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Fish welfare in aquaculture: From physiology to molecular activities and new tools for study innovative diets, social and spatial stress

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Abstract: Guaranteeing a high quality of life for animals has recently become a matter of increasing concern. Welfare assessment has been well-developed for terrestrial species, mainly for those kept in captivity, but the current state of the art is less well-characterized for aquatic animals. The classical methodologies utilised to date, such as the kind of behavioural observation widely used for terrestrial animals, are not appropriate for improving our knowledge of the well-being of aquatic animals if used alone, mainly due to the large number of species and the difficulty of obtaining comparative results among the different taxa of interest. Among different approaches, the evaluation of internal responses inside organisms can be carried out using different physiological and biochemical tools. This thesis presents methodologies and results of studies aimed at validating physiological and immunity parameters as markers of stress in the evaluation of fish welfare, with a particular focus on two important species in aquaculture, *Sparus aurata* and *Dicentrarchus labrax*. Fish were exposed to different conditions, and their welfare status was evaluated. An approach based on physiological markers was introduced to investigate the effects of the surgical implantation of electronic tags to provide telemetry for aquaculture study purposes. Indeed, the use of telemetry to study aquatic organisms has developed rapidly and its utilization needs to be better understood. Nutrition and food quality are further critical aspects for farmed animals. Indeed, aquaculture, both conventional and organic, has increased widely in recent years and has attracted the attention of various stakeholders. Physiological stress indicators, growth performance, and swimming activity data obtained by acoustic transmitters are good indicators for welfare assessment, and here they have been used to evaluate the effect of different aquaculture methodologies, in particular on fish fed with different diets. In addition, social stress and territoriality are relevant factors to evaluate for gregarious species that may have consequences on animals farmed in captivity conditions. These aspects may impair the ability of fish to respond to various stimuli, such as pathogens and environmental variations, with negative influences. In this thesis, we evaluate the effects of social stress on gilthead bream through behavioural observation supported by the evaluation of physiological and immunological-cellular parameters, such as cortisol, glucose, lactate, osmolarity, and phagocytosis.

Keywords: Welfare, Physiology; Growth; Telemetry; Tag; Conventional or organic diets; Aquaculture; Social hierarchy; Territoriality; *Sparus aurata*; *Dicentrarchus labrax;* Stress; Cortisol; Behaviour; Gilthead sea bream; European sea bass; Hormones; Phagocytosis

Directly connected papers with the thesis

Alfonso, S., Zupa, W., Manfrin, A., Fiocchi, E., Dioguardi, M., **Dara, M.**, Lembo, G., Carbonara, P., Cammarata, M., Cammarata, M., 2020. Surgical implantation of electronic tags does not induce medium-term effect: Insights from growth and stress physiological profile in two marine fish species. Animal Biotelemetry 8, 4–9. https://doi.org/10.1186/s40317-020-00208-w (CHAPTER1)

Carbonara, P., Alfonso, S., Dioguardi, M., Zupa, W., Vazzana, M., **Dara, M.,** Spedicato, M.T., Lembo, G., Cammarata, M., 2021. Calibrating accelerometer data, as a promising tool for health and welfare monitoring in aquaculture: Case study in European sea bass (*Dicentrarchus labrax*) in conventional or organic aquaculture. Aquaculture Reports 21, 4–13. https://doi.org/10.1016/j.aqrep.2021.100817 (CHAPTER2)

Carbonara, P., Zupa, W., Bitetto, I., Alfonso, S., **Dara, M.,** Cammarata, M., 2020. Evaluation of the effects of the enriched-organic diets composition on european sea bass welfare through a multi-parametric approach. Journal of Marine Science and Engineering 8, 1–23. https://doi.org/10.3390/jmse8110934 (CHAPTER3)

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Other relevant papers:

Cammarata, M., Benenati, G., **Dara, M.,** Parisi, M.G., Piazzese, D., Falco, F., Stabili, L., 2019. *Sabella spallanzanii* mucus contain a galactose-binding lectin able to agglutinate bacteria. Purification and characterization. Invertebrate Survival Journal 16, 15–24. https://doi.org/https://doi.org/10.25431/1824-307X/isj.v0i0.15-24

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Summary

Chapter 1

1.Introduction

1.1. Welfare

The concept of "animal welfare" refers to the physical and psychological well-being of animals, and the study of this topic has primarily developed during the last half century. Human-animal interactions, in particular domestication and breeding, date back to ancient time. Humankind, indeed, has reared and domesticated animals, mainly birds and mammals, for millennia for a variety of purposes, such as food, clothing, agricultural work, pets; but research centred on animals as sentient organisms, capable of suffering, only started during the 20th century, probably due to our better understanding of animals' motivation, cognition, and behavioural complexity (Broom, 2011). Nowadays, the need to improve the efficiency of this interaction has led different stake holders to reconsider the value of animal welfare. Research on animal welfare, initially centred on livestock and laboratory animals, has extended also to fish, other vertebrates, and even invertebrate groups (e.g., cephalopods, crustaceans, and others) (Benn *et al.*, 2019; Branson, 2008; Carere and Mather, 2019; Diggles, 2019). Unfortunately, the concept of welfare is not clearly defined for animals, and different ideas have been proposed to explain it. It is generally associated with three different aspects of their life: the correct physiological functioning of the organism; the natural environment of the animal; its feelings/emotional state (Huntingford *et al.*, 2006a; Kristiansen and Bracke, 2020). In the breeding sector, animal welfare is associated with satisfying the "five principles of freedom" described by the Farm Animal Welfare Council (Manteca *et al.*, 2012; Webster, 2005), with the aim of guaranteeing the basic necessities of animals without negative experiences:

1. The animal is free from hunger, thirst, and malnutrition, because it has ready access to drinking water and a suitable diet.
2. The animal is free from physical and thermal discomfort because it has access to shelter from the elements and a comfortable resting area.
3. The animal is free from pain, injury, and disease, thanks to suitable prevention and/or rapid diagnosis and treatment.
4. The animal is able to express most of its normal behavioural patterns, because it has sufficient space, proper facilities, and the company of other animals of its kind.

5. The animal does not experience fear or distress, because the conditions needed to prevent mental suffering have been ensured.

Therefore, animals must be free from hunger, thirst, discomfort, pain, disease, fear, and anguish, and they must be free to engage in their natural behaviour. But even in this case the definition of well-being is rather vague and poorly defined (Manteca *et al.*, 2012; Webster, 2005).

Various stakeholders, including governments, have recently been paying attention to the management of terrestrial farming ecosystems, but even more to aquatic ecosystems and the welfare of farmed animals. Indeed, in Europe, fish, have only recently been included in the group of animal considered sentient, along with for mammals, birds, and reptiles (Algers *et al.*, 2009; The Council of the European Union, 1997). However, the goal of including all aquatic taxa with human-interaction has posed difficulties due to the lack of sufficient scientific evidence regarding the definition of what animal sentience means (2010/63/EU, 2010; Browman *et al.*, 2019).

Different approaches have been considered for defining "animal sentience" in order to include different aquatic taxa in animal welfare regulations, but in some cases, such as the invertebrates, there have been some difficulties in terms of application (Browman *et al.*, 2019). Some researchers have pointed toward the investigation of these animals' neuroanatomical structure in order to individuate the sensory neurons which perceive stimuli and respond to painful stimuli, called nociceptors (Fiorito *et al.*, 2015; Kristiansen and Bracke, 2020; C B Schreck *et al.*, 2016). However, the ideas of pain, suffering, and consciousness remain to be investigated and better-defined for humans and, even more, for animals. A further approach is the ethological one, a well-established area of study of animal behaviour, in particular for terrestrial farmed animals due to our long historical knowledge of them. Indeed, a number of abnormal behaviours have been listed which indicate the status of an animal's welfare. With aquatic organisms, however, this approach is not always easy to apply; indeed, few species can be easily observed and their behaviour described (Browman *et al.*, 2019). Due to the vast number of aquatic taxa, vertebrates and invertebrates, and different inter and intra species responses, studying the behaviour of each species becomes a very hard, laborious, and time-consuming task. Despite different authors having previously treated this topic from different points of view (Broom, 2002; Browman *et al.*, 2019; Fiorito *et al.*, 2015; Huntingford *et al.*, 2006a; Kristiansen and Bracke, 2020; Martins *et al.*, 2012; Carl B. Schreck *et al.*, 2016; Webster, 2005), these studies were mainly

based on neuroanatomical analogies between human and animals, mental capabilities, behavioural alteration, perception of pain and/or suffering.

Obtaining consistent data that allow us to properly evaluate the welfare of aquatic animals, avoiding inconsistent outcomes, is necessary to focus on objectively measurable welfare indicators such as behaviour, physiology, growth, fecundity, health, and stress (Arlinghaus *et al.*, 2009). As a consequence, more species-specific research is required in order to correctly apply these indicators (Bøtner *et al.*, 2012).

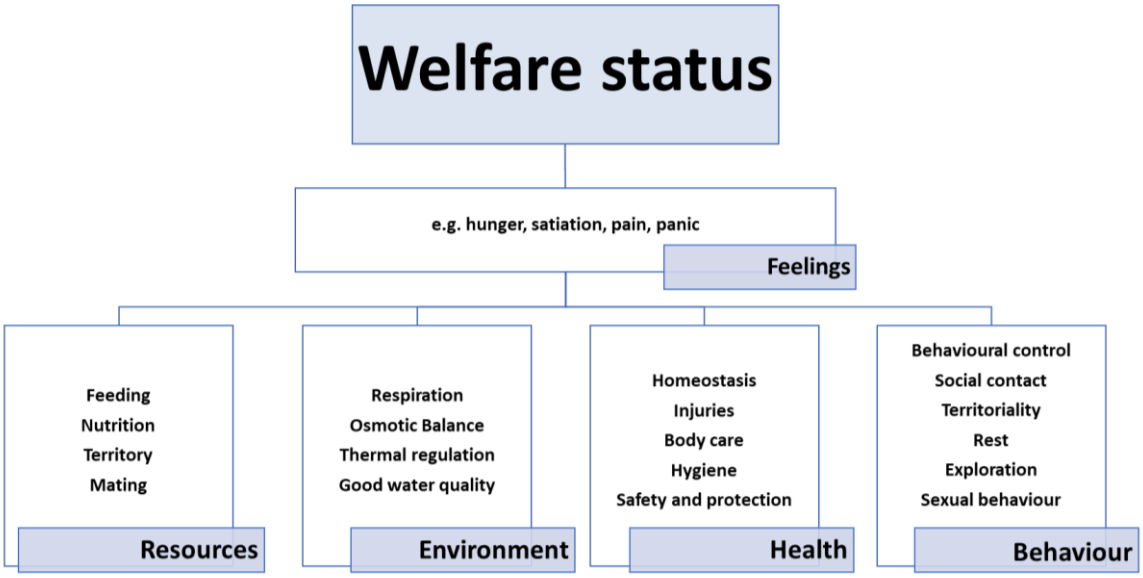

Figure 1 The welfare needs of fish can be placed into several different broad categories. The degree of fulfilment of these needs affects their mental state and thereby the welfare status of the animals Adapted from (Mellor *et al.*, 2009*).*

Homeostasis, nowadays, is a well-known concept; it consists of a series of biochemical mechanisms devoted to maintaining the internal functioning equilibrium in living organisms. These physiological responses, even though correlated to the previously mentioned processes (i.e., pain, sentience, suffering), are independent. It is possible to establish a baseline relation to the welfare of the species, and each variation may indicate an imbalance that could be considered an adverse condition. Of course, even for this approach it is not possible to obtain a universal pattern for all aquatic species, and it is fundamental to have a deep knowledge of the physiology of each species. Among all the different above-mentioned approaches, this, the evaluation of the physiological processes, may be considered a relevant field that deserves to be investigated and utilized in the welfare assessment of fish, in particular in the aquaculture sector (Gesto, 2022; Jerez-Cepa and Ruiz-Jarabo, 2021).

Beyond the definition considered, the welfare of fish intended for human use is critically important for several reasons (Seibel *et al.*, 2020), and it is at the centre of interest of different sectors where animals play a role as central actor. Welfare, indeed, has to be guaranteed for all animals involved in zoos and aquariums, where they are at the centre of the exhibition, and the interest of the keepers is to maintain them in good conditions with their natural behaviour as an attraction for visitors (Olivotto *et al.*, 2011) . Good conditions and a good welfare status are of fundamental importance when talking about animals involved in scientific research experiments and guarantee that results are not impaired by problematic factors (Ruiz-Jarabo *et al.*, 2020, 2019b; Vargas-Chacoff *et al.*, 2020). In relation to fish farming, appropriate welfare conditions have fundamental importance in the fishery and aquaculture (F&A) sectors. Aquatic animals which are not chronically stressed present better growth rates, are less prone to diseases, and the final product maintains higher quality features (Sneddon *et al.*, 2016). In addition, avoiding unnecessary animal suffering during the capturing, rearing, and slaughtering of fish is important according to current ethical standards regarding the use of animals. Moreover, welfare is also critical for the economic implications for farmers, which include that fish growth is highly dependent on the welfare status of the fish, the need to optimize feed expenses and keep expenses for disease care low, and to keep economic value for the final product higher as we know that stress affects the quality of the flesh. Regarding this last point, indeed, slaughter is a critical aspect in F&A since the quality of the flesh is affected when the animal is stressed before its death (Barragán-Méndez *et al.*, 2018; Commission, 2017; Zhang *et al.*, 2017). All the previous listed aspects are greatly affected by stress, and it is understandable that there is a shared interest among farmers, researchers, aqua-culturists, and ornamental fish keepers that fish held in captivity live well, and it is in the interest of commercial fishers that fish captured in a trawl maintain high flesh quality and, thereby, receive a high market price. Recently, one more interest has been added, which is related to a growing insistence on the part of consumers that the organisms that are farmed are well treated.

All these reasons explain the increasing interest in fish welfare research. Still, in spite of this growing interest, research studies on fish welfare represent less than 10% of the studies about general animal welfare. Finally, even strict regulations about fish utilization issued by national and international organizations, based on both ethical standards and available information on fish physiology and behaviour, constitute important legal reasons to maintain fish welfare at adequate standards (Falaise, 2019).

1.2. Current knowledge gaps related to fish welfare

Despite fish welfare becoming a hot topic of late, currently there are a number of gaps in our knowledge about fish, both in wild and artificial conditions. As mentioned, various sets of recommendations or guidelines have been published by researchers (Noble *et al.*, 2020, 2018) or other institutions, such as the Royal Society for the Prevention of Cruelty to Animals (RSPCA) in the UK (Kristiansen and Bracke, 2020; RSPCA, 2020). Certainly, the protection of fish by national regulations is not uniform, but it is on the increase and such guidelines may constitute the basis of new regulations for fish welfare monitoring in captivity.

Current research seeks to answer questions about better welfare conditions under which to keep fish in captivity. These seemingly simple questions do not have easy resolutions. For example, there are questions about the most adequate conditions for maintaining fish; or questions regarding which indicators need to be considered to guarantee good welfare, or how the indicators should be evaluated and quantified; or questions about the validity of these variables, considering inter or intra species variability and how to compare different groups of fish from different sites/farms and/or environmental conditions. Indeed, the principal difficulty is to find reliable indicators that allow us to evaluate the conditions of fish, in particular after long periods of exposure to inadequate conditions. Acute stress and related welfare problems are relatively easy to detect, but the difficulty lies in detecting evidence of the effects of chronic disturbances.

1.3. Stress Physiology

Schreck and Tort defined stress as "the physiological cascade of events that occurs when the organism is attempting to resist death or re-establish homeostatic norms in the face of insult" (Schreck and Tort, 2016). Homeostasis is the capability of the organisms to maintain all the fundamental parameters which ensure survival and the proper functioning of vital processes (pH, osmolarity, energy metabolites, pO_2) in equilibrium. Maintaining equilibrium is of fundamental importance in restoring conditions after disturbances from stressful events and deviations from the baseline. The system, regulated by biochemical reactions, involves enzymes, hormones, transporters, and proteins, and it requires a synchronized action of allostatic change enabling a return to the optimal physiological level (Chrousos, 2009; McEwen and Wingfield, 2003).

Endocrine cascades control stress physiology in teleosts (Gorissen and Flik, 2016). Physiological responses to stress may ideally be divided into three groups, primary,

secondary, and tertiary, with sequential activation related to the intensity and duration of stress (Barton, 2002).

The primary response to stress is initiated and coordinated by two neuroendocrine axes (Fig.2), the hypothalamic-pituitary-interrenal (HPI) system and sympatho-chromaffin tissues, (Mommsen *et al.*, 1999; Carl B. Schreck *et al.*, 2016) and include the release into the bloodstream of neuroendocrine hormones such us catecholamines (Malham *et al.*, 2002; Reid *et al.*, 1998) and corticosteroids for vertebrates (Fiorito *et al.*, 2015; Ruiz-Jarabo *et al.*, 2019a; Wendelaar Bonga, 1997). The presence of these hormones in the circulatory system induces the activation of the secondary stress responses, including increasing heart and respiration frequency rates and mobilizing energy metabolites to cover the demand for energy and oxygen imposed by the stressor (Barragán-Méndez *et al.*, 2020, 2018; Costas *et al.*, 2011; Fiorito *et al.*, 2015; Wedemeyer *et al.*, 1990).

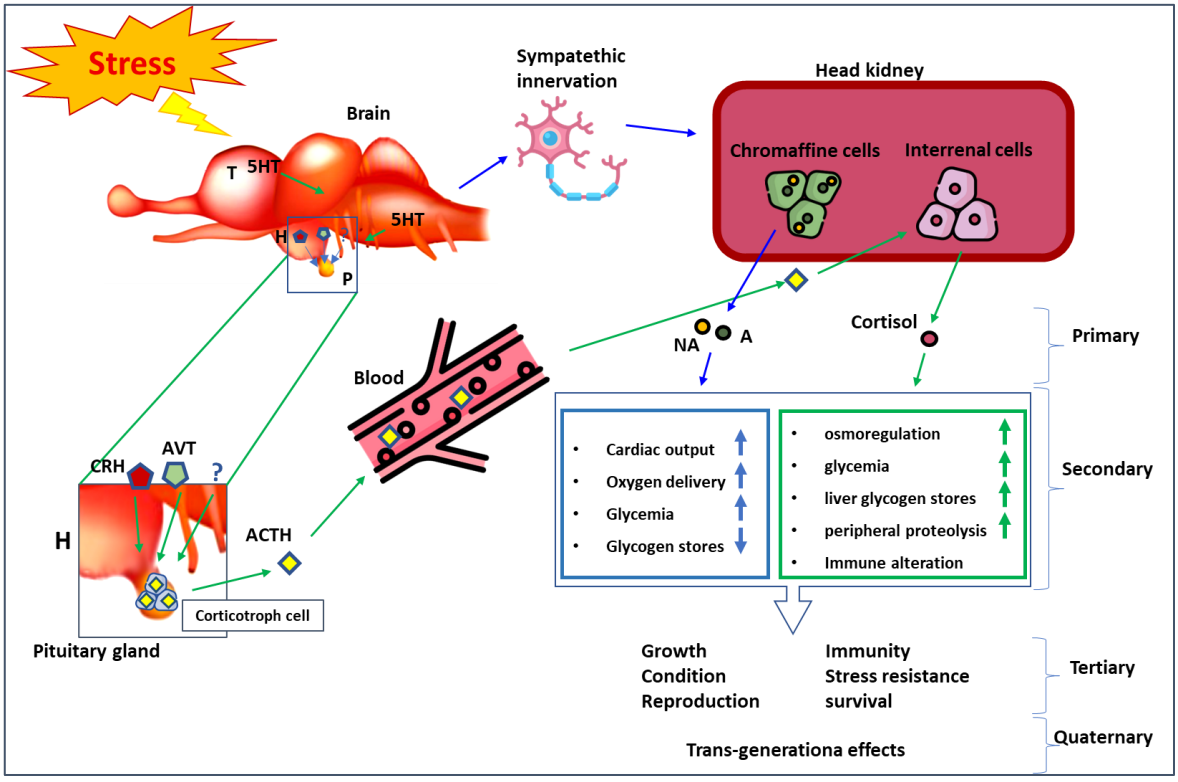

Figure 2 Main neuroendocrine pathways involved in fish stress responses. The two neuroendocrine routes are indicated by blue (the brain-sympathetic nervous system- chromaffin cells axis, BSC) and green lines (the hypothalamus-pituitary-interrenal cells axis, HPI). 5HT, serotonin; A, adrenaline; ACTH, adrenocorticotropic hormone; AVT, arginine vasotocin; CRH, corticotropin-releasing hormone; H, hypothalamus; NA, noradrenaline; P, pituitary; T, telencephalon. Image adapted from (Gesto, 2022).

If the stress is perdurable, it can lead to the activation of the tertiary responses, causing the collapse of energy stores and affecting the immune system, behaviour and fitness, and in extreme cases causing the death of the animal (Arjona *et al.*, 2009; Fiorito *et al.*, 2015;

Wedemeyer *et al.*, 1990). As a consequence, teleosts experience metabolic disorders, lower growth rates, immune-deficiencies, impaired development, reproductive disruptions, alteration of behavioural and social skills that clearly compromise their welfare (Fiorito *et al.*, 2015; Jerez-Cepa and Ruiz-Jarabo, 2021).

1.4. Primary, secondary, and tertiary physiological indicators

In the three different steps of the physiological response to stress, between the molecular and whole animal levels, it is possible to individuate some indicators that can be used as tools for welfare evaluation. It is important to underline that the evaluation of a single indicator does not give enough information and that it is better to integrate information obtained from the evaluation of different indicators. Evaluation can begin after the activation and mobilization of a series of molecules identified as primary indicators (catecholamines and stress hormones), followed by secondary indicators (e.g., changes in glucose, ion balance, acid–base balance, immunological functions, or other indicators of energetic metabolism) (Schreck and Tort, 2016).

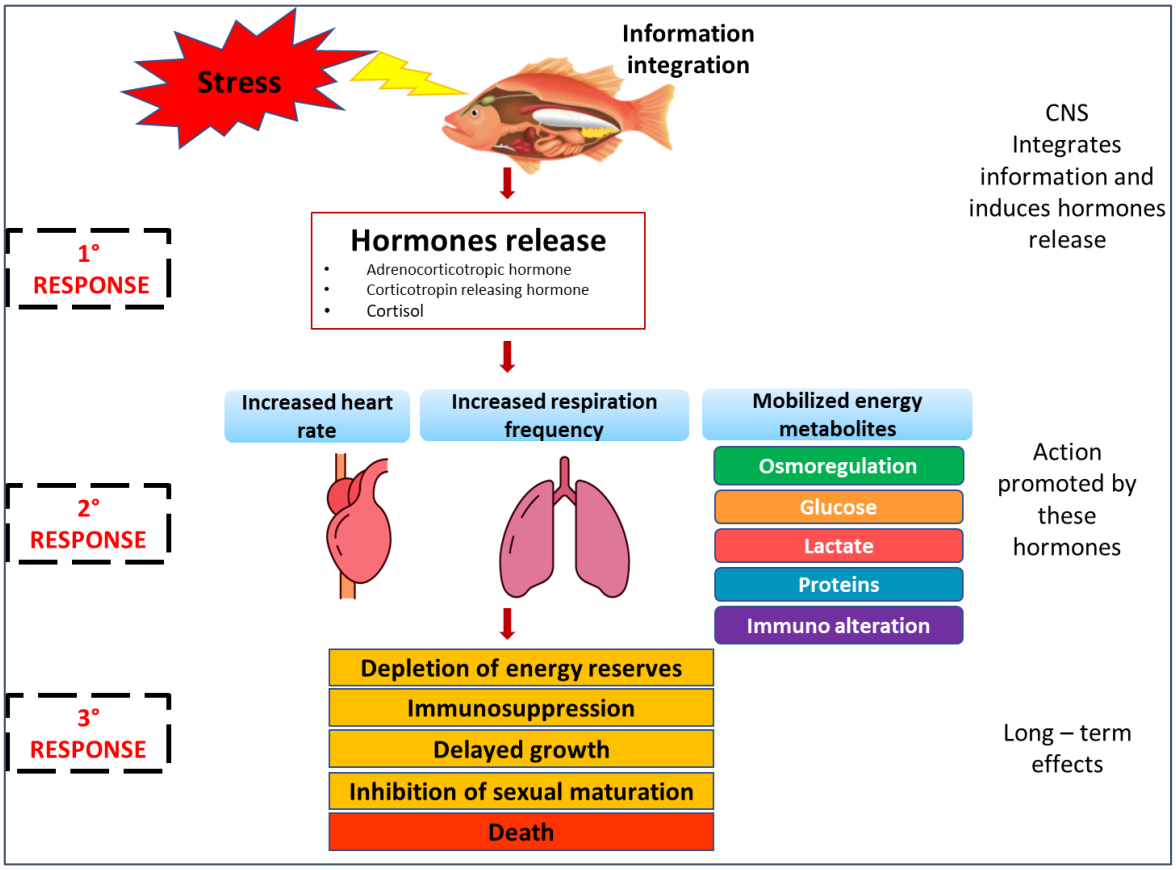

Figure 3 Physiological stress responses in aquatic animals. CNS (Central Nervous System). Adapted from (Jerez-Cepa and Ruiz-Jarabo, 2021).

These indicators are useful for assessing fish welfare. The choice of the appropriate indicators in relation of the stress suffered from the organisms is of particular importance. Catecholamines, for example, provide the fastest primary response but are difficult to measure because they respond quickly and may be influenced by capture and handling (Pottinger, 2008; Reid *et al.*, 1998); they can be used as good indicators in laboratory conditions but not for field investigations. Differently from catecholamines, cortisol, the so-called stress hormone, is the most common stress indicator used. It responds more slowly than catecholamines and can be quantified in laboratory or field conditions. (Barton, 2002; Mommsen *et al.*, 1999; Pottinger, 2008; Romero and Reed, 2005). It can be used to obtain basal and post-stress levels. Cortisol is also involved in different molecular responses. It has a role in stimulating the expression of several classes of proteins, such as metallothionein, ubiquitin, and heat shock proteins (HSPs), by interacting with heat shock factors (HSFs) (Basu *et al.*, 2001; Kassahn *et al.*, 2009; Vamvakopoulos and Chrousos, 1994; Vijayan *et al.*, 2003). It can be used to obtain basal and post-stress levels. The secondary indicators include glucose elevation, lactate elevation, osmolality and specific ion changes, and leukocyte activity. Glucose elevation is caused by the increase of catabolism and glucose release in circulation due to stress (Barton, 2002); lactate elevation, is related to anaerobic metabolism caused by low levels of oxygen in body tissues (hypoxia) or exercise stressors (Wood *et al.*, 2006); variations in osmolality may be triggered by the release of catecholamines and their effect on higher heart rate and gill permeability (Sopinka *et al.*, 2016); the leukocytes, cells of the immune system, may reflect acute and/or chronic stress exposure, with consequent alteration of their functions (Davis *et al.*, 2008). The tertiary step, also called the whole-organism level, includes a plethora of indicators correlated to welfare status and fish conditions: growth, dimension, weight, organo-somatic index, disease resistance, alteration of metabolism, indicator of swimming activity, cardiac functions, oxygen consumption, recovery ratio, behaviour, mortality (Adams *et al.*, 1993; Brodeur *et al.*, 2001; Jain *et al.*, 1998; Sadoul and Vijayan, 2016; Wedemeyer *et al.*, 1990). Differently from primary and secondary stress indicators which mainly relate to the physiology of the animals, tertiary stress response is mainly related to ethology and ecology, even though it is strictly correlated to certain physiology aspects, such as indicators related to cardiac activity and metabolism. Recently, the presence of an additional kind of response, called quaternary stress response, has been proposed in which stress effects are trans-generational and transmitted to the progeny through genetic and epigenetic mechanisms (Faught *et al.*, 2020; Ryu *et al.*, 2018; Vera-Chang *et al.*, 2018).

System	Parameters	References
Acid-base balance	H^+, OH^-, HCO_3^{-}, $P0_4^{2-}$, SO_4^{2-}	(Evans *et al.*, 2005; Tresguerres and Hamilton, 2017)
Hydric-ionic balance	H_2O, osmolality, Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+}, others	(Foster *et al.*, 2010; Freire *et al.*, 2008; Hwang *et al.*, 2011; Mccormick, 2011)
O_2 (CO_2) transport	Haemoglobin/hemocyanin, haematocrit	(Barragán-Méndez *et al.*, 2019; Jensen *et al.*, 1997; Wells, 2009)
Energy management	Glucose, lactate, amino acids, triglycerides, free fatty acids, etc.	(Speers-Roesch and Treberg, 2010; Storey and Storey, 1983)
Immune system (Innate)	Physical barriers, cell-cell mediated defence (phagocytosis), humoral defence (antimicrobial enzymes, non-specific proteins, complement system), inflammation	(Adachi *et al.*, 2003; Decker and Jaenicke, 2004; Gestal and Castellanos-Martínez, 2015; Loker *et al.*, 2004; Smith *et al.*, 2019; Vazquez *et al.*, 2009)
Immune system (adaptative)	Cell-mediated defence (B and T lymphocytes)	(Smith *et al.*, 2019)
Free radicals balance	Oxidative stress system	(Del Rio *et al.*, 2005; Lushchak, 2011; McCormick and Bradshaw, 2006; Porte *et al.*, 2006; van der Oost *et al.*, 2003)

Table 1 Main physiological parameters of aquatic animals' homeostasis. Adapted from (Jerez-Cepa and Ruiz-Jarabo, 2021).

1.5. Telemetry

Current human knowledge about animal farming and captivity conditions has been obtained mainly from the direct observation of, and interaction with, terrestrial animals; however, we must consider the many potential difficulties that arise when attempting to obtain direct information on populations of animals living underwater and evaluate their health status (Bjelland *et al.*, 2015).

To overcome these difficulties, technology has been developed to provide appropriate identification systems which facilitate the collection of the required data. Acoustic telemetry is a method initially and widely utilized for terrestrial animals which has recently been applied to underwater environments. It is a method for remote sensing where individual fish are equipped with electronic transmitters containing sensors that measure variables and transmit the information to data receiver units using acoustic signals. Initially, this technique was used for wild fish research, but nowadays it is utilized within aquaculture-related research permitting us to obtain and record different kind of information, such as individual depth movements, positions in the 3D environment, swimming activity, muscle activity, respiration rates and feed intake. Currently, acoustic telemetry is the only method which allows the collection of continuous data from fish reared in cages. While other methods, such as cameras or sonar, collect behavioural information, acoustic telemetry has the advantage of collecting physiological data because the transmitters are placed in or on the specimen (Føre *et al.*, 2018). Despite these advantages, the application of transmitters requires careful handling, and the surgical application of transmitters creates the risk of influencing the conditions of the fish, thus altering the collected information.

Chapter 2

2.1. State of the art

Due to an increasing demand for fish products and a decrease in natural resources, the aquaculture sector has grown rapidly over the past few decades, and it has also served to limit the impoverishment of natural fish populations; it now represents more than 50% of total fish production (FAO, 2020).

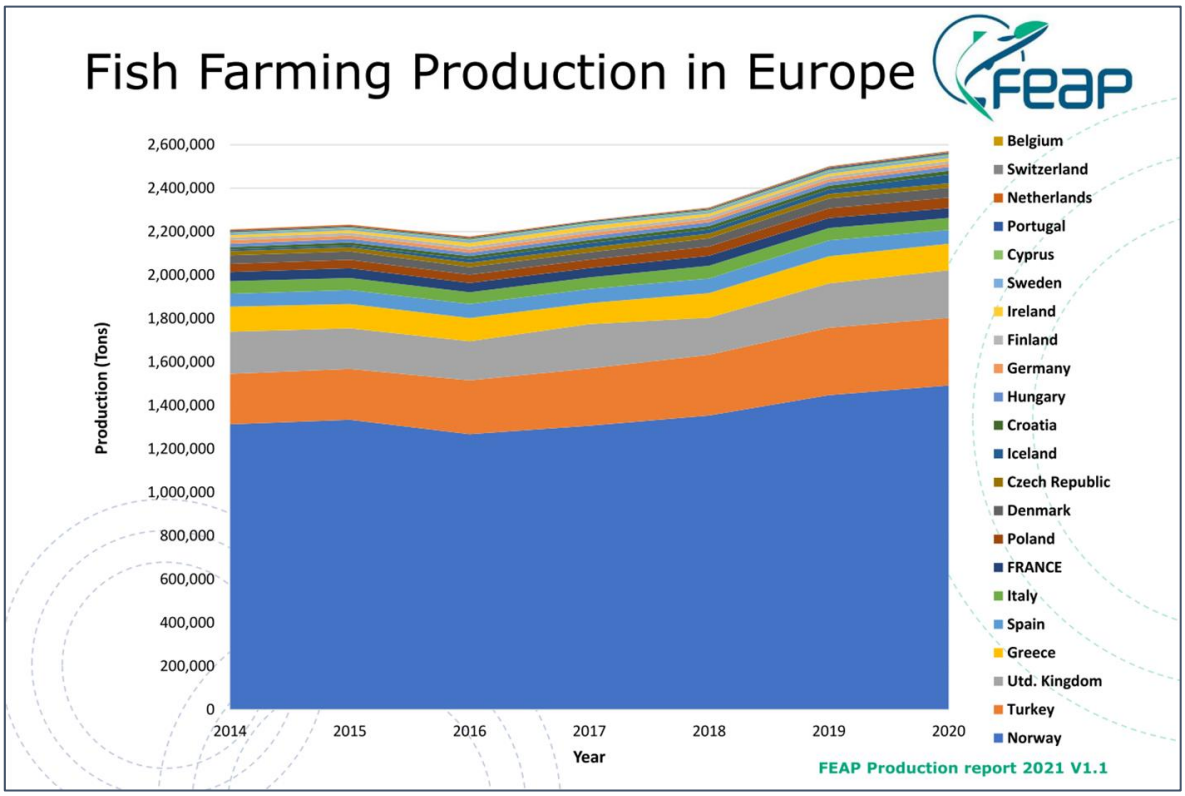

Figure 2 Fish farming production in EU countries in recent years (FEAP, 2021).

The total European production of fish by aquaculture was estimated to be 2,570,650 tons in 2020. The main species produced are salmon, trout, sea bream, sea bass and carp, making up 95% of total European production in 2020.The following graphics provide an overview of the key production sectors, analysed by country and by species (FEAP, 2021).

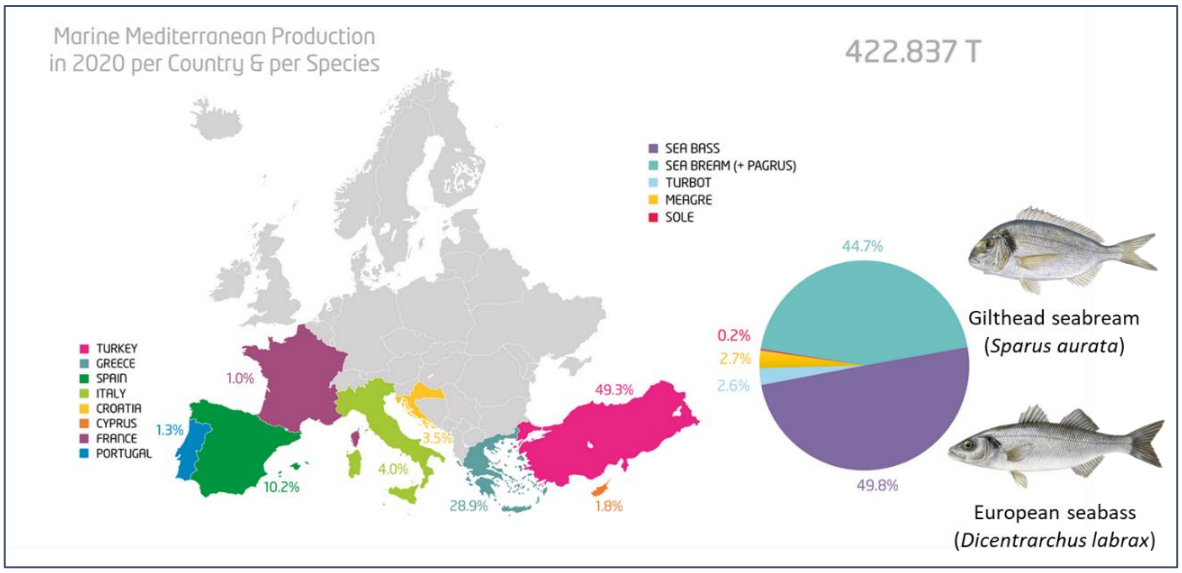

Figure 3 Marine Mediterranean production in 2020 per country and species; adapted from (FEAP, 2021).

Consequently, as above described, good fish welfare is strictly correlated with good rearing conditions, and it is understandable that the welfare of farmed fish is important for the market, as well as being a matter of increasing public concern (Braithwaite and Huntingford, 2004; Cerqueira *et al.*, 2020). In captivity, the environment available for rearing fish is very different from the environment in which their wild counterparts live (Gross, 1998). Good food quality is readily available, as fish are protected from natural predators and disease and do not have to compete for mates. However, the physical environment is much reduced, as fish are disturbed by rearing activities and are often restricted at high densities within limited and crowded spaces, with the consequent risk of spreading disease and increased social interaction, including with aggressive fish. However, assessing fish welfare is a complex task that requires an integrative overview, ranging from physiology to behaviour and biological performances (Huntingford *et al.*, 2006b; Martos-Sitcha *et al.*, 2020; Schreck and Tort, 2016; Sneddon *et al.*, 2016).

Figure 4 European bass sea and gilthead sea bream on the fish counter of a market.

The structure of this Ph.D. thesis is made up of five chapters, which may be divided into two main blocks. The first is composed of studies about the application of new telemetry tools and the evaluation of alternative diets in order to face the new challenges that the aquaculture sector presents; in the second block, the effects of social stress and territoriality on the welfare of reared animals are studied. The two species involved in the experimentations are *Dicentrarchus labrax* and *Sparus aurata*, two important fish for European aquaculture which are mainly reared in the Mediterranean region, respectively known as the European sea bass and gilthead sea bream (FEAP, 2021).

Chapter 3 introduces the use of stress and physiological markers in order to validate the telemetry and the surgical implantation of electronic tags for aquaculture purposes. Indeed, its application to aquatic organisms has developed rapidly, and physiological sensors have been increasingly used as tools for fish welfare monitoring. However, for the technology to be used as a reliable welfare indicator, it is important that the tagging procedure not disrupt fish physiology, behaviour and performance. In this chapter, medium-term data on the physiological stress profile and growth performance after surgical tag implantation are shown for the two species at the centre of this thesis. **Chapter 4** focuses on nutrition and the quality of the food supplied to fish in captivity, one of the critical aspects to analyse for farmed animals. Both the conventional and organic aquaculture sectors have grown rapidly over the past few years, and more recently, animal welfare has attracted increased attention on the part of both consumers and governments. In this chapter, in addition to the classical welfare indicator measurements (physiological stress indicators and growth performance), the swimming activity data recorded by the acoustic transmitters introduced in chapter 3 have been used to evaluate the welfare of European sea bass fed with a conventional or organic diet. **Chapter 5** continues to centre on the nutrition of fish reared in artificial conditions. This chapter presents a study carried out on three groups of European sea bass fed for seven months with either a conventional diet or two different organic diets containing organic vegetables and a natural antioxidant compound. The two organic diets differed in terms of raw proteins, fish oil and lipid contents. Sea bass welfare conditions were assessed in relation to these three diets using 16 different indicators in a multiparametric approach in order to obtain a comprehensive picture of sea bass physiological state. In **chapter 6**, the focus shifts to a different kind of stress in gregarious species. Social stress, indeed, may have consequences on animals kept in crowded conditions, especially in captivity. It may impair the ability of fish to respond to various stimulus, such as pathogens or environmental variations. In this chapter, the effects of social stress on gilthead bream were investigated.

To study the effects of social stress, biochemical and immunological-cellular parameters, such as cortisol, glucose, lactate, osmolarity and phagocytosis, were evaluated 24 h after the establishment of a social hierarchy in a group of three fish, and the values obtained were correlated to social rank. Social hierarchy was determined and characterized by behavioural observation (aggressive acts and feeding order), and a social rank was assigned to each specimen (dominant or subordinates). In **chapter 7**, the focus continues on social stress of a gregarious species which lives in small groups. Here, gilthead sea bream behaviour under social stress was examined, and the mechanisms involved in the establishment of hierarchy was determined by using two experimental models which underline the importance of territoriality. To study the effects of social stress and territoriality in both models, behavioural observation was used, integrated with the evaluation of cellular and physiological biochemical characteristics such as phagocytosis, cortisol, glucose and osmolarity.

Chapter 3

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Animal Biotelemetry

SHORT COMMUNICATION Open Access

Surgical implantation of electronic tags does not induce medium-term effect: insights from growth and stress physiological profile in two marine fish species

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3.1. Background

Over the past decades, telemetry applied to aquatic organisms has greatly developed in terms of tag miniaturization, battery life, software and hardware [1]. These tags are precious tools for the characterization and monitoring of behaviour in a wide range of organisms, including fish [2]. Moreover, electronic tags can also be equipped with environmental sensors that can record diverse data, such as temperature, depth and salinity, while monitoring physiological parameters, such as heart and ventilation rates or muscle activity [3–6]. Although these physiological sensors have mainly been used in the wild in the context of conservation and ecology, they have progressively been employed in aquaculture, serving as welfare indicators of common stressors (e.g. slaughtering practices, water quality and stocking density) [4, 7–9].

Telemetry studies assume that tagged fish are physiologically representative of the entire population. Therefore, it is essential that the tag does not negatively affect growth performance, physiology and survival. The implantation method and site and the tag's size are important factors for preventing the disruption of the physiological state, normal movement, and growth performance of tagged fish [10–13] and avoiding bias in the collected data. The maximum tag weight generally considered acceptable is no more than 2% of the fish's body weight in air (the so-called "2% rule") [10, 11]. However, in some cases, the "2% rule" is not enough to avoid negative effects on the fish's health and welfare, such as stress, inflammation or obstruction of internal organs, or on its buoyancy and swimming performance [10, 14]. In particular, stress is considered as "a condition induced by a factor (a stressor) that evokes an endocrine response (e.g. cortisol release) that could be beneficial

as well as disadvantageous" [15]. Thus, due to many factors listed above, surgical implantation of electronic tag may induce stress for fishes. Most of our knowledge about the link between surgical implantation of electronic tag and stress is mainly based on salmonids [14, 16, 17]; therefore, more species-specific information is needed. In this study, we collected data from two different experiments, on the European sea bass (*Dicentrarchus labrax*) and the sea bream (*Sparus aurata*), two of the most important species for European aquaculture [18, 19], aiming to evaluate growth performance and the physiological stress profile of tagged fish at least 46 days after intraperitoneal surgical implantation. Their physiological stress profile was assessed by comparing the means of plasma stress indicator values (cortisol, glucose and lactate levels) with those of untagged fish, while growth was assessed by comparing the specific growth rates (SGR) between tagged and untagged fish.

3.2. Methods

3.2.1. Animals

Sea breams (mean weight ± SD: 314.6 ± 49.1 g) were obtained from the commercial hatchery Ittica Caldoli (Lesina, Italy). After 3 weeks of acclimation, ID100 radio frequency identification (RFID) tags (Trovan, Nether- lands) were implanted in the fish, which were then separated into three fiberglass tanks of 1.2 m^3 (n = 115 fish per tank; ~ 30 kg/m^3), forming triplicates. The implantation of pit-tag was performed under anaesthesia conditions (hydroalcoholic clove oil solution; 30 mg/L) under the skin in the region near the first dorsal fin. The fish were reared in marine water at a constant temperature of 18 °C, salinity of 35 PSU and a pH of 7.1. The water was completely replaced three times a day, and the oxy- gen levels were continuously monitored by an automatic system programmed to maintain the dissolved oxygen concentration above 5 ± 1 ppm. European sea bass fish (mean weight ± SD:335.5 ± 62.4 g) were obtained from the commercial hatchery Panittica Pugliese SpA (Torre Canne, Italy). After 3 weeks of acclimation, RFID tags (ID100) were implanted in the fish, which were then separated into three fiberglass tanks of 1.2 m^3 (n = 35 fish per tank; ~ 10 kg/m^3), forming triplicates. The implantation of pit-tag in sea bass was performed under similar conditions (anaesthesia and area of implantation) as for sea bream. The fish were left undisturbed for 2 months before the start of the experiment. The water parameters (temperature, salinity, and oxygen) were constant and similar to those for the sea breams. Throughout the experimental period, all fish were exposed to a 12L:12D photoperiod and were fed 1% of their body mass using commercial feed (Skretting Marine 3P, Italy) dispensed by automatic feeders for 3 h every morning.

3.2.2. Experimental procedure

At the beginning of the experiment (t0; Fig. 1), the fish were gently removed from their rearing tanks and anaesthetized with a hydroalcoholic clove oil solution (30 mg/L) [16, 17]. Morphometric parameters (body weight and total length) were recorded to calculate the SGR (see "Growth measurements and SGR calculations" section).

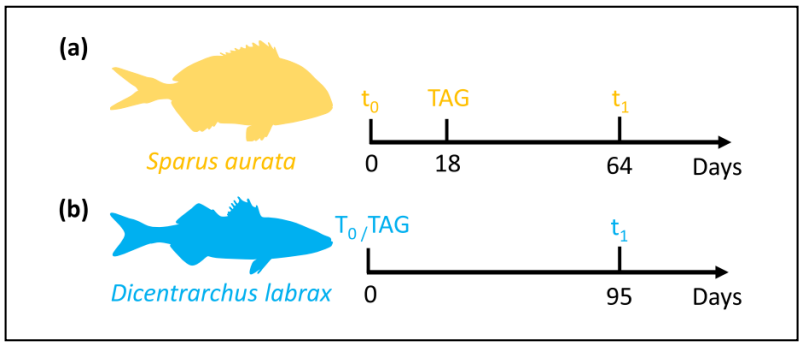

Figura 1 Time course schedule (days) of the experimental procedure for sea bream (Sparus aurata; yellow) and European sea bass (Dicentrarchus labrax; blue). T0 and t1 represent the beginning and the end of the experiment, corresponding to the first and fin final measurement for SGR calculation. TAG represents the period of implantation of accelerometers tag

3.2.3. Tag implantation

At the beginning of the experiment (Day 0) for sea bass and 18 days later for sea breams (Day 18) (Fig. 1), V9AP acoustic accelerometer tags (Vemco Systems Inc., Nova Scotia, Canada) were implanted in nine randomly selected sea bass and five randomly selected sea breams (at least two fish from each tank, except one fish from one tank for the sea bream experiment), as described in Carbonara *et al.* [7]. Briefly, the fish were subjected to fasting for 24 h before implantation and were anaesthetized using a hydroalcoholic clove oil solution in doses of 30 mg/L [20, 21]. The transmitter was inserted into the body cavity through a 1.5 cm incision. The incision was then carefully sutured, and the fish were injected with antibiotic (sodic ampicillin–cloxacillin; 1 mg/kg 24/h) [22] before being returned to their home tanks until the end of the experiment (t1; Fig. 1). The mean tag weight in air accounted for 1.63% ± 0.32 and 0.90% ± 0.21 of the sea bream and sea bass body mass, respectively. All tagged fish recovered within a few days, and no mortality linked to the surgical procedure was observed [7]. To evaluate possible tag effects, 12 untagged sea breams and 9 untagged sea bass were randomly selected as controls (at least three fish per tank; Table 1) and were monitored during the experimental period.

Species	Status	*N*	Mass at t_0 (g)	Mass at t_1 (g)
Sea bream (*Sparus aurata*)	Tagged	5	312.6 ± 48.2	407.8 ± 52.4
	Untagged	12	309.4 ± 65.3	389.5 ± 90.8
European sea bass (*Dicentrarchus labrax*)	Tagged	9	423.8 ± 80.7	466.9 ± 79.5
	Untagged	9	425 ± 76.4	479.2 ± 71.4

Table 1 Sample size and mean masses of tagged and untagged sea breams and European sea bass. t_0: beginning of the experiment; t_1: end of the experiment

3.2.4. Growth measurements and SGR calculations

At t1 (Days 46 and 95 after tagging the sea breams and sea bass, respectively; Fig. 1), the tagged and untagged fish were once again gently removed from their rearing tanks and anaesthetized with clove oil solution as described above. Their body weight was measured (in grammes) to calculate the differences in SGR between t0 and t1. The SGR was calculated according to the following equation [23]:

$$SGR = 100 * (\ln W_{t1} - \ln W_{t0}) * T^{-1},$$

where W is the total weight at the end (t1) and the beginning of the experiment (t0), and T is the number of feeding days between t0 and t1.

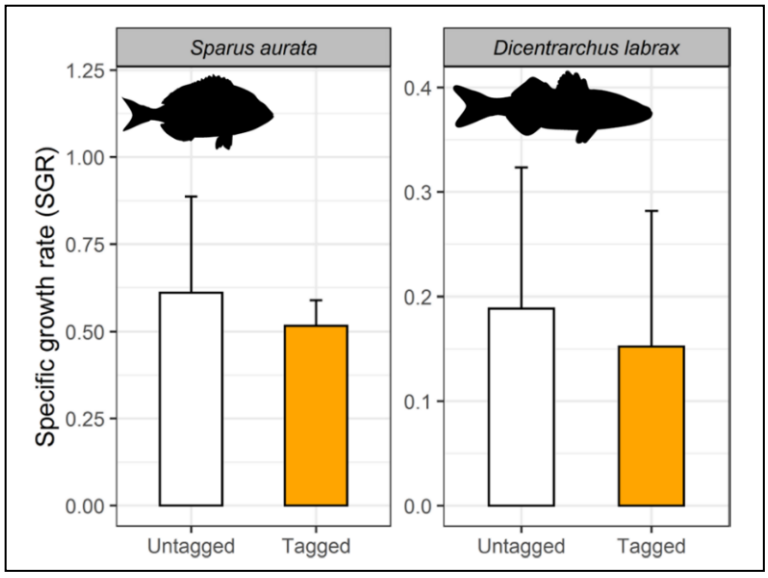

Fig. 2 Specific growth rate (SGR; mean ± SD) of untagged (white bars; n = 12 sea bream and n = 9 European sea bass) and tagged fish (orange bars; n = 5 sea bream and n = 9 European sea bass) in sea bream (Sparus aurata) and European sea bass (Dicentrarchus labrax). See main text for statistics

3.2.5 Blood sampling and stress indicator analysis

After the morphometric measurements (2–3 min after anaesthesia inducement), blood samples of 0.5 mL were immediately taken from the first branchial arch of the tagged and

untagged fish using a heparinized syringe. The samples were then centrifuged at 15,000g for 3 min, and plasma was collected and stored at − 20 °C until further processing, described below.

The plasmatic cortisol, glucose and lactate concentrations were measured as described in Carbonara *et al.* [7]. Briefly, the cortisol concentration was determined using solid-phase competitive chemiluminescent enzyme immunoassays with a cobas Cortisol II kit (Roche, Switzerland). The glucose and lactate concentrations were determined using kits 17630H and 17285 (Sentinel Diagnostics, Italy), respectively, based on the enzymatic colorimetric Trinder reaction (GOD/PAP for glucose and PAP for lactate).

3.2.6 Statistical analysis

Statistical analyses were performed using the R software version 3.6.2 [24] at a 95% level of significance. Homoscedasticity of the data was a priori tested using the Shapiro–Wilk test. The appropriate statistical test (either the Wilcoxon test or the t test) was then performed to compare the SGRs and physiological stress indicators (cortisol, glucose and lactate) between the tagged and untagged fish of each species.

3.3. Results

In terms of growth performance, the SGR was similar between the tagged and untagged fish for both the sea bream ($W = 38$, $p = 0.44$) and the sea bass ($t = -0.58$, $p = 0.56$; Fig. 2) between t0 and t1, which correspond to a period of 64 days for the sea breams and 95 days for the sea bass. At t1, the plasma concentrations of stress indicators were overall similar between the tagged and untagged fish of both species (Fig. 3). More specifically, the plasma cortisol concentration showed no statistically significant differences either in the sea breams ($W = 32$, $p = 0.88$) or in the sea bass ($t = 0.94$, $p = 0.36$; Fig. 3a). The levels of the secondary stress indicators (i.e. glucose and lactate) were also similar both in the sea breams ($W = 25.5$, $p = 0.67$ for glucose and $t = 1.04$, $p = 0.33$ for lactate) and in the sea bass ($W = 39$, $p = 0.93$ for glucose and $t = 1.18$, $p = 0.26$ for lactate; Figs. 3a, 3b).

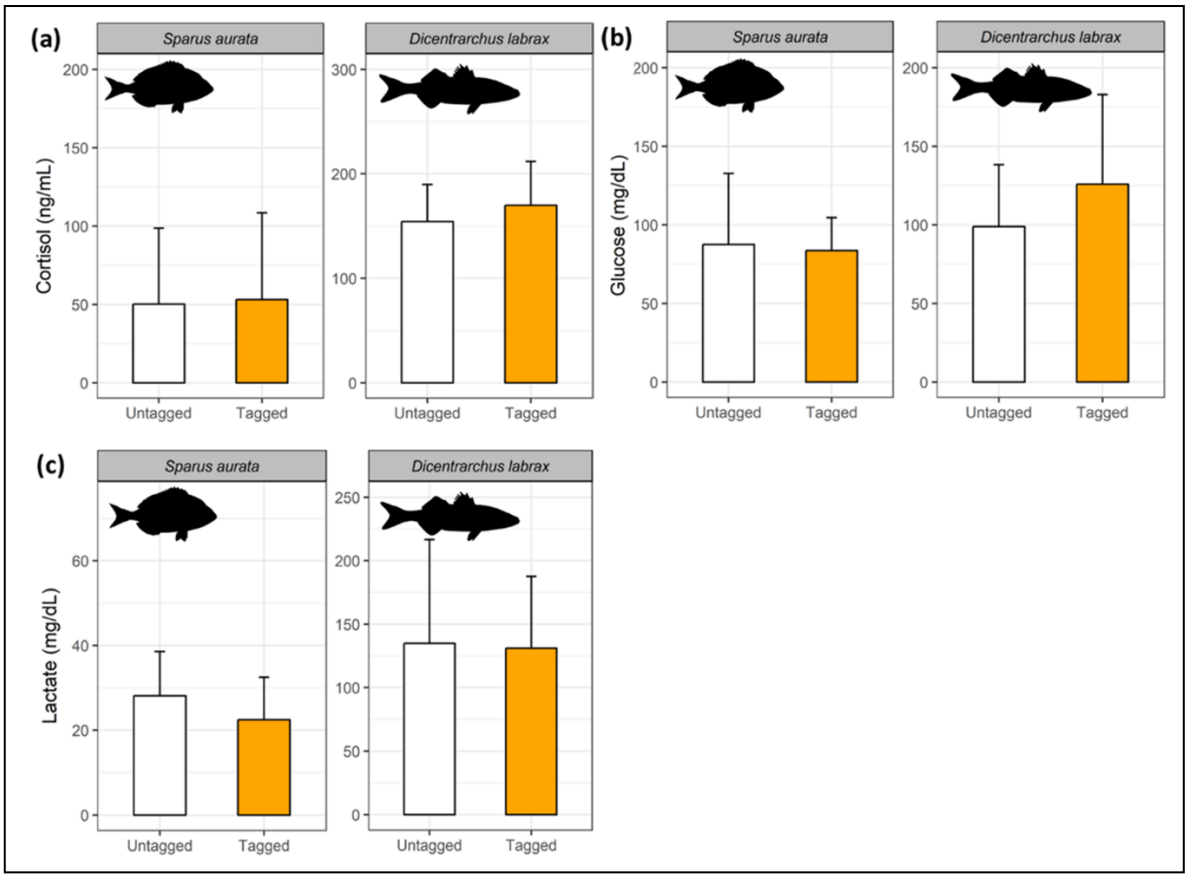

Fig. 2 Stress physiological profile of untagged (white bars; n = 12 sea bream and n = 9 European sea bass) and tagged fish (orange bars; n = 5 sea bream and n = 9 European sea bass) at t1. a Cortisol (ng/ mL), b glucose (mg/dL) and c lactate (mg/L). Values are mean ± SD. See main text for statistics

3.4. Discussion

Our results show that after a relatively long period (46 days for the sea bream and 95 days for the sea bass) following surgical implantation of accelerometer tags, the tagged fish were comparable with the untagged fish in terms of both growth and stress physiology in aquaculture conditions. To our knowledge, this is the first report concerning stress physiological indicators for the sea bream and the European sea bass, two important species for European marine aquaculture. These findings support the use of accelerometer tags in these two species in aquaculture conditions. Surgical implantation of accelerometer tags is perceived as a stressor for fish, causing cortisol release into the blood [25], which is the main stress hormone in teleost fishes [26]. It is a relatively acute response of organisms coping with stressors before regaining homeostasis, but it may last only a few days, depending on the species. For instance, in rainbow trout (*Oncorhynchus mykiss*), a heart rate increase was observed during the first 72 h following surgical implantation of a heart rate sensor, after which it was stabilized [27], suggesting that fish regain homeostasis relatively quickly after this stressful event. Jepsen *et al.* [25] reported similar observations in Chinook salmon, where physiological stress indicators were higher up to 24 h following tag implantation, but

were comparable with those of untagged fish at most 7 days later. In our experiments, 46 and 95 days after tag implantation in sea breams and sea bass, respectively, the levels of all monitored stress indicators (cortisol, glucose and lactate) were found to be similar to those of untagged fish and consistent with the levels reported in the literature regarding these species [7, 28]. Our results confirm that tag implantation does not induce chronic stress in either the sea bream or the sea bass, as observed in various other fish species [25, 29]. It is thus important to emphasize that tag implantation does not exert long-term adverse effects on a high-stress responder species such as the European sea bass [30–32]. Nonetheless, although we did not directly investigate the acute stress response to tag implantation by measuring physiological stress indicators after the surgical procedure, we did observe that generally, the tagged fish did not eat for 2 to 4 days post-operatively (personal observations), probably because of surgery-induced stress. Indeed, stress and growth are closely related; stress is known to inhibit food intake and, consequently, limit the energy available for biological processes, including growth [33]. Therefore, it appears that acute stress is indeed induced by tag implantation, but it only lasts a few days in these species. Moreover, this period of no food intake has no long-term consequences on growth, as shown by the similar SGRs between the tag and untagged fish of both species. It has been demonstrated in different fish species that when the "2% rule" is applied, growth performance is generally not impacted [11, 25, 34]. The similar growth rates between tagged and untagged fish can be explained by compensatory growth, which is a period of unusually rapid growth following a period of undernutrition [35]. It is noteworthy that we observed similar growth rates between the tagged and untagged fish in two different stocking densities (~ 10 kg/m^3 for the sea bass and ~ 30 kg/m^3 for the sea bream), which suggests that tagged fish can compensate growth and continue their normal life under different rearing conditions.

3.5. Conclusion

In conclusion, surgical implantation of accelerometer tags does not cause medium-term changes in the stress physiological profile and growth of either sea breams or sea bass reared in a controlled environment. Future studies are needed to investigate exactly how long these species take to recover from stress induced by tag implantation and thus be considered "normal" fish, displaying normal behaviour (e.g. feeding) and basal levels of stress indicators. Our study confirms (i) that the implanting process of accelerometer tags does not affect the basic growth and stress physiological indicators of tagged fish and (ii) that tagged fish can be sampled 46 or 95 days post-surgery for sea bream and seabass, respectively,

during experiments and considered representative of the population, as they display growth and physiological parameters comparable to those of untagged fish.

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Chapter 4

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Calibrating accelerometer data, as a promising tool for health and welfare monitoring in aquaculture: Case study in European sea bass (*Dicentrarchus labrax*) in conventional or organic aquaculture

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Abstract

Both the conventional and organic aquaculture sectors have grown rapidly over the past few years. Moreover, welfare has attracted increased attention on the part of both consumers and governments. However, fish welfare assessment is complex and thus needs to adapt measurements that are easily applicable to aquaculture conditions. In this study, in addition to classical welfare indicator measurements (physiological stress indicators and growth performance), we recorded the swimming activity data using acoustic transmitters to evaluate the welfare of European sea bass (*Dicentrarchus labrax*) fed a conventional vs. an organic diet. Prior feeding trial, the swimming activity recorded by tag has been calibrated with water speed during critical swimming speed (Ucrit) tests. This calibration allowed us to increase the power of the recorded data, providing information on swimming activity with respect to the Ucrit value and on the metabolic cost of swimming. After a four-month experimental period, physiological stress indicators and growth performance did not differ significantly between the two diet groups. However, we observed a subtle difference in swimming activity: the fish in the organic diet group were more active during the feeding period in the morning. All indicators considered, our results suggest that an organic diet does not incur higher metabolic costs and does not affect the welfare of the European sea bass. Moreover, this study shows that the use of acoustic transmitters previously calibrated with physiological indicators, such as Ucrit, is a promising tool for welfare monitoring in aquaculture conditions.

4.1. Introduction

Due to an increasing demand for fish products and a decrease in natural resources, the aquaculture sector has grown rapidly over the past decades, now representing more than 50

% of total fish production (FAO, 2018). Fish production from organic aquaculture has also increased rapidly (Gambelli *et al.*, 2019; Gould *et al.*, 2019). Organic aquaculture may contribute to addressing environmental issues related to the aquaculture sector – for example, by replacing fish protein content with proteins and oils from land-based agriculture, thus preventing overfishing for the production of fish feed (Gould *et al.*, 2019; Turchini *et al.*, 2009). However, the replacement of fish protein in feed formulations must be measured, as total substitution can disrupt physiological processes and growth performance (Borquez *et al.*, 2011; Geay *et al.*, 2011), causing fish health and welfare issues, mainly in the farming of carnivorous fish species (e.g. sea bass, *Dicentrarchus labrax* and rainbow trout, *Oncorhynchus mykiss*). Besides the development of aquaculture, fish welfare has also attracted increased attention on the part of both consumers and governments and has become a critical point to consider in the growth of this sector (Ashley, 2007; FSBI, 2002; Huntingford and Kadry, 2008), especially organic aquaculture (Lembo *et al.*, 2019). However, assessing fish welfare is complex and requires an integrative overview, from physiology to behaviour and biological performances (Huntingford *et al.*, 2006; Martos-Sitcha *et al.*, 2020). Overall, fish welfare can be closely linked to stress (Prunet *et al.*, 2012; Schreck and Tort, 2016; Sneddon *et al.*, 2016). Thus, plasma cortisol, end product of the hypo- thalamus–pituitary–interrenal (HPI) axis, and secondary stress response indicators, such as blood glucose and lactate levels, may be used as welfare indicators (Barton, 2002). However, stress does not always mean compromised welfare, as it can also be a response to predation, competition or environmental changes (Alfonso *et al.*, 2020a; Barton, 2002; Gorissen and Flik, 2016). Thus, a short-term stress response (e.g. elevation of cortisol levels) can be viewed as adaptive, allowing fish to cope with stressors and preserve both individuals and populations. Chronic or repeated stress, on the other hand, can lead to dysfunctions and compromise welfare. Therefore, stress indicators alone may be insufficient for properly evaluating fish welfare (Ellis *et al.*, 2012; Raposo de Magalhães *et al.*, 2020; Sadoul *et al.*, 2021; Sadoul and Geffroy, 2019). Consequently, many innovative approaches using fish spontaneous swimming activity and/or behaviour as a reliable proxy of fish welfare have been developed (Martins *et al.*, 2012). This can be achieved using classical video recordings (Alfonso *et al.*, 2020b; Sadoul *et al.*, 2017, 2014; Stien *et al.*, 2007) but also acoustic transmitters that record several variables, such as positioning, speed or acceleration. Having previously been widely used for monitoring natural fish populations for conservation purposes (McKenzie *et al.*, 2016), these transmitters are now being increasingly used in aquaculture contexts for fish welfare monitoring (Carbonara *et al.*, 2020a, 2020b, 2019a;

Gesto *et al.*, 2020; Muñoz *et al.*, 2020). Indeed, their use appears promising for monitoring welfare, as they allow evaluations of the behaviour of free-swimming fish over long periods (Halachmi *et al.*, 2019) without affecting welfare and biological performance (Alfonso *et al.*, 2020c; Jepsen *et al.*, 2011). It is important to emphasize that tag implantation does not affect fish welfare and biological performances only if some conditions are respected, i.e. the tag has not to exceed 2% of the fish's body weight in air, the position has to be thought to not disturb normal fish movements, and the tag implantation has not to obstruct the internal organs (Bridger and Booth, 2003; Jepsen *et al.*, 2005; Smircich and Kelly, 2014). The signals recorded by the transmitter can be prior calibrated with other physiological variables, such as muscle activity and swimming performance, thereby increasing the power of the phys- iological data obtained (Clark *et al.*, 2010; Martos-Sitcha *et al.*, 2019; Wilson *et al.*, 2013; Zupa *et al.*, 2015). Swimming performance and aerobic/anaerobic metabolism use are of primary importance in assessing the physiological state of fish and their ability to cope with stressors (Lembo *et al.*, 2007; Zupa *et al.*, 2015). The critical swimming speed (Ucrit) achieved by a fish during a swimming test provides information on swimming performance, as well as its maximum metabolic rate (MMR) (Norin and Clark, 2016). The MMR indicates the capacity for energy usage by aerobic pathways under different environmental conditions (Brett, 1964; Norin and Clark, 2016). Previous evidence on European sea bass shown that the MMR is generally achieved before the Ucrit (Claireaux *et al.*, 2006; Zupa *et al.*, 2015), suggesting that near this threshold, the supplementary energy requirement necessary to sustain the increasing swimming activity of fish is mainly fuelled by anaerobic metabolism. As observed by Claireaux *et al.* (2006), before the Ucrit was reached, the maximum swimming speed (Umax) of sea bass is reached, and fish use anaerobic metabolism to display a burst swimming mode, and the MO2 usually levelled off or decreased slightly. This state may be detrimental to fish health and welfare if repeated or sustained for long time, as observed by Carbonara *et al.* (2015) regarding high stocking density. Moreover, the environmental conditions, included the feed, have an impact on the fish activities and performance (Claireaux and Lefrançois, 2007). Therefore, calibrating the acceleration data recorded by acoustic transmitters with swimming performance during a Ucrit test may offer more precise information on the swimming activity, aerobic/anaerobic metabolism and life energy cost of free-swimming fish (Carbonara *et al.*, 2020b; Zupa *et al.*, 2015). In fish, the activity of red muscles, supported by aerobic metabolism, increases with speed until it reaches a maximum and is maintained at that level even if the swimming speed increases further (Rome *et al.*, 1992). On the other hand, white muscle recruitment, supported by

anaerobic metabolism, follows an exponential pattern (Burgetz *et al.*, 1998; Zupa *et al.*, 2015). Muscle recruitment appears to be species-specific, as indicated by the different placement and amount of slow-twitch aerobic (for sustained swimming) and fast-twitch anaerobic muscle fibres (recruited during fast starts) in different species (Ellerby *et al.*, 2000). In the European sea bass, we have previously observed that the activity of red muscles increases with speed to a maximum and is maintained at that level until the end of the Ucrit (Zupa *et al.*, 2015), following a classical activation pattern. On the other hand, white muscle activation follows an exponential pattern, with the start of increase at approximately 65 % of the Ucrit to compensate for the reduction of red muscle activity recruitment (Zupa *et al.*, 2015). Thus, information about swimming performances may offer valuable insights into the metabolic costs of swimming related to both aerobic and anaerobic metabolism. This can be valuable in free swimming fish under different aquaculture rearing conditions, including diet regime.

In this study, we first calibrated the values of acceleration recorded by acoustic transmitters with the swimming velocity in a swimming chamber, obtaining a baseline of fish swimming performance to determine the Ucrit. Then, we used the recorded swimming activity data, as a proxy of the metabolic cost of swimming of European sea bass and be used for health and welfare monitoring of European sea bass fed a conventional and an organic diet. Indeed, organic aquaculture is a production method that combines preservation of natural resources and the use of renewable products (Lembo and Mente, 2019). Organic feed is composed of a greater percentage of proteins and oils from land-based agriculture (Gould *et al.*, 2019). However, the partial substitution of fishmeal and fish oil with plant proteins and oils was found to be a promising more environmental friendly alternative to fish protein and oil on one hand (Lembo and Mente, 2019), but from the other hand, it can negative impact the natural physiological processes and so also the growth performances (Geay *et al.*, 2011; Montero and Izquierdo, 2010). Therefore, we also measured fish growth performance and classical physiological stress indicators at the end of the experimental period. The results obtained from both kinds of data were considered together in the framework of welfare assessment in organic aquaculture of European sea bass, a key species of European marine aquaculture (Vandeputte *et al.*, 2019).

4.2. Materials and methods

Care and handling of fish were accomplished in accordance with the recommendations 2007/526/EU C(2007) 2525 on the accommodation and care of animals used for

experimental and other scientific purposes. The experimental protocol was approved by the ethic committee on the animal experiments of COISPA.

4.2.1. Fish holding and diet

Juvenile (approximately 1 year old) European sea bass specimens were purchased from the commercial farm Ittica Caldoli (Lesina, Italy) and maintained at a stocking density of ~10 kg/m^3 at salinity of 35 PSU, pH of 7.30 ± 0.05, temperature of 18 ± 1 ◦C and oxygen saturation above 90 % for two weeks in a 12Light:12Dark photoperiod (light from 06:00 am to 06:00 pm). The fish were reared in a flow-through system with water replacement of 25 L/min and fed commercial feed (Skretting Marine 3 P, Italy) amounting to 1 % of their body mass. After this two- weeks period, the fish were randomly separated into six fiberglass tanks of 1.2 m^3 (n = 35 per tank; 10 kg/m^3) with the same water quality and photoperiod (specified above) but fed two different commercial diets (a conventional and an organic diet) in three replicates each. Some additional fish remained in the initial batch and fed commercial feed until the calibration of accelerometer tag (see Section 2.3). The conventional feed used was Performance Mare (NaturAlleva, Italy), and the organic feed was Acquabiomare 15 PLUS (NaturAlleva, Italy) (Table 1). In the organic feed, 51.13 % of the feed is composed from contents from agricultural origin (80 % of the protein and fats). The fishmeal and fish oil contribute to 18 % and 7% of the total content of the diet, asserting the diet as organic according to EU regulation (Busacca and Lembo, 2019). In comparison, in the conventional feed, the fishmeal and fish oil account for about 30 % and 12 % of the content of the total content of the diet respectively. In both diet groups, the fish were fed feed amounting to 1% of their body mass administered by automatic feeders for six hours a day six days a week throughout the experimental period (133 days) from 07:00 am to 01:00 pm. Morphometric parameters were assessed, and blood samples were taken from a subsample of fish as a baseline for each diet at the beginning of the experiment (t0; details are provided in the following Sections 2.2. and 2.5). The fish weight in the conventional and organic diet groups was similar at the beginning of the experiment (mean ± standard error of the mean [SEM] = 336.05 ± 19.41 and 356.76 ± 7.83 g, respectively).

Diet composition	Conventional diet	Organic diet
Crude protein (%)	43	43
Crude oils and fats (%)	21	15
Raw cellulose (%)	2.0	2.9
Ashes (%)	9.0	11.5
Vitamin A (UI/kg)	7515	6938
Vitamin D3 (UI/kg)	2505	2313
Phosphorus (%)	1.1	1.5
Sodium (%)	0.2	0.8
Calcium (%)	1.4	2.1

Tab 1 Compositions of the conventional and organic diets used in the experiment.

4.2.2. Experimental procedure, growth performance and sampling

Growth performance was measured at three sampling points over the experimental period: the first day of the experiment (t0), the day 74 (t1) and 133 (t2) after the beginning of the experiment in all fish (n = 35 per tank). For each measurement, fish were gently caught from their rearing tanks and anaesthetized with a hydro-alcoholic clove oil solution (30 mg/L) (Iversen *et al.*, 2003). The morphometric parameters measured were body weight and total length. The mean specific growth rate (SGR) in each tank was calculated based on the mean fish mass as follows:

$$SGR = 100 * \frac{(\ln Wt2 - \ln Wt0)}{(t2 - t0)} \qquad (1)$$

where W is the mean fish weight (in grams) per tank at t0 (W_{t0}) and t2 (W_{t2}). The feed conversion ratio (FCR) was calculated as the ratio of the feed supplied (in kilograms of dry weight) per biomass of weight gained (in kilograms), and the protein efficiency ratio (PER) was calculated as the ratio of the total biomass in each tank to the total proteins assumed.

4.2.3. Tag calibration using a critical swimming speed (Ucrit) trial

For the calibration of the accelerometer tags, 10 fish from the batch fed commercial feed (i.e. Skretting Marine 3 P) were subjected to a Ucrit trial. The mean body weight of the specimens subjected to the Ucrit trial (mean ± SEM = 398.64 ± 25.93 g) did not differ significantly from that of the fish in which tags were implanted (see Section 2.4) during the diet experiment (mean ± SEM = 447.61 ± 16.57 g; $p > 0.05$). In addition, the tag did not exceed 2% of the fish's body weight in air (i.e., 1.53 and 1.36 % for calibration and diet experiment respectively), in order to do not alter fish physiology and normal behaviour of tagged fish during the experiment (Bridger and Booth, 2003; Jepsen *et al.*, 2005; Smircich

and Kelly, 2014). The Ucrit tests were performed in a Blazka-type respirometer as previously described (Carbonara *et al.*, 2006). The chamber volume was 120 L with a respirometer 123 cm long with an inner diameter of 24 cm. This swimming chamber was built to generate a laminar water flow in the inner part of the tube (Carbonara *et al.*, 2006). After the V9A acoustic accelerometer tags (VEMCO, Nova Scotia, Canada; see Section 2.4 for details on the surgical procedure) were implanted, the fish were left undisturbed for at least five days to ensure full recovery prior to the Ucrit trial. Twenty-four hours before the trial, the fish were subjected to fasting to ensure a post-absorptive state during the test (McFarlane *et al.*, 2004). On the trial day, the fish were gently caught from their rearing tanks and placed in the chamber for a maximum acclimatization time of one hour. After acclimatization, the fish were left at a velocity of 0.1 m/s for 30 min before the start of the trial. The protocol consisted in an increase in water velocity of 0.1 m/s every 15 min until fatigue ensued and the test was terminated. Fatigue was indicated by the caudal fin touching the grid for at least 5 s (Carbonara *et al.*, 2010). The Ucrit was calculated as described by Brett (1964), and a solid blocking effect correction was performed according to the equation Smit *et al.* (1971). During the swimming trial, the accelerometer tags were programmed to record data at a sampling rate of 10 Hz (10 measurements per second), which were sent to an acoustic receiver (VR2W; VEMCO) located near the swimming chamber. The tag returned an 8-bit value that represents the root mean square (RMS) acceleration resulting from the contribution of two axes (vertical and lateral directions of movement), every 30 s on average. The tag algorithm was designed to provide data with a more precise measurement of tailbeat activity, excluding the forward/backward component of the movement. The adimensional value obtained can be converted in acceleration using the following equation (acceleration (m/s^2) = 0.01955 (x), where x is the adimensional value returned by tags). In this study, adimensional value of acceleration data (RMS) were provided without conversion to real acceleration values, and later called swimming activity (expressed in arbitrary units; AU). To calibrate the swimming activity data with the water velocity, the recorded data were averaged for each water velocity increment (from 0.1 m/s to fatigue).

4.2.4. Tag implantation and recording of swimming activity

At t1 (74 days after the beginning of the experiment), following the morphological measurements, a subsample of three fish per tank (n = 9 per diet) was randomly selected for implantation of acoustic accelerometer tag (V9A; Vemco) to measure swimming activity as described in Alfonso *et al.* (2020c). Briefly, fish were subjected to a 24h fast before tag implantation. The gills were continuously irrigated with a hydro-alcoholic clove oil solution

(30 mg/L) during the entire surgical procedure. The transmitter was inserted into the body cavity through an incision 1.5 cm long. The body cavity was then carefully closed using sutures. The fish then received antibiotic injections (sodic-ampicillin–- cloxacillin; 1 mg/kg 24 h^{-1}) (Lembo *et al.*, 2008) before being returned to their home tanks. All fish recovered within a few days, and no mortality related to the surgical operation was observed, regardless of the diet treatment. The accelerometer tags were programmed to record data with a sampling rate of 10 Hz (10 measurements per second) during the whole experiment duration. The tag IDs and coded acceleration values were stored in the memory of submergible acoustic receivers (VR2W) located in each tank. Swimming activity (AU) were recorded for 43 days, from 82 to 125 days after the beginning of the diet treatments. The fish were allowed an interval of eight days between the surgical procedure and the start of data collection to fully recover from the surgery and display normal behaviour during data collection.

4.2.5. Physiological measurements

At t0 and t2, a subsample of fish (n = 9 fish at t0 and n = 5 fish per tank, 15 per diet at t2) was randomly selected for blood sampling. After 2–3 min under anaesthetic, the blood samples were taken from the first branchial arch using a heparinized syringe. The samples were analysed to assess the basal levels of the following physiological indicators: cortisol, glucose, lactate, haematocrit (HCT), haemoglobin (Hb), red blood cell count (RBCC) and lysozyme. The quantification of these parameters was performed as described by Carbonara *et al.* (2019a). Briefly, HCT was determined using a heparinized micro-haematocrit tube filled with blood directly from the syringe needle, which was then centrifuged at 15,000×g for 3 min and immediately read. HCT was expressed as the red blood cell percentage of the entire blood volume. The RBCC was performed in a Bürker counting chamber under a light microscope (Nikon 400E, Japan). Hb was measured using a commercial kit (H7379; Sigma, USA). The remaining blood was centrifuged at 15,000×g for 3 min to obtain plasma samples, which were stored at −20 ◦C until further analysis. Plasma cortisol was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (InterMedical, Italy) for microplate readers (k = 450 nm) following the manufacturer's instructions. Plasma glucose and lactate concentrations were measured using commercial kits (17630H and 17285, respectively; Sentinel, Italy) based on enzymatic colorimetric Trinder reactions (GOD/PAP for glucose and PAP for lactate). Plasma lysozyme concentrations were measured using a turbidimetric assay modified for a microplate reader (Carbonara *et al.*, 2019b).

2.6. Statistical analyses

Statistical analyses were performed using R software (R Core Team, 2021). All analyses were performed at a 95 % significance level. Values were expressed as means ± standard errors (SE) unless otherwise indicated. Data normality was assessed using the Shapiro–Wilk test, following which an appropriate statistical test was performed. The Wilcoxon rank sum test was used to investigate differences in the SGR, FCR and PER between the two diet groups. For the calibration of swimming activity data as a function of water speed during the Ucrit trial, linear and exponential models were tested. Model showing the lowest AIC has been selected for the calibration (Akaike, 1973). A generalized linear mixed model (GLMM) was used to compare the swimming activity between the two diet groups, with the diet (conventional or organic) and the time of day (day- or night-time) as fixed factors and the fish ID as a random factor, using the package lme4 (Bates *et al.*, 2014). An analysis of frequency distribution between the two diets has been carried out on the whole data set of swimming activity values recorded by tags during the experiment, merging by slot of 10 the swimming activity values (i.e., 0–10, 11–20, […], 241–250, 251–255). The statistical analysis has been carried out using Pearson's Chi-squared test, and pairwise comparisons have been then carried out to compare slot by slot the two diets using comparisons using pairwise chi-squared tests with p value adjusted by the Bonferroni method. The proportion of acceleration values above the Ucrit value during the experiment was also analysed using the diet and time of day as fixed factors and the fish ID as a random factor. Both GLMM analyses were performed using the gamma distribution family and logarithmic link. If significant, the GLMM analyses were followed by Tukey's honest significant difference (HSD) post-hoc test (Lenth *et al.*, 2019). A visual inspection of the residuals showed no violation of the statistical assumptions by the two models. It should be noted that one individual in the conventional diet group was excluded from the statistical analyses because of accelerometer tag acquisition defect, resulting in 17 fish (eight in the conventional and nine in the organic diet group). Finally, analysis of variance (ANOVA) was performed to evaluate differences between the two diet groups (conventional or organic) and the control group at the start of experiment t0, in normally distributed physiological parameters (RBCC, HCT, Hb and lysozyme), and the Kruskal–Wallis test was used for non-normally distributed variables (cortisol, glucose and lactate). When significant, ANOVA or Kruskal-Wallis was followed by Tukey's HSD test.

4.3. Results

4.3.1. Growth performance

There were no statistically significant differences between the two groups in SGR, FCR and PER at the end of the experimental period (Table 2; $p > 0.05$ for all).

Diet	Mass at t_0 (g)	Mass at t_1 (g)	Mass at t_2 (g)	SGR*	FCR*	PER*
Conventional	336.05 ± 19.41	378.37 ± 29.66	412.79 ± 26.80	0.20 ± 0.02	4.99 ± 0.39	2.15 ± 0.17
Organic	356.76 ± 7.83	402.96 ± 30.58	440.02 ± 3.95	0.20 ± 0.03	5.45 ± 1.68	2.15 ± 0.72

*Table 2 Growth performance in the conventional and organic diet groups (n=3 tanks per diet). All values are means ± standard errors. t0, Day 1 of the experiment; SGR, specific growth rate; FCR, feed conversation ratio; PER, protein efficiency ratio. *Calculated between t_0 and t_2*

4.3.2. Calibration of swimming activity values with water velocity during the Ucrit test

The mean absolute Ucrit value was 1.32 ± 0.11 m/s, which is corresponding to a relative Ucrit of 4.08 ± 0.15 BL/s. Exponential model showed the lowest AIC value compared to linear one (926.9 for exponential vs. 934.4 for linear). Thus, the recorded swimming activity was fitted with an exponential model using the water velocity ($p < 0.001$; $R2 = 0.71$; Table 3). Using this model, the swimming activity value at which the fish reached the Ucrit value was determined to be 122.1 (Fig. 1). This value was then used for further analysis of the swimming activity data of the tagged fish in the two diet groups during the experiment (see Section 3.3).

Parameter	Estimate	Standard Error	t-value	p-value
α	16.264	1.648	9.869	<0.001
β	0.494	0.036	13.772	<0.001

Table 3 Estimation of the parameters and Standard Error of the exponential model ($y=\alpha*e^{\beta*x}$) for swimming activity as a function of water speed (BL s^{-1}) during the Ucrit test ($R^2=0.67$).

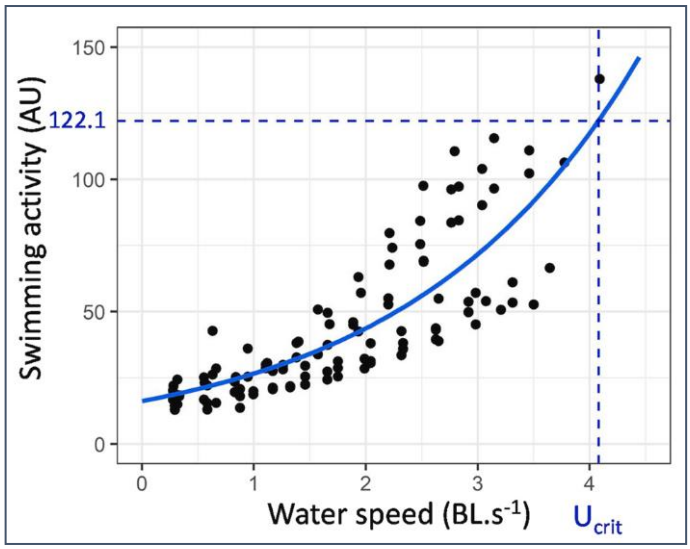

Fig. 1. Exponential model fitting the swimming activity (in arbitrary units; AU) recorded by accelerometer tags as a function of water speed (Body length, BL s−1) during the Ucrit trial (n = 10 fish). The black dots represent individual values, and the The black dots represent individual values, and the blue line represents the model curve based on the parameters displayed in Table 3. The dashed blue lines represent the correspondence of the Ucrit value to the swimming activity according to the exponential model.

4.3.3. Swimming activity

Daytime and diet, as well as the interaction between the two parameters, had significant effects on the swimming activity of the fish (Table 4). The sea bass showed a diurnal activity pattern, even though the differences in swimming activity between day and night were relatively small (Fig. 2A). During the daytime, the fish in the organic diet group displayed more intense swimming activity than those in the conventional diet group (Fig. 2B), especially during the first hours of daylight (Fig. 2A). In both groups, the mean swimming activity levels were far below the corresponding Ucrit value (46.3 ± 22.5 AU and 51.4 ± 24.2 AU for the conventional and organic diet respectively), representing 37.9 % of the value in the conventional diet group and 42.1 % of the value in the organic diet group during the daytime, but overall displaying high variability throughout the experiment (Fig. 2C). However, the frequency distributions were found different between the two diets during the whole experiment (χ^2 = 4310, df = 25, $p < 0.001$; Fig. 3A). In more details, higher data frequency for low swimming activity values (<20 AU) has been found in fish fed organic diet ($p < 0.05$; Fig. 3C). For values ranged from 21 to 50 AU, higher data frequency has been observed in fish fed conventional diet ($p < 0.05$; Fig. 3C), while from 51 to 120 AU higher data frequency has been again recorded in fish fed organic diet than fish fed with conventional one ($p < 0.05$; Fig. 3A, D). For high swimming activity values (>140 AU), no difference in data distribution has been observed between the two diet regimes ($p > 0.05$; Fig. 3D). Indeed, the recorded swimming activity values above the calibrated Ucrit value

(122.1 AU) represented a small proportion of all recorded values (approximately 1.4 %; Fig. 3A), and did not differ significantly between the two diet groups (GLMM, t = 0.57, p = 0.57), regardless of the time of day (GLMM, t = 0.15, p = 0.88) or interaction of both factors (GLMM, t = -0.07, p = 0.95; Fig. 3B).

Fixed effects	Estimate	Standard error	t-value	p-value
Intercept	3.844	0.021	187.222	<0.001
Diet	0.086	0.028	3.048	0.002
Time of day	−0.038	0.002	−16.636	<0.001
Diet–time of day interaction	−0.046	0.003	−13.880	<0.001

Random effects	Estimate	Standard error
Fish ID (n = 17)	0.003	0.058
Residuals	0.249	0.499

Table 4 Outputs of the generalized lineax mixed model (GLMM) for swimming activity as a function of diet (conventional or organic) and time of day (day-or night-time). The reference factor level for diet is conventional. The reference factor level for the time of day is daytime.

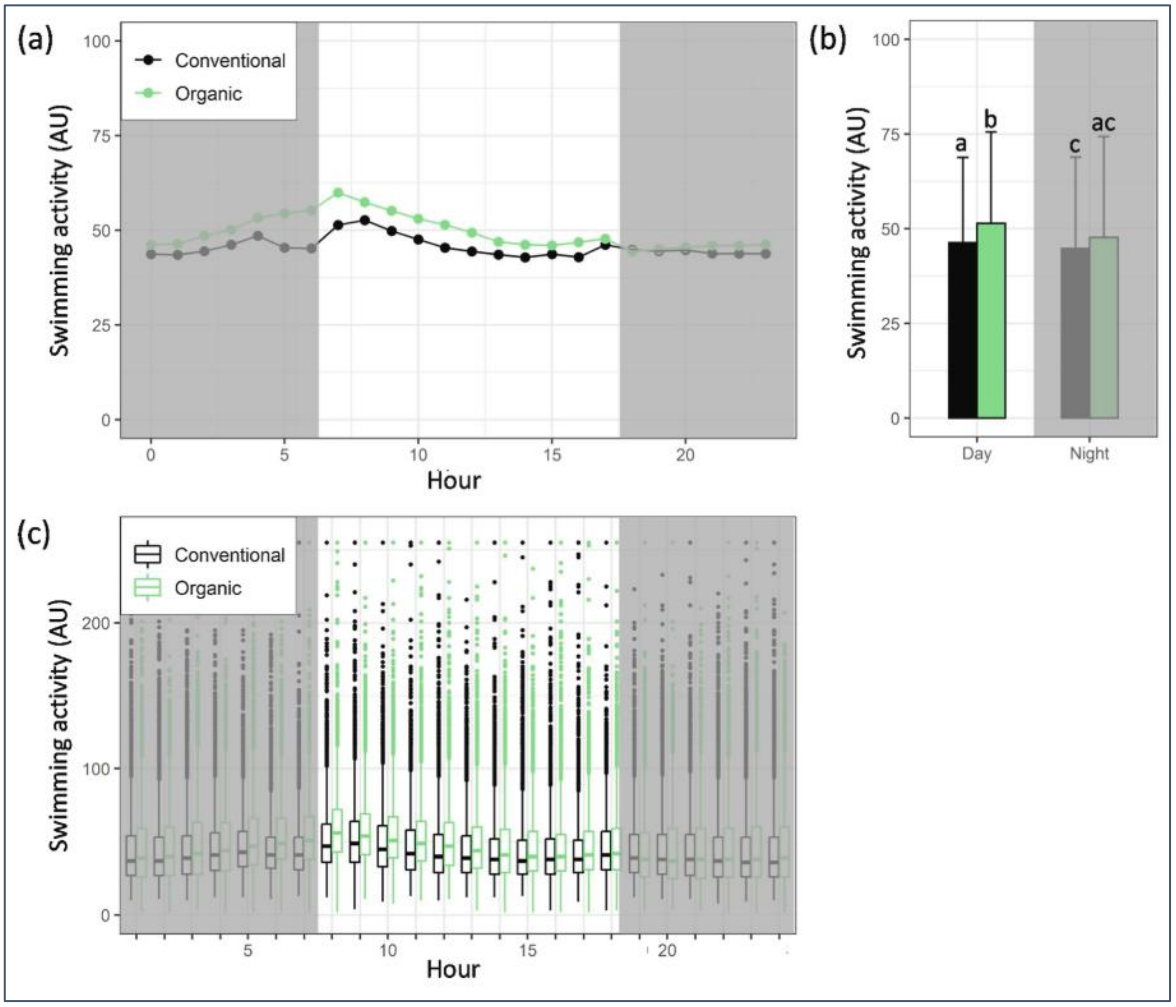

Fig. 2. (a) Means of swimming activity (in arbitrary units; AU) as a function of hours per day in the conventional (black; n = 8 fish) and organic diet (green; n = 9 fish) groups. (b) Means ± standard errors of swimming activity as a function of the time of day (day- and night-time) and diet. The light grey areas indicate

the night periods. The different letters indicate statistically significant differences between the two diet groups and/or time of day (GLMM followed by Tukey's HSD post-hoc test; p < 0.05; see Table 4 for details). (c) Boxplot of the swimming activity (in arbitrary units; AU) as a function of hours per day in the conventional (black; n = 8 fish) and organic diet (green; n = 9 fish) groups. The central line in each boxplot indicates the median, and the boxes on either side represent the quartiles, with the whiskers covering 95 % of the values. Outliers are represented by points.

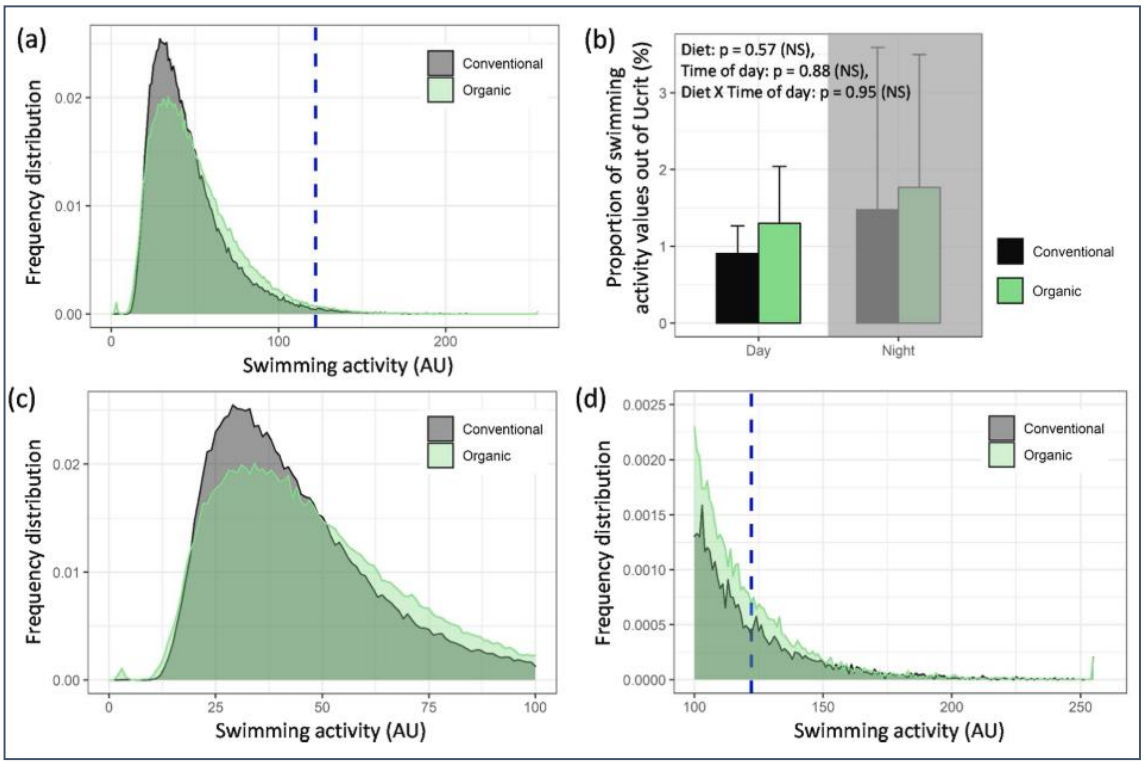

Fig. 3. (a) Frequency distribution of the swimming activity values (in arbitrary units; AU) as a function of conventional (black distribution; n = 8 fish) and organic (green distribution; n = 9 fish) diets. The dashed blue line corresponds to the swimming activity value at the Ucrit. i.e., 122.1 AU). (b) Mean ± standard error of the percentage of swimming activity values above the Ucrit as a function of the time of day (day- and night-time), diet and interaction of both factors. P values are indicated for each factor, as well as the significance. NS = not significant (GLMM; p < 0.05). Panels (c) and (d) are zooms to the frequency distribution of swimming activities values displayed in panel (a) from 0 to 100 for panel (c) and 100 to 260 AU for panel (d).

4.3.4. Physiological parameters

No significant differences were observed between the two diet groups in any physiological indicators of stress and welfare at the end of the experiment (Fig. 4; $p > 0.05$ for all). However, the RBCC, lactate, Hb and lysozyme levels were significantly higher at the end than at the beginning of the experiment in both groups (Fig. 4; $p < 0.05$ for all), The HCT levels were significantly higher only in the organic diet group ($p < 0.05$).

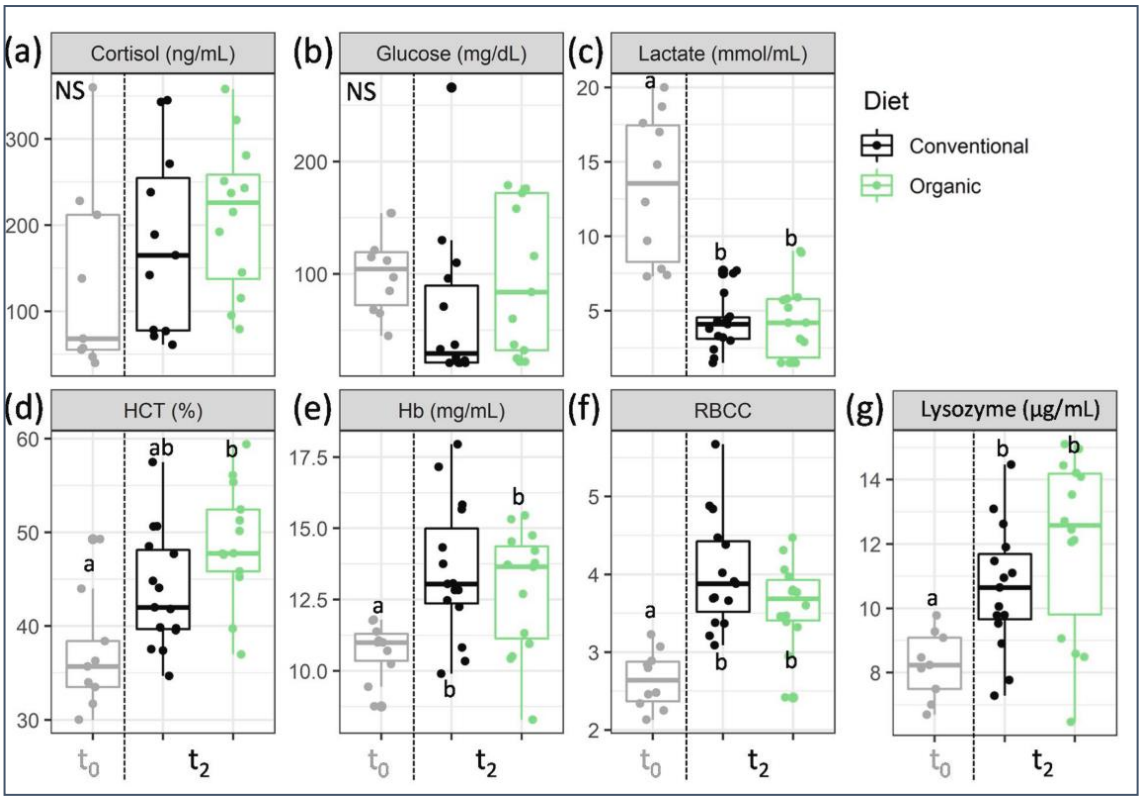

Fig. 4. Physiological parameters measured in blood samples at the start (t0, grey; n = 9) and the end of the experiment (t2) in the conventional (black; n = 15) and organic diet (green; n = 15) groups. (a) Cortisol (ng/mL), (b) Glucose (mg/dL), (c) Lactate (mmol/mL), (d) Haematocrit (HCT; %), (e) Haemoglobin (Hb; mg/mL), (f) Red blood cell count (RBCC; 106 cells/mm^3) and (g) Lysozyme (µg/mL). The central line in each boxplot indicates the median, and the boxes on either side represent the quartiles, with the whiskers covering 95 % of the values. All values are represented by coloured points. The letters indicate statistically significant differences between the groups (i.e., control at t0, and conventional and organic at t2). NS = not significant (ANOVA or Kruskal–Wallis test followed by Tukey's HSD post-hoc test; $p < 0.05$).

4.4. Discussion

In this study, we evaluated some physiological indicators of health and welfare, swimming activity and growth performance of European sea bass under two different feed regimes (conventional and organic). Swimming activity was measured using acoustic transmitters previously calibrated with the swimming performances during Ucrit trial, allowing better qualification of swimming activity during the experimental period according to the diet. In particular, due to a recent increase in fish production from organic aquaculture and the importance of fish welfare in this context, it is important to address this question in one of the most important farmed fish species of European marine aquaculture, the European sea bass. In teleost fishes, cortisol is the main stress hormone end product of the HPI axis (Sadoul and Geffroy, 2019). It triggers physiological and behavioural responses, and is involved in other physiological functions, such as growth and reproduction (Sadoul and Vijayan, 2016; Schreck and Tort, 2016). In our experiment, neither cortisol levels nor levels

of the secondary stress indicators (glucose, lactate and haematological parameters) were affected by either feeding regime. However, it is important to note that lactate decreased in both groups compared to the levels measured at the beginning of the experiment, whereas the haematocrit, haemoglobin and lysozyme concentrations and RBCC increased. Since the water temperature and photoperiod remained constant throughout the experimental period, these changes may be attributed to stress caused by the transfer of the fish to our facility. In line with the physiological data, the growth performance indicators (SGR, FCR and PER) during the experimental period were similar in the two groups, suggesting that the organic diet did not induce chronic stress, with long-term detrimental effects on growth (Sadoul and Vijayan, 2016). It is well known that plasma stress physiological indicators, such as cortisol, glucose and lactate, and haematological parameters may be insufficient for a proper assessment of fish welfare under experimental conditions that may induce chronic stress (Ellis *et al.*, 2012; Raposo de Magalhães *et al.*, 2020; Sadoul and Geffroy, 2019). Moreover, rearing procedures, including feed, may change the energy demands (Attia *et al.*, 2012; Carbonara *et al.*, 2020b), which are, in turn, reflected in changes in swimming behaviour (Martins *et al.*, 2012). Therefore, data from accelerometer tags, if appropriately calibrated, could be used as a measure of swimming behaviour in captivity and thus as an indicator of fish well-being (Carbonara *et al.*, 2020a, 2019b, 2015; Zupa *et al.*, 2021). For this reason, besides monitoring classical welfare indicators, we also monitored the swimming activity of the fish using acoustic transmitters. Calibrating the swimming activity recorded by acoustic transmitters with the water speed in swimming tunnel allowed us to determine swimming activity value corresponding to the critical swimming speed (the Ucrit) of the fish. One could say that values obtained in swimming tunnel may be different from spontaneous activity, since swim tunnel conditions may differ from natural swimming conditions, and may cause abnormal behaviour or induce stress (Nelson *et al.*, 2002). Nevertheless, it was previously reported that the range of activity levels achieved during the swim trials successfully spanned the range of activity exhibited by the fish in the holding tank in sea bass (Wright *et al.*, 2014). In addition, we also observed that values of MO_2 in free-swimming fish estimated from the calibration of accelerometer tag signal in swimming tunnel were in the range of what has been measured in the swimming tunnel in rainbow trout (Zupa *et al.*, 2021). Even these results are promising for using the calibration of signals from accelerometer tag in swimming tunnel, it has to be emphasized that forced swimming trials is not always representative of natural swimming conditions, and MO2 can be overestimated, due do possible stress induced by such swimming conditions. Further studies have to elucidate in which way values can

differ from forced to free-swimming conditions. In this study, during the feeding trial, the European sea bass displayed a diurnal pattern of swimming activity regardless of diet, with the highest activity observed at the beginning of the day during feeding, but also in anticipation of feeding before sunrise (~2 h before feeding). This diurnal pattern has already been described in individuals of this species living in groups (Anras *et al.*, 1997), although different activity patterns may be observed depending on environmental factors (Anras *et al.*, 1997; Helfman, 1978). However, in our study, the average swimming activity of the fish fed the organic diet was more intense than that of the fish fed the conventional diet during the daytime, while no differences were observed during the night-time. In addition, the analysis of the frequency distribution of swimming activity values recorded by the tag in free-swimming fish, revealed that higher data frequency has been found for low swimming activity values (<21 AU) in fish fed organic diet. For medium values (from 21 to 50 AU), higher data frequency has been observed in fish fed conventional diet, while from medium/high value (from 51 to 120 AU), higher data frequency has been again recorded in fish fed organic diet than fish fed with conventional one. For high swimming activity values (>140 AU), no difference in data distribution has been observed between the two groups, suggesting near and above swimming activity values corresponding to the Ucrit value (122.1 AU), no difference exist between fish fed two diets. Near and above the Ucrit value, swimming activity is mainly fuelled by anaerobic metabolism in many species, including in sea bass (Zupa *et al.*, 2015), which may affect the health and welfare of the fish if repeated or sustained for long time. More intense swimming activity requires greater use of anaerobic metabolism, reducing the metabolic energy reserves available for growth, reproduction, or coping with stressful situations. In a previous study on the European sea bass, we observed that white muscle (i.e. anaerobic metabolism) activity starts at approximately 65 % of the Ucrit value (corresponding to ~0.85 m/s or 2.7 BL/s) (Zupa *et al.*, 2015). Indeed, before the Ucrit was reached, the MO2 usually levelled off or decreased slightly, and sea bass starts to use anaerobic metabolism to display a burst swimming mode (Umax; ~0.9 m/s) (Claireaux *et al.*, 2006). Values of 0.85−0.9 m/s corresponds to swimming activity values recorded by the tag of ~62−65 AU. In our experiment, we observed that fish fed the organic diet displayed higher frequency of values for this swimming activity range, but were on average similar between the two diets. As, explained above, more intense swimming activity, especially at this level, implies higher metabolic costs (Carbonara *et al.*, 2015, 2006), which may affect growth or stress coping abilities. Nevertheless, in this study, we observed neither decrease in growth, or physiological disruptions in the organic diet group, which suggests that the

metabolic costs are relatively low and/or compensated by the organic diet, provided that it is well balanced in terms of protein, lipid and amino acid contents (Carbonara *et al.*, 2020b; Mente *et al.*, 2019). Calibrating the accelerometer tags also allowed us to estimate the amount of energy reserves (anaerobic metabolism) that the fish could use to cope with stress, which is crucial for animal welfare (Huntingford *et al.*, 2006; Korte *et al.*, 2007). Further, it allowed us to identify the acceleration values above the Ucrit, providing information about events of intense swimming activity potentially related to stress during the experimental period. By isolating these values, we were able to determine whether the fish in the two diet groups faced a similar number of stress events. Even if, higher frequency of medium-high swimming activity value has been observed near the Ucrit threshold, the fish in the organic diet group did not show a higher proportion of swimming activity values above the Ucrit. This suggests that the more intense swimming activity observed in this group was not linked with increased anaerobic metabolism due to stress. The similar lactate levels in the two groups lend weight to this conclusion. In terms of day/night patterns of swimming activity, the main difference between the two diet groups in this study was observed during the feeding period in the morning, while the swimming activity is overall similar the rest of the day. This suggests that the difference was probably due to more intense competition for organic feed. Overall, we also observed that the frequency of swimming activity values recorded by tag differed between the two diets but were on average similar between the two diets. That did not seem to affect sea bass health and welfare since the physiological indicators and biological performance were similar in the two diet groups throughout the experimental period. In summary, two main conclusions can be drawn from this study's findings. First, the use of acoustic transmitters previously calibrated with physiological indicators, such as the Ucrit, appears to be promising for real-time welfare monitoring in aquaculture. The precision of such calibrations of swimming activity may be enhanced by including other parameters, such as oxygen consumption and muscle activity, or other indicators such as Umax to better link the swimming performances with aerobic and anaerobic metabolism. Real-time monitoring of fish's behaviour and physiological state offers new possibilities for welfare monitoring in the aquaculture sector (e.g. Brijs *et al.*, 2019, 2018; Gesto *et al.*, 2020; Muñoz *et al.*, 2020), especially with recent advancements in data transmission through acoustic instead of radio channels, which provides greater applicability on production scales (Halachmi *et al.*, 2019). Second, based on all the indicators considered, well-balanced organic diet does not seem to negatively affect the health and

welfare of the European sea bass, which suggests that organic aquaculture may address challenges of the sector without compromising fish welfare.

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Chapter 5

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Article

Evaluation of the Effects of the Enriched-Organic Diets Composition on European Sea Bass Welfare through a Multi-Parametric Approach

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Abstract: Three groups of European sea bass (*Dicentrarchus labrax*) were fed for seven months, with either a conventional diet or two different organic diets, which contain organic vegetables and a natural antioxidant compound. The two organic diets differed themselves in terms of raw proteins, fish oil, and lipid contents. Sea bass welfare condition was assessed in relation to these three diets, using 16 different indicators. These were: swimming activity (recovery test, muscle activity), haematological and serological stress indicators (haematocrit, haemoglobin, red-blood-cell count, cortisol, glucose, lactate), aspecific immunity parameter (lysozyme), indicators of exposure to organic contaminants (7-ethoxyresorufin-O-deethylase and glutathione-S-transferase), and growth parameters (weight gain, specific growth rate, feed conversion ratio, protein efficiency ratio, and hepato-somatic index). Most of these parameters individually did not give consistent responses, but their integration can provide an accurate evaluation of the fish welfare conditions among the three diet experimental groups. The multiparametric approach outlined a comprehensive picture of sea bass physiological state. The principal component analysis and the multi-criteria-decision-analysis were found to be useful tools for an integrated fish welfare assessment, highlighting that the best welfare condition was achieved in the experimental group fed with the protein-rich organic diet.

Keywords: European sea bass; multiparametric approach; muscle activity; organic; welfare

5.1. Introduction

Aquaculture plays a crucial role for supplying the increasing demand of animal protein to feed the growing world population [1]. However, the future expansion of aquaculture remains hampered by the demand for wild forage fish for the production of feed used for

aquaculture, among other factors. Indeed, the capacity of wild fisheries to cope with the increasing demand for fishmeal and fish oil has reached the limit of sustainability [1]. Moreover, fishmeal and fish oil derived from the wild can be considered a vehicle of contamination as organochlorines, such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAH), and dioxins, principally accumulate in the lipidic fraction [2]. These contaminants are attracting particular attention since exposure through diet can trigger a long-lasting effect on fish physiology, behaviour, and ultimately growth and reproduction [3]. As a result, aquaculture tends to move toward more sustainable and organic sources of fish meal and oil [4,5], even if the challenge of the transition is still huge [6]. Organic aquaculture is an alternative production that combines environmental friendly practices, the maintenance of biodiversity, preservation of natural resources, high animal welfare standards, and production methods in line with defined standard of quality, using natural substances and processes [7]. Organic feed is composed of a greater percentage of proteins and oils from land-based agriculture combined with natural antioxidants [4,8]. The partial substitution of fishmeal and fish oil with plant proteins and oils was found to be a promising alternative to fish protein (and oil) already adopted for commercial diets [9–11], while total substitution can disrupt physiological processes and growth performances [12,13]. Currently, soybean meal represents the predominant choice in terms of vegetable protein source, considering its relative high protein content and suitable amino acid profile [14]. Nevertheless, some limitations still exist regarding the soybean meal percentage that can be tolerated in fish feed formulation, especially for carnivorous fishes. For European sea bass (*Dicentrarchus labrax*), which is a key species of European marine aquaculture [15,16], a partial substitution of fish meal with raw plant material can be used, showing interesting results concerning the physiological and growth performances, along with an improved flesh quality [17,18]. These results are promising to support the transition towards more sustainable aquaculture, which would mitigate the environmental issues while ensuring good growth performances and the welfare of farmed fish. In the last decades, the welfare of farmed fish received considerable attention, becoming a key

point of the aquaculture [19–24]. The basis for fish welfare is that fish are sentient animals capable of experiencing good or bad feelings or emotional states [25]. Most of the animal welfare definitions could be linked to biological functions and/or feelings [22], although this has been recently questioned [26]. In the first case, the welfare is correlated with physiological state including the stress response measured for example by blood parameters (e.g., cortisol, glucose, lactate). In the second case, the welfare is more linked with avoiding

negative (e.g., pain, fear, hunger) experiences measured, for example, by behavioural parameters (e.g., swimming activity, energetic balance) [27]. However, addressing fish welfare remains a complex issue due to the difficulty of finding realistic and objective indicators that can describe the overall response of the organisms in captivity [21,28]. In teleost fishes, the welfare is closely linked with the stress response [29]. Thus, the measure of cortisol, the major end-product of the hypothalamo–pituitary–interrenal (HPI) axis, is commonly used as a proxy of welfare in the aquaculture research [30,31]. In addition, changes in metabolism (e.g., lactate, glucose), hematological (e.g., hematocrit, hemoglobin), and immunity (lysozyme) features, constituting the secondary stress response [32,33], are also commonly used for welfare assessment (e.g., [34–40]). However, stress does not always mean suffering or low welfare [28]. Indeed, the stress could also be seen as an adaptive function that, in the short term, allows fish to preserve both single individual and population [28]. However, a prolonged stress condition is known to trigger higher cortisol levels in plasma that may result in an energy imbalance [41]. From this perspective, the cortisol could be used as the welfare indicator, but needs to be coupled with more integrative welfare indicators [42,43], such as behaviour [44–46], swimming [47–49], or growth performances, which constitute the tertiary stress response [32]. In particular, the measurement of muscular activity, through electromyography (EMG), appeared to be a sensitive and promising method to assess the fish welfare status [34,50]. Previous studies have considered only specific aspects of the effects of diets with a partial substitution of proteins and antioxidants, such as feed conversion, immunity, or flesh quality. Thus, the main scope of the present study is a deeper investigation of the effect of the two organic diets (Dana Fish DAN-EC 1650TMand Dana Fish DAN-EC 2640TM) on the welfare of the European seabass in comparison to a conventional one (DAN-EX 1754TM). To do so, a holistic approach was adopted, including the measurement of primary (cortisol), secondary (i.e., lactate, glucose, hematologic parameters, lysozyme), and ultimately tertiary (i.e., swimming performances, muscular activity and growth parameters) stress response indicators. In parallel, we assessed the 7-ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferase (GST) enzymatic activities, as a functionality index of the hepatic microsomal mixed-function oxygenase (MFO) system [51,52], to assess possible effects of pollutant contamination through diet. In this study, multi-parametric analysis approaches were performed to obtain a better understanding of the effectiveness of a holistic approach to quantify welfare in organic aquaculture.

5.2. Materials and Methods

5.2.1. Ethical Consideration

Care and handling of fish were accomplished in accordance with the recommendations 2007/526/EU C2007 2525 on the accommodation and care of animals used for experimental and other scientific purposes. The experimental protocol was approved by the ethic committee on the animal experiments of COISPA. All the fish manipulations were performed on fish completely anaesthetized (stage 4: loss of reflex activity and no reaction to strong external stimuli; [53]) with a 30 mg L^{-1} of clove-oil to minimize pain and discomfort of fish. Survival after manipulations was 100%.

5.2.2. Experimental Design and Rearing Conditions

Adult sea bass (1.3 years old) were purchased in 2007 from a commercial hatchery (Panittica Pugliese S.p.A., Fasano, Italy) The fish came from the same eggs batch (wild broodstock) and they were left undisturbed during 2 months before the following experimental procedure. Water temperature (18 ± 0.5◦C), O2 concentration (5.30 ± 0.16 ppm), pH (7.09 ± 0.02), photoperiod (12:12 D:L), and water recirculation (10 L min−1) were maintained constant during the entire duration of the experiment and did not differ between the different diets. Fish were fed by automatic feeders for a period of three hours (09:00–12:00 h) six days per week at 0.9% of the tank biomass. After the acclimation period, fish were split in nine fibreglass tanks of 1.2 m^3 (n = 3 for each diet), as reported in Table 1. Fish mean weights and mean total lengths were not statistically different among the different diet conditions (Kruskal–Wallis test: $p > 0.05$ for both, Table 1). The three groups of fish were fed for about seven months (from December 2007 to June 2008—208 days) with three different diets (see Table 2).

Diet	Tank	*n*	Mean Weight (g)	Biomass (kg)	Mean Length (cm)	Density (kg m^{-3})
DAN-EX 1754 (Conventional)	1	42	297.8 ± 15.4	12.51	302 ± 6.00	10.43
	2	41	336.4 ± 24	13.79	313 ± 7.93	11.49
	3	42	324.9 ± 16.6	13.65	312 ± 5.40	11.38
DAN-EC 1650 (Organic 1)	4	42	317.5 ± 14.1	13.34	308 ± 4.15	11.12
	5	42	312.7 ± 21.4	13.13	295 ± 9.69	10.94
	6	42	339.1 ± 28.9	14.24	315 ± 9.78	11.87
DAN-EC 2640 (Organic 2)	7	42	327.8 ± 17.5	13.77	305 ± 6.85	11.48
	8	40	319.3 ± 23.6	12.77	306 ± 7.75	10.64
	9	41	322.2 ± 13.3	13.2	306 ± 7.46	11

Table 1 Mean ± SEM of the morphometric and rearing variables for the three tanks of each diet at the beginning of the experiment. Fish mean weights and mean total lengths were not statistically different in the nine experimental tanks (Kruskal-Wallis, p > 0.05).

The first group was fed with a conventional diet (Dana Fish DAN-EX 1754TM, later called conventional) and served as a control, while the other two groups were fed with two different organic diets (Dana Fish DAN-EC 1650TM and Dana Fish DAN-EC 2640TM later called respectively organic diet 1 and 2; Table 2). The two organic diet according with the EU regulation (EC, 1358/2014) for organic aquaculture comprise <25% of fishmeal and <10% of fish oil (23% and 4% in the diet organic 1 and 13% and 9% in organic diet 2 respectively of fishmeal and fish oil). The two organic diets contained a greater content in vegetable proteins than the conventional diet (41.4% for organic diet 1 and 43.9% for organic diet 2 vs. 34.8% for conventional). Moreover, the vegetable component in organic diets coming from organic agriculture. The antioxidant compound used in the organic diets was the rosemary essential oil (200 g kg^{-1}) while Butylated hydroxytoluene (BHT) was used in the conventional diet. The two organic diets differed each other both in proportion of the crude proteins (50% and 40% respectively for organic diet 1 and organic diet 2) and total lipids percentages (16% and 26%, respectively for organic diet 1 and organic diet 2; Table 2).

Raw Material (%)	Conventional		Organic 1	Organic 2
Rough proteins	54		50	40
Gross fats	17		16	26
Rough ashes	9.16		10.59	8.64
Rough fibers	2.01		2.89	3.39
Phosphorus	1.27		1.74	1.31
Additives				
Copper (mg kg^{-1})	5		5	5
Vitamin A (I.U. g^{-1})	0.72		0.72	0.72
Vitamin D3 (I.U. g^{-1})	0.11		0.11	0.11
Vitamin E (mg kg^{-1})	198		198	198
BHT (mg kg^{-1})	79		0	0
Rosmary essential oil (mg kg^{-1})	0		200	200
Proteic compounds (%)		**Proteic compounds in organic diets (%)**		
Fish flour	54.65	Fish flour	56.88	38.25
Soybean proteic concentrate	15	Roasted organic soy extract	10.49	11.10
Fish oil	10.07	Fish oil	5.23	17.29
Wheat	8.83	Organic wheat	12.50	12
Wheat gluten	4	Extract of organic colza	2.20	5.99
Hulled roasted soy flour	4	Roasted organic soybeans	10	11.10
Peas	3	Extract of organic sunflower seed	2.20	3.76
Vitamin pre-mix	0.45	Vitamin pre-mix	0.50	0.50

BHT—Butylated hydroxytoluene.

Table 1 Composition of the three experimental diets: conventional (DAN-EX 1754) and two organic diets (Organic diet 1 and 2, DAN-EC 1650 and DAN-EC 2640 respectively).

5.2.3. Blood and Hepatic Sampling and Morphometric Procedures

First, at the beginning of the experiment, fish (n = 20) were randomly and gently caught from their stock and anesthetized using clove oil as previously described. When fish were anesthetized (~after 3 min in anesthesia bathing), blood samples were taken using heparinized syringes (needle 23 G). Two intermediate blood samplings were then performed over the experiment (at 70 and 130 days after the beginning of the experiment) on seven fish from each tank (n = 21 fish per diet) and at the end of the experiment (208 day after), eight fish per tank were sampled (i.e., n = 24 fish per diet). In parallel, liver tissues were extracted from 20 fish at the beginning of the experiment and from 5 fish per tank at the end of the experiment (n = 45 fish in total). Fish euthanasia was realized using an overdose of clove oil (60 mg L^{-1}). For each fish, the blood sample taken was circa 0.5 mL. For each sample, 5 µL was directly stored at −20 ◦C for the haemoglobin (Hb) determination, other 5 µL were diluted in 1 mL of Hendriks solution for erythrocyte counts (RBCC) and stored at +4 ◦C, and about 25 µL to fill microhaematocrit tubes Haematocrit (Hct) quantification. The remaining part of the blood sample was centrifuged at 2000× g for 3 min to obtain plasma which was stored at – 80 °C for cortisol, lysozyme, glucose and lactate further quantification. The hepatic samples were stored at – 80 °C before the analysis. The sea bass hepatic cytosolic andmicrosomal fractions were prepared as reported by Corsi *et al.* [54]. The hepatic sample were used to quantify the 7-ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferase (GST) enzymatic activities, as functionality index of the hepatic microsomal mixed-function oxygenase (MFO) system. Finally, n = 10 fish per tank, at the beginning and in the intermediate sampling, and n = 20 fish per tank at the end were used for morphometric measurements: total length measured to the nearest 0.5 cm, total weight measured to the nearest 0.1 g, gutted weight to the nearest 0.01 g and liver weight to the nearest 0.01 g. The calculation of growth parameters is further presented in the Section 2.6.

5.2.4. Quantification of the Haematological and Serological Parameters and Enzymatic Activities

The Hb concentration was determined using cyanmethemoglobin method with a commercial kit (Sigma, St. Louis, Missouri, USA). Hct was determined using a micro-haematocrit tube filled directly by the syringe needle, centrifuged (15,000× g for three minutes) and immediately read. The number of RBCC was determined using a Bürker counting chamber under a light microscope (Nikon 400E, Nikon, Tokio, Japan) at 40× magnification. Plasma glucose and lactate concentrations were determined using a commercial kit (SentinelTM, Milan-Italy) based on the enzymatic-colorimetric Trinder

reaction (GOD/PAP glucose and PAP lactate). Lysozyme concentration was measured using turbidimetric assay modified for microplate reader [41]. Plasma cortisol concentration was quantified by high performance liquid chromatography according to Vissali *et al.* [55] and modified for European sea bass as reported by Carbonara *et al.* [41]. EROD activity was determined, into the hepatic microsomal fraction, according to Burke & Mayer [56]. GST activity was determined by the hepatic cytosolic fraction and measured using spectrophotometer as described by Habig *et al.* [57], modified for microplate readers. By using a Shimadzu UV-160A visible spectrometer (Shimadzu, Kyoto, Japan) and bovine serum albumin as standard, the total amount of proteins was determined according to the procedure described by Bradford [58].

5.2.5. Critical Swimming Speed Tests and EMG Activity Monitoring

In order to monitor fish red muscle activity, a total of n = 18 individuals (n = 2 per tank; 6 per diet) were surgically implanted with CEMG-R11-25 (Lotek Wireless™; 12 g in air) and ~3–4% of fish weight radio transmitters as described in Lembo *et al.* [59]. Although a ratio between tag and weight of the fish not greater than 2% is generally considered optimal, this ratio is species specific and, in some cases, such as sea bass, it was observed that a ratio of 3–4% did not affect growth and swimming behaviour [34]. Specimens were randomly selected for the surgical implantation of EMG radio tags and were fasted 24 h before the surgical procedure [60]. Fish were gently caught from their rearing tank and bathed into anaesthetic about 5 min until loss of reflex activity and no reaction to strong external stimuli [53]. After this period gills were continuously irrigated with anaesthetic solution to proceed the surgery. EMG-tags were implanted into the peritoneal cavity through a 3 cm incision located 4–5 cm posterior to the pelvic girdle. The gold electrodes of the sensor were inserted in the red muscle band by mean of a hollow needle. The incision was closed with four independent sutures [34]. The surgery lasted on average 5 min, followed by a recovery time of about 10 min. After that, all fish were recovered successfully. Each tagged fish was treated with antibiotic injections (sodic-ampicillin-cloxacillin 1 mg kg^{-1} 24 h^{-1}) for 3 days after the surgery as described in Lembo *et al.* [61]. The fish resumed feeding circa 5 days after the surgery. The two gold tipped electrodes positioned in the red muscle of the fish allowed to decode the electromyographical signals (EMG) [62]. In the EMG-tag, the changing voltage in muscle activity was corrected, summed and stored every five seconds. Then, the average value was transmitted to a radio receiver as an entire adimensional number ranging from 0 to 50 [50], allowing for real time monitoring of each free swimming fish. After this period all the fish were considered completely recovered from the surgery [63], and were

subjected to a critical swimming speed test (Ucrit) in a Blažka style swimming chamber to calibrate EMG signals with the Ucrit test as described in Carbonara *et al.* [34]. Briefly, Ucrit is a swimming test in which fish swim in a chamber at increasing speed step (0.1 m s−1 every 10 min) until the fatigue is reached, determining the critical swimming speed of the challenged fish (Ucrit) [59]. The calibration gives the possibility to correlate each single swimming level to an activity index expressed as the EMG level [50]. In particular, the EMG level at the Ucrit speed represents the threshold limit of the aerobic muscular activity [34]. After the calibration in the critical swimming speed, fish were released in the experimental tanks and the EMG signals were recorded during the feeding period (9 AM–1 PM) and no-feeding period (2 PM–6 PM; starvation period). Daily average index of muscle activity is expressed as a ratio between the value of EMG recorded in tank and EMG value at Ucrit (EMG/ EMG at Ucrit). The measurement of EMG value lasted for 110 days. Finally, at the end of the experiment, three fish per tank (n = 9 per diet treatment) were randomly selected to perform a recovery test, as described in Carbonara *et al.* [41]. The recovery test consists in submitting the fish to a second critical swimming test, one hour after the first one, to estimate both relative Ucrit1, Ucrit2 and the recovery ratio value (RR = Ucrit2/Ucrit1). The latter is a useful index to assess the fish capacity to restore the metabolic glycogen storage in muscle, after a first Ucrit test was already performed [64].

5.2.6. Growth Parameters Calculations

Fish growth performances were estimated for each tank (n = 3 tanks per diet treatment) as follow. Specific growth rate (SGR) was calculated according to the following equation [65]:

$$SGR = 100 \times (\ln W_{t2} - \ln W_{t1}) \times T^{-1} \quad (1)$$

where W is the total weight of the fish respectively at the end (t2) and the beginning (t1) of the experiment and T is the number of feeding days. The food conversion ratio (FCR) was calculated as the ratio of the feed supplied (kg of dry weight) per biomass of weight gained (kg). The protein efficiency ratio (PER) was calculated as the biomass gained (kg) divided by the total amount of proteins (kg) administered during the trial. The effects of the different treatments were also evaluated considering the hepato-somatic index (HSI), calculated as the liver weight divided by the gutted body weight of fish and expressed as percentage.

5.2.7. Statistical Analyses

Statistical analyses were performed using the R software [66] excepted otherwise mentioned, and were carried out at the 95% level of significance. All the data are reported

as mean ± SEM (standard error of the mean). All the data were prior checked for normality (Shapiro–Wilk test) and homogeneity of variance (Levene test). The mean EMG values per day, the Ucrit1 and Ucrit2 values, growth parameters and biomarkers data were statistically analysed with one-way ANOVA or Kruskal–Wallis' test depending on the condition of application. For the haematological and serological parameters, a two-way ANOVA was performed using the diet and time as fixed factors. Tukey's post-hoc test was then applied to highlight differences between the diet groups. The correlation between daily mean EMG values within diet treatments overtime, for the two registered periods, were first evaluated using the Pearson's correlation test. The regressions of the two data collection periods (feeding and fasting) were then compared in each group by the analysis of covariance (ANCOVA). The comparisons between the recovery ratio (RR) values (Ucrit2/Ucrit1) and the value 1 (RR in fish completely recovered after the first Ucrit) in each diet group and the comparisons between Ucrit1 and Ucrit2 were performed using a Wilcoxon's test. Furthermore, a principal component analysis (PCA) was performed on sixteen of the parameters monitored (Hb, Hct, RBCC, SGR, HSI, FCR, PER, RR,EMG (feeding period), GST, EROD, gained weight, glucose, lactate, cortisol, lysozyme) in the three experimental groups using FactoMineR package [67]. The relevant dimensions of the PCA were selected using the acceleration factor method [68]. The PC score of the relevant axes were then downloaded and, Kruskal–Wallis test was performed and followed by Tukey test.

Among the 16 parameters used in PCA, seven were selected based on significant differences of these parameter between the diets (cortisol, glucose, lysozyme, SGR, HSI, RR, and EMG) to perform a multicriteria decision analysis (MCDA) by means of a non-structural fuzzy decision support system (NSFDSS), a decision-setting model used for ranking a set alternatives on the basis of agreed-upon criteria [69]. In this case, the decisions to be ranked are the three diets, and the decision factors (names thereafter also criteria) are 7 selected parameters. The non-structural fuzzy decision support system (NSFDSS) is a method for multi-criteria decision analysis (MCDA), belonging to the methods of deterministic preference modelling [69–73]. NSFDSS is used for ranking a set of possible decisions on the basis of agreed-upon decision factors. This multi-criteria decision technique, although generally applied in participatory management of natural resources [69,74], was considered particularly suitable to be applied to derive the ranking of the diets on the basis of observations of the 7 parameters, because based on a simpler scoring scale respect to other techniques (0, 0.5, 1). The NSFDSS requires, as other MCDA tools, that the problem is structured into different steps: decomposition, comparative judgment, and

synthesis of priorities. Thus, first a goal (fish welfare) is outlined then a decision tree is built moving downward to the lower levels to reaching more specific decision factors. The decision factors (level 1) or criteria deemed to be important for contributing to the goal are thus elicited; in our case the criteria are represented by the selection of the seven physiological parameters. Then, a number of different alternatives (level 2) are also defined to reach the goal. In our case, they are the diets. These alternatives are scored against each decision factor and then pairwise comparisons are made also between decision factors. The last step is the synthesis of priorities. Local priorities are multiplied by the priority of their corresponding criterion on the level above and then weighted by means of classification of criteria, constructing a sort of composite priority. In the NSFDSS, a comparative judgement and a synthesis of priorities were made. The former was performed by the construction of pairwise comparisons among the diets: each diet was compared with the others on the basis of each shared criterion; then, pairwise comparisons were made also among criteria. While, during the step of priorities synthesis, the local priorities were multiplied by the priority of their corresponding criterion and then weighted by means of classification of criteria as a sort of composite priority. The results of pairwise comparisons among diets according to the behaviour of each physiological parameter in relation to sea bass welfare. Moreover, the same analysis was conducted in the hypothesis of the equivalence of the criteria in the ranking of diets ("equal importance of criteria" hypothesis), in order to evaluate the differences of the results between the two hypotheses. The MCDA was performed using a freeware Excel sheet online [75].

5.3. Results

5.3.1. Haematological and Serological Parameters

No significant differences were found in the Hct values following the administration of the three diets during all experimental duration (ANOVA, $p > 0.05$; Figure 1a). Moreover, the haematocrit values decreased over the experimental period in all treatments, reaching levels significantly lower than those found at the beginning of the experiment (ANOVA, $p < 0.05$). The RBCC values showed a general increase for all the groups in the first two months in comparison with the beginning of the experiment (ANOVA, $p < 0.05$). In particular, this increment was significant for fish fed with the conventional diet and with the organic diet 2 (Tukey HSD, $p < 0.05$; Figure 1b). The Hb concentration showed a trend similar to the Hct and RBCC parameters, showing a significant increase in the first two months period for all the experimental groups (ANOVA, $p < 0.05$), and thereafter a

significant reduction between the first and the second period of the experiment in fish fed with organic diet 1 (ANOVA, $p < 0.05$; Figure 1c).

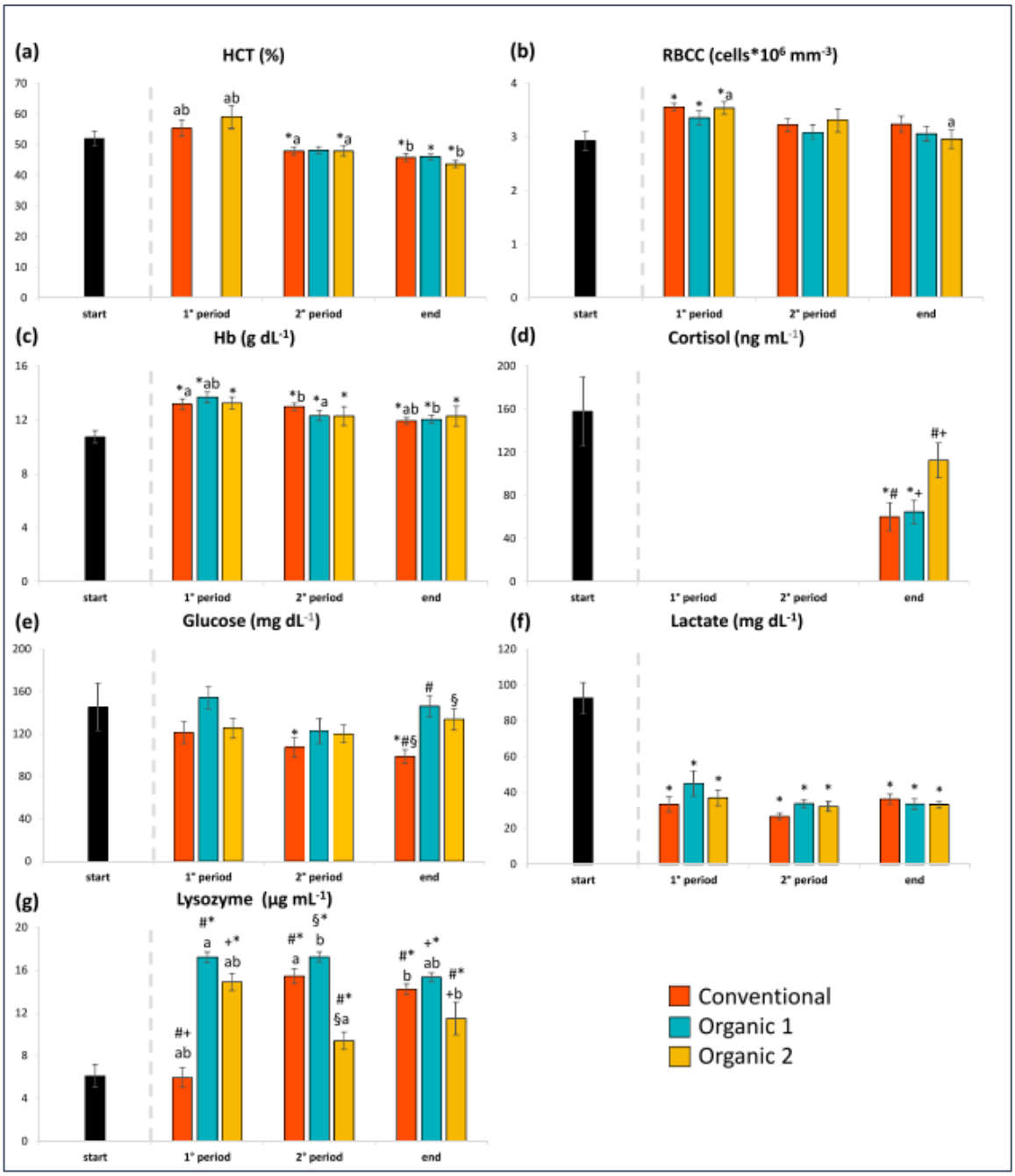

Fig. 1 Mean±SEMof the haematological and serological parameters measured over the experimental duration (start, 1° period, 2° period and end of the experiment) among the different diets. (a) HCT (%); (b) RBCC (cells 10^6 mm^{-3}); (c) Hb (g dL^{-1}); (d) Cortisol (ng mL^{-1}); (e) Glucose (mg dL^{-1}); (f) Lactate (mg dL^{-1}); (g) Lysozyme (µ mL^{-1}). An asterisk (*) denotes a significant difference from the "start" value. Similar symbols (#, +, §) denote significant difference in treatments comparisons. Similar letters (a, b) denote significant differences in time-points comparisons (Tukey HSD, $p < 0.05$).

Initial plasma cortisol concentration was significantly higher than the level recorded at the end of the experiment for the conventional diet and the organic diet 1 (Kruskal–Wallis

test, $p < 0.05$; Figure 1d). At the end of the experiment, fish fed with a higher protein level (conventional and organic diet 1) showed the lower cortisol concentration than fish fed with the Organic diet 2 (Tukey HSD, $p < 0.05$). Plasma glucose levels showed a high variability within each diet group (Kruskal–Wallis, $p < 0.05$; Figure 1e). In particular, glucose concentration in fish fed with the conventional diet showed a progressive significant reduction of glucose levels (ANOVA, $p < 0.05$; Figure 1e). In all sampling points, the glucose level of the conventional diet group was significantly lower than the other experimental groups (Tukey HSD, $p < 0.05$). Lactate level was significantly higher for the first period than the following ones in all the experimental groups (ANOVA; $p < 0.05$; Figure 1f), showing a decrease over time. No significant differences between the diet treatments were found in each sampling point (ANOVA, $p > 0.05$ for each diet; Figure 1f). Finally, the lysozyme concentration in all the diets was significantly higher at the end of the experiment than at the beginning (Kruskal–Wallis test, $p < 0.001$; Figure 1g). As for cortisol, the higher lysozyme levels were found in fish fed with diets rich in proteins (i.e., conventional and organic diet 1; Tukey HSD, $p < 0.05$; Figure 1g).

5.3.2. Physiological Parameters—Critical Swimming Speed and EMG

The average index of muscle activity (EMG) resulted significantly different among the three experimental diet groups but there were no significant differences between recording periods (i.e., feeding and starvation) (Kruskal–Wallis test, $p < 0.05$; Figure 2).

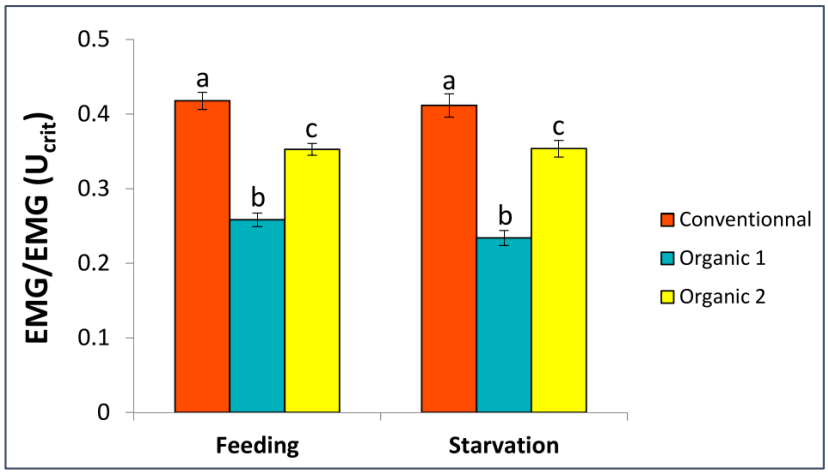

Fig.2. Mean ± SEM of the red muscle activity recorded (EMG)/red muscle activity at the Ucrit (EMG Ucrit) during the feeding starvation periods for the three diets. Different letters indicate significant difference between diets (Tukey, $p < 0.05$).

The post hoc test revealed that fish fed with the organic diet 1, richer in proteins, showed the lowest EMG value during feeding and starvation periods while the conventional ones showed the highest EMG value (Tukey HSD, $p < 0.05$). The daily mean value of EMG

showed a significant correlation for the three diets with the time both for the feeding and starvation periods (Spearman rho test, $p < 0.05$ for all; Figure 3). In particular, the analysis of the direction of the Spearman correlation indicated that the EMG values have a significant negative trend during the experiment for fish fed by the diet Organic 1 (feeding period: $p < 0.001$; Starvation period: $p < 0.001$), while both the diet conventional and organic diet 2 showed positive trends during the experiment (feeding period: $p < 0.001$; starvation period: $p < 0.05$ for both) (Figure 3).

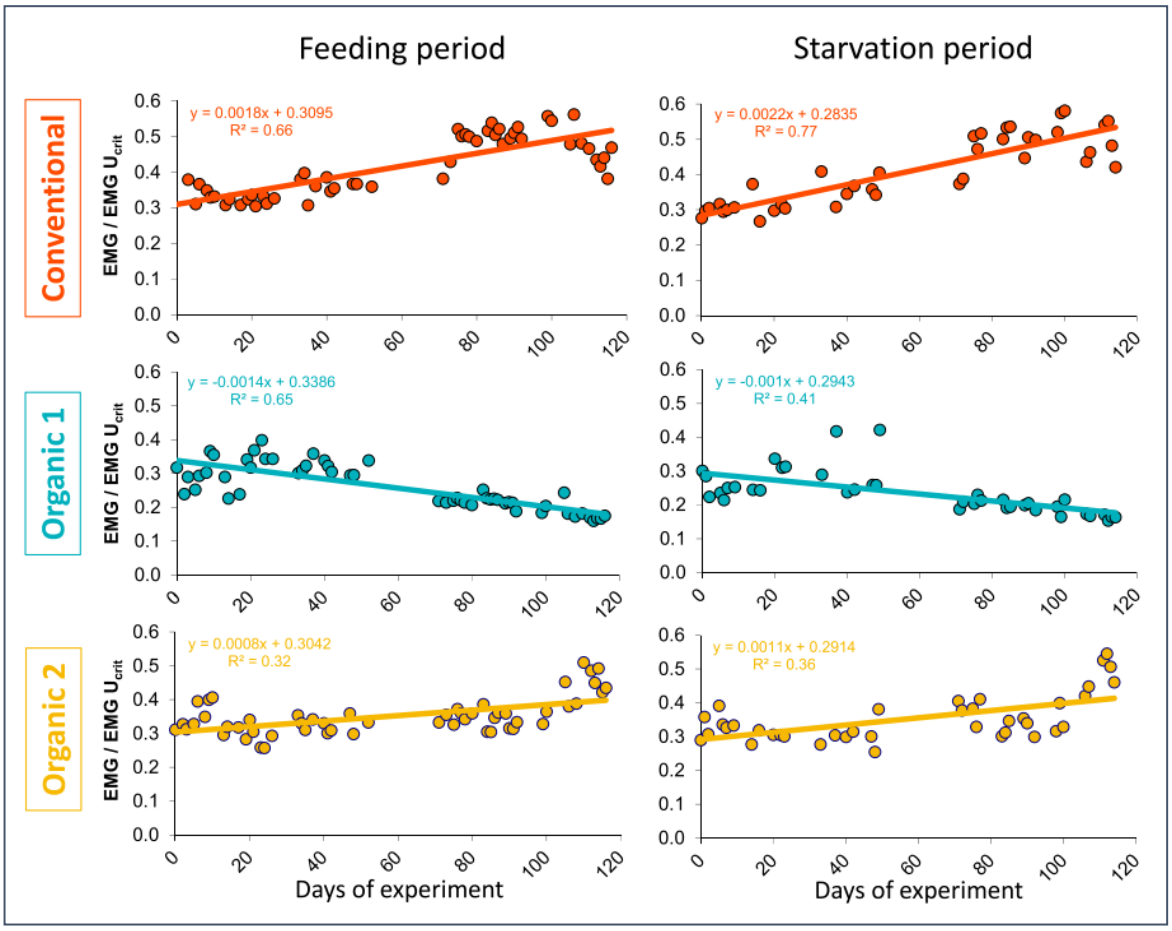

Fig. 3. Mean daily red muscle activity values (EMG/EMG Ucrit) during the feeding and starvation periods for the three diets (Conventional, red; organic diet 1, blue and organic diet 2, yellow). Equation and R2 are reported for significant correlation (Spearman's correlation, $p < 0.05$).

The analysis of covariance between the feeding and starvation periods showed that the slopes were significantly different between the three diets (ANCOVA, Fcalc > Fcrit), while the values of the intercepts were significantly different only for fish fed by the diet Organic 1 (ANCOVA, Fcalc > Fcrit). The average values of the two consecutive critical swimming speed tests (Ucrit) and the recovery ratio are reported in the Table 3.

Diet	Mean Weight (g)	U_{crit1} (BL s^{-1})	U_{crit2} (BL s^{-1})	Recovery Ratio
Conventional	403.7 ± 8.9	4.1 ± 0.14	3.8 ± 0.15	0.93 ± 0.03 *
Organic 1	416.9 ± 19.1	3.9 ± 0.10	3.8 ± 0.14	0.99 ± 0.02
Organic 2	403.8 ± 15.14	3.6 ± 0.10	3.5 ± 0.02	0.98 ± 0.02

*Table 2. Mean ± SEM of the relative Ucrit1 (BL s^{-1}), Ucrit2 (BL s^{-1}) and the recovery ratio values for the diet treatments. *—indicates the recovery ratio values significantly different from 1 (Wilcoxon test, $p < 0.05$).*

First, there is no difference for both Ucrit1 and Ucrit2 values between the three diets (Kruskal–Wallis, $p > 0.05$ for all). Although, fish fed the conventional diet did not show a significant difference between the Ucrit1 and Ucrit2 values (Wilcoxon test, $p > 0.05$), this result was not confirmed by the analysis of RR. Indeed, only the fish fed with the conventional diet displayed a RR lower than 1 (Wilcoxon test, $p < 0.05$).

5.3.3. Specific Biomarkers—EROD and GST

All three experimental diets induced a decrease of the EROD activity over time, especially for fish fed with the conventional diet and the organic diet 2 (Kruskal–Wallis test, $p < 0.05$; Table 4). No significant differences were found between the three diets, and no significant differences were observed over time for the GST activity regardless of diet (Kruskal–Wallis test, $p > 0.05$ for all; Table 4).

Parameter	Start	Conventional	Organic 1	Organic 2
EROD (nmol min^{-1} mg^{-1})	50.1 ± 3.58	* 37.5 ± 4.96	47.3 ± 3.28	* 38.4 ± 3.38
GST (nmol min^{-1} mg^{-1})	60.0 ± 2.66	56.3 ± 4.21	58.2 ± 5.65	50.4 ± 4.65
HSI (%)	1.03 ± 0.05	*, #, + 1.66 ± 0.06	*, #, § 1.99 ± 0.07	*, +, § 2.3 ± 0.09
TL (mm)	306.9 ± 2.4	#, + 350 ± 2.3	# 357± 2.7	+ 360 ± 2.6
Weigth (g)	321.9 ± 6.7	#, + 509.9 ± 11.43	# 546.7 ± 12.76	+ 545.6 ± 11.76
SGR (%)		#, + 0.29 ± 0.011	# 0.33 ± 0.01	+ 0.33 ± 0.005
FCR		5.31 ± 0.680	4.92 ± 0.213	5.06 ± 0.356
PER (%)		36.01 ± 4.37	40.82 ± 4.29	49.90 ± 3.54
Survival rate (%)		82	78	77

Table 3 Table 4. Mean±SEM of EROD and GST activities and the growth parameters during the experimentation for the three diets (Conventional, organic 1 and 2). An asterisk (*) denotes a significant difference from the "start" value (Tukey, $p < 0.05$). Moreover, the symbol #, +, § denote significant differences among the diets at end of the experiment.

5.3.4. Growth Parameters

At the end of the experiment, hepato-somatic index (HSI) was higher than the initial value in all groups (ANOVA, $p < 0.001$). In addition, significant differences were also found

between all diets for other growth parameters indexes (Tukey HSD, $p < 0.05$ for all; Table 4). In more details, the SGR was significantly lower in fish fed with the conventional diet in comparison to fish fed by the two organic diets (Kruskal–Wallis, $p < 0.05$). On the contrary, the FCR and PER were not influenced by the diets (Kruskal–Wallis, $p > 0.05$, Table 4). Fish fed with the two organic diets showed better growth performances both in terms of length and weight gained compared to fish fed with the conventional diet (Tukey HSD, $p < 0.05$ for all; Table 4).

5.3.5. PCA and NSFDSS Results

According to the acceleration factor method, the relevant components of the PCA were the first three, together explaining 56.9% of the data variability (Table 5). The first component of the PCA, which explained 27.3% of the observed data variability, was mainly driven by the SGR, weight gain, FCR, PER, EMG and EROD activity. Individual displaying high values on this component are those that showed better PER, a lower FCR, better growth performances, but higher EROD activity (Table 5). This suggests that individuals with high values on the first component are those displaying higher welfare status. For the component 2 of the PCA, which explained 16.7% of the observed data variability, it was mainly driven by cortisol and lysozyme concentration, EROD activity and PER (Table 5). Individual displaying high value on this component are those with high plasmatic cortisol and low lysozyme concentration, low EROD activity and high PER (Table 5). Thus, we can consider that individuals with high values on the second component are individuals displaying lower welfare status. Finally, the component 3 of the PCA, which explained 13.2% of the observed data variability, was mainly driven by Hb, RBCC, cortisol, and glucose. Individual displaying high value on this component also displayed higher levels of RBCC, HB, cortisol, glucose, and GST. Since high values on this component are linked both to high cortisol/glucose values and high HB/RBCC values, the link between this component and welfare is more delicate to clearly establish.

Variables	Component 1	Component 2	Component 3
HCT			0.38
RBCC		0.23	**0.54**
HB		0.26	**0.52**
Cortisol	0.19	**0.58**	**0.52**
Glucose	0.31	0.2	**0.63**
Lactate			
Lysozyme		**−0.51**	−0.29
EROD	**0.64**	**−0.67**	0.31
GST	−0.17	**−0.83**	**0.5**
HSI	0.41	0.32	
SGR	**0.98**		
RR	0.16		0.31
EMG	**−0.54**	0.45	
PER	**0.67**	**0.59**	−0.41
FCR	**−0.95**	0.26	
Weight gain	**0.98**		
Variance explained (%)	27.3	16.4	13.2

Table 4. Contribution of the sixteen variables to the different three first components of the principal component analysis (PCA) and data variance explained by each component. Only significant contributions are shown in the table ($p < 0.05$). Bold indicate a contribution higher than $|0.5|$.

Looking to the individual positioning of the PCA (Figure 4a), the two-organic diet treatments had close and positive values on the first component of the PCA while the conventional diet is located far away from these two groups, displaying negative values on the first component (Figure 4a). Even if the PC scores of the two organic diets are close on the first component, the organic diet 1 showed higher PC score values than the organic diet 2, meaning higher welfare level regarding to the first component (Tukey, $p < 0.05$; Figure 4b). On the second component, the conventional diet is located between the two organic diets (Figure 4a) with higher values for the organic diet 2 and lower values for organic diet 1. Statistical analysis of the PC scores of the component 2 revealed significant differences between all diet treatments with lower values for the organic diet 1, meaning a higher welfare level for this group (Figure 4c).

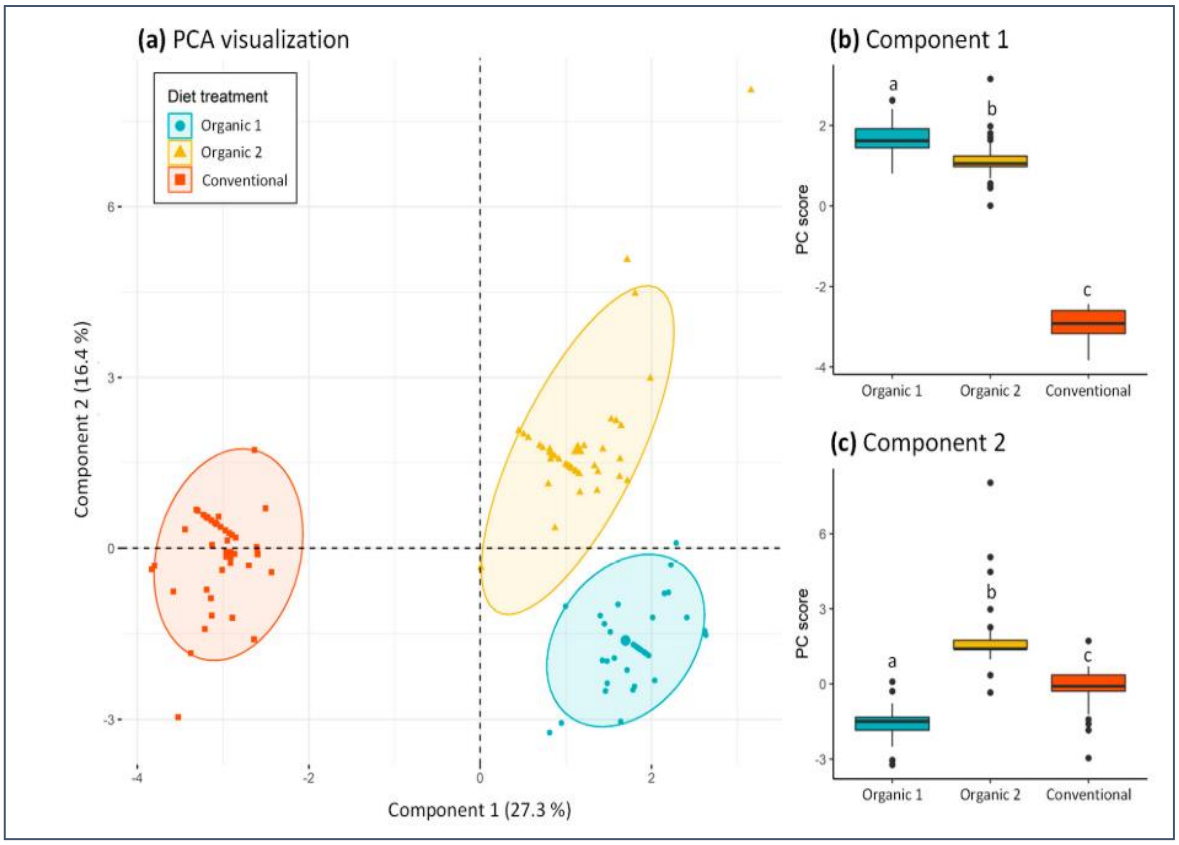

Fig. 4. Principal component analysis. (a) Visualization of the individual positioning of the individual on the PCA as a function of diets. Confidence ellipses drawn around the levels of the categorical variable diet treatment with a confidence level of 0.95; (b) Individual PC score for the first component as a function of diet treatment; (c) Individual PC score for the first component as a function of diet treatment. Both for (b,c), different letters indicate groups significantly different (Tukey HSD, $p < 0.05$).

Moreover, according to the NSFDSS analysis, the best score relative to the welfare status of fish was achieved by the organic diet richer in proteins (Organic diet 1, score = 1.000; Table 6), confirming the results of the PCA analysis, followed by the conventional diet (0.953). In Table 7 are reported the scores of the three diets respect to each of the 7 parameters considered. The results showed that respect to EMG, cortisol and lysozyme the conventional diet is equivalent to Organic 1, both in the highest position, while for RR and HIS, the conventional diet is in highest position. On the other hand, respect to SGR and glucose, the Organic 1 and 2 are equivalently in highest position. In the Hypothesis 1, the NSFDSS showed that the parameter characterized by highest score is EMG, followed by RR, while glucose and lysozyme have null score (Table 8). The same ranking among the diets was obtained in the two hypotheses (hypothesis 1: ranked criteria; hypothesis 2: equal importance of criteria), in which the organic diet 1 and conventional diets showed the best and second score respectively. The organic diet 2 showed a lower score in both cases (Table 6).

Diet	Hypothesis 1	Hypothesis 2
Organic 1	1.000	0.928
Conventional	0.953	0.901
Organic 2	0.428	0.520

Table 5 Diagnostic frame of the scores obtained by each diet treatment in the two study hypotheses by means of the NSFDSS. The case with the ranking of criteria is named Hypothesis 1 and the "equal importance of criteria" hypothesis is called Hypothesis 2.

Diet	EMG	RR	Cortisol	SGR	HSI	Glucose	Lysozyme
Conventional	1	1	1	0.333	1	0.333	1
Organic 1	1	0.333	1	1	0.538	1	1
Organic 2	0.333	0.333	0.333	1	0.25	1	0.333

Table 6. Diagnostic frame of the scores obtained for the three diets respect to each criterion (parameter) by means of the NSFDSS. These scores are the same for Hypothesis 1 and Hypothesis 2.

Parameters (Criteria)	Score
EMG	1
RR	0.333
Cortisol	0.176
SGR	0.111
HSI	0.053
Glucose	0.00
Lysozyme	0.00

Table 7. Diagnostic frame of the scores obtained for the criteria (parameters) by means of the NSFDSS in the case of Hypothesis 1.

5.4. Discussion

Aims of the study were first to define the physiological effects of the diets in sea bass, looking to different parameters monitored individually. Then, a multi parametric approaches have been conducted to have a whole organism view using PCA and MCDA analyses. During the experiment, sixteen different parameters commonly used to evaluate fish welfare condition were considered: haematological (Hct, Hb, RBCC), plasmatic (glucose, lactate, cortisol, lysozyme), muscle activity (EMG, recovery tests), specific biomarkers (EROD, GST), and growth performances (gained weight, SGR, HIS, FCR, PER). All these parameters could bring an overview for evaluating the welfare status of fish in response to different diets (conventional: DAN-EX 1754TM and two organic diets 1 and 2: DAN-EC 1650TM and DAN-EC 2640TM respectively). Feeding composition is essential to regulate the specific needs of the species, and thus are important for assuring fish welfare under aquaculture conditions. For carnivorous fish species, such as European sea bass, the use of

fish meal (or fish oil) is important for protein acquisition, but have to be controlled, due both to the decrease of wild fish stock and levels of pollutants in food [76]. In the present study, we followed the EROD and GST activities, both of which are specific biomarkers of the exposure to dioxins and dioxin-like compounds, and polycyclic aromatic hydrocarbons (PAH) [51,77], including in European sea bass [78]. The values of the EROD and GST activities for fish fed with commercial diets seemed comparable to both the values measured in the present study [78–80]. Therefore, a possible environmental contamination from dioxins and dioxin-like compounds can be excluded. However, the activity of EROD was inhibited following the exposure to the conventional diet and the organic diet 2, while it did not for fish fed with the organic diet 1. This inhibition may be due to exposure to other chemicals which are present in these diets [51,81]. However, at the end of the experiment, the levels of both EROD and GST are similar between the three diet treatments, suggesting that the changes in physiological parameters discussed below are probably not related to potential pollutant exposure through diet. Cortisol is the major stress hormone in teleost fishes [31], activating of physiological and behavioural responses modulating growth and reproduction [33,82]. In our study, the higher initial level of cortisol observed may be likely due to a stress condition attributable to the transport and the adaptation to new rearing conditions, since European sea bass is known to be high stress responder [83,84]. At the end of the trial, the cortisol level was significantly lower both in conventional diet and organic diet 1 than in the organic diet 2. However, since the high variability of cortisol values and great number of different stimuli that could influence their concentration, this hormone should not be used alone as operational welfare indicator [34,43]. By considering the secondary stress responses, the plasmatic glucose showed a sharp decrease from the beginning to the end of the experimental period in fish fed with the conventional diet similarly to cortisol level pattern; but not for the organic diets. Moreover, the higher initial level of lactate also seems to be related to the high cortisol level at the beginning of the experiment. Then, the lactate levels did not vary significantly in response to the different diets. Moreover, during the first two months of our experiment, the haematological parameters shown a global increase in all diet groups. Among the haematological parameters, RBCC and Hb seemed to be more stable. However, data resulting from the present work did not show any significant relationship between the haematological parameters and the diet composition, as reported by Mourente *et al.* [85] for diets differing in their lipid origin (animal vs. vegetable). Then, lysozyme is used as a general indicator of fish nonspecific immunity [86,87], which can also be modulated by cortisol level [34,41,88].

In this study, fish fed with the diets containing higher protein concentrations (conventional and organic diet 1) showed higher plasmatic lysozyme levels, as previously reported in European sea bass [89]. Together, these results confirm that the relative high protein content in these diets is essential for fishes to create such a level of nonspecific immunity and cope with potential diseases [90,91]. In contrast to physiological blood indicators, the use of swimming performances to assess fish welfare in relation to the diet is still at its embryonic stage, as demonstrated by the limited amount of studies [60,92–94]. The Ucrit test is widely used as indicator of swimming performances of fish [34]. However, a single Ucrit test is not really as sensitive to metabolic disturbances as the determination of the recovery ratio (RR), using two consecutive Ucrit tests [34,64]. Exhaustive exercise depletes glycogen storages, as a response to the physiological increase of cortisol levels to cope with the increased energy requested. Thus, healthy fish should rapidly restore metabolic energy after a test of critical swimming speed and should be able to perform a similar Ucrit, showing a RR value close or equal to one. On the contrary, physiological disturbances, such as stress, generate high level of cortisol, that inhibits muscle glycogen synthesis [95], impairs the recovery and the RR value is significantly lower than one [41]. Interestingly, in this study, the RR was lower for fish fed by the conventional diet than for organic diets, suggesting a lower capacity to cope with stress and ensure good growth performances [82,96]. EMGs record bioelectrical voltage changes, which are proportional to the degree and duration of muscle tension, as well as to the energetic demand of the individual for swimming and living activities [97]. Telemetry technology has produced specific EMG tags, which are useful for evaluating fish activity and energy in response to environmental conditions during free swimming in real time [50,61]. The quantitative monitoring of muscular activity achieved through EMG has already been successfully assessed in relation to different aquaculture conditions, such as starvation and/or feeding periods, transportation activities, rearing densities [34,50,98]. Thus, a change of the fish muscular activity (EMG) shows a sensible response of the organism to the environmental factors. In this view, the muscle activity, expressed as EMG, has been demonstrated as a sensitive index of fish welfare [34,50,99]. In our work, the initial mean daily EMG values were similar for the three diets (roughly around 30%), indicating a similar physiological condition at the beginning of the experiment. During the experimental period, fish fed with organic diet 1 showed a significant negative trend, while the other two diet groups showed positive trends both during feeding and starvation sampling period. Considering that a lower proportion of saturated fatty acid and a higher amount of linoleic acid in the diet can affect the cardiac function [100,101], these results indicated how the

three diets could have a significant influence on the muscle activity (EMG). Moreover, the decrease of EMG over time was representative of lower use of white muscle [59]. This physiological situation minimizes the use of anaerobic reserves, placing fish in favorable conditions to cope with stress. In contrast, the other two experimental groups (conventional and organic diet 2) used a larger proportion of anaerobic metabolism (up to 50%), that stands for a lower storage of metabolic energy that should be useful to cope with stressful situations. Then, the measurement of HSI, which is used as biomarker of the hepatic functionality, was found comparable with the literature for sea bass [102,103]. We observed higher values for fish fed with the two organic diets than those individuals fed with the conventional one. In the case of the organic diet 2, it was probably due to the higher lipid concentration [85,103,104]. In addition, the increment of the HSI for fish fed with the organic diet 1 could be attributed to the different protein sources (animal vs. vegetable) or to the different antioxidant compounds [105]. However, this hypothesis should be demonstrated with specific analysis of the effect of rosemary essential oil on the hepatic functionality. Concerning growth parameters, it is difficult to compare with the literature because in general a smaller size is used to amplify the growth differences over time. Nevertheless, after one year of monitoring, Kavadias & Dessypris [106] reported a comparable SGR value for fish ranging from 300 to 400 g. In the present study, both the SGR and the weight gained at the end of the experiment were higher in the organic diets than in the conventional one. These results are consistent with the results and expectations looking to the EMG and the RR data. However, the link between the swimming performances, growth and blood parameters are not completely consistent when studied individually. Indeed, when there is a high number of variables (i.e., 16 in this study), it could be difficult to rise a clear conclusion on the welfare status of fish because some variables are going in one sense and others are going in the opposite sense. Therefore, in this study, we performed a multi-parametric analysis using both a principal component approach (PCA), as already shown to be efficient in this context [37,107], and a multi criteria decision analysis (MCDA), which appears to be an innovative approach in the context of animal welfare [108]. The PCA allowed us to discriminate the effects of the three different diets upon all the sixteen parameters used. Overall, we found that low EMG values were correlated with better growth (Component 1 of the PCA) and low cortisol values were correlated with higher lysozyme values (Component 2 of the PCA). These correlations between the variables drove the different components of the PCA, allowing to discriminate the better welfare status for fish depending on their diet treatment (i.e., low EMG values and better growth for component 1, low cortisol

concentration and high lysozyme for component 2). Fish fed by the two organic diets were clearly different from fish fed by the conventional one, overall displaying lower EMG values and higher growth performances (PCA component 1), suggesting better welfare status. Moreover, the organic diet 1 differed from organic diet 2, displaying higher plasmatic lysozyme concentration and lower cortisol concentration (PCA component 2), suggesting higher welfare status. Using the PCA method, fish fed with organic diet 1 showed better welfare status on the two components of the PCA, clearly suggesting the best welfare status for this diet treatment. However, fish fed with the conventional diet showed controversial results, such as good plasmatic parameters (glucose, lactate, cortisol) and worse physiological parameters (EMG and recovery), and SGR. In the MCDA, the effects of the diets were analysed not only in terms of differences between the experimental groups as in the case of PCA, but above all in terms of effects on welfare. Thus, among the 16 parameters monitored in the study not all captured a difference (significant difference among the diets at the end of the experiment) in the experimental context. For this reason, we chose for the multicriteria decision analysis only the parameters which at the end of the experiment showed significant differences between the experimental groups (diets), which were related to welfare, and which therefore contributed more than the others to discriminate the effects of the three diets on sea bass welfare. Therefore, for example in the case of lysozyme if the fish fed with the organic diet 1, at the end of the experiment, showed greater lysozyme level in comparison with the other diets, this was evaluated positively (see Section 2.7, Tables 6 and 7) with respect to welfare, as well as a higher cortisol in one of the experimental groups was evaluated negatively with respect to welfare. In this perspective, the MCDA analysis allowed us a more effective evaluation of the three diets in term of "welfare effect". Thus, the seven parameters that were shown to be more sensitive to the different diet compositions (cortisol, glucose, lysozyme, SGR, HSI, RR, and EMG) were integrated in a diagnostic frame to perform a MCDA by means of a non-structural fuzzy decision support system (NSFDSS). Higher welfare score was found in fish fed by the organic diet 1, confirming the results highlighted by the PCA. The low cortisol level, the high lysozyme concentration, the swimming activity (mostly sustained by the aerobic metabolism), the greater metabolic recovery capacity, and the better growth performances (SGR) are in agreement with a better physiological condition. On the contrary, fish fed with the organic diet 2 showed the lowest welfare score, with the prevalence of negative effects on welfare, suggesting that the conventional diet is more appropriate than this one for European sea bass aquaculture. Fish welfare evaluation remains a complex issue to be evaluated using a single parameter and/or

group of parameters. Indeed, haematological parameters in fish lack a threshold reference levels useful to univocally diagnose impaired welfare [109]. In disturbance conditions (e.g., not appropriate feed), fish are faced with higher living costs [30], reducing the reserve energy budget intended to cope with stressful situations, and this affects the fish well-being. The complementary use of functional-based parameters (e.g., haematological and biochemical profile) and feeling-based parameters (e.g., behavioural profile) could give a more comprehensive view, validating the diagnosis of fish welfare induced by culture practices [34,110]. Overall, results of this work indicate that the organic diet, if it is well balanced, could be a good trade-off in term of growth performance and physiological fish welfare. In term of sustainability, aquaculture has a lower carbon footprint in comparison to other protein production systems (e.g., cattle, pork, poultry) [111]. In particular, organic aquaculture, due the higher component of vegetable origin, has the potential to effectively address the sustainability challenges that humans are facing [112].

5.5. Conclusions

In conclusion, both in terms of growth performances and physiological welfare status, this study supports the transition towards organic aquaculture for European sea bass, by nevertheless choosing the diet adapted to the need of the species. This transition towards organic agriculture can also benefit humans by providing higher quality products, and thus enhancing health [113]. The multiparametric approach has enabled to outline a comprehensive picture of the physiological state of sea bass fed with three different diets. Even if not all of the sixteen parameters gave globally consistent response, the use of all the parameters gave a strong decision criterion. The parameters that gave a whole organism response, such as EMG, recovery ratio, and growth parameters proved to be sensitive to assess welfare condition [34,110]. Other physiological indicators, such as cortisol concentration, glucose, or lysozyme, are important for welfare assessment, even if these parameters are highly variable (e.g., [43] for cortisol). Finally, the PCA and MCDA methods appeared to be powerful tools to assess welfare in aquaculture using a multi-parametric approach, as recommended by Huntingford *et al.* [28].

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Chapter 6

fishes

MDPI

Article

Effects of Social Hierarchy Establishment on Stress Response and Cell Phagocytosis in Gilt-Head Sea Bream (*Sparus aurata*)

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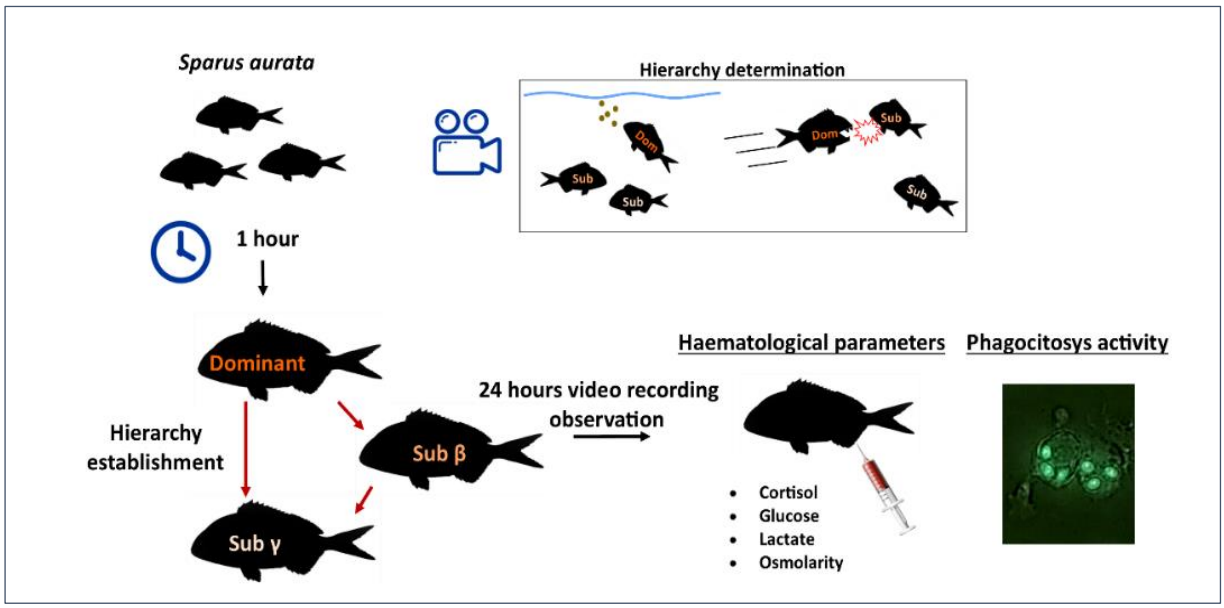
Abstract: Social stress can affect the ability of fish to respond to various stressors, such as pathogens or environmental variations. In this paper, the effects of social stress on gilt-head bream (*Sparus aurata*) were investigated. To study the effects of physiological stress, we evaluated biochemical and cellular parameters, such as cortisol, glucose, lactate, osmolarity, and phagocytosis, 24 h after the establishment of social hierarchy in a group of three fish. Social hierarchy was determined and characterized by behavioral observation (aggressive acts and feeding order) of the specimens (dominant: "α"; subordinate: "β" and "γ"). After the establishment of social hierarchy, we observed that, overall, levels of plasma cortisol and other biochemical plasmatic stress markers (glucose and lactate) were higher in subordinate individuals than in dominant individuals. In addition, the modulation of phagocytic activity of the peritoneal exudate cells (PECs) demonstrated that social stress appeared to affect immune response. Finally, principal component analysis clearly separated the subordinate fish groups from the dominant groups, based on stress markers and the phagocytic activity of peritoneal exudate cells. This study contributes to current knowledge on gilt-head sea bream, helping to understand the link between social stress, behavior, and physiology of this species, relevant in the aquaculture sector, where fish are subjected to several kinds of stress.

Keywords: gilt-head bream; social stress; cortisol; phagocytosis; immunity

Graphical abstract



6.1. Introduction

Currently, there is a growing interest in fish welfare, both in fisheries and aquaculture [1]. In the aquaculture framework, the environment available to reared fish is very different from the environment in which their wild counterparts live [2]. Good food quality is readily available, as fish are protected from natural predators and disease and do not have to compete for mates. However, the physical environment is much simpler, as fish are disturbed by rearing activity and often restricted at high densities to limited and crowded spaces, with the consequent risk of disease spread and increased social interaction, including with aggressive fish. Because good fish welfare is correlated with good overall production, the welfare of farmed fish is important for the market, as well as being a matter of increasing public concern [3].

One of the important aspects to consider when evaluating aquaculture is the behavioral profile and interaction between animals [4]. Indeed, the social environment of a species can be a considerable source of stress, impairing the welfare status of fish, and social relationships can impact both mental and physical health [5]. Dominant–subordinate relationships can affect physiological status and animal responsiveness [6]. Social stress can be considered the result of physical contact between animals (high-density and agonistic interaction) and psychological components, such as hierarchical instability and submission [7]. Social interactions reflect agonistic competition for access to limited resources [8]. Responses to social stress depend on the life history of the species, sex, and age [9]. Social

interactions between conspecifics are, for some fish species, dynamic processes, where subordinates frequently try to become dominants and dominants try to maintain their status by using direct attack or displaying cues to others [10]. Dominant and subordinate can be distinguished by characteristic behavioral differences in activity, feeding, and aggression, with subordinates being less active and aggressive and consuming less food. In addition to behavioral differences, dominant and subordinate fish can also differ from each other in their stress response [11]. In teleost fish, stress response is divided into different levels: primary, secondary, and tertiary. The primary response is coordinated by the neuroendocrine axes, which, in teleost fish, comprise the hypothalamic–pituitary–interrenal (HPI) axis and sympatho-chromaffin tissues and leads to increased levels of adrenocorticotropic hormone, cortisol, and catecholamine in the blood [12]. The increase in plasma cortisol levels under stress conditions typically causes an increase in plasma glucose and lactate levels, while the increase in plasma glucose is initially generated by catecholamine-mediated glycogenolysis and, in later stages, cortisol-mediated gluconeogenesis [13]. The increase in lactate concentration, as muscle lactate, is formed during anaerobiosis and is released into the plasma [13]. In the literature, it is reported that when fish cope with stress, an increase in osmolarity levels is observed in the plasma [14]. Indeed, secondary stress responses include changes in the concentration of circulating ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, and Cl^-). Prolonged stress exposition could also affect the tertiary response, including behavior, immune response, growth, and fitness [6]. Unnecessary stress can be considered deleterious, and, indeed, it can be seen from an adaptive point of view, which temporarily allows fish to cope with environmental changes, safeguarding single specimens and populations [15]. Cortisol, the major stress hormone in fish, mediates several physiological processes, such as glucose metabolism, ionic and osmotic regulation, and immune response. It plays a pivotal role in stress response through its action on both aerobic and anaerobic metabolism, osmoregulation, carbohydrate metabolism, immunity, and appetite [13,16]. Corticosteroid hormones play a central role in behavioral and neuroendocrine control in vertebrate species [17]. On the contrary, chronic stress, which is associated with elevated plasma cortisol levels, can result in a compromised physiological state. High levels of cortisol are considered a causal factor in many of the deleterious effects of stress in reared fish, such as reduced immune competence, reduced growth, flesh quality, or impaired reproduction [18,19]. For this reason, the mechanisms involved in stress coping strategies in fish are receiving significantly more attention. Most of the responses in organisms are species specific; therefore, it is necessary to investigate responses in different reared species.

Physiological differences are widely demonstrated to be correlated, in part, to different social positions in fish, and the consequent variation in response to changes in social status is well documented [11,20]. In aquaculture, inadequate rearing conditions may cause an increase in blood plasma cortisol levels, which can affect both the behavior and physiology of farmed species. During social interaction, the HPI axis is activated, resulting in variation in blood cortisol levels [7]. Social status (i.e., dominant or subordinate) is inextricably linked to hormonal regulation, such as testosterone and cortisol [21]. Different studies correlate social status and/or aggressive behavior with hormones, such as testosterone and cortisol, in gilt-head sea bream (*Sparus aurata*) [22–24]. In social animals, interactions between conspecifics are often initiated to obtain a higher position in the hierarchical structure. Physiological consequences of social interaction have been observed in the subordinate fish of gregarious species. The defeat of socially subordinate fish is perceived as a strong stressor [11,25], and it could also affect the secondary and tertiary stress responses. For instance, it has been demonstrated that physiological changes in subordinate fish might affect appetite and reduce aggression [26]. Moreover, contact between conspecific fish does not promote habituation [27], and prolongated stress exposition, as mentioned below, could ultimately trigger the tertiary stress response, with severe consequences on animals and aquaculture services. Indeed, aggressivity has been linked with several issues in aquaculture, such as decreased feed intake, growth dispersion, chronic stress, and disease vulnerability [28]. Therefore, understanding the aggressive behavior of farmed fish, as well social interaction, is of great importance for the aquaculture practice, in order to both improve animal welfare and productivity [29].

Sea bream is a protandric hermaphrodite species, born as males and becoming female at about 3 years old, living either solitarily or in small aggregations. Mainly carnivorous, sea bream is one of the most important fish of Mediterranean marine aquaculture [30]. The important development and wide spreading of seabream aquaculture is due to its features; the robustness, plasticity, diet adaptability and illness resistance renders it able to adapt to a wide range of environmental conditions. These characteristics, in addition to significant technological advances in reproduction, using artificial conditions, permitted the expansion of this industry [31]. In that species, the overall dominant fish carry out more aggressive acts and bite at food particles more often than the subordinate, resulting in higher relative specific growth rate. Goldan, and Popper [32] concluded that direct competition for food is probably the major social mechanism regulating growth in small groups of juveniles of this species, when food is limited and defendable. Recently, Arechavala-Lopez et al [33] showed that

effects of social hierarchy could be modulated by aquaculture management in sea bream. For instance, self-feeding reinforces the social hierarchy, which might lead to a higher competitiveness for resources among fishes, increasing the social hierarchy and, therefore, stress, when compared to hand feeding. Therefore, understanding the link between aggressive and feeding behaviours with the social hierarchy and stress physiology of sea bream is of great interest for the aquaculture sector, in order to find the best farming practices for the species [23].

In Cammarata *et al.* [34], we have previously demonstrated, using the paired fish model, that the established hierarchy between two specimens of gilt-head bream, determines a change of the principal biochemical (cortisol, glucose and osmolarity) and cellular (phagocytic activity) parameters in subordinate individuals, in a short period of time after pairing (at 24 h). The biochemical and cellular stress markers considered (e.g., cortisol, glucose, lactate) were higher in subordinate individuals than in dominant [34]. This has also been reported for cortisol in groups of two, five or ten sea breams by Montero *et al.* [35]. As reported by Vazzana *et al.*, stressors affect the immuno-cells activity, such as the respiratory burst and cytotoxic activity of leucocytes in the head kidney and peritoneal cavity of fish [36]. Moreover, *in vivo* and *in vitro* experiments showed that increased cortisol levels act on phagocytic activity via the cytosolic receptor DlGR1 in sea bass [37]. Peritoneal exudates cells (PECs) play important roles in both non-specific and specific immune mechanisms [38,39], and the peritoneal exudate has been used as a source of leucocytes for studies of innate immunity, including phagocytosis and cytotoxicity [40,41]. In this study, introducing one extra specimen in the experimental design, with respect to the previous work, we increased the complexity of the interactions among the individuals of the gilt-head bream. The social ranks were determined using various parameters, such as aggression and feeding priority, with a novelty of a clear determination of sequential hierarchy, with dominant α, subordinate β and γ. After the establishment of the hierarchy, we examined the plasma levels of stress parameters (cortisol, glucose, lactate, osmolarity) and the phagocytic activity of the PECs to compare physiological status between dominant α and subordinates β and γ. Well established physiological and immunity bioindicators (plasma concentrations of cortisol, glucose, lactate, osmolality and major ions, and phagocytosis) were monitored in this study [42].

Overall, throughout the behavioural observation of sea bream, and analysis of relevant plasmatic biochemical indicators, this study aims to contribute to the improvement of knowledges on an important marine farmed species—the gilt-head sea-bream. This study

would help to understand the link between social stress, behaviour and physiology of this species in a confined environment, such as in aquaculture environments, where fish are subjected to several kinds of stress.

6.2. Materials and Methods

6.2.1. Animals

Twenty-seven male specimens (125 ± 25 g body weight) of the seawater proterandrous teleost gilt-head seabream were obtained from a commercial fish farm (Ittica Trappeto, Sicily, Italy). Fish were selected to have the same weight, age and sexual maturity in order to reduce variability and obtain comparable results. The experimental design involved the observation of specimens of gilt-head bream in glass tanks (200 L) in which the fish were placed simultaneously in groups of three. The experiment was replicated nine times, each time three different specimens, resulting in the monitoring of 27 fish in total. The tanks were obscured on three sides to eliminate re-flection or external disturbance on fish. Experiments were carried out after an acclimatising period of one week [34]. The animals were subdivided and placed in tanks in groups of three. The sampling and analysis were done for each group at 24 h after hierarchical establishment. The hierarchy had been clearly distinguished after one hour for each experiment. New fish were utilised for the replicate experiments in order to avoid influences in new hierarchical scheme. No fish died or showed signs of disease during the experiments.

6.2.2. Experimental Conditions and Behaviour Observation

The aquaria seawater was monitored daily and maintained at an average temperature of 18 ± 1 °C, at a salinity of 38 ± 1%, oxygen 6 ± 0.5 mg L−1 by Dissolved Oxy-gen Meter (Thermo Fisher Scientific, Inc., Waltham, Massachusetts) and total ammonia-nitrogen concentration of <0.2 mg L−1 by commercial kit (Tetra, Spectrum Brands, Blacksburg, VA, USA) under a photoperiod of 12 h dark and 12 h light. The fish were fed with commercial pellet diet (Skretting USA, Tooele, Utah) once a day ad libitum. To be able to identify the three individuals in an objective manner the fish were marked as follows: a fish was clipped at the dorsal fin, another was clipped on the caudal fin, and one was not clipped [43]. Fin clipping is a technique extensively used to recognize individuals within a group; it is commonly used in breeding work and animal tracking without affecting behaviour [44–46]. The behavioural changes were recorded to assign a hierarchical position to each individual and observed to detect changes in the social status until the social positions were established. Every group was observed for 24 h and recorded by a digital camera for the data acquisition

(Panasonic sdr-h85 hybrid; Panasonic corporation, Kadoma, Japan) during light period. The choice of 24-h monitoring period following introduction of fish into experiment tank was chosen based on previous study on sea bream showing that the hierarchy establishment occurs after 24 h of cohabitation with a duration of at least 6 months [34]. The method used for behavioural monitoring was continuous sampling. For each specimen, 15 videos of 15-min each were randomly sampled during daylight, resulting in the monitoring of 225 min for each fish. During the monitoring period, all behavioural acts were recorded using the Behavioral Observation Research Interactive Software v. 7.12.2 (BORIS) [47–49]. The individual behaviour was examined by the continuous check of the different behaviour categories, and individuals from each group were distinguished as dominant α, subordinate β and subordinate γ. High social status has been correlated with increased aggressiveness and preferential access to food [48]. To define this social distinction, the number of aggressive acts (A+) were observed and defined as a bite or a rapid approach without biting that resulted in the displacement of the subordinate [50] classifiable as: charge, nip, chase, butting, mouth fighting and circling [51,52]. Feeding order (FO) of each group was determined according to the methods of McCarthy *et al.* [48] counting the number of accessions to feeding area for all the specimens. Data are expressed as percentage of total interactions among fish.

6.2.3. Blood Sampling and Peritoneal Cell Preparation

After 24 h of cohabitation, the fish were anaesthetised with 0.05% w/v MS222 (3-aminobenzoicacid ethyl ester, Merck & Co., Darmstadt, Germany) in seawater for three minutes. After the anaesthetic exposure, the blood samples were collected via caudal venepuncture into heparin-coated syringes (2500 IU mL−1 heparin sodium salt, Merck & Co., Darmstadt, Germany) and centrifuged (10,000 g for 2 min). Plasma was extracted and stored at −80 °C for later analysis of cortisol, glucose, lactate and osmolarity levels. The peritoneal exudates cells (PECs) were obtained as follows: the fish were anesthetized and after disinfection of the body ventral surface with 70% ethyl alcohol, the peritoneal cavity was injected with 15 mL of isotonic (370 mOsm kg−1) medium (Leibovitz L15 medium containing 2% foetal calf serum, 100 units penicillin mL−1, 100 units streptomycin mL−1 and 10 units heparin mL−1, Merck & Co., Darmstadt, Germany). During the procedure fish gills were wetted by continued water flow by a pipe inserted in mouth during the ten minutes. Ventral surface was disinfected again with 70% ethyl alcohol. After massaging the ventral surface for 10 min, the medium containing the PECs was collected by withdrawing with a syringe needle from the peritoneal cavity. The PECs were isolated by centrifugation at 400

g for 10 min at 4 °C. The dead cells were determined by light microscopy after addition of 0.01% trypan blue to the medium.

6.2.4. Plasmatic Biochemical Parameters

The concentrations of total cortisol were measured in the plasma sample using a commercially available kit (Intermedical Diagnostics srl, Villaricca, Italy) according to the manufacturer's instructions and confirmed by radioimmunoassay (RIA)[53]. The glucose and lactate plasma levels were determined using the Accutrend Plus Kit (Roche Diagnostics Rotkreuz, Switzerland) according to the manufacturer's instructions. The osmolarity of the plasma samples was measured using a freezing-point depression osmometer type 4b (Hermann Roebling MESSTECHNIK, Berlin, Germany).

6.2.5. Phagocytosis Assay

The method established in Cammarata *et al.* [34] using *Saccharomyces cerevisiae* (Merck & Co., Darmstadt, Germany) as a target for evaluating the per-centage of phagocytosis, was performed with slight modifications. Briefly, yeast was prepared in distilled water as a 0.25% (w/v) solution (approximately 1 × 107 yeast mL−1), autoclaved for 15 min, washed 2 times at 2000 g at 4 °C for 5 min and incubated for 1 h at 20 °C with eosin Y (4-Bromo-fluorescein) to a final concentration of 0.05%. The yeast was washed four times in distilled water and resuspended to a final concentration of 0.0125% w/v in phosphate buffered saline (PBS: 103.6 mM NaCl, 1.46 mM KH_2PO_4, 0.8 mM Na_2HPO_4, 2.6 mM KCl, 0.9 mM $CaCl_2$ and 0.49 mM MgCl2, pH 7.4) and stored at −20 °C for a maximum of 2 weeks. The yeast suspension was added (v/v) to 100 µL of leucocyte suspension (2.5×10^6) and placed in a 1 mL plastic tube. The mixture was incubated for 30 min at 20 °C with gentle stirring. To observe the phago-cytosis, 50 µL of a quenching solution (QS) (2 mg mL^{-1} trypan blue and 2 mg ml^{-1} crystal violet in 0.02 M citrate buffer, pH 4.4 containing 33 mg mL^{-1} NaCl) was added to the reaction mixture. The slides were examined under a microscope equipped with a Normarski interferential contrast device and fluorescence apparatus (450–490 nm filter) (Diaplan, Leica, Wetzlar, Germany). A total of 200 cells, 35 cells on each slide, were counted. The results were expressed as a percentage of cells containing yeasts.

6.2.6. Statistical Analyses

Statistical analyses were performed using the R software version 4.0.4 [54] and carried out at the 95% level of significance. Generalized linear mixed model (GLMM) was carried out using the library lme4 [55]. The principal component analysis (PCA) was performed

using packages ade4 [56] and FactoMineR library [57]. The data are expressed as mean ± standard deviation unless otherwise mentioned. All the recorded plasmatic biochemical markers were analysed as function of social rank (fixed factor) and experimental tank (i.e., replicate; random factor) one by one using GLMM. When social status appeared significant, Tukey HSD post-hoc test was carried out to deter-mine differences between groups (i.e., dominant α, subordinate β and subordinate γ). GLMM was fitted using gaussian distribution family and log link for all variables. Log cortisol data were analysed instead of raw cortisol data in GLM to better fit model assumptions. Visual inspection of the residuals revealed no violation of the statistical assumptions by the model. In addition, to examine the interrelation between the two sets of variables (stress plasmatic biochemical markers and phagocytic activity of the PEC) and social status, principal component analysis (PCA) was performed for multiple groups of principal component analysis including five variables (cortisol, glucose, lactate levels, osmolarity and phagocytosis). The relevant dimensions of the PCA were selected using the acceleration factor method [58]. Individual PCA scores of fish were downloaded for each relevant component of the PCA, and then analysed as function of fish social position using Kruskal–Wallis test followed by Tukey HSD when significant.

6.3. Results

6.3.1. Determination of Social Hierarchy

The social hierarchy was established after about an hour of cohabitation and interaction and remained unchanged throughout the 24-h period of observation. During the experimentation, we did not identify any fish behavioural differences or particular hierarchical positions attributable to the different clipped fins (Table 1). This is consistent with previous works, demonstrating that fin clipping does not induce negative effects for fish (e.g., [43,46,59]).

Type of Mark	Dom α	Sub β	Sub
Dorsal fin	4	2	3
Caudal fin	3	3	3
No cut	2	4	3
Total	9	9	9

Dom = Dominant; Sub = subordinate.

Table 1. Hierarchical classification of the fish according to the mark position (dorsal fin, caudal fin, or no mark).

Social status and hierarchical position have been correlated with increased aggressiveness and preferential access to the food (Table 2). As shown in Table 2, the percentage of

aggressive acts for each hour (A+) and preferential access to the food (FO) allowed us to distinguish the fish, identifying them as dominant α or β and γ subordinates in each group, attributing a social status in the hierarchy to each fish, with the order of: dominant α < subordinate β < subordinate γ ($p < 0.001$). The hierarchy was maintained for the total observation time.

	Aggressive Acts (A+) Mean (%) ± SD	**Preferential Food Accession (FO) Mean (%) ± SD**
Dom (α) > Sub (β)	98 ± 3%	100 ± 0%
Dom (α) > Sub (γ)	85.7 ± 4%	100 ± 0%
Sub (β) > Sub (γ)	81 ± 4%	98 ± 1%

Dom = Dominant; Sub = subordinate.

Table 2. Mean percentage (%; ±SD) of aggressive acts (A+) and preferential access at the food (FO) during experimental period in sea bream (Sparus aurata). Nine fish were monitored for each social status (dominant (α), subordinate (β and γ).

6.3.2. Stress Biochemical Profile Related to Social Position

After 24 h of cohabitation-interaction among the three sea breams, the cortisol levels in plasma appeared different, depending on the social rank acquired by fish ($p < 0.001$ for subordinate β and subordinate γ, respectively, vs. dominants; Figure 1b); the highest levels were measured in subordinate individuals, following the order: dominant α < subordinate β < subordinate γ ($p < 0.001$ for all). In a similar way, the lactate level in plasma is affected by the social position ($p < 0.001$ for subordinate β and sub-ordinate γ, respectively, vs. dominants; Figure 1d); the highest levels were measured in subordinate individuals, following the order: dominant α < subordinate β < subordinate γ ($p < 0.001$ for all). The plasmatic glucose level is also affected by the social position gained, 24 h after introduction in the novel tank ($p < 0.001$ for subordinate β and subordinate γ, respectively, vs. dominants; Figure 1c). This time, the greater glucose plasmatic level is still observed in subordinate fish γ, but with a different order: subordinate β < dominant α < subordinate γ ($p < 0.001$ for all; Figure 1c). The pattern of osmolarity, depending on social position, has been found similar to that of plasmatic glucose level ($p < 0.001$ for subordinate β and subordinate γ, respectively, vs. dominants; Figure 1c), with the following order: subordinate β < dominant α < subordinate γ ($p < 0.001$ for all; Figure 1e).

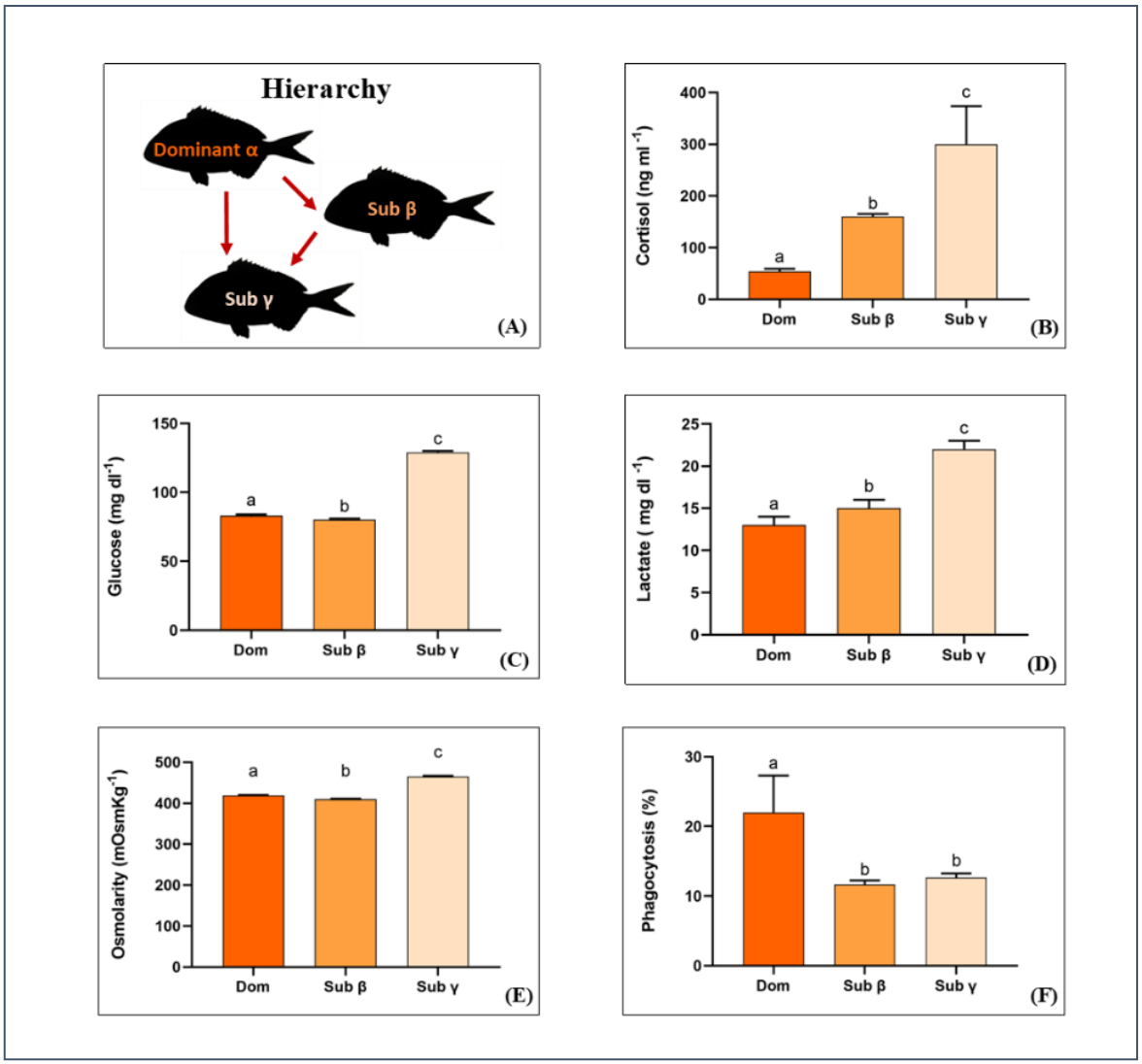


Figure 1. Mean (±SD) of plasmatic parameters of dominant (α) (orange histogram) (n = 9), subordinate (β) (pale orange histogram) (n = 9), and subordinate (γ) (pinkish histogram) (n = 9) sea bream (Sparus aurata). The panel (A) presents the social relationship between dominant (α) and subordinates (β and γ). Arrow indicates social domination from an individual toward the other. (B) Cortisol concentration (ng mL−1), (C) glucose concentration (mg dL−1), (D) lactate concentration (mg dL−1), (E) osmolarity level (mOsmKg−1) and (F) Phagocytosis (%). Different lowercase letters over the bars indicate significant statistical difference between groups, same lowercase letters over the bars indicate absence of statistical differences (GLMM followed by Tukey HSD post-hoc test, $p < 0.05$).

6.3.3. Phagocytic Activity Related to Social Position

The percentage value of the PECs' phagocytic activity is also affected by the social position of sea bream ($p < 0.001$ for subordinate β and subordinate γ, respectively, vs. dominants; Figure 1f). Post-hoc test indicates that, after 24 h of cohabitation-interaction between specimens, the phagocytic activity of the dominant is higher compared to both the subordinate β and γ ($p < 0.001$ for both), while it remains significantly unchanged between the subordinates β and γ fish ($p = 0.85$; Figure 1f).

6.3.4. Principal Component Analysis of Plasmatic Biochemical and Cellular Parameters

The PCA analysis was performed on five monitored physiological variables (Table 3). According to the acceleration factor method, the relevant components of the PCA were the first two, together explaining 93.4% of the data variability. The first component of the PCA, which explained 75.3% of the observed data variability, is mainly driven by the variables related to fish stress response, including cortisol, glucose and lactate plasmatic levels, and osmolarity (Table 3). The phagocytosis also significantly drives component 1, but less than the other variables (Table 3). Individuals displaying high values on this component are those that showed higher levels of glucose, cortisol, and lactate (Table 3), suggesting more stressed individuals, while the second component of the PCA, which explained 18.1% of the observed data variability, is driven only by the variable phagocytosis (Table 3). Individuals displaying high values on this component are those that showed higher level of glucose, cortisol, and lactate (Table 3).

Variables	Component 1	Component 2
Cortisol	**0.89**	−0.15
Glucose	**0.96**	0.25
Lactate	**0.96**	0.04
Osmolarity	**0.94**	0.31
Phagocytosis	**−0.50**	**0.85**
Variance explained (%)	75.3	18.1

Table 3. Contribution of the five variables to the two first components of the principal component analysis (PCA) and data variance explained by each component. Bold values indicate a variable contribution higher than |0.5| to the component.

Looking at the individual positioning on the first component of the PCA (Figure 2a), fish from the subordinate group ϒ displayed greater values than fish from the sub-ordinate group β ($p < 0.001$), displaying greater values than dominant fish ($p = 0.04$). The second component of the PCA is also affected by the social position of the sea bream ($p < 0.001$). In more detail, the subordinate fish β displayed lower values on the second component of the PCA than both dominant fish and subordinate ϒ ($p < 0.001$ and $p = 0.007$, respectively). Dominant and subordinate ϒ are displaying similar values in component 2 of the PCA ($p = 0.48$). Overall, the results of the PCA support the previous results, suggesting that subordinate sea breams displayed higher level of stress than dominant ones in triads.

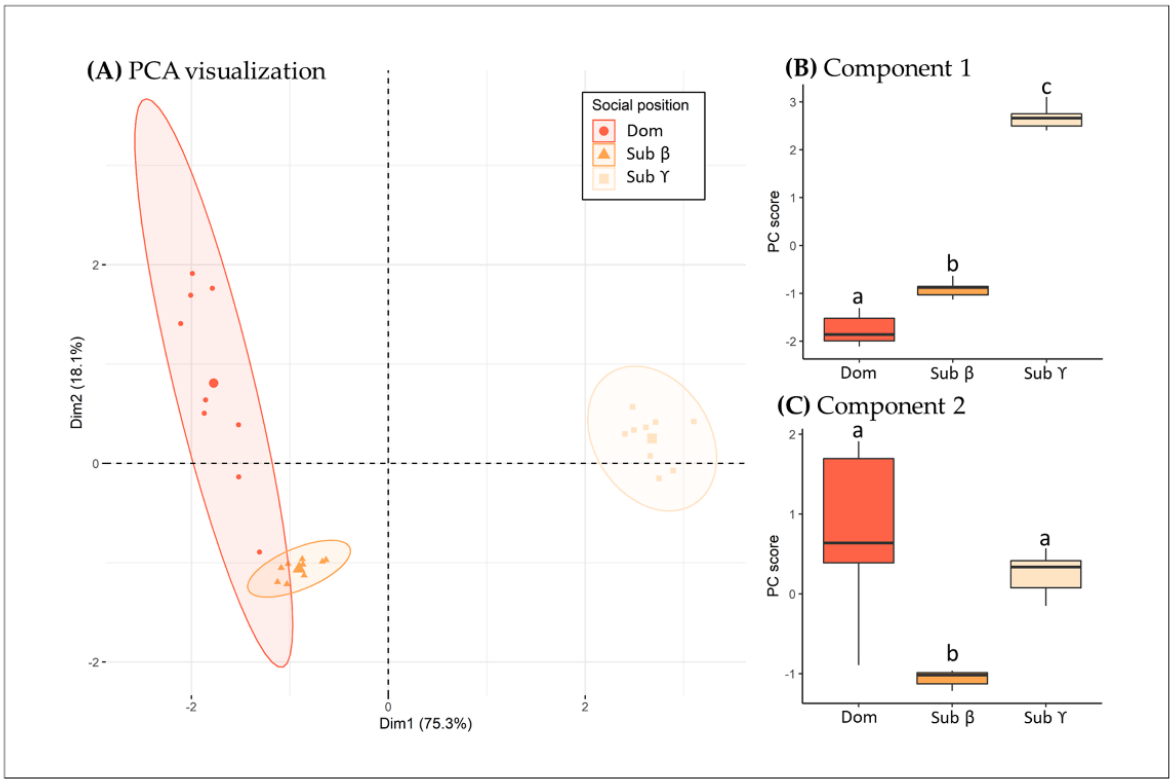


Figure 2. Principal component analysis. (A) Scatter plot of the principal component analysis in- cluding the haematological parameters and immune activities. Cortisol concentration (ng mL−1), glucose concentration (mg dL−1), lactate concentration (mg dL−1), osmolarity level (mOsmKg−1) and phagocytosis (%) according to social status of sea bream (Sparus aurata). Orange colour represents dominant fish (α) (n = 9), pale orange represents subordinate fish (β) (n = 9) and pink represents subordinate fish (γ). Confidence ellipses drawn around the levels of the categorical variable social status with a confidence level of 0.95. (B) Individual PC score for the first component as a function of diet treatment; (C) individual PC score for the first component as a function of diet treatment. For (B,C) different lowercase letters over the bars indicate significant statistical difference between groups, same lowercase letters over the bars indicate absence of statistical differences (Tukey HSD, p < 0.05).

6.4. Discussion

In this study, we provide valuable insights into the dominance and social interactions of sea bream, aiming to establish the link between social interactions and behavioural patterns (feeding order and aggressivity), and stress physiological and immunity blood indicators. Here, with respect to previous work (Cammarata *et al.* [34]), we modified the experimental design, introducing one more fish to the observation group than the paired model previously used to evaluate the social dynamics, in a more com-plex pattern between fish that were confined, but maintaining a restricted number of specimens [21].

Following the establishment of hierarchy (24 h after introduction in tank), greater levels of cortisol and glucose, lactate and osmolarity were observed in fish, regardless their social position, which is indicative of stress. In previous work, the basal plasmatic cortisol and glucose concentrations were 18 ± 3 ng mL−1 and 50 ± 4 mg dL−1, respectively, in sea bream, while in paired fish, the concentrations of cortisol were 114 ± 5 ng mL−1 for the

dominant and 204 ± 4 ng mL−1 for the subordinate, and the concentrations of glucose were 130 ± 8 mg dL−1 for dominant and 117 ± 7 mg dL−1 in subordinate, clearly supporting the induction of stress state flowing the establishment of social hierarchy, as already demonstrated in paired sea bream [34]. In the current study, stress state appeared to be greater in subordinate fish (β and γ) than in dominant ones, because higher levels of cortisol, glucose, lactate and osmolarity were measured in these fish. In addition, cortisol, glucose, lactate and osmolarity values were significantly higher in subordinate fish γ compared to fish β. This is also consistent with previous work carried out in sea bream, where subordinate fish were characterized by the elevation of plasma cortisol levels, in groups of two, five or ten fish [35]. Overall, this is consistent with results in other fish species, such as rainbow trout or sea bass [11,27,60], and in fishes in general, as recently reviewed by Bessa *et al.* [21], which showed that dominants generally exhibit lower basal cortisol levels than subordinates in a small group. This effect is, however, modulated by many factors, such as group size, habitat temperature, fish size, sexual maturity. All these factors, in larger groups, contribute to the complexity with which social hierarchies can elicit stress.

Pottinger and Carrick [61], with their study on rainbow trout, and Fatsini *et al.* [8], with their study on Senegalese sole (*Solea senegalensis*), used the classical paired model and reported that fish position within a tank, locomotor activity, agonistic behaviour, feeding, and plasma cortisol levels, are useful criteria for the determination of social dominance. Here, the dominant gilt-head seabream individuals showed a highly aggressive attitude and monopolized the access to food, with respect to the subordinates. These latter fish showed behavioural inhibition, such as suppressed aggression and low competition for feed intake with the dominant [11,62]. Feed utilization and lower growth performances have also been demonstrated in subordinate sea bream [32,35]. Low feed intake could not, however, only be the result of monopolization from the dominant. Indeed, it has been widely demonstrated, with studies on different species, that the dominant and subordinate relationships could have consequences for the animals' physiological status. Behaviour may affect glucocorticoid levels, and, in many cases, when changes in behaviour and glucocorticoid hormones co-occur, causes and effects cannot be easily disentangled [11,63] However, many examples in vertebrates are often characterised by chronically high plasma cortisol levels, coupled with suppressed aggressiveness and reduced food intake [61,64,65]. As reported by Gregory and Wood, chronic exposure at the cortisol level influences behaviour activities; for example, the research of food [66]. Confirming the data in Cammarata *et al.* [34] obtained by studies using a paired model, we observed that the establishment of the hierarchy in sea bream

involves not only behavioural patterns, such as aggressiveness and the order of food access, but also different stress physiological profiles. It was also demonstrated, in that species, as well as in rainbow trout, that more aggressive individuals also display lower cortisol response when coping with other stress events, such as confinement or restraining tests [23]. In this study, we also find that the glucose levels measured were increased in subordinate fish. Glucose release in blood is generally associated with the secondary stress response being modulated by the action of cortisol, which influences glucidic metabolism in fish [6]. Mommsen *et al.* [13] reported that plasmatic, hepatic and muscular glucose levels in teleosts might not be univocally correlated with the stress condition (i.e., cortisol level). Further, in this paper, we have observed an increase in lactate levels in the blood of subordinate individuals compared to dominant ones. An increase in blood lactate level is generally reported as a secondary response to stress [67,68]. The secondary responses are, in part, triggered by the cortisol increase (primary response). Cortisol plays a role in stimulating some processes (e.g., gluconeogenesis) and inhibiting others (e.g., digestion). According to Peters *et al.* [69], increased plasma glucose could be originated catabolizing the hepatic glycogen. Overall, it indicates the mobilisation of the required energy to face and fight future interactions with other fish to maintain the top hierarchical position. Changes in plasmatic lactate level, as a product of the anaerobic metabolism, suggest that energy could be subtracted from muscle and/or the reproductive system [70]. Since the increased glucocorticoid levels exhibited during stress might also serve to mitigate defence mechanisms [71], we examined the correlation between the social rank in the hierarchy establishment, as a stress source, and its effects on innate immunity.

It is well known that continuous agonistic interactions between conspecifics constitute social stress in fishes, which translates as chronic cortisol elevation [27,35,60]. Cammarata *et al.* [34] demonstrated the cortisol effect on gilthead sea bream PECs, treating cells with three different cortisol concentrations and showing a dose-dependent decrease in cells' phagocytic activity. Moreover, *in vivo* and *in vitro* experiments showed that increased cortisol levels affect phagocytosis via the cortisol cytosolic receptor DlGR1 (*Dicentrarchus labrax* glucocorticoid receptor1) in European sea bass [36]. This receptor was localised in the head, kidney, spleen, gills, intestine, heart and liver tissues [37,72], highlighting the crucial role of cortisol in the regulation of homeostasis. It is known that stress-induced hormonal responses, lead to osmotic imbalances in fish [14]. Thus, stress causes the elevation of plasma cortisol and electrolyte loss in freshwater fish [73,74]. In agreement with previous studies, in this work, we have also observed a significant increase in the levels of

osmolarity in subordinate individuals compared to dominant α. Moreover, we have evaluated the effects of social stress on the phagocytic activity of peritoneal cavity cells. In Cammarata *et al.* [34], we proved that cohabitation and hierarchy have a physiological effect, after a 24 h pairing period, affecting the PECs, with respect to phagocytic activity. Further, we showed, after this cohabitation-interaction period, PECs' phagocytic activity was significantly higher in the dominant fish, with respect to subordinates β and γ, highlighting a rapid (24 h) and strong effect of social interaction on the peritoneal exudate leucocytes responses. Indeed, the social stress mainly affects the PECs' response in subordinate individuals, as revealed by phagocytosis and respiratory burst activity [34,75]. In addition to PEC, Montero *et al.* [35] also demonstrated in sea bream that lysozyme activity can be reduced in subordinates. Reduced lysozyme activity in subordinate fish has also been observed in other fish species, such as sea bass or Nile tilapia [11,76]. Overall, this indicates that subordinate sea bream displays reduced immunity status, indicative of chronic stress, which could be deleterious in case of disease outbreak [35]. In addition to immunity markers (e.g., PECs, lysozyme, enzyme activities), further experiments could study specific immunological response to bacteria/virus, depending on fish social rank.

In this paper, the testosterone levels, which have a role in the regulation of cellular immune functions [77], have not been evaluated. However, sea bream is a proterandrous hermaphrodite species. At the stage examined in this study (i.e., 125 ± 25 g), fish are all immature males. Further experiments could also study possible changes in testosterone levels, following the establishment of social hierarchy in sea bream, as well as how hierarchy establishment works later in sea bream life, when males become mature or become female, as well as the underlying physiology.

The results of the PCA analysis, overall, support the analysis of the different variables one by one. Indeed, the first component of the PCA, significantly driven by all variables (cortisol, glucose, lactate, osmolarity and phagocytosis) and explaining a high percentage of data variability (i.e., 75.3%), is significantly affected by social rank. Insights obtained from the second component of the PCA are few, since only phagocytosis is significantly driving the component overall, explaining the low percentage of data variability (18.1%). These results moderate, in some way, the result from the GLM analysis of the percentage of phagocytosis, depending on the social rank of sea bream, where subordinate β and ϒ displayed similar levels of phagocytosis, both lower than the level measured in the dominant fish. Other indicators of immunity, such as oxidative stress markers, cellular cytotoxic activity, could be measured in the future to bring further insights on immunity differences,

according to social rank in sea beam. Overall, the differences observed between the groups, measured both in GLMM and PCA analyses, could be attributed to different allostatic load and adaptation time in the responses, indicating that these could be used as allostatic load biomarkers of social stress responses and impact on fish health. Our results show that stress physiological status can be determined from social interactions and from the territorial disputes activating the stress response, through cortisol release in gilt-head bream, as occurs in response to other stressors and in vertebrates [78].

Interestingly, as for aggressivity, the boldness behaviour has also been observed to affect the growth of sea bream, reared at different density over long periods of time [79]. These results may be correlated with the aggressive behaviour of dominant sea bream (i.e., eat first and more), coupled with different physiological features, depending on the boldness/aggressiveness level [23,80]. In the rainbow trout, Gesto *et al.* [81] also found that fish with different abilities compete for food, showing different behavioural responses to cope with hypoxia and ammonia exposure. In addition, stress physiological responses toward other stressors (e.g., confinement, netting) could be different, depending on fish social rank. Thus, the behavioural indicators of social position (e.g., boldness, aggressive behaviour, feeding behaviour) in sea bream could be a relevant proxy of the physiological status and stress response [23,80,82].

Furthermore, since it has been demonstrated that hierarchy is a cause of chronic stress, our data support the need to find solutions to mitigate its effects on the condition of the fish, preserving the aquaculture final product from its consequent negative effect. Solutions could be to improve the rearing condition of fish, implementing the rearing methods, as well as structural complexity of rearing tanks, which in fish farming are under-implemented [83]. For instance, self-feeding reinforces the social hierarchy, which might lead to a higher competitiveness for resources among fishes, in-creasing the social hierarchy and, therefore, stress, when compared to hand feeding. Thus, hand feeding could reduce the deleterious effect of social hierarchy, but this is not really feasible in intensive farming conditions. Increasing the complexity of the physical environment can also lead to benefits for the species, such as a reduction in aggressivity and related stress [84]. Indeed, an articulated space, introducing the possibility to avoid direct interaction, to defend territory, to escape during social conflict, investigate and interact with enrichment, could reduce stress. Studies report the relevant importance of environment on gilt-head sea bream behaviour and aggressivity; for example, the presence of blue or red-brown substrate on the tank bottom resulted in suppression of aggressive behaviour, compared to green substrate and no-

substrate tanks [85]. This hypothesis is supported by Arechavala-Lopez *et al.*, which demonstrated the influence of environmental enrichment on the enhancement of cognition, exploratory behaviour and brain physiological functions of sea bream [86].

6.5. Conclusions

In conclusion, in this study, the links between behaviour, stress physiological profile (cortisol, glucose and lactate) and immunity, in relation to social hierarchy, were investigated in gilt-head bream triads for the first time. We confirmed previous work in pairs, subordinate sea bream displayed greater stress level (plasmatic level of cortisol, glucose, and lactate), as well lower immunity (lower percentage of phagocytosis) than dominant fish. In this optic, further research is needed to study the hierarchic relationship in different rearing conditions, such as larger and sex-mixed groups and in an enriched environment. Further, this would help to improve the health and welfare of farmed sea bream by finding best management practices, depending on the need of the species.

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Article

The role of spatial exploration, territorial dominance and social stress on Seabream (*Sparus aurata*) welfare.

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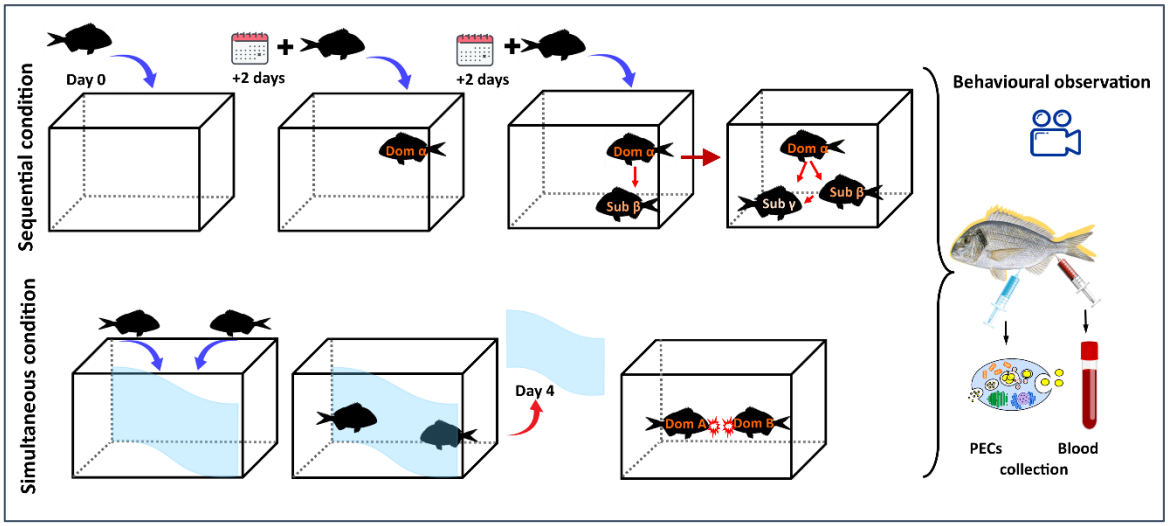
Abstract: Territoriality, spatial and social hierarchy are strictly related in gregarious fish. In this study, we investigate the formation of the social organization and the role of the territoriality in gilthead seabream (Sparus aurata) using two experimental approaches. In the first approach, three fish were placed sequentially in the aquarium with an interval of two days (sequential model), while in the second (simultaneous model), two fish were simultaneously placed in an aquarium divided by a septum which was removed after a period of time. To study the effect of social stress and the spatial perception in the two models, we performed well known behavioral observation (aggressive act and feeding priority) integrated with the evaluation of stress physiological and cellular parameters such as phagocytosis, cortisol, glucose, and osmolarity. After the establishment of the social hierarchy in the "sequential model", we observed that the levels of cortisol and of the immunological cell mediated marker were higher in subordinate individuals, than in the dominant individuals. We observed a different modulation of phagocytic activity of the peritoneal cavity cells demonstrating the social stress act on the immune response. Differently from the first model no differences were found between two fish involved in the simultaneous model, where both "confused" fish acted as dominant, defending their territory.

Keywords: *Sparus aurata*; social stress; dominance; Hierarchies; cortisol; phagocytosis.

Graphical abstract



7.1. Introduction

Due to an increasing demand for fish products and a decrease in natural resources, the aquaculture sector has grown rapidly over the past decades [1]. And guaranteeing a high quality of life for animals is even more recently a matter of concern, including in fisheries and aquaculture [2]. Fish welfare is overall correlated with good production, better quality of the meat, lower treatments and cares and lowered cost of production. Although farms are generally organized to optimize intensively growth [3], aquaculture conditions, including social interactions, can induce chronical stress [4] which is a factor often underrated. Welfare of farmed fish is important both for the market and for consumers [5,6], and could be ensured avoiding unnecessary animal suffering during rearing, handling and/or slaughtering. Fish welfare is important according to current ethics standards on the use of animals.

Methods for evaluating the welfare states are currently being developed and the evaluation of internal responses in the organisms can be carried out using analytical tools [7]. The later includes the monitoring of the stress responses, including the stress hormones measurement [6,8,9]. Links between stress response and immunity are of relevant interest and appear to be great tool in improving animal welfare comprehension and knowledge [10–12].

Also in teleost fishes, stress response is divided into three different levels: primary, secondary, and tertiary. The primary response to stress is initiated and coordinated by a two neuroendocrine axes, the hypothalamic-pituitary-interrenal (HPI) system and the sympatho-chromaffin tissues, finally leading to the increase levels of cortisol, major stress hormone in teleost fishes, and catecholamines in blood stream [13,14]. The increase in plasma cortisol levels under stress conditions typically induces an increase in plasma glucose and lactate

levels, while the increase in plasma glucose is initially generated by catecholamine-mediated glycogenolysis in later stages is cortisol-mediated gluconeogenesis [13]. Also, fish are coping with stressors including changes in the concentration of plasma ions [15]. It is worth mentioning that stress is not inherently bad and an acute stress response can be viewed as an effective way to promote a physiological/behavioral changes to adapt to the environment [16]. But if stress exposure is repeated or prolonged, it could induce the tertiary response, including behavioral changes, immune response, growth/reproduction alterations [10].

In gregarious species social environment is a considerable source of stress. Indeed, animals are organized in territories, and interact socially to establish and maintain hierarchical dominance ranking [17]. The social interactions in many animals are overall structured around dominance relationships or hierarchies where the position in the hierarchy governs access to resources such as food, water, space and, ultimately, individual fitness and/or reproductive success [18]. Social interactions between conspecifics are dynamic processes where dominants try to maintain their status by using direct attack or displaying signs to the others [19]. Dominant-subordinate relationships, causing social stress, can have consequences representing a powerful stressor leading to changes in animal behavior and physiology [8,9,20–24].

Territoriality is a common features fishes, related to social organization, present in a wide number of groups. Territory defense involves physical display and the disputes between intra and or inter specific individuals [25,26]. In sea bream, the dominant fish are distinguished by more aggressive acts on subordinates and first access at food [8,9]. Direct competition for food is retained probably the major social mechanism regulating growth in small groups of juveniles of this species, when food is limited and defendable [27]. Recently, has been demonstrated that effects of social hierarchy could be modulated by aquaculture management in sea bream [28]. Therefore, understanding the link between aggressive and feeding behaviors with the social hierarchy and stress physiology of sea bream is of great interest for the aquaculture sector, in order to find the best farming practices for the species [29].

In previous works, we examined social stress in Seabream (Sparus aurata), key species of the European marine aquaculture [30,31]. We found that, social interaction of two or three animals induces stress, increasing blood cortisol level and affecting immunity features, such as peritoneal exudate cells (PECs) activity which play important roles in both non-specific and specific immune mechanisms [8,9,32].

In nature, the development of dominance-subordinate relationship and hierarchy onset as adaptative strategy may act to reduce aggression but in fish farmed in captivity it may cause in severe chronic stress, compromising fish welfare. For that, in this work, we explore the role of environmental and territory perception on the hierarchy determination by using two experimental models where the fish are either inserted in a time-sequential manner for territorial evaluation or simultaneously in an aquarium divided by a divisor panel. In the first model, called "sequential model", the fish were placed sequentially in the same aquarium, in the second model called "simultaneous model" two fish were placed contemporarily in the same aquarium. By these two different experimental designs and an integrative approach, we examined the process of the hierarchy establishment trough behavioral observations (using parameters such as aggression and feeding priority) and investigated relevant biomarker of stress (plasma cortisol, glucose and osmolarity level) and immunity cells activity. This study would contribute to understand the link between hierarchy formation spatial and social stress, behavior, and physiology.

7.2. Materials and Methods

7.2.1 Animals

Gilthead seabream (Sparus aurata), 150 ± 20 g mean body weight were obtained from a commercial fish farm (Ittica Trappeto, Sicily, Italy) and transferred to the laboratory aquarium (200L) for acclimatation (15 days). All the specimens used in this study are all males not yet sexually mature, indeed gilthead seabream is a protandric hermaphrodite species which born as males and become female about 3 years old (from 500-600 g) [30]. Water quality was maintained similar for rearing tanks and experimental tanks during the whole experiment, and checked every day: temperature of 18 ± 1°C, salinity of 38 ± 1%, oxygen 6 ± 0.5 mg L−1 by Dissolved Oxygen Meter (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and total ammonia-nitrogen concentration of <0.2 mg L−1 by commercial kit (Tetra, Spectrum Brands, Blacksburg, VA, USA) under a photoperiod of 12 h dark and 12 h light. The fish were fed with a commercial pellet diet (Trouvit, Hendrix SpA, Italy) once a day ad libitum. The fish were distinguished by slight morphological differences, such as opercular spots, facial structure, and possible small external differences of the tail, dorsal or pectoral fins. No fish died or showed signs of disease during the experiments.

7.2.2 Sequential model

In the experimental set, named "sequential model", the specimens were sequentially placed in the aquarium (200L) with a difference period of two days. Briefly, as shown in figure 1,

the first fish was introduced in the experimental tank, after two days, a second fish was added, and after two more days a third fish was introduced. Each group was observed for a period of 15 days to assess the social rank among the specimens, and at 15th day, blood sampling and PECs collection has been performed after the monitoring for further biochemical and phagocytosis analysis (see specific sections for procedure details). Experiments was repeated 6 times, resulting in the monitoring of 27 specimens for sequential model.

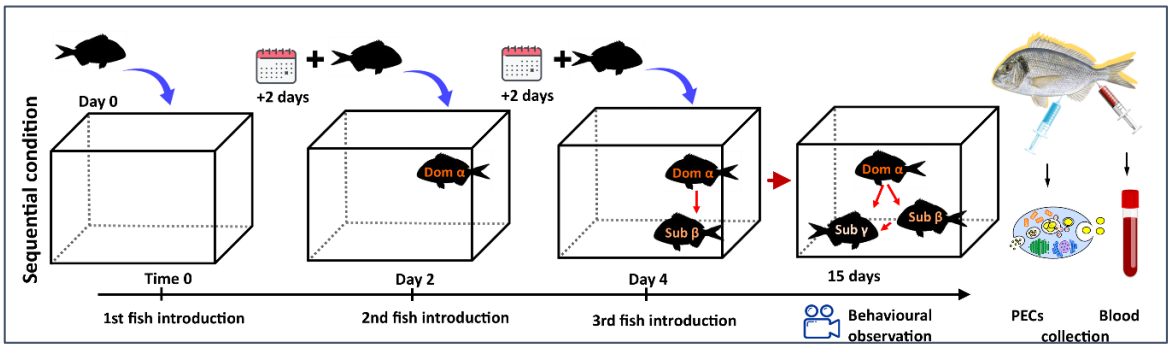


Fig. 1. Fig. 1 Sequential model. The fish were placed in the aquarium with difference of two days. The order of introduction determines the hierarchy in the triplet of specimens. Each group was observed for a period of 15 days in order to assess the hierarchical organization among the specimens and at 15th day were performed blood sampling for biochemical analysis.

7.2.3 Simultaneous model

In the "simultaneous model", as illustrated in the Figure 2, two fish (called A and B) were introduced in an aquarium (200 L) divided in two equal sectors by a transparent curved plexiglass septum. The two spaces resulted interpenetrating and the separator allowed the water communication between the two chambers. The two fish, one for each space, were placed in the aquarium at the same time. During these 4 days, thanks to the transparency of the divisor, the fish had the visual perception of the other one. After the fourth day the dividing panel was removed, and the behavior has been observed for further five days. After these five observational days the blood and PECs were collected after the monitoring for further biochemical analysis (see specific sections for procedure details). Experiment was repeated 9 times, resulting in the monitoring of 18 specimens for simultaneous model. In the control experiment, following our previous paper of Cammarata *et al.* (2012) two fish were placed simultaneously in a regular aquarium, with any divisor, and cohabited the same space. Blood and PECs were collected for the analysis at the same endpoint of the experimental

fish. Experiment was repeated 9 times, resulting in the monitoring of 18 specimens for control experiment.

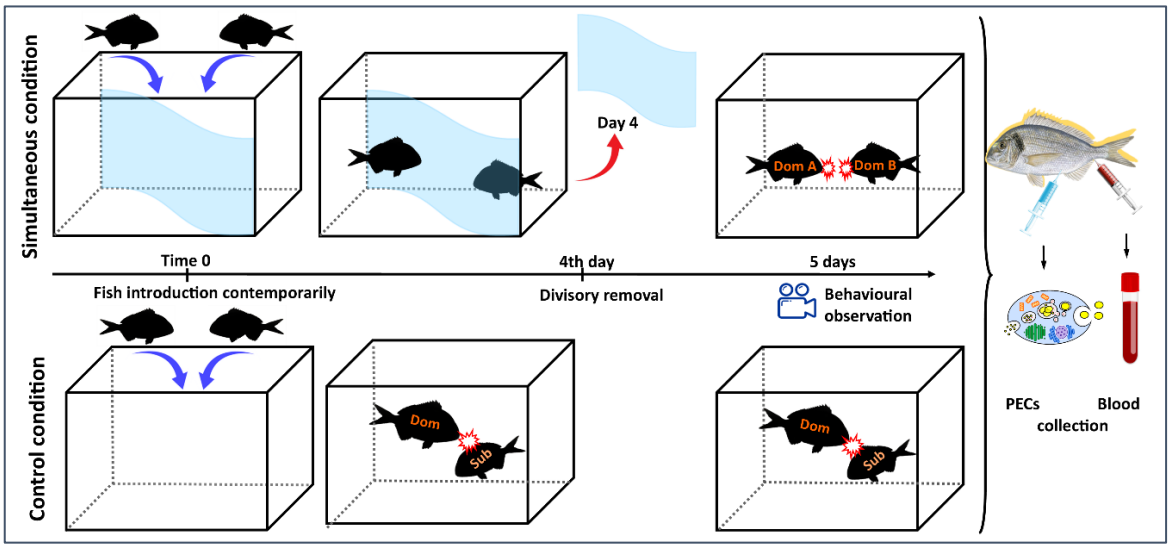


Fig 2 Simultaneous model. The fish were placed simultaneously in the aquarium divided by the transparent septum in two spaces. After 4 days the septum was removed, and each fish acted as dominant defending its territory. In the control between the two fish placed in the aquarium, with any divisor, was immediately established a clear hierarchy.

7.2.4 Behavioural observations

The behavioral changes were recorded to assign a hierarchical position to each individual fish. Specimens were observed to detect changes in the social status until the social positions were established, every group was observed and recorded by a digital camera (Panasonic sdr-h85 hybrid) for the data acquisition during light period [8,9]. During the surveys all recorded behavioral acts were analyzed using the Behavioral Observation Research Interactive Software v. 7.12.2 (BORIS). Individuals from each group were distinguished according to McCarthy *et al.* (1999) as dominant, subordinate "β" or subordinate ''γ'' [33]. Dominant position has been correlated with increased aggressiveness and preferential access to the food. To define this social distinction, the number of aggressive acts (A+) were observed and defined as a bite or a rapid approach without biting that resulted in the displacement of the subordinate [34], and the feeding order (FO) of each group was determined.

7.2.5 Blood sampling

At the end of the two model experiments (i.e. sequential and simultaneous model), the fish were anesthetized with 0.05 % w/v MS222 (3-aminobenzoicacid ethyl ester; (Merck & Co., Darmstadt, Germany); about 0.5-1 ml of blood was collected from the caudal vein into a sterile plastic syringe containing 0.2 ml of heparin within three minutes. The blood was then

centrifuged at 800 g for 10 min at 4 °C and plasma was retrieved before proceeding to biochemical analysis.

7.2.6 Cell suspensions and PEC

Following blood sampling, the body ventral surface was disinfected with 70 % ethyl alcohol, the peritoneal cavity was injected with 15 ml of isotonic (370 mOsm kg-1) medium (Leibovitz L15 medium containing 2 % fetal calf serum, 100 units penicillin ml-1, 100 units streptomycin ml-1 and 10 units heparin ml-1). The culture medium components were purchased from Sigma. After massaging the ventral surface for 10 min, the medium containing the PECs was collected, and the PECs were isolated by centrifugation at 400 g for 10 min at 4 °C. The dead cells were determined by light microscopy after addition of 0.01 % trypan blue to the medium.

7.2.7 Haematological parameters

The levels of total cortisol were measured in the plasma sample using a commercially available kit (Intermedical Diagnostics srl, Villaricca, Italy) based on ELISA technique according to the manufacturer's instructions and confirmed by radioimmunoassay (RIA) [35]. The osmolarity of the plasma samples was measured using a freezing-point depression osmometer type 4b (Hermann Roebling Messtechnik, Berlin, Germany). The glucose plasma levels were determined using the Accutrend Plus Kit (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's instructions.

7.2.8 Phagocytosis assay

The method pr the phagocytosis assay was performed as established in Cammarata *et al.*, (2012) using yeast, Saccharomyces cerevisiae (Merck & Co., Darmstadt, Germany), as target for evaluate the percentage of phagocytosis [8]. Briefly, yeast cells were prepared in distilled water as a 0.25 % (w/v) solution (approximately 1 x 107 yeast ml-1), autoclaved for 15 min, washed 2 times at 2000 g at 4 °C for 5 min and incubated for 1 h at 20 °C with eosin Y (4-Bromo-fluorescein) to a final concentration of 0.05 %. The yeast was washed four times in distilled water and resuspended to a final concentration of 0.0125 % w/v in phosphate buffered saline (PBS: 103.6 mM NaCl, 1.46 mM KH_2PO_4, 0.8 mM Na_2HPO_4, 2.6 mM KCl, 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$, pH 7.4) and stored at -20 °C for a maximum of 2 weeks. The yeast suspension was added (v/v) to 100 µl of leucocyte suspension (2.5 x 106) and placed in a 1 ml plastic tube. The mixture was incubated for 30 min at 20 °C with gentle

stirring. To indicate the activation of phagocytosis, 50 µl of a quenching solution (QS) (2 mg ml-1 trypan blue and 2 mg ml-1 crystal violet in 0.02 M citrate buffer, pH 4.4 containing 33 mg ml-1NaCl) (Hed, 1986) was added to the reaction mixture. The slides were examined under a microscope equipped with a Normarski interferential contrast device and fluorescence apparatus (450-490 nm filter) (Diaplan, Leica, Wetzlar, D). The results of phagocytosis were expressed as the percentage of cells containing yeast cells.

7.2.9 Statistical analyses

Statistical analyses were performed using Graphpad Prism 8.0.2 software, and a probability level of $p < 0.05$ was considered significant. Anova Test followed by Tukey HSD post-hoc test, were performed to assess differences between dominant and subordinate specimens.

7.3. Results

7.3.1 Sequential model: Behavioural observation, establishment of a social hierarchy, blood stress parameter and phagocytosis activity evaluation

The sequential experimental model (Figure 1) involved the observation of triplets of specimens in which individuals were added to the tank in a sequential manner with a temporal distance of two days of each other. As shown in Table 2, the percent of aggressive acts (A+) and the preferential access to the food (FO) distinguished ($p < 0.001$) the fish as either dominant or β or γ subordinates in each group (A+: Dom>Sub (β)= 95.7±5 %, FO: 100±0 %; Dom>Sub(γ)= 85±5 %, FO=100±0 %; Sub(β)>Sub(γ)=90±5 %; FO=95±3 %).The hierarchy state has been established based on the time of exploration that determines the acquisition of territoriality. In fact, the first fish of the sequence was always dominant, the second fish became always the subordinate β, and the last fish of the sequence became in all the experiments the subordinate γ (Table 1).

Sequential model	Aggressive acts (A+) Mean % ± SD	Preferential food accession (FO) Mean % ± SD
Dom > Sub (β)	95.7±5 %	100±0 %
Dom > Sub (γ)	85±5 %	100±0 %
Sub (β) > Sub (γ)	90±5 %	95±3 %

Dom=Dominant; Sub=subordinate

Table 1: Mean percentage (±SD) of aggressive acts (A+) and preferential access at the food. (FO) for the "sequential model" in which the three fish were sequentially located.

Plasma cortisol levels are correlated with social status; highest levels are displayed by *subordinate* individuals ($p<0.001$, Figure 3). After 15 days, the cortisol concentrations peaked in both the *subordinate* types (β: 198±19 ng ml^{-1}; γ: 405±39 ng ml^{-1}) compared to the *dominant* type (Dom=107±16 ng ml^{-1}). Also, cortisol level was significantly higher in *subordinate γ* than *subordinate β* ($p<0.001$, Figure 3). Glucose values were higher in the *dominant* specimens compared to *subordinate β* ($p<0.05$) but not compared to *subordinate γ* ($p>0.05$). Also *subordinates β* and *γ* displayed similar levels of glucose (Figure 3). Osmolarity values were lower in both *subordinates β* and *γ* than in dominant, but levels were not significantly different between *subordinates β* and *γ* (Figure 3). The phagocytosis activity of the peritoneal cavity cells significantly increased in *subordinate γ* compared to *dominant* ($p<0.05$; β =9±5% and Dom=5±2%) but the phagocytosis activity of PEC of *subordinate β* (7±4%) did not differ from activity of both *dominant* and *subordinate γ* ($p>0.05$; Figure 3).

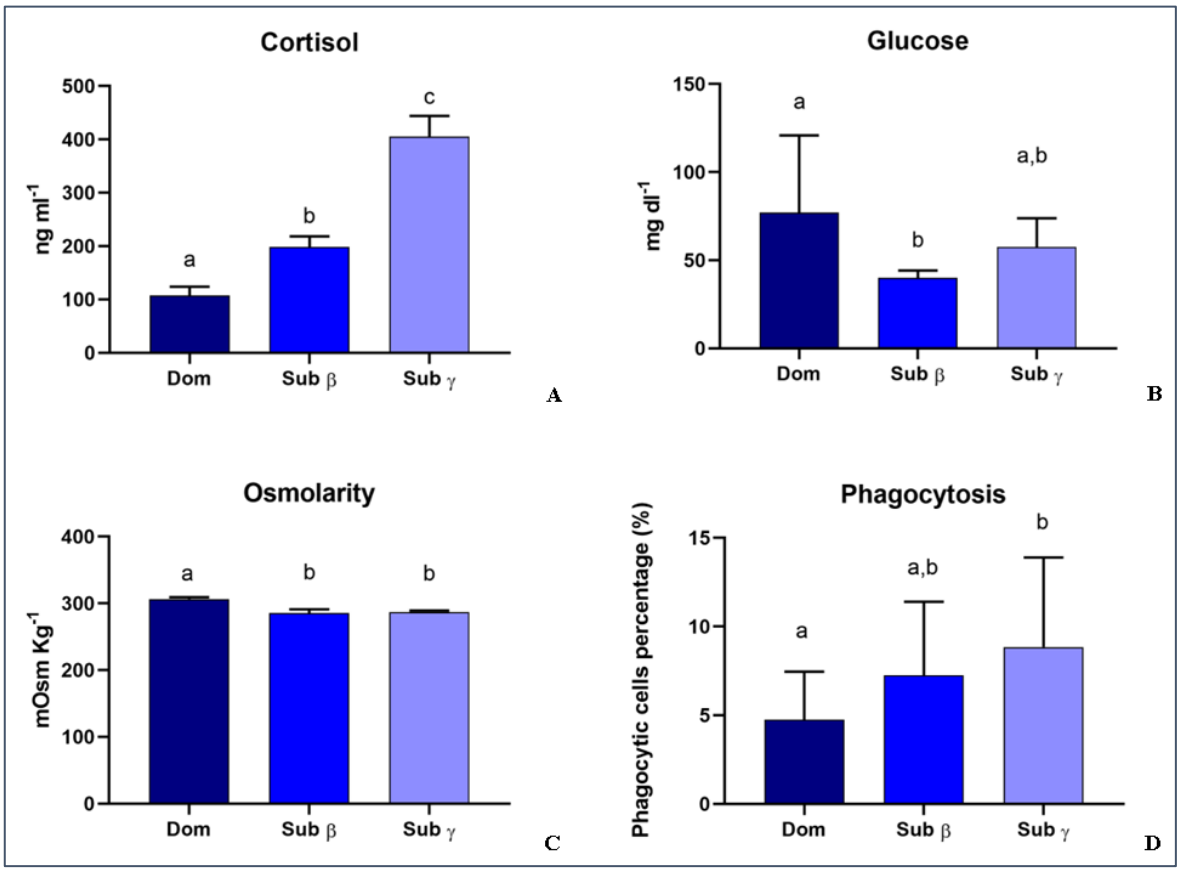


Fig.3. The values of plasma parameters of fish involved in sequential model experiments (n=9 fish per social group). Mean (±SD) of plasmatic parameters of dominant (Dark blue histogram), subordinate (β) (electric blue histogram), and subordinate (γ) (violet histogram) sea bream (Sparus aurata). The panels present (A) Cortisol concentration (ng mL^{-1}), (B) glucose concentration (mg dL^{-1}), (C) osmolarity level (mOsm Kg^{-1}) and (D) Phagocytosis (%). Different letters over the bars indicate significant statistical difference between groups (Anova Test followed by Tukey HSD post-hoc test, $p < 0.05$).

7.3.2 Simultaneous model: behavioural observation, establishment of a social hierarchy, blood stress parameter and phagocytosis activity evaluation

After the fourth day, subsequently removing the divisor, trough observation of the fish in the experimental tank has been possible to establish that both fish (A and B) believed themself the dominant at the top of social rank (confused fish from now called Dom A and Dom B) and had the same bold behaviors. They attacked each other in the same quantity, and they feed for first every time, defending their perceived own territory from intrusion of the other fish, and trying to affirm their dominance of their space (Table 2). The behavior in experimental tank resulted highly different respect the behavior of animal placed in control tank at the same time. These latter, indeed, showed a clear hierarchy with dominant and subordinate specimens. The social hierarchy was established after about an hour and remained unchanged throughout all the period of observation of 9 days. In Table 3 is indicated that the interaction between the two fish were almost all the aggressive act from the dominant versus the subordinate, and that for the totality the dominant feed always for first. Behavioural observations were supported by biochemical analysis. Cortisol, glucose, osmolarity and phagocytic activity were all at the same level for the fish Dom A and Dom B (Figure 4).

Territoriality model	***Aggressive acts (A+) Mean % ± SD***	***Preferential food accession (FO) Mean % ± SD***
Dom A > Dom B	50 ± 5 %	50 ± 0 %
Dom B > Dom A	50 ± 5 %	50 ± 0 %
Dom contr>Sub contr	94 ± 3%	100 ± 0%

Dom=Dominant; Sub=subordinate

Table 3: Mean percentage (±SD) of aggressive acts (A+) and preferential access at the food. (FO) for the "simultaneous model" in which the three fish were sequentially located.

In the simultaneous model, the hierarchy has been evaluated in comparison to control. Lower cortisol value was measured in the dominant control fish compared to subordinate control fish and Dom A and Dom B ($p<0.001$; figure 4). Plasma cortisol level in subordinate control fish is similar to values measured in both fish Dom A and of Dom B. Higher values of glucose and osmolarity were found in both dominant and subordinate control fish than in both the Dom A and Dom B ($p<0.05$, Figure 4). Also, the glucose and osmolarity levels did not differ between dominant and subordinate control fish, as well between fish Dom A and Dom B ($p>0.05$, Figure 4). About phagocytic activity the dominant control fish displayed

similar levels of fish Dom A and Dom B ($p>0.05$, Figure 4) while as expected subordinate control fish displayed lower phagocytic activity value than Dom A and Dom B ($p<0.001$, Figure 4).

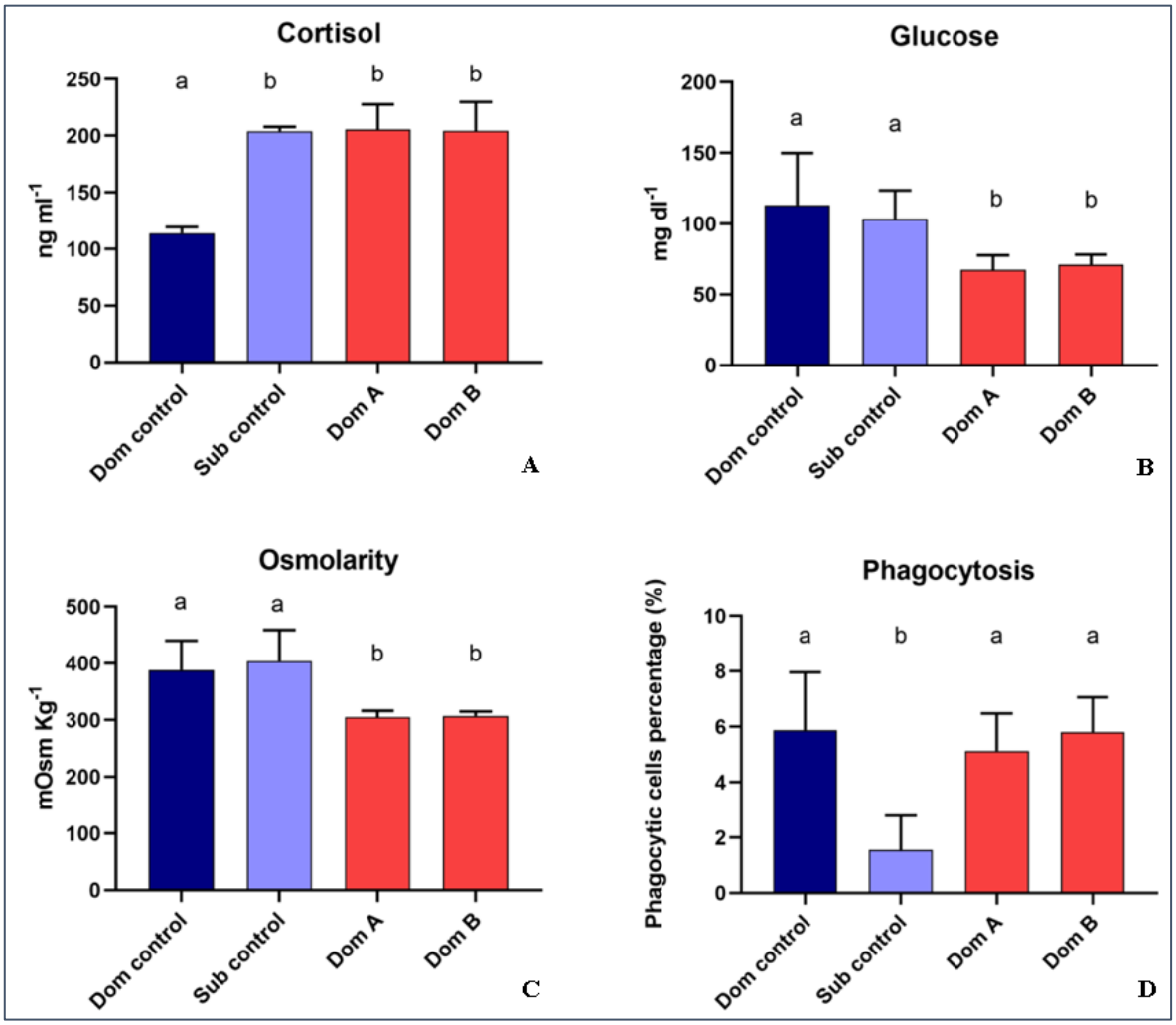


Fig.4. The values of plasma parameters of fish involved in the simultaneous model experiments (n=9 fish per social group). Mean (±SD) of plasmatic parameters of control fishes, dominant (dark blue histogram), subordinate (violet histogram), and confused dominant fish (A and B) (red histogram) sea bream (Sparus aurata). The panels present (a) Cortisol concentration (ng mL^{-1}), (b) glucose concentration (mg dL^{-1}), (c) osmolarity level (mOsm Kg^{-1}) and (d) Phagocytosis (%). Different letters over the bars indicate significant statistical difference between groups (Anova Test followed by Tukey HSD post-hoc test).

7.4. Discussion

In different fish species, have been demonstrated the correlation between social position, activity behaviour and physiological fish conditions and that the position within a tank, locomotor activity, agonistic behaviour, feeding, and plasma cortisol levels, are useful criteria for the determination of social dominance [36,37].

In previous studies, we provided valuable insights into the social interactions among small groups of sea bream and described the link between social interactions, behavioral patterns

(feeding order and aggressivity) and physiological stress and immunity blood indicators [8,9].

In this work, the goal is reached by using two different experimental approaches named "sequential" and "simultaneous" models with different modality of fish introduction in the tanks to comprehend the role of time dedicated to spatial exploration and sociality in the hierarchical organization and its effect on the status of the fish. By these two different experimental designs and an integrative approach, we examined this trough behavioural observations and investigated relevant biomarker of stress and immunity cells activity.

The sequential model demonstrated the importance of spending time in exploration of the territories for the establishment of a dominance hierarchy. Indeed, we have shown that the first fish that was placed in the tank became the dominant in every case (9 replicates), the second fish placed after two days became the subordinate β and the third fish placed after four days became the subordinate γ. It evidences that the time expended for space exploration is crucial in the formation of the social hierarchy as example of sensitization, a non-associative learning, in which the progressive amplification of a response follows repeated stimulus [38]. The more time spent exploring corresponds to a greater sense of territoriality, which results in the dominance hierarchy as example of environmental influences, spatial memory, and manipulation of the environment [39].

After 15 days following introduction of the three animals sequentially, increased levels of plasma cortisol were measured in the subordinate specimens (β and γ) respect in the dominant (γ > β >Dom). Also, level of phagocytic activity was greater in subordinate γ than dominant specimens but the level in subordinate β was not different than the two other social groups. These results are consistent with Cammarata *et al.* (2012) and Dara *et al.* (2022), which showed the same modulation response of PECs in sea breams while using paired experimental model. According to our previous studies, this highlights a difference in the response in specimen showing parametric and physiological alterations caused by social stress in this gregarious species. It should be noted that in this study, individuals who assert themselves dominant after the establishment of the social status almost always win the next battle for social dominance.

After evidenced the role of the time of each fish expended in exploration and territoriality acquisition that leads to a hierarchy formation, by the second model (simultaneous model), we wanted to investigate more in dept the role of territorial exploration evidencing the relationship between the time expended in exploration of the territory, territoriality, and social rank. The two fish placed in the aquarium divided by the panel had the same time to

explore the territory acquiring the dominance of their space. Once removed the panel, the two fish, manifested their territoriality, competed aggressively to defend "their" territory and tried always to feed for first. Both the fish acted as dominant and confirmed the hypothesis of the importance of the time invested in exploration to acquire the territoriality and dominance role. Differently respect the control condition, where the two fish were placed contemporary in the same space, and after a short period was observed a hierarchy onset, with a dominant and a subordinate individual, that was maintained during all the experimentation [8,9].

Here the dominant specimens of both experiments showed a highly aggressive behaviour and monopolized the access to food, with respect to the subordinates specimens in the sequential model and in the subordinates control specimens of the simultaneous model that showed behavioural inhibition, such as suppressed aggression and low competition for feed intake with the dominant [8,9,40,41]. Behaviour may affect glucocorticoid levels, and, in many cases, when changes in behaviour and glucocorticoid hormones co-occur, causes and effects cannot be easily disentangled [42,43]. We observed, also here, that the onset of the hierarchy in sea bream involves different behavioural patterns, such as aggressiveness and the order of food access accompanied by also different stress physiological profiles, both in sequential experiments and in control condition of the simultaneous experiments, whereas, as the same manner of the behavioural acts, also the cortisol level maintained the same level for the specimen, named "A" and "B", of the simultaneous model.

The plasma glucose levels measured were also influenced by social position in the sequential experiments. About the simultaneous experiments, as the same for cortisol, the glucose of the dominant like confused fish was at the same level, whereas it was slightly lower in the subordinate of the control condition, as previously observed in [9]. Glucose release is modulated by the action of cortisol, and increase of glucose level in blood is generally linked to stress condition [13]. Overall, increase of glucose indicates the mobilisation of the required energy to face and fight future interactions with other fish to maintain the top hierarchical position. It is also known that stress-induced hormonal responses, lead to osmotic imbalances in fish [15]. The levels of plasma osmolarity in subordinate specimen of the sequential model, is lower than the dominant. Differently osmolarity level was at the same level also for the experiments in the simultaneous experiments.

Cammarata *et al.* (2012), demonstrated the cortisol effect on gilthead sea bream PECs, treating cells with three different cortisol concentrations and showing a dose-dependent decrease in cells' phagocytic activity, in agreement with previous studies, we proved that

cohabitation and hierarchy have a physiological effect, influencing in the same manner the PECs, with respect to phagocytic activity. On the contrary in the simultaneous experiments the phagocytic activity of the PECs of the subordinate. The two dominant confused fish, also in this case, showed the same phagocytic activity comparable with the dominant fish of the control condition highlighting the effect of social interaction on the peritoneal exudate leucocytes responses. Indeed, the social stress may affect the immunity effector, such as the PECs' response as revealed by phagocytosis and respiratory burst activity [8]. In addition to PECs, Montero *et al.* [44] also demonstrated in sea bream that lysozyme activity can be reduced in subordinates. Reduced lysozyme activity in subordinate fish has also been observed in other fish species, such as sea bass or Nile tilapia [40,45].

Since stress is well known to affect growth and fitness [22,23], such study could be of commercial interest to the aquaculture industry. Economically important fish species incur size down-regulation and susceptibility to disease of which evidence suggests may be stress related, including related to social interactions and social stress [46,47].

Also, fish with different abilities compete for food (and social rank), showing different behavioural responses to cope with further stressors, confinement or netting could be different, depending on fish social rank [48,49]. Interestingly, aggressivity of fish is generally correlated with boldness behaviour, including in sea bream, and coupled with different physiological features and divergent response to stressors [29,42,50]. High-throughput tests for boldness screening in seabream existing (e.g. risk-taking test) [29,50], boldness (as a proxy of aggressivity and social rank) could be used for selecting fish better adapted to different aquaculture practices, and so avoid deleterious welfare issues.

Furthermore, since social organization and the time of spatial exploration may be cause of chronic stress with its negative consequences on fishes, our work evidence the importance to have a greater focus on these two aspects in the rearing conditions finding solutions to mitigate its effects and to preserve the quality of aquaculture final product. [51–54].

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Chapter 8

Final considerations

As has been extensively described, aquaculture has recently seen a rapid expansion, becoming an important source of seafood for the consumers (FEAP, 2021). In spite of this, modern intensive fish aquaculture has had to deal with emerging biological, economic and social challenges that may influence the ability to maintain ethically sound, productive and environmentally friendly fish production.

Acoustic telemetry has the advantage of collecting information and physiological data with real-time acquisition because transmitters are placed in or on the specimen (Føre *et al.*, 2018). Despite these advantages, the application of transmitters requires careful handling, and the surgical application of transmitters creates the risk of influencing fish welfare conditions and affecting the collected information.

Table 1 summarizes the main results obtained in the studies presented in the chapters of this thesis. In particular, in chapter 3 the results of the study aimed at investigating the effects of the surgical implantation of accelerometer tags and the manipulation on exemplars of sea breams or sea bass reared in a controlled environment are presented. Here, the results of this study confirm that the manipulation and surgical implantation of accelerometer tags does not cause medium-term changes affecting the basic growth and stress physiological indicators of tagged fish, and they further confirm that tagged fish can be sampled and considered representative of the population as they display growth and physiological parameters comparable to those of untagged fish. This confirms that this technology, applied with appropriate measures, is a useful tool in the aquaculture framework, allowing us to obtain real-time information. Indeed, acoustic telemetry may have many different applications for monitoring fish, and this promotes the expansion of the use of this technology to a wide number of studies, for example, to monitor different experimental groups with different rearing conditions or under different diets. Organic aquaculture is a method for farming fish based on organic principles. It has become popular very recently thanks to consumer concerns about the harmful impact of aquaculture on both themselves and the environment. Indeed, it combines the preservation of natural resources and the use of renewable products. Aqua-cultured animals' nourishment is based on organic feed composed in large part of proteins and oils obtained from land-based agriculture. The partial substitution of animal proteins and lipids in feed with vegetable proteins and lipids may be considered a promising, environmentally friendly alternative on the one hand, but on the other hand, if it is administered over the long term, it may affect the natural physiology of

the processes impacting fish health (Lembo and Mente, 2019). Aquaculture has grown rapidly over the past few years and, consequently, fish welfare has attracted increased attention from different kinds of stakeholders. However, fish welfare assessment is complex and thus needs to adapt measurements that are easily applicable to aquaculture conditions. The effects of different rearing conditions, conventional or organic methodologies, are addressed in chapters 4 and 5. Here, the conditions, growth performance and physiology of fish after an experimental period in which they were nourished with diets of different formulations have been evaluated. Results from the application of radiotelemetry reported in these chapters support the utilization of this technology in aquaculture contexts for the evaluation of welfare in relation to different conditions, when coupled with other welfare indicators. Here it is used to assess the conditions in relation to different diets supplied to the two aqua-cultured species, European sea bass and gilthead sea bream.

From the findings presented in chapter 4, it is possible to report two main conclusions. Firstly, the use of acoustic transmitters, opportunely calibrated with physiological indicators, is a promising monitoring tool in aquaculture for real-time fish welfare evaluation. The precision of this technology may be augmented by integrating different parameters with swimming activity and other parameters, such as oxygen consumption, muscle activity and metabolic indicators. Secondly, based on information obtained from all the indicators investigated, it seems that a well-balanced organic diet does not have a negative impact on the health and welfare of the European sea bass, suggesting that organic aquaculture can face the challenges of the sector, maintaining high standards for fish welfare. In chapter 5, European sea bass welfare conditions were assessed after having been fed with either a conventional diet or two different organic diets (different in terms of raw proteins, fish oil and lipid contents) containing organic vegetables and a natural antioxidant compound for seven months. The effects of the different diets were assessed through multiple different indicators: indicators of swimming activity, haematological and serological stress indicators, immunity parameters, indicators of exposure to organic contaminants, and growth parameters have enabled us to obtain a global view of the physiological state of the sea bass. This study, both in terms of growth performance and physiological welfare status, supports the transition towards organic aquaculture, here in particular for the European sea bass, but it could be expanded to other species considering the appropriate diet adapted to the needs of the species. Further, the transition towards organic agriculture can also benefit humans by providing higher quality products (Mie *et al.*, 2017). Even though not all of the sixteen parameters gave globally consistent responses, the use of all the parameters furnished a

strong decision criterion. The parameters that gave a whole organism response, such as EMG, recovery ratio and growth parameters proved to be sensitive to assessing welfare conditions (Carbonara *et al.*, 2019, 2015). Cortisol concentration, glucose, lysozyme and other physiological indicators considered in this study, despite a certain variability, made important contributions to welfare assessment (Ellis *et al.*, 2012). Finally, the PCA and MCDA methods turned out to be powerful tools for assessing welfare in aquaculture using a multi-parametric approach (Huntingford *et al.*, 2006a).

A further important, but often not sufficiently considered, aspect for aquaculture covered in this thesis is relate to the effect of cohabitation in the same crowd spaces and the effect social stress on the welfare of individuals of gregarious species. In chapter 6 and 7 have been studied the effect of a hierarchical organization and the mechanisms that lead at its establishment through behavioural observation (aggressivity and feeding order) coupled with analysis of physiological marker of stress (cortisol, glucose, lactate, osmolarity, and phagocytosis activity). Indeed, since it has been demonstrated that hierarchy is a cause of chronic stress it can affect the welfare of the animal acting on their homeostasis causing serious deleterious effect on medium-long term on different biological function (e.g. feeding, mating, fitness, immune response). In chapter 6, are presented the results about the study clarifying the links between behaviour, stress physiological profile and immunity, in relation to social hierarchy. This connection previously investigated in paired fish has been now for the first time investigated in gilt-head bream triads. Subordinate sea bream seems to be more stresses, indeed they displayed greater stress level (higher plasmatic level of cortisol, glucose, and lactate), as well lower immunity (lower percentage of phagocytosis) than dominant fish (Cammarata *et al.*, 2012).

In chapter 7 are presented results of a study aimed to investigate the processes involved in the social organization among specimens of a group of gilthead seabream and the role of the territoriality in its formation. For that here have been used two experimental approach: in the first, a group of three fish were placed in the same aquarium sequentially with an interval of two days, the called *"sequential model"*, and in the second, called *"simultaneous model"*, two fish were simultaneously placed in an aquarium opportunely modified, it was divided by a septum which impeded the physical interaction among the fish and that was removed after a period of time. To study the effect of social stress and the territoriality acquisition in the two models, have been performed behavioural observation integrated with the evaluation of physiological and cellular parameters such as phagocytosis, cortisol, glucose, and osmolarity. After the establishment of the social hierarchy in the *sequential*

model, the levels of cortisol and others biochemicals stress markers were higher in subordinate individuals than in the dominant. We observed a different modulation of phagocytic activity of the peritoneal cavity cells demonstrating the social stress affection on the immune response. Differently from the first model no differences were found between two specimens involved in the *simultaneous model*, where both "confused" fish acted as co-dominant, defending in the same manner their perceived territory, and monopolizing the access to food. In this chapter, underlying the importance of the time dedicate to exploration of the territory and territoriality we provided insight about the hierarchy process formation linking with the stress physiological profile (cortisol, glucose and osmolarity) and immunity effector (PECs). Here we confirmed data obtained in previous chapter, showing that social stress exerts effect on subordinate sea bream from dominant fish.

In this optic, from results presented in these two last chapters, further research is needed to study the hierarchic relationship in different rearing conditions, such as larger and sex-mixed groups and in an enriched environment. Further, this would help to improve the health and welfare of farmed sea bream by finding best management practices, depending on the need of the species. Since it has been demonstrated that hierarchy is a cause of chronic stress, these data support the need to find solutions which contribute to reduce the chronic stress or to mitigate its effects on the condition of the fish, preserving the aquaculture final product from its consequent negative effect. Solutions could be to improve the rearing condition of fish, implementing the rearing methods, as well as structural complexity of rearing tanks, which in fish farming are under-implemented (Arechavala-Lopez *et al.*, 2019). Different other studies, indeed, support the relevant importance of environment on gilt-head sea bream behaviour and aggressivity demonstrating the influence of environmental enrichment on the enhancement of cognition, exploratory behaviour and brain physiological functions of sea bream (Arechavala-Lopez *et al.*, 2020; Batzina and Karakatsouli, 2012).

	Species	Tools	Methods	Analysis	Results
Chapter 3	*S. aurata* *D. labrax*	Acoustic accelerometer tags	Telemetry; growth performance; physiological approach	Cortisol, glucose, lactate levels, specific growth rates	No significant differences among the tagged and untagged fish groups.
Chapter 4	*D. labrax*	Acoustic accelerometer tag; Blažka swimming chamber; different diets	Telemetry; morphometric; physiological; immunological parameters approach	Swimming tests; morphometric measures, cortisol, glucose, lactate, haematocrit, haemoglobin, red blood cell count, lysozyme measurements	Organic diet does not affect the welfare of the European sea bass. The use of previously calibrated acoustic transmitters is a promising tool.
Chapter 5	*D. labrax*	Different diets; radio transmitters; Blažka chamber	Telemetry; morphometric, physiological, immunological approach	Recovery test, muscle activity, haematocrit, haemoglobin, red-blood-cell count, cortisol, glucose, lactate, lysozyme; indicators of exposure to organic contaminants; growth index, feed conversion and protein efficiency ratio, hepato-somatic index	PCA and MCDA: useful for fish welfare assessment. Best welfare condition was achieved in fish fed with the protein-rich organic diet. EMG, recovery ratio and growth parameters proved to be sensitive to assessing welfare condition. Cortisol, glucose, and lysozyme are important for welfare assessment, even though highly variable.
Chapter 6	*S. aurata*	Aquariums- arena; recording camera	Behavioural observation; physiological, immunological approach	Behavioural observation, haematological and cellular analysis: cortisol, glucose, lactate and phagocytosis activity	Links between behaviour, stress physiological profile and immunity, in relation to social hierarchy, subordinate sea bream displayed greater stress levels, as well as lower immunity than dominant fish.
Chapter 7	*S. aurata*	Aquariums- arena; recording camera	Differential fish introduction; behavioural observation; physiological, immunity approach	Behavioural observation, haematological and cellular analysis: cortisol, glucose, phagocytosis activity	The time expended in territory exploration is fundamental for social establishment; demonstrated social rank and physiological immunological profile relation

Table 6 Main results obtained in chapters 3,4,5,6,7

In conclusion, by the new findings presented through the chapters of this thesis, schematically summarized in Table 1, the validity of using the presented methodologies is evident. All these results confirm that the physiological approach to assessing fish welfare conditions is a rich and effective investigative toolbox, and its efficacy is empowered when coupled with integrative methods, such as telemetry, that allow us to obtain a global and real-time overview of fish health and welfare conditions after different stress exposures. Furthermore, organic aquaculture may address challenges of the sector without affecting fish welfare, being a good compromise between producer and fish needs. Moreover, this study demonstrates the importance of an aspect which is often underrated in the aquaculture sector: indeed, sociality and the onset of social hierarchy cause stress that may became chronic affecting long-term fish welfare. This suggests that it may be necessary to identify the best management procedures that can effectively deal with this critical point.

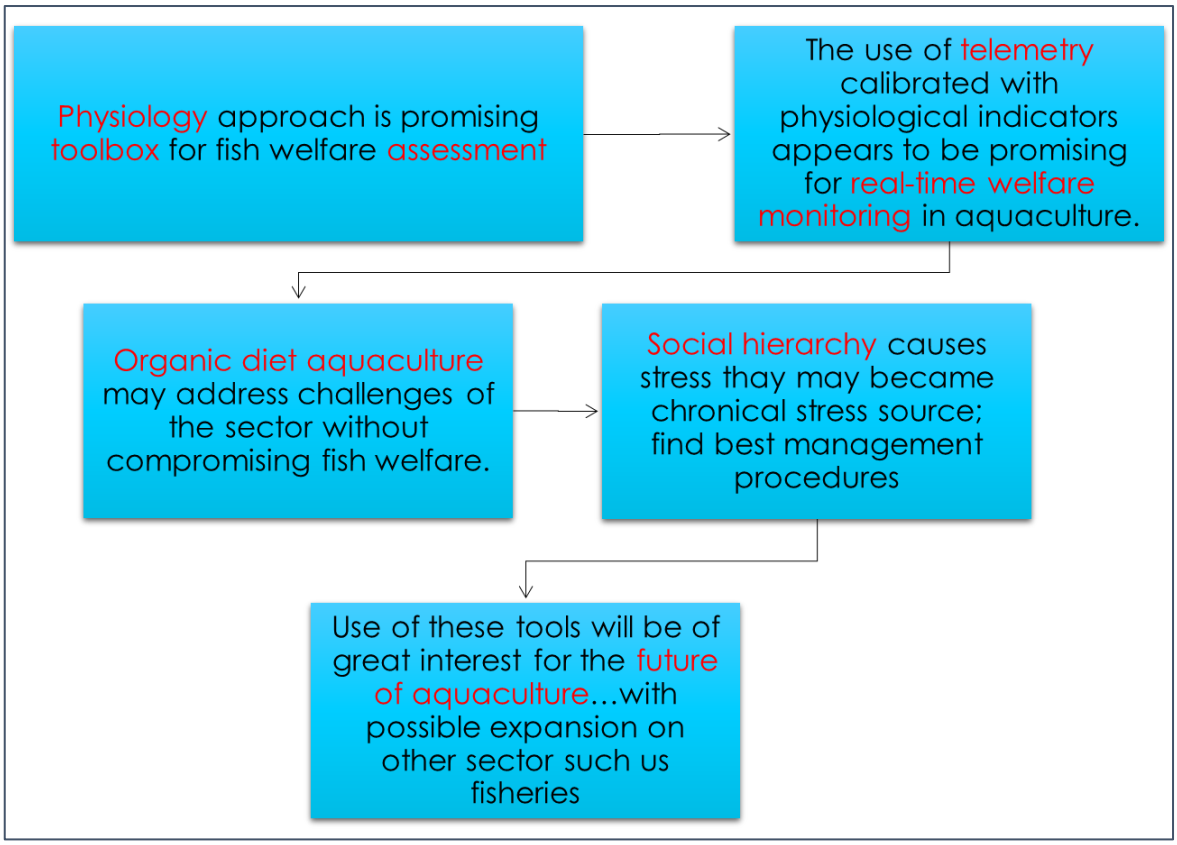

Figure 5 Schematic summary of the conclusion

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