plasmatic parameters
- Glucose
- Lactate
- pO_2, pCO_2, HCO_3, TCO_2, SO_2, base excess, pH
- Cortisol

3. Antimicrobial activities
- Peroxidase
- Bactericidal
- Antiprotease
- Protease

Blood
Skin mucus

SUMMARY

The main objective of this thesis has been to improve the welfare at slaughter in five fish species (gilthead seabream, Atlantic salmon, Nile tilapia, European seabass and rainbow trout) in experimental and industrial farm conditions by using clove essential oil (CEO) nanoencapsulated in β-cyclodextrins (β-CD) as a novel anaesthetic. The methodology was first applied in gilthead seabream specimens, in which the novel anaesthetic was compared with the most usually used stunning method in aquaculture (cooling in ice slurry). We tested **i)** a range of CEO doses (from 5 to 60 mg/kg), a range of temperatures (from - 8.0 to 29.0 °C), two different types of ice (liquid and crushed) and two different farm conditions (experimental and industrial). The novel anaesthetic was then tested in several fish species under different farm conditions, such as type of water (sea or fresh water) and different temperatures. For this, the dose of CEO encapsulated in the β-CD was adjusted according to the species and size of the specimens. In addition, we tested **ii)** CEO+β-CD in industrial farm conditions on gilthead seabream that had never been anesthetized in their productive cycle, as their size had always been calculated with underwater cameras, and **iii)** a dose of 20 mg of eugenol (EU) nanoencapsulated in β-CD in seawater at 27.8-28.0 °C on seabream in experimental farm conditions.

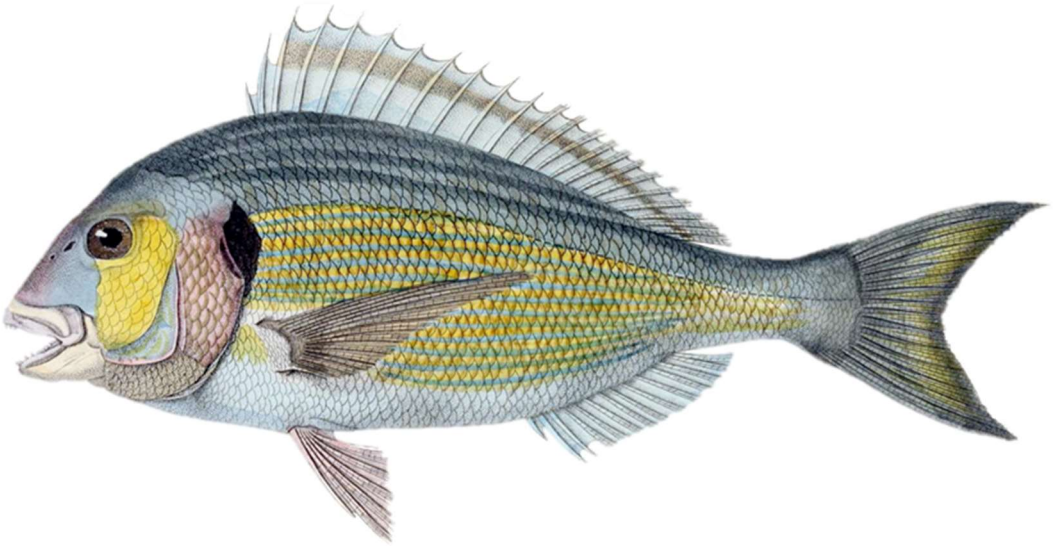
All the research described was financed by projects coordinated and realized by the companies CUBIPLAYA S.L. and Pescados de Acuicultura de Murcia S.L. (PESCAMUR), in collaboration with Universidad Politécnica de Cartagena, and was carried out in the Research Group of "Innate Immune System of Teleost Fish", from Department of Cell Biology and Histology of the University of Murcia.

The first aim of this work was to determine the time needed to induce two stages of anaesthesia: i) the total loss of equilibrium, defined as the total loss of muscle tone and balance with a low, regular opercular rate, and ii) the loss of reflex activity, defined as the total loss of reactivity, with slow and irregular opercular movements and slow heart rate. In all the fish species studied, the CEO+β-CD treatment allowed us to reduce the dose of CEO needed to induce the loss of reflex activity under experimental and industrial farm conditions at different temperatures and independently of the type of water used, as it was equally effective in sea and fresh water. In addition, the nanoencapsulation of EU in β-CD reduced the time needed to induce anaesthesia compared to EU or CEO alone.

The second aim of this thesis was to determine the level of stress induced by the novel stunning method in different fish species. For that, we analysed the levels of glucose, lactate, cortisol, pO_2, pCO_2, HCO_3, TCO_2, SO_2, base excess and pH in the plasma and the skin mucus in almost all the species studied and the level of expression of *RyR3* gene in the skeletal muscle of gilthead seabream. Taking into account the data obtained in gilthead seabream specimens treated under different conditions (type of ice, temperature and dose), we concluded that CI at - 0.2 °C containing a low dose of CEO+β-CD (15 mg/kg) is the method that produced the lowest stress response. Interestingly, gilthead seabream specimens that had never been anesthetised before behaved in a similar way when stunned with CEO+β-CD as other fish that had been anesthetised during their productive cycle. In addition, in all the species studied we found that a low dose of CEO+β-CD used to control or reduce the metabolic alteration induced by the stress response was enough to assume that the same fish would not suffer irreversible lesions. Thus, 40 mg CEO+β-CD/L water, 60 mg CEO+β-CD/L water, 5 mg CEO+β-CD/kg ice, 5 mg CEO+β-CD/kg ice are the optimal doses that triggered the lowest stress response in Atlantic salmon, Nile tilapia, European seabass and rainbow trout, respectively. However, when EU+β-CD was tested in gilthead seabream, the data suggest a strong stress response that leads to physiological alterations.

The third aim of this thesis was to determine the consequences of the novel anaesthetic method for the immune response of fish. For this, we analysed the expression level of the genes coding for two cytokines (*il1-β* and *tgf-β*) and a heat sock protein (*Hsp70*) in the head-kidney (the main haematopoietic tissue in fish) of gilthead seabream. We also analysed several innate immune activities (peroxidase, bactericidal, anti-protease and protease) in the plasma and skin mucus of all the species. Our data revealed that the doses of CEO+β-CD mentioned above for each species altered some innate immune parameters in plasma and skin mucus. However, impairment of the immune response can be discounted as the bactericidal activity was not affected in most of the species. The exception was Nile tilapia in which we observed a level of innate activities that pointed to an immune-suppression effect at the optimal dose of CEO+β-CD. Similarly, gilthead seabream specimens treated with EU alone or EU+β-CD showed immune-suppression, which was not observed in specimens treated with CEO alone or CEO+β-CD.

I.Introduction



I.1. Aquaculture sector

Aquaculture is considered, according to The Food and Agriculture Organisation (FAO) of The United Nations, as "The farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated, the planning, development and operation of aquaculture systems, sites, facilities and practices, and the production and transport".

Over the last half-century, aquaculture has become the fastest growing food production sector (Broitman *et al*., 2017), and nowadays surpasses the fishery capture (Béné *et al*., 2015; Froehlich *et al*., 2018; Longo *et al*., 2019; Naylor *et al*., 2000; Valderrama & Anderson, 2010) to meet the growing demand of fish for human consumption (Béné *et al*., 2015; FAO, 2018, Ottinger *et al*., 2018). Because of the aquaculture technology development, fish is one of the most traded food commodities around the World (Allison, 2011; Hill, 2005; Metian *et al*., 2019; Zhou, 2017), being fish every day more accessible to many developing countries (Toufique & Belton, 2014). In fact, aquaculture is the World's most diverse food production sector (FAO, 2018), with 171 million tonnes (MT) of total fish production, where the 88 % (over 151 MT) of total were utilized for direct human consumption (FAO, 2018) (**Figure 1**).

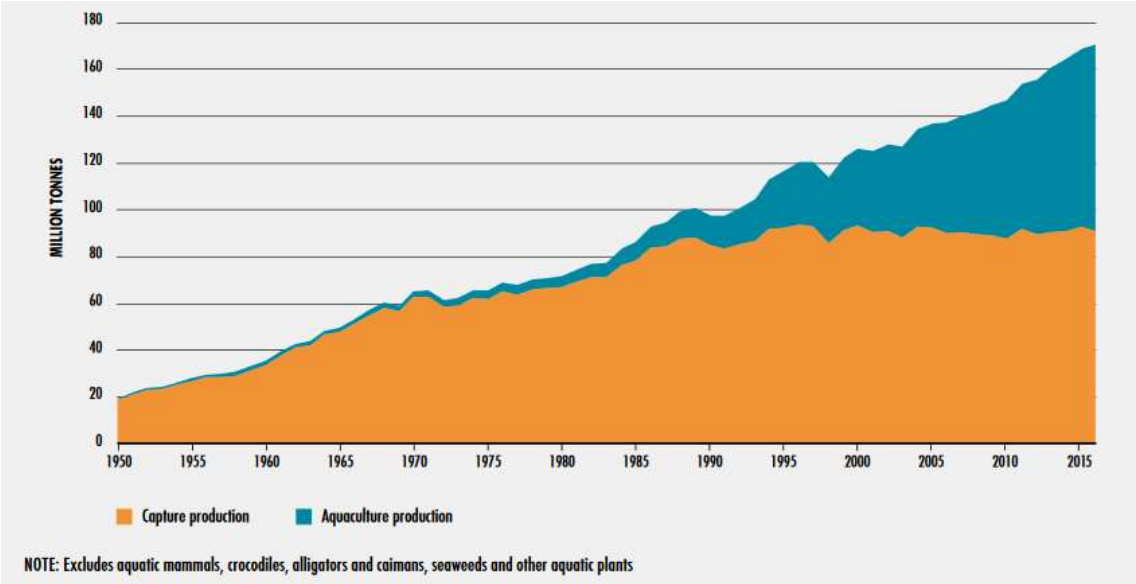


Figure 1. Global capture fisheries and aquaculture production, 1950-2016 (FAO, 2018).

Nowadays, Asia is the first continent on the list with the highest world aquaculture production. The rest of aquaculture production is distributed across America, Europe, Africa and Oceania (APROMAR, 2019). Egypt, Nigeria, Chile, India, Indonesia, Vietnam, Bangladesh and Norway are the major producing countries that have strengthened the world production over the past two decades (FAO, 2018). Due to this high geographical distribution in worldwide aquaculture production, there is a great diversity of farmed fish species around the world, considering as global species to such as Nile Tilapia, Atlantic salmon and rainbow trout (FAO, 2018; Tveteraas, 2015).

As shown in **table 1**, Nile Tilapia is one of the most worldwide farm fish species with a production of 4.20 MT, following by Atlantic salmon with 2.24 MT, and rainbow trout with 8.14 T (FAO, 2018).

Table 1. The most 20 species produced in World aquaculture (2010-2016). (FAO, 2018) (x 1000 T)

	Species item	2010	2012	2014	2016
	Finfish				
1	Grass carp, *Ctenopharyngodon idellus*	4362	5018	5539	6068
2	Silver carp, *Hypophthalmichthys molitrix*	4100	4193	4968	5301
3	Common carp, *Cyprinus carpio*	3421	3753	4161	4557
4	**Nile tilapia, *Oreochromis niloticus***	**2537**	**3260**	**3677**	**4200**
5	Bighead carp, *Hypophthalmichthys nobilis*	2587	2901	3255	3527
6	Carassius spp.	2216	2451	2769	3006
7	Catla, *Catla catla*	2977	2761	2770	2961
8	Freshwater fishes nei, *Osteichthyes*	1378	1942	2063	2362
9	**Atlantic salmon, *Salmo salar***	**1437**	**2074**	**2348**	**2248**
10	Roho labeo, *Labeo rohita*	1133	1566	1670	1843
11	Pangas catfishes nei, Pangasius spp	1307	1575	1616	1741
12	Milkfish, *Chanos chanos*	809	943	1041	1188
13	Tilapias nei, *Oreochromis* (=Tilapia) spp.	628	876	1163	1177
14	Torpedo-shaped catfishes nei, Clarias spp.	353	554	809	979
15	Marine fishes nei, *Osteichthyes*	477	585	684	844
16	Wuchang bream, *Megalobrama amblycephala*	652	706	783	862
17	**Rainbow trout, *Oncorhynchus mykiss***	**752**	**883**	**796**	**814**
18	Cyprinids nei, Cyprinidae	719	620	724	670
19	Black carp, *Mylopharyngodon piceus*	424	495	557	632
20	Snakehead, *Channa argus*	377	481	511	518

The European production was approximately 7,034,104 T (1.2 % of the total world fish production in 2018), where 1,353,201 tonnes belongs to aquaculture products and 5,680,902 T came from capture fisheries (FAO, 2018).

In Europe, Atlantic salmon (*Salmo salar* L.) is the most produced specie with 209.180 T, followed by rainbow trout (*Oncorhynchus mykiss* W.) with 185.316 T, gilthead seabream (*Sparus aurata* L.) with 95.390 T and European seabass (*Dicentrarchus labrax* L.) with 79.350 T (APROMAR, 2018; 2019; Di Marco *et al*., 2017,). The Mediterranean Sea contribute significantly to the european economy of aquaculture production, where gilthead seabream and European seabass are the two most important cultured fish there (APROMAR, 2019; Basurco *et al*., 2011; Fernandez Sanchez *et al*., 2018; Grigorakis, 2017; Grigorakis *et al*., 2002; Oliva-Teles *et al*., 2011). The biggest producer countries of these two species are Turkey, Greece and Spain (Arabaci *et al*., 2010; Fisheries and aquaculture in Europe, 2012a).

Spain is the Member State of the European Union with the highest aquaculture harvest, with 311,032 T in 2017 (23.0 % of the total for the Union), followed by the United Kingdom and France (APROMAR, 2019). The aquaculture production in Spain (Galicia, Andalucía, Region of Murcia, Castilla and Leon, Valencia, Cataluña, and the Canary Islands) achieved 348,395 T in 2018 (APROMAR, 2019; Instituto Geografico Nacional, 2016) (**Figure 2**).

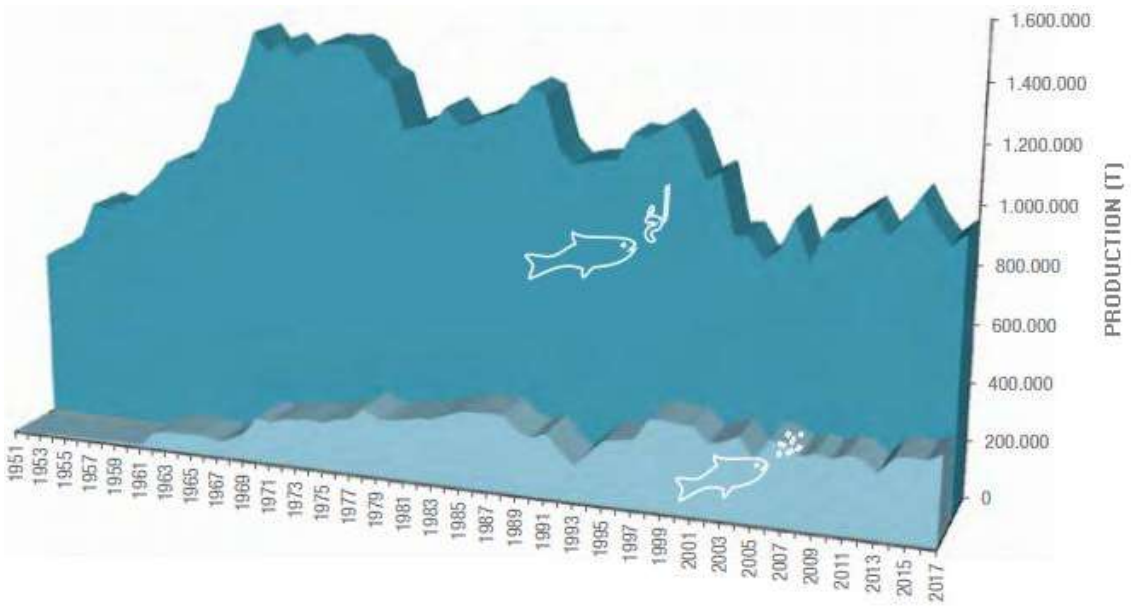


Figure 2. Evolution of total aquatic production (aquaculture and fisheries) in Spain (T) from 1950-2017 (APROMAR, 2019).

In Spain, these two fish species represent approximately the 90–95 % of total production of seawater (SW) farm fish (Di Marco *et al*., 2017) with a total of 37,390 T in 2018 (APROMAR, 2019) (**Figure 3**). Regarding to freshwater (FW) species, the species mainly produced in Spain is rainbow trout (APROMAR 2018; ESACUA, 2015; FAO, 2019; Muñoz-Lechuga, 2018).

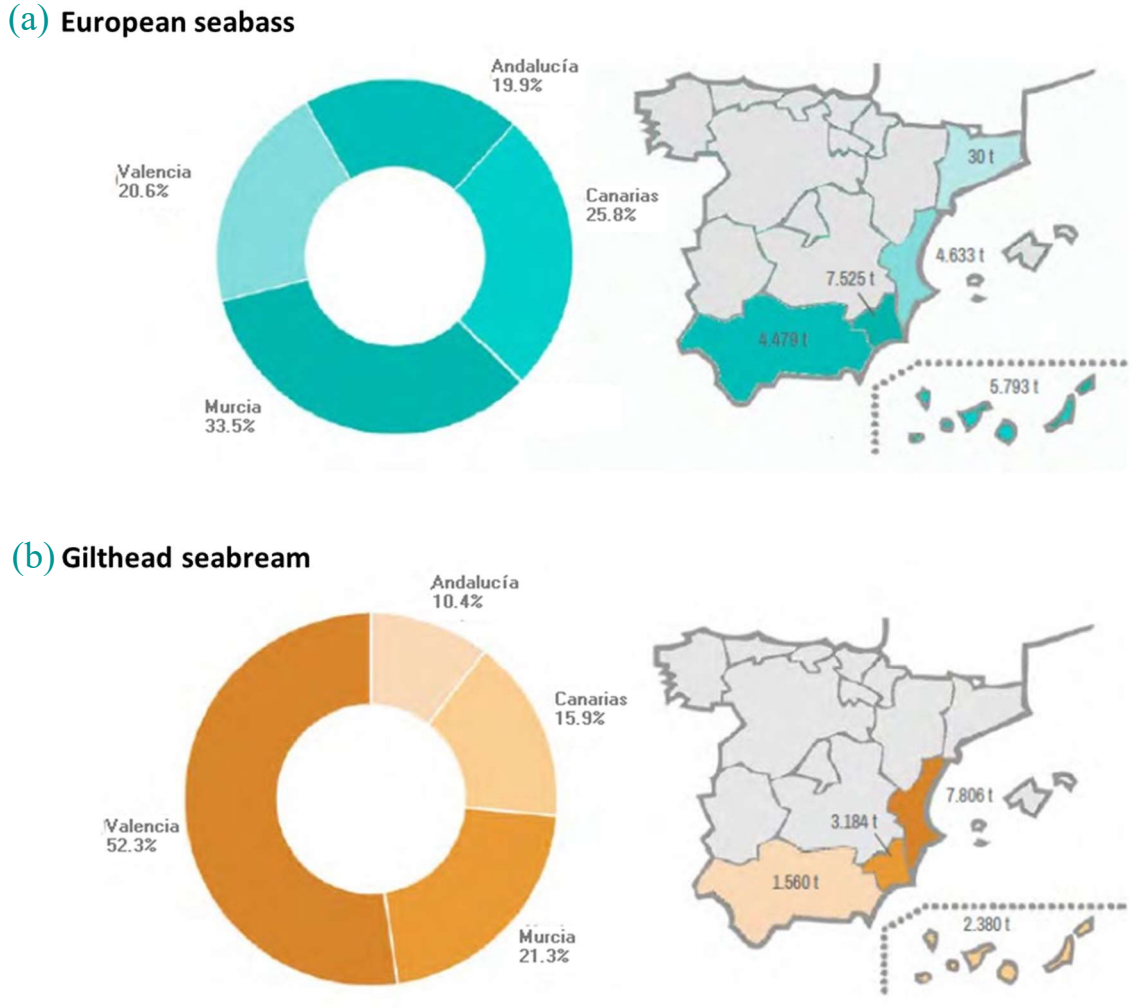


Figure 3. Distribution percentage productions (tonnes) of European seabass **(a)** and Gilthead seabream **(b)** in Spain by Autonomous region for 2018 (APROMAR, 2019).

Based on the general importance of European and Spanish aquacultural production, different species of interest were chosen. Thus, gilthead seabream, Atlantic salmon, Nile tilapia, European seabass and rainbow trout were studied.

I.1.1. Gilthead seabream (*Sparus aurata*)

The gilthead seabream (*Sparus aurata* Linnaeus, 1758) is a marine teleost that belongs to the Sparidae family and to the genus Sparus (Basurco & Abellán, 1999; Moretti *et al*., 1999; Pavlidis & Mylonas, 2011), found along the Eastern Atlantic coasts, from the United Kingdom to the Canary Islands (FAO, 2019a; Fisheries and aquaculture in Europe, 2012a). It is predominantly carnivorous, but accessorily herbivorous (Froese & Pauly, 2006). It has euryhaline and eurythermal habits (Mancera *et al*., 1995), so it could be found in both marine and brackish water environments such as coastal lagoons and estuarine areas, in particular during the initial stages of its life cycle (FAO, 2019a; Fisheries and aquaculture in Europe, 2012; Moretti *et al*., 1999). Young fish remain in relatively shallow areas (up to 30 m), whereas adults can reach deeper waters, generally not more than 50 m depth (FAO, 2019a; Moretti *et al*., 1999).

Gilthead seabream specimens are born in the open sea during October-December. In early spring, juvelines migrate towards protected coastal water to find abundant trophic resources and milder temperatures. Gilthead seabream grows rapidly in the summer months when the sea temperature is high (Huidobro & Tejada, 2004). In late autumn they return to the open sea, where the adult fish breed (FAO, 2019a). The commercial size is reached after one and a half years in culture (Fisheries and aquaculture in Europe, 2012a). Nevertheless, the sexual maturity develops in males at 2 years of age (20-30 cm) and turn into females at 3-4 years (33-40 cm) of life, what means that is a protandrous hermaphrodite (FAO, 2019a; Moretti *et al*., 1999; Mylonas *et al*., 2011).

The gilthead seabream had been used in many studies with special focus on reproductive, but also on physiology, nutrition and immune and stress responses (Ibarz *et al*., 2010; Mylonas *et al*., 2011). Moreover, it has been shown that the welfare of gilthead seabream is altered through chronic stressors (environment noise, salinity, aquaculture management methods, etc) (Altimiras *et al*., 1994; Filiciotto *et al*., 2017; Tort *et al*., 1996) but under an acute stress situation is able to recover after a period of 4–24 h, depending on the type and intensity of the stressor (Molinero *et al*., 1997). Nevertheless, when the stressor is applied daily for short periods, gilthead seabream specimens tent to adapt and are not significantly affected (Sunyer *et al*., 1995).

I.1.2. Atlantic salmon (*Salmo salar*)

The Atlantic salmon belong to the Salmonidae family and is native to the basin of the North Atlantic Ocean, from the Arctic Circle to Portugal in the eastern Atlantic, in Iceland and southern Greenland, and from the Ungava region of northern Quebec to the Connecticut River (ASF, 2018; Ferguson *et al*., 2007; Scott & Crossman, 1973).

In the natural environment, this anadromous fish (fish born in FW who spend most of their lives in SW and return to FW to spawn) undergo a physiological process called smoltification that drives physiological and behavioral changes (Bigelow, 1963), such as increased salinity tolerance, olfactory sensitivity, metabolic rate, scope for growth, and altered hemoglobin and visual pigments that prepares them for their life at sea (Bigelow, 1963; McCormick *et al*., 1998). This process is performed during limited period (the physiological "smolt window") and regulated by environmental conditions such as temperature, food, and predators (an ecological "smolt window"). Smolt development is adversely affected by acidity, pollutants, and improper rearing conditions.

After spending 1–2 years at sea, the specimens return to their FW rivers to spawn. At sea, the Atlantic salmon prefers temperatures from 4 to 12 ºC but can withstand short periods of time at lower (- 0.7 ºC) or upper (27.8 ºC) lethal temperatures (Bigelow, 1963). Thus, Atlantic salmon culture requires both FW and SW facilities. Interestingly, the Atlantic salmon when is in culture increase their rates of growth and reduces the FW stage to less of 1 year and the marine phase to a period between 10 to 15 months (FAO, 2019d) (FAO, 2019d). Regarding the optimal stocking density, the theoretical maximum stocking density in sea-cages had been suggested to be between 15 and 25 kg/m^3, but it is normaly culture with higher densities at more than 80 % of the salmon farms (CIWF, 2009; Rosten *et al*., 2007).

I.1.3. Nile tilapia (*Oreochromis niloticus*)

The Nile tilapia belongs to the cichlidae family and is an omnivorous grazer (FAO, 2019e), found in several FW habitats such as rivers, streams, canals, lakes and ponds distributed worldwide (FAO, 2019e; Popma & Lovinsh, 1995).

Sexual maturity is reached at an age of 5-6 months (FAO, 2019e). This is a polygynous species whose males mate with several females and for this issue; they interact

aggressively, establishing a territorial based hierarchy based on one dominant male and several subordinate males. When social stability is altered, prolonged fighting behaviour emerge (Gonçalves-de-Freitas *et al*., 2019).

Nile tilapia usually lives in shallow water of temperature ranges from 31 to 36 ºC, but is also tolerates lower or upper temperatures, such as 11-12 ºC or 42 ºC, respectively (FAO, 2019e). This tolerance to non-optimal temperatures is genetically based (Cnaani *et al*., 2000), but is also induced by abiotic (the season or several water parameters such as hardness, salinity, dissolved oxygen and so on) and by biotic (age, size, sex and condition of the fish) factors (Chervinski, 1982). In culture conditions, the manipulation of some environmental factors could help to increase the tolerance of tilapia to low temperature (Atwood *et al*., 2003). Conversely, light intensity and background colour can affect feed detection, feeding success and food conversion rate of cultured fish, influencing fish growth and mortality rates (Jentoft *et al*., 2006). In fact, the stress triggered by background colour or light intensity may also affect their swimming performance, the activity levels and the habitat utilization (Papoutsoglou *et al*., 2005; Schreck *et al*., 1997).

I.1.4. European seabass (*Dicentrarchus labrax*)

European seabass belongs to the Moronidae family and is an euryhaline and eurythermal fish (FAO, 2019b; Frimodt, 1995). It is naturally found in coastal waters down to about 100 m depth, in estuaries, and occasionally in coastal lagoons, (FAO, 2019b; Frimodt, 1995), but they migrate offshore when the weather is cold appearing in deep waters (FAO, 2019b). European seabass habits in the north-east Atlantic Ocean from Morocco to Scotland and Norway, in the Black Sea and in the Mediterranean Sea (Pickett & Pawson, 1994). In wild, European seabass breed only once per year. In the Mediterranean Sea, the breeding season takes place in winter (December to March), while in the Atlantic Ocean occurs at the beginning of the summer (around June) (Muus & Nielsen 1999). The sexual maturity of males is attained between 2 to 4 or 4 to 7 years of age in the Mediterranean or Atlantic populations, respectively. In contrast, the females reach sexual maturity from 5 to 8 years of age (36-46 cm length) (FAO, 2019b; Pawson & Pickett, 1996). European seabass specimens migrate mainly depending on water temperatures but, in general, in response to seasonal changes in their environment. Thus, the North East Atlantic population make extensive migrations to southwest to areas within the western English Channel to spawn in warm water

during the spring. Then, they spend their summer and autumn months in the southern North Sea and finally, they move north wards again to summer feeding grounds (Pawson *et al*., 2007). Similarly, the migration route along the Mediterranean Sea goes from Almeria-Oran front to east of Gibraltar strait (Tine *et al*., 2014).

Under farming conditions, maturity is reached at a much earlier age than in wild conditions where annual spawning occurs during winter or early spring (FAO, 2019b). Despite the fact that female seabass grows faster than males, reaching differences of around 30 % in body weight (Gorshkov *et al*., 2004), the optimal commercial size (around 300-500 g) is achieved from a year and a half to two years of culture depending on water temperature (Fisheries and aquaculture in Europe, 2012b; Papadaki *et al*., 2005).

The successful farming of seabass requires a good management of the feeding strategy in function of the environmental conditions (Begout-Anras, 1995). Regarding stress responses, European seabass is able to tolerate adverse environmental conditions (Pichavant *et al*., 2003), such as constant high or low temperatures (Varsamos *et al*., 2006), fasting feeding periods (Rubio *et al*., 2010) or handling procedures commonly applied during its rearing (Morales-Nin *et al*., 2011). But it also has a sensibility to repeated acute stress situations or chronic stress that trigger high levels of cortisol and impairs seabass performance (Fanouraki & Mylonas, 2011; Millot *et al*., 2010; Papoutsoglou *et al*., 1998; Santos *et al*., 2010; Varsamos *et al*., 2006). In fact, reproductive dysfunctions and a quite sensitivity to disease outbreaks downfall the seabass culture (FAO, 2016).

I.1.5. Rainbow trout (*Oncorhynchus mykiss*)

The rainbow trout belongs to the salmonidae family and is an anodromous fish species which is native from the Northwest coast of North America, ranging from Alaska to Mexico (Bobe *et al*., 2016; FAO, 2019c; Fisheries and aquaculture in Europe, 2012c), but it is cultured in nearly every country worldwide (Barnhart, 1969).

Rainbow trout has a rapid growth and maturation (FAO, 2019c), achieving 7-10 kg within 3 years in SW, but when culture in FW can only attain 4-5 kg in the same time (FAO, 2019c; Fisheries and aquaculture in Europe, 2012c; Froese & Pauly, 2009). In nature, this specie only spawn once, in spring (January-May), although selective breeding and photoperiod adjustment has developed hatchery strains that can mature earlier and spawn all

year round (FAO, 2019c). Regarding temperature tolerance, the greater growth capacity is reached when the daily temperatures are from 13 to 15 ºC (Bear *et al*., 2007).

Despite the control of several variables such as size, age, diet, water temperature, water quality, and holding facilities that can be maintained in rainbow trout culture facilities (Barnhart, 1969), studies about the stress responsiveness capacity of this species are being performed (Trenzado *et al*., 2006). Thus, it is known that rainbow trout is tolerant to a wide range of environments and handling protocols (FAO, 2019c), but high stocking density causes stress, disease and problems such as fin erosion (Wedemeyer, 1996). This stress condition also reduces growth and feed conversion efficiency (North *et al*., 2006).

I.2. Stunning and slaughter of farmed fish

In aquaculture, slaughter method is the last of three stages of harvesting farmed fish (the period of food withdrawal to empty the gut, the collection and movement of fish to the point of slaughter and the process of slaughter) and is considered one of the most stressful stages for farmed fish (Kuhn & Smith, 2017; Lines & Spence, 2014). Because there is a broad scientific consensus that fish are sentient and have the capacity to suffer (Brown, 2015), slaughter methods should offer a rapid loss in sensibility and consciousness in terms of fish welfare (Zhang *et al*., 2017). Thus, the Europe Union Directive on the protection of animals at slaughter applies that "Animals shall be spared any avoidable excitement, pain or suffering during movement, restraint, stunning, slaughter or killing" (Van de vis *et al*., 2003). As consequence, the aquaculture industry also showed its concern in the ethical and welfare issues related to fish husbandry (Ribas *et al*., 2007). The acceptable slaughter methods must render the animal insensible immediately and should be carried out without causing avoidable pain or suffering (Savina *et al*., 1995).

Depending on the specie, fish are slaughtered and processed individually or collectively (Kuhn & Smith, 2017), but slaughter is generally a two-stage process: **1)** A previous stunning to make the animal insensible to pain and **2)** fish death (Lines *et al*., 2003). The first stages of slaughter must be minimised to prevent any recovery of consciousness before death occurs (Kuhn & Smith, 2017; Lines *et al*., 2003).

Nowadays, there is a significant necessity of improving the slaughter management studying the close relationship between fish welfare, the physiological stunning and death processes and the final quality of the flesh. Several stunning/slaughtering methods are used for aquaculture purposes, although some of them are considered stressful as fish undergo long periods of agony and different levels of stress (Baldi, *et al*., 2018; Håstein, 2004;). Most of them are briefly described below:

The electrical stunning, which main principle is to pass an electric current through the brain, powerful enough to cause an epileptic-like fit, resulting in an immediate unconsciousness and insensibility to pain. If the current flows are long enough, the fish will die before the brain is able to recover sensibility (Benson, 2006). When applied properly, it is a rapid stunning method (Oliveira-Filho *et al*., 2016; Van De Vis *et al*., 2003; Zampacavallo *et al*., 2014).

The CO_2 narcosis, which is applied in a water bath saturated with CO_2 that is dissolved in water giving H_2CO_3 in equilibrium with HCO_3^- and H^+. This exerts a lowering effect on blood pH and, in this way, a toxic effect on the brain (Poli *et al*., 2005).

The percussive stunning method is widely used in commercial aquaculture. The fish are hit on the head with a rapidly moving. When force enough is applied the concussion can be irrecoverable. But when it is not immediate, fish are hit more than once (Roth *et al*., 2007; Wall, 2001). The careless handling of this technique can cause eye damage, bruising, and downgrading of the fish (Qin et *al*., 2016).

Bleeding and gutting trigger fish death due to the anoxia caused by blood loses (Borderıas & Sanchez-Alonso, 2010). Thus bleeding is applied cutting the gills with a sharp knife, the throat (ventral aorta) or the arteries in the neck (dorsal aorta) to accelerate death and drain the majority of blood from the muscle (Botta *et al*., 1986; Borderías & Sanchez-Alonso, 2010). Gutting is usually done by cutting the belly, and when it is open it should be thoroughly washed to remove traces of blood and debris and to wash bacteria and intestinal content out of the gut cavity, skin, and gills of the fish (Borderías & Sánchez-Alonso, 2010). The main reason for gutting is to prevent autolytic spoilage rather than bacterial spoilage (Shewan, 1961).

The asphyxia on air causes acute hypoxia that turn into physiological stress and physical damage to the gill lamellae that increases with the time of air exposure (Cook *et al*., 2015). Asphyxia on ice immediately decreased fish oxygen levels and body temperature reducing fish metabolic rate and movements (Poli *et al*., 2005). Both methods, of asphyxia are the most stressful killing methods as it takes long time until death is confirmed (Simitzis *et al*., 2013; Tulli *et al*., 2015).

Other method is the **thermal shock on a mixture of ice and water**, that avoid the asphyxia but quickly reduces the brain function and shorten the loss of consciousness time compared with air asphyxia (Poli, 2009). However, cold-shock stress also occurs due to the fact that the difference in temperature have to be of at least 10 °C lower than the optimal temperature of culture (Donaldson *et al*., 2008; Madeira *et al*., 2017; Omeji *et al*., 2017; Poli, 2009). Nowadays, the direct immersion in containers filled with ice-water slurry (ice flakes and water ranging from 1:2 to 3:1 ratio) is the most commonly used method by fish farmers for stunning/slaughtering. As these methods, it is not exempt of stress and suffer; the combination of chilling on ice/water slurry preceded by anaesthesia could be a great improve on the welfare of farmed fish (Concollato *et al*., 2015; Panagiotis *et al*., 2013).

I.2.1. Anaesthesia

Anaesthetics are physical or chemical agents that firstly induce a calming effect and subsequently the loss of mobility, equilibrium, consciousness, and finally, the loss of reflex actions, allowing overcome the stressful procedures (Javahery *et al*., 2012; Summerfelt & Smith, 1990).

I.2.1.1. Stages of anaesthesia in fish

Different anaesthesia stages can be observed through swimming activity, balance, respiratory frequency, as well as reactions to external stimuli (Easy *et al*., 2009). The anaesthesia-induction stages suggested by Keene *et al*. (1998) are divided in five different conditions: 0) Normal, 1) Light sedation, 2) Deep sedation, 3) Partial loss of equilibrium, 4) Total loss of equilibrium, 5) Loss of reflex reactivity and 6) Medullary collapse (**Figure 4**).

Stages	Descriptor	Behaviour exhibited
0	Normal	Reactive to external stimuli; opercular rate and muscle tone normal.
1	Light sedation	Slight loss of reactivity to external visual and tactile stimuli; opercular rate slightly decreased; equilibrium normal.
2	Deep sedation	Total loss of reactivity to external stimuli except strong pressure; slight decrease in opercular rate; equilibrium normal.
3	Partial loss of equilibrium	Partial loss of muscle tone; swimming erratic; increased opercular rate; reactive only to strong tactile and vibrational stimuli.
4	Total loss of equilibrium	Total loss of muscle tone and equilibrium; slow but regular opercular rate; loss of spinal reflexes.
5	Loss of reflex reactivity	Total loss of reactivity; opercular movements slow and irregular; heart rate very slow; loss of all reflexes.
6	Medullary collapse	Opercular movements cease; cardiac arrest usually follows quickly.

Figure 4. Behavioural observation of different anaesthesia stages (Modifed from Hikasa *et al*., 1986).

I.2.1.2. Anaesthetic absorption in fish

The gills of fish are the main route of entry and the excretion of anaesthetics (Ferreira *et al*., 1984; Summerfelt & Smith, 1990;).

Gills in fish are arranged as a series of cartilaginous gill arches that provide the physical support for the delicate gill filaments also termed primary lamellae that are projected posterior-laterally in two rows of regularly spaced filaments (Lumb, 2016). The filaments are dorsoventrally flattened, and their surface area is greatly increased by folding to form secondary lamellae. In teleost fish, the gill arches are supported by cartilaginous elements partially calcified that are also present in the filaments and together with the abductor muscles have a role in the positioning of the gills in the water flow (Wilson & Laurent, 2002). The functional unit of the gill are the filaments which are formed by lamellae where the gas exchange takes place. The primary lamella is attached to a stem where two primary blood vessels, efferent artery a vein run in parallel. They are further subdivided into capillaries and sub-capillaries while entering into secondary lamellae (Evans *et al*., 2004), which are arranged in a rack like structure as shown in **Figure 5**. Thus, these secondary lamellae provide a shuttle barrier between the aquatic environment and the fish's blood running in vascular network encased with a velocity enough to allow maximum diffusion of gas/solute from water flowing in inter lamellar spaces (Hughes & Morgan, 1973).

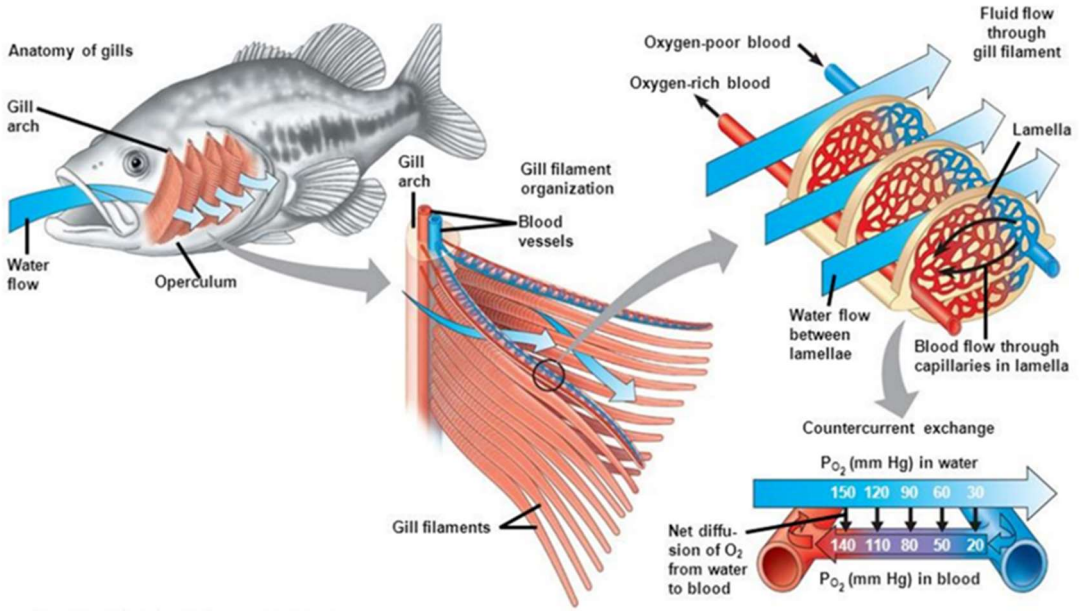


Figure 5. Schematic of multi scale architecture of fish gill demonstrating exchange of oxygen from ambient water to fish body (Kumar et al., 2018).

The movement of an anaesthetic compound through the gills is presumably regulated by factors such as branchial ventilation, perfusion and effective exchange area. At high temperatures, the increases of gill ventilation and the cardiac rate would enhance the permeability of the gill to an anaesthetic and in turn increasing the efficacy of the anaesthetic (Sladky *et al*., 2001). Once the anesthetic goes through the gills and is absorbed by the the gill epithelium, it enters the bloodstream and is distributed through the body reaching the fat and the brain; rapidly affecting the central nervous system (Ferreira *et al*., 1984; Summerfelt & Smith, 1990).

I.2.1.3. Anesthesic agents used in aquaculture

The choice of an anesthetic agent should take into account several factors: efficacy, availability, cost-effectiveness, ease of use and safety for fish, humans and the environment (Cho & Heath., 2000; Iversen *et al*., 2003; Mylonas *et al*., 2005). However, very little is known about anaesthesia in fish; the multiple species and different management protocols make it very difficult to found possible generalisations (Readman *et al*., 2013). There is an extensive list of anaesthetics commonly used in aquaculture industry (Palić *et al*., 2006; Small *et al*., 2003). The anesthetics can be divided into natural and synthetic agents. The synthetic agents are expected to produce more residues, while the natural agents are supposed to be more reliable and have a promising future (Purbosari *et al*., 2019). Some synthetic anesthetics usually applied in fish have been resuming in **Table 2**.

Table 2. List of non-natural anesthetics used in fish.

Anesthetic	Use	Concentration (mg/L)	Characteristics	References
Ether and quinaldine solutions	Fish anaesthetic	15-60	- May cause mild muscle contractions - Recovery usually rapid	Allen & Hunn, 1986.
Benzocaine	Fish anaesthetic	25-100	- Rapid induction - Good recovery time	Ross & Ross, 2008. PennState., 2010.
2-phenoxyethanol	Anesthetic in veterinary medicine and surgery as well as aquaculture	200-600	- Efficacy - Safety - Fast induction time - Short recovery time	Ghanawi *et al*., 2013.
Tricaine methane sulfonate (MS-222)	Fish anaesthetic	25-100	- The exposure times range from seconds or few minutes for high concentrations up to hours for low concentrations - Approved by the FDA* for use on food fish	Davidson *et al*., 2000. FDA, 2011. Topic Popovic et al., 2012.
AQUI-S®	Fish anesthetic in aquaculture, during handling events, and in live fish transport	10-20	- Effective at low concentrations - Approved by the FDA* for use on food fish	Davidson *et al*., 2000. FDA, 2011. Kolarevic & Terjesen, 2014.

* Food and Drug Administration (USA)

In the other hand, natural anesthetics are usually obtained from terrestrial plants. Among them, the most used anesthetics in the aquaculture industry are CEO and EU as CEO active compound (**Figure 6**). The CEO, an essential oil obtained from *Syzygium aromaticum*, is considered noncarcinogenic, non-mutagenic and "Generally recognized as safe" (GRAS) substance by the FDA (Nagababu & Lakshmaiah, 1992).

In addition, CEO contains bioactive compounds with antioxidant, antimicrobial, anti-stress and immune-stimulating properties (Bento *et al*., 2013; Concollato *et al*., 2016; Hernández-Sánchez *et al*., 2017; Li *et al*., 2016b; Teixeira *et al*., 2014; Wang *et al*., 2018). Such properties make CEO a very competitive product (Readman *et al*., 2013). CEO and EU are considered highly efficient to induce anesthesia and safer for human consumption, and the composition of CEO is mainly EU (70–90 %) (Dehghani *et al*., 2017; Li *et al*., 2016a). Other components of CEO are β-caryophyllene, α-humelene and eugenyl acetate (Li *et al*., 2016; Majeed *et al*., 2016), which may have synergistic effects with EU (Dehghani *et al*., 2017). Nowadays, CEO is used as an anaesthetic for many species of fish worldwide (Fujimoto *et al*., 2017; Taylor & Roberts, 1999) because it is highly effective even in low doses due to its high lipid solubility (Hunn & Allen, 1974; Javahery *et al*., 2012; Soltanian *et al*., 2018; Suprapto *et al*., 2017) and guarantees a product of good final quality for direct human consumption (Ribas *et al*., 2007).

As EU (2-methoxy-4-prop-2-enyl-phenol) is the primary active ingredient of CEO (70–90 %) and has been used as a general sedative for fish (Park *et al*., 2009). EU is mainly used in continental aquaculture as anaesthetic when harvesting, slaughter and transport of fish as it is very effective inducing anesthesia (Li *et al*., 2016c; Tarkhani *et al*., 2016). EU, as occurs with CEO, has anti-bacterial, anti-fungal, anti-parasitic and anti-helminthic properties (Li *et al*., 2016c). Interestingly, the risk of the presence of EU in fish flesh has been reported to be negligible (Ke *et al*., 2018).

Despite of their good qualities for fish anesthetics, both CEO and EU have a narrow safety limit for fish as slight differences in dose might result in fish death (Bodur *et al.,* 2018; Purbosari *et al*., 2019). In addition, relative long recovery time and adverse effect on fish flavour are clear disadvantages for using these natural oils for farming fish (Sutili *et al*., 2014).

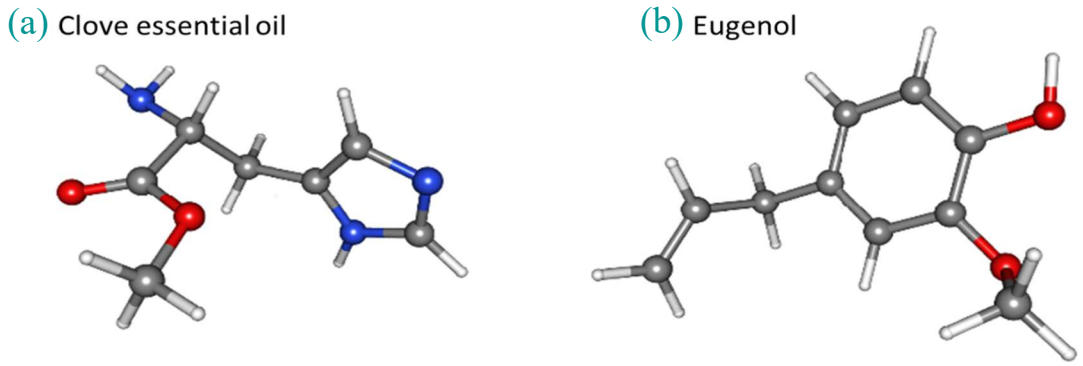


Figure 6. Interactive chemical structure of clove essential oil **(a)** and eugenol **(b)**. (National Center for Biotechnology Information. PubChem Database, 2019).

I.2.2. Cyclodextrins

The natural cyclodextrins (CD) are produced during the degradation of starch in the enantiomerically pure dextrorotatory state by the action of CD glycosyltransferase, an enzyme produced by several microbial organisms such as *Bacillus macerans*. They are widely used in foods, cosmetics, pharmaceuticals and agrochemicals due to their excellent ability to form molecular inclusion complexes with volatile or labile substances and to stabilize them. The CDs are industrially produced from enzymatically hydrolyzed starch and its production reaches more than 350.000 T per year (Okabe, 1993).

The CDs are formed by several glucopyranose units joined together with α (1 → 4) bonds. The amounts of glucose molecules involved in their structure are normally used to classify them into three groups. Those consisting of 6, 7 or 8 glucose units are called α-cyclodextrin (α-CD), β-cyclodextrin (β-CD) or γ-cyclodextrin (γ-CD), respectively (Kfoury *et al*., 2015; Okabe, 1993). The most stable three-dimensional configuration for these nonreducing cyclic oligosaccharide takes the form of a toroid (**Figure 7**) with the larger and smaller openings presenting secondary and primary hydroxyl groups, respectively, to the solvent environment. The interior of the CD molecule is a chiral, nonpolar, hydrophobic cavity that readily forms stable inclusion complexes with a wide range of chemical substances (Fravel, 2008).

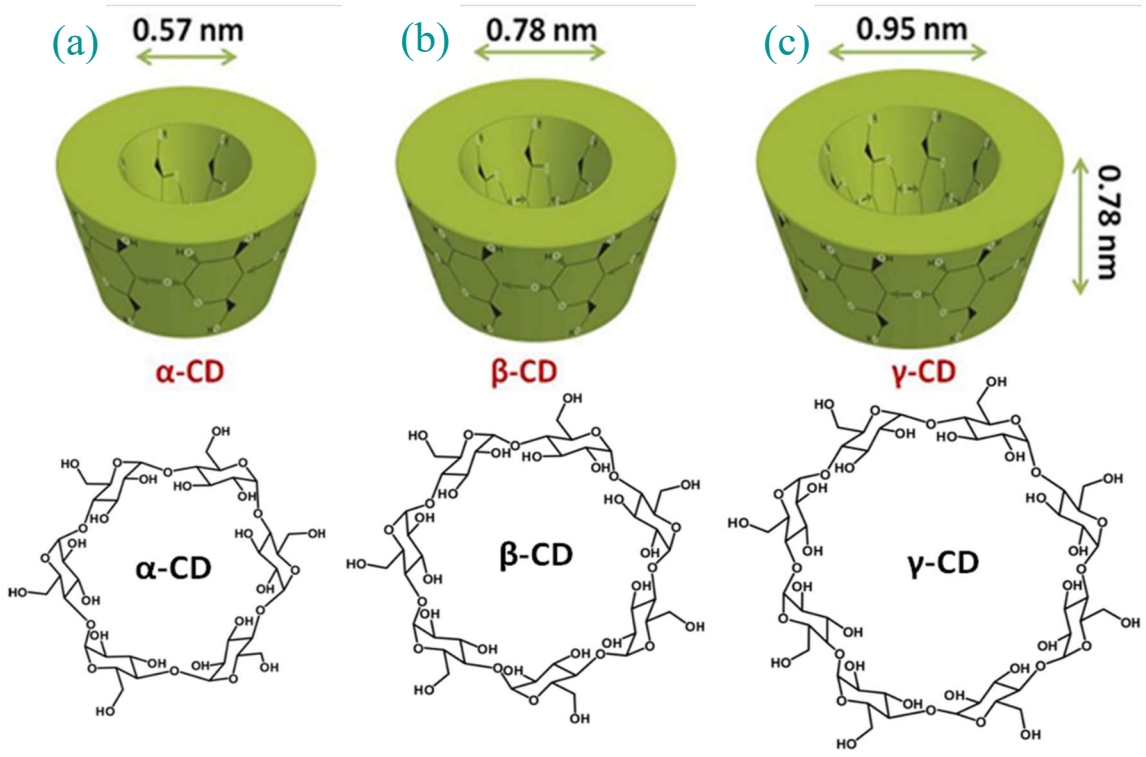


Figure 7. The molecular structure of different cyclodextrins (CDs): **(a)** α-CD, **(b)** β-CD, and **(c)** γ-CD (Celebioglu *et al*., 2017).

The α-CD has the smallest dimension **(Figure 7)** being most suitable for encapsulating apolar molecules of five or fewer carbons (Neoh *et al*., 2007). They act like nuclei and generate a favourable driving force for the final complexation at low temperature (Fleury *et al*., 2005).

The β-CDs form inclusion complex with a large variety of small molecules (Salgado *et al*., 2017). They have the lowest water solubility of all CD (Astray *et al*., 2009), due to the formation of rather stable intramolecular hydrogen bonds (Davis & Brewster, 2004; Miiller & Brauns, 1985). β-CD are the most accessible, the lowest-priced and generally the most used to prepare inclusion products in food and pharmaceutical science (Mele *et al*., 2002; Muñoz-Botella *et al*., 1995). They had received extensive attention in the pharmaceutical field to improve the aqueous solubility, chemical stability, dissolution and release rates of various drug molecules (Cabral-Marques, 1994; Saenger, 1980; Uekama *et al*., 1978). In addition, the complexation may also suppress the volatility and unpleasant odours or tastes of the chemicals and preserves them from oxidation, heat and light degradation, evaporation and moisture (Buschmann *et al*., 1991; Hernández-Sánchez *et al*., 2017; Munhuweyi *et al*., 2017).

The cavity size of γ-CDs is larger than that of α- and β-CDs **(Figure 7)**, allowing the complexion of large molecules (Rekharsky & Inoue, 1998).

To limit the degradation/loss of some components of essential oils during processing or storage conditions, and to control their delivery at the desired time and site, the encapsulation is considered beneficial prior to use in foods or beverages (Marques, 2010). The CD complexes with a wide variety of hydrophobic guest molecules (Del Valle, 2004; Welliver, 2007), including flavours, fragrances and essential oils (Celebioglu *et al*., 2017) are chemically produced by means of weak forces, such as van der Waals forces, dipole-dipole interactions, and hydrogen bonding (Hedges *et al*., 1995). The complexation of essential oils with CD increases their solubility and stability in the presence of light, high temperature and oxidizing conditions, and decreases their volatility (Del Valle, 2004). In aqueous solutions, the non-polar cavity of CD is occupied by water molecules. Thus, the main driving force of complex formation is the release of enthalpy-rich water molecules from this cavity. Water molecules are displaced by more hydrophobic guest molecules present in the solution to favour an apolar–apolar association and a decrease in cyclodextrin ring strain, resulting in a more stable lower energy state (Mazzobre *et al*., 2010) (**Figure 8**).

The hydrophilic outer surface structure and high molecular weight of CDs prevent them from penetrate into lipophilic membranes leaving biological membranes intact (**Figure 8**) (Jansook *et al*., 2018). Applications of CD in food, pharmaceutical, agricultural and chromatographic techniques are growing in importance as their toxicity is low and they are biodegradable (Wenz, 1994). In fact, their low price provides motivation for the discovery of new applications as could be the improvement of anaesthetics for fish (Del Valle, 2004; Welliver, 2007). The aim of the encapsulation in CD emerged as a potential tool to improve essential oils solubility, bio-availability and to avoid the adverse effect to flesh quality (Babaoglu *et al*., 2016; Kfoury *et al*., 2015).

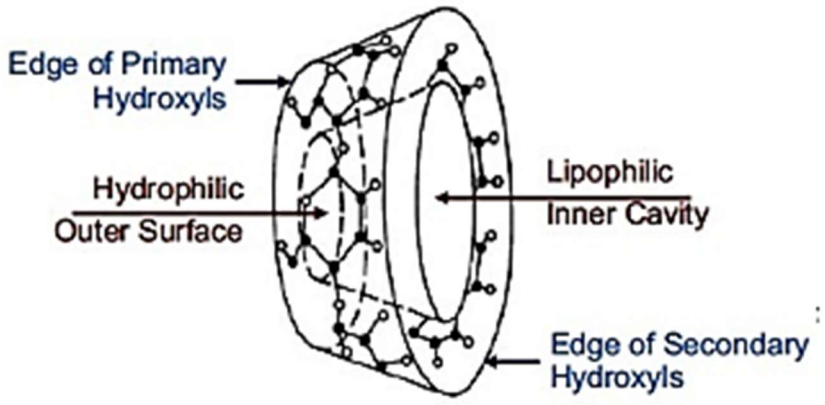


Figure 8. 3D structural formula of β-CD, showing the lipophilic inner cavity and the hydrophilic outer surface (Scarpignato, 2013).

I.3. Stress response in fish

Fish are stressed in aquaculture operations through handling, transportation, crowding and pre-slaughter procedures (Bagni *et al*., 2007; Ellsaesser & Clem., 1986; Guardiola *et al*., 2016; Iversen *et al*., 1998; Iwama *et al*., 1997; Ruane *et al*., 1999; Stevens *et al*., 2017; Vijayan *et al*., 1997). At the same time, fish possess a set of physiological strategies, allowing them to respond to stressors (Chreck & Tort., 2016; Naour *et al*., 2017). The stress conditions in fish can be detected at three different levels:

I.3.1. First level: neuroendocrine response

The primary stress response involves the neuroendocrine responses: **(i)** the release of catecholamines from chromaffin tissue (Cowan *et al*., 2017; Kim & Kang, 2016; Reid *et al*., 1998), and **(ii)** the stimulation of the hypothalamic-pituitary-interrenal (HPI) axis (Chreck & Tort., 2016; De Mercado *et al*., 2018; Mommsen *et al*., 1999; Vijayan *et al*., 1997).

Firstly, the release of catecholamines (adrenaline and noradrenaline and their precursor dopamine) from chromaffin tissue immediately occurs upon a stress stimulus (Chreck & Tort., 2016; De Mercado *et al*., 2018; Mommsen *et al*., 1999; Vijayan *et al*., 1997). The chromaffin tissue (the adrenal medulla homologue in fish) is located in the anterior region of the kidney in fish (Barton, 2002; Pankhurst., 2011; Pickering, 1993; Reid *et al*., 1998; Sampaio & Freire., 2016). The increase in the circulating levels of catecholamine hormones, subsequently modifies the cardiovascular and respiratory functions (Martínez-Porchas *et al*., 2009).

The activation of the HPI axis takes more time upon a stressor (Cowan *et al*., 2017; Kim & Kang, 2016). A stress condition stimulates the release of corticotrophin-releasing hormone (CRH) from the hypothalamus in the brain and induce the secretion of adrenocroticotropin (ACTH) in the corticotropes cells of the pituitary which in turn stimulates the synthesis and release of corticosteroid hormones by the interrenal tissue located in the anterior kidney of fish (Joseph *et al*., 2015; Thang *et al*., 2017). The cortisol is the main corticosteroid hormone in fish (Barton *et al*., 1998). The secretion of cortisol is slower than catecholamines, but its effects are more prolonged as cortisol concentrations only return to pre-stress levels within hours of exposure to an acute stressor (Pickering, 1993).

The cortisol levels also are elevated when a continuous stress condition (chronic stress) occurs (Braithwaite & Ebbesson, 2014). Thus, the elevation of the cortisol levels in plasma of fish is used as an indicator of the stress level of the fish (Barcellos *et al*., 1999 reviewed in Barton, 2002; Hossain *et al*., 2017; Kim *et al*., 2017; Pickering., 1993).

I.3.2. Second level: biochemical and physiological response

Secondary responses are the metabolic effects of the hormones released by the neuro-endocrine system during the primary response (Barton & Iwama, 1991). During stressful situations, some biochemical and physiological effects comprise metabolic changes (increased glucose and lactate in blood), osmoregulatory disturbance, changes in hematological features, cellular changes and changes in the immune response (Barnes, 2002).

The cathecolamines mediates the changes related to the diffusion and concentration of oxygen in blood. Thus, the ventilatory rates and the capacity of blood to transport oxygen is modify upon stress. These effects are orchestrated by two types of receptors that bind catecholamines: α- and β- adrenergic receptors (AR), (Bernier *et al*., 2009). Hypoventilatory responses appear to be mediated via α-AR (Peyraud-Waitzenegger, 1979). However, the compensatory biochemical and physiological mechanisms to alleviate the disruptive effects of stress on blood oxygen transport (Perry & Reid, 1993), is mediated via β-AR (Peyraud-Waitzenegger, 1979).

Subsequently, an increase in plasma cortisol levels, is generally followed by changes in the blood and tissue chemistry (Inoue *et al*., 2008), in the hydromineral and metabolic balance (Arends *et al*., 1999), in several hematological features, and in the heat-shock or stress proteins (HSP) levels (Mommsen *et al*. 1999). These biological effects of cortisol are triggered through glucocorticoid and mineralocorticoid receptors (Bury & Sturm 2007; Escriva *et al*., 2004).

Regarding the blood chemistry, two indicators of stress are the most commonly used, the increases in glucose and lactate plasma levels.

The three main hormones released upon stress (adrenaline, noradrenaline and cortisol) are involved in the increase of the glucose levels in blood (Barton, 2002; Reid *et al*., 1998; Sampaio & Freire., 2016).

Adrenaline and noradrenaline are involved in the immediate production of glucose after stress (glucogenesis), whereas cortisol, either directly and/or indirectly, is important for the long-term regulation of glucose in fish (Goldstein *et al*., 1992; Iwama *et al*. 1999) by means of altering the gluconeogenesis (the formation of glucose) and glycogenolysis (the breakdown of the molecule glycogen into glucose) pathways (Iwama *et al*.1995). Cortisol stimulates the gluconeogenesis in liver leading to an increase of glucose in blood (Wedemeyer *et al*. 1990), but also in other tissues such as brain, gills, erythrocytes, and gonads (Vijayan *et al*., 1997). This way, the increases in the glucose production lead to have enough energy through glycolysis (the production of energy stores in form of ATP from glucose) according with the demand of fish in stress situations (Brownscombe *et al*., 2016; Martínez-Porchas *et al*., 2009) (**Figure 9**).

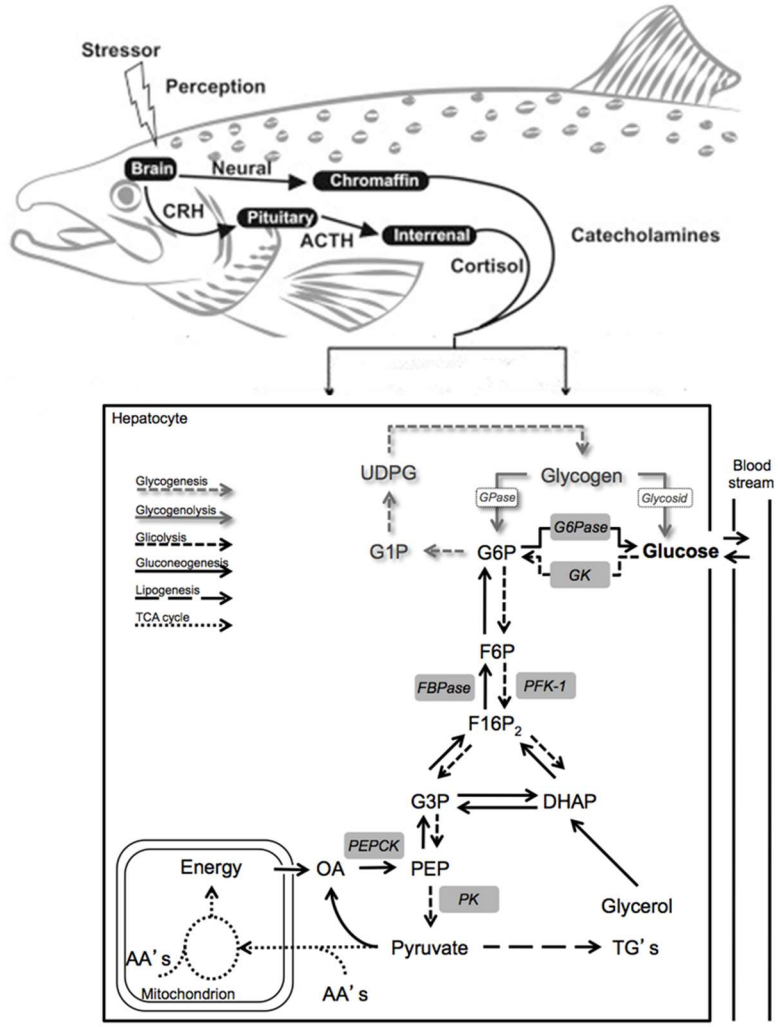


Figure 9. The secondary stress response in fish. Briefly view of the dynamics of cortisol and catecholamine in the production of glucose. (Modifed from Martinez-Porchas *et al*., 2009; Schreck *et al*., 2016).

When lactate is high in blood is due to a shift to an anaerobic glycolysis (the enzymatic breakdown of glucose by way of production of lactic acid and energy stored in high-energy phosphate bonds of ATP) that normally occurs when hypoxia takes place and high energy mobilization and utilization is required (Barton & Iwama, 1991; Grutter & Pankhurst, 2000; Lim & Hur., 2018; Sumpter, 1997; Wood *et al*.,1983). Lactate generation require that glucose is degraded until ATP and pyruvate which is transformed by the lactate dehydrogenase (LHD) in lactate. Any condition raising the concentration of pyruvate further enhances lactate production without altering the kinetic rates between substrates (Grutter & Pankhurst, 2000). All tissues produce lactate, but skeletal muscle, because of its large mass, has the greatest potential (Grutter & Pankhurst, 2000; Lim & Hur., 2018; Sumpter, 1997). The lactate has to be excreted, but it could not occur via the gills, so the swimming muscles of fish contain an unusual form of alcohol dehydrogenase (ADH) that can convert the lactic acid through pyruvate production into ethanol and CO_2. Both molecules are easily transported via the blood stream to the gills and then excreted. (Gleeson, 1996) (**Figure 10**).

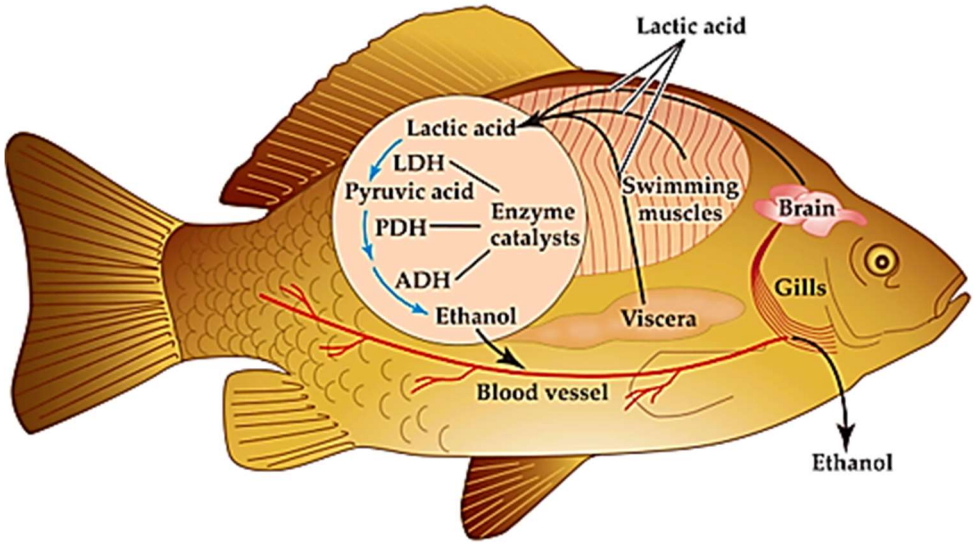


Figure 10. The production of lactic acid via anaerobic glycolysis in skeletal muscle of fish (http://www.bio.miami.edu/dana/360/360F16_8print.html).

These physiological indicators (glucose and lactate) are measured in plasma as they provide a snapshot of the metabolims (Barton, 2002; Pottinger, 2008). The benefit of sampling plasma for measuring physiological parameters is that the reference values for several species had been studied and there are commercially available assays to facilitate data generation (Beecham *et al*., 2006; Wells & Pankhurst, 1999).

In fish, middle or long periods of stress make ATP content in the muscle decreases to below a critical level, and the bond between actin and myosin becomes irreversible. At the same time, the use of high amounts of ATP and the production of energy decreases the pH of the muscle due to the generation of H^+ ions (produced when ATP is hydrolyzed) and lactic acid (Gasco *et al*., 2014; Lyu *et al*., 2015). This way, muscle lactate and H^+ ions are released to the bloodstream (Wood, 1991) decreasing the pH (Hochachka & Mommsen, 1983; Wood, 1991). Moreover, the excretion of lactate (Hostetler *et al*. 1969) leads ultimately to the formation of CO_2 (Jorfeldt, 1970). High concentrations of CO_2 limit the capacity of fish blood to carry O_2 by lowering blood pH (Wurts & Durborow, 1992) and increasing the amount of HCO_3 in blood (Heming *et al*., 1987).

Regarding the heat-shock or stress proteins (HSPs), they are known to be activated upon stress stimuli such as temperature changes (Barton, 2002; Brownscombe *et al*., 2016; Jiang *et al*., 2016; Sampaio & Freire, 2016). These proteins are sensitive biomarkers of changes in homeostasis mainly in liver and kidney tissues (Bly *et al*., 1997; George et al., 1999; Kim & Kang, 2016). Specifically, the Hsp70 protein family consist of both inducible and constitutive forms, which help in stress tolerance by increasing the chaperon activity in the cytoplasm (Lindquist & Craig, 1998). They are considered as molecular chaperons for their function in protecting the cellular compartment from thermal stress (Galt et al., 2018; Sørensen *et al*., 2003). The assistance of Hsp70 proteins is further divided into 3 activities including: **(i)** supporting proteins folding to native state, **(ii)** avoidance of aggregation of proteins and **(iii)** solubilisation and refolding of aggregated proteins (Cheng *et al*., 2017). (**Figure 11**).

Regarding the effect of Hsp70 proteins in the cell membrane, they help proteins to move across membranes by using ATP binding and hydrolysis and improve the overall integrity of cellular proteins (Mishra & Palai, 2014; Yokota & Fujii, 2010).

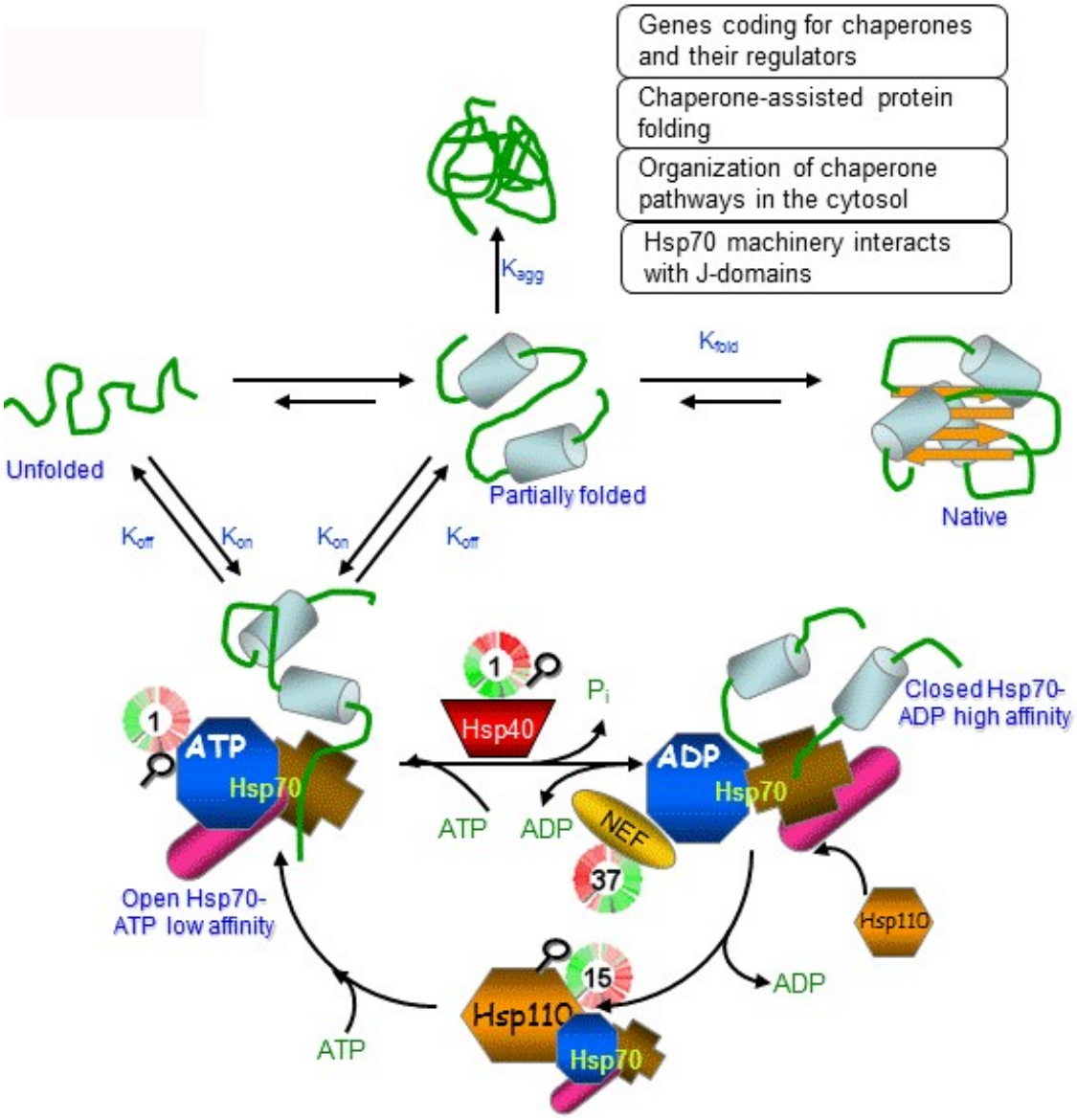


Figure 11. The Hsp70 chaperone cycle (Adapted from Hartl *et al*., 2011).

I.3.3. Third level: change in the whole animal

The prolonged activation of the stress response is regarded as the tertiary response level (Barton *et al*., 1987; De Mercado *et al*., 2018). Thus, when fish are under chronic stress situations, such as conditions of intensive culture (harvesting, stunning, slaughtering) (Hultmann *et al*., 2016; Refaey *et al*., 2017; Tulli *et al*., 2015), it results on a decrease in disease resistance, impaired reproduction, a reduced growth and changes in swimming capacity and behaviour of fish and finally impairs survival (Barton *et al*., 2002).

I.4. Immune system of fish

The immune system can be defined as a complex system that protects the organism against organisms or substances that might cause infection or disease (Flajnik & Pasquier, 2008). The immune system of fish is based on humoral and cellular responses, both innate and adaptive (Dixon & Stet., 2001; Rubio-Godoy, 2010). Innate response generally precedes the adaptive one and also plays a fundamental role in the organization of the acquired immune response and the maintenance of homeostasis (Fearon, 1997) (**Figure 12**). The innate immune system is characterized by protect an organism against infection without any prior exposure to the pathogen, acting as the first line of defence against foreign agents until the specific response is activated. The specific (adaptive or acquired) immune system, however, is activated by the innate response (Rauta *et al*., 2012), and specifically responds to an invading pathogen and upon repeated exposure of it (Rauta *et al*., 2012; Secombes & Ellis., 2012). This response is mediated by humoral factors (antibodies) and T-cell antigen receptor (TCR) specificities that are clonally expressed on B and T lymphocytes (Holland & Lambris 2002; Matsunaga *et al*., 1998; Rauta *et al*., 2012; Secombes & Ellis, 2012).

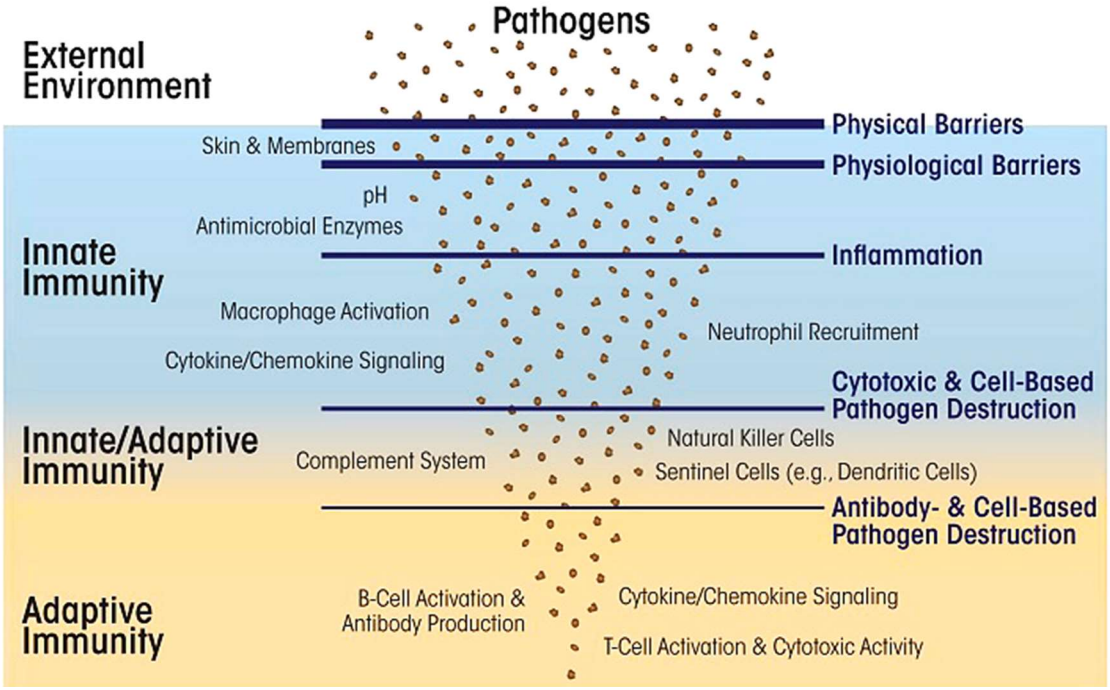


Figure 12. Overview of the immune system in fish (Spiering, 2015).

I.4.1. Innate immune system of fish

Innate (also known as natural or non-specific) immune system recognizes regions in molecules called pathogen associated molecular patterns (PAMPs) from infectious agents or microorganisms of normal microbiota, such as lipopolysaccharide, peptidoglycan, bacterial DNA or viral RNA, or other molecules found in multicellular organism's membranes known as "non-self". The PAMPs are usually highly preserved portions of the molecules during the evolution of species and are found in the greater part of microorganisms. The innate immune system has a primary importance in vertebrates, especially in fish (Ellis, 2001; Uribe *et al*., 2011). It is characterized by being the first to send the defend signals to protect the host from infection and include a rich and powerful array of mechanisms, that appear to be more potent in fish than in higher vertebrates (Tort *et al*., 2004). Innate immunity in fish is divided into physical barriers, cellular and humoral components (Dixon & Stet, 2001; Magnadóttir, 2006; Uribe *et al*., 2011; Valenzuela *et al*., 2017).

The main tissues related to the immune system in fish are the head kidney (the cephalic portion of the kidney), the thymus, the spleen and a diffuse mucosa-associated lymphoid tissue (MALT) located in several organs such as skin, gut, nose and gills (Lin *et al*., 2005; Salinas *et al*., 2015; Zapata *et al*., 1996). Regarding the head kidney, is the main myelopoietic organ in fish, although it also possesses lymphopoietic *foci* (Lin, *et al.*, 2005). Thus, the head kidney displays lymphoid functions as primary lymphoid organ (where lymphocyte maturation occurs) or even as secondary lymphoid organ (where mature lymphocytes establish contact with antigens) since some cells containing bacterial antigen have been observed in this tissue upon bacterial infection in some species (Chaves-Pozo, *et al.*, 2005a). Moreover, data related to dendritic cells (DCs) and DCs markers support the role of head kidney as secondary lymphoid tissue in fish (Zoccola, *et al.*, 2015). However, the thymus, located near the gills is the primary source of mature T lymphocytes, being the spleen (the main secondary organ in fish), located in the peritoneal cavity, composed by T and B lymphocytes (Zapata, *et al.*, 1996). In addition, the MALT is also part of the secondary lymphoid tissue (Salinas, 2015).

I.4.1.1. Physical barriers

Fish are constantly immersed in an aquatic environment and as a result are continuously exposed to potential pathogens or other harmful agents. The first line of defense in fish innate immune system are physical barriers that prevent the entry of pathogens, which includes the fish scales, the epithelial cells, which line the skin, gills, and the alimentary tract and the mucus that wrap these epithelia (Magnadottir, 2006 2010).

Regarding the skin, this tissue is characterized as a mucosal tissue in fish (Parra *et al*., 2015). Fish mucus contains a wide range of immune substances, in particular, lectins, pentraxins, lysozyme, peroxidase, proteases and antiproteases complement proteins, antibacterial peptides and IgM, that inhibit pathogen entry or induce pathogen elimination (Firdaus-Nawi & Zamri-Saad, 2016; Fischer *et al*., 2006; Saurabh & Sahoo, 2008; Valenzuela *et al*., 2017).

I.4.1.2. The cells of the innate system

Cellular response is defined by the physical barrier and specialized cells, which are capable of killing and digesting pathogens if the latter breaches the physical barriers (Aoki *et al*., 2008; Frøystad *et al*., 1998).

The cells of the innate immune system of teleost fish are phagocytic cells and natural cytotoxic cells (NNCs). The phagocytic cells of the innate system of fish (granulocytes and mononuclear phagocytes), recognize and eliminate invading organisms and other molecules from damaged tissues (Magnadottir, 2010; Secombes & Fletcher, 1992). Granulocytes destroy any potential pathogens and also recruit other immune cells to the site of infection (Matsuyama & Iida, 1999). However, macrophages protect the host by phagocytizing foreign material, being an important part of the cellular immune system of fish (Weeks & Warinner, 1984).

The NCCs in fish are an array of cells including monocyte-macrophages, granulocytes, platelets, mammalian natural killer NK cells and/or lymphocytes which have the ability to kill tumour cells, xenogeneic cells, virus-infected cells and parasites (Cuesta*, et al.*, 1999; Ellis, 2001; Fischer*, et al.*, 2006; Manning, 1998; Meseguer*, et al.*, 1996; Secombes, 1996). These cells are the functional equivalent of mammalian-like NK cells (Evans *et al*., 1984; Evans & Jaso-Friedmann, 1992). They are commonly found in lymphoid tissues, such as the anterior kidney and spleen, but are rarely found in the blood

(Graves *et al*., 1984; Shen *et al*., 2002; Xu *et al*., 2013). The NCCs also provide an essential cytokine response during bacterial infections in teleost fish (Aoki *et al*., 2008; Evans & Cooper, 1990; Evans & Jaso-Friedmann 1992, 1993; Frøystad *et al*., 1998).

I.4.1.3. Humoral factors

The humoral response in teleost fish includes a large number of non-specific defense substances, such as growth inhibitors, various lytic enzymes and components of the complement pathways, agglutinins and precipitins (primarily lectins), natural antibodies, cytokines, chemokines and antibacterial peptides (Magnadóttir, 2006).

These substances are predominantly proteins or glycoproteins and non-specifically inhibit the growth of infectious microorganisms (Yano, 1996). These humoral plasma levels are considered good indicators when determining the immune response of fish that could be exposed to stressful conditions (Sunyer *et al*., 1995).

I.4.1.3.1. Lytic enzymes

Peroxidase is an important enzyme that uses hydrogen peroxide and produces ions to form chlorides and chloramines that are also highly toxic and important microbicidal agents in an immune defence pathway known as respiratory burst (Whyte, 2007). Peroxidase is present in serum but also in mucus where is essential for mucosal immunity and skin defense (Guardiola *et al*., 2014).

Proteases are a group of proteins responsible for the hydrolysis of peptide bonds (Streit *et al*., 1995) that are often responsible for degrading pathogens and other foreign substances (Ingram, 1980; Braun *et al*., 1990). In addition, they also function indirectly by activating and enhancing the production of various immunological components such as complement, immunoglobulin and antimicrobial peptides (Cho *et al*., 2002). They are classified based on the chemical nature of the groups responsible for catalysis: serine, cysteine, aspartic and metalloproteases (Hartley, 1960). When proteases are present in plasma and mucus, play an important role in immune system of fish, where antiprotease can inhibit proteases (Streit *et al*., 1995). Antiproteases play a crucial role in inhibiting the toxic proteases produced by pathogens (Alexander & Ingram, 1992). There are two basic functional classes of protease inhibitors: **(i)** the active site inhibitors, which bind to the active

site of the target protease and inactivate its ability to hydrolyze all substrates and **(ii)** the α2-macroglobulins, which operate by the unique process of molecular entrapment (Barrett & Starkey, 1973). Several protease inhibitors are present in the serum and other body fluids of fish to maintain body fluid homeostasis (Bowden *et al*., 1997). This way, a correct balance between protease and antiprotease activities is needed to preserve the correct functionality of any system (Streit *et al*., 1995).

I.4.1.3.2. Antibacterial peptides

Fish secrete different kinds of antimicrobial peptides (AMPs), which are major components of certain phagocytic cells, especially granulocytes and macrophages and they are used to defence against many pathogens, such as bacteria, virus, fungi, protozoa, and even tumor cells (Elsbach & Weiss, 1988; Mihajlovic & Lazaridis, 2010; Rajanbabu & Chen., 2011; Silphaduang & Noga, 2001). Due to the low molecular weight and anionic characteristics of AMPs, they directly interact with the pathogens leading them to membrane disruption and a quick death (Patrzykat & Douglas, 2003).

Furthermore, AMPs are involved in inflammation and also in the regulation of the innate immune system (Cuesta *et al*.,2008).

I.4.1.3.3. Complement system

Complement system is composed by 35 individual proteins and it is an important component of the innate immune system due to its function in the organism defense, such as cellular activation, phagocytosis, chemotaxis, inflammatory reaction and lysis of foreign cells and pathogens (Nakao et al., 1997; Sunyer *et al*., 2003).

Activation of the complement system of teleost fish can be achieved through all three pathways: alternative pathway (C3 and factors B and D), classical pathway (C1, C4, C2 and C3, where C1 primarily recognizes antigen bound antibodies and activates C4 and C2 in this order) and lectin pathway (C1 replaced by a complex of the mannan-binding lectin (MBL) and MBL-associated serine proteases (MASPs)) (Matsushita *et al*., 2000; Sunyer *et al*., 2003).

I.4.1.3.4. Natural antibodies

Natural antibodies are produced without any apparent specific antigen stimulation and they could be found in the serum of healthiest vertebrates (Boes, 2000). Natural antibodies in fish play an important role in their innate/acquired immune defence, mainly the defence against bacterial and viral infections and the recognition of foreign tissue and substances (Gonzalez *et al*., 1989; Michael, 1969).

I.4.1.3.5. Lectins

In fish, lectins are proteins that binds carbohydrates in a specific way and induce their agglutination and induce the opsonization, phagocytosis and activation of the complement system (Drickamer & Taylor, 1993; Sharon & Lis, 1989).

I.4.1.3.6. Lysozymes

Fish lysozyme is widely distributed through the body and has an important role in the defence against Gram-positive and Gram–negative bacteria as hydrolyses the peptidoglycan of bacteria cell walls (Saurabh & Sahoo, 2008). In addition, lysozymes promote phagocytosis by directly activating polymorphonuclear leucocytes and macrophages or indirectly acting as an opsonin or activating the complement system (Magnadottir, 2006; Saurabh & Sahoo, 2008).

I.4.1.3.7. Cytokines

Cytokines are secreted proteins with growth, differentiation and activation functions that regulate the nature of immune responses. Through multiple signalling pathways of immune-related genes, cytokines are able to induce the immune response. Cytokines are produced by macrophages, lymphocytes, granulocytes, DCs, mast cells, and epithelial cells. Cytokines have multiple overlapping and sometimes contradictory functions. Although their classification is sometimes difficult to establish, based on their functions, we can distinguish pro- and anti-inflammatory or innate and adaptive immunity-related cytokines (Aoki *et al*., 2008; Salazar-Mather & Hokeness, 2006; Savan & Sakai, 2006; Wang *et al*., 2011). The most typical pro-inflammatory cytokines present in fish are interleukin il-1β and tumor necrosis factor (TNF). The il-1 is the common name for a family of proteins known to have multiple

functions in mammals, but in general induces a cascade of reactions that leads to inflammation (Dinarello, 1997). Different members of this protein family have been clone in teleost suggested that these molecules are evolved for at least 350 million years (Scapigliati *et al*., 2004). The il-1β is produced by a wide range of cell types after activation of host pattern recognition receptors (PRRs) by PAMPs. The il-1β is synthesised as a precursor lacking a signal peptide and is required to be processed to release active protein. The il-1β precursor can be cleaved either intracellularly or extracellularly (Afonina *et al*., 2015; Vojtech *et al*., 2012). The biological activity of il-1β has been studied in several fish species, such as gilthead seabream (Pelegrin *et al*., 2001), Nile tilapia (Lee *et al*., 2006), European seabass (Scapigliati *et al*., 2001), and rainbow trout (Zou *et al*., 1999) and as occurs in mammals, triggered the molecular pathways of inflammation (Dinarello, 1997; Lu *et al*., 2008; Secombes *et al*., 2011). The il-1β receptor complex comprises a ligand specific chain (il-1R1 or il-1R2) and a common chain shared by different members of the il-1 family (Palomo *et al*., 2015; van der Meer *et al*., 1988). The binding of il-1β to its receptor triggers complex intracellular pathways resulting in the activation of new genes or in the modification of protein (Scapigliati *et al*., 2004) including the production enhance and secretion of itself (Dinarello, 2009).

The anti-inflammatory cytokines are known to regulate cell development, proliferation, differentiation, migration, and survival in various leukocyte lineages including lymphocytes, dendritic cells, NK cells, macrophages and granulocytes (Dhandapani & Brann 2003; Li & Flavell, 2008). This way, it affects cell growth and differentiation, extracellular matrix regulation, wound healing and immune function (Basile *et al*., 1998; Wan & Flavell, 2007). Major anti-inflammatory cytokines include several interleukins such as il-4, il-10, il-11 and il-13, the interferon-alpha (IFN-α) and the transforming growth factor-β (tgf-β). These molecules are characterized by inhibiting the release of pro-inflammatory cytokines, inducing the production and release of IL-1 receptor antagonist (IL-1ra) and soluble TNF receptor (sTNFR) and limiting some of the proinflammatory activities of il-1 and TNF. Focusing on the tgf-β, in the mammalian immune system, the dominant role of this cytokine is to maintain immune tolerance and suppress autoimmunity (Harms *et al*., 1999; Saxena *et al*. 2008; Zuckerman *et al*., 2001). High levels of tgf-β have been found in several disease states associated with immunosuppression in mammals (Harms *et al*., 1999). In fish, high level of this cytokines are being recorded during the latest

stage of infection (Tafalla *et al*., 2005). Although, little studies had been performed in fish about the functional role of tgf-β, tgf-β1 are recently reported to exerts powerful immune depressing effects on activated leukocytes, as it does in mammals (Kadowaki *et al*., 2008; Yang *et al*., 2008).

I.5. The effect of stress response on the immune system efficacy

The immune system is usually influenced by a wide range of acute or chronic stress factors in fish (Costas *et al*., 2011; Tort, 2011; Yarahmadi *et al*., 2016). Regarding the MALT, the stressing agents may activate the local receptors and generate a first response in a particular tissue, mostly in the portals of entry, the mucosal tissues (Parra *et al*., 2015). These tissues produce locally several mediators, including hormones and other peptides such as cytokines that create a tightly connexion between the immune and the neuroendocrine systems (Khansari *et al*., 2017; Makrinos & Bowden, 2016; Rodríguez-Quiroga *et al*., 2017 Wunderink *et al*., 2011; Yada & Tort, 2016), triggering subsequently the activation of the brain pituitary-head kidney axis and in turn a global neuroendocrine stress response (**Figure 13**). Some effects of stress, mostly due to cortisol release, on immune responses described in fish are: increase of neutrophils, reduction of lymphocyte number and antibody responses, reduction of complement activity, and decrease in the production of some cytokines (Parra *et al*., 2015). The lysozime activity is altered upon a stress situation depending on the type of stressors, intensity and its duration (Yildiz, 2006).

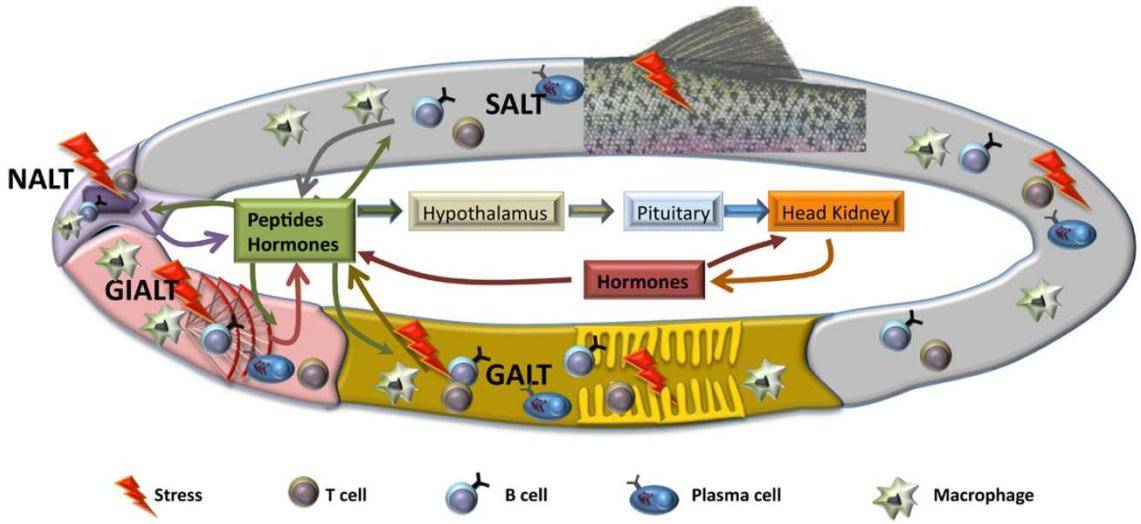


Figure 13. Mucosal responses to stress in fish. SALT, skin-associated lymph tissue; GALT, gut-associated lymph tissue; NALT, nose-associated lymph tissue; GIALT, gill-associated lymph tissue (Parra *et al*., 2015).

Thus, increase of cortisol is related with an inhibition of the release of IL1-β during the resolution of inflammation (Castillo et al. 2009; De Mercado et al., 2018; Elenkov & Chrousos, 1999; Mizock, 2009; Tort, 2011) and decreases in the production of TGF-β (Chen, 2003).

Other stress effect on the immune system that might impair the competence (Pickering & Pottinger, 1989; Tort *et al*., 2004) are inhibition of lysozyme, complement proteins, or antimicrobial peptides coding genes in liver, while the gene expression levels of proinflamatory cytokines are up-regulated in head-kidney and spleen (Teles *et al*., 2013).

I.6. The effect of stress response on muscle physiology

During stress situations, there is a loss of water and lipids contains by the fish muscle (Jørpeland *et al*., 2015). It is generally thought that abnormally rapid post-mortem skeletal muscle metabolism causes pale, soft and exudative meats (Strasburg & Pale, 2009). Often leads to nucleotide breakdown, muscle pH falls (Lefevre *et al*., 2016) and changes in skin (Cassol *et al*., 2015; Erikson *et al*., 2017). The condition is associated with an excess of calcium ions (Ca^{2+}) in the cytosol (Oda *et al*., 2009). Contraction in the muscle is due to neurostimulation through the motor endplate which triggeres the release of calcium from the sarcoplasmic reticulum to the sarcoplasm of the muscular cell. The physiological consequences of Ca^{2+} level increase prior to slaughter include a rise in muscle hypermetabolism which leads to post-mortem warming and acidosis in the muscle tissue (Droval *et al*., 2012).

The ryanodine receptors (RyR) are large homotetrametric protein complexes (Darbandi & Franck, 2009) that play an essential role in most cell types including neurons, muscle cells, and epithelial cells. Mediating the release of calcium (Ca^{+2}) from the endoplasmic and sarcoplasmic reticulum into the cytosol. Thus RyR convert different extracellular stimuli into intracellular calcium signals (Palade *et al*., 1989) **(Figure 14).**

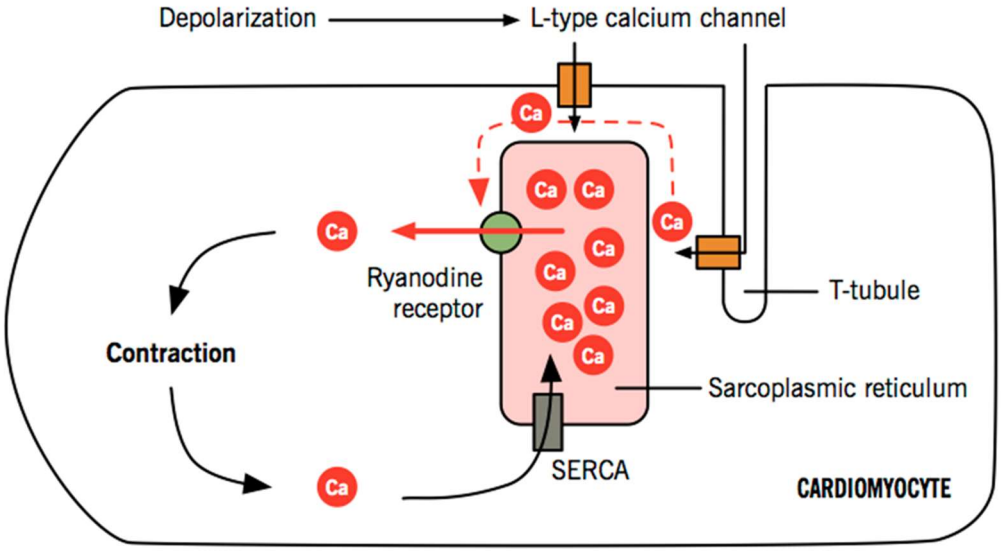
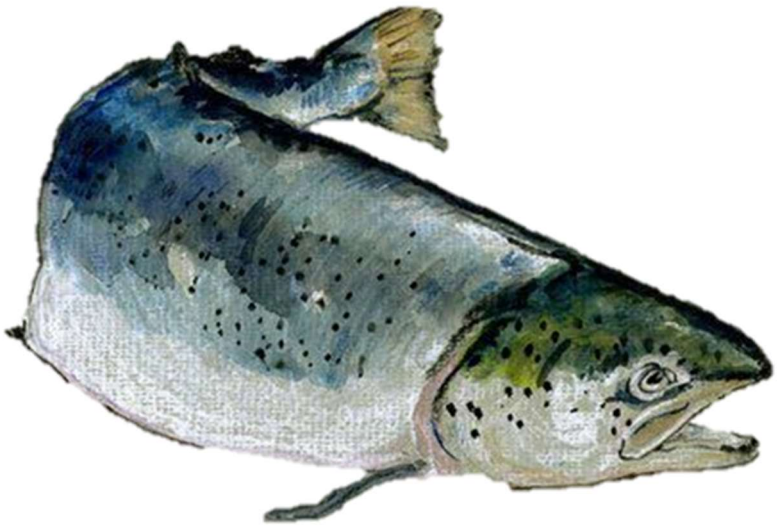


Figure 14. Receptor ryanodine (RyR) induces calcium release in skeletal muscle of fish (Ikonnikov & Wong, 2013).

Three homologous isoforms of RyR were identified in non-mammalian tissues based on molecular, immunological, biochemical, and physiological results (Airey *et al*., 1993). Adult mammals predominantly express RyR1 isoform in the skeletal muscle. While non-mammalians including fish express in their skeletal muscles the two isoforms, RyR1 and RyR3 (Franck *et al*., 1998). The RyR2 isoform is expressed primarily in the cardiac tissue of teleost fish (Darbandi & Franck, 2009). The two RyR isoforms (1 and 3) are encoded by different genes (Strasburg &Pale, 2009) and are present in similar quantities in the muscle of fish (Airey *et al*., 1993). The RyR3 is the least studied ryanodine receptor, and consequently little is known about its function (Supnet *et al*. 2009), but it is possible that the RyR3 channels may be activated by the increase in Ca^{2+} concentration provided by the initial opening of the RyR1. The lower sensitivity of RyR3 channels to inactivation at high Ca^{2+} concentration may be relevant to maintain a sustained Ca^{2+} release after deactivation of RyR1 (Schneider, 1994). These properties may comply with RyR3 contributing to the Ca^{2+} release from the skeletal sarcoplasmic reticulum (Bertocchini *et al*., 1997; Schneider, 1994).

The pre-slaughter stress induces a vigorous usage of the skeletal muscle leading to anaerobic metabolism and a decrease on muscle pH and increasing in Ca^{2+} release that in turn produce acidosis in the muscle and a heavy loss of quality in the fish flesh (Poli *et al*., 2005). Recent results obtained in Nile tilapia at pre-slaughter conditions, provided clear evidence that the stress and the RyR3 coding gene expression are inversely correlated (Goes *et al*., 2015).

II.Objectives



The main objective of this thesis is to improve the welfare at slaughtering time, at experimental and industrial farm conditions, of five fish species of high economic value for European aquaculture sector, and with different rearing characteristics (gilthead seabream, Atlantic salmon, Nile tilapia, European seabass and rainbow trout) by using clove essential oil nanoencapsulated in β-cyclodextrins as anaesthetic at the lowest but effective dose. For that the following specific objectives are proposed:

1. To determine the time needed for reaching two anesthesia-induction stages as the loss of equilibrium and the loss of reflex activity.
2. To analyze the level of some plasmatic parameters considered as stress markers such as glucose, lactate and cortisol as well as other plasmatic parameters related to metabolic unbalance upon stress (pO_2, pCO_2, HCO_3, TCO_2, SO_2, base excess and pH).
3. To study the mRNA levels of some inflammatory related genes in head kidney or intracellular Ca^{2+} trafficking in skeletal muscle.
4. To analyze the effect on the innate immune system, through the analysis of humoral activities (peroxidase, bactericide, antiprotease and protease) and the total level of proteins in plasma and skin mucus.

III.Material & methods

III.1. Essential oils inclusion complex preparation

The CEO+β-CD inclusion complex powder was prepared following the kneading method (Marques, 2010). The ice crystals containing CEO+β-CD were manufactured by adding and dissolving the CEO+β-CD powder in running water before freezing in a commercial crushed ice (CI) machine (Scotsman, Milan, Italy), or in a commercial liquid ice (LI) machine (Mejisa, Alicante, Spain) (**Figure 14a**). Moreover, EU+β-CD inclusion complex powder was prepared following the same methodology described before (**Figure 14b**). The different CEO+β-CD or EU+β-CD doses are expressed in mg/kg (mg of CEO or EU per kg of ice crystals) or in mg/L (mg of CEO or EU per L of SW or FW), depending on the treatment.

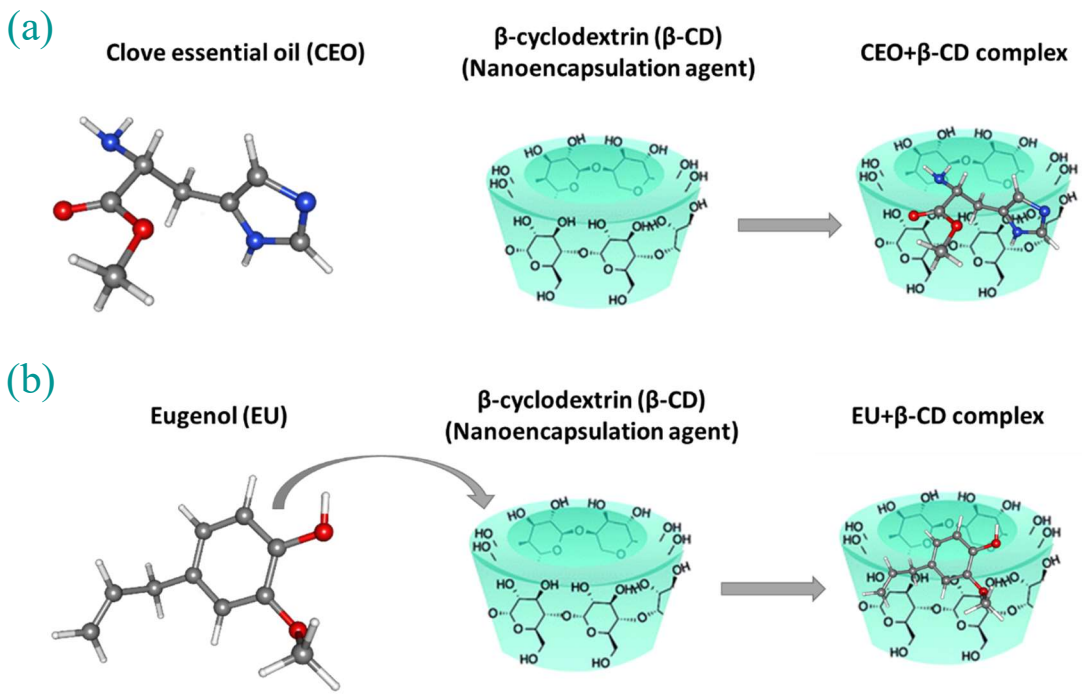


Figure 14. Inclusion complex formation of CEO **(a)** or EU **(b)** nanoencapsulated in β-cyclodextrins (β-CD).

III.2. Animals and experimental design

Gilthead seabream, European seabass and rainbow trout specimens used in this study were handled in accordance with the guidelines of the European Union Council (2010/63/UE), Committee on the Ethics of Animal Experiments of the IEO (REGA: ES300261040017) and the approval of the Ministry of Water, Agriculture and Environment of the Autonomous Community Region of Murcia (Spain; A13160508). Atlantic salmon and Nile tilapia were handled according to the guidelines of the Norwegian Food Safe Authority (Mattilsynet: FOTS ID 18273) and approved by the Nord University (Bodø, Norway) Ethics Committee.

Under experimental or industrial farm conditions, fish were anesthetized due to the routine management and control processes. Exceptionally, in a study with gilthead seabream under industrial farm conditions, fish were monitored by cameras during rearing and they are not exposed to anaesthesia until sampling time. All specimens used in this thesis were fed *ad libitum* three times per day and fasted for 24 h before sampling.

The currently slaughtering method used in the aquaculture industry is hypothermia in seawater (SW) containing CI (ratio of 1:1 or 1:2, weight:weight: water:ice). In this study, same method was used in gilthead seabream, European seabass and rainbow trout as control group. Nevertheless, Atlantic salmon and Nile tilapia treated under experimental farm conditions were compared with two different control groups: fish placed on SW or FW, alone or containing 240 mg β-CD/L water.

All the stunning assays developed in this study (**Table 3**) were carried out prior to killing.

As previously mentioned, gilthead seabream is one of the most relevant species in terms of production and commercial value in the aquaculture industry of the Mediterranean area (APROMAR, 2017). In this sense, we have developed most of the methodology applied in this study in this specie under experimental and industrial farm conditions it has been slightly modified and used in the other four species studied, Atlantic salmon and Nile tilapia, under experimental farm conditions, and European seabass and rainbow trout, under industrial farm conditions. These four species have been also selected in this study due to its importance at National and European aquaculture production.

II.2.1. Gilthead seabream

The trials with gilthead seabream were undertaken between September 2015 and December 2017 under experimental and industrial farm conditions.

III.2.1.1. Experimental farm conditions

The specimens were bred and kept at the Oceanographic Centre of Murcia (Instituto Español de Oceanografía, IEO) (Mazarrón, Murcia, Spain) (n=32; 773.02±287.43 g body mass, bm) and at the Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA) (San Pedro del Pinatar, Murcia, Spain) (n=44; 763.17±370.85 g bm). Fish were kept in terrestrial SW tanks with a flow-through circuit, suitable aeration and filtration system and natural photoperiod. The temperature of SW ranged from 14.6 to 17.8 °C in winter to 27.3 to 28.0 °C in summer.

Different concentrations of CEO+β-CD (15, 20, 30, 40 or 60 mg) included in SW at 27.3-28.0 °C or in different type of ice (CI or LI) at different temperatures (- 0.2, - 1.5, - 5.0, or - 8.0 °C) were used. The following stunning treatments were carried out using CI at - 0.2 °C with an ice crystals size of 20 mm mixed with SW (ratio 1:1), as control condition:

1. SW at 27.3-28.0 °C containing 20, 40 or 60 mg of CEO+β-CD/L SW.
2. CI at - 0.2 °C containing 15, 20, 30 or 60 mg of CEO+β-CD/kg ice mixed with SW (ratio 1:1).
3. LI at - 1.5 °C containing 0, 15, 30 or 60 mg CEO+β-CD/kg ice mixed with SW (ratio 1:1).
4. LI at - 5.0 °C containing 0, 15, 20, 30, 40 or 60 mg of CEO+β-CD/kg ice mixed with SW (ratio 1:1).
5. LI at - 8.0 °C containing 15, 30 or 60 mg CEO+β-CD/kg ice mixed with SW (ratio 1:1).

In addition, EU in SW at 27.3-28.0 °C was also tested:

6. SW at 27.3-28.0 °C containing 20 mg EU/L SW or 20 mg EU+β-CD/L SW. In this case, the control group was treated with 20 mg CEO/L SW.

III.2.1.2. Industrial farm conditions

The specimens were growth and kept in 150 m^3 net-tanks in the open sea, far from the coast, in fish farm of Servicios Atuneros del Mediterráneo S.L. company (San Pedro del Pinatar, Murcia, Spain) (n=108; 747±250g bm). The different stunning treatments were conducted using one of the onboard tanks of the catch vessel (with a capacity of 250 kg of fish) for each treatment. The SW temperature ranged from 14.5 to 21.5 °C through the year. The following treatments were carried out using smaller concentrations of CEO+β-CD (5, 10 or 15 mg/kg) and smaller size of ice crystals (15 mm) than the previously used in the experimental farm conditions:

A) Fish that have been anesthetized due to the routine management and control processes

7. CI at 1.5-2.5 °C with crystals ice of 20 mm size mixed with SW (ratio 1:2), as control condition, or containing 5 mg CEO+β-CD/kg ice (15 or 20 mm of ice crystals size).
8. CI at 1.0-2.0 °C with ice crystals of 20 mm size mixed with SW (ratio 1:1), as control condition, or containing 10 mg CEO+β-CD/kg ice (15 or 20 mm of ice crystal size).
9. CI at 1.0-2.0 °C with ice crystals of 20 mm size mixed with SW (ratio 1:1), as control condition, or containing 15 mg CEO+β-CD/kg ice (15 or 20 mm of ice crystal size).

B) Fish that have been monitored by cameras during rearing and had never been exposed to anaesthesia until sampling

10. CI at 1.0-2.0 °C with ice crystals of 20 mm size mixed with SW (ratio 1:1), as control condition, or containing 20 mg CEO+β-CD/kg ice.

III.2.2. Atlantic salmon

The trial with Atlantic salmon was undertaken in March 2019 under experimental farm conditions.

Fish were growth and kept in 15 m^3 terrestrial tanks at Mørkvedbukta forskningsstasjon (Nord University, Bodø, Norway) facilities, with a flow-through circuit, suitable aeration and filtration system and natural photoperiod (n=75; 2323.85±85.53 g bm). The SW temperature of all tanks ranged from 4.0 to 6.0 °C.

Different treatments were carried out at this growing condition using three concentrations of CEO+β-CD (40, 50 or 60 mg/kg) dissolved in SW (4.0 to 6.0 °C). Treated groups were compared with two control groups: **a)** fish placed only on SW and **b)** fish placed on SW with 240 mg β-CD/L SW.

III.2.3. Nile tilapia

The trial with Nile tilapia was undertaken in March 2019 under experimental farm conditions.

All fish were bred and kept in 15 m^3 terrestrial tanks of Mørkvedbukta forskningsstasjon (Nord University, Bodø, Norway) facilities, with a flow-through circuit, suitable aeration and filtration system and natural photoperiod (n=75; 677.14±28.82 g bm). The FW temperature of all tanks ranged from 27.0 to 29.0 °C.

The different treatments were carried out using different concentrations of CEO+β-CD (20, 40 or 60 mg/L) dissolved in FW (27.0 to 29.0 °C). These treated groups were compared with two control conditions: **a)** fish placed only on FW, and **b)** fish placed on FW with 240 mg β-CD/L FW.

III.2.4. European seabass

Two trials with European seabass were undertaken in July 2018 under industrial farm conditions.

All fish were bred and kept in 150 m^3 net-tanks in the open sea of a Spanish company, Servicios Atuneros del Mediterráneo S.L. (San Pedro del Pinatar, Murcia, Spain), far from the coast farm. The SW temperature ranged from 27.3 to 28.0 °C. The SW temperature ranged from 27.3 to 28.0 °C. European seabass (n=20 of 395.62±57.78 g bm) were stunned with CI at - 0.2 °C with ice crystal of 20 mm of size mixed with SW (ratio 1:1), as control condition, or containing low concentrations of CEO+β-CD/kg ice:

1. CI at - 0.2 °C containing 5 or 10 mg of CEO+β-CD/kg ice mixed with SW (ratio 1:1).
2. CI at - 0.2 °C containing 15 or 30 mg of CEO+β-CD/kg ice mixed with SW (ratio 1:1).

III.2.5. Rainbow trout

The trial was undertaken in October 2018 under industrial farm conditions.

All specimens were kept in 300 m^3 water tanks of Piscifactorias Andaluzas S.A. (Loja, Granada, Spain), with a flow-through circuit, suitable aeration and filtration system and natural photoperiod (n=40; 652.62±31.50 g bm). The FW temperature ranged from 16.0 to 18.0 °C.

The treatment was conducted with fish stunned with CI at - 0.2 °C with ice crystal of 20 mm of size mixed with FW (ratio 1:1), as control condition, or including 5, 15 or 35 mg CEO+β-CD/kg ice mixed with FW (ratio 1:1).

Table 3. Summary of the pre-slaughter stunning procedures applied to farmed gilthead seabream (*Sparus aurata,* Linnaeus, 1758), Atlantic salmon (*Salmo salar,* Linnaeus, 1758) and Nile tilapia (*Oreochromis niloticus,* Linnaeus, 1758), European seabass (*Dicentrarchus labrax,* Linnaeus, 1758) and Rainbow trout (*Oncorhynchus mykiss,* Walbaum, 1792)

		Stunning treatment	Seawater/Freshwater temperature	Ice type	Ice-crystals size (mm)	water:ice ratio (w/w)	Treatment temperature	CEO+β-CD (mg CEO/Kg ice)	CEO (mg/L)	EU+β-CD (mg/L)	EU (mg/L)	β-CD (mg/L)
Gilthead seabream, *Sparus aurata* (Linnaeus, 1758)	Experimental farm conditions	1	27.3-28.0 °C	n/a	n/a	1:0	27.3-28.0 °C	0, 20, 40, 60(*)				
		2	14.6-17.8 °C	Crushed	20	1:1	-0.2 °C	0, 15, 20, 30, 40, 60				
		3	14.6-17.8 °C	Liquid	0.5	1:1	-1.5 °C	0, 15, 30, 60				
		4	14.6-17.8 °C	Liquid	0.5	1:1	-5.0 °C	0, 15, 20, 30, 40 60				
		5	14.6-17.8 °C	Liquid	0.5	1:1	-8.0 °C	0, 15, 30, 60				
		6	27.3-28.0 °C	n/a	n/a	1:0	27.3-28.0 °C		20	20	20	
	Industrial farm conditions	7	21.5 °C	Crushed	20	1:1	1.0-2.0 °C	0, 5				
		8	21.5 °C	Crushed	15	1:1	1.0-2.0 °C	5				
			21.5 °C	Crushed	20	1:1	1.0-2.0 °C	0, 10				
			21.5 °C	Crushed	15	1:1	1.0-2.0 °C	10				
		9	14.5 °C	Crushed	20	1:2	1.5-2.5 °C	0, 15				
			14.5 °C	Liquid	0.5	1:2	1.0-2.0 °C	15				
		10	14.5 °C	Crushed	20	1:2	1.0-2.0 °C	0, 20				
Atlantic salmon, *Salmo salar* (Linnaeus, 1758)	Experimental farm conditions	1	4.0-6.0 °C	n/a	n/a	1:0	4.0 - 6.0 °C	0, 40, 50, 60(*)				240
Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758)	Experimental farm conditions	1	27.0-29.0 °C	n/a	n/a	1:0	27.0- 29.0 °C	0, 20, 40, 60(*)				240
European seabass, *Dicentrarchus labrax* (Linnaeus, 1758)	Industrial farm conditions	1	27.3-28.0 °C	Crushed	20	1:1	-0.2 °C	0, 5, 10, 15, 30				
Rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792)	Industrial farm conditions	1	16.0-18.0 °C	Crushed	20	1:1	-0.2 °C	0, 5, 15, 35				

n/a: not applicable; (*) mg of CEO/L of sea or fresh water.

III.3 Anaesthesia-induction stages

The efficacy of the anaesthetics depends on the species, body size as well as the density of fish and the water quality in the bath (e.g., hardness, temperature, or salinity) (Iwama *et al*., 1989). To determine the anaesthesia-induction stages in each experimental condition (see below), we have followed the description of Keene *et al*. (1998). Briefly, the total loss of equilibrium is when the total loss of muscle tone and balance is achieved, showing a slow, regular opercular rate and a loss of spinal reflexes, while the loss of reflex activity is defined as the total loss of reactivity, when the opercular movements are slow and irregular, the heart rate is very slow and the fish lost all reflexes.

All tested fish were placed individually into the anaesthetic baths supplied with aeration. The observer was recording the times required to reach these two induction stages of anaesthesia using a chronometer. At each treatment, when the fish reached loss of reflex activity, it was removed from anaesthetic bath by net to the sampling area and samples were taken.

III.3.1. Under experimental farm conditions

i) Gilthead seabream. Treated with different doses of CEO+β-CD (15, 30 or 60 mg/kg ice) included in different types of ice at different temperatures (CI, 20 mm crystal size, at - 0.2 °C; LI, 0.5 mm size at - 1.5, - 5.0 or - 8.0 °C) were observed to determine the two anesthetic parameters previously indicated. In this case, fish were captured from the original tank and placed in a 200 L bucket filled with seawater and optimal aeration. Then the fish were placed individually in a 40 L bucket filled with the treatment. Five fish per group were analyzed.

Similarly, we also analyzed the anesthesia induction time in gilthead seabream specimens treated with EU+β-CD at 20 mg/L SW, 20 mg of EU/L SW or 20 mg CEO/L SW at 27.3-28.0 °C.

ii) Atlantic salmon specimens were reared in 150 m^3 terrestrial tanks (4.0-6.0 °C). Five different 40 L buckets were prepared with the different treatments (40, 50 or 60 mg CEO+β-CD/L SW at 4.0-6.0 °C) and control (**a)** only SW, or **b)** SW containing 240 mg β-CD/L SW at 4.0-6.0 °C). Five fish were removed from the original terrestrial tanks, and placed into treatment or control baths. The observer timed the anesthesia induction times in the five fish at the same time during 4 or 8 min maximum for the loss of equilibrium and the loss of reflex activity, respectively. This was repeated 15 different times (n = 75 fish; 15 fish per group).

iii) Nile tilapia were reared in 15 m^3 terrestrial tanks. Five different 20 L FW buckets were prepared with the different treatments (20, 40 or 60 mg CEO+β-CD/L FW at 27.0-29.0 °C) and control conditions (**a)** only FW, or **b)** FW containing 240 mg β-CD/L FW at 27.0-29.0 °C). Five fish were captured from the original tanks, where they were raised, and placed individually in the tanks with the treatments. The observer checked the induction time in the five fish at the same time during 4 or 8 min maximum for the loss of equilibrium and the loss of reflex activity, respectively. This was repeated 15 different times (n = 75 fish; 15 fish per group).

III.3.2. Under industrial farm conditions

iv) Gilthead seabream. No data were obtained for gilthead seabream due to the boat conditions were not had yet adapted for this study.

v) European seabass specimens were extracted directly from the floating cages located in the open sea and placed in the anaesthetic bath. Tanks of 40 L SW with CI at - 0.2 ºC containing 0 or 30 mg CEO+β-CD/kg ice were prepared and 4 fish were placed in each tank at the same time and the observer annotated the time needed to reach each stage of anaesthesia for each fish.

vi) Rainbow trout under terrestrial industrial farm conditions were captured from the original tanks and placed individually into 40 L FW tanks with CI at - 0.2 °C containing 0, 5, 15 or 35 mg CEO+β-CD/kg ice. The induction time of anaesthesia were observed fish by fish. Ten fish per anesthetic concentration were studied.

III.4. Samples collection

(i) **Plasma.** Blood samples were extracted from the caudal vein, using lithium heparin coated syringes immediately after death of individuals. After that, plasma was obtained by centrifugation at 10,000 xg during 5 min at 4 °C. Then, the samples were immediately frozen and stored at - 80 °C until used.

(ii) **Skin mucus** was collected by gentle scraping of the dorsolateral surface of each specimen using a cell scraper with enough care to avoid contamination with blood and/or urinogenital and intestinal excretions. Then, cell-free skin mucus was centrifugated and stored as previously described for blood.

(iii) **Tissues samples for gene expression.** Approximately 0.5 g of muscle and 0.1 g of head kidney from gilthead seabream were taken. Every tissue was put into a 1.5 mL Eppendorf and filled with 500 µL of RNAlater and stored at - 80 °C until used.

III.5. Plasmatic parameters

The levels of glucose (ng/mL), lactate (mmol/L), pO_2 (mmHg), pCO_2 (mmHg), HCO_3 (mmol/L), TCO_2 (mmol/L), SO_2 (%), base excess (mmol/L) and pH were analyzed in all plasma samples by a Portable Clinical Analyzer (i-STAT, Abbot, Unit City, CA), using i-STAT CG4+ disposable cartridges and following the manufacturer's instructions.

The cortisol levels in fish plasma samples were analyzed by ELISA (CO103S, CALBIOTECH), according to the manufacturer's instructions. Briefly, 25 µL of each cortisol standard solution (0, 20, 50, 100, 200, 400 and 800 ng/mL) or fish plasma sample were added to a 96-wellplate. Then, 200 µL of Cortisol Enzyme Conjugated buffer diluted with assay diluents buffer (1:21) were added to all wells and incubated for 1 h at room temperature (20-25 °C). Finally, the wells were washed and the reaction revealed with 100 µL of tetramethylbenzidine (TMB) enzyme substrate. The enzymatic reaction was visualized by color change and was stopped by adding 50 µL of 2 M of sulfuric acid. Absorbance was read in a spectrophotometer at 450 nm on a SPECTROstar® Omega microtiter plate reader within 20 min of adding the stop solution. The absorbance value obtained was inversely proportional to the concentration. The concentration of cortisol was calculated according to the standard curve. All the parameters were analyzed in duplicate for each sample.

III.6. Analysis of gene expression

Total RNA was extracted from head kidney and muscle samples of gilthead seabream specimens stunned with CI or LI at different temperatures and with different concentrations of CEO+β-CD, using TRIzol Reagent (Invitrogen) and following the manufacturer's instructions. Total RNA was quantified using a spectrophotometer (NanoDrop, ND-1000) and treated with DNase I (amplification grade, 1 unit/µg RNA, Invitrogen). The SuperScript IV RNase H-Reverse Transcriptase (Invitrogen) was used to synthesize first strand cDNA with an oligo (dT) 18 primer from 1 µg of total RNA, at 50 °C for 50 min. The β-*actin* (*actb*) gene expression was analyzed by conventional PCR to determine the efficacy of the retro-transcription using an Eppendorf Mastercycle Gradient Instrument (Eppendorf). Reaction mixtures in a total volume of 25 µL (1.25 µL DMSO, 1.25 µL 50 mM $MgCl_2$, 0.13 µL 10 mM dNTPs, 2.5 µL 10X Buffer Taq Pol, 1 µL primer forward (F) and 1 µL primer reverse (R) at 10 µM, 0.1 µL Taq pol 5 U/mL, 1 µL cDNA and 16,77 µl H_2O) were incubated for 2 min at 95 °C, followed by 30 cycles of 45 s at 95 °C, 45 s at the specific annealing temperature (55 °C), 45 s at 72 °C, and finally 10 min at 72 °C. The PCR products were run on a 1.2 % agarose gel (1.2 % agarose and 0.5 µg/mL of RedSafe (Biotium)). The bands were visualized with UV light and then, photographed.

Table 4. Gene accession numbers and primer sequences used for gene expression analysis.

Gene ID	Accession Nº	Name	Nucleotidesequence (5'-3')	Use
actb	X89920	F3	ATCGTGGGGCGCCCCAGGCACC	Conventional PCR
		R3	CTCCTTAATGTCACGCACGATTTC	
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC	
		R	CTTCTGCCTGTTGAGGAACC	
il1-β	AJ277166	F2	GGGCTGAACAACAGCACTCTC	Real-time PCR
		R3	TTAACACTCTCCACCCTCCA	
tgf-β1	AF424703	F	AGAGACGGGCAGTGAAAGAA	
		R	GCCTGAGGAGACTCTGTTGG	
Hsp70	EU805481	F	TCATCAACGAGCCAACTGCT	
		R	GATGGGTATCTCCGGCAGTG	
RyR3	SRP062962	F	TCAACGATGTCTCCGAGTCC	
		R	TGCAGCAGTCGGCATTCG	

The expression profile of several genes in head kidney and muscle were analyzed by real time PCR. In the head kidney samples, the genes coding for a pro-inflammatory cytokine, *il1-β*, an anti-inflammatory cytokine, *tgf-β1*, and a stress-related protein, *Hsp70* were analyzed. In the case of the muscle samples, the levels of expression of *RyR3* gene at each condition were studied. The expression levels of all the genes were analyzed by real-time PCR, performed with an ABI PRISM 7500 instrument (Applied Biosystems) using 5 µL of SYBR Green PCR Core Reagents (Applied Biosystems), 0.6 µL of each of the specific primers for each of the genes analyzed (Invitrogen) at 5 µM and 4.4 µL H_2O to reach a final volume of 10 µL. Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. For each gene analyzed, the gene expression was corrected by *the ribosomal protein S18* gene (*rps18*) content in each sample. The gilthead seabream specific primers used are shown in **Table 4**. In all cases, each PCR was performed in triplicates and repeated at least twice, being the variation between samples less than 2 % for the *rps18* gene expression levels.

III.7. Antimicrobial activities

Some antimicrobial activities were determined in plasma and skin mucus samples. Thus, natural peroxidase, bactericidal, antiprotease and protease activity assays were conducted.

III.7.1 Peroxidase activity

The peroxidase activity levels in plasma and skin mucus were measured according to a protocol previously described (Quade & Roth, 1997). Briefly, 5 µL of plasma or skin mucus was diluted with 45 µL of Hank's buffer (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates (Nunc) and mixed with 100 µL of 10 mM TMB solution containing 10 µL of 0.015 % H_2O_2 as substrate. The color-change reaction was stopped after 15 min by adding 50 µL of 2 M sulphuric acid and the optical density (OD) was read at 450 nm using a plate reader (MultiskanGo, Thermo Fisher Scientific). Wells with HBSS but without sample were used as blanks. Samples were run in triplicates. One unit was defined as the amount of activity producing an absorbance change of 1 and the activity was expressed as U/mL of plasma or skin mucus.

III.7.2 Bactericidal activity

The pathogenic marine bacteria *Vibrio harvey*i (strain Lg 16/ 100) was grown in agar plates at 25 °C in tryptic soy agar (TSA, Sigma). Then, fresh single colonies of 1-2 mm were diluted in 5 mL of tryptic soy broth (TSB; Laboratorios Conda), cultured for 16 h at 25 °C on an orbital incubator at 200-250 rpm and adjusted to 10^8 bacteria/mL with TSB. The absorbance of bacteria cell cultures was measured at 600 nm and used to know the concentration based on growth curves.

The antibacterial activity of plasma and skin mucus was determined by evaluating their effects on the bacterial growth of *V. harvevi* curves using a method previously described (Sunyer & Tort, 1995). Aliquots of 10 µL of the bacterial dilutions of *V. harvevi* (1/10) were placed in flat-bottomed 96-well plates and incubated with 10 µL of plasma or skin mucus during 2 h at room temperature. Then, 150 µL of TSB were added and the absorbance of the samples was measured at 620 nm every 30 min intervals during 36 h at 25 °C. Samples without bacteria were used as blanks (negative control). Samples without plasma or skin mucus were used as positive controls (100 % growth or 0 % antibacterial activity). Bactericidal activity was calculated as % of bacterial growth inhibition per mL of plasma or skin mucus.

III.7.3. Antiprotease activity

Antiprotease activity was determined by the ability of plasma or skin mucus to inhibit proteinase K activity using a modified protocol previously described (Ellis, 1990). Briefly, 10 µL of each sample were incubated during 10 min at room temperature with the same volume of 2 mg/mL proteinase K solution (Applichem) in 0.01 M PBS. After adding 100 µL of 0.01 M PBS and 125 µL of 2 % azocasein (Sigma) dissolved in 0.01 M PBS (Sigma), samples were incubated during 2 h at room temperature. Afterwards, 250 µL of 10 % trichloroacetic acid (TCA) were added and an additional incubation during 30 min at room temperature was done. The mixture was then centrifuged at 6,000 xg for 5 min and 100 µL of the supernatants were transferred to a flat-bottomed 96-well plate. One hundred µL of 1 N NaOH were added and the OD read at 450 nm using a plate reader. For a positive control (100 % of anti-protease activity), 0.01 M PBS replaced samples and proteinase K; and for a negative control (0 % of anti-protease activity) 0.01 M PBS replaced only the sample.

Samples were run in duplicates. The percentage of inhibition of proteinase K activity for each sample was calculated as [100 - (% of the activity of the negative control)]. Results were expressed as % in plasma or skin mucus.

III.7.4. Protease activity

Protease activity in plasma and skin mucus was determined as the percentage of hydrolysis of azocasein using a modified protocol previously described (Charney & Tomarelli, 1947). Briefly, 10 µL of plasma or skin mucus were incubated with 100 µL of 0.01 M PBS and 125 µL of 2 % azocasein (Sigma) dissolved in 0.01 M PBS for 24 h at room temperature. The reaction was stopped by adding 10 % TCA for 30 min at room temperature. The mixture was centrifuged at 6,000 xg for 5 min and 100 µL of the supernatants were transferred to a flat-bottomed 96-well plate. One hundred µL of 1 N NaOH were then added and the OD read at 450 nm using a plate reader. For a positive control, 10 µL of 2 mg/mL proteinase K (AppliChem) in 0.01 M PBS replaced the sample (100 % of activity), and for a negative control, 0.01 M PBS replaced the sample (0 % of activity). Samples were run in duplicates. The percentage of protease activity for each sample was calculated as % of the activity of the positive control. Results were expressed as % in plasma or skin mucus.

III.8. Total proteins

This determination was carried out following the method of Bradford (1976). Briefly, a standard curve was made with bovine serum albumin (BSA, Sigma) diluted in PBS at different concentrations (4, 8, 12, 16 and 20 µg/mL). The samples (plasma or skin mucus) were diluted in PBS at the optimal dilution (1:100 or 1:50, respectively). Then, 160 µL of samples or standards were placed in triplicates in a 96-well plate and mixed with 40 µL of Bradford reagent (Sigma). After 45 min of incubation at room temperature (20-25 °C), the absorbance was read in a spectrophotometer at 595 nm. Results were expressed as µg/mL in plasma or skin mucus.

III.9. Statistical analysis

The data from the experiments that have only two experimental groups were analyzed accordingly with a Student-t test. The data from the experiments with three or more experimental groups were analyzed accordingly with One-way analysis of variance (ANOVA) and the subsequent Bonferroni post-hoc test to determine differences between groups.

The critical value for statistical significance was taken as $p \leq 0.05$. All statistical analyses were carried out using the IBM SPSS Statistic 23 program.

IV.Results

IV.1. Clove essential oil nanoencapsulated in β-CD

IV.1.1. Stages of anaesthesia

The first aim of this study was to determine the time needed to achieve the anesthesia-induction stages in fish exposed to different concentrations of CEO nanoencapsulated in β-CD at experimental and industrial farm conditions. In this way, we have observed the behavior of gilthead seabream, Atlantic salmon and Nile tilapia under experimental farm conditions and European seabass and rainbow trout under industrial farm conditions, was observed. The time that fish take to loss of equilibrium and the loss of reflex activity were observed following the descriptions of Keene *et al*. (1998). On the one hand, when fish began to turn around by themselves into the water and their opercular rate slow, the observer indicated the start of loss of equilibrium. On the other hand, when had an irregular opercular movement and the body of fish is completely quiet and face up, the observer indicated the start of loss of reflex activity, what it means that the anaesthesia has been totally induced.

In general, the anaesthesia-induction stages in gilthead seabream were achieved faster in fish treated with CEO+β-CD (15, 30 or 60 mg/kg) incorporated in CI (at - 0.2 °C) or LI (at - 1.5, - 5.0 or - 8.0 °C), compared with fish treated under control condition, CI at - 0.2 °C (**Figure 16**).

First of all, fish treated with different doses of CEO+β-CD incorporated into CI at - 0.2 °C, achieved the loss of equilibrium at 3.05±0.55 min (15 mg/kg), or 2.74±0.42 min (60 mg/kg), while fish treated under control condition (CI at - 0.2 °C) showed the loss of equilibrium after a longer time, 5.60±0.47 min **(Figure 16a)**. Moreover, no differences were observed in time of loss of equilibrium between control group and fish treated with CI at - 0.2 °C, or LI at - 1.5 °C **(Figure 16b)**, neither between fish treated with LI at - 1.5 °C alone or containing CEO+β-CD (15, 30 or 60 mg/kg) **(Figure 16b)**. However, the loss of equilibrium was achieved in a shorter period of time in fish treated with CEO+β-CD (15, 30 or 60 mg/kg) incorporated in LI at - 1.5 °C (2.17±0.61, 1.55±0.48 and 2.14±0.21 min, respectively) (Figure 16b), at - 5.0 °C (1.10±0.48, 0.58±0.40 and 0.93±0.27 min, respectively) (Figure 16c) and at - 8.0 °C (0.73±0.18, 0.52±0.10 and 0.68±0.11 min, respectively), compared to control group (CI, - 0.2 °C) **(Figure 16d)**.

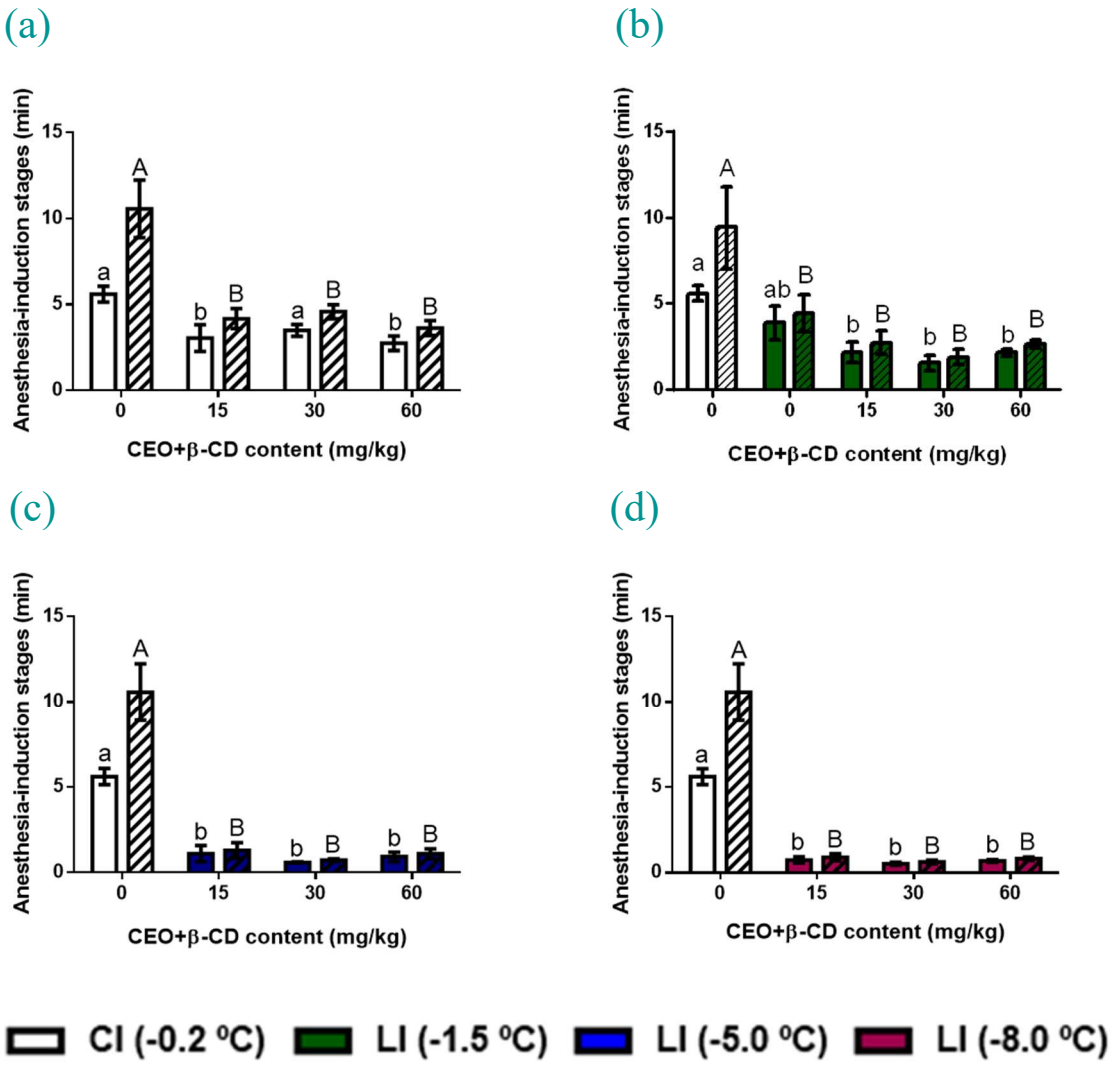


Figure 16. The anesthesia-induction stages in gilthead seabream treated with CEO+β-CD under experimental farm conditions. Fish were exposed to CI at - 0.2 °C mixed with SW (ratio 1:1), as control condition. **(a)** CI at - 0.2 °C containing 15, 30 or 60 mg CEO+β-CD/kg ice mixed with SW (ratio 1:1); **(b)** CI at - 0.2 °C or LI at - 1.5 °C containing 0, 15, 30 or 60 mg CEO+β-CD/kg ice mixed with SW (ratio 1:1); **(c)** CI at - 0.2 °C or LI at - 5.0°C containing 15, 30 or 60 mg CEO+β-CD/kg ice mixed with SW (ratio 1:1) or **(d)** CI at - 0.2 °C or LI at - 8.0 °C containing 15, 30 or 60 mg CEO+β-CD/kg ice mixed with SW (ratio 1:1). Plain bars represent the loss of equilibrium; striped bar represent the loss of reflex activity. Each value represents the mean ± S.E.M. of n=4 fish per group in each sampling condition. Letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

Regarding to the loss of reflex activity, it was achieved in a shorter period of time in fish treated with CEO+β-CD (15, 30 or 60 mg/kg) incorporated in CI at - 0.2 °C (4.17±0.58, 4.57±0.40 and 3.63±0.10 min, respectively) compared to control group (CI at - 0.2 °C) (10.57±1.67 min) (Figure 16a). Differently to the loss of equilibrium, differences in the loss of reflex activity between fish from control condition, CI at - 0.2 °C (10.57±1.67 min), and fish treated with LI at - 1.5 °C (4.44±1.07 min) were observed (Figure 16b). Moreover, the loss of reflex activity was achieved in a shorter time in fish treated with 15, 30 or 60 mg CEO+β-CD/kg incorporated in LI at - 1.5 °C (2.72±0.67, 1.87±0.45 and 2.66±0.25 min, respectively) **(Figure 16b)**, - 5.0 °C (1.29±0.45, 0.73±0.10 and 1.10±0.27 min, respectively) (Figure 16c) and - 8.0 °C (0.89±0.20, 0.62±0.11 and 0.81±0.10 min, respectively), compared to control fish (CI at - 0.2 °C) **(Figure 16d)**. Similar to the loss of equilibrium, no differences were observed in the time of loss of reflex activity in fish treated with LI at - 1.5 °C or containing CEO+β-CD (15, 30 or 60 mg/kg) **(Figure 16b).**

In case of Atlantic salmon and Nile tilapia studied under experimental farm conditions, fish were kept during 4 min of observation, without achieving the loss of equilibrium in the two control conditions used (Atlantic salmon, SW, 4.0-6.0 °C, with or without 240 mg β-CD/L; Nile tilapia, FW, 27.0-28.0 °C, with or without 240 mg β-CD/L) **(Figure 17)**. When CEO+β-CD was added in SW or FW, the time of loss of equilibrium was shorter than 4 min in both species. In Atlantic salmon, the decrease in time of the loss of equilibrium was correlated with the increase doses of CEO+β-CD (40 mg/L, 2.39±0.13 min; 50 mg/L, 1.73±0.12 min; 60 mg/L, 1.21±0.12 min) **(Figure 17a)**. In Nile tilapia, the loss of equilibrium in a shorter time than 4 min in fish treated with 40 and 60 mg of CEO+β-CD/L (1.89±0.14 min and 1.29±0.12 min, respectively), but no in fish treated with 20 mg CEO+β-CD/L (Figure 17b). Looking closer the time of loss of reflex activity in both species, control groups were observed until 8 min, with no loss of reflex activity achieved (Atlantic salmon, SW, 4.0-6.0 °C, with or without 240 mg β-CD/L; Nile tilapia, FW, 27.0-28.0 °C, with or without 240 mg β-CD/L) **(Figure 17)**. However, the time of loss of reflex activity was below 8 min in both species after the treatment with CEO+β-CD **(Figure 17)**.

Atlantic salmon treated with 40 or 50 mg CEO+β-CD/L achieved the loss of reflex activity in similar times (5.53±0.23 and 4.47±0.19 min, respectively), while the shortest one was achieved in fish treated with 60 mg/L (3.37±0.19 min) **(Figure 17a)**.

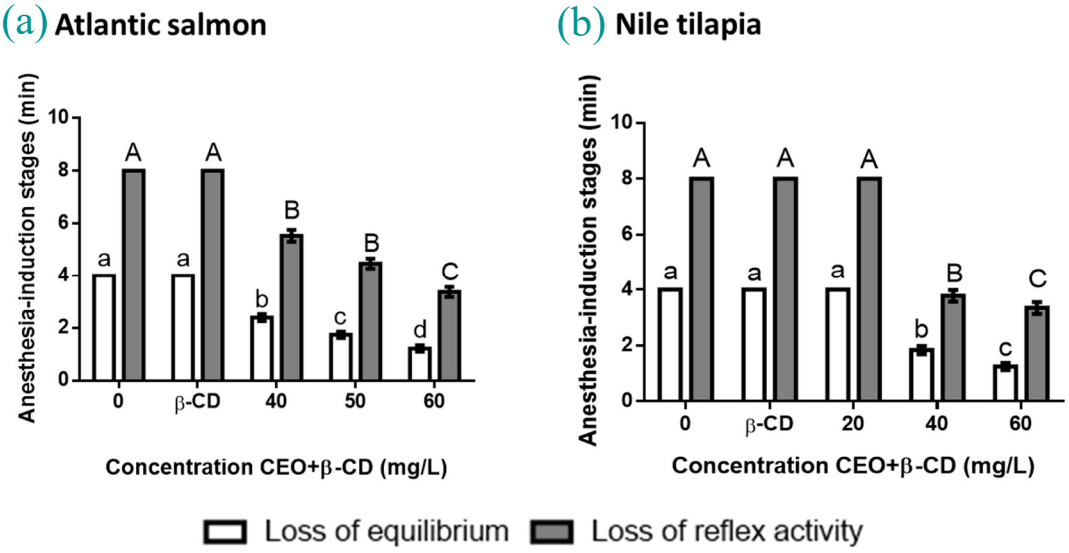


Figure 17. The anesthesia-induction stages in Atlantic salmon (a) and Nile tilapia (b) with CEO+β-CD under experimental farm conditions. Atlantic salmon were treated with SW at 4-6 °C and Nile tilapia with FW at 27.0-28.0°C, with or without 240 mg β-CD/L water (control conditions). Fish were stunned with 40, 50 or 60 mg CEO+β-CD/L water in Atlantic salmon (a) or with 20, 40 or 60 mg CEO+β-CD/L water in Nile tilapia (b). Each value represents the mean ± S.E.M. of n=15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test (P≤ 0.05).

In European seabass studied at industrial farm conditions, the loss of equilibrium and loss of reflex activity started in a shorter period of time in fish treated with 30 mg CEO+β-CD/kg (1.28±0.08 and 3.01±0.10 min, respectively), compared to control group (CI at - 0.2 °C; 2.49±0.17 and 3.42±0.02 min, respectively) **(Figure 18a)**. In rainbow trout, the loss of equilibrium and loss of reflex activity were achieved in a shorter time in fish treated with different doses of CEO+β-CD incorporated in CI at - 0.2 °C (5 mg/kg, 1.92±0.22 and 5.89±0.80min; 15 mg/kg, 1.27±0.27 and 3.53±0.17 min; and 35 mg/kg, 0.50±0.10 and 1.79±0.25 min, respectively) compared with control fish (CI at - 0.2 °C; 7.95±1.70 and 22.04±3.66 min, respectively**) (Figure 18b)**. But not differences were found between fish treated with different doses of CEO+β-CD **(Figure 18b)**.

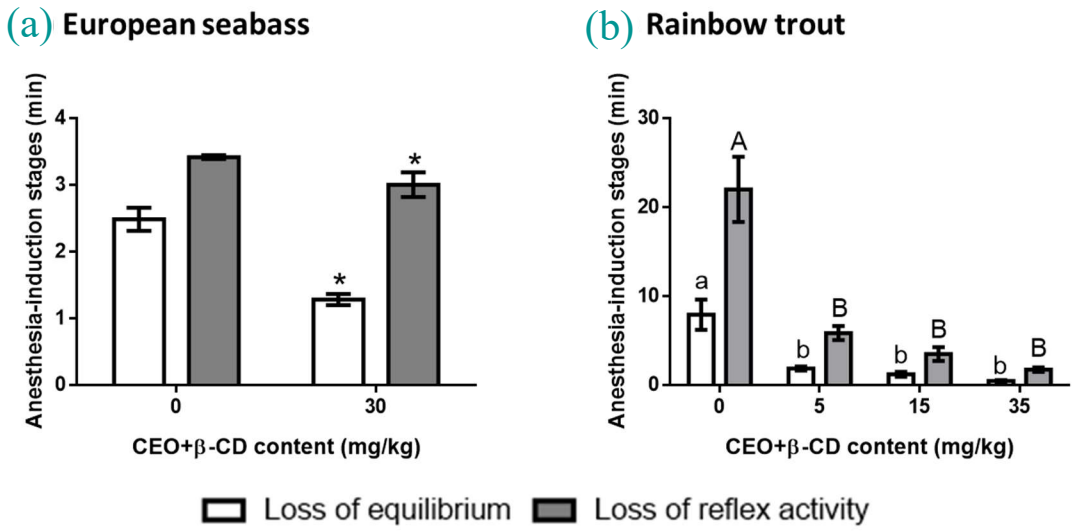


Figure 18. The anesthesia-induction stages in European seabass (a) and rainbow trout (b) with CEO+β-CD under industrial farm conditions. (a): European seabass stunned with CI at -0.2 °C with ice crystals of 20 mm size mixed with SW (ratio 1:1) (control condition) or 30 mg CEO+β-CD/kg ice. (b): Rainbow trout stunned with CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (ratio 1:1) (control condition) or 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=4-10 fish per group in each sampling condition. Asterisks (*) indicate statistically significant differences between groups according to t-test Student ($P \leq 0.05$) (a) and the letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$) (b).

IV.1.2. Plasmatic parameters

The second aim of this thesis was to study the effect of CEO nanoencapsulated in β-CD on stress in experimental and industrial farm conditions. In this way, we have used gilthead seabream in both farm conditions, Atlantic salmon and Nile tilapia in experimental farm conditions, and European seabass and rainbow trout in industrial farm conditions to stun at slaughtering time. In order to perform this study, some plasmatic parameters considered as stress indicators were analysed, such as glucose (ng/mL), lactate (mmol/L), pO_2 (mmHg), pCO_2 (mmHg), HCO_3 (mmol/L), TCO_2 (mmol/L), SO_2 (%), base excess (mmol/L), pH and cortisol (ng/mL).

IV.1.2.1. Glucose

In gilthead seabream, under experimental farm conditions, CEO+β-CD was incorporated into SW at 27.3-28.0 °C, CI at - 0.2 °C or LI at - 1.5, -5.0 or - 8.0 °C, and in all cases, CI at - 0.2 °C was used as control condition **(Figure 19)**. All the fish stunned with CEO+β-CD showed lower glucose level in plasma than those of the control, except when LI at - 1.5 °C was used. Thus, the plasmatic glucose level of fish treated with 20, 40 or 60 mg CEO+β-CD/L of SW at 27.3-28.0 °C decreased when compared with fish treated with CI at - 0.2 °C, although no differences between the different CEO+β-CD concentrations used were observed (**Figure 19a**). Moreover, fish treated with 15, 20, 30 or 60 mg CEO+β-CD/kg incorporated in CI at - 0.2 °C also showed a decrease in the plasmatic glucose level compared with fish treated with CI at - 0.2 °C, although in this case differences between the different CEO+β-CD concentrations used were observed; being the lowest glucose level achieved with 15 mg CEO+β-CD/kg followed by 30 mg CEO+β-CD/ kg (**Figure 19b**). When fish were treated with LI at - 1.5 °C no differences were observed between different experimental groups: CI at - 0.2 °C alone or LI at - 1.5 °C alone or containing 15, 30 or 60 mg of CEO+β-CD/kg (**Figure 19c**). However, when fish were treated with LI at - 5.0 °C alone or containing CEO+β-CD, a decrease was observed in the glucose plasmatic level compared with the level of the control condition (CI at - 0.2 °C); being 15 and 30 mg CEO+β-CD/kg concentrations which recorded lowest levels (**Figure 19d**). These last concentrations (15 and 30 mg CEO+β-CD) also decrease the glucose level of fish when incorporated in LI at - 8.0 °C compared with the level of the control conditions (CI at - 0.2 °C) (**Figure 19e**).

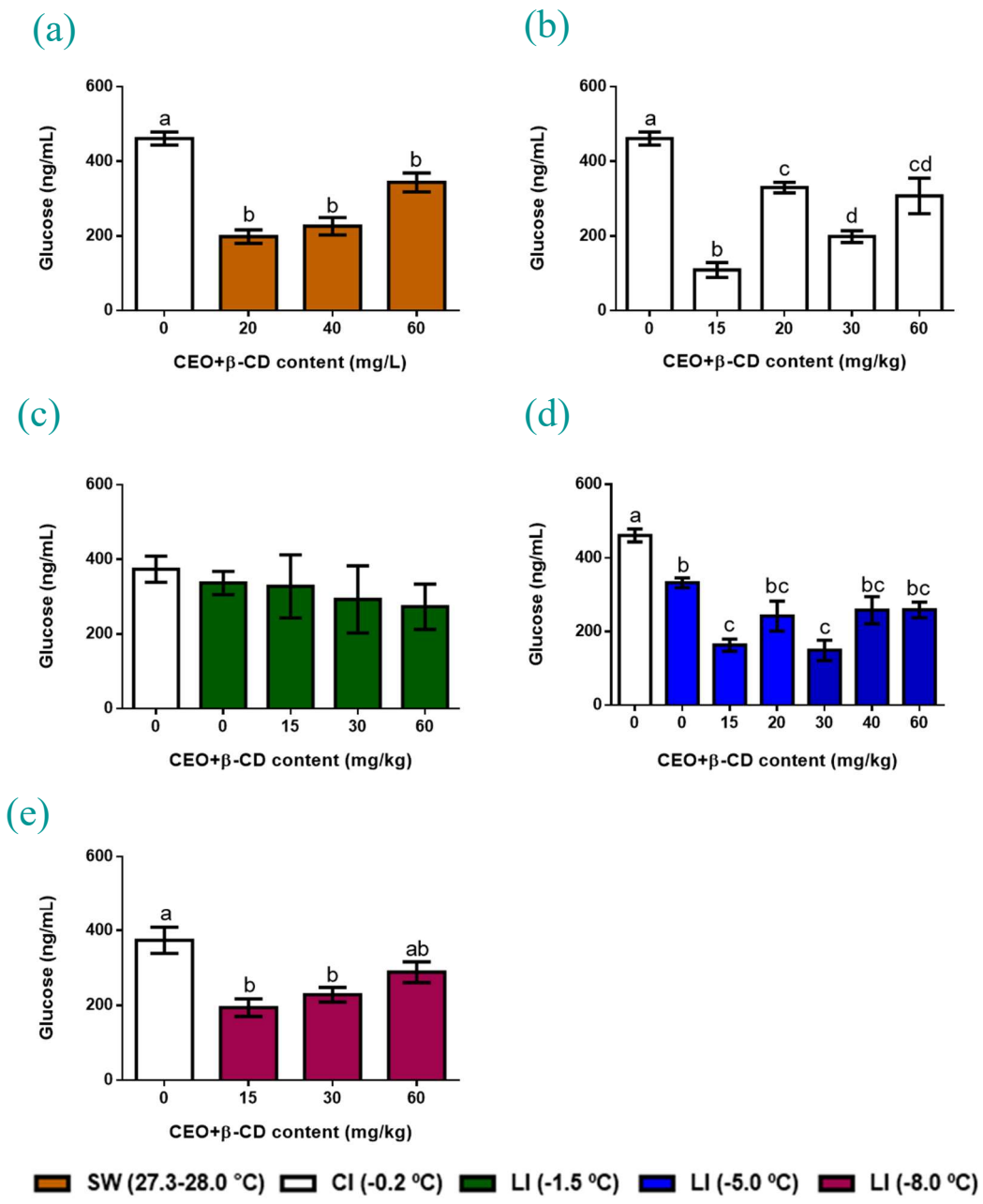


Figure 19. Plasmatic glucose level of gilthead seabream stunned with CEO+β-CD incorporated in SW (a), CI (b), or LI (c, d, e) under experimental farm conditions. Control group: CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (ratio 1:1). **(a)** CI as control condition or SW at 27.3-28.0 °C containing 20, 40 or 60 mg CEO+β-CD/L SW. **(b)** CI as control condition or containing 15, 20, 30 or 60 mg CEO+β-CD/kg ice. **(c)** CI as control condition or LI at - 1.5 °C mixed with SW (ratio 1:1), alone, or containing 15, 30 or 60 mg CEO+β-CD/kg ice. **(d)** CI as control condition or LI at - 5 ºC mixed with SW (ratio 1:1) alone or containing 15, 20, 30, 40 or 60 mg CEO+β-CD/kg ice. **(e)** CI as control condition or LI at - 8 ºC mixed with SW (ratio 1:1) with 15, 30 or 60 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

Gilthead seabream, under industrial farm conditions, treated with low concentrations of CEO+β-CD incorporated in CI (15 or 20 mm of crystal size) at 1.0-2.0 °C (5 mg/kg, **Figure 20a**; 10 mg/kg, **Figure 20b**; 15 mg/kg, **Figure 20c**) showed a decrease in the plasmatic glucose level compared with the level of the fish treated with CI at 1.0-2.0 °C (20 mm of crystal size) (control group). However, no differences in the plasmatic glucose level were observed between the two ice crystals size (15 and 20 mm) (**Figure 20a-c**).

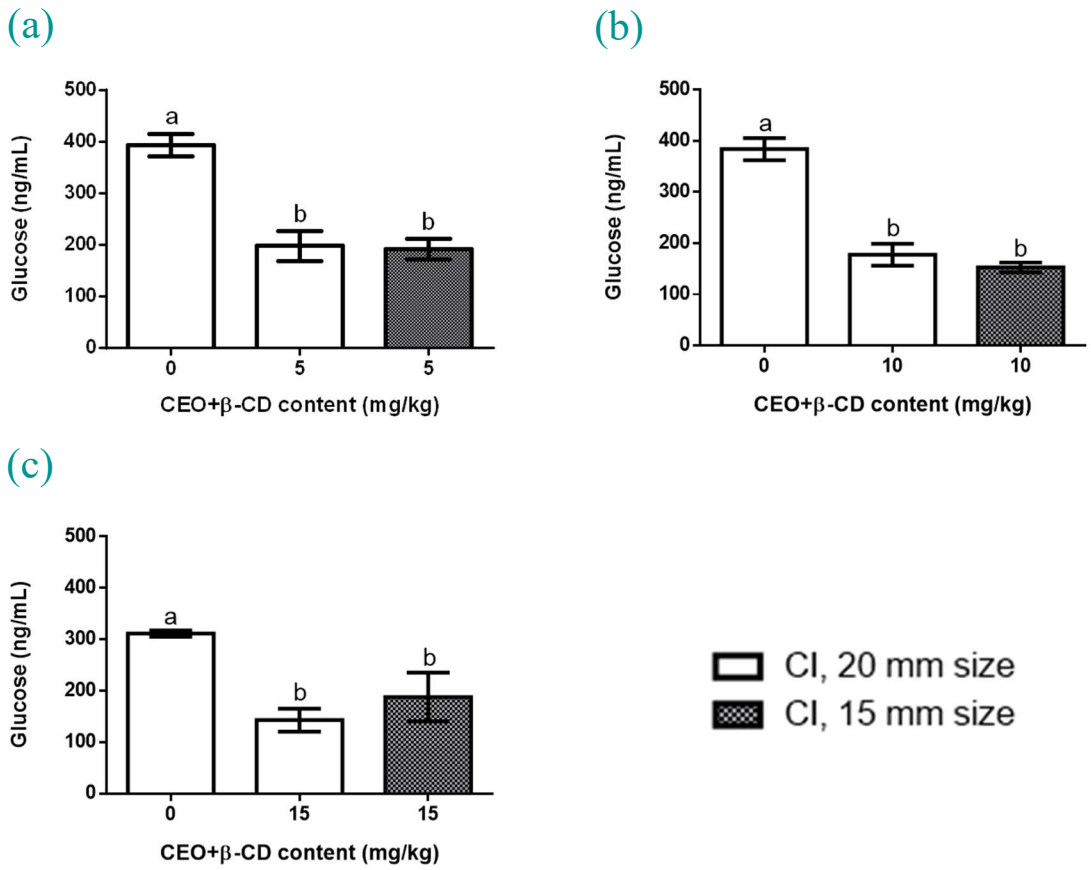


Figure 20. Plasmatic glucose level in gilthead seabream stunned under industrial farm conditions with CEO+β-CD incorporated in crushed ice of different sizes of ice crystals (20 or 15 mm). CI at 1.0-2.0 °C (ice crystals of 20 mm size) mixed with SW (ratio 1:2) (control condition). **(a)** CI in control condition or CI of 20 or 15 mm ice crystals size containing 5 mg CEO+β-CD/kg. **(b)** CI in control condition or CI of 20 or 15 mm ice crystals size containing 10 mg CEO+β-CD/kg. **(c)** CI in control condition or CI of 20 or 15 mm ice crystals size containing 15 mg CEO+β-CD/kg groups. Each value represents the mean ± S.E.M. of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In Atlantic salmon and Nile tilapia, the plasmatic glucose level also decreased after stunning the fish with CEO+β-CD/L of water under experimental farm conditions compared to their respective control groups (Atlantic salmon, 4.0-6.0 °C, with or without 240 mg β-CD; Nile tilapia, FW, 27.0-29.0 °C, with or without, 240 mg β-CD) (**Figure 21**).

Interestingly, higher concentrations of CEO+β-CD were needed to observe a decrease in the plasmatic glucose level in Atlantic salmon (50 or 60 mg CEO+β-CD/L, **Figure 21a**) and Nile tilapia (60 mg CEO+β-CD/L, **Figure 21b**) compared to those needed in gilthead seabream also at experimental farm conditions (see point I.1.2.1.1).

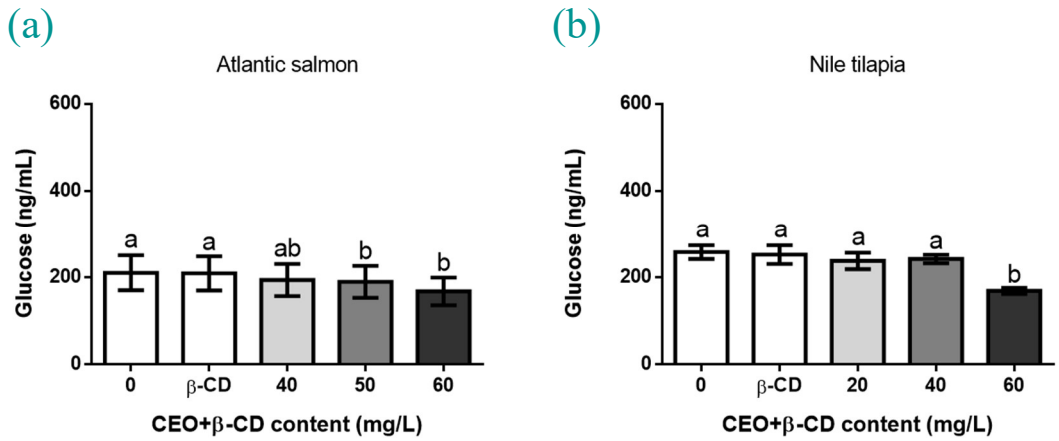


Figure 21. Plasmatic glucose level in Atlantic salmon (a) and Nile tilapia (b) stunned with CEO+β-CD under experimental farm conditions. (a) Atlantic salmon were treated with SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L water (control 2) or 40, 50 or 60 mg CEO+β-CD/L water. (b) Nile tilapia were treated with FW at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L water (control 2) or 20, 40 or 60 mg CEO+β-CD/L water. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In European seabass and rainbow trout, at industrial farm conditions, the plasmatic glucose level also decreased after applying CEO+β-CD compared with the level of the control fish (CI at - 0.2 °C) (**Figure 22**). Thus, in European seabass, 15 or 30 mg CEO+β-CD/kg of ice decreased the plasmatic glucose level, while no effect was observed with the lowest concentrations used (5 or 10 mg/kg) (**Figure 22a**). In the other hand, rainbow trout needed 35 mg CEO+β-CD/kg to decrease the plasmatic glucose level, while no effect was observed with the lowest concentrations used (5 or 15 mg/kg; **Figure 22b**).

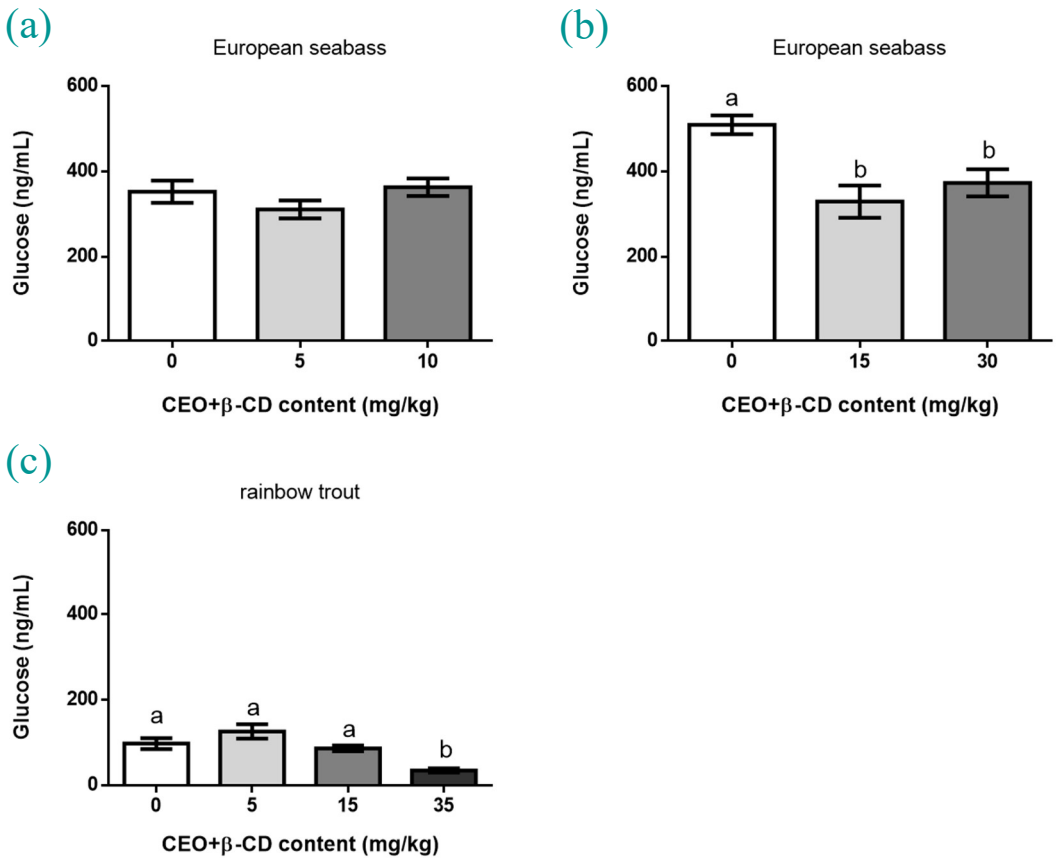


Figure 22. Plasmatic glucose level in European seabass (a) and rainbow trout (b) stunned with CEO+β-CD under industrial farm conditions. European seabass and rainbow trout were stunned with CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (European seabass)/FW (rainbow trout) (ratio 1:1) (control condition). **(a, b)** CI in control condition or containing 5, 10, 15 or 30 mg CEO+β-CD/kg ice. **(c)** CI in control condition or containing 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.1.2.2. Lactate

In some of the experimental farm conditions assayed with gilthead seabream, plasmatic lactate level was reduced when CEO+β-CD was included in SW, CI or LI at - 5.0 ºC or - 8.0 °C but not at - 1.5 °C, and in all cases compared with the level of the fish from the control group (CI at - 0.2 °C). When fish were treated with SW at 27.3-28.0 °C, only the lowest concentration of CEO+β-CD (20 mg/L) used triggered lower lactate (**Figure 23a**). However, when CEO+β-CD was incorporated into CI (- 0.2 °C), a lower concentration of CEO+β-CD (15 mg) decreased the lactate level (**Figure 23b**). Curiously, LI at - 1.5 °C promoted an increase in the plasmatic lactate level, although when 15 or 30 mg CEO+β-CD/kg was incorporated in LI, a decrease in the plasmatic lactate level was observed; however, no differences between the plasmatic level of control condition and LI at - 1.5 ºC with 15, 30 or 60 mg CEO+β-CD/kg) were observed (**Figure 23c**). In contrast, no differences in the plasmatic lactate level between control condition and LI at - 5.0 °C alone stunned fish were observed, while a decrease of this level was recorded when 15, 20 or 40 mg CEO+β-CD/kg was incorporated in the LI at - 5.0 °C (**Figure 23d**). When CEO+β-CD was incorporated in LI at - 8 °C, only the lowest concentrations assayed (15 or 30 mg CEO+β-CD/kg) decreased the plasmatic lactate level (**Figure 23e**).

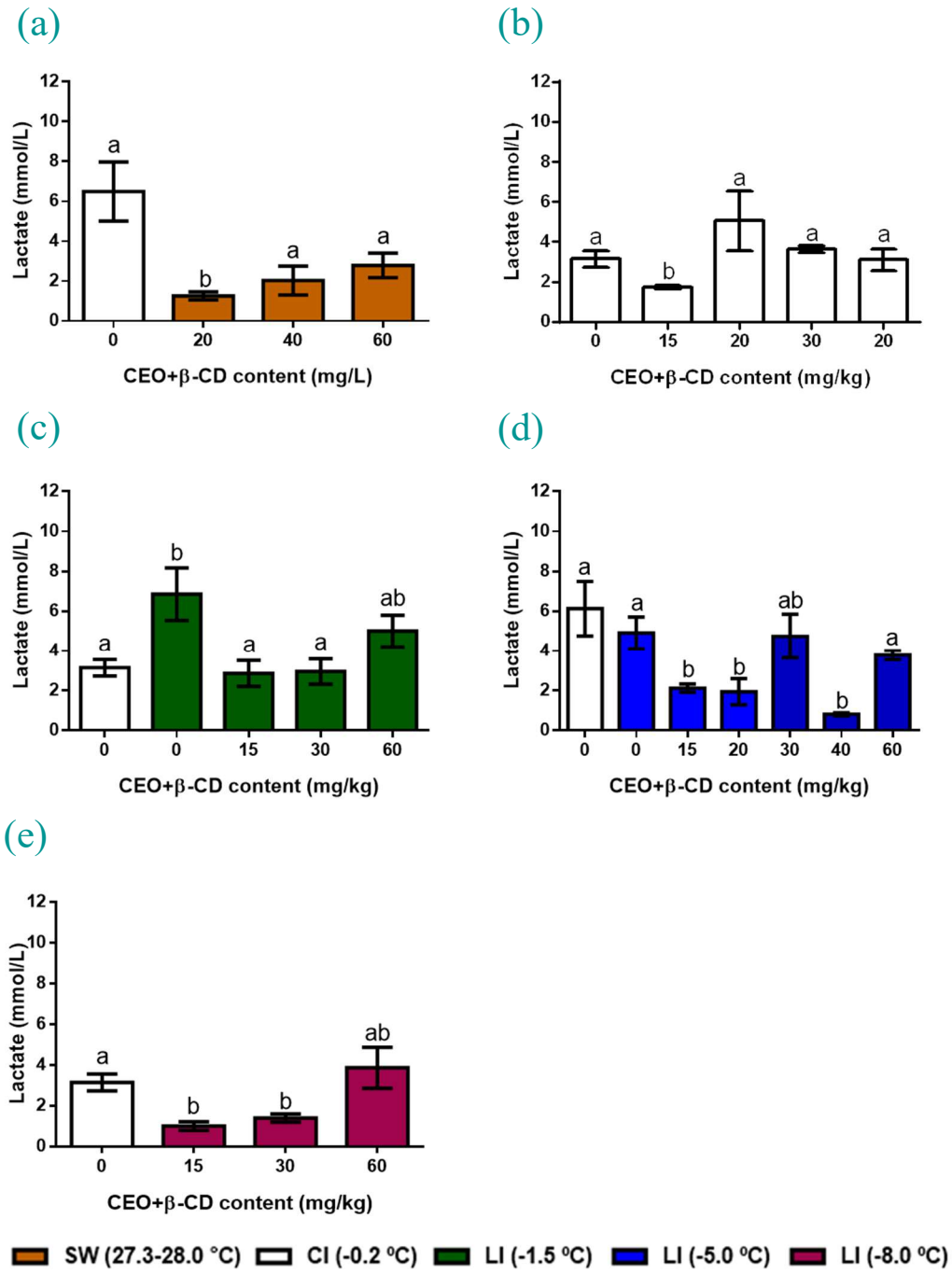


Figure 23. Plasmatic lactate level of gilthead seabream stunned with CEO+β-CD incorporated in SW (a), CI (b), or LI (c, d, e) under experimental farm conditions. Control condition: CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (ratio 1:1). **(a)** CI in control condition or SW at 27.3-28.0 °C containing 20, 40 or 60 mg CEO+β-CD/L SW. **(b)** CI in control condition or containing 15, 20, 30 or 60 mg CEO+β-CD/kg ice. **(c)** CI in control condition or LI at - 1.5 °C containing 0, 15, 30 or 60 mg CEO+β-CD/kg ice mixed with SW (ratio 1:1). **(d)** CI in control condition or LI at - 5 ºC containing 0, 15, 20, 30, 40 or 60 mg CEO+β-CD/kg ice mixed with SW. **(e)** CI in control condition or LI at - 8 ºC containing 15, 30 or 60 mg CEO+β-CD/kg ice mixed with SW (ratio 1:1). Each value represents the mean ± S.E.M of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In gilthead seabream, under industrial farm conditions, no differences were observed in the plasmatic lactate level between fish treated with 5 or 10 mg CEO+β-CD/kg (CI, 20 or 15 mm ice crystal size, 1.0-2.0 °C) compared with the level of the fish from the control group (CI, 20 mm ice crystal size, 1.0-2.0 °C) (**Figure 24a, b**). However, those fish treated with 15 mg CEO+β-CD/kg (CI, 20 or 15 mm ice crystal size, 1.0-2.0 °C) had lower lactate level than those fish from the control group (CI, 20 mm ice crystal size, 1.0-2.0 °C; **Figure 24c**).

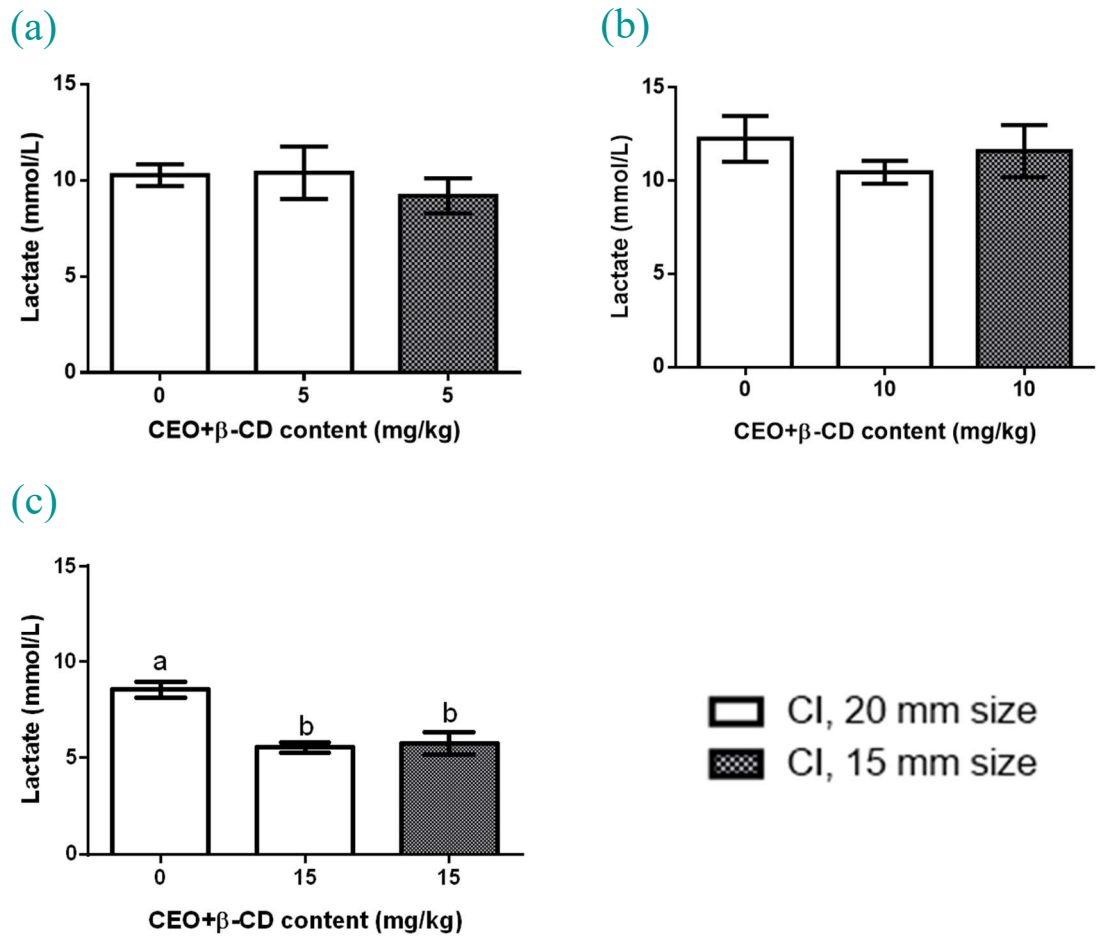


Figure 24. Plasmatic lactate level in gilthead seabream stunned with CEO+β-CD incorporated in different sizes of ice crystals (20-15 mm) under industrial farm conditions. Control condition: CI mixed with SW (ratio 1:2) at 1.0-2.0 °C with ice crystals of 20 mm size. **(a)** CI in control condition or containing 5 mg CEO+β-CD/kg ice of 20 or 15 mm ice crystal size. **(b)** CI in control condition or containing 10 mg CEO+β-CD/kg ice of 20 or 15 mm ice crystal size. **(c)** CI in control condition or containing 15 mg CEO+β-CD/kg ice of 20 or 15 mm ice crystal size. Each value represents the mean ± S.E.M. of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

Atlantic salmon treated with 40 or 50 mg CEO+β-CD/L underwent an increase in the plasmatic lactate level compared to fish from the control condition (SW at 4.0-6.0 °C; **Figure 25a**). Moreover, 240 mg β-CD/L also increased this level compared to fish from the control condition (SW at 4.0-6.0 °C; **Figure 25a**). Differently, the plasmatic lactate level decreased in Nile tilapia treated with 20, 40 or 60 mg CEO+β-CD/L compared to the level observed in fish from the control conditions (FW at 27.0-29.0 °C with or without 240 mg β-CD/L) (**Figure 25b**). However, no differences in the plasmatic lactate level between the different concentrations of CEO+β-CD/L used were observed (**Figure 25b**).

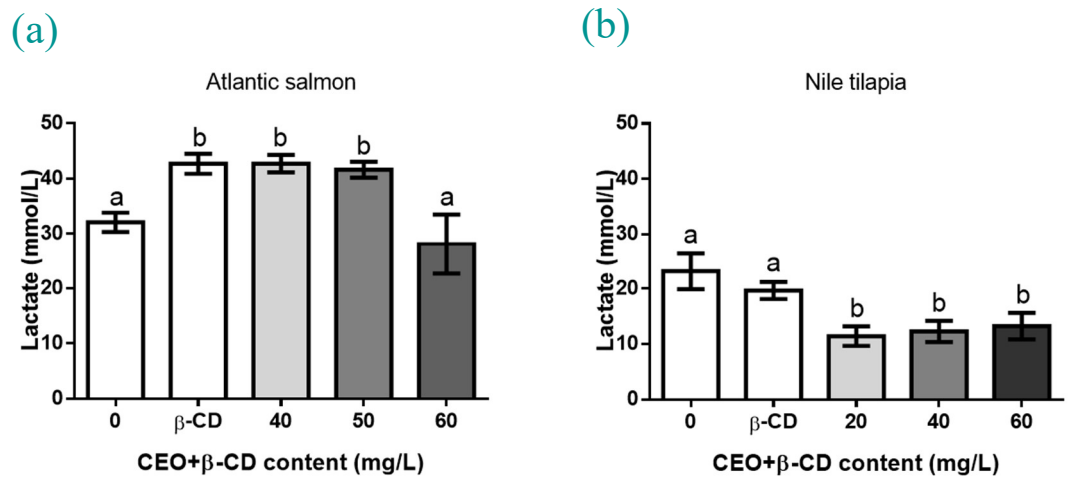


Figure 25. Plasmatic lactate level in Atlantic salmon (a) and Nile tilapia (b) stunned with CEO+β-CD under experimental farm conditions. (a) Atlantic salmon treated with SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L (control 2) or 40, 50 or 60 mg CEO+β-CD/L of SW. (b) Nile tilapia treated with FW at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L (control 2) or 20, 40 or 60 mg CEO+β-CD/L of SW. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In European seabass, only when 30 mg CEO+β-CD/kg was incorporated in CI at - 0.2 ºC, an increase in the plasmatic lactate level was detected compared to the level of the fish from the control group (CI at - 0.2 °C; **Figure 26a, b**). In rainbow trout, no differences in the plasmatic lactate level between fish treated with CEO+β-CD/kg (5, 15 or 35 mg) and those of the fish from the control group (CI at - 0.2 °C) were observed (**Figure 26b**).

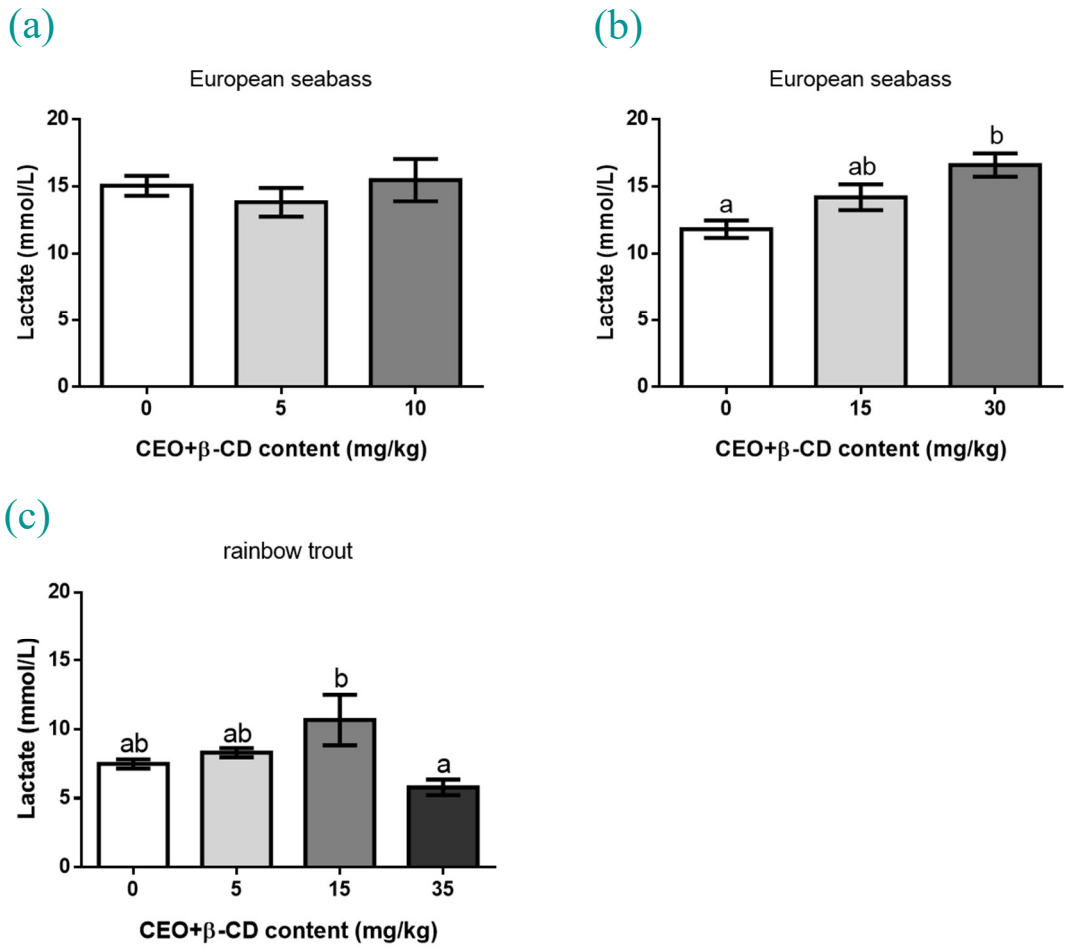


Figure 26. Plasmatic lactate level in European seabass (a, b) and rainbow trout (b) stunned with CEO+β-CD under industrial farm conditions. European seabass and rainbow trout stunned with CI mixed with SW (ratio 1:1) at - 0.2 °C with ice crystals of 20 mm size (control condition). **(a, b)** European seabass treated with CI in control condition or containing 5, 10, 15 or 30 mg CEO+β-CD/kg ice. **(c)** Rainbow trout treated with CI in control condition or containing 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.1.2.3. Other plasmatic parameters

The plasmatic level of pO_2 (mmHg), pCO_2 (mmHg), HCO_3 (mmol/L), TCO_2 (mmol/L), SO_2 (%), base excess (mmol/L) and pH have been analyzed in the same experiments in which we have analyzed the level of glucose and lactate. However, only few modifications have been observed in some determined conditions.

In gilthead seabream under experimental farm conditions (control condition, CI at - 0.2 ºC) (**Table 5**), no variations in the level of any of these plasmatic parameters were observed when CEO+β-CD was incorporated in SW at 27.3-28.0 °C or in CI at - 0.2 °C compared in both cases with the level of the control group. However, when CEO+β-CD was incorporated in LI at different temperatures, variations in the level of several plasmatic parameters were observed depending on the temperature and the concentration used. Thus, only 60 mg CEO+β-CD incorporated in LI at -1.5 °C increased the plasmatic level of pO_2 and pCO_2 (241.75±4.71 and 29.52±2.87 mmHg, respectively) compared to the level in control fish (228.33±9.02 and 16.53±1.11 mmHg, respectively). When using a lower temperature (LI at - 5.0 °C), the plasmatic level of pO_2 decreased to the level of control (228.33±9.02 mmHg) when fish were stunned with 15 or 60 mg CEO+β-CD (196.25±2.25 or 203.75±7.76 mmHg, respectively), while the plasmatic level of pCO_2 increased with 30 or 60 mg CEO+β-CD (21.45±1.64 or 20.93±0.41 mmHg, respectively) compared to the level of control (16.53±1.11mmHg). Moreover, the control group and the groups treated with LI at - 1.5 °C or LI at - 5 °C recorded similar levels of all the plasmatic parameters analyzed. In contrast, when the temperature of LI achieved - 8.0 °C, other plasmatic parameters different from pO_2 and pCO_2 were also modified. Thus, the plasmatic level of pO_2 decreased or increased after 30 or 60 mg CEO+β-CD (195.75±11.77 or 259.51±5.72 mmHg), respectively), compared with the level of fish from the control group (228.33±9.02 mmHg). However, 60 mg CEO+β-CD were needed to increase the plasmatic level of pCO_2 (24.55±1.90 mmHg) compared with control fish (16.53±1.11 mmHg). In addition, the plasmatic level of HCO_3 decreased when 15 or 30 mg CEO+β-CD was used (10.31±1.75 or 9.25±0.38 mmol/L, respectively) compared with the level of the fish from the control group (13.16±0.29 mmol/L) and the base excess decreased from - 10.00±0.57 mmol/L in control fish to - 15.42±0.33, - 17.34±0.25 or - 14.98±0.85 mmol/L in 15, 30 or 60 mg CEO+β-CD/kg treated fish, respectively. Any of the conditions assayed modified the plasmatic levels of TCO_2, SO_2 or pH in gilthead seabream under experimental farm conditions.

Table 5. Plasmatic parameters level in gilthead seabream stunned with CEO+β-CD under experimental farm conditions.

Stunning treatments	CEO+β-CD (mg/kg or mg/L)	pO_2 (mmHg)	pCO_2 (mmHg)	HCO_3 (mmol/L)	TCO_2 (mmol/L)	SO_2 (%)	Base Excess (mmol/L)	pH
1	0 CI	228.33 ± 9.02^{a}	16.53 ± 1.11^{a}	13.16 ± 0.29^{a}	13.66 ± 0.33^{a}	100^{a}	-10.00 ± 0.57^{a}	7.51 ± 0.02^{a}
	20	214.66 ± 18.67^{a}	14.93 ± 0.54^{a}	9.40 ± 0.61^{a}	10.00 ± 0.56^{a}	100^{a}	-15.33 ± 1.20^{a}	7.41 ± 0.04^{a}
	40	210.66 ± 11.36^{a}	16.23 ± 0.98^{a}	10.23 ± 0.84^{a}	10.66 ± 0.66^{a}	100^{a}	-14.34 ± 1.20^{a}	7.41 ± 0.03^{a}
	60	230.33 ± 10.47^{a}	15.43 ± 0.62^{a}	10.96 ± 1.45^{a}	11.67 ± 1.45^{a}	100^{a}	-13.00 ± 2.08^{a}	7.45 ± 0.04^{a}
2	0 CI	228.33 ± 9.02^{a}	16.53 ± 1.11^{a}	13.16 ± 0.29^{a}	13.66 ± 0.33^{a}	100^{a}	-10.00 ± 0.57^{a}	7.51 ± 0.02^{a}
	15 CI	196.25 ± 0.48^{a}	14.60 ± 0.67^{a}	9.23 ± 0.37^{a}	9.50 ± 0.50^{a}	100^{a}	-16.00 ± 0.82^{a}	7.39 ± 0.03^{a}
	20 CI	235.33 ± 23.24^{a}	13.93 ± 103^{a}	16.16 ± 0.30^{a}	16.66 ± 2.73^{a}	100^{a}	-7.33 ± 1.33^{a}	7.47 ± 0.12^{a}
	30 CI	191.50 ± 14.16^{a}	19.95 ± 2.84^{a}	12.13 ± 1.66^{a}	12.75 ± 1.55^{a}	100^{a}	-13.00 ± 1.58^{a}	7.39 ± 0.01^{a}
	60 CI	180.75 ± 10.90^{a}	19.23 ± 3.21^{a}	10.87 ± 1.04^{a}	11.50 ± 0.96^{a}	100^{a}	-14.25 ± 1.49^{a}	7.35 ± 0.05^{a}
3	0 CI	228.33 ± 9.02^{a}	16.53 ± 1.11^{a}	13.16 ± 0.29^{a}	13.66 ± 0.33^{a}	100^{a}	-10.00 ± 0.57^{a}	7.51 ± 0.02^{a}
	0 LI	235.25 ± 11.37^{a}	22.35 ± 2.89^{a}	10.60 ± 1.03^{a}	11.00 ± 1.50^{a}	100^{a}	-16.00 ± 0.91^{a}	7.29 ± 0.02^{a}
	15 LI	230.25 ± 4.10^{a}	18.75 ± 1.73^{a}	9.77 ± 0.52^{a}	10.50 ± 0.65^{a}	100^{a}	-16.25 ± 1.50^{a}	7.33 ± 0.02^{a}
	30 LI	237.75 ± 6.26^{a}	22.75 ± 1.53^{a}	11.25 ± 1.18^{a}	12.00 ± 1.22^{a}	100^{a}	-15.12 ± 0.87^{a}	7.31 ± 0.06^{a}
	60 LI	241.75 ± 4.71^{b}	29.52 ± 2.87^{b}	12.73 ± 0.96^{a}	13.50 ± 0.95^{a}	100^{a}	-14.20 ± 0.99^{a}	7.30 ± 0.08^{a}
4	0 CI	228.33 ± 9.02^{a}	16.53 ± 1.11^{a}	13.16 ± 0.29^{a}	13.66 ± 0.33^{a}	100^{a}	-10.00 ± 0.57^{a}	7.51 ± 0.02^{a}
	0 LI	215.00 ± 15.00^{a}	14.25 ± 1.05^{a}	11.70 ± 0.63^{a}	12.50 ± 0.50^{a}	100^{a}	-11.50 ± 1.50^{a}	7.52 ± 0.05^{a}
	15 LI	196.25 ± 2.50^{b}	14.80 ± 1.14^{a}	8.65 ± 1.07^{a}	9.25 ± 1.11^{a}	100^{a}	-14.00 ± 0.13^{a}	7.35 ± 0.04^{a}
	20 LI	188.00 ± 1.52^{a}	13.80 ± 1.56^{a}	10.90 ± 1.85^{a}	11.33 ± 2.02^{a}	100^{a}	-12.00 ± 2.52^{a}	7.50 ± 0.03^{a}
	30 LI	214.75 ± 6.08^{ac}	21.45 ± 1.64^{b}	11.05 ± 1.07^{a}	11.75 ± 1.25^{a}	100^{a}	15.34 ± 0.45^{a}	7.35 ± 0.03^{a}
	40 LI	197.00 ± 6.72^{a}	13.40 ± 0.84^{a}	11.93 ± 0.75^{a}	12.66 ± 0.76^{a}	100^{a}	-10.33 ± 1.04^{a}	7.55 ± 0.05^{a}
	60 LI	203.75 ± 7.76^{c}	20.93 ± 0.41^{b}	9.25 ± 1.06^{a}	10.00 ± 1.10^{a}	100^{a}	-16.23 ± 0.49^{a}	7.28 ± 0.05^{a}
5	0 CI	228.33 ± 9.02^{a}	16.53 ± 1.11^{a}	13.16 ± 0.29^{a}	13.66 ± 0.33^{a}	100^{a}	-10.00 ± 0.57^{a}	7.51 ± 0.02^{a}
	15 LI	241.75 ± 13.42^{a}	20.45 ± 2.28^{a}	10.31 ± 1.75^{b}	10.75 ± 1.79^{a}	100^{a}	-15.42 ± 0.33^{b}	7.31 ± 0.03^{a}
	30 LI	195.75 ± 11.77^{b}	14.33 ± 0.78^{a}	9.25 ± 0.38^{b}	9.75 ± 0.48^{a}	100^{a}	-17.34 ± 0.25^{b}	7.42 ± 0.01^{a}
	60 LI	259.51 ± 5.72^{c}	24.55 ± 1.90^{b}	14.71 ± 0.93^{a}	15.51 ± 1.04^{a}	100^{a}	-14.98 ± 0.85^{b}	7.39 ± 0.01^{a}

Stunning treatments: CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW: ratio 1:1 (control condition).

(1) CI in control condition or SW at 27.3-28.0 °C containing 20, 40 or 60 mg CEO+β-CD/L SW.
(2) CI in control condition or containing 15, 20, 30 or 60 mg CEO+β-CD/kg ice.
(3) CI in control condition or LI at - 1.5 °C containing 0, 15, 30 or 60 mg CEO+β-CD/kg ice mixed with SW (ratio 1:1).
(4) CI in control condition or LI at - 5 ºC containing 0, 15, 20, 30, 40 or 60 mg CEO+β-CD/kg ice mixed with SW (ratio 1:1).
(5) CI in control condition or LI at - 8 ºC containing 15, 30 or 60 mg CEO+β-CD/kg ice mixed with SW (ratio 1:1).

Each value represents the mean ± S.E.M of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

Under industrial farm conditions (control condition, CI, at 1.0-2.0 ºC, 20 mm of crystal size) and using crystals ice of different sizes, CEO+β-CD promoted modifications in some plasmatic parameters in gilthead seabream (**Table 6**) different from those observed under experimental farm conditions (**Table 5**). Thus, 5 mg CEO+β-CD only increased the pH (6.93±0.02 mmHg) when CI of 15 mm of crystal size was used compared with the pH of the control condition or the pH of 5 mg CEO+β-CD (CI of 20 mm ice crystal size: 6.67±0.01 mmHg). However, 10 mg CEO+β-CD only decreased the plasmatic pO_2 and pCO_2 level when the size of the ice crystals was 15 mm (238.65±9.14 and 51.32±2.89 mmHg, respectively) compared with the level of the fish from the control group (CI of 20 mm ice crystal size: 479.41±20.62 and 68.14±4.13 mmHg, respectively). Moreover, 15 mg CEO+β-CD with ice crystal size of 20 mm decreased the plasmatic HCO_3 level (6.25±0.09 mmol/L) and increased the plasmatic pH (7.47±0.06) compared to the levels of the control group (CI of 20 mm of ice crystal size; 6.52±0.07 mmol/L and 7.17±0.06, respectively). In contrast 15 mg CEO+β-CD with crystals size of 15 mm only increased the pH (7.49±0.06) compared with the the level of the control (7.17±0.06). No variations were observed in the level of TCO_2, SO_2 or base excess in any of the conditions assayed at industrial farm conditions.

Table 6. Plasmatic parameters level in gilthead seabream stunned with CEO+β-CD under industrial farm conditions.

Stunning treatments	CEO+β-CD (mg/kg)	pO_2 (mmHg)	pCO_2 (mmHg)	HCO_3 (mmol/L)	TCO_2 (mmol/L)	SO_2 (%)	Base Excess (mmol/L)	pH
7	0 (20 mm)	500.64 ± 23.40^{a}	72.47 ± 5.97^{a}	8.28 ± 0.67^{a}	10.60 ± 0.81^{a}	100^{a}	-28.4 ± 0.81^{a}	6.67 ± 0.01^{a}
	5 (20 mm)	462.42 ± 33.55^{a}	77.74 ± 11.71^{a}	8.05 ± 1.06^{a}	10.25 ± 1.43^{a}	100^{a}	-24.56 ± 1.39^{a}	6.48 ± 0.11^{a}
	5 (15 mm)	443.02 ± 21.35^{a}	70.85 ± 3.12^{a}	7.98 ± 0.44^{a}	10.98 ± 1.27^{a}	100^{a}	-23.76 ± 1.22^{a}	6.93 ± 0.02^{b}
8	0 (20 mm)	479.41 ± 20.62^{a}	68.14 ± 4.13^{ab}	7.24 ± 0.68^{a}	9.24 ± 0.73^{a}	100^{a}	-27.41 ± 0.93^{a}	6.54 ± 0.08^{a}
	10 (20 mm)	490.87 ± 36.25^{a}	77.34 ± 10.27^{a}	7.74 ± 0.65^{a}	9.46 ± 0.93^{a}	100^{a}	-27.46 ± 0.68^{a}	6.62 ± 0.07^{a}
	10 (15 mm)	238.65 ± 9.14^{b}	51.32 ± 2.89^{b}	6.88 ± 0.61^{a}	8.69 ± 0.67^{a}	100^{a}	-27.68 ± 0.76^{a}	6.73 ± 0.03^{a}
9	0 (20 mm)	199.00 ± 3.08^{a}	24.70 ± 1.53^{a}	6.52 ± 0.07^{a}	8.00 ± 0.41^{a}	100^{a}	-22.75 ± 0.63^{a}	7.17 ± 0.06^{a}
	15 (20 mm)	187.75 ± 3.75^{a}	21.25 ± 3.38^{a}	6.25 ± 0.09^{b}	9.25 ± 1.30^{a}	100^{a}	-21.00 ± 0.41^{a}	7.47 ± 0.06^{b}
	15 (15 mm)	183.42 ± 1.98^{a}	23.29 ± 2.21^{a}	6.10 ± 0.08^{a}	8.76 ± 1.27^{a}	100^{a}	-19.44 ± 0.87^{a}	7.49 ± 0.06^{b}

Stunning treatments: CI at 1.0-2.0 °C with ice crystals of 20 mm size mixed with SW (ratio 1:2) (control condition).

(7) CI in control condition or containing 5 mg CEO+β-CD/kg ice of 20 or 15 mm ice crystal size.
(8) CI in control condition or containing 10 mg CEO+β-CD/kg ice of 20 or 15 mm ice crystal size.
(9) CI in control condition or containing 15 mg CEO+β-CD/kg ice of 20 or 15 mm ice crystal size.

Each value represents the mean ± S.E.M. of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

Atlantic salmon specimens treated under experimental conditions (**Table 7**) with 50 or 60 mg CEO+β-CD/L showed an increase in the plasmatic level of pO_2 (222.11±2.02 or 223.30±3.14 mmHg, respectively) compared to both control groups (SW, 4.0-6.0 °C, 203.40±4.26 mmHg, or SW, 4.0-6.0 °C, containing 240 mg β-CD/L, 204.21±2.25 mmHg). However, the plasmatic level of HCO_3, TCO_2, base excess and pH decreased in those fish (HCO_3, 8.94±0.18 or 8.53±0.18 mmol/L; TCO_2, 9.93±0.18 or 9.46±0.22 mmol/L; base excess, - 21.41±0.29 or -21.60±0.25 mmol/L; pH, 7.06±0.01 or 7.06±0.01) compared to the levels of control fish (SW, 4.0-6.0 °C, or SW, 4.0-6.0 °C containing 240 mg β-CD (HCO_3, 9.98±0.27 or 10.04±0.25 mmol/L; TCO_2, 10.73±0.28 or 11.02±0.29 mmol/L; base excess, - 19.07±0.40 or - 19.73±0.34 mmol/L; pH, 7.15±0.02 or 7.13±0.04). Interestingly, the plasmatic levels of pCO_2 was only altered when SW, 4.0-6.0 °C containing 240 mg β-CD/L was used (33.33±0.96 mmHg) compared with the level of the fish treated with SW, 4.0-6.0 °C, alone (29.12±0.91 mmHg). No variations were observed in the level of SO_2 in any of the conditions assayed.

Table 7. Plasmatic parameters level in Atlantic salmon stunned with CEO+β-CD under experimental farm conditions.

Stunning treatments	CEO+β-CD (mg/L)	β-CD (mg/L)	pO_2 (mmHg)	pCO_2 (mmHg)	HCO_3 (mmol/L)	TCO_2 (mmol/L)	SO_2 (%)	Base Excess (mmol/L)	pH
1	0	240	203.40 ± 4.26^{a}	29.12 ± 0.91^{a}	9.98 ± 0.27^{a}	10.73 ± 0.28^{a}	100^{a}	-19.07 ± 0.40^{a}	7.15 ± 0.02^{a}
	40		204.21 ± 2.25^{a}	33.33 ± 0.96^{b}	10.04 ± 0.25^{a}	11.02 ± 0.29^{a}	100^{a}	-19.73 ± 0.34^{a}	7.13 ± 0.04^{a}
	50		204.67 ± 1.81^{a}	30.52 ± 0.62^{ab}	9.86 ± 0.16^{a}	10.73 ± 0.18^{a}	100^{a}	-19.53 ± 0.27^{a}	7.12 ± 0.01^{a}
	60		222.11 ± 2.02^{b}	31.39 ± 0.84^{ab}	8.94 ± 0.18^{b}	9.93 ± 0.18^{b}	100^{a}	-21.41 ± 0.29^{b}	7.06 ± 0.01^{b}
			223.30 ± 3.14^{b}	29.79 ± 0.74^{ac}	8.53 ± 0.18^{b}	9.46 ± 0.22^{b}	100^{a}	-21.60 ± 0.25^{b}	7.06 ± 0.01^{b}

Stunning treatment: SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L (control 2) or with 40, 50 or 60 mg CEO+β-CD/L of SW.

Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In Nile tilapia, under experimental farm conditions, stunned with different concentrations of CEO+β-CD/L incorporated in FW at 27.0-28.0 °C some altered plasmatic levels were recorded (**Table 8**). The plasmatic level of pO_2 decreased in fish treated with 40 mg of CEO+β-CD (181.67±7.05 mmHg), while base excess levels (- 15.67±0.71 mmol/L) and pH (7.42±0.03) increased compared with the level of the control group (FW at 27.0-28.0 °C) (200.40±5.07 mmHg, - 18.47±0.95 mmol/L or 7.15±0.02 respectively). Interestingly, the plasmatic level of TCO_2 increased in fish treated with FW containing 240 mg β-CD/L (15.84±1.68 mmol/L) compared with the level of the fish from the rest of the groups assayed. In addition, base excess level in fish treated with 60 mg CEO+β-C (15.54±0.61 mmol/L) also increased compared with the level in control fish (FW, 27.0-28.0 °C: - 18.47±0.95 mmol/L). No variations were observed in the level of pCO_2, HCO_3 and SO_2 in any of the conditions assayed.

Table 8. Plasmatic parameters level in Nile tilapia stunned with CEO+β-CD under experimental farm conditions.

Stunning treatments	CEO+β-CD (mg/L)	β-CD (mg/L)	pO_2 (mmHg)	pCO_2 (mmHg)	HCO_3 (mmol/L)	TCO_2 (mmol/L)	SO_2 (%)	Base Excess (mmol/L)	pH
1	0	240	200.40 ± 5.07^{a}	17.41 ± 1.03^{a}	8.22 ± 0.41^{a}	8.80 ± 0.45^{a}	100^{a}	-18.47 ± 0.95^{a}	7.29 ± 0.04^{a}
	20		187.79 ± 1.93^{ab}	15.84 ± 1.68^{a}	8.68 ± 0.39^{a}	15.84 ± 1.68^{b}	100^{a}	-17.59 ± 0.43^{ab}	7.33 ± 0.03^{ab}
	40		196.62 ± 2.99^{ab}	14.84 ± 0.67^{a}	8.42 ± 0.28^{a}	8.84 ± 0.30^{ac}	100^{a}	-16.92 ± 0.58^{ab}	7.36 ± 0.21^{ab}
	60		181.67 ± 7.05^{b}	13.91 ± 1.04^{a}	8.68 ± 0.38^{a}	9.20 ± 0.43^{ac}	100^{a}	-15.67 ± 0.71^{b}	7.42 ± 0.03^{b}
			198.43 ± 1.80^{ab}	16.21 ± 0.62^{a}	9.11 ± 0.26^{a}	9.57 ± 0.22^{ac}	100^{a}	-15.54 ± 0.61^{b}	7.63 ± 0.24^{ab}

Stunning treatment: FW at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L (control 2) or 20, 40 or 60 mg CEO+β-CD/L of FW. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition.

The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In European seabass, under industrial farm conditions, only few plasmatic parameters varied upon stunning treatments (control condition, CI, - 0.2 ºC) (**Table 9**). In fact, only the plasmatic pH was up regulated in fish stunned with 5 and 10 mg CEO+β-CD (7.24±0.02 and 7.25±0.02, respectively), compared with the pH of the control group (7.13±0.04).

Table 9. Plasmatic parameters level in European seabass stunned with CEO+β-CD under industrial farm conditions.

Stunning treatments	CEO+β-CD (mg/kg)	pO_2 (mmHg)	pCO_2 (mmHg)	HCO_3 (mmol/L)	TCO_2 (mmol/L)	SO_2 (%)	Base Excess (mmol/L)	pH
1	0	252.89 ± 7.60^a	17.69 ± 1.26^a	5.69 ± 0.34^a	6.44 ± 0.28^a	100^a	-23.78 ± 0.97^a	7.12 ± 0.05^a
	15	262.75 ± 5.63^a	19.81 ± 1.53^a	6.21 ± 0.31^a	6.75 ± 0.28^a	100^a	-23.25 ± 0.32^a	7.11 ± 0.02^a
	30	261.03 ± 4.97^a	19.79 ± 1.08^a	5.40 ± 0.14^a	6.11 ± 0.24^a	100^a	-25.11 ± 0.29^a	7.04 ± 0.04^a
	0	234.30 ± 4.73^a	17.75 ± 1.41^a	5.97 ± 0.19^a	6.40 ± 0.22^a	100^a	-22.91 ± 0.75^a	7.13 ± 0.04^a
	5	232.75 ± 4.45^a	15.73 ± 1.08^a	6.71 ± 0.24^a	7.25 ± 0.32^a	100^a	-20.51 ± 0.45^a	7.24 ± 0.02^b
	10	244.71 ± 3.61^a	14.97 ± 0.59^a	6.51 ± 0.18^a	7.10 ± 0.18^a	100^a	-20.80 ± 0.35^a	7.25 ± 0.02^b

Stunning treatment: CI at - 0.2 °C of 20 mm crystals ice size mixed with SW (ratio 1:1) (control condition) or containing 5, 10, 15 or 30 mg CEO+β-CD/kg ice.

Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In rainbow trout, under industrial farm conditions, no differences in any plasmatic parameter level between fish treated with different concentrations (5, 15 and 35 mg of CEO+β-CD/kg in CI at - 0.2 °C) and control fish (CI at - 0.2 °C) were observed (**Table 10**).

Table 10. Plasmatic parameters level in rainbow trout stunned with CEO+β-CD under industrial farm conditions.

Stunning treatments	CEO+β-CD (mg/kg)	pO_2 (mmHg)	pCO_2 (mmHg)	HCO_3 (mmol/L)	TCO_2 (mmol/L)	SO_2 (%)	Base Excess (mmol/L)	pH
1	0	271.51 ± 4.25^a	30.59 ± 1.62^a	11.74 ± 0.78^a	12.81 ± 0.85^a	100^a	-16.51 ± 1.23^a	7.19 ± 0.03^a
	5	284.13 ± 5.76^a	28.23 ± 2.88^a	12.38 ± 0.56^a	13.25 ± 0.52^a	100^a	-14.63 ± 0.96^a	7.26 ± 0.04^a
	15	268.88 ± 8.66^a	30.01 ± 3.01^a	13.41 ± 0.99^a	14.51 ± 1.52^a	100^a	-15.38 ± 1.22^a	7.24 ± 0.08^a
	35	286.67 ± 8.69^a	31.05 ± 2.21^a	14.11 ± 0.70^a	15.01 ± 0.73^a	100^a	-12.88 ± 1.07^a	7.26 ± 0.04^a

Stunning treatment: CI at - 0.2 °C of 20 mm crystals ice size mixed with FW (ratio 1:1) (control condition) or containing 5, 15 or 35 mg CEO+β-CD/kg ice.

Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.1.2.4. Cortisol

In gilthead seabream, under experimental farm conditions, no differences in plasmatic cortisol level were observed when 20 mg CEO+β-CD/L was incorporated in SW at 27.3-28.0 °C (**Figure 27a**), while this level decreased when 15 or 20 mg CEO+β-CD/kg was incorporated in CI (- 0.2 °C), in both cases compared with the level recorded in the control group (CI at - 0.2 °C) (**Figure 27b**). In contrast, no differences were observed in fish treated with 15 mg CEO+β-CD/kg and incorporated in LI at - 1.5 °C compared with fish treated with LI at - 1.5 ºC alone (**Figure 27c**).

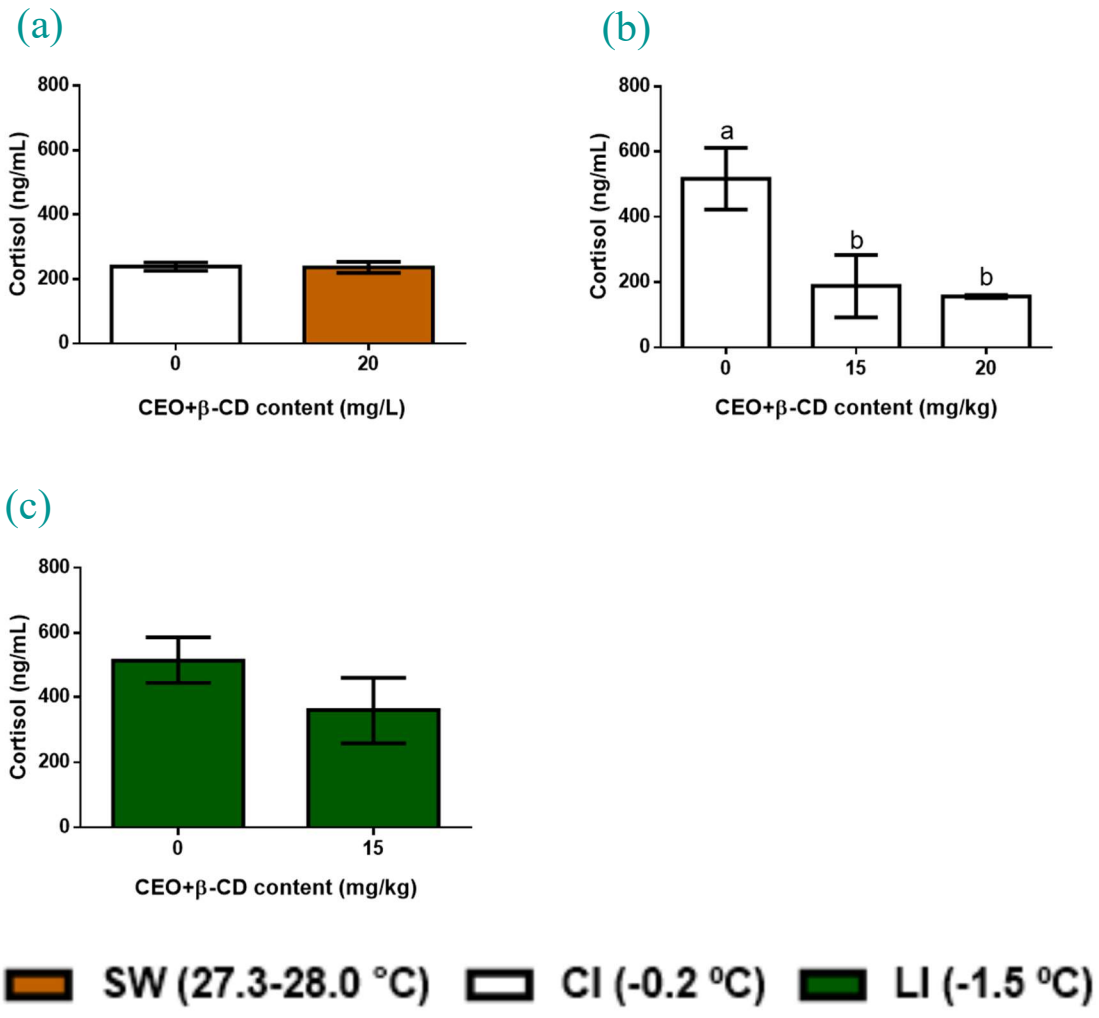


Figure 27. Plasmatic cortisol level of gilthead seabream stunned with CEO+β-CD incorporated in SW (a), CI (b), or LI (c) under experimental farm conditions. Fish stunned with **(a)** CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (ratio 1:1) (control condition) or SW at 27.3-28.0 °C containing 20 mg CEO+β-CD/L SW. **(b)** CI in control condition or containing 15 or 20 mg CEO+β-CD/kg ice. **(c)** LI at - 1.5 °C mixed with SW (ratio 1:1), alone, or with CEO+β-CD at 15 mg/kg ice. Each value represents the mean ± S.E.M of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In gilthead seabream, under industrial farm conditions, no differences in the plasmatic cortisol level between fish treated with low concentrations of CEO+β-CD/kg (5, 10 or 15 mg) incorporated in CI with crystals ice of 15 or 20 mm size and fish from control condition (CI, ice size of 20 mm, 1.0-2.0 °C) were observed (**Figure 28**).

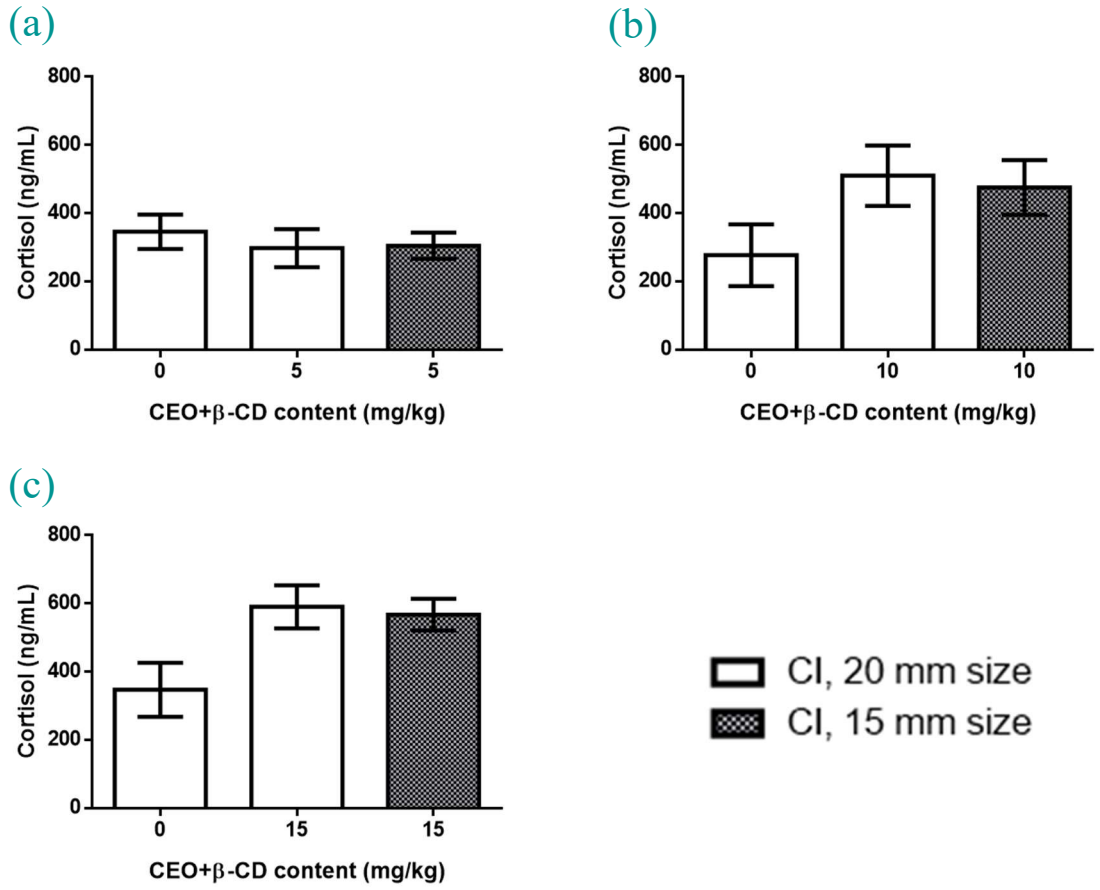


Figure 28. Plasmatic cortisol level of gilthead seabream stunned with CEO+β-CD incorporated in different sizes of ice crystals (20-15 mm) under industrial farm conditions. CI at 1.0-2.0ºC with ice crystal size of 20 mm mixed with SW (ratio 1:2) (control condition). **(a)** CI in control condition or containing 5 mg CEO+β-CD/kg of ice of 20 or 15 mm ice crystal sizes. **(b)** CI in control condition or containing 10 mg CEO+β-CD/kg of ice of 20 or 15 mm ice crystal sizes. **(c)** CI in control condition or containing 15 mg CEO+β-CD/kg of ice of 20 or 15 mm ice crystal sizes. Each value represents the mean ± S.E.M. of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In Atlantic salmon specimens, under experimental farm conditions, 40 mg CEO+β-CD/L SW decreased the plasmatic cortisol level compared with the level of the fish from the control groups (SW at 4.0-6.0 °C, with or without, 240 mg β-CD/L) (**Figure 29a**). However, no differences were observed between control and treated (50 or 60 mg CEO+β-CD/L) fish groups (**Figure 29a**).

In Nile tilapia, however, only the highest concentration (60 mg of CEO+β-CD/L) used decreased the plasmatic cortisol level compared to the level of fish from the control groups (FW at 27.0-28.0 °C, with or without, 240 mg β-CD/L) (**Figure 29b**) or from the groups treated with the other two concentrations assayed (20 or 40 mg of CEO+β-CD/L) (**Figure 29b**).

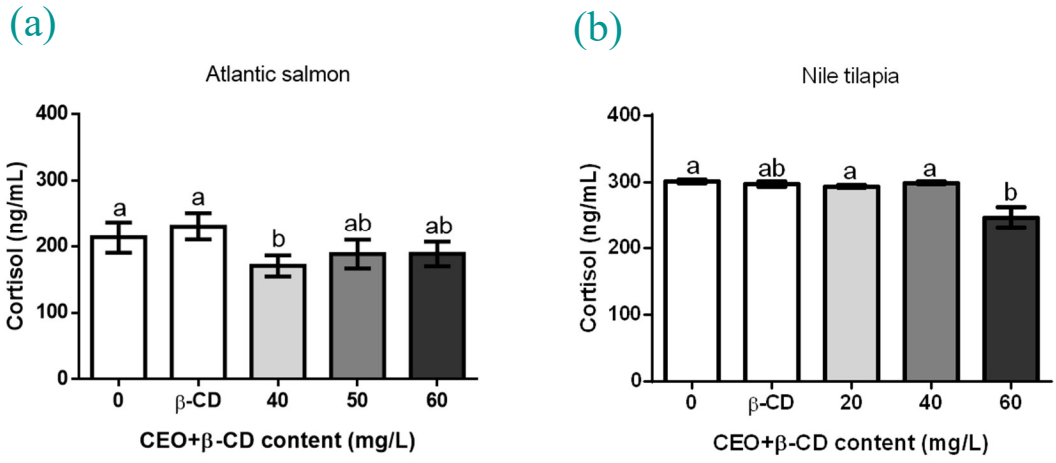


Figure 29. Plasmatic cortisol level Atlantic salmon (a) and Nile tilapia (b) stunned with CEO+β-CD under experimental farm conditions. **(a)** Atlantic salmon treated with SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L SE (control 2) or 40, 50 or 60 mg CEO+β-CD/L water. **(b)** Nile tilapia treated with FW at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L FW (control 2) or 20, 40 or 60 mg CEO+β-CD/L water. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In European seabass, the plasmatic cortisol level decreased only with the lowest concentration assay of CEO+β-CD/kg used (5 mg) compared to the level of the control fish (CI at - 0.2 °) (**Figure 30a, b**).

In addition, a reduction in the level of plasmatic cortisol level occurred in rainbow trout treated with 5 or 15 mg of CEO+β-CD/kg compared to the level of the fish from the control group (CI at - 0.2 °C) (**Figure 30c**).

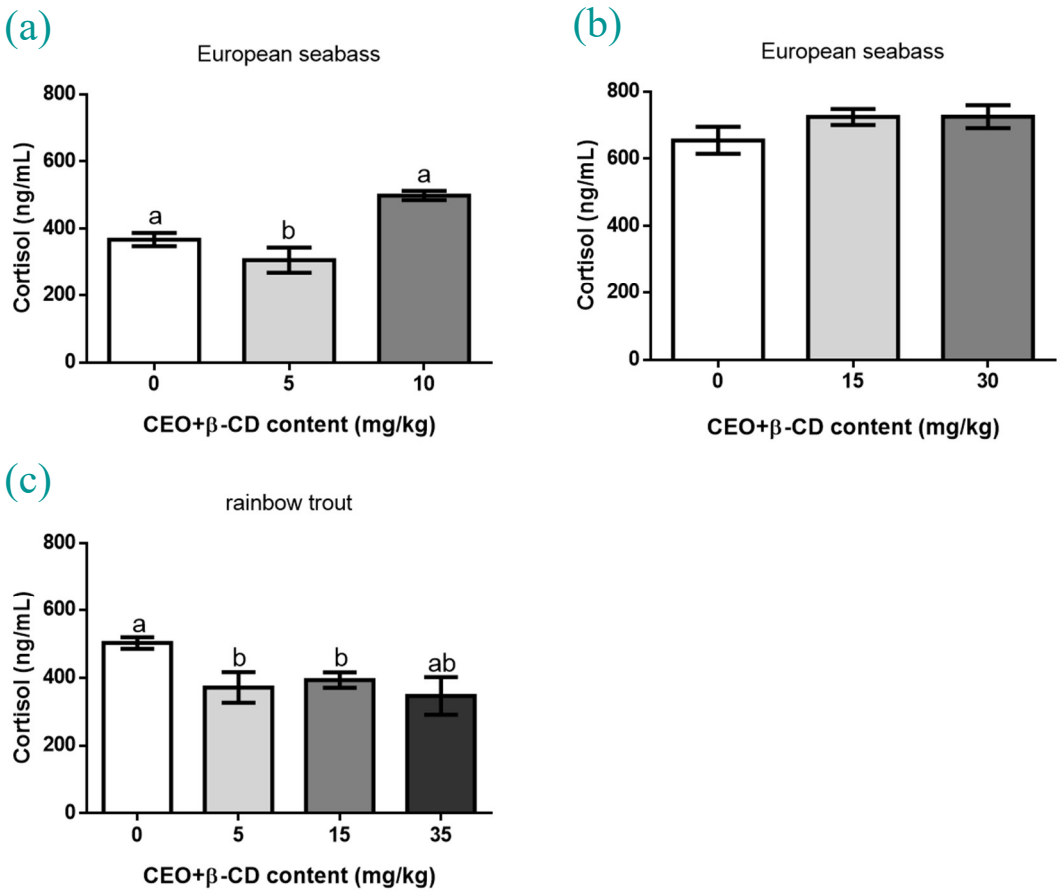


Figure 30. Plasmatic cortisol level in European seabass (a, b) and rainbow trout (c) stunned with CEO+β-CD under industrial farm conditions. European seabass and rainbow trout stunned with CI mixed with SW (ratio 1:1) at - 0.2 °C with ice crystals of 20 mm size (control condition). **(a, b)** European seabass treated with CI in control condition or containing 5, 10, 15 or 30 mg CEO+β-CD/kg ice. **(c)** Rainbow trout treated with CI in control condition or containing 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$)

IV.1.3. Inflammatory cytokines in the head kidney and ryanodine receptor in skeletal muscle in gilthead seabream

To fulfill the third objective of the thesis, the expression level of the genes that code for a pro-inflammatory *il1-β* and an anti-inflammatory *tgf-β1* cytokines, and a heat shock protein (*Hsp70*) was analyzed in the head kidney of gilthead seabream under experimental farm conditions. In addition, the muscle mRNA profile of *RyR3* gene that code for a ryanodine receptor involved in the control of intracellular Ca^{+2} release, was also analyzed.

No differences in the mRNA expression level of *il1-β* gene was observed between fish stunned with 15 mg CEO+β-CD/kg (CI at - 0.2 °C or LI at - 1.5 °C) and their respective control groups (CI at - 0.2 °C or LI at - 1.5 °C, respectively); neither between both control groups (CI at -0.2 °C and LI at - 1.5 °C) (**Figure 31a**).

Curiously, the mRNA expression level of *tgf-β1* gene decreased in fish treated with 15 mg CEO+β-CD/kg (CI at - 0.2 ºC) compared to the level of the control group (CI at - 0.2 °C) (**Figure 31b**). In contrast, when LI at - 1.5 °C was used, no differences in the transcription level of *tgf-β1 gene* occurred between fish stunned with or without 15 mg CEO+β-CD/kg (**Figure 31b**).

Similarly, to *il1-β* gene, the expression pattern of *Hsp70* gene was not altered by 15 mg CEO+β-CD/kg (CI at - 0.2 °C or LI at - 1.5 °C) compared to their respective control (CI at - 0.2 °C or LI at - 1.5 °C) (**Figure 31c**). Moreover, no differences between both control groups (CI at - 0.2 °C and LI at - 1.5 °C) were observed (**Figure 31c**).

However, the mRNA expression level of *RyR3* gene in skeletal muscle increased only when 15 mg CEO+β-CD/kg was incorporated into LI at - 1.5 °C compared to the level of the control group (LI at - 1.5 °C) (**Figure 31d**). Moreover, no differences between both control groups (CI at - 0.2 °C and LI at - 1.5 °C) were observed (**Figure 31d**).

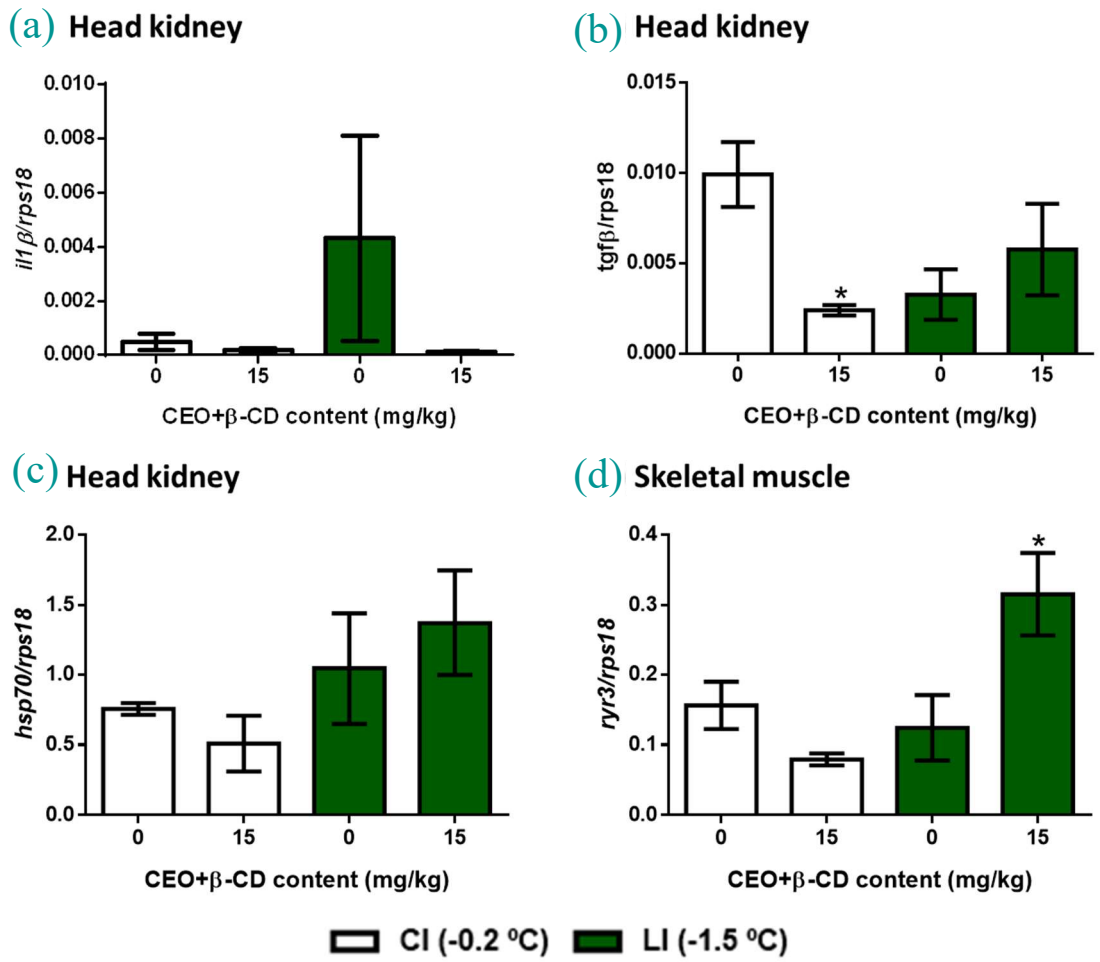


Figure 31. The expression level of *il1-β* (a), *tgf-β1* (b) and *Hsp70* (c) genes in head kidney and of *RyR3* (d) gene in muscle of gilthead seabream stunned with CEO+β-CD in experimental farm conditions. CI at - 0.2 °C containing 0 (control condition) or 15 mg CEO+β-CD/kg ice mixed with SW (ratio 1:1) or LI at - 1.5 ºC containing 0 (control) or 15 mg CEO+β-CD/kg ice mixed with SW (ratio 1:1). Each value represents the mean ± S.E.M. of n=4-12 fish per group in each sampling condition. Asterisks (*) indicate statistically significant differences between experimental and control groups according to a Student-t test ($P \leq 0.05$).

IV.1.4. Humoral activities

The fourth aim of this study was to determine whether the innate immune system is affected by the stunning treatments. For that, we analyzed some humoral activities, such as peroxidase, bactericidal, antiprotease and protease, in plasma and skin mucus of gilthead seabream, European seabass, rainbow trout, Atlantic salmon and Nile tilapia.

IV.1.4.1. Peroxidase activity

In gilthead seabream, the plasmatic peroxidase activity level decreased when treated with CEO+β-CD in experimental (**Figure 32a, b**) and industrial farm conditions (**Figure 32c**) at different concentrations of CEO+β-CD (20 mg/L or 15 and 20 mg/kg of CI at - 0.2 °C under experimental conditions; 15 mg/kg of CI at 1.0-2.0 °C under industrial conditions) in all cases compared with the level of the fish from the respective control group (CI at - 0.2 °C, experimental conditions, or at 1.0-2.0 °C, industrial conditions).

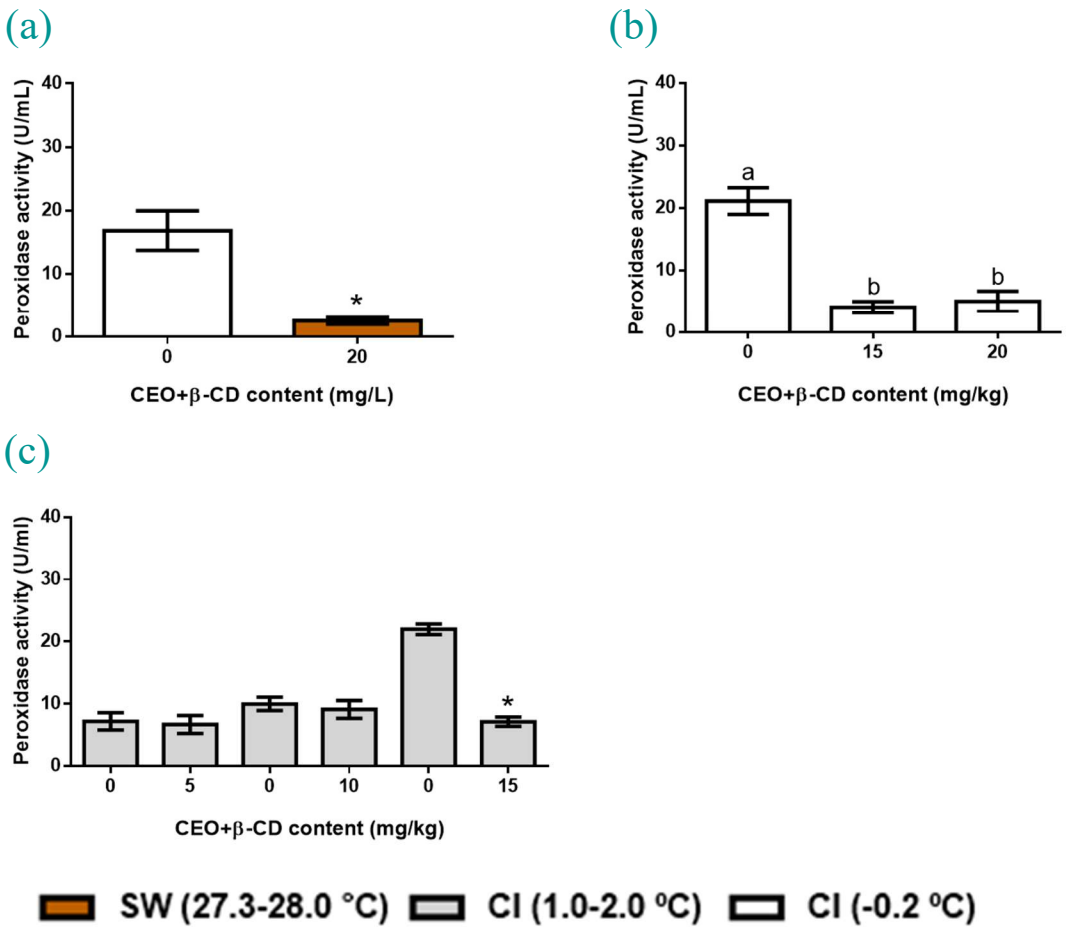


Figure 32. Peroxidase activity level in plasma of gilthead seabream stunned with CEO+β-CD under experimental (a, b) and industrial (c) farm conditions. In experimental farm conditions, fish were stunned with CI at - 0.2 °C with 20 mm ice crystal size mixed with SW (ratio 1:2) (control condition). **(a)** CI in control condition or with SW at 27.3-28.0 °C containing 20 CEO+β-CD/L SW. **(b)** CI in control condition or containing 15 or 20 mg CEO+β-CD/kg ice. In industrial farm conditions, fish were stunned with CI at 1.0-2.0 °C of 20 mm ice crystal size mixed with SW (ratio 1:2) (control condition). **(c)** CI in control condition or containing 5, 10 or 15 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10 fish per group in each sampling condition. Asterisks (*) indicate statistically significant differences between experimental and control groups according to a t-Student test (a, c) and letters indicate statistically significant differences between groups accordingly with one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$) (b).

In Atlantic salmon, no differences in the plasmatic peroxidase activity level between fish treated with CEO+β-CD/L (40, 50 or 60 mg; SW, at 4.0-6.0 °C) and control fish (SW at 4.0-6.0 °C with or without 240 mg CEO+β-CD/L) were observed (**Figure 33a**). Moreover, no differences were observed betwwen the two control conditions assayed (**Figure 33a**).

However, the plasmatic peroxidase activity level of Nile tilapia increased only with the highest concentration of CEO+β-CD used (60 mg/L), compared with the level observed in fish treated with the lowest concentrations (20 or 40 mg CEO+β-CD/L) and both control conditions (FW at 27.0-29.0 °C, with or without 240 mg β-CD/L) (**Figure 33b**).

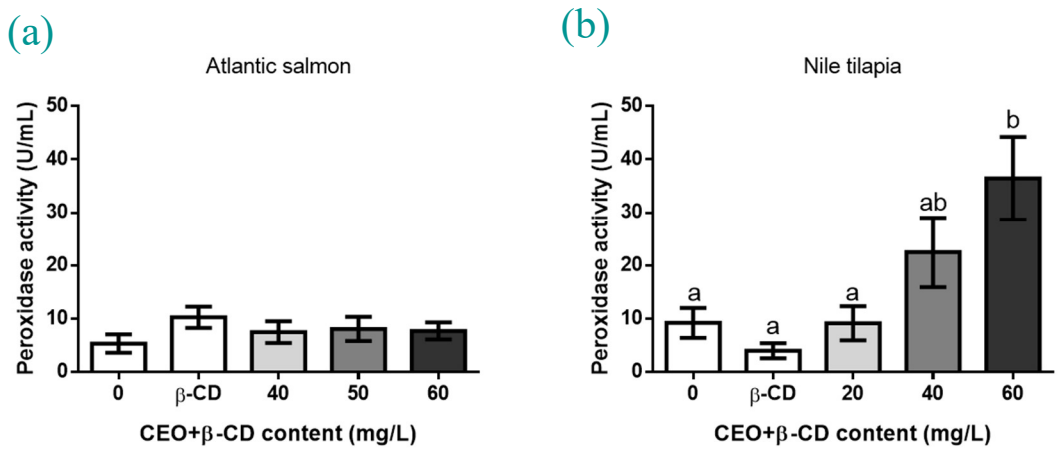


Figure 33. Peroxidase activity level in plasma of Atlantic salmon (a) and Nile tilapia (b) stunned with CEO+β-CD under experimental farm conditions. (a) Atlantic salmon treated with SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L SW (control 2) or 40, 50 or 60 mg CEO+β-CD/L SW. (b) Nile tilapia treated with FW at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L FW (control 2) or 20, 40 or 60 mg CEO+β-CD/L FW. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

Regarding the peroxidase activity levels in skin mucus, no differences were found between the different groups studied neither in Atlantic salmon (**Figure 34a**) nor in Nile tilapia (**Figure 34b**).

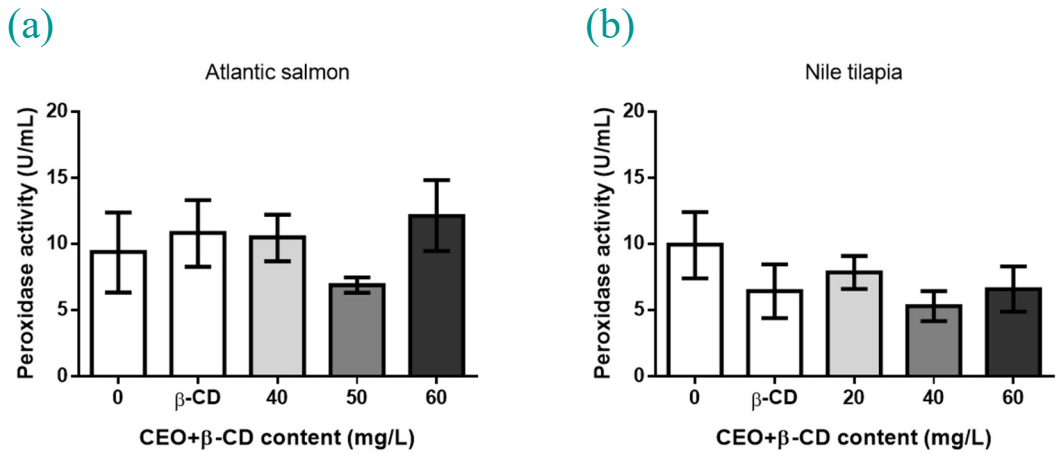


Figure 34. Peroxidase activity level in skin mucus of Atlantic salmon (a) and Nile tilapia (b) stunned with CEO+β-CD under experimental farm conditions. **(a)** Atlantic salmon treated with SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L SW (control 2) or 40, 50 or 60 mg CEO+β-CD/L SW. **(b)** Nile tilapia treated with FW at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L FW (control 2) or 20, 40 or 60 mg CEO+β-CD/L FW. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In European seabass, the plasmatic peroxidase activity level decreased when 30 mg of CEO+β-CD/kg were included in the CI at - 0.2 °C compared with the level of the control conditions (CI at - 0.2 ºC) (**Figure 35b**), while no effect was observed with 5, 10 (**Figre 35a**) or 15 mg CEO+β-CD/kg (**Figure 35b**).

However, in rainbow trout, the plasmatic peroxidase activity levels decreased with the lowest concentrations of CEO+β-CD/kg used (5 or 15 mg), while no effect was observed with 35 mg CEO+β-CD/kg (**Figure 35c**); in all cases compared to fish treated with CI at - 0.2 °C, as control group.

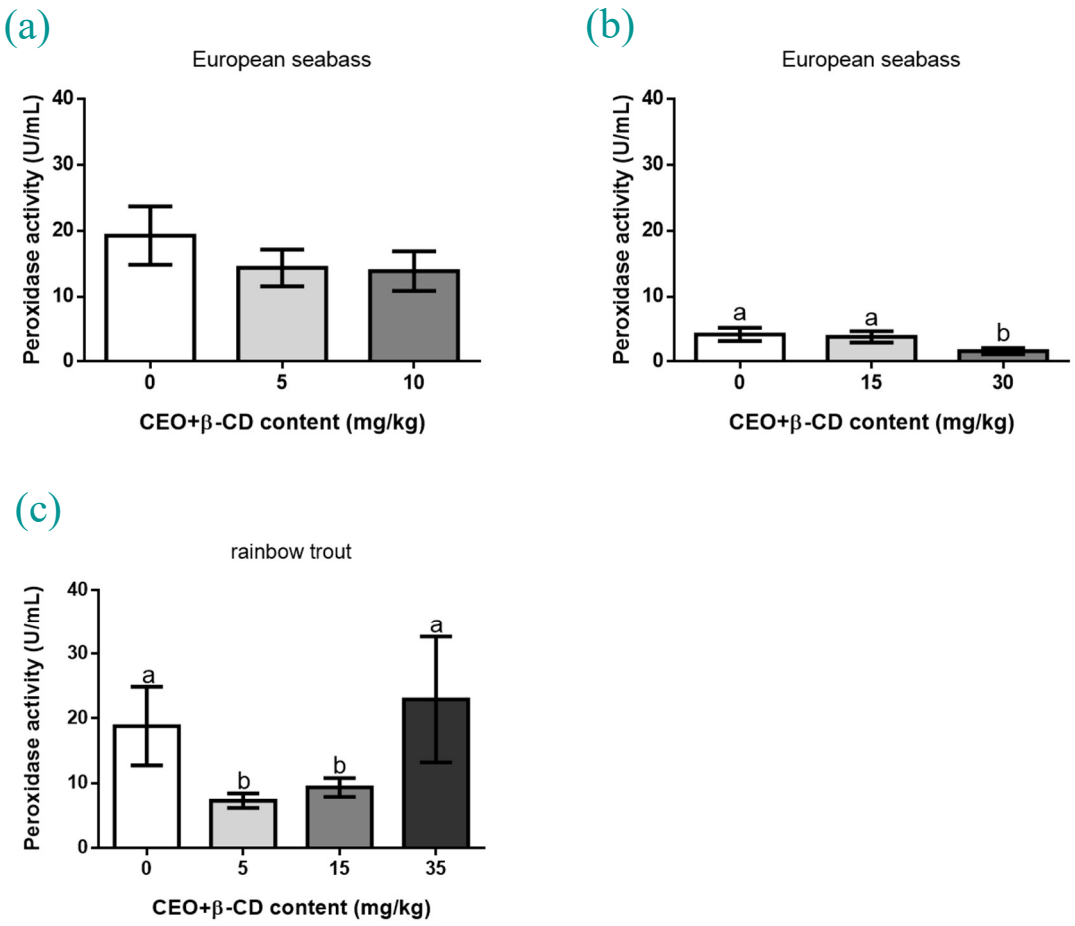


Figure 35. Peroxidase level in plasma of European seabass (a, b) and rainbow trout (c) stunned with CEO+β-CD under industrial farm conditions. European seabass and rainbow trout stunned with CI at -0.2 °C of 20 mm ice crystal size mixed with SW (European seabass)/ FW (rainbow trout) (ratio 1:1) (control condition). **(a, b)** European seabass treated with CI in control condition or containing 5, 10, 15 or 30 mg CEO+β-CD/kg ice. **(b)** Rainbow trout treated with CI in control condition or containing 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

As occurred with the plasmatic level, the peroxidase activity level in the skin mucus of European seabass decreased only with the highest concentrations of CEO+β-CD used (15 or 30 mg/kg) (**Figure 36b**), while in the skin mucus of rainbow trout only the lowest concentration used (5 mg/kg) promoted a decrease (**Figure 36b**); in all cases compared to fish treated with CI at - 0.2 °C, as control group.

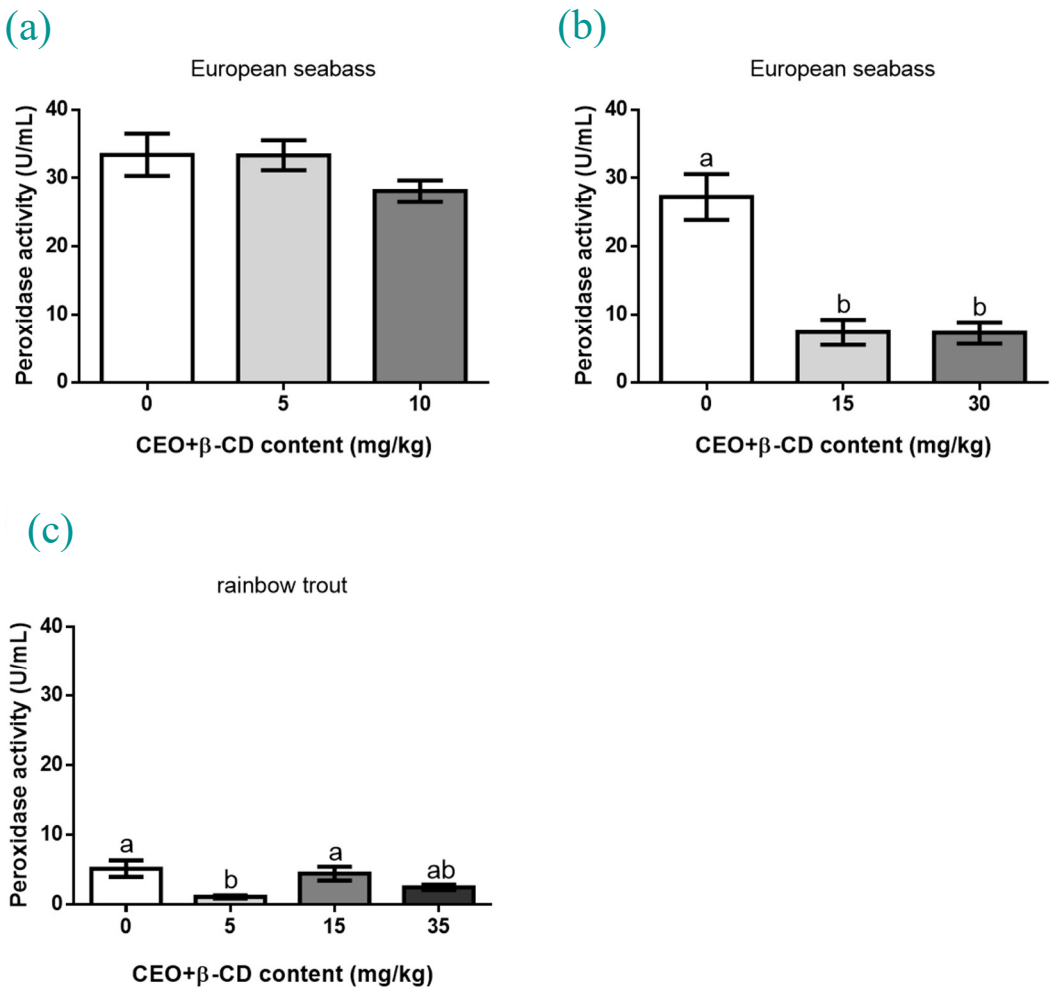


Figure 36. Peroxidase activity level in skin mucus of European seabass (a, b) and rainbow trout (b) stunned with CEO+β-CD under industrial farm conditions. European seabass and rainbow trout stunned with CI at - 0.2 °C of 20 mm ice crystal size mixed with SW (European seabass)/ FW (rainbow trout) (ratio 1:1) (control condition). **(a, b)** European seabass treated with CI in control condition or containing 5, 10, 15 or 30 mg CEO+β-CD/kg ice. **(c)** Rainbow trout treated with CI in control condition or containing 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.1.4.2. Bactericidal activity

Interestingly, in gilthead seabream, CEO+β-CD did not modify the plasmatic bactericidal activity level in plasma in experimental (**Figure 37a, b**) or industrial farm conditions (**Figure 37c**) in any of the conditions assayed.

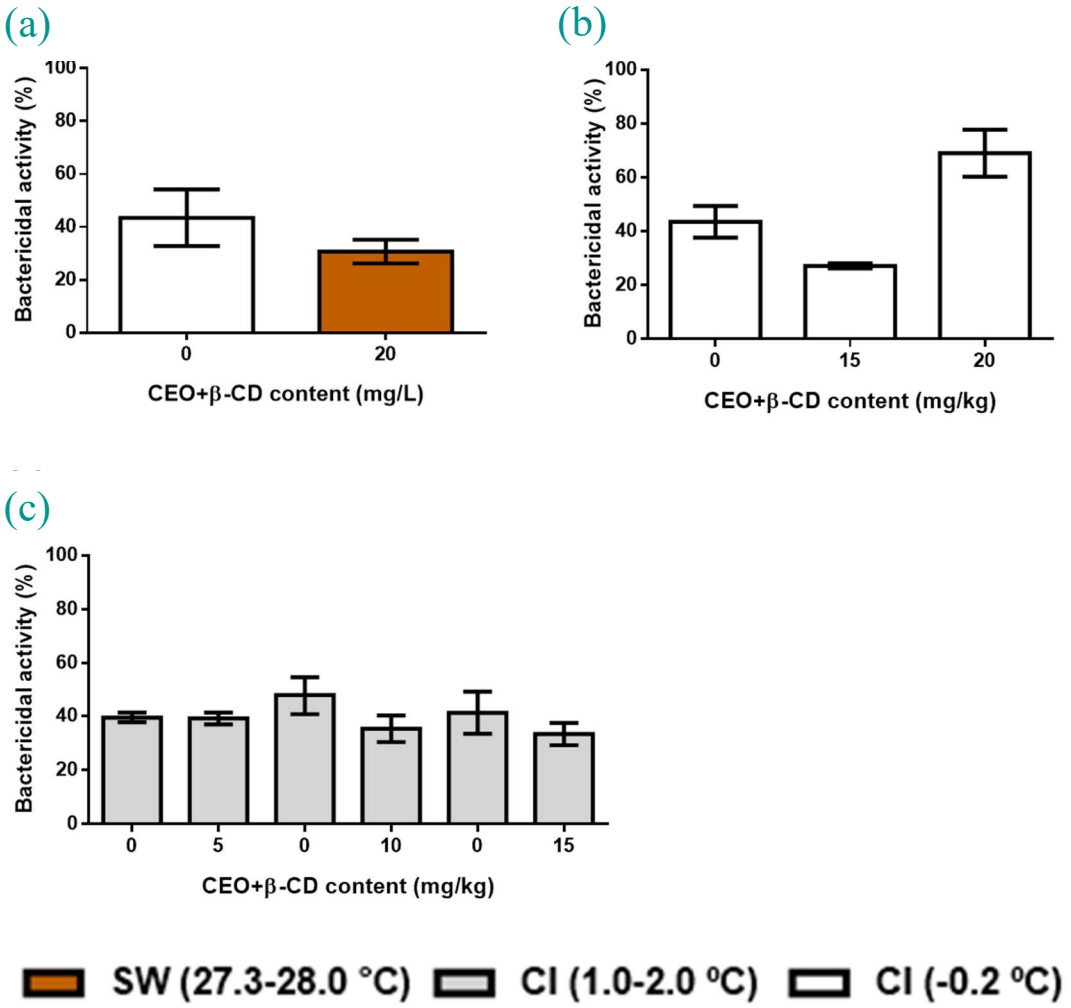


Figure 37. Bactericidal activity level in plasma of gilthead seabream stunned with CEO+β-CD under experimental and industrial farm conditions. In experimental farm conditions, fish were stunned with CI at - 0.2 °C ice crystals of 20 mm size mixed with SW (ratio 1:2) as control condition. (a) CI in control condition or with SW at 27.3-28.0 °C containing 20 CEO+β-CD/L SW. **(b)** CI in control condition or containing 15 or 20 mg CEO+β-CD/kg ice. In industrial farm conditions, fish were stunned with CI at 1.0-2.0 °C with ice crystals of 20 mm size mixed with SW (ration 1:2) as control condition. **(c)** CI in control condition or containing 5, 10 or 15 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10 fish per group in each sampling condition. Asterisks (*) indicate statistically significant differences between experimental and control groups according to a Student-t test (a, c) and letters indicate statistically significant differences between groups accordingly with one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$) (b).

In Atlantic salmon (**Figure 38a**) and Nile tilapia (**Figure 38b**), under experimental farm conditions, as occurred in giltehead seabream, CEO+β-CD did not modify the plasmatic bactericidal activity level compared with the level of their respective control groups (Atlantic salmon, SW at 4.0-6.0 ºC with or without 240 mg β-CD/L; Nile tilapia, FW at 27.0-29.0 °C with or without 240 mg β-CD/L). Moreover, no differences between the two control conditions were observed in both species (**Figure 38a, b**).

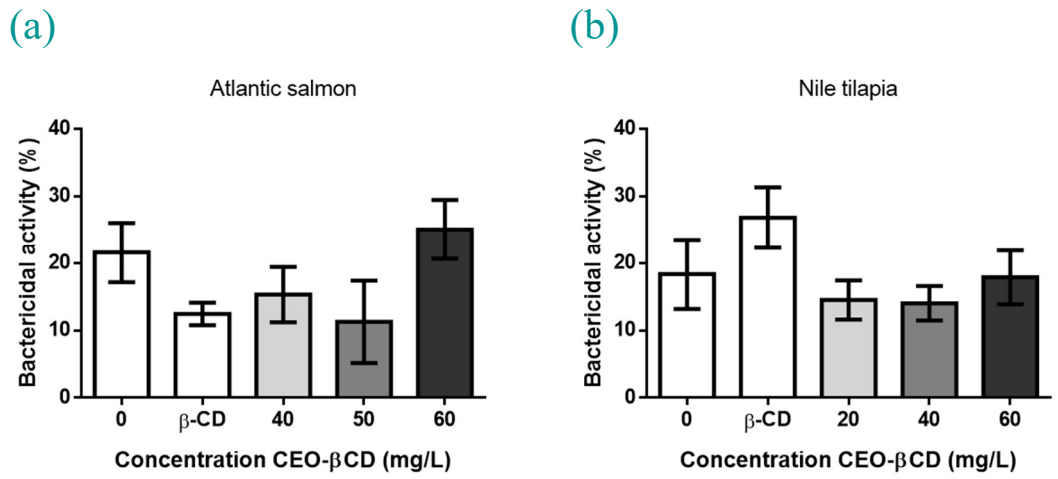


Figure 38. Bactericidal activity level in plasma of Atlantic salmon (a) and Nile tilapia (b) stunned with CEO+β-CD under experimental farm conditions. **(a)** Atlantic salmon treated with SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L SW (control 2) or 40, 50 or 60 mg CEO+β-CD/L SW. **(b)** Nile tilapia treated with FW at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L FW (control 2) or 20, 40 or 60 mg CEO+β-CD/L FW. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

Regarding the skin mucus, in Atlantic salmon, CEO+β-CD did not modify the bactericidal activity level compared with the level of the control conditions (**Figure 39a**). However, in Nile tilapia, a decrease of the bactericidal activity level of fish treated with 60 mg of CEO+β-CD/L was detected compared with the two control groups and the other CEO+β-CD/L doses assayed (**Figure 39b**).

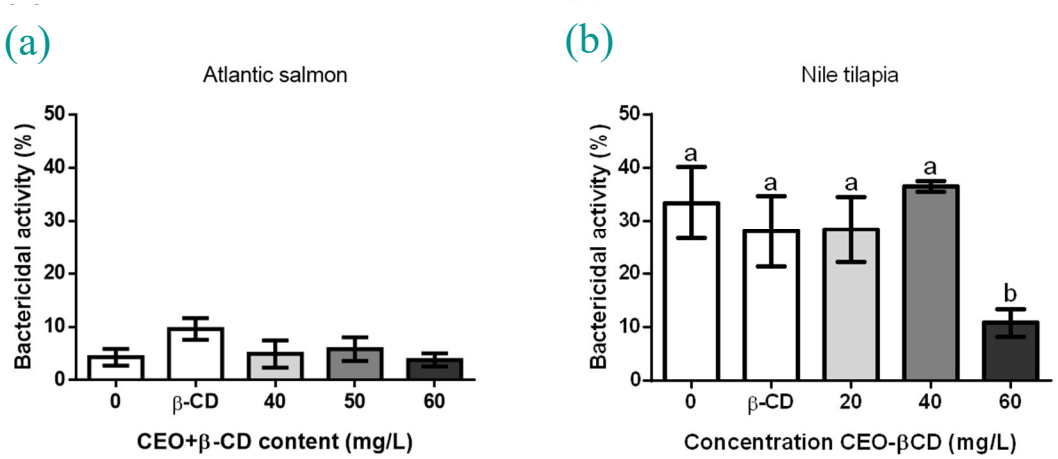


Figure 39. Bactericidal activity level in skin mucus of Atlantic salmon (a) and Nile tilapia (b) stunned with CEO+β-CD under experimental farm conditions. (a) Atlantic salmon treated with SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L SW (control 2) or 40, 50 or 60 mg CEO+β-CD/L SW. (b) Nile tilapia treated with at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L FW (control 2) or 20, 40 or 60 mg CEO+β-CD/L FW. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In European seabass, no significant differences were found in the lowest doses (5 and 10 mg/kg**) (Figure 40a)**, only the highest concentrations of CEO+β-CD used (15 and 30 mg/kg) decreased the plasmatic bactericidal activity level, compared to the level of the fish from the control group (CI at - 0.2 °C) (**Figure 40b**).

However, in rainbow trout, CEO+β-CD included in CI did not produce any modification in the plasmatic bactericidal activity at any concentration assayed compared with control fish (CI at - 0.2 °C) (**Figure 40c**).

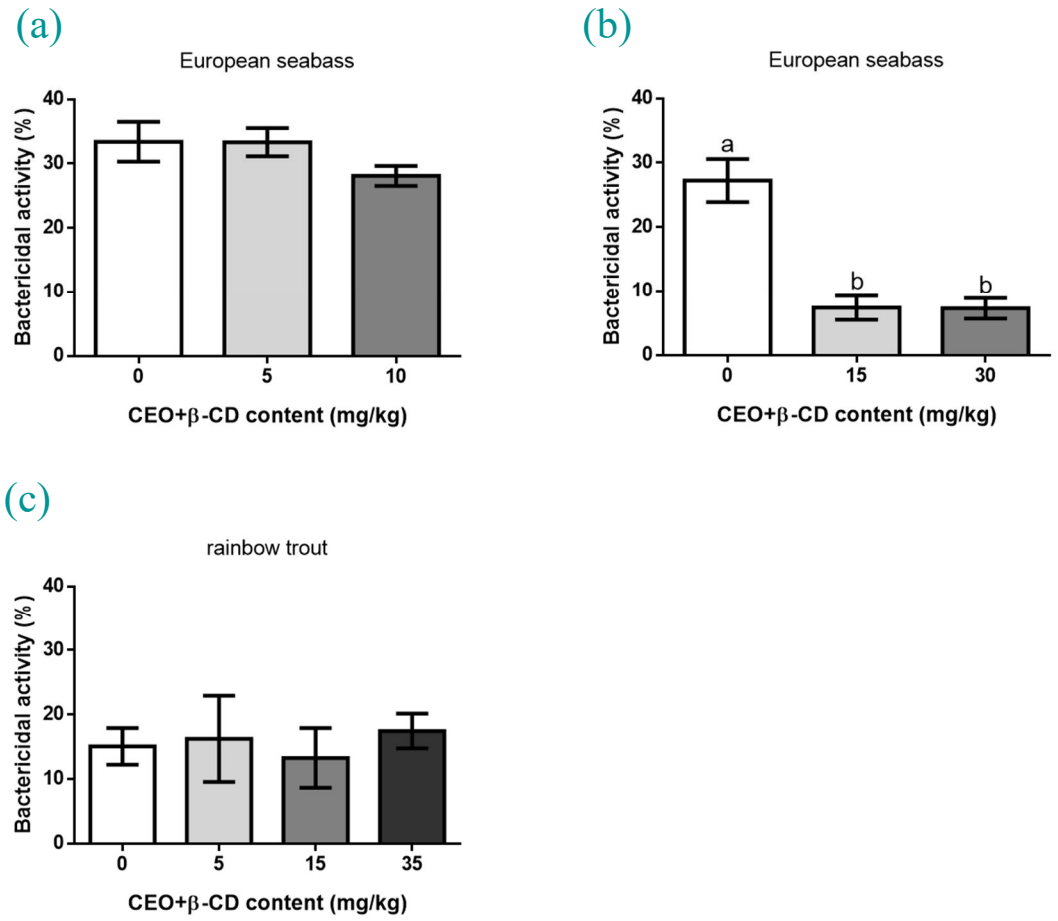


Figure 40. Bactericidal activity level in plasma of European seabass (a, b) and rainbow trout (c) stunned with CEO+β-CD under industrial farm conditions. European seabass and rainbow trout stunned with CI at - 0.2 °C of 20 mm ice crystal size mixed with SW (European seabass)/ FW (rainbow trout) (ratio 1:1) (control condition). **(a, b)** European seabass treated with CI in control condition or containing 5, 10, 15 or 30 mg CEO+β-CD/kg ice. **(c)** Rainbow trout treated with CI in control condition or containing 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$)

In the skin mucus of European seabass, only the lowest concentrations of CEO+β-CD used (5 and 10 mg/kg) decreased the bactericidal activity level compared to the level of the fish from the control group (CI at -0.2 °C) (**Figure 41a)** while no significant differences were found at highest doses (15 and 30 mg/kg) **(Figure 41b).**

In contrast, when 5 and 15 mg of CEO+β-CD/kg were used in rainbow trout, the bactericidal activity levels increased in the skin mucus compared with the level of the fish from the control group (CI at -0.2 °C), while no effect was observed with 35 mg CEO+β-CD/kg (**Figure 41c**).

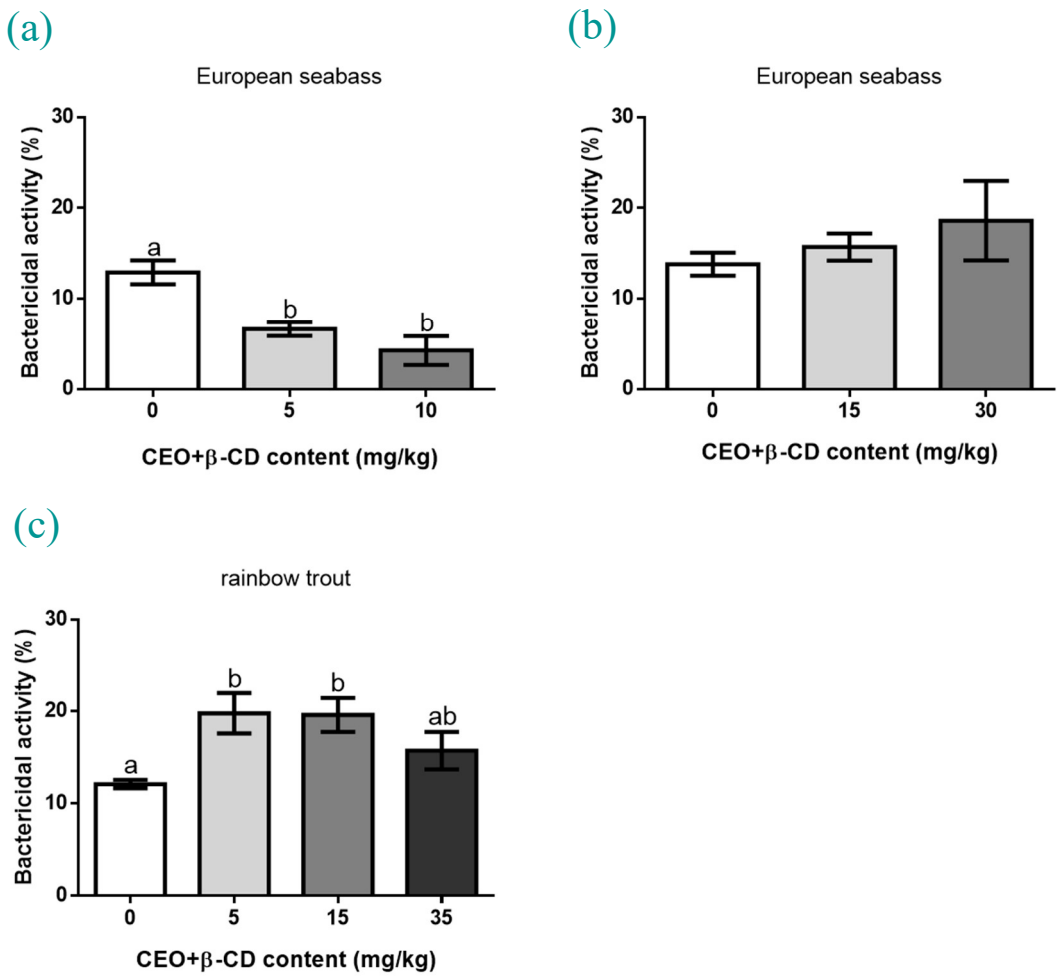


Figure 41. Bactericidal activity level in skin mucus of European seabass (a, b) and rainbow trout (c) stunned with CEO+β-CD under industrial farm conditions. European seabass and rainbow trout stunned with CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (European seabass)/ FW (rainbow trout) (ratio 1:1) (control condition). **(a, b)** European seabass treated with CI in control condition or containing 5, 10, 15 or 30 mg CEO+β-CD/kg ice. **(c)** Rainbow trout treated with CI in control condition or containing 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.1.4.3. Antiprotease activity

In gilthead seabream, under experimental farm conditions, CEO+β-CD incorporated in water at 27.3-28.0 °C did not alter the plasmatic antiprotease activity level compared to the level of the fish from the control group (CI at - 0.2 °C) (**Figure 42a**). Interestingly, when 20 mg CEO+β-CD/kg was incorporated in CI at - 0.2 °C, the plasmatic antiprotease activity level increased, compared to the level of the control condition (CI at - 0.2 °C) (**Figure 42b**). In contrast, under industrial farm conditions, the antiprotease activity level decreased when fish were stunned with 15 mg CEO+β-CD compared to the level of the fish from the control group (CI at 1.0-2.0 °C) (**Figure 42c**).

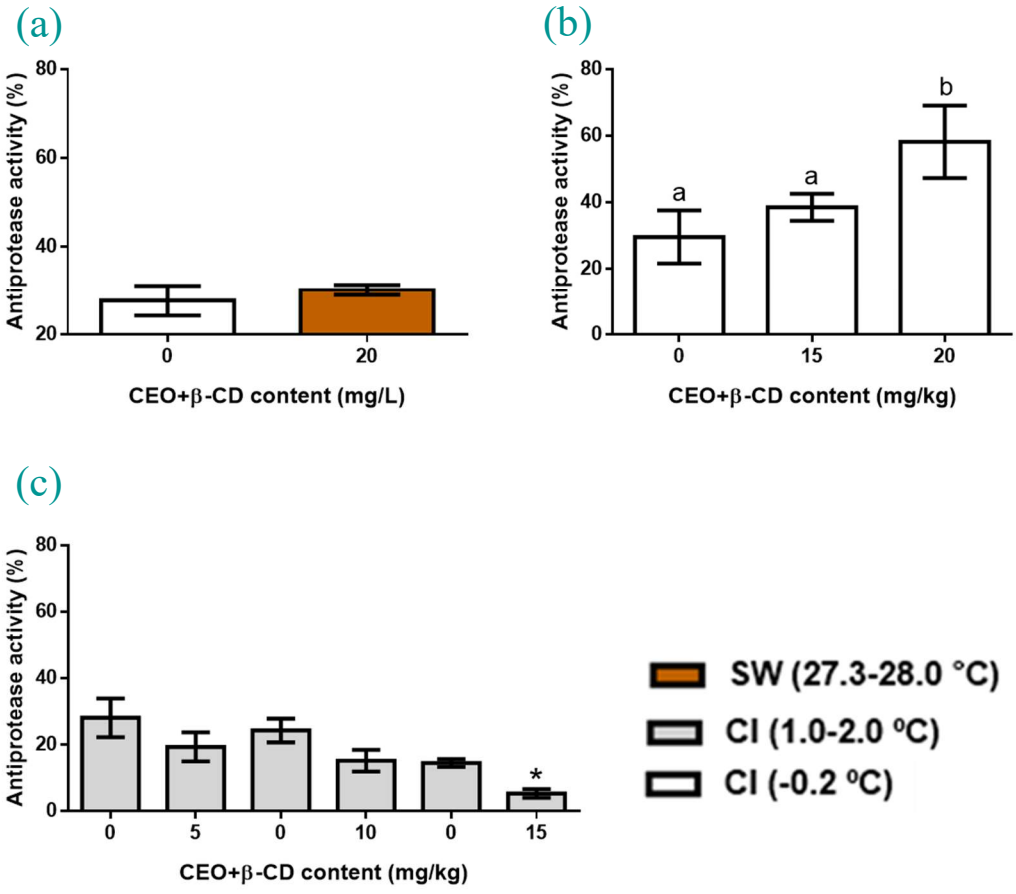


Figure 42. Antiprotease activity level in plasma of gilthead seabream stunned with CEO+β-CD under experimental and industrial farm conditions. In experimental farm conditions, fish were stunned with CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (ratio 1:2) (control condition). **(a)** CI in control condition or with SW at 27.3-28.0 °C containing 20 CEO+β-CD/L SW. **(b)** CI in control condition or containing 15 or 20 mg CEO+β-CD/kg ice. In industrial farm conditions, fish were stunned with CI at 1.0-2.0 °C with ice crystals of 20 mm size mixed with SW (ratio 1:2) (control condition). **(c)** CI in control condition or containing 5, 10 or 15 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10 fish per group in each sampling condition. Asterisks (*) indicate statistically significant differences between experimental and control groups according to Student-t test (a, c) and letters indicate statistically significant differences between groups accordingly with one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$) (b).

In Atlantic salmon and Nile tilapia, under experimental farm conditions, the plasmatic antiprotease activity level decreased in fish stunned with CEO+β-CD compared with the two control groups (SW at 4.0-6.0 °C, with or without 240 mg β-CD, or FW at 27.0-29.0 °C, respectively). Thus, this decrease was observed only with 60 mg CEO+β-CD/L in Atlantic salmon (**Figure 43a**), while it was observed with the highest and lowest concentration of CEO+β-CD (20 and 60 mg/L) in Nile tilapia (**Figure 43b**).

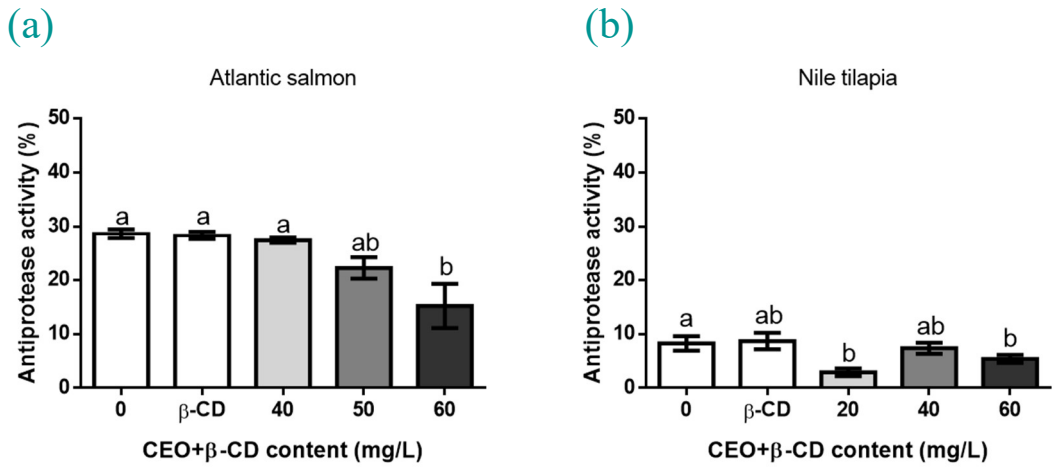


Figure 43. Antiprotease activity level in plasma of Atlantic salmon (a) and Nile tilapia (b) under experimental farm conditions. (a) Atlantic salmon stunned with SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L SW (control 2) or 40, 50 or 60 mg CEO+β-CD/L SW. (b) Nile tilapia stunned with FW at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L FW (control 2) or 20, 40 or 60 mg CEO+β-CD/L FW. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

Regarding the plasmatic antiprotease activity level of skin mucus, in Atlantic salmon, CEO+β-CD did not modify this level compared with the level of the control conditions (**Figure 44a**). Although a decrease and an increase was detected in skin mucus of Nile tilapia treated with 40 or 60 mg of CEO+β-CD/L, respectively, compared to fish treated with 50 mg/L and control groups (FW at 27.0-29.0 °C, with or without 240 mg β-CD/L) (**Figure 44b**).

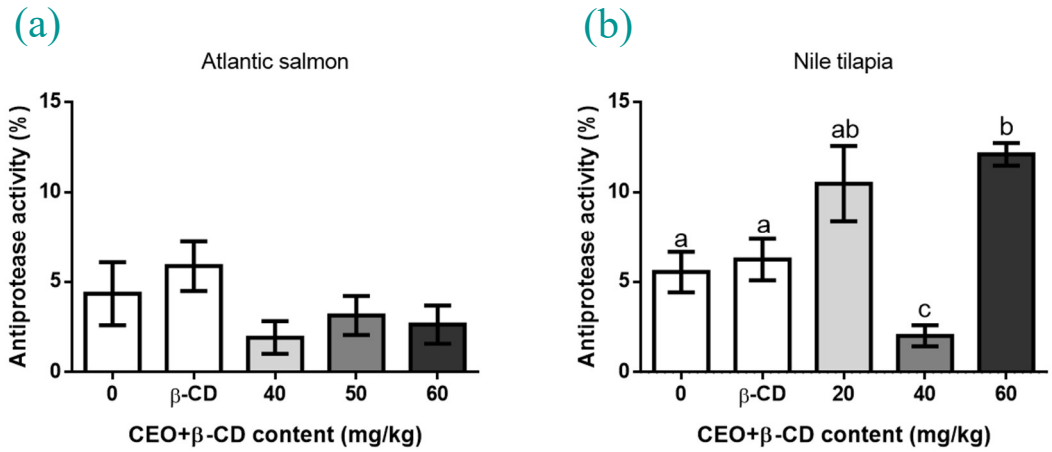


Figure 44. Antiprotease activity level in skin mucus of Atlantic salmon (a) and Nile tilapia (b) stunned with CEO+β-CD under experimental farm conditions. **(a)** Atlantic salmon treated with SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L SW (control 2) or 40, 50 or 60 mg CEO+β-CD/L SW. **(b)** Nile tilapia treated with FW at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L FW (control 2) or 20, 40 or 60 mg CEO+β-CD/L FW. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In European seabass, under industrial farm conditions, a decrease in the plasmatic antiprotease activity level was observed when 30 mg CEO+β-CD/kg was used compared with the level of the fish from the control group (CI at - 0.2 °C) (**Figure 45a, b**). In contrast, the plasmatic antiprotease activity level was similar in all the groups of the rainbow trout conditions, treated with CEO+β-CD/kg and control (**Figure 45c**).

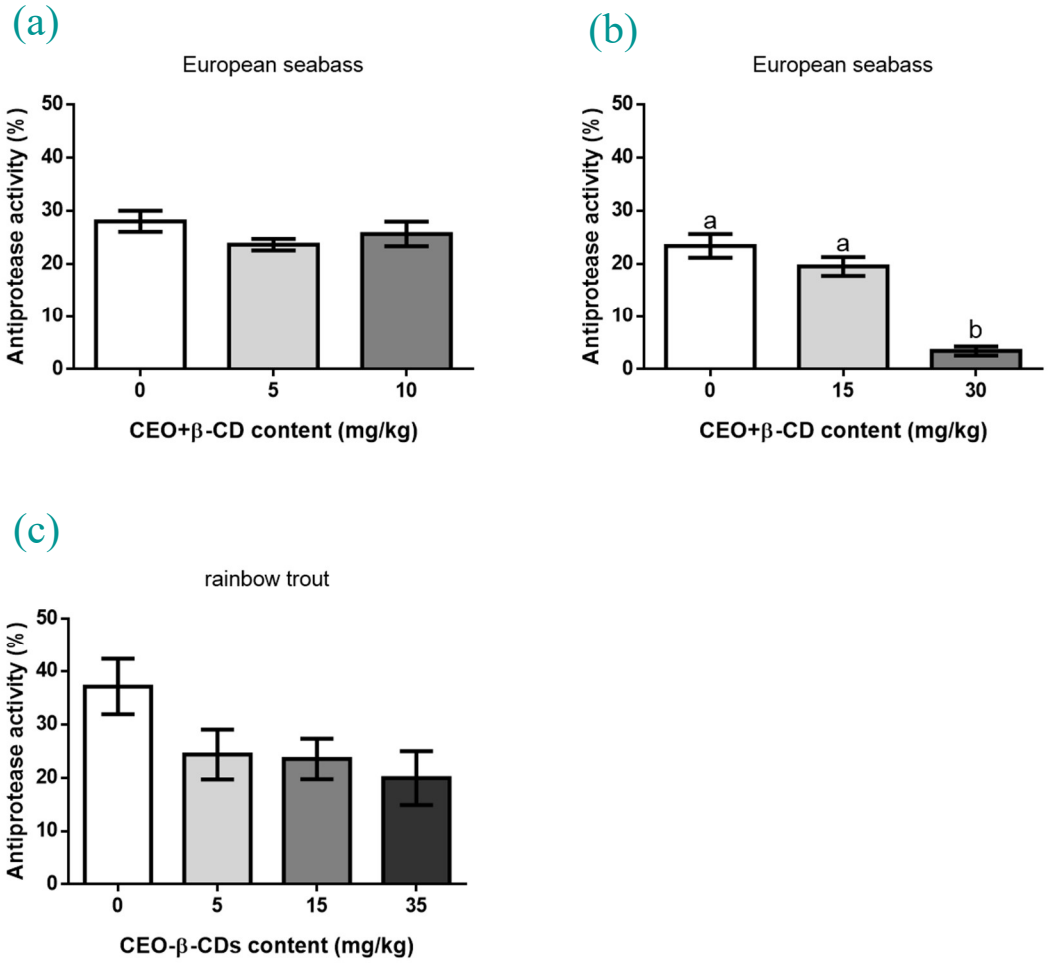


Figure 45. Antiprotease activity level in plasma of European seabass (a, b) and rainbow trout (c) stunned with CEO+β-CD under industrial farm conditions. European seabass and rainbow trout stunned with CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (European seabass)/FW (rainbow trout) (ratio 1:1) (control condition). **(a, b)** European seabass treated with CI in control condition or containing 5, 10, 15 or 30 mg CEO+β-CD/kg Ice. **(c)** Rainbow trout treated with CI in control condition or containing 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

Regarding skin mucus, a completely blockage of the antiprotease activity level was observed when 5 or 10 mg of CEO+β-CD/kg was used (**Figure 46a**). In contrast, the highest concentrations used (15 or 30 mg) did not alter this activity compared with the level of the control (CI at - 0.2 ºC) (**Figure 46b**).

In rainbow trout skin mucus, the antiprotease activity level was not detected in any condition assayed, treated with CEO+β-CD or control (**Figure 46c**).

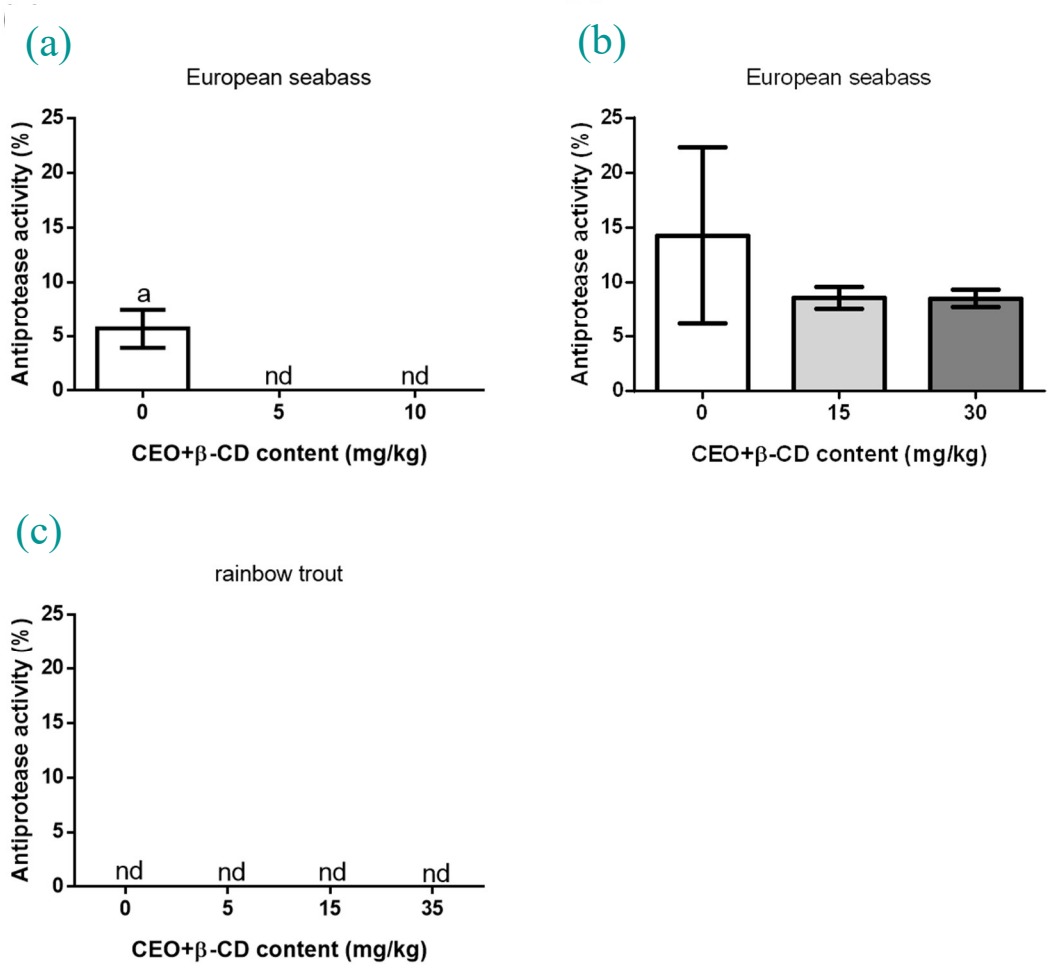


Figure 46. Antiprotease activity level in skin mucus of European seabass (a, b) and rainbow trout (c) stunned with CEO+β-CD under industrial farm conditions. European seabass and rainbow trout stunned with CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (European seabass)/ FW (rainbow trout) (ratio 1:1) (control condition). **(a, b)** European seabass treated with CI in control condition or containing 5, 10, 15 or 30 mg CEO+β-CD/kg ice. **(c)** Rainbow trout treated with CI in control condition or containing 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.1.4.4. Protease activity

In gilthead seabream, under experimental farm conditions, 20 mg/L of SW at 27.3-28.0 °C (**Figure 47a**) and 15 or 20 mg of CEO+β-CD/kg of CI at - 0.2 °C (**Figure 47b**) did not alter the plasmatic protease activity level compared with the control condition (CI at -0.2 °C).

Nevertheless, when fish were stunned under industrial farm conditions, the plasmatic protease activity level decreased when fish were treated with 10 mg of CEO+β-CD/kg (CI at 1.0-2.0 °C) compared with the level of the fish from the control group (CI at 1.0-2.0 ºC) (**Figure 47c**) but no with 5 or 15 mg of CEO+β-CD/kg (**Figure 47c**).

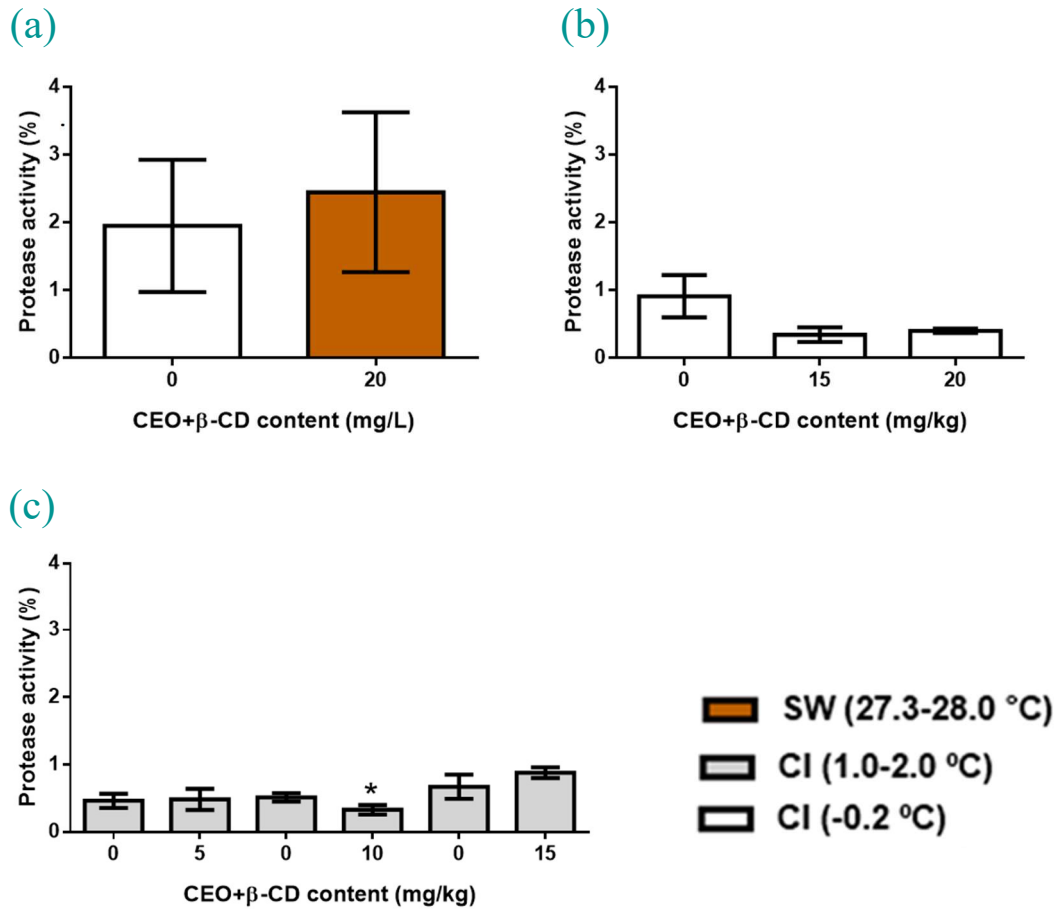


Figure 47. Protease activity level in plasma of gilthead seabream stunned with CEO+β-CD under experimental and industrial farm conditions. In experimental farm conditions, fish were stunned with CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (ratio 1:2) (control condition). **(a)** CI in control condition or with SW at 27.3-28.0 °C containing 20 CEO+β-CD/L SW. **(b)** CI in control condition or containing 15 or 20 mg CEO+β-CD/kg ice. In industrial farm conditions, fish were stunned with CI at 1.0-2.0 °C with ice crystals of 20 mm size mixed with SW (ratio 1:2) (control condition). **(c)** CI in control condition or containing 5, 10 or 15 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10 fish per group in each sampling condition. Asterisks (*) indicate statistically significant differences between experimental and control groups according to a Student-t test (a, c) and letters indicate statistically significant differences between groups accordingly with one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$) (b).

In Atlantic salmon and Nile tilapia, no differences in plasmatic **(Figure 48)** or skin mucus **(Figure 49)** protease activity level between the different experimental groups occurred neither in Atlantic salmon **(Figures 48a, 49a)** nor Nile tilapia **(Figures 48b, 49b)**.

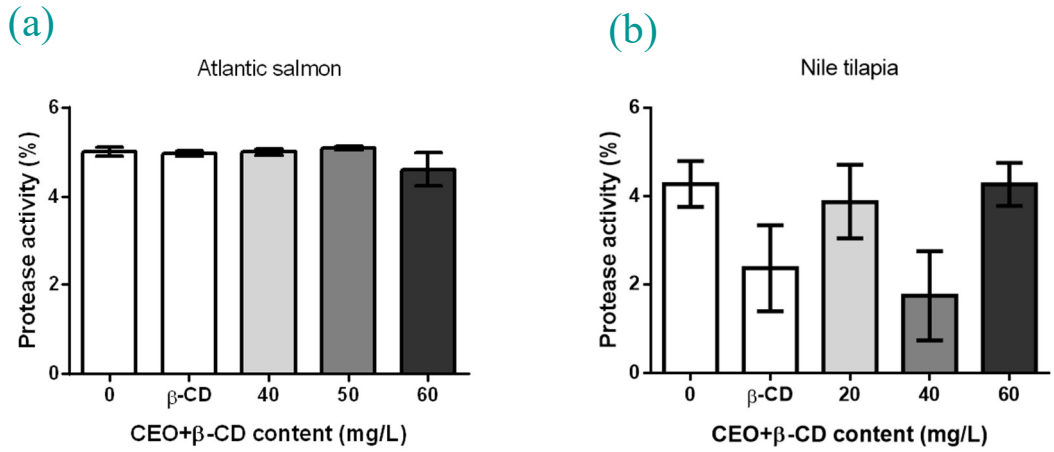


Figure 48. Protease activity level in plasma of Atlantic salmon (a) and Nile tilapia (b) stunned with CEO+β-CD under experimental farm conditions. (a) Atlantic salmon stunned with SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L SW (control 2) or 40, 50 or 60 mg CEO+β-CD/L SW. (b) Nile tilapia stunned with FW at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L FW (control 2) or 20, 40 or 60 mg CEO+β-CD/L FW. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

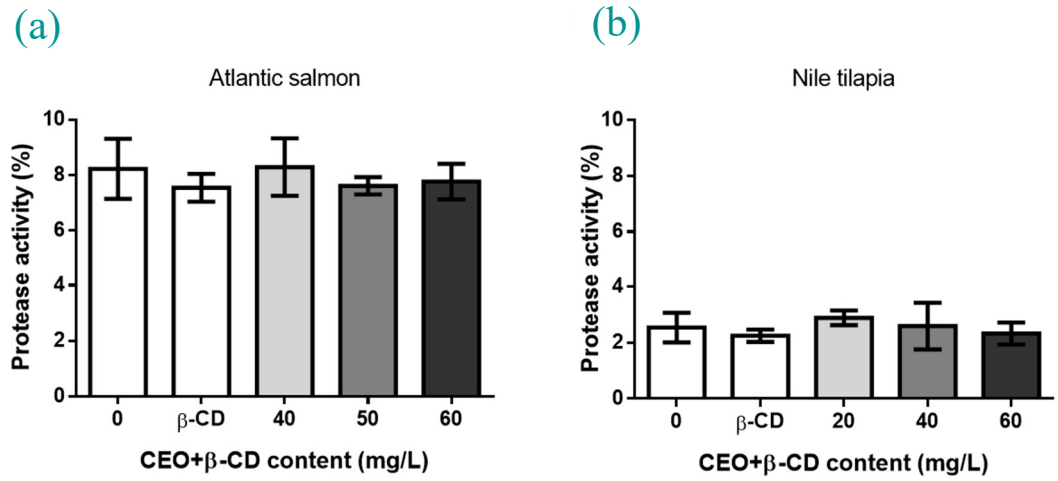


Figure 49. Protease activity level in skin mucus of Atlantic salmon (a) and Nile tilapia (b) stunned with CEO+β-CD under experimental farm conditions. (a) Atlantic salmon stunned with SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L SW (control 2) or 40, 50 or 60 mg CEO+β-CD/L SW. (b) Nile tilapia stunned with FW at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L FW (control 2) or 20, 40 or 60 mg CEO+β-CD/L FW. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In European seabass, the plasmatic protease activity level decreased when treated with 15 mg of CEO+β-CD/kg, compared to the level of the fish from the control group (CI at - 0.2 °C) (**Figures 50a, b**). However, plasmatic protease activity level was not detected in rainbow trout in any of the conditions assayed, treated with CEO+β-CD/kg or control (**Figure 50c**).

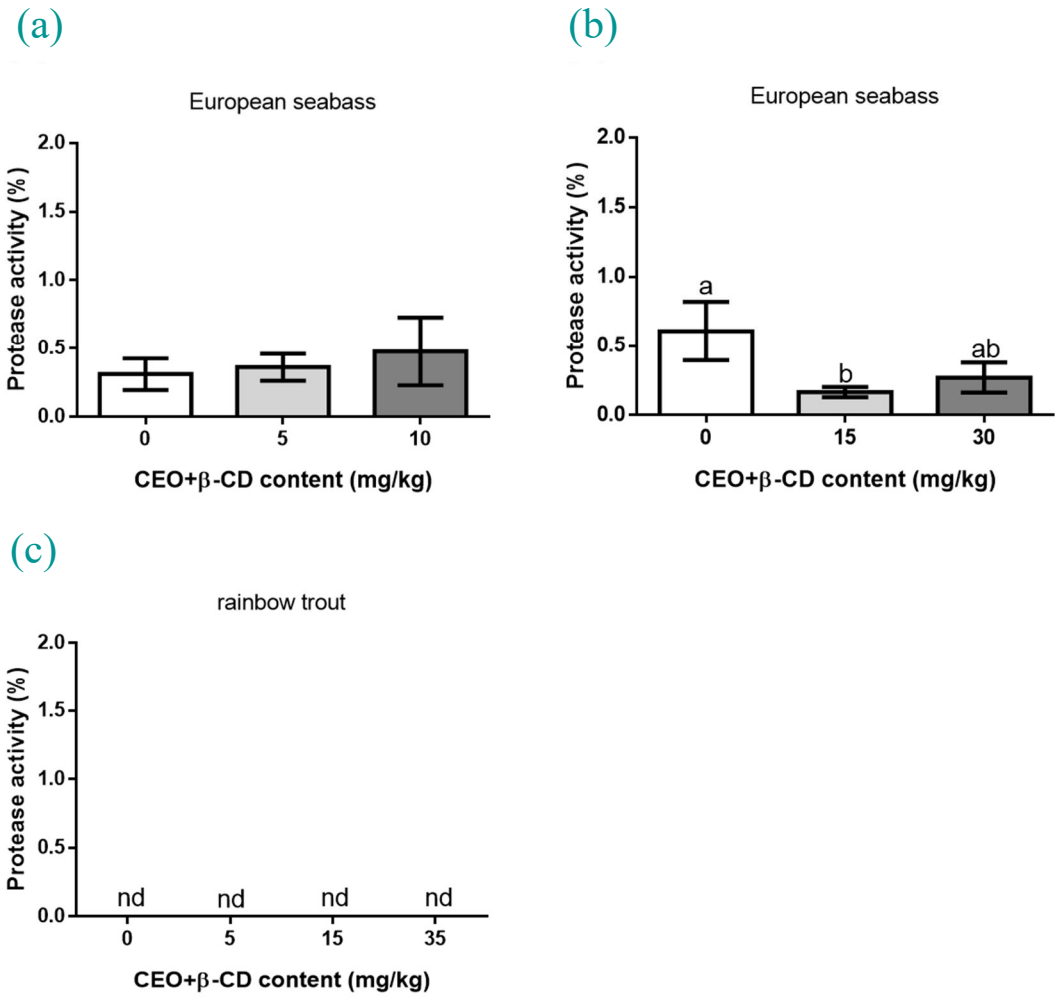


Figure 50. Protease activity level in plasma of European seabass (a, b) and rainbow trout (b) stunned with CEO+β-CD under industrial farm conditions. European seabass and rainbow trout stunned with CI at - 0.2 °C with ice crystals of 20 mm size (mixed with SW (European seabass)/ FW (rainbow trout) (ratio 1:1) control condition). **(a)** European seabass treated with CI in control condition or containing 5, 10, 15 or 30 mg CEO+β-CD/kg ice. **(b)** Rainbow trout treated with CI in control condition or containing 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In European seabass (**Figures 51a, b**) and rainbow trout (**Figure 51c**), protease activity level in skin mucus was not detected in any of the conditions assayed, treated with CEO+β-CD/kg or control

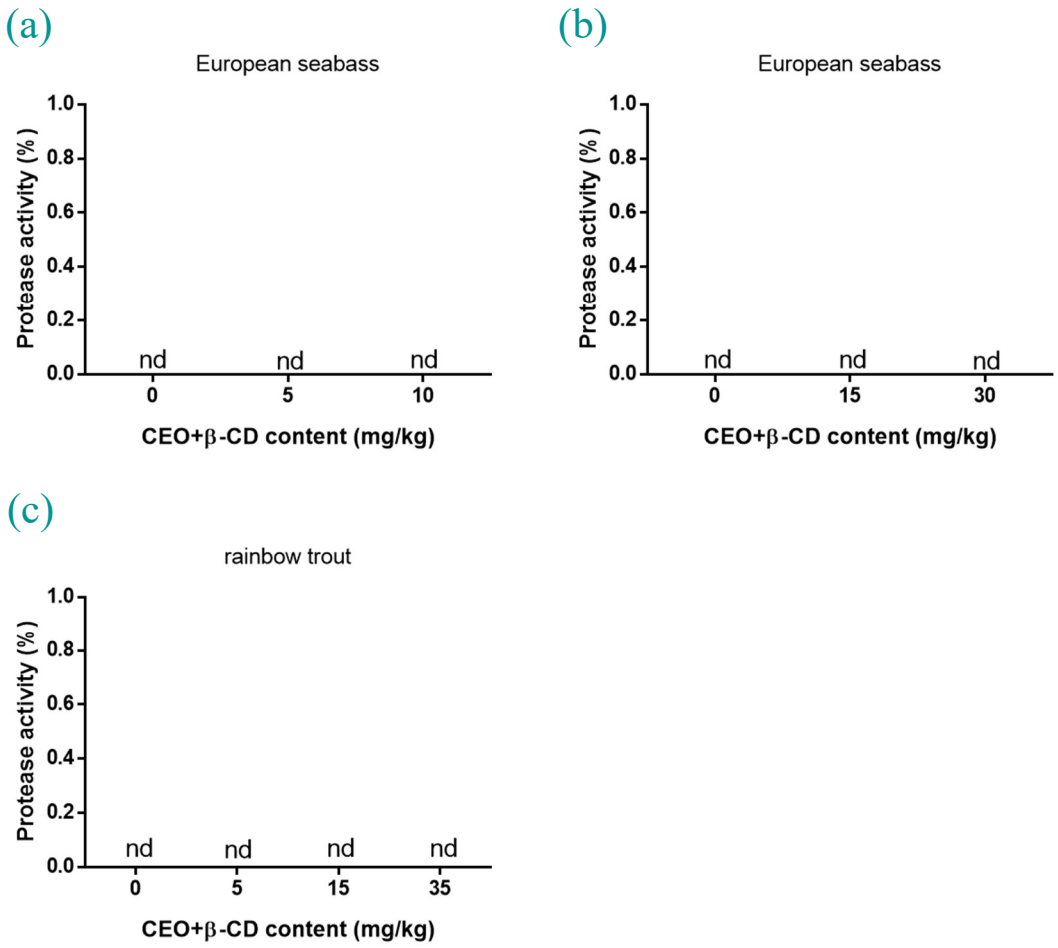


Figure 51. Protease activity level in skin mucus of European seabass (a, b) and rainbow trout (c) stunned with CEO+β-CD under industrial farm conditions. European seabass and rainbow trout stunned with CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (European seabass)/ FW (rainbow trout) (ratio 1:1) (control condition). **(a)** European seabass treated with CI in control condition or containing 5, 10, 15 or 30 mg CEO+β-CD/kg ice. **(b)** Rainbow trout treated with CI in control condition or containing 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.1.5. Total proteins

In gilthead seabream, under experimental farm conditions, no modifications in the plasmatic total proteins level of fish stunned with 20 mg of CEO+β-CD/L of SW at 27.3-28.0 °C compared with the level of the fish from the control group (CI at - 0.2 ºC) (**Figure 52a**). However, when 20 mg CEO+β-CD/kg was incorporated in CI at - 0.2 °C, an increase in the plasmatic total proteins level was observed compared with the level of the fish from the control group (CI at - 0.2 °C) (**Figure 52b**), although no differences exist when fish were stunned with 15 mg of CEO+β-CD/kg (**Figure 52b**).

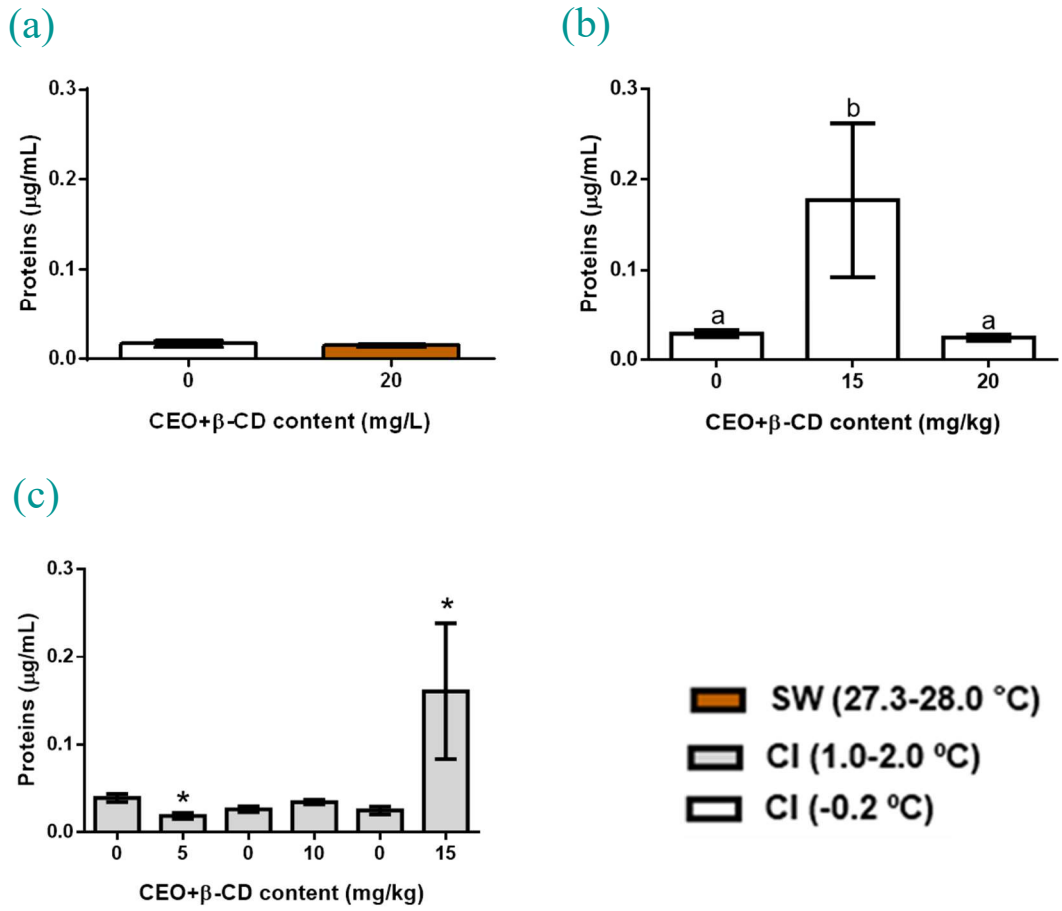


Figure 52. Total proteins level in plasma of gilthead seabream stunned with CEO+β-CD under experimental and industrial farm conditions. In experimental farm conditions, fish were stunned with CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (ratio 1:2) as control condition**.** **(a)** CI in control condition or SW at 27.3-28.0 °C containing 20 mg CEO+β-CD/L SW. **(b)** CI in control condition or containing 15 or 20 mg CEO+β-CD/kg ice. In industrial farm conditions, fish were stunned with CI at 1.0-2.0 °C with ice crystals of 20 mm size mixed with SW (ratio 1:2) as control condition. **(c)** CI in control condition or containing 5, 10 or 15 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10 fish per group in each sampling condition. Asterisks (*) indicate statistically significant differences between experimental and control groups according to t-test Student ($P \leq 0.05$).

In Atlantic salmon, the plasmatic total proteins level was similar in all the different experimental groups, treated with CEO+β-CD/L and controls (**Figure 53a**). However, in Nile tilapia only the highest concentration used, 60 mg CEO+β-CD/L, increased the plasmatic total proteins level compared with the level of the fish from the control group (FW at 27.0-29.0 °C, containing 240 mg β-CD) or treated with 20 mg CEO+β-CD/L, but not compared to fish treated with FW at 27.0-29.0 °C, without β-CD (**Figure 53b**).

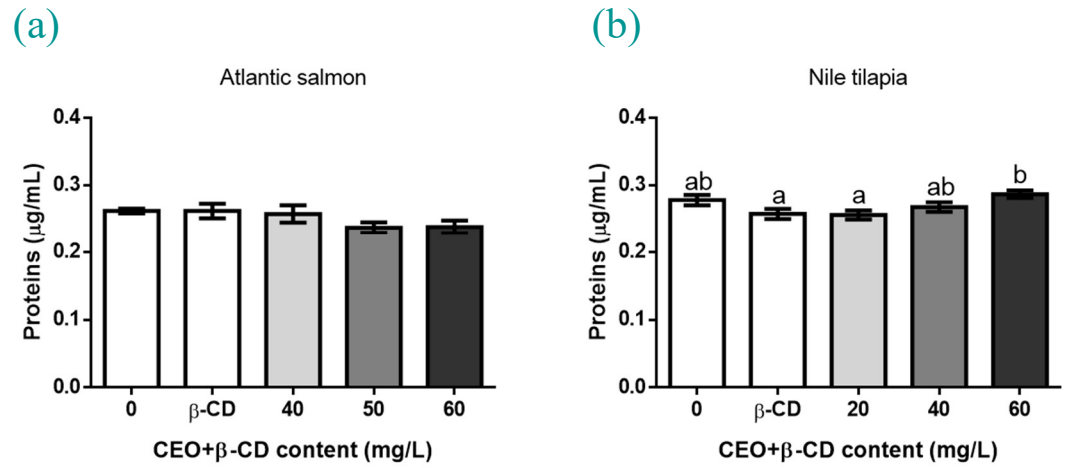


Figure 53. Total proteins level in plasma of Atlantic salmon (a) and Nile tilapia (b) stunned with CEO+β-CD under experimental farm conditions. (a) Atlantic salmon treated with SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L SW (control 2) or 40, 50 or 60 mg CEO+β-CD/L SW. (b) Nile tilapia treated with FW at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L FW (control 2) or 20, 40 or 60 mg CEO+β-CD/L FW. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

Regarding skin mucus, concerning the total proteins level, no differences occurred between fish treated with CEO+β-CD/L and controls groups, neither in Atlantic salmon (**Figure 54a**) nor in Nile tilapia (**Figure 54b**).

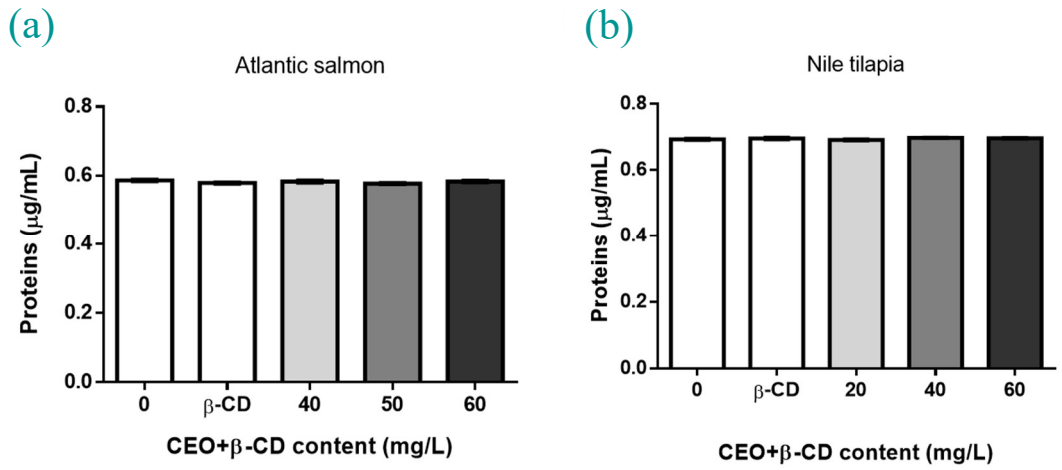


Figure 54. Total protein level in skin mucus of Atlantic salmon (a) and Nile tilapia (b) stunned with CEO+β-CD under experimental farm conditions. (a) Atlantic salmon treated with SW at 4.0-6.0 °C alone (control 1) or containing 240 mg β-CD/L SW (control 2) or 40, 50 or 60 mg CEO+β-CD/L SW. (b) Nile tilapia treated with FW at 27.0-29.0 °C alone (control 1) or containing 240 mg β-CD/L FW (control 2) or 20, 40 or 60 mg CEO+β-CD/L FW. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

In European seabass, only the lowest concentrations of CEO+β-CD/kg (5 and 10 mg) used promoted an increase in the plasmatic total proteins level compared with the level of the control conditions (**Figure 55a, b**). However, in rainbow trout no differences in the plasmatic total proteins level was observed between the different groups analyzed, treated with CEO+β-CD and control (**Figure 55c**).

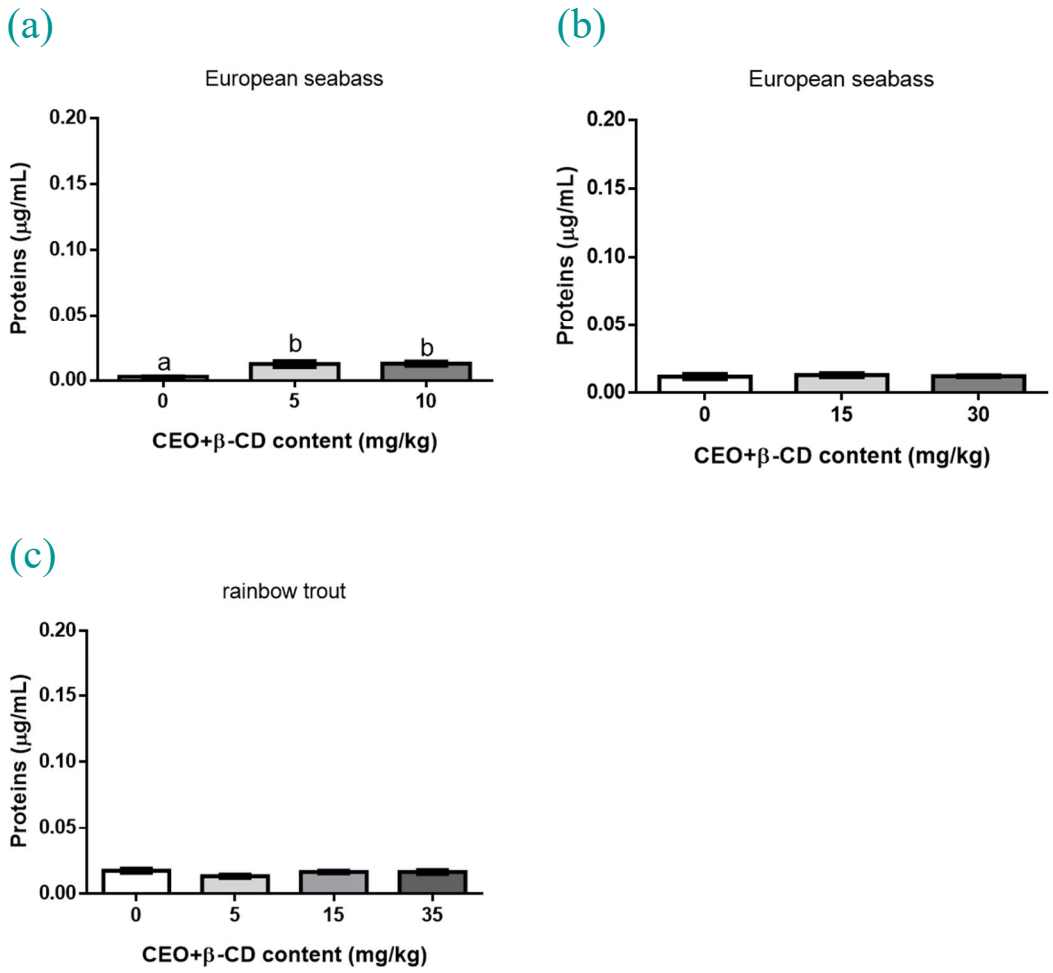


Figure 55. Total proteins level in plasma of European seabass (a, b) and rainbow trout (c) stunned with CEO+β-CD under industrial farm conditions. European seabass and rainbow trout stunned with CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (European seabass)/ FW (rainbow trout) (ratio 1:1) (control condition). **(a)** European seabass treated with CI in control condition or containing 5, 10, 15 or 30 mg CEO+β-CD/kg ice. **(b)** Rainbow trout treated with CI in control condition or containing 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

However, in the skin mucus, a decrease in the total proteins level was detected in European seabass treated only with 30 mg CEO+β-CD/kg, compared with the control group (CI at - 0.2 °C) (**Figure 56a, b**). In contrast, in rainbow trout no differences occurred between the groups studied, treated with CEO+β-CD/kg and control (**Figure 56c**).

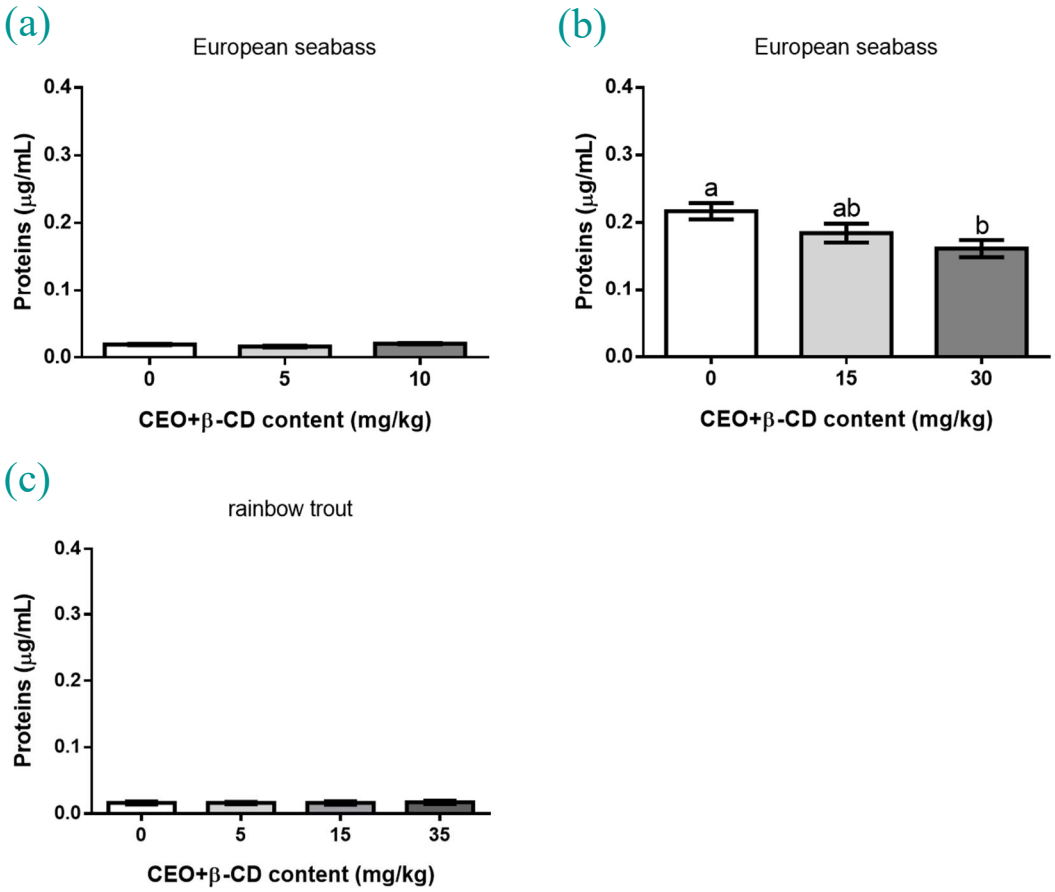


Figure 56. Total proteins level in skin mucus of European seabass (a, b) and rainbow trout (c) stunned with CEO+β-CD under industrial farm conditions. European seabass and rainbow trout stunned with CI at - 0.2 °C with ice crystals of 20 mm size mixed with SW (European seabass)/ FW (rainbow trout) (ratio 1:1) (control condition). **(a)** European seabass treated with CI in control condition or containing 5, 10, 15 or 30 mg CEO+β-CD/kg ice. **(b)** Rainbow trout treated with CI in control condition or containing 5, 15 or 35 mg CEO+β-CD/kg ice. Each value represents the mean ± S.E.M. of n=10-15 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.2. Innovative management of gilthead seabream under industrial farm conditions

A group of gilthead seabream farmed under innovative industrial conditions were analyzed. This group of fish were handled using underwater cameras during their productive cycle, so these fish had never been previously anaesthetized. These fish were treated with CI at 1.0-2.0 °C with ice crystals of 20 mm size, with CEO or without (control group) 20 mg CEO+β-CD/kg.

IV.2.1. Plasmatic parameters

The effect of CEO+β-CD on some plasmatic parameters related to stress such as glucose (ng/mL), lactate (mmol/L), pO_2 (mmHg), pCO_2 (mmHg), HCO_3 (mmol/L), TCO_2 (mmol/L), SO_2 (%), base excess (mmol/L), pH and cortisol was determined in this group of fish.

IV.2.1.1. Glucose and lactate levels

The plasmatic glucose level decreased in those fish treated with 20 mg CEO+β-CD/kg incorporated in CI at 1.0-2.0 °C compared with the level of the fish from the control group (CI at 1.0-2.0 °C) (**Figure 57a**), while no effect was observed in the plasmatic lactate level (**Figure 57b**).

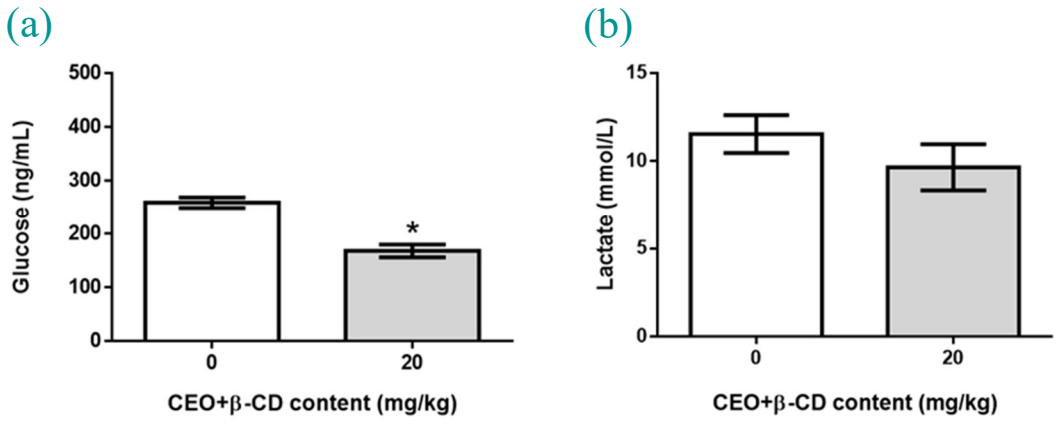


Figure 57. Plasmatic glucose (a) and lactate (b) level of gilthead seabream that had never been anesthetized before stunned with CEO+β-CD under industrial farm conditions. Fish were stunned with CI at 1.0-2.0 ºC with ice crystals of 20 mm size mixed with SW (ratio 1:2) as control condition or containing 20 mg CEO+β-CD/kg ice. Asterisk (*) indicate statistically significant differences between groups according to Student-t test ($P \leq 0.05$).

IV.2.1.2. Other plasmatic parameters

A decrease in the plasmatic level of pO_2, HCO_3 and TCO_2 was observed in fish treated with 20 mg CEO+β-CD/kg (310.00± 1.35 mmHg, 5.22±0.52 mmol/L and 5.83±0.48 mmol/L, respectively) compared with the level of the fish from the control group (357.17±14.72 mmHg, 7.38±0.49 mmol/L and 8.17±0.54 mmol/L, respectively). No variations were observed in the plasmatic level of pCO_2, SO_2, base excess and pH (**Table 11**).

Table 11. Plasmatic parameters after stunning treatments in gilthead seabream specimens, that have never been exposed to anaesthesia until sampling, under industrial farm conditions.

Stunning treatments	CEO+β-CD (mg/kg)	pO_2 (mmHg)	pCO_2 (mmHg)	HCO_3 (mmol/L)	TCO_2 (mmol/L)	SO_2 (%)	Base Excess (mmol/L)	pH
10	0	357.17 ± 14.72^a	29.35 ± 3.02^a	7.38 ± 0.49^a	8.17 ± 0.54^a	100^a	-14.03 ± 2.15^a	7.24 ± 0.02^a
	20	310.00 ± 11.35^b	22.52 ± 1.84^a	5.22 ± 0.52^b	5.83 ± 0.48^b	100^a	-15.12 ± 1.42^a	7.11 ± 0.01^a

Stunning treatment: CI at 1.0-2.0 ºC with ice crystals of 20 mm size mixed with SW (ratio 1:2) as control condition or containing 20 mg CEO+β-CD/kg ice. The letters indicate statistically significant differences between groups according to according to t-test Student ($P \leq 0.05$).

IV.2.1.3. Cortisol levels

The plasmatic cortisol level decreased in fsh treated with 20 mg CEO+β-CD/kg incorporated in CI at 1.0-2.0 °C, compared with the level of fish treated under control conditions (CI at 1.0-2.0 °C) (**Figure 58**).

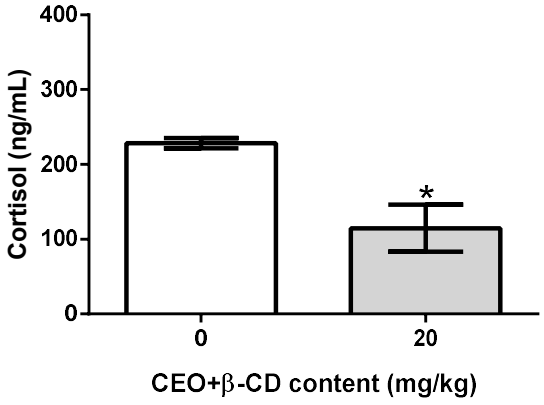


Figure 58. Plasmatic cortisol level of gilthead seabream, that had never been anesthetized before, stunned with CEO+β-CD under industrial farm conditions.CI mixed with SW (ratio 1:2) at 1.0-2.0 ºC with ice crystals of 20 mm size as control condition or containing 20 mg CEO+β-CD/kg ice. (*) indicate statistically significant differences between groups according to Student-t test ($P \leq 0.05$).

IV.2.2. Humoral activities

Then, peroxidase, bactericidal, antiprotease and protease activities, in plasma and skin mucus of gilthead seabass that had not been anaesthetized before this trial were analyzed.

IV.2.2.1. Peroxidase activity

Curiously, the plasmatic peroxidase activity levels were not altered respect to the control (CI at 1.0-2.0 °C) levels when 20 mg CEO+β-CD/kg CI was used (**Figure 59a**), but decreased in the skin mucus (**Figure 59b**).

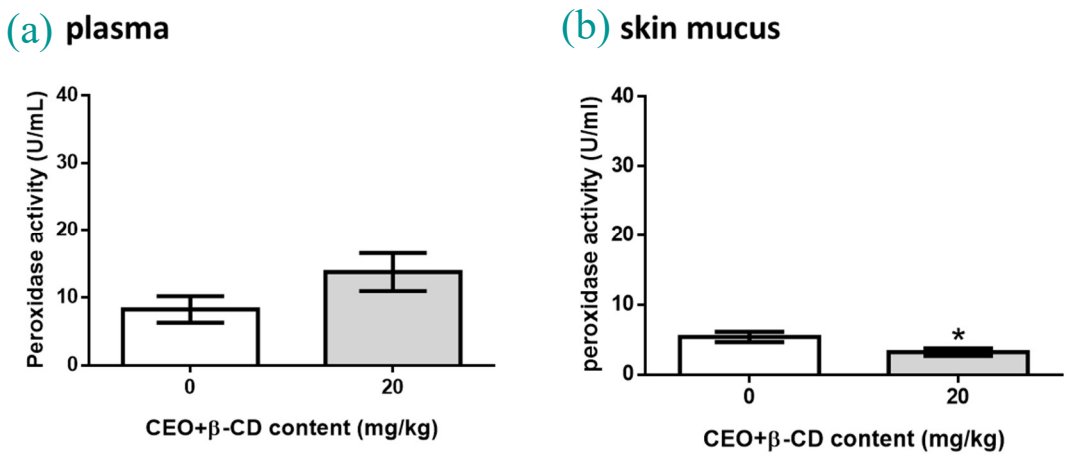


Figure 59. Peroxidase activity level in plasma (a) and skin mucus (b) of gilthead seabream that had never been anesthetized before this trial and stunned with CEO+β-CD under industrial farm conditions. CI at 1.0-2.0 ºC with ice crystals of 20 mm size mixed with SW (ratio 1:2) treated with CI in control condition or containing 20 mg CEO+β-CD/kg ice. (*) indicate statistically significant differences between groups according to t-test Student ($P \leq 0.05$).

IV.2.2.2. Bactericidal activity

The bactericidal activity level was no modified neither in plasma nor in skin mucus when 20 mg CEO+β-CD/kg was incorporated in CI at 1.0-2.0 ºC compared to the level of the fish from the control group (CI at 1.0-2.0 °C) (**Figure 60a, b**).

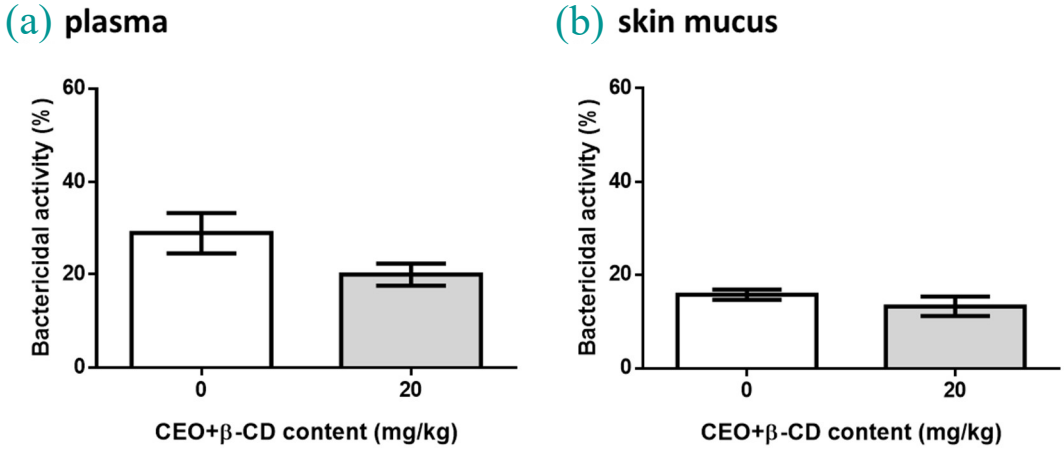


Figure 60. Bactericidal activity level in plasma (a) and skin mucus (b) of gilthead seabream that had not been anesthetized before this trial and stunned with CEO+β-CD under industrial farm conditions. CI at 1.0-2.0 ºC with ice crystals of 20 mm size mixed with SW (ratio 1:2) as control condition or containing 20 mg CEO+β-CD/kg ice. (*) indicate statistically significant differences between groups according to Student-t test ($P \leq 0.05$).

IV.2.2.3. Antiprotease activity

The antiprotease activity level was no modified neither in plasma nor in skin mucus when 20 mg CEO+β-CD/kg was incorporated in CI at 1.0-2.0 ºC compared to the level of the fish from the control group (CI at 1.0-2.0 °C) (**Figure 61a, b**).

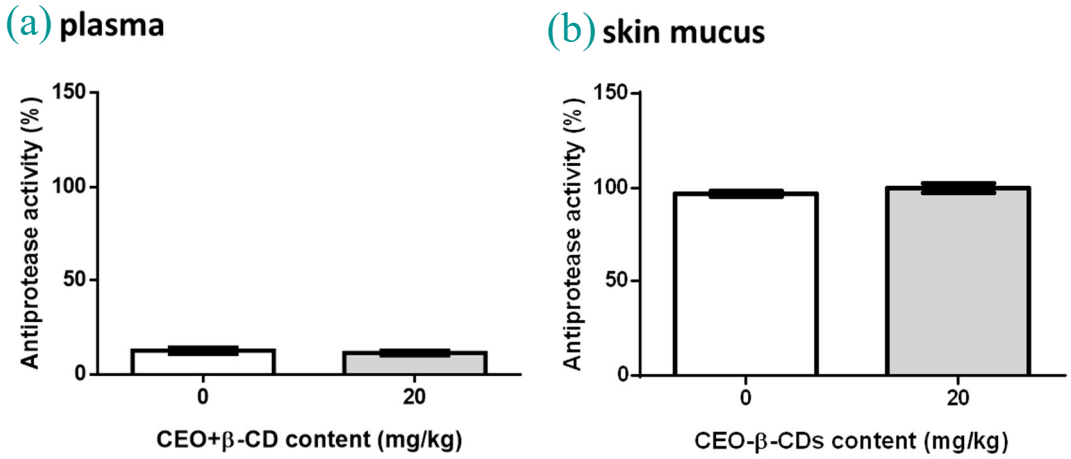


Figure 61. Antiprotease activity level in plasma (a) and skin mucus (b) of gilthead seabream that had never been anesthetized before this trial and stunned with CEO+β-CD under industrial farm conditions. CI at 1.0-2.0 ºC with ice crystals of 20 mm size mixed with SW (ratio 1:2) as control condition or containing 20 mg CEO+β-CD/kg ice. (*) indicate statistically significant differences between groups according to a Student-t test ($P \leq 0.05$).

IV.2.2.4. Protease activity

The protease activity level was no modified neither in plasma nor in skin mucus when 20 mg CEO+β-CD/kg was incorporated in CI at 1.0-2.0 ºC compared to the level of the fish from the control group (CI at 1.0-2.0 °C) (**Figure 62a, b**).

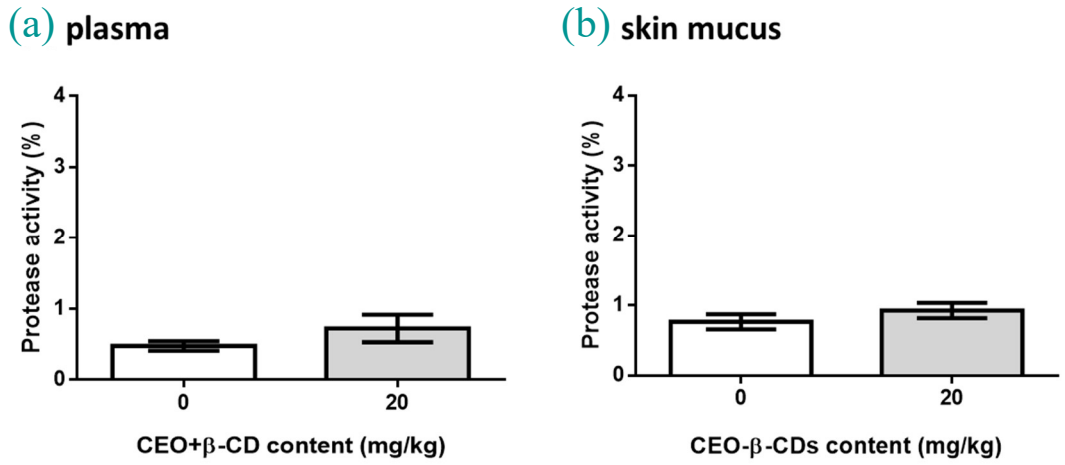


Figure 62. Protease activity level in plasma (a) and skin mucus (b) of gilthead seabream that had never been anesthetized before this trial and stunned with CEO+β-CD under industrial farm conditions. CI at 1.0-2.0 ºC with ice crystals of 20 mm size mixed with SW (ratio 1:2) treated as control condition or containing 20 mg CEO+β-CD/kg ice. (*) indicate statistically significant differences between groups according to t-test Student ($P \leq 0.05$).

IV.3. Eugenol nanoencapsulated in β-CD in gilthead seabream under experimental farm conditions

IV.3.1. Stages of anaesthesia

The anesthesia-induction stages were analyzed in gilthead seabream specimens using 20 mg of EU nanoencapsulated in β-CD and incorporated in SW at 27.3-28 °C under experimental farm conditions and compared with fish treated with 20 mg EU alone or 20 mg CEO alone incorporated in SW at 27.3-28 °C.

The lowest time needed to loss equilibrium was achieved in fish treated with EU+β-CD (6.43±0.07 min) followed by EU alone (12.61±0.06 min) and CEO alone (21.05±2.39 min) (**Figure 63**). However, the time to achieve the loss of reflex activity was similar in fish treated with EU alone or EU+β-CD (19.24±0.10 min) but lower than the time recorded in fish treated with CEO (29.76±2.77 min) (**Figure 63**).

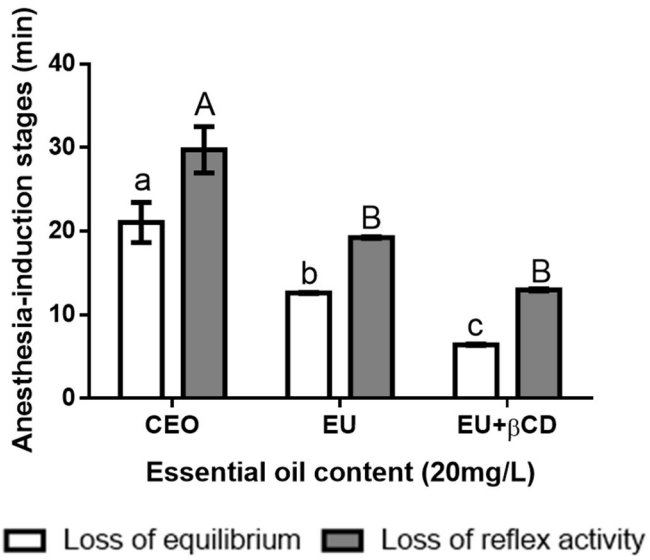


Figure 63. The anesthesia-induction stages in gilthead seabream stunned with EU+β-CD under experimental farm conditions. Fish were exposed to SW at 27.3-28 °C containing 20 mg CEO/L SW as control group, 20 mg EU/L SW or 20 mg EU+β-CD/L SW. Each value represents the mean ± S.E.M. of n=10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.3.2. Plasmatic parameters

Some plasmatic parameters considered as stress indicators, such as glucose (ng/mL), lactate (mmol/L), pO_2 (mmHg), pCO_2 (mmHg), HCO_3 (mmol/L), TCO_2 (mmol/L), SO_2 (%), base excess (mmol/L), pH and cortisol (ng/mL), were analyzed.

IV.3.2.1. Glucose and lactate levels

Plasmatic glucose level decreased in fish treated with EU or EU+β-CD compared with the level of the fish treated with CEO (**Figure 64a**). However, no differences between fish treated with EU or EU+β-CD were observed (**Figure 64a**). In contrast, the use of EU, with or without β-CD, did not affect plasma lactate level, compared to fish treated with CEO (**Figure 64b**).

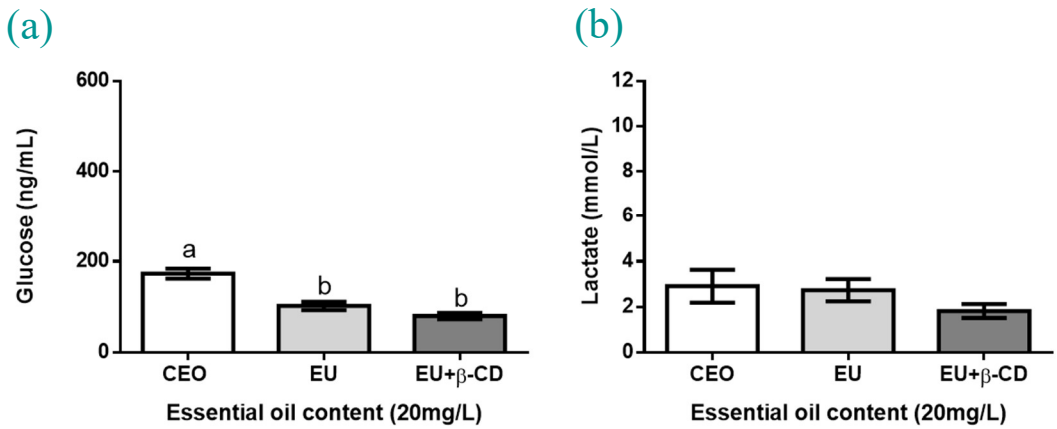


Figure 64. Plasmatic glucose (a) and lactate (b) level of gilthead seabream stunned with EU+β-CD under experimental farm conditions. Fish stunned with SW at 27.3-28.0 °C containing 20 mg CEO/L SW, 20 mg EU/L SW or 20 mg EU+β-CD/L SW. Each value represents the mean ± S.E.M of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.3.2.2. Other plasmatic parameters

EU or EU+β-CD showed similar level of pCO_2, HCO_3 and TCO_2, while being lower than the level of fish treated with CEO. However, fish treated with EU+β-CD showed the lowest pH (5.31±0.03) followed by fish treated with EU (5.61±0.04) and CEO (6.63±0.03). No differences were observed between these three groups analyzed in the level of pO_2, SO_2 and base excess (**Table 12**).

Table 12. Plasmatic parameters level in gilthead seabream stunned with EU+β-CD under experimental farm conditions.

Stunning treatment	CEO (mg/L)	EU (mg/L)	EU+β-CD (mg/L)	pO_2 (mmHg)	pCO_2 (mmHg)	HCO_3 (mmol/L)	TCO_2 (mmol/L)	SO_2 (%)	Base Excess (mmol/L)	pH
6	20			294.78±8.39a	78.64±5.65a	7.96±0.27a	10.22±0.31a	100a	-27.44±0.45a	6.63±0.03a
		20		310.16±3.94a	153.02±6.64b	11.17±0.37b	12.67±0.49b	100a	-26.50±0.22a	5.61±0.04b
			20	322.28±17.46a	141.89±7.54b	10.60±0.46b	12.67±0.46b	100a	-26.57±0.39a	5.31±0.03c

Stunning treatment: SW at 27.3-28.0 °C containing 20 mg CEO/L SW, 20 mg EU/L SW or 20 mg EU+β-CD/L SW. Each value represents the mean ± S.E.M of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.3.2.3. Cortisol levels

No differences in plasmatic cortisol level between fish treated with CEO, EU or EU+β-CD were observed (**Figure 65**).

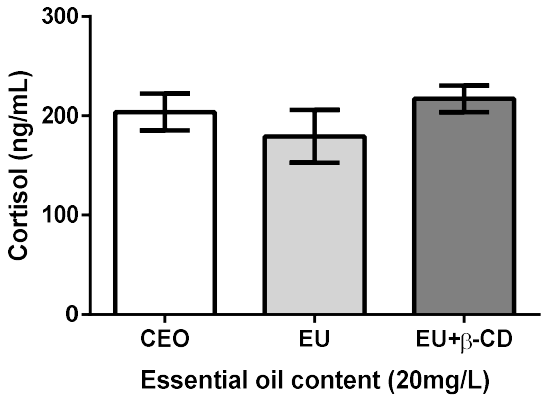


Figure 65. Plasmatic cortisol level of gilthead seabream stunned with EU+β-CD under experimental farm conditions. Fish stunned with SW at 27.3-28.0 °C containing 20 mg CEO/L SW, 20 mg EU/L SW or 20 mg EU+β-CD/L SW. Each value represents the mean ± S.E.M of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.3.3. Humoral activities

Then, peroxidase, bactericidal, antiprotease and protease activities levels, in plasma and skin mucus of gilthead seabass treated with EU, with or without β-CD, under experimental farm conditions and compared with CEO treatment.

IV.3.3.1. Peroxidase activity

Plasmatic peroxidase activity levels decreased in fish treated with EU+β-CD compared to fish treated with EU alone (**Figure 66a**), although no differences were observed between these two groups of fish and fish treated with CEO alone (**Figure 66a**).

In the skin mucus, however, this activity was similar in fish treated with EU+β-CD and EU alone (**Figure 66b**), while was lower in fish treated with EU+β-CD than in fish treated with CEO alone (**Figure 66b**).

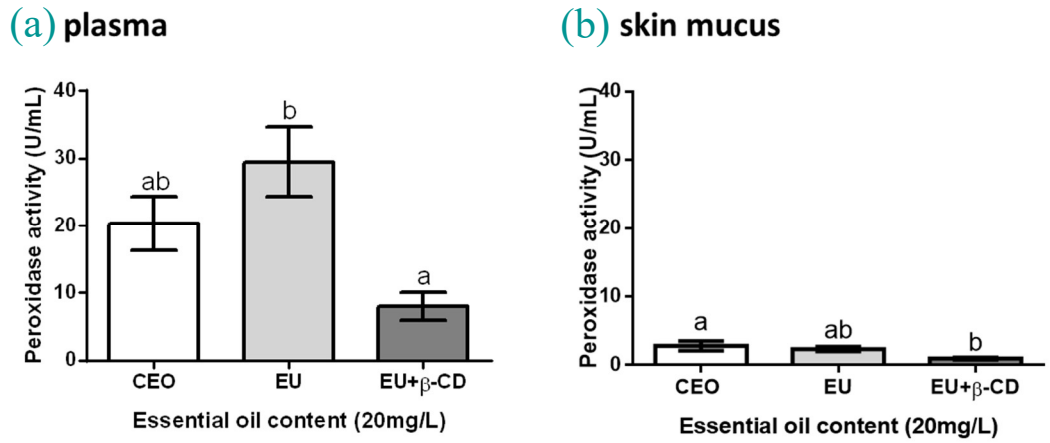


Figure 66. Peroxidase level in plasma (a) and skin mucus (b) of gilthead seabream stunned with EU+β-CD under experimental farm conditions. Fish stunned with SW at 27.3-28.0 °C containing 20 mg CEO/L SW, 20 mg EU/L SW or 20 mg EU+β-CD/L SW. Each value represents the mean ± S.E.M of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.3.3.2. Bactericidal activity

Plasmatic bactericidal activity level decreased in fish treated with EU or EU+β-CD compared with fish treated with CEO (**Figure 67a**), although no differences were observed between these two groups of fish (**Figure 67a**).

In the skin mucus, however, the bactericidal activity levels decreased in fish treated with EU+β-CD compared with fish treated with CEO (**Figure 67b**), although no differences were observed between fish treated with EU, with or without β-CD (**Figure 67b**).

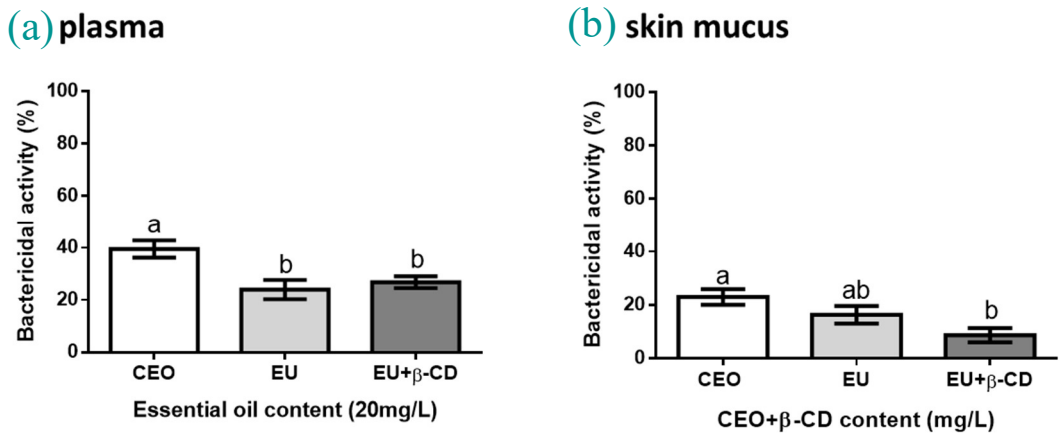


Figure 67. Bactericidal level in plasma (a) and skin mucus (b) of gilthead seabream stunned with EU+β-CD under experimental farm conditions. Fish stunned with SW at 27.3-28.0 °C containing 20 mg CEO/L SW, 20 mg EU/L SW or 20 mg EU+β-CD/L SW. Each value represents the mean ± S.E.M of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.3.3.3. Antiprotease activity

The antiprotease activity levels were not affected by any of the treatment used neither in plasma (**Figure 68a**) nor skin mucus (**Figure 68b**).

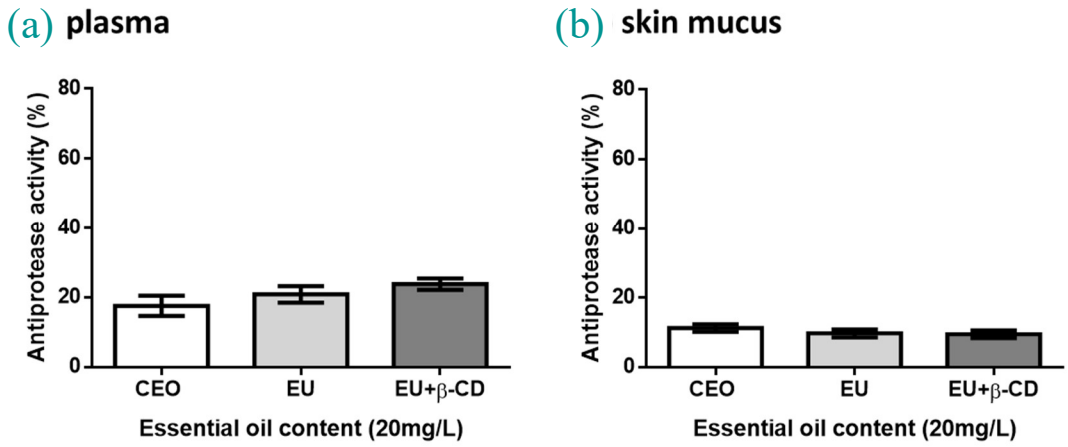


Figure 68. Antiprotease activity level in plasma (a) and skin mucus (b) of gilthead seabream stunned with EU+β-CD under experimental farm conditions. Fish stunned with SW at 27.3-28.0 °C containing 20 mg CEO/L SW, 20 mg EU/L SW or 20 mg EU+β-CD/L SW. Each value represents the mean ± S.E.M of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

IV.3.3.4. Protease activity

Similarly, no differences in the protease activity levels occurred neither in plasma (**Figure 69a**) nor skin mucus (**Figure 69b**).

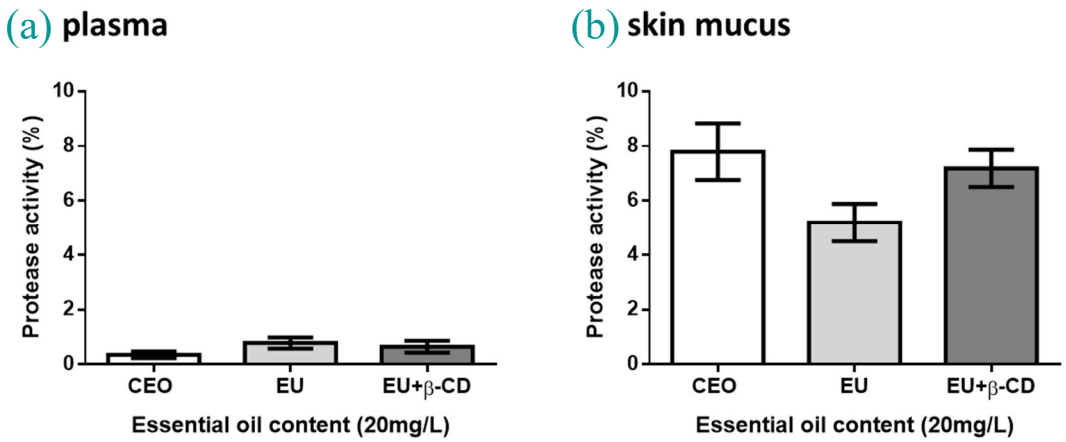


Figure 69. Protease activity level in plasma (a) and skin mucus (b) of gilthead seabream stunned with EU+β-CD under experimental farm conditions. Fish stunned with SW at 27.3-28.0 °C containing 20 mg CEO/L SW, 20 mg EU/L SW or 20 mg EU+β-CD/L SW. Each value represents the mean ± S.E.M of n=4-10 fish per group in each sampling condition. The letters indicate statistically significant differences between groups according to one-way ANOVA and Bonferroni post-hoc test ($P \leq 0.05$).

V.Discussion

In the aquaculture industry, fish are stunned using a wide range of methods, some of which are considered unacceptable to other vertebrates (Lines and Spence, 2014). Thus, stuning methods ranging from cooling in ice of storage, air suffocation, anoxia, overcrowding, electric charges, to nitric oxide or CO_2 narcosis, have been studied in different species and culture conditions in order to determine the effect on stress and welfare of fish and the flesh quality (Acerete *et al*., 2009; Bagni *et al*., 2007; Castro *et al*., 2017; Lambooij *et al*., 2008; Lines *et al*., 2003; Oliveira Filho *et al*., 2015; Proctor *et al*., 1992; Robb *et al*., 2002; Robb and Roth, 2003; Tejada and Huidobro, 2002; Van de Vis *et al*., 2003; Wang *et al*., 2017; Wills *et al*., 2006; Zampacavallo *et al*., 2015).

In this thesis, five fish species of high economic interest in the marine and continental aquaculture have been studied. The induction stages of anaesthesia, the most remarkable stress plasmatic parameters in fish, and some innate immune activities have been analysed upon stunning gilthead seabream and European seabass, the most important species cultured in the Mediterranean area (APROMAR, 2019; Mendes, 2019), in Atlantic salmon and rainbow trout which are the most economically important species worldwide (FAO, 2017; Tikeiogly, 2000), and in Nile tilapia, a key species for future sustainable aquaculture due to its unique characteristics (Yue *et al*., 2016). To this end, a new method of stunning based on the application of nanoencapsuled CEO in β-CD has been compared with live cooling in ice slurry, that is probably the most used method in different species in the aquaculture sector. Moreover, the efficacy of EU (the active compound of CEO), nanoencapsulated or not in β-CD, with CEO was also studied.

V.1. Clove oil nanoencapsulated in β-CD as anesthetic agent

V.1.1. Induction time of anesthesia stages

To evaluate the efficacy of this new method of stunning, and if it offers a rapid loss in sensibility and consciousness in terms of fish welfare, the time needed to reach the loss of equilibrium and the loss of reflex activity in all species studied, was observed.

An effective dose of an anesthetic agent would be able to induce the loss of reflex activity within 3 minutes after exposure (Massee *et al*., 1995), taking into account that the efficacy of these agents depend on the dose and the fish species, but it is also influenced by

environmental factors such as salinity, pH, oxygen level and water temperature (Sylvester & Holland, 1982). Fish anesthetics have been used in a considerable number of studies, some of them focussed in CEO (Del Valle, 2004; Lopez-Canovas *et al*., 2019; Welliver, 2007). In this thesis, the link between the time of anaesthesia-induction stages and the different temperatures used during the stunning method, such as the blood acid-base status of fish was analyzed. It was shown that the increased temperature leads to acidemia and hypercapnia that stimulate hyper-ventilation, which in turn decreased induction and recovery time for anaesthetics taken up or eliminated through the gills (Aguiar *et al*., 2002).

The results of this thesis showed that farmed gilthead seabream under experimental farm conditions, and stunned with LI at - 1.5 ºC needed a shorter time to loss reflex activity than when the traditional stunning method (CI at - 0.2 °C) is used; however, this decreases not occurred with LI at - 5 ºC or - 8 ºC. Interestingly, when CEO+β-CD was incorporated in these last conditions (LI at - 5 ºC or - 8 ºC), the time of anaesthesia-induction stages (loss of equilibrium and reflex activity) was significantly shorter (less than 5 minutes) than when CEO+β-CD was incorporated in CI at - 0.2 ºC or LI at - 1.5 ºC (around 5 minutes). So, the release of the nanoencapsulated CEO in β-CD might be faster due to low temperatures (- 1.5 or - 8 °C), leading to times of anesthesia-induction stages in shorter than traditional stunning methods used in aquaculture industry. Moreover, different doses of CEO+β-CD (15, 30 or 60 mg/kg) in CI or LI at different temperatures, did not promoted differences in anaesthesia induction time between the different doses, while the results of Farid *et al*. (2008) and Hoskonen & Pirhonen (2004) shown a lower induction time when the dose of CEO was increased from 5 to 20 °C in several salmonids and cyprinids fish species. Our results demonstrated that a low dose of CEO nanoencapsulated in β-CD (15 mg/kg ice) incorporated in LI at low temperatures (- 1.5 ºC or - 8 ºC) was more efficient and generated lower induction time of anaesthesia than higher doses of CEO (30 or 60 mg/kg ice) at higher temperatures (CI at - 0.2 ºC) or than cooling the fish with CI or LI alone. However, Mylonas *et al*. (2005) observed, in gilthead seabream stunned with CEO diluted in water at different temperatures, a decrease in temperature with an extension of induction time. This difference could be explained by the ability of β-CD to control the release of CEO and to improve its water solubility (Brewster & Loftsson, 2007; Capelezzo et al., 2018).

Similarly to gilthead seabream, in Atlantic salmon and Nile tilapia, the use of CEO+β-CD in SW at 4-6 °C or FW at 27-28 ºC, respectively, allow to reduce the dose of CEO. Thus, Atlantic salmon specimens treated with 40, 50 or 60 mg CEO+β-CD/L achieved the loss of equilibrium in a period of time below to 3 minutes, while previous studies found the effective dose of CEO for different species of salmonids between 80-100 mg/L to achieve time of anaesthesia of around 3 minutes (Iversen *et al*., 2003; Woody *et al*., 2002). Our results showed that 40 mg CEO+β-CD/L was the minimum dose in Nile tilapia to achieve the anaesthesia-induction at 3.77±0.20 minutes. Simões *et al*. (2011) proposed that the most appropriate dose of CEO to induce deep anesthesia in Nile tilapia was 90 mg/L. So, our data showed that the use of β-CD help to reduce the dose of CEO by half under experimental farm conditions.

In fish treated under industrial farm conditions, the use of β-CD also allow to reduce the amount of CEO needed to reach the loss of reflex activity in less than 3 minutes. Thus, previous studies fixed the optimal dose of CEO between 40 and 100 mg/L for gilthead seabream and European seabass (Javahery *et al*., 2012; Mylonas *et al*., 2005), while for rainbow trout the optimal dose was around 50 mg/L (Ghanawi *et al*., 2013; Perdikaris *et al*., 2010; Takatsuka *et al*., 2019). In our study, European sea bass and rainbow trout specimens were anaesthetized with lower doses of CEO (30 and 5 mg CEO+β-CD/kg ice, respectively) than the doses described in previous studies for each specie (Ghanawi *et al*., 2013; Javahery *et al*., 2012; Mylonas *et al*., 2005; Perdikaris *et al*., 2010; Takatsuka *et al*., 2019).

In general, our results showed that nanoencapsulation of CEO in β-CD allow reducing the dose of CEO used to induce a rapid loss of reflex activity in several fish species treated under experimental or industrial farm conditions at different temperatures and independently of the type of water used, as it was effective in both, seawater and freshwater. This way, offering a rapid loss in sensibility and consciousness in terms of fish welfare.

V.1.2. Plasma parameters as stress indicators

In most cases, the effectiveness of anaesthetics used in aquaculture also depends on how it generates a response to stress in fish (Thomas & Robertson, 1991; Hoseini *et al*., 2018). The increase in plasmatic glucose, lactate and cortisol has been related to a stress response (Barry *et al*., 1993; Begg and Pankhurst, 2004; Biron and Benfey, 1994; Dabrowska

et al., 1991; Webb *et al*., 2007; Wells *et al*., 1984). Moreover, the plasma concentration of pO_2 tends to increase immediately after a stress response and in turn the plasma concentrations of pCO_2 and HCO_3 decreased (Cech *et al*., 1996). Interestingly, the increase of glucose, lactate and cortisol are also an indicative of oxidative stress and, subsequently, tissue damage (Saccol *et al*., 2016). On the other hand, low concentrations of HCO_3 lead to decrease in plasma pH due to the enhancement of the anaerobic metabolism and the hypoxia during anesthesia, and it has also been related with branchial lesions (Black & Connor, 1964; Gabriel *et al*., 2009).

In this work, the different levels of glucose, lactate, cortisol and other stress plasmatic parameters have also been analyzed in order to determine the methods with less metabolic alterations in several fish species under different culture conditions. Thus, several experiments performed with gilthead seabream stunned with different concentrations of CEO+β-CD at different temperatures and types of ice under experimental and industrial farm conditions were tested at plasmatic glucose and lactate decreased when CEO+β-CD was applied at different temperatures (SW at 27.3-28.0 ºC, CI at - 0.2 ºC or LI at - 5 ºC or - 8 ºC), but not with LI at - 1.5 ºC, always compared to CI at - 0.2 ºC alone. However, plasmatic cortisol levels only decreased when fish were treated with SW and CEO+β-CD added to CI at - 0.2 ºC, but not in fish treated with SW and CEO+β-CD incorporated to LI at - 1.5 ºC incorporating the complex. Our data related to glucose levels were similar than previous studies in which 55 mg/L of CEO in water at different temperatures (from 22 ºC to 2 ºC) reduced the glucose levels in gilthead seabream (Bahi *et al*., 2018). Nevertheless, the encapsulation of CEO in β-CD allowed using lower doses of anaesthetic. In addition, other plasmatic levels (pO_2, pCO_2, HCO_3, TCO_2, SO_2, base excess and pH) were only modify when CEO+β-CD was incorporated in LI at different temperatures, but not when the anaesthetic complex was added to CI at - 0.2 ºC. Taking all this data into account, it could be concluded that a low dose of CEO+β-CD (15 mg/kg) incorporated to CI at - 0.2 ºC is the stunning method that kept the three parameters (glucose, lactate and cortisol) levels low and do not modify the rest of the plasmatic parameters analyzed, indicating a low stress response in the stunned fish. The use of LI during slaughter promoted cloudy eyes, which significantly reduces the commercial value of the fish (Davis & Parker, 1991; Huidobro *et al*., 2001), Also, in this thesis, the use of LI supponed a high difficulty of working in industrial facilities, so LI would not be recommended as a method of stunning fish.

In addition, we analysed the expression pattern of RyR3 coding gene in muscle upon stunning and found that the *RyR3* expression increased after stunning with 15 mg of CEO+β-CD/kg ice at - 1.5 ºC but not when 15 mg of CEO+β-CD/kg ice at - 0.2 ºC was used. The RyR3 protein allows the rapid release of Ca^{+2} (Protasi *et al*., 2000). The increase of the Ca^{+2} level before slaughter leads to post-mortem heating and acidosis in the muscle affecting the quality of the fillet (Droval *et al*., 2012). So all this data together supports the idea that LI would not be recommended as a method of stunning fish before slaughtering and that CI at - 0.2 ºC might be more adequate. Interestingly, when CI of different sizes of ice crystals (20 or 15 mm) were used with 15 mg CEO+β-CD/kg under industrial farm conditions a decrease of glucose and lactate levels, and also an increase in pH was observed. However, cortisol level was not altered. Moreover, lower doses of CEO+β-CD incorporated in CI of either 20 or 15 mm of ice crystal ice, only reduced the glucose level but not lactate or cortisol level. Furthermore, alterations in pO_2, pCO_2 or pH were observed after use low doses of CEO+β-CD incorporated in CI of 15 mm of ice crystal ice.

In general, we could conclude that the optimal method for gilthead seabream under experimental or industrial farm conditions might be 15 mg CEO+β-CD/kg ice incorporated in CI (20 mm) at - 0.2 ºC.

The novel stunning method used in this thesis was also tested in a group of farmed gilthead seabream under industrial farm conditions, that was handled with an innovative underwater cameras system during their productive cycle. Thus, this group of fish never been previously anaesthetized until stunning process, using SW and 20 mg of CEO β-CD/kg added in CI at 1-2 ºC. These fish showed a decrease of plasmatic glucose and cortisol level, compared with control fish (SW and CI at 1-2 ºC). Although decreased occurs in the plasmatic level of HCO_3 and TCO_2, the low level of glucose and cortisol together with the fact that no changes were observed in plasmatic lactate level and that pO_2 decreased instead of increased, point to the fact that fish experiment low levels of stress after be exposed to the novel stunning method with a low dose of CEO β-CD into CI at 1-2 ºC mixed with SW. So, our data indicated that the behaviour between the fish that never been previously anaesthetized and fish that had been anesthetized several times along their productive cycle, is quite similar after they were stunned with CEO+β-CD added into CI-SW at 1-2 ºC.

All these results obtained with gilthead seabream lead us to kept working with the anaesthetic complex incorporated into CI (20 mm) at - 0.2 ºC with other fish species under experimental farm conditions. Thus, in case of Atlantic salmon stunned with SW at 4-6 ºC and different doses of CEO+β-CD/L (40, 50 or 60 mg), a decrease of plasmatic glucose, lactate and cortisol level was observed. Differently, the plasmatic glucose only decreased in fish stunned with doses between 50 or 60 mg CEO+β-CD/L, while cortisol only decreased in fish stunned with the lower dose (40 mg CEO+β-CD/L). Similary, a decrease or no effects on plasmatic cortisol levels of Atlantic salmon specimens has been reported after exposure them to different doses of CEO (Iversen *et al*., 2003; Small, 2003; Palic *et al*., 2006; Hur *et al*., 2019). In addition, our data showed a decrease in plasmatic levels of pO_2 in fish stunned with 50 or 60 mg CEO+β-CD/L, while HCO_3, TCO_2 and pH decreased. This could suggest a higher stress response in fish stunned with the higher doses. Conversely, none of the other plasmatic parameters (pO_2, pCO_2, HCO_3, TCO_2, SO_2, base excess and pH) were altered in fish stunned with the lower dose (40 mg CEO+β-CD/L) compared to control group. In general, all those data indicate that stunning with SW and 40 mg CEO+β-CD/L is the method than triggered a low stress response in Atlantic salmon specimens. Surprisingly, the lactate level of Atlantic salmon specimens increased when fish were exposed to SW and β-CD alone, keeping it high even when CEO+β-CD was incorporated at 40 or 50 mg/L. However, the group of Atlantic salmon stunned with SW and 60 mg CEO+β-CD/L showed similar lactate level than control, and lower than the group of fish stunned with SW and β-CD alone, as also occurs with pCO_2. In fact, previous studies reported that the plasmatic lactate level normally increased with different anesthetic, including CEO (Iversen *et al*., 1998, 2003; Skjervold *et al*., 2001; BjÓrlykke *et al*., 2011). Looking closer to our data, they could suggest that a right dose of CEO nanoencapsulated in β-CD might be useful for controlling the plasmatic lactate and pCO_2 levels during stunning in Atlantic salmon.

In Nile tilapia, we observed a reduction in the plasmatic glucose, lactate and cortisol level when 60 mg CEO+β-CD/L was used, but only the lactate level decreased to control level with lower doses (20 or 40 mg CEO+β-CD/L) as also occurs with the pCO_2 and the base excess, while the pH slightly increased. Navarro *et al*., (2016) also showed a low glucose level in Nile tilapia exposed to high concentration of menthol 75 mg/L, and eugenol 20 mg/L compared with fish no anaesthetized. Interestingly, a decrease in glucose levels of Nile tilapia after exposure with CEO (50-60 mg/L) has been already described in previous studies

(Simões *et al*., 2011). Regarding cortisol, other studies suggested that CEO (30 mg/L) induce high stress level leading to greater mortalities upon handle Nile tilapia specimens (Sink *et al*., 2007). However, our data showed that 60 mg CEO+β-CD/L reduced cortisol level and the pH so help to decrerase the stress response of fish, even when reduced the plasmatic pCO_2 level, an issue related with stress responses (Cech *et al*., 1996). Interestingly, and as occurs in Atlantic salmon, the β-CD alone increased the TCO_2 of blood in Nile tilapia, but the incorporation of CEO induces its reduction to similar levels of control fish.

European seabass farmed under industrial contiditions and stunned with 15 and 30 mg CEO+β-CD/kg ice showed lower glucose level, slightly higher lactate level and similar cortisol level than control (stunned with CI alone). As occurred in rainbow trout, the decrease of glucose and the increase of lactate might be due to a slower blood circulation induced by the stunning (Iversen *et al*., 2003). But, the cortisol level was not modified compared to control so a stress response might be occurring and could alter the metabolism of fish. This is support by the fact that the other plasmatic parameters analyzed were similar to control level. However, a lower dose 5 mg CEO+β-CD/kg ice decreased the cortisol level and up-regulated the pH of blood without modifying the other plasmatic parameters, suggesting these data that 5 mg CEO+β-CD/kg ice is the optimal for European seabass as reduce the stress response and preserve a normal metabolism that in turn might result in a better quality of the flesh and a welfare of the fish.

Also under industrial farm conditions, 35 mg CEO+β-CD/kg ice at - 0.2 ºC used to stun rainbow trout specimens showed a significant decrease of the glucose level compared to control. Conversely the level of lactate and cortisol kept similar to control as well as the rest of the parameters studied. In concordance with our data, other studies performed with 30 mg/L of CEO and several species of trout also found that the glucose level decreased while the rest of stress parameters kept steady (Bahrekazemi and Yousefi, 2017; Velíšek *et al*., 2005). Interestingly, other studies performed in carp or trout with different doses of CEO, also found increased in the plasmatic glucose level immediately after the stunning (Holloway *et al*., 2004; Morgan *et al*., 2004; Velisek *et al*., 2005a, b). However, when lower doses (5 and 15 mg CEO+β-CD/kg ice) were used, the lactate level increased up to control level, while the cortisol level decreased compared to control. Taking into account that when a fish is stunned with an anesthetic the circulation may decreases and reduce the availability of oxygen in the tissues resulting in the production of lactate (Iversen *et al*., 2003), our data

suggested that the lowest doses of CEO+β-CD applied to rainbow trout might reduce the stress response of the fish as decrease the cortisol level, even when increased the lactate level. In fact, neither pO_2, pCO_2, HCO_3 nor pH are modified upon stunning with 5 mg CEO+β-CD/kg ice, so the metabolic problems related to the unbalance of these parameters might be low enough to avoid irreversible lesions.

As conclusion, our results found a low dose of CEO+β-CD which control or reduce the metabolic alteration induced by stress responses. It is enough to hypothesis that those will not have irreversible lesions in all species studied.

V.1.3. Immune response indicators

The stress response and its negative consequences for the immune system have been widely described in fish (Tort, 2011). Thus, the immune response is also considered a secondary response to a stressful environment (Nardocci *et al*., 2014; Ni *et al*., 2014). Such knowledge is of great importance since stressors can damage the general state of health, increase susceptibility to pathogens and reduce resistance to diseases (Khansari *et al*., 2017). To date, there are not enough studies that clearly link anesthetics and immune system disturbance. In fact, there is no studies on the effects of essential oils encapsulated in β-CD on the immune system of farmed fish. So, we analyzed in the head kidney of gilthead seabream, the expression level of the coding genes of a pro-inflammatory (il-1β) and an anti-inflammatory (tgf-β) cytokines, as well as the Hsp70, a thermal shock protein that is strongly induced in response to environmental stress (Yamashita *et al*., 2010). Previous studies showed that after stressor stimuli, cortisol had a clear inhibitory effect on both pro-inflammatory (il-1β, il-6, tnf-α) and anti-inflammatory (il-10, tgf-β1) cytokines (Khansari et al., 2017; Teles et al., 2017). However, our data showed that CEO+β-CD did not alter the expression level of *il-1β* gene, but induce a decrease of the *tgf-β* expression level when CI at - 0.2 ºC containing 15 mg CEO+β-CD/kg ice was used, which was linked with a reduction of plasmatic cortisol level. Although further studies are needed, the knowledge of how the stunning methods alter the inflammatory response of fish might be of importance when handling and transport of fish in the aquaculture enterprises. Moreover, the level of expression of *Hsp70* were not modified when 15 mg of CEO+β-CD were incorporated in CI at - 0.2 ºC. Taking into account that 15 mg of CEO+β-CD/kg ice at - 0.2 ºC decreased the level of cortisol, we can discard that these treatments triggered an intense stress response as thermal shock proteins are normally used as effective stress biomarkers (Sørensen *et al*., 2003).

As previously mention, anaesthesia can depress the immune system in fish, although CEO is considered safe as it does not cause immunosuppression in fish (Tort *et al*., 2002). Because of that, several humoral innate immune activities (peroxidase, bactericidal, antiprotease and protease activities) and total proteins in the plasma and skin mucus of the different fish species where analyzed in order to determine that CEO nanoencapsulated with β-CD keep its safety condition were analyzed. Although we have performed the study using several doses of CEO nanoencapsulated with β-CD, we next discuss the effect of the optimal doses defines for each specie accordingly with the stress parameters previously discuss in this study.

Thus, we found that the optimal doses defined for gilthead seabream (15 mg/kg ice) and rainbow trout (5 mg/kg ice) drastically reduced the plasmatic peroxidase activity. However, the optimal doses defined for Atlantic salmon (40 mg/L) and European sea bass (5 mg/kg) did not modify this activity, while the dose defined for Nile tilapia (60 mg/L) drastically increased peroxidase activity in plasma. These data suggest that in some species CEO+β-CD could block the peroxidase secretion of blood immediately after the stunning process resulting in lower level of peroxidase activity, but not in others. In the case of Nile tilapia, the increase indicates an enhancement of the secretion of this enzyme to the plasma. Similarly, Guardiola *et al*. (2016) detected a significant increase in the level of peroxidase activity in skin mucus of fish exposed to stressful conditions. So the optimal stunning conditions defined in this study might be stressful for Nile tilapia but not for the rest of the species studied.

Because of the bacterial infection susceptibility in many species of fish increases under stressful situations (Ortuño *et al*., 2001; Thompson *et al*., 1993; Walters & Plumb, 1980; Wise *et al*., 1993;), we decided to evaluate if the optimal doses defined in this study of CEO+β-CD would modify the bactericidal function. On the one hand, our results indicated that the plasmatic bactericidal activity of gilthead seabream, Atlantic salmon or Nile tilapia was not modified upon any of the treatment assayed. Similarly, previous studies performed in gilthead seabream showed that CEO is a safe anaesthetic that it does not cause immune depression in anesthetized fish (Bressler & Ron, 2004). On the other hand, European sea bass specimens stunned with 5 mg CEO+β-CD/kg ice showed normal bactericidal activity level in plasma but not in skin mucus where this activity was reduced. In contrast, the bactericidal activity measured in the skin mucus of rainbow trout was increased upon

stunning with the optimal dose of 5 mg CEO+β-CD/kg ice. Other studies have suggested that a marked decrease in plasma bactericidal activity during stress could be the result of an increase in cortisol level (Pickering and Pottinger, 1989). In concordance, our results showed an increase in the cortisol level of rainbow trout stunned with 5 mg CEO+β-CD/kg ice, while in a preview study has been suggested that 25 mg/L of CEO avoid possible immunosuppression in rainbow trout (Soltani & Mirzargar, 2013). However, our data showed that the nanoencapsulation of CEO in β-CD allowed the use of a lower dose (5 mg/kg ice) to the bactericidal activity was enhanced in rainbow trout.

Plasma protease and antiprotease activities are also important humoral innate activities to fights against infections (Ingram, 1980). In this study, we observed increases or decreases of these activities depending on the specie and the doses analyzed. Thus, gilthead seabream, Atlantic salmon, European sea bass and rainbow trout specimens stunned with the optimal doses for each specie (15 mg CEO+β-CD/kg ice at - 0.2 ºC, 40 mg CEO+β-CD/L, 5 mg CEO+β-CD/kg ice and 5 mg CEO+β-CD/kg ice, respectively) showed similar antiprotease and protease levels than control fish. However, the optimal doses in Nile tilapia (60 mg CEO+β-CD/L) decreased the antiprotease activity in plasma and increased in skin mucus, while this dose did not modify the protease activity in any tissues. Previous studies reported changes in the protease level in skin mucus after a stressful condition (Easy & Ross, 2010) as well as decreases in plasma antiprotease/protease level ratio could indicate a weakness of the immune system (Bowden *et al*., 1997). So, the decreases in the antiprotease activity, although when protease activity was not altered, might point out to an immunosuppression even when the conditions were not dramatically stressful for fish. So the optimal dose defined for Nile tilapia based on stress parameters previously, might generate immunosuppression regarding antiprotease/protease ratio in plasma, but not in the rest of the species and their respective optimal doses. Further studies including experimental infections will be mandatory to clarify the relation between these parameters in blood and skin mucus and a subsequent immunosuppression in order to establish the adequate doses for each species for handling or transport fish. So, taking all the data into account for the most species studies and the optimal doses defined in this study, we could rule out the impairment of the innate immune response and suggest that low level of plasma peroxidase activity could reduce tissue oxidative stress and positively influence meat quality either in experimental or industrial farm conditions.

Total plasma protein level may reflect the metabolic, nutritional and immune status of fish (Barton *et al*., 2002; Tahmasebi-Kohyani *et al*., 2012). In this study, no significant differences were found between fish treated with CEO+β-CD and control fish in Atlantic salmon, Nile tilapia and rainbow trout, as previously reported (Barton *et al*., 2002; Deriggi *et al*., 2006). However, the optimal doses (15 mg CEO+β-CD/kg ice and 5 mg CEO+β-CD/kg ice) defined for gilthead seabream and European sea bass increased the protein plasmatic levels. Jeney *et al*., (1997) founded significantly lower plasma protein level in fish exposed to stressful situations, so our data suggest that the anesthesia complex had a significantly anti-stressful effect in gilthead seabream and European sea bass. When analyzing fish that have never been anesthesiazed during their productive cycle we found that non activity analyzed (peroxidase, bactericida, antiprotease and protease) were modified in plasma, while only the peroxidase activity was slightly decreased in skin mucus. So as also observed for the stress indicator, this fish never stunned with CEO showed similar responses than other fish stunned with CEO several times during their life cycle and the immune-suppression could be discarded.

In conclusion, the optimal dose CEO+β-CD for each specie, although altered some innate immune parameter in plasma or skin mucus, might not be considered an immune-suppressed anaesthetic. So our results could conclude that CEO+β-CD is an anaesthetic with high potential to reduce stress in fish at slaughter and to avoid immune impairment when handling and transport fish.

V.2. Eugenol nanoencapsulated in β-CD as anaesthetic agent in gilthead seabream

2.1. Induction time of anesthesia stages

EU, similar to CEO, is rapidly absorbed and metabolized after oral administration, and it is almost completely excreted in the urine within 24 h (Fischer *et al*., 1990). Thus, EU has long been considered safe for laboratory use (Liu & Gibson, 1977). As CEO, EU is effective at different doses in fish species (Javahery *et al*., 2012). But, in general, higher doses of EU are needed to achieve the anesthesia stages at least in a period of time considered as low as CEO. In this study, a low dose of EU (20 mg/L) has been compared with EU+β-CD or with CEO alone. It was showed that farmed gilthead seabream treated with EU+β-CD

achieved a lower time of anaesthesia induction than fish treated with EU or CEO alone at 27.8-28.0 ºC. However, CEO+β-CD achieved a faster time (3.05±0.55 min) of induction anesthesia stages than EU+β-CD (19.24±0.10 min). This could be due to the temperature of the water/ice. In general, our data demonstrated that essential oils encapsulated in β-CD significantly decrease the time of anesthesia inductions in stunning at slaughtering.

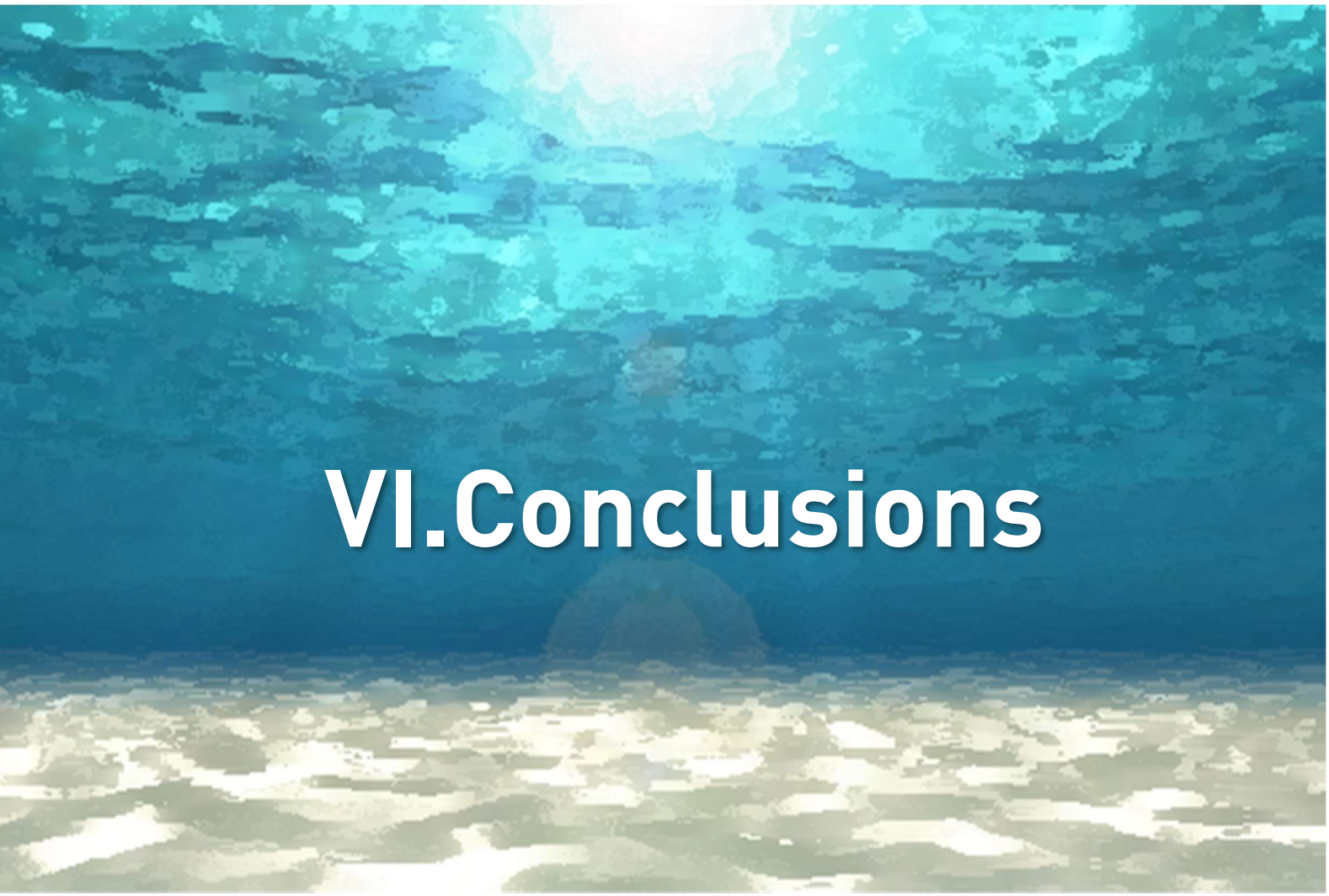
2.2. Plasma parameters as stress indicators

Anaesthetics based on EU appear to be promising as a stress-reducing sedative for some species of fish because whether used properly this chemical could improve animal welfare and survivability during and after common aquaculture-related incidents (Iversen *et al*., 2009). In this study, plasmatic glucose level decreased in gilthead seabream treated with 20 mg/L, with or without encapsulating in β-CD, compared to fish treated with CEO alone incorporated in seawater. In juvenile specimens of *Lophiosilurus alexandri* decreases in plasma glucose also occurs upon stunning with doses from 2 to 6 mg/L of EU (Favero *et al*., 2019).

Regarding plasmatic lactate and cortisol levels no differences were observed in gilthead seabream specimens treated with EU compared to CEO as also was described for lactate previously in several fish species (Mirghaed *et al*., 2018; Yousefi *et al*., 2019) or for glucose in rainbow trout (Holloway *et al*., 2004). However, decreases in cortisol level of rainbow trout have been described after stunning with 150 mg EU/L (Holloway *et al*., 2004). In fact, it has been proposed that EU reduced the release of plasma cortisol during anesthesia (Renault *et al*., 2011). Interestingly, an increase in plasmatic pCO_2, HCO_3 and TCO_2 level was detected in fish treated with EU 20 mg/L, with or without encapsulating in β-CD, compared to fish treated with the same dose of CEO. Similarly, increases in HCO_3 plasmatic level were found in *Leiarius marmoratus* treated with EU 80 mg/L (Honorato *et al*., 2014). The decrease in pCO_2 and HCO_3 plasmatic level and pH are related to functional problems due to stress responses (Barry *et al*., 1993; Black and Connor, 1964; Begg and Pankhurst, 2004; Biron and Benfey, 1994; Cech *et al*., 1996; Dabrowska *et al*., 1991; Gabriel *et al*., 2009; Saccol *et al*., 2016; Webb *et al*., 2007; Wells *et al*., 1984;). Our data showed increases in the plasmatic level of pCO_2 and HCO_3 and a decrease of pH in both groups treated with EU, nanoencapsulated or not with β-CD. So we cannot discard a stress response with physiological alterations upon treatment with EU.

2.3. Immune response indicators

Our data showed a down regulation of several humoral innate activities in the fish treated with EU compared to fish treated with CEO. Thus, plasma bactericidal and skin mucus peroxidase and bactericidal activities were reduced compared to CEO treatment. Interestingly, any of the previous stunned treatment applied in gilthead seabream at several temperatures and farm conditions altered the plasmatic bactericidal activity that was always around 40% as the bactericidal activity of gilthead seabream stunned with CEO alone. However, when EU was applied alone or nanoencapsulated in β-CD, this activity dropped to around 20%. Moreover, this activity drops down to 20% in the skin mucus when EU+β-CD was used. When taking into account that fish handling could produce skin lesions that could positively influence the appearance of pathological outbreaks together with our data, we should conclude that CEO nanoencapsulated in β-CD could be a better anesthetic than EU as allow to control the stress response and avoid immune-suppression much better than EU. The inhibition of the bactericidal activity could be equilibrated with increases in the antiprotease and protease activity as these activities in fish body fluids are involved in the inhibition of bacterial growth in fish tissues (Lamas and Ellis, 1994). However, our data showed similar level of these activities in CEO and EU alone or EU+β-CD treated fish either in plasma or in skin mucus. So, we have to conclude that EU showed a bigger potential capacity to alter fish physiology leading to stress and immune-suppression responses more easily than CEO. In fact, other studies suggest that EU inhibits the production of proteases in fish tissues (Thoroski *et al*., 1989; Wendakoon & Sakaguchi, 1993).



VI.Conclusions

1 Nanoencapsulation of clove oil in β-cyclodextrins achieved a rapid loss in sensibility and consciousness in terms of welfare in gilthead seabream, Atlantic salmon and Nile tilapia under experimental farm conditions and in European seabass and rainbow trout under industrial farm conditions by comparing to the standard conditions of stunning in each specie without anaesthesics, being the concentratin of clove oil needed different between the species.

2 A reduction of plasmatic glucose, lactate and cortisol levels in gilthead seabream under experimental or industrial farm conditions indicates that 15 mg of clove oil nanoencapuslated in β-cyclodextrins included in crushed ice at - 0.2 ºC is the best stunning condition.

3 The optimal condition for stunning Atlantic salmon or Nile tilapia specimens under experimental farm conditions inducing a low stress response is 40 mg or 60 mg of clove oil nanoencapuslated in β-cyclodextrins included in sea or fresh water, respectively.

4 European seabass and rainbow trout at industrial farm conditions showed a low stress response and similar levels of the metabolic parameters after stunning with 5 mg of clove oil nanoencapuslated in β-cyclodextrins and included in crushed ice at - 0.2 ºC.

5 The optimal concentrations of clove oil nanoencapuslated in β-cyclodextrins, previously reported for the different species used in this study, differently alter their humoral innate immune activities in serum or skin mucus, but not suppress them.

6 Gilthead seabream specimens that had been never exposed to anesthesia do not have a different stress response than other fish that had been anesthetized several times along their productive cycle when stunned with clove oil nanoencapuslated in β-cyclodextrins.

7 Low doses of eugenol oil nanoencapuslated in β-cyclodextrins and incorporated in seawater at 27.0 to 28.0 ºC, decrease the time of anesthesia induction stages in stunning at slaughtering of gilthead seabream, treated under experimental farm conditions, but might induce a stress response with physiological alterations, including humoral innate immune suppression.

VI.Resumen en catellano

1. Introducción

La acuicultura se ha convertido en el sistema de producción de alimentos de más rápido crecimiento (Broitman *y col*., 2017) para poder así satisfacer la creciente demanda de pescado para consumo humano (Ottinger *y col*., 2018), donde el bienestar de los animales es una preocupación especial que exige el cuidado adecuado para permitir un estrés mínimo (Savina *y col*., 2016).

En acuicultura, los peces están expuestos a diversos factores estresantes (Wendelaar-Bonga, 1999), como los métodos de sacrificio, que son motivo de preocupación, ya que hay evidencia sustancial de que los peces pueden experimentar dolor y sufrimiento innecesarios durante este proceso (Hedrick *y col*., 2019). A pesar de que los métodos de sacrificio son muy diversos, la mayoría de los peces cultivados suelen ser sacrificados sin aturdimiento previo (Cooke, 2018) sumergiéndolos en una mezcla de hielo y agua. Este tratamiento puede crear estrés innecesario para los peces (Lerfall *y col*., 2015), lo que implica una respuesta fisiológica que genera inicialmente la elevación en los niveles del cortisol. Como consecuencia, se generan cambios relacionados con el metabolismo, tales como el aumento en los niveles de glucosa y lactato (Zhang *y col*., 2018). Además, en los peces existe una correlación entre la ausencia de expression del gen RyR3 y la rapidez del ciclo de contracción-relajación, necesaria para la liberación rápida y controlada de Ca^{2+} en el músculo, afectando por tanto a la calidad del filete (Chiang *y col*., 2004).

El sistema inmune también se ve influenciado por una serie de factores estresantes ya que existe una conexión con el sistema neuroendocrino (Rodríguez-Quiroga *y col*., 2017) a través de la activación del eje HPI (Cockrem *y col*., 2019) y la secreción de cortisol (Cockrem *y col*., 2019) con el sistema inmune (Rodríguez-Quiroga *y col*., 2017). Cuando existen altas concentraciones de cortisol, se inhiben varias respuestas inmunes en los peces (Harris y Bird, 2000). Los estudios relacionados con el sistema inmune en los peces se han centrado esencialmente en el riñón cefálico (Zapata y Amemiya, 2000) ya que este órgano está estrechamente vinculado con la respuesta al estrés y los sistemas nervioso, endocrino e inmune, dando mecanismos de regulación cruzada en este tejido (Tort, 2011). Las citoquinas, tanto proinflamatorias como la interleucina 1β (*Il-1β*), como antiinflamatotias como el factor de crecimiento transformante beta 1 (*tgf-β1*), regulan la respuesta inmune y son alteradas ante situaciones de estrés (De Mercado *y col*., 2018).

Por ello, el procedimiento de sacrificio desarrollado en la acuicultura debe basarse en el principio de que el animal debe morir rápidamente con un mínimo de dolor o sufrimiento (Veldhuizen *y col*., 2018). Como solución preventiva al impacto negativo que supone una situación estresante, existe el uso de aceites esenciales como anestésicos (Souza *y col*., 2019), como por ejemplo el aceite esencial de clavo (Javahery *y col*., 2012) o el eugenol (Roubach *y col*., 2005). A pesar de considerarse anestésicos naturales y efectivos, además de apropiados para el uso comercial en diferentes métodos de acuicultura (Javahery *y col*., 2012), ambos aceites esenciales presentan una serie de desventajas: (i) insolubilidad en agua, (ii) eficacia a altas concentraciones, (iii) posibles cambios drásticos en el aroma y sabor o las propiedades sensoriales de los alimentos a los que se agrega (Hill *y col*., 2013). Las β-ciclodextrinas (β-CD), oligosacáridos cíclicos de origen natural, pueden resolver estos problemas porque son capaces de formar complejos de inclusión sólidos con una amplia variedad de moléculas huésped hidrófobas, como los aceites esenciales (Fravel, 2008).

2. Objetivos

Esta tesis tiene como objetivo principal mejorar el bienestar animal en el sacrificio de cinco especies de peces (dorada, salmón Atlántico, tilapia del Nilo, lubina europea y trucha arcoíris) cultivados en condiciones experimentales o industriales, utilizando las dosis posiblemente más bajas de aceite esencial de clavo encapsulado (AECe) en β-ciclodextrinas. Para eso, proponemos los siguientes objetivos específicos:

1. Analizar la inluencia del AECe sobre los estados de inducción de anestesia en peces: pérdida de equilibrio y pérdida de actividad reflejo.

2. Determinar la inluencia del AECe sobre los niveles plasmáticos de los principales marcadores de estrés usados en peces (glucosa, lactato y cortisol), además de otros parámetros plasmáticos (pO_2, pCO_2, HCO_3, TCO_2, SO_2, exceso de base y pH).

3. Determinar la inluencia del AECe sobre la expresión génica de citoquinas proinflamatorias y antiinflamatorias, así como proteínas de choque térmico en el riñón cefálico, y la expresión del gen receptor rianodina involucrado en la liberación intracelular del Ca^{+2} en el músculo, en dorada.

4. Estudiar el efecto del AECe sobre la posible modulación del sistema inmune innato, a través del análisis de actividades humorales (peroxidasa, bactericida, anti-proteasa y proteasa) y los niveles totales de proteínas, en plasma y moco.

3. Material y métodos

Con el fin de estudiar los objetivos anteriormente expuestos, se realizarón varios experimentos con diferentes concentraciones de CEO+β-CD, que varió entre las especies tratadas en función del tamaño y las características de las distintas especies. Las condiciones de cultivo fueron experimentales (dorada, salmón del Atlántico y tilapia del Nilo) y/o industriales (dorada, lubina europea y trucha arcoíris) y se ensayaron diferentes temperaturas de anestesiado (desde - 8.0 a 29.0 °C), tipos de hielo (liquido o picado), tamaños del hielo (20 o 15 mm) y el tipo de agua (salada o dulce).

Además, en el caso de la dorada también se utilizó como anestésico EU+β-CD en condiciones experimentales de cultivo a 20 mg/L de agua de mar. Un último experimento se realizó en condiciones de cultivo industrial con peces que nunca habían sido anestesiados con CEO en su ciclo productivo, ya que siempre se habían manejado con cámaras submarinas, usando 20 mg de CEO+β-CD /kg de hielo.

Durante el anestesiado, con o sin CEO+β-CD se analizaron los tiempos necesarios para alcanzar la perdida de equilibrio a partir de la observación de una disminución en la tasa opercular y una posición invertida del pez y la perdida de la actividad refleja, observando una inmovilidad total del pez y por tanto verificando la inducción profunda de la anestesia tal y como ha sido anteriormente descrito (Keene *y col*., 1998).

Una vez anestesiados, se obtuvo el moco de la piel y el plasma, así como fragmentos de riñón cefálico y músculo de los peces. En el moco de piel y plasma, se analizaron los niveles de glucosa, lactato y cortisol, además de otros indicadores de cambios metabólicos relacionados con el estrés (pO_2, pCO_2, HCO_3, TCO_2, SO_2, exceso de base y pH). Además, se determinaron posibles alteraciones en la respuesta inmune innata a través del estudio de los niveles de las actividades peroxidasa, bactericida contra *Vibrio harveyi*, antiproteasa y proteasa, además de los niveles totales de proteínas según la metodología previamente descrita (Sunyer y Tort, 1995).

En las muestras de riñón cefálico obtenidas en dorada, se analizaron los niveles de transcripción de tres genes: dos citoquinas, una proinflamatoria (*il-1β*), otra antiinflamatoria (*tgf-β1*) y una proteína de choque térmico (*Hps70*). En las muestras de músculo se analizaron los niveles de expresión del receptor de rianodina (*RyR3*).

4. Resultados y Discusión

Los tiempos de inducción de la anestesia fueron significativamente más bajos en todas las especies tratadas con bajas concentraciones de CEO+β-CD o EU+β-CD, en comparación con los peces control. Además, se consiguió una anestesia profunda en menos tiempo y usando unas concentraciones más bajas que otros autores (Hoskonen y Pirhonen, 2004). De forma similar, se comprobó que los tiempos de inducción fueron más cortos a bajas temperaturas que a altas temperaturas. Durante la anestesia por hipotermia se altera la frecuencia respiratoria. Como el estado ácido-base de la sangre de los peces depende de la temperatura, el aumento de la temperatura conduce a acidemia e hipercapnia que estimulan la hiperventilación, lo que a su vez disminuye los tiempos de inducción y recuperación de los anestesiantes que se toman o eliminan a través de las branquias (Aguiar *y col*., 2002).

El segundo objetivo de este estudio fue determinar que permitiría disminuir los niveles de estrés. Así, tanto en condiciones experimentales como industriales, se observó una disminución significativa en niveles plasmáticos de glucosa y lactato en peces tratados con bajas concentraciones de CEO+β-CD o EU+β-CD, en comparación con peces control. Los estudios de Beecham *y col.* (2006), indican que altos niveles de glucosa, lactato y cortisol en plasma en los peces era significativo de una exposición a una situación de estrés. Por lo que el uso de CEO encapsulado permitió la disminución de los niveles plasmáticos de estrés en peces tratados con concentraciones más bajas de anestésicos usados en otros estudios (Fernandes *y col*., 2016).

Curiosamente, observamos que los niveles de ARNm de *tgf-β1* en dorada disminuyeron con la presencia de CEO+β-CD incorporado en el hielo picado, acompañado de una reducción en los niveles plasmáticos de cortisol. Esto podría señalar la importancia de los procedimientos de aturdimiento durante la manipulación o el transporte de peces. También se observaron niveles más altos de expresión de ARNm de *RyR3* en el músculo de peces aturdidos con hielo líquido que en peces aturdidos con hielo picado; además, estos altos niveles se redujeron cuando el anestésico CEO+β-CD se incorporó en hielo líquido, pero no cuando se incorporó en hielo picado. Tal aumento en los niveles de Ca^{+2} antes del sacrificio implica un aumento en el hipermetabolismo muscular, lo que lleva a un calentamiento post mortem y acidosis en el músculo, afectando así a la calidad del filete. Por

lo tanto, la posible modulación de los niveles de expresión de *RyR3* es un punto interesante al evaluar nuevos métodos de aturdimiento (Droval *y col*., 2012).

La relación existente entre estrés y la incapacidad de desarrollar una respuesta inmune competente es ampliamente conocida en peces (Dunier y Siwicki, 1993). Nuestros resultados indican que el uso de CEO+β-CD a dosis óptimas para cada especie podría considerarse que no genera inmunodepresión, aunque alteró algún parámetro inmune innato en plasma o en el moco de la piel. Así, concluimos que CEO+β-CD es un anestésico con potencial para reducir el estrés en los peces en el sacrificio y evitar el menoscabo inmunitario debido a la manipulación o transporte.

5. Conclusiones

1. La nanoencapsulación de aceite de clavo en β-ciclodextrinas logra una rápida inducción de la anestesia, mejorando el bienestar en la dorada, salmón del Atlántico y tilapia del Nilo en condiciones de cultivo experimental y en lubina europea y trucha arcoiris en condiciones de cultivo industrial, comparado con las condiciones estándar de aturdido en cada especie sin anestesia, siendo la concentración de aceite de clavo necesaria diferente entre las especies.

2. Una reducción de los niveles plasmáticos de glucosa, lactato y cortisol en dorada en condiciones experimentales o industriales indica que el uso de una dosis de 15 mg de CEO+β-CD incluido en una mezcla de agua y hielo picado a - 0.2 ºC es la condición óptima de aturdimiento, obtenida después de una evaluación intensiva de distintas condiciones de aturdimiento que incluyen diferentes temperaturas, tipos y tamaños de hielo.

3. La dosis óptima para aturdir ejemplares de salmón del Atlántico o tilapia del Nilo en condiciones experimentales induciendo poco estrés es de 40 mg o 60 mg de CEO+β-CD agua de mar o agua dulce, respectivamente.

4. En condiciones de cultivo industriales, tanto la lubina europea como la trucha arcoiris muestran una respuesta al estrés reducida y niveles similares a los controles de los parámetros metabólicos analizados cuando se aturden con 5 mg de CEO+β-CD incluido en hielo picado y mezclado con agua a - 0.2 ºC.

5. Las concentraciones óptimas de CEO+β-CD, previamente determinadas para las diferentes especies utilizadas en este estudio, alteran de manera diferente las actividades inmunes innatas humorales en suero y moco de la piel, pero no las suprimen.

6. Individuos de dorada que nunca han sido anestesiados con CEO (durante su ciclo de cultivo) no tienen una respuesta al estrés cuando son aturdidos con CEO+β-CD diferente a la de otros peces que sí han sido anestesiados (varias veces) a lo largo de su ciclo productivo.

7. Dosis bajas de CEO+β-CD o EU+β-CD e incorporadas en agua de mar entre 27.0 y 28.0 ºC, disminuyen el tiempo de inducción de anestesia de individuos de dorada tratados en condiciones experimentales, pero podría inducir una respuesta de estrés con alteraciones fisiológicas, destacando la supresión de la respuesta inmune humoral innata.

VIII.References

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