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Review

Fish Welfare in Aquaculture: Physiological and Immunological Activities for Diets, Social and Spatial Stress on Mediterranean Aqua Cultured Species

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Abstract: Welfare assessment currently is less well-characterized for aquatic animals and the classical methodologies used for terrestrial animals are not adequate to improve our knowledge about fish well-being. Among different approaches, the status of organism responses can be carried out using different physiological and biochemical tools. Here, we present the state of the art regarding fish welfare, methodologies, and experimental results with a particular focus on two important Mediterranean aquaculture species, *Sparus aurata* and *Dicentrarchus labrax*. We introduce an approach using physiological stress-indicators, growth performance and swimming activity to investigate the effects of the implantation of electronic tags to facilitate the application of telemetry for aquaculture purposes. The application of telemetry to research on aquatic organisms has expanded recently, and its utilization needs to be better understood. The mentioned approaches have been discussed for application in different aquaculture methodologies. Moreover, social stress and territoriality are relevant factors in the evaluation of gregarious species that may have consequences on the conditions of animals farmed in captivity. These aspects, that may impair the ability of fish to respond to various stimuli or negatively influence the flesh quality, here are analysed through behavioural observation, flanked by the physiological and immunological approach.

Keywords: welfare; physiology; growth; telemetry; organic aquaculture; social hierarchy; territoriality; *Sparus aurata*; *Dicentrarchus labrax*

Key Contribution: The new insights presented affirm the validity of using the physiology to assess the welfare of farmed fish, thus contributing to the improvement and development of fisheries and aquaculture sector, and pointing to the need to analyse certain, sometimes underrated, aspects that can influence fish production and contributing to the achievement of the blue economy goals.

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1. Introduction

1.1. Welfare

The concept of “animal welfare” refers to the physical and felt well-being of animals, and its study has mostly developed during the last half-century. Human-animal interactions, in particular domestication and breeding, date back to ancient times. Humankind, indeed, has reared and domesticated animals, mainly birds and mammals, for millennia for different purposes, such us food, clothing, agricultural work, pets; but research centred on animals as sentient organisms capable of suffering, only began during the 20th century, probably due to our better understanding of animal motivation, cognition and behavioural complexity [1]. Nowadays, the need to improve the efficiency of this interaction is leading different kinds of stakeholders to reconsider the value of animal welfare. Research on animal welfare, which initially centred on livestock and laboratory animals, has been extended to fish, other vertebrates and even invertebrate groups (e.g., cephalopods, crustaceans and others) [2–5]. Unfortunately, the concept of animal welfare is not clearly defined, and different ideas have been proposed. It is generally associated with three different aspects of their lives: the organism's correct physiological functioning, natural environment and feeling/emotional state [6,7]. In the breeding sector, animal welfare is associated with the "Five principles of freedom" described by the Farm Animal Welfare Council [8,9], 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 express their natural behaviour. As indicated in Figure 1, welfare status can be imagined as the top of a pyramid resting on a base composed of the needs of fish that can broadly be categorised into different categories, and the fulfilment of these categories contributes to animals welfare.

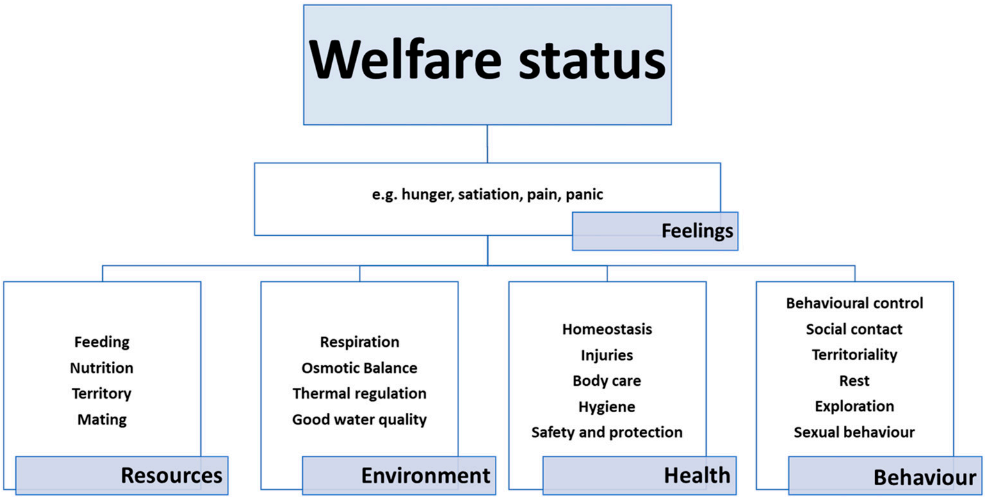

Figure 1. The welfare needs of fish can broadly be categorised into different categories. The degree of fulfilment of these needs affects their mental state and thereby the welfare status of the animals. This figure is based on information from several sources, including [10].

Among different stakeholders, governments have also recently started paying attention to the management of terrestrial farming ecosystems, as well as aquatic ecosystems and the welfare of farmed animals. In Europe, indeed, fish have only recently been included in the groups of animals considered sentient, along with mammals, birds and reptiles [11,12]. With the idea of including all aquatic taxa with human-interactions arose difficulties due to the lack of sufficient scientific evidence; thus, it became necessary to define what animal sentience means [13,14].

Different approaches have been considered for defining "animal sentience" in order to include different aquatic taxa under animal welfare regulations but, in some cases, like that of invertebrates, there have been some difficulties related to their application [13]. Some researchers have pointed to the investigation of the neuroanatomical structure of these animals in order to individuate the sensory neurons, called nociceptors, with the role of perceiving stimuli and responding to painful stimuli [7,15,16]. However, the ideas of pain, suffering and consciousness need to be better investigated and defined for humans, still 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 about it. Indeed, a number of abnormal behaviours indicative of animal welfare have been enumerated. With aquatic organisms, however, this approach is not always easy to apply; indeed, few species can be easily observed and their behaviour described [13]. Due to the vast number of aquatic taxa, both vertebrates and invertebrates, and the different inter and intra specific responses, studying the behaviour of each species becomes a very hard, laborious and time-consuming task. Despite the attempts of different authors to study this topic from different point of views [6–8,13,15,17–19], these studies are mainly based on neuroanatomical analogies between human and animals, mental capabilities, behavioural alteration, perception of pain and/or suffering. To obtain consistent data that allow us to properly evaluate the welfare of aquatic animals, while avoiding inconsistent outcomes, it is necessary to focus on objectively measurable welfare indicators such as behaviour, physiology, growth, fecundity, health and stress [20]. As a consequence, more species-specific research is required in order to correctly apply these indicators [21].

Nowadays, homeostasis is a well-known concept; it consists of a series of biochemical mechanisms devoted to maintaining the internal functional equilibrium of living organisms. These physiological responses, even though they correlate to the previously-mentioned processes (i.e., pain, sentience, suffering), are independent of them. It is possible to establish a baseline related to the welfare of a 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, biology and ecology of each species. Among all the different above-mentioned approaches, the evaluation of physiological processes should be considered a relevant field that deserves to be investigated and utilized in the welfare assessment of fish, in particular in the aquaculture industry.

Beyond the definition considered, the welfare of fish intended for human use is critically important for several reasons [22] and is a centre of interest for different sectors in which animals play a central role. Indeed, welfare 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 as their natural behaviour as an attraction for visitors [23]. Good conditions and welfare are of fundamental importance for experimental animals involved in scientific research, guaranteeing results that are not impaired by problematic factors [1,7,12,21].

In relation to fish farming, appropriate welfare conditions are of fundamental importance for aquaculture sectors. Aquatic animals that are not chronically stressed present better growth rates, are less prone to disease and the final product maintains high quality features [24]. Besides, avoiding unnecessary animal suffering during the capture, rearing, and slaughter of fish is important according to current ethical standards regarding the use of animals. Moreover, it is also critical for the economic implications for farmers, as fish growth is highly dependent on their welfare status; it is also important for the optimization of feed expenses, low disease-related care costs, and avoiding economic losses. Indeed, regarding the latter point, it is critical for aquaculture in terms of the slaughter of animals since the quality of the meat is affected when an animal is stressed before its death (e.g., low oxygen concentration, increased of pH) [25–28]. All the previous listed aspects are greatly affected by stress, and it is understandable that there is a shared interest amongst fish 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 maintain high flesh quality and, thereby, obtain a high market price. Recently, one more interest has been added which is related to a growing insistence by consumers that farmed organisms be well treated. All these reasons explain the increasing interest in fish welfare research, and 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 [29]. Unfortunately, despite these regulations, there are some slaughtering methods in aquaculture plants that use physiological responses against inducing exogenous stress, such as CO_2 narcosis or electricity, to increase the marketability of seafood products, causing a great source of avoidable stress, for stunning method in farmed fish to improve the colour of fillets [30].

The different arguments treated below, and schematized in Table 1, are articulated around the main topics related to fish welfare. Firstly, we present studies on the application of new telemetry tools and on the evaluation of alternative diets to improve the aquaculture sector, solving the newer challenges related to fish welfare. Subsequently, we examine the effects of social stress and territoriality on the welfare of farmed animals. Indeed, Section 2.1 introduces the use of stress and physiological markers in order to validate 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 section, medium-term data on physiological stress profiles and growth performance after surgical tag implantation are shown for gilthead sea bream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) [31]. Sections 2.2 and 2.3 focus 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. The diets administered in the studies differed in terms of raw protein, fish oil and lipid contents. Seabass welfare conditions were assessed in relation to these three diets using many different indicators in a multiparametric approach in order to obtain a comprehensive picture of the physiological state of the seabass [32,33]. In Section 2.4, 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 stimuli, such as pathogens or environmental variations. In this section, the effects of social stress on gilthead bream were investigated through biochemical and immunological-cellular parameters, 24 h after the establishment of a social hierarchy in a group of three fish, and the results 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 subordinate) [34]. Subsequently, social stress was investigated in depth along with the mechanisms involved in the establishment of hierarchy by using two experimental models which underlined the importance of territoriality, territorial exploration and sensitization in the formation of a hierarchy. To study the effects of social stress and territoriality, behavioural observation was used, integrated with the evaluation of cellular and physiological biochemical characteristics [35].

1.2. Current Knowledge Gaps Related to Fish Welfare

Despite fish welfare becoming a hot topic, currently there are a number of gaps in our knowledge about fish, both in wild and artificial conditions. As noted previously, several sets of recommendations or guidelines have been released, published by researchers [36,37] or other institutions such as the RSPCA (Royal Society for the Prevention of Cruelty to Animals) in the UK [7,38]. Certainly, the protection of fish by national regulations is not uniform, but it is essentially increasing and those guidelines may constitute the basis of new regulations for monitoring fish welfare in captivity.

Table 1. List of the paper and scheme of the mains results presented.

Paper	Species	Tools	Methods	Results
[31]	*S. aurata* *D. labrax*	Acoustic accelerometer tags	Telemetry; growth; physiological approach	No significant differences among the tagged and untagged fish groups.
[32]	*D. labrax*	Acoustic accelerometer tag; Blažka swimming chamber; diets	Telemetry; morphometry; physiological, immunological approach	Organic diet does not affect the welfare of the European sea bass. Previously calibrated acoustic transmitters are a promising
[33]	*D. labrax*	Different diets; radio transmitters; Blažka chamber	Telemetry; morphometric, physiological, immunological approach	EMG, recovery ratio, growth Cortisol, glucose, and lysozyme proved to be sensitive to assessing welfare
[34]	*S. aurata*	Aquariums-arena; recording camera	Behavioural observation; physiological, immunological approach	Links between behaviour, stress physiological profile and immunity, in relation to social hierarchy
[35]	*S. aurata*	Aquariums-arena; recording camera	Behavioural observation; physiological, immunological approach	Exploration time is fundamental for hierarchy; demonstrated social rank and physiological immunological profile relation

Current research is seeking to answer questions about the best welfare conditions for keeping fish in captivity—simple questions without easy solutions, for example: questions about the best conditions for maintaining fish; which indicators to consider in order to guarantee good welfare; how these indicators should be evaluated and quantified; the validity of these variables considering inter or intra specific variability; how to compare different groups of fish from different sites/farms and/or environmental conditions. Indeed, the principal difficulty is to identify reliable indicators that allow the evaluation of 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 challenge is to show the effects of chronic disturbances [39–41].

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" [42]. Homeostasis is the capability of organisms to maintain all the fundamental parameters which ensure survival and the proper functioning of vital processes (pH, osmolarity, energy metabolites, pO_2) at equilibrium. Its maintenance is of fundamental importance in restoring conditions after disturbance by a stressful event and deviation from the baseline. The system, regulated by biochemical reactions, involves enzymes, hormones, transporters and proteins and requires the synchronized action of allostatic changes enabling the return to optimal physiological levels [43,44].

Endocrine cascades control stress physiology in teleosts [45]. Physiological responses to stress may ideally be divided in three groups with sequential activation related to the intensity and duration of stress: primary, secondary and tertiary [46]. The main neuroendocrine pathways involved in fish stress responses and their effects are schematically represented in Figure 2. Here, blue and green lines indicate the brain-sympathetic nervous system-chromaffin cell axis (BSC, in blue) and the hypothalamus-pituitary-interrenal cell axis (HPI, in green) and the different types of responses induced after activation.

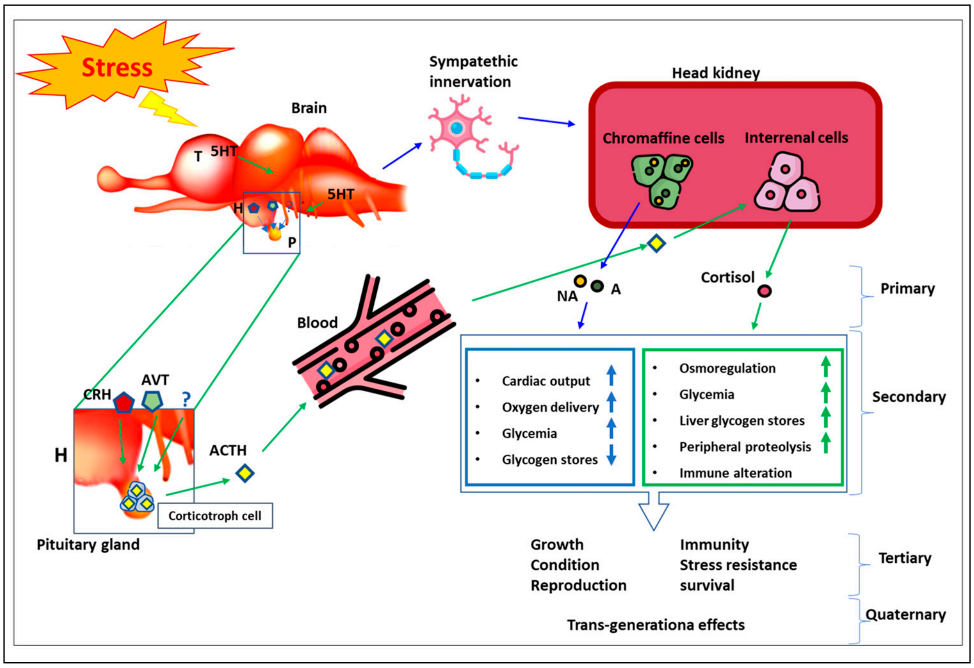

Figure 2. Main neuroendocrine pathways involved in fish stress responses and their effects. The two neuroendocrine routes are indicated by blue lines (the brain-sympathetic nervous system-chromaffin cell axis, BSC) and green lines (the hypothalamus-pituitary-interrenal cell axis, HPI). 5HT, serotonin; A, adrenaline; ACTH, adrenocorticotropic hormone; AVT, arginine vasotocin; CRH, corticotropin-releasing hormone; H, hypothalamus; NA, noradrenaline; P, pituitary; T, telencephalon. This figure is based on information from several sources, including [47].

The primary response to stress is initiated and coordinated by a two neuroendocrine axes, the hypothalamus-pituitary-interrenal (HPI) system and the sympatho-chromaffin tissues [17,48], and it includes the release into the bloodstream of neuroendocrine hormones such us catecholamines [49,50] and corticosteroids in vertebrates [15,51,52]. The presence of these hormones in the circulatory system induces the activation of secondary stress responses, including increased heart and respiration frequency rates and mobilizing energy metabolites to cover the demand for energy and oxygen imposed by the stressor [15,25,53–55]. If the stress is lasting, it can lead to the activation of 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 [15,55,56]. As a consequence, as described in Figure 3, teleost experience metabolic disorders, lower growth rates, immune-deficiencies, impaired development, reproductive disruptions and altered behavioural and social skills that clearly compromise their welfare and, in the worst case, lead to death [15,57].

1.4. Physiological Indicators

In the three different steps of the physiological response to stress, between the molecular and whole animal levels, it is possible individuate some indicators that can be used as tools for welfare evaluation. Is important to underline that the evaluation of a single indicator alone does not provide evaluable information and that it is better to integrate information obtained from the evaluation of various indicators. As reported in Table 2, several studies report a number of good physiological indicators. After HPI axis activation, a series of molecules identified as primary indicators (catecholamine and stress hormones) are mobilized, followed by secondary indicators (e.g., changes in glucose, ion balance, acid–base balance, immunological functions or other indicators of energetic metabolism) [42]. These indicators are useful tools for assessing fish welfare. The importance of choosing the appropriate indicators in relation to the stress suffered by the organisms is vital. 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 [50,58]; they can be used as good indicators in laboratory conditions but are inadequate for field investigations. Differently from catecholamines, cortisol, the so-called stress hormone, is the common stress indicator used. It responds more slowly than catecholamines and can be quantified under both laboratory and field conditions [46,48,58,59]. Cortisol can be used to obtain basal and post-stress levels and is also involved in different molecular responses. It has a role in the stimulation of the expression of several classes of proteins, such as metallothionein, ubiquitin and HSPs, by interacting with heat shock factors (HSFs) [60,61]. The stress hormone also binds with glucocorticoid receptors and interacts by activating transcription factors [62,63].

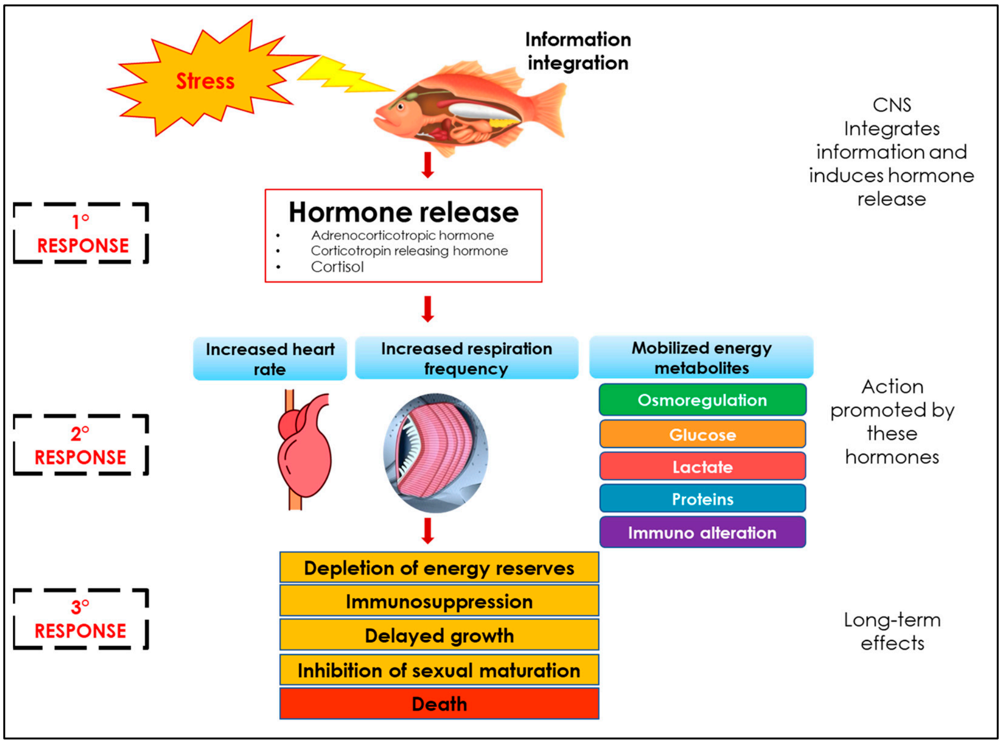

Figure 3. Physiological stress responses in aquatic animals. CNS, central nervous system. This figure is based on information from several sources, including [57].

The secondary indicators include glucose elevation, lactate elevation, changes in osmolality, specific ions and leukocyte activity. Glucose elevation is caused by increased catabolism and glucose release in circulation due to stress [46]; lactate elevation is related to anaerobic metabolism caused by low levels of oxygen in body tissues (hypoxia) or exercise stressors [64]; osmolality may be altered by the release of catecholamines and their effects on higher heart rate and gill permeability [65]; leukocytes, immune system cells, may reflect acute and/or chronic stress exposure with altered functioning [66]. The tertiary step, also called whole-organism, includes a plethora of indicators correlated to welfare status and fish conditions: growth, dimensions, weight, organo-somatic index, disease resistance, metabolism alteration, indicators of swimming activity, cardiac function, oxygen consumption, recovery ratio, behaviour and mortality [55,67–71]. Differently from primary and secondary stress indicators, which mainly relate to animal physiology, the tertiary stress response is mainly related to ethology and ecology, even though they are strictly correlated to certain physiological aspects, such as indicators related to cardiac activity and metabolism. Recently, the existence of a further kind of response, called quaternary stress response, has been proposed and which suggests that stress effects are trans-generational and transmitted to progeny through genetic and epigenetic mechanisms [72–74].

Table 2. Main physiological parameters of aquatic animal homeostasis. Adapted from (Jerez-Cepa and Ruiz-Jarabo, 2021).

System	Parameters	References
Acid-base balance	H^+, OH^-, $HCO_3{}^-$, $PO_4{}^{2-}$, $SO_4{}^{2-}$	[75–77]
Hydric-ionic balance	H_2O, osmolality, Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+}, others	[78–81]
O_2 (CO_2) transport	Haemoglobin/haemocyanin, haematocrit	[82–84]
Energy management	Glucose, lactate, amino acids, triglycerides, free fatty acids, etc.	[77,85,86]
Immune system (innate)	Physical barriers, cell-cell mediated defence (phagocytosis), humoral defence (antimicrobial enzymes, non-specific proteins, complement system), inflammation	[31,33–35,87–95]
Immune system (adaptative)	Cell-mediated defence (B and T lymphocytes)	[92]
Free radical balance	Oxidative stress system	[96–100]
Others	Hormones, temperature, etc.	[97,100]

1.5. Stress Assessment via Molecular Approach

At this point of this review is clearly established the role that stress play on the wellness of fish. Stressogenic stimuli, homeostatic perturbations, are detectable at the molecular level through the gene analysis of genes whose expression responds to an environmental stressor and are considered as "inducible" or on the contrary "constitutively expressed" gene. The main goal is to individuate and select an informative panel of such inducible genes that are quantifiable after induction following certain stimuli. Recording multiple, informative stress-biomarker genes gains more importance for the aquaculture industry with regard to fish welfare and consumer opinion. The physiological responses to stressors, including hormonal profiles and associated tissue responsiveness, main topic of this review, have been extensively studied in teleosts, but the molecular mechanisms associated with this adaptive response are not well understood. Studies of genes involved in stress responses and profiling is conducted in cortisol-responsive organs that are referred to as "frequent targets for stress responses" [101]. Among different organs, one candidate for this purpose is the liver. As liver is a key organ for metabolic adjustments to stressors and also is a major target for cortisol action, the genomic studies about stress and glucocorticoid regulation addressed this organ. Different studies have identified several genes that are up- or down-regulated after an acute stressor exposure in fish [101,102]. Gills are also retained as organs sensitive to and have been considered in several investigations of stress in teleost [103–107]. It is important to studies genes that are well-known responders to stress in general. As our interest is in molecular responses linked to cortisol triggered pathway in fish, we should focus on gene expression changes after acute or chronic stressor exposure. It is well known that acute stressor exposure causes transient elevation in plasma cortisol levels, which is re-established during recovery from stress in fish [108]. In teleosts, are involved in cortisol signalling pathways different glucocorticoid receptors (GRs) and one mineralocorticoid receptor (MR) [109,110]. On ligand binding, cortisol-GR complex translocates to the nucleus and binds to specific DNA region activating or repressing the glucocorticoid responsive genes [109]. Despite several studies have utilized mammalian models, only few studies have been carried out in fish using physiologically relevant models [63,110,111]. A further well-established metabolic response to stress is elevation in plasma glucose concentration. One of the features of liver metabolism is de novo glucose synthesis to provide glucose for extra-hepatic tissues during stress [48]. The rapid output of glucose in response to stress is involve adrenergic signaling and activation of the glycogenolytic pathway, the maintenance of plasma glucose levels, involves upregulation of the gluconeogenic pathway [48,112]. Hence, several genes encoding proteins are involved in glycolysis and gluconeogenesis during stressor stimuli or stressor condition. The fundamental regulatory enzymes involved in gluconeogenesis pathways are phosphoenolpyruvate carboxy-kinase and glucose-6-phosphatase. Indeed, transcripts for both these enzymes were shown to be elevated, in conjunction with enhanced glucose production, during recovery from an acute stressor exposure in *Oncorhynchus mykiss*, linking gene expression to functional changes in liver gluconeogenic capacity [113,114]. These results confirm the upregulation of enzymes activated during gluconeogenesis as a key aspect of the stress during recovery process. While lactic acid, glycerol and amino-acids (aa) are substrates for gluconeogenesis in the liver, aa from peripheral proteins stores are the principal substrate for stress-induced gluconeogenesis in teleost [48]. Hence, glutamine synthetase, arginase and cathepsin D transcripts were upregulated in liver after stressor exposure, supporting upregulation of the proteolytic pathways [113]. Globally analysed, these results suggest molecular regulation of enzymes fundamental for energy substrate mobilization and utilization during stress adaptation in fish. Muscles, among the already mentioned, are target tissue good to investigate the stress molecularly. In a study on transport stress on *Argyrosomus regius* the authors Bortoletti and colleagues [115] among the different organs, considered liver and muscle the sites of choice for evaluating its gene expression as they are directly and strongly involved in protein catabolism and gluconeogenesis. The aim of the study was to investigate the muscle cortisol levels, the expression of glucocorticoid receptor in muscle and liver coupled with the cellular distribution of heat shock protein 70, 4-hydroxy-2-nonenal, nitrotyrosine and 8-hydroxy-2′-deoxyguanosine in several tissues by immunohistochemical methodologies in the teleost fish meagre (*Argyrosomus regius*) exposed to transportation stress. The cortisol level trend observed in this study is highly coherent and supported by the molecular results. In fact, has been evidenced that the highest expression of glucocorticoid receptor gene was detected in muscle of animals just after loading on the truck. These results confirm the correlation between cortisol and the intracellular glucocorticoid receptor levels that leads to an increase in the GR mRNA levels [112,116].

The recent diffusion of cDNA microarray technology revolutionized the field of functional genomics by revealing global gene expression changes and consequently also in response to stressor exposures including fish [117–120]. Recently, several species-specific cDNA microarrays have been developed for teleosts and increasingly being used to reveal global gene expression patterns in response to stressor exposure and/or hormonal treatment [121]. Transcriptome profiling demonstrated to be a reliable methodological approach in functional genomics and gene profiling with reference to cells, tissues, organismal systems, or physiological conditions [122,123]. In the last years, in the framework of next-generation sequencing (NGS) technology has been developed methods for quantifying transcriptomes [124,125] and studies aimed to understand the response to stress at the transcriptome level are helpful to comprehend the molecular basis of adaptations.

1.6. Stress Assessment via Histological Approach

Stress and its effect on organisms may be further investigated by the histological approach analysing the status of organs and tissues [98,126–128]. The histology approach, indeed, has been mainly utilised in studies on wild fish [128–134] but rarely has been applied in studies on farmed fish [135,136]. The histological examens can be carried out on different target, tissues or organs according to the different goal of the studies. The sample, pieces of gills, liver, kidney, intestine skin etc. are fixed in fixative solution, embedded in paraffin and further processed for histological analysis by a microtome obtaining section of 5µm thickness and subsequently stained. Histological changes can be assessed and classified according to different categories. For example, Saraiva et al. (2015) [137] used the semi-quantitative system proposed by Bernet et al. (1999) [138]. Briefly, according to this method, histological changes are categorized into different reaction patterns such us circulatory, regressive, progressive, inflammatory and neoplastic and each pattern may contain different alterations per organ. These alterations are assessed individually using a score value ranging from 0 (unaltered) to 6 (severe/diffuse occurrence) and to each alteration can be assigned an importance factor ranging from 1 to 3 (from minimal pathological importance to marked pathological importance). The gills are the most delicate structures of the teleost and are very sensitive to environmental conditions and pathogens, including parasites. Many studies report histological alteration caused by different factors both biotic and abiotic on the gills. Example of biotic factors are the parasites, e.g., *Diplectanum aequans,* members of the class Monogenea, is likely the most common detected parasite on gilt-head seabream that causes frequently hyperaemia, haemorrhages, oedema, hyperplasia, leucocyte infiltration and gill necrosis [139–142]. The parasite copepod *Lernanthropus kroyeri* causes oedema, hyperplasia and necrosis [142,143]. Among the abiotic factor that cause the same alteration on the gills can be addressed toxic pollutant present in the waters such as cadmium and herbicide [144]. About kidney, Kurtovic et al. (2008) [145] reports to a higher number of melano-macrophage centres and atrophy of glomerulus, typical of the organ examined from farmed seabass. Also extensive administration of antibiotic therapy is considered cause of renal tubular degeneration and circulatory disturbances like haemorrhages [146,147]. Necrosis of renal haematopoietic tissue occurs in several biotic and toxic situations [146]. Nephrocalcinosis is frequently associated with fish farming, and commonly addressed to insufficient levels of calcium and magnesium administered through alimentation, exposure to high levels of CO_2, excessive use of antibiotic [146]. In the liver, cytoplasmatic hepatocytes alteration is a very early and unspecific signal of disturbance [148]. Many studies report that fish exposed to different kind of toxicant manifest hypertrophy, vacuolar degeneration and increase of lipid droplets in hepatocytes [148–151]. Similar effects has been observed in farmed fish feed with inadequate commercial feed which causes lipid droplet accumulation, hepatocyte membrane disruption and vacuolization causing further circulatory disturbances [135,152,153]. One other important tissue for histological studies is the intestinal epithelium. It is an important site for the absorption of nutrients, it is involved in immunity, in osmotic balance and in recycling of enzymes and macronutrients [154–156]. The indiscriminate use of vegetables matters in the food for farmed fish can impact the gut integrity favouring the deleterious effect of gut pathogens [154,157–160]. Integrity and health of intestine is a key factor for the growth and welfare of farmed fish [126,146,154]. Among the different parasites, in European seabass, *Shaerospora dicentrarchi* and the myxosporean *Enteromyxum leei* cause serious disorders in intestine [161–163]. Refaey and colleagues investigated the relationship between the physiological changes and the growth at different stocking density of channel catfish (*Ictalurus punctatus*) studying the structure of the intestine [164]. They have found that the overall structure of intestine was affected by the high stocking density of farms that caused shortening of villi length and reducing numbers and sizes of goblet cells, affecting the absorption surface of the intestine of fish respect to those reared in medium or low stocking density [165]. Conforto et al. [166] investigated histologically the effect of bacterial infection on different tissues: gills, liver and skins. In this study have been observed different, vasodilation with blood congestion, epithelial uplift, lamellar disorganization, and aneurysm of the primary filament, in the gills of European eel, *Anguilla anguilla,* challenged with *Vibrio anguillarum*. Therefore, has been shown that the bacteria infection damaged epithelial cells [167]. In eels challenged with *Tenacibaculum soleae*, cellular hypertrophy and hyperplasia, and partial fusion of secondary lamellae were observed. Eels showed also similar cytopathic effects of *Senegalese sole* challenged with *Tenacibaculum maritimum* [168]. The liver of eel challenged with *T. soleae* showed cells with irregular nuclei, sometimes hypertrophic coherent with the lesions reported in hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) challenged with *Edwardsiella ictalurid* and in the liver of Koi carp (*Cyprinus carpio*) infected with *Myxobolus* sp. parasite [169,170]. In the same study eels infected with *V. anguillarum*, was observed a general loss of hepatic tissue structure and an increase in hepatic sinusoidal spaces and the presence of pyknotic nuclei in hepatocytes. Regarding the skin both pathogenic bacteria, *V. anguillarum* and *T. soleae* induced only an overspread presence of goblet cells [166].

The advantage of the use of histopathology as biomarker lies in the possibility to be coupled with other approaches like the molecular and the physiological approach that is the main topic of this review. However, it is fundamental to consider that healthy fish are not characterised by absence of pathology identifiable by histology, but that they may present mild structural alteration or moderate inflammatory reactions [171].

1.7. Social Stress and Adaptative Stress Coping Styles

Corticosteroid hormones play a central role in behavioural and neuroendocrine control in vertebrate species [47,172]. Cortisol, the major stress hormone in fish, plays a pivotal role in stress response through its action on both aerobic and anaerobic metabolism, osmoregulation, carbohydrate metabolism, immunity and appetite [48,173]. 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 farmed fish, such as reduced immune competence, reduced growth, flesh quality or impaired reproduction [174,175]. For this reason, the mechanisms involved in stress coping strategies in fish have been receiving significantly more attention. Most of the responses in organisms are species specific; therefore, it is necessary to investigate responses in different reared species.

As mentioned below, repeated contact between conspecific fish does not cause habituation [176], and prolonged stress exposure could ultimately trigger the tertiary stress response, with different and severe consequences on animals and aquaculture services. In artificial environment fish are often restricted at high densities to limited and crowded spaces and forced to continuous contact, with the consequent increased social interaction, including the aggressive ones, risk of disease outbreak, competition for the resources and accumulation of organic waste dissolved in the water. The social environment, indeed, can be a considerable source of stress, and social relationships can impact both mental and physical health [177] affecting numerous physiological functions, including hematologic features [46], metabolism [71,178,179] immunity [180], behavioural responses [181] and ultimately performances, such as growth and reproduction [67,182]. Animals are often organized into territories and interact socially to establish and maintain hierarchical dominance ranking [177]. The intraspecific social interactions in many animals are primarily structured around dominance relationships or hierarchies in which an animal's position within the hierarchy determines access to resources, such as food, water, space and, ultimately, individual fitness and/or reproductive success [183]. 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 [184]. These relationships can affect physiological status and animal responsiveness [57]. In nature, the development of dominant–subordinate relationships and the onset of hierarchy as an adaptative strategy may act to reduce aggression, but in farmed fish, due to the continuous stimuli, it may cause chronic stress, compromising fish welfare. High stocking density which causes chronic crowding stress in fish and potentially affecting fish health reducing growth and affects the immune system in several species. High stocking density is an aquaculture-related situation and the effect on the growth performance, physiological and immunological response of fish may vary depending on many factors such as fish species, body size, age, rearing condition, and so on. And the adverse impacts of high stocking density have been reported on some fish species like blunt snout bream (*Megalobrama amblycephala*), Amur sturgeon (*Acipenser schrenckii*), turbot (*Scophthalmus maximus*), juveniles of thick-lipped grey mullet (*Chelon labrosus),* Atlantic salmon (*Salmo salar*), common carp (*Cyprinus carpio*) or gilthead seabream (*Sparus aurata*) [185–193]. Aggressivity, among the others effect caused by supernumerary specimens held at high density (e.g., space limitation for swimming in no adequate rearing tanks, or, poor water quality, malnutrition or scarce feeding) has been linked to several issues in aquaculture, such as decreased feed intake, growth dispersion, chronic stress, immune-compromission and consequently disease vulnerability, up to physical aggression and cannibalism behaviours (e.g., carnivorous fish manifest cannibalism at a high rate as an aggressive behavior if they are not fed properly or sort in aquaculture sectors) [194].

Therefore, understanding the aggressive behaviour of farmed fish, as well their social interactions, is of great importance for aquaculture practice in order to improve both animal welfare and productivity [195] in a historical moment in which increasing consumer awareness of sustainability, safety and quality issues is driving demand for traceability systems and certification schemes for a growing range of fish and fish products.

During the last few decades, great interest has been directed toward the causes and consequences of consistent individual behavioural and physiological variations, leading researchers to postulate the existence of stress-coping styles (SCSs): "a coherent set of individual physiological and behavioural differences in stress responses consistent across time and context" [196]. They have been described in various animal species, including fish, as a continuum between two extreme phenotypes, called proactive and reactive (proactive BOLD and reactive SHY) [197–200]. From a behavioural point of view, proactive fish are generally bolder, more active and aggressive than reactive ones [197–199,201,202]. These divergent behavioural responses are generally correlated with the physiological mechanisms of stress response [197–199,201,202], resulting in distinct adaptive responses to cope with stressors. In a recent study, Carbonara et al. (2020) described physiological performance using several indicators (e.g., haematocrit, cortisol, adrenalin, noradrenalin, glucose, lactate, lysozyme), including growth performance and swimming activities of gilthead sea bream at different stocking densities, depending on SCSs. Overall, personality screening using the risk-taking test made it possible to highlight the physiological differences between sea bream from divergent SCSs. These physiological divergences between SCSs, mainly highlighted using the Principal Component Analysis (PCA) approach, seem to be progressively lost in response to stocking densities. They are nevertheless important in highlighting differences in the physiological parameters (noradrenaline, lactate, glucose, and red blood cells count) of fish in response to these different stocking densities by using the PCA approach. Moreover, the swimming activity of sea bream was different regarding SCS and stocking densities. Given that higher swimming activity is linked to a higher cost of life, the results suggest that SHY individuals are more able to cope with higher densities, while BOLD individuals are more able to cope with low densities. This observation agrees with the idea that BOLD behaviour (and the other characteristics linked to a proactive coping style) is more adaptive to a stable environment, while SHY behaviour is adaptive to a fluctuating environment (possibly, low vs. high density). The personality analysis may be helpful in the aquaculture context by selecting the most adapted fish to the rearing conditions (i.e., bold in low density and shy in high density), even though no differences were observed in terms of growth performance as it relates to SCS. SCSs also play a role in the stress/welfare state at the individual level. The two divergent phenotypes show a different sensibility to stressors [198,199,201–203], with significantly different physiological responses [204].

1.8. Aquaculture: The State of the Art

The main challenge that the world's fisheries and aquaculture (F&A) industry is facing is growing demand due to constant population growth in a context where environmental pressure and social imbalances are becoming more and more serious. United Nations Sustainable Development Goal (SDG) 14, called "Life below water" [205], established goals for the contribution of F&A to food security and nutrition in order to gain different benefits and ensure economic, social and environmental sustainability [206]. Globally, catches in the fisheries sector have remained constant during the last few decades, while an increase in fish production, thanks to the rapid growth of aquaculture, has been registered: indeed, while aquaculture provided only 7% of fish for human consumption in 1974, due to increasing demand for fish products and a decrease in natural resources, this share has increased to 50% of total fish production in recent years [207,208]. Total European fish production by aquaculture was estimated to be 2,875,732 tons in 2021. The main species produced are salmon, trout, sea bream, seabass and carp, which represented 95% of total European production in 2020. The following graphic in Figure 4 provides an overview of

European producer countries and the production, in tons, in the years between 2015 and 2021, evidencing a constant increase [209].

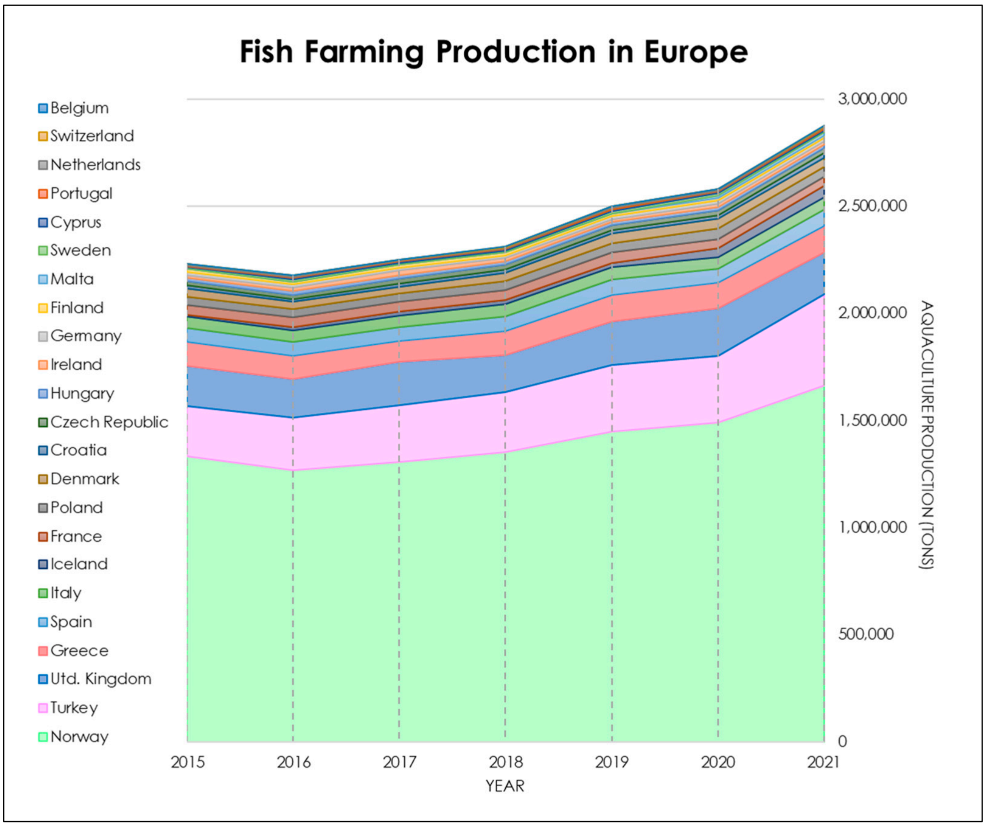

Figure 4. Fish farming production in Europe countries in recent years This figure is based on information provided by the FEAP Member Associations or national authorities and from [209].

Consequently, as described above, 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 [210,211]. In captivity, the environment available for rearing fish is very different from the environment in which their wild counterparts live [212]. 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 activities and often restricted at high densities to 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, from physiology to behaviour and biological performance [6,24,42,213].

Figure 5 indicates the marine Mediterranean production in 2020 per country and species, detailing the Mediterranean farmer countries and the percentage of the species mainly raised. The two most important fish for European aquaculture which are mainly raised in the Mediterranean region are the European seabass and the gilthead sea bream [214], which are also the two species involved as a model in the following studies.

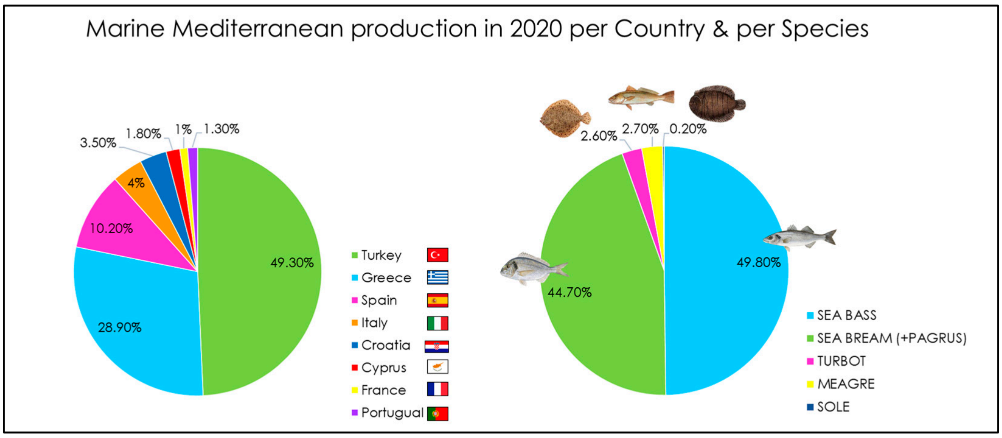

Figure 5. Marine Mediterranean production in 2020, per country and species. This figure is based on information provided by the FEAP Member Associations or national authorities and from [209,214].

2. Fish Welfare Assessment

2.1. Telemetry as a New Tool in the Field of Fish Welfare Assessment

One of the first difficulties to tackle is acquiring information and data on the animals. To date, human knowledge of animal farming and captivity conditions has been obtained mainly from direct observation and interaction with terrestrial animals and, as can be imagined, many difficulties arise when obtaining direct information from populations of animals living underwater to evaluate their health status [215]. To overcome these difficulties, technologists have developed identification systems to facilitate the collection of the required data, finding acoustic telemetry to be well-suited to the task. Acoustic telemetry is a widely used method for terrestrial animals which has recently begun to be applied to underwater environments. It is a method for remote sensing in which individual fish are equipped with electronic transmitters containing sensors that measure variables and transmit the information to data receiver units by acoustic signals; Figure 6 presents a simple representation of the basic approach, in which an acoustic tag records and transmits information to an acoustic receiver.

Aquatic organism telemetry has significantly advanced over the past few decades in terms of tag size, battery life, software and hardware [216]. The characterization and monitoring of behaviour in a variety of organisms, including fish, can be done with the use of these tags [217]. Moreover, environmental sensors that can capture a variety of information, including temperature, depth, and salinity, as well as physiological characteristics like heart and breathing rates or muscle activity, can be added to electronic tags [178,218–220]. Although these physiological sensors have primarily been applied in the wild for the sake of ecology and conservation, they have increasingly been used in aquaculture as welfare indicators of common stressors (such as slaughtering procedures, water quality and stocking density) [178,204,221–224].

Telemetry studies assume that all tagged fish are physiologically typical of the population. The tag, and the surgical implantation, must therefore not have a negative impact on growth performance, physiology or survival. For tagged fish to maintain their physiological state, normal movement patterns and growth performance, as well as to prevent bias in the data collected, the implantation method, place and tag size are crucial considerations [225–228]. The "2% rule" states that the maximum tag weight should not be greater than 2% of the fish's dry body weight [225,226]. The "2% rule" may not always be sufficient to prevent detrimental consequences on a fish's health and welfare, such as stress, inflammation of internal organs, obstruction of internal organs or negative impacts on buoyancy and swimming performance [225,229]. Stress is specifically defined as "a situation created by a factor (a stressor) that stimulates an endocrine response (e.g., cortisol release) that could be favourable as well as detrimental" [45]. Hence, the surgical insertion of an electronic tag, as can be observed in Figure 7, may cause stress in fish as described above even if it is carried out with the appropriate procedures. Further research on specific species is required, as the majority of our understanding of the relationship between the surgical implantation of electronic tags and stress is based on salmonids [229–231].

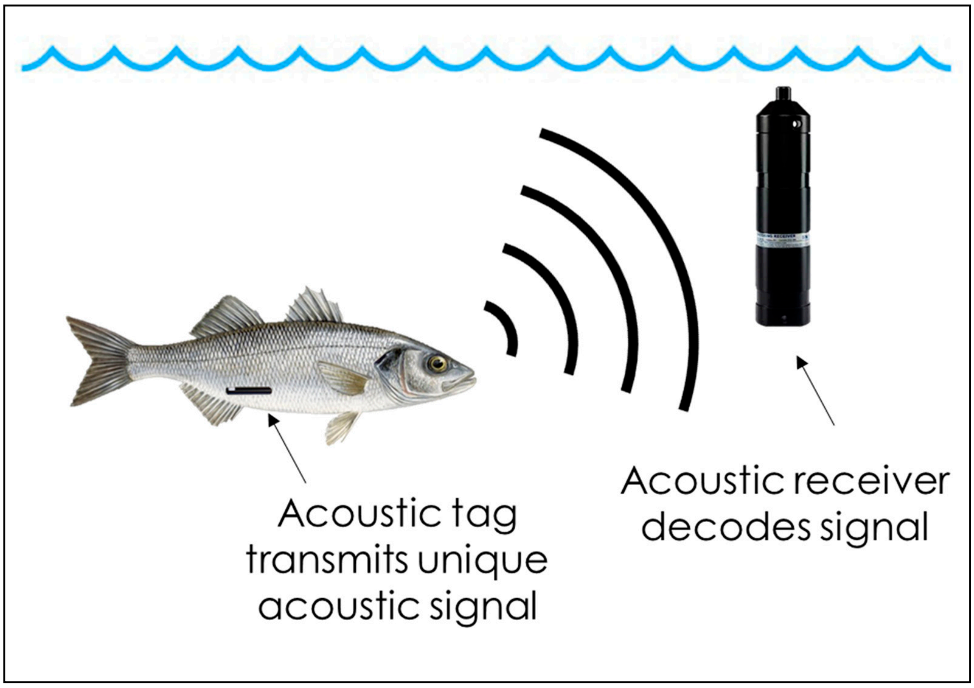

Figure 6. Schematic representation of the signal transmission from an acoustic tag to an acoustic receiver.

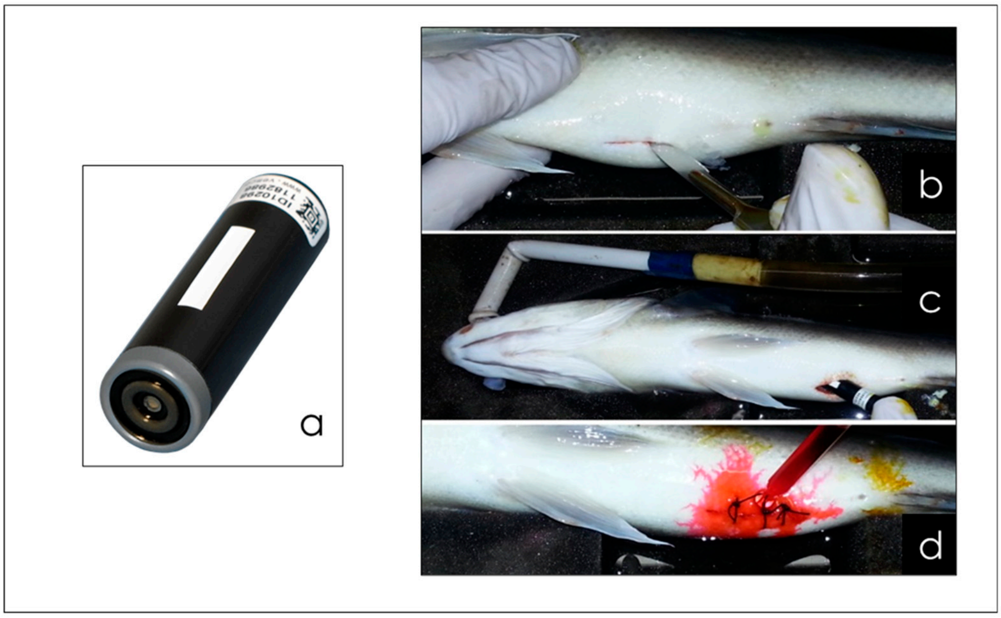

Figure 7. Surgical placement of the acoustic transmitter (**a**, at left) in the abdominal cavity. (**b**) ventral opening by scalpel; (**c**) placement of the transmitter; (**d**) closure of the cut with stitches and application of disinfectant.

In our recent studies [31,32], we have confirmed (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 after medium-long periods post-surgery for sea bream and seabass, respectively, as they displayed growth and physiological parameters comparable to those of untagged fish. In conclusion, the surgical implantation of accelerometer tags does not cause medium-term changes in the physiological stress profile and growth of either sea bream or seabass reared in a controlled environment. Future studies are needed to investigate exactly how long these species take to recover from the stress induced by tag implantation and thus be considered "normal" fish, displaying normal behaviour (e.g., feeding) and basal levels of stress indicators. Figure 8 shows the timeline of the period between the implantation of the accelerometer tag and the sampling point after 46 and 95 days, and the indicator used in [31] to assess the impact of the implantation.

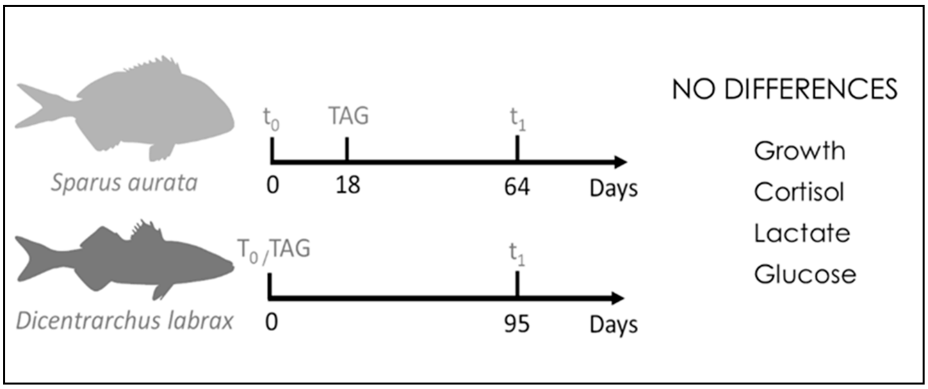

Figure 8. Timeline of the experimentation and results obtained.

2.2. Application of Telemetry for Fish Welfare Assessment in Aquaculture Farms

Due to an increasing demand for fish products and a decrease in natural resources, the aquaculture sector has grown rapidly over recent decades and now represents more than 50% of total fish production [207]. Fish production from organic aquaculture has also increased rapidly [232,233]. 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 [233,234]. However, the replacement of fish protein in feed formulations must be measured, as total substitution can disrupt physiological processes and growth performance [235,236], causing fish health and welfare issues, mainly in the farming of carnivorous fish species (e.g., seabass and rainbow trout, *Oncorhynchus mykiss*). Besides the development of aquaculture, fish welfare has also attracted increased attention from both consumers and governments and has become a critical point to consider in the growth of this sector [237–239], especially organic aquaculture [240].

However, assessing fish welfare is complex and requires an integrative overview, from physiology to behaviour and biological performance [6,213]. Overall, fish welfare can be closely linked to stress [24,42,241]. Thus, plasma cortisol, the end product of the HPI axis, and secondary stress response indicators, such as blood glucose and lactate levels, may be used as welfare indicators [46]. However, stress does not always mean compromised welfare, as it can also be a response to predation, competition or environmental changes [45,46,242]. 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 [173,242–244]. Consequently, many innovative approaches using spontaneous swimming activity and/or behaviour as a reliable proxy of fish welfare have been developed [18]. This can be achieved using traditional video recordings [34,35,245–248] or by acoustic transmitters that record several variables, such as positioning, speed and acceleration. Having previously been widely used for monitoring natural fish populations for conservation purposes [249], these transmitters are now being increasingly used in aquaculture contexts for fish welfare monitoring [33,204,222,250,251]. Indeed, their use appears promising for monitoring welfare, as they allow the evaluation of the behaviour of free-swimming fish over long periods [252] without affecting welfare and biological performance [31,253].

The signals recorded by the transmitter can be calibrated beforehand, along with other physiological variables, such as muscle activity and swimming performance, thereby increasing the power of the physiological data obtained [254–258]. Swimming performance and aerobic/anaerobic metabolism are of primary importance in assessing the physiological state of fish and their ability to cope with stressors [224,242,256,257,259]. 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) [260]. The MMR indicates the capacity for energy usage by aerobic pathways under different environmental conditions [260,261]. Previous evidence on European seabass has shown that the MMR is generally achieved before the Ucrit [256,262], suggesting that near this threshold, the supplementary energy requirement necessary to sustain the increased 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 seabass is reached, and fish use anaerobic metabolism to display a burst swimming mode, and the oxygen consumption rate (MO2) usually levelled off or decreased slightly. This state may be detrimental to fish health and welfare if repeated or sustained for a long time, as observed by Carbonara et al. (2015) regarding high stocking density.

Moreover, environmental conditions, including food availability, have an impact on fish activities and performance [33,263]. 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 [222,256]. 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 [264]. On the other hand, white muscle recruitment, supported by anaerobic metabolism, follows an exponential pattern [256,265]. 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 [266]. In the European seabass, it has been observed that red muscle activity increases with speed to a maximum and is maintained at that level until the end of the Ucrit [256], following a classical activation pattern. On the other hand, white muscle activation follows an exponential pattern, with the increase starting at approximately 65% of the Ucrit to compensate for the reduction of red muscle activity recruitment [256]. Thus, information about swimming performance 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 their diet regime. Helping to tackle the main obstacle of the lack the exact behavior of fish, in different conditions, due to their high diversity and diversity of response.

It has been demonstrated that certain physiological indicators of health and welfare on European seabass, in particular swimming activity and growth performance, can be used for evaluating the welfare after two different feed regimes (conventional and organic). Swimming activity was measured using acoustic transmitters previously calibrated with swimming performances during a Ucrit trial, allowing better qualification of swimming activity during the experimental period according to 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 for the European seabass, one of the most important farmed fish species of European marine aquaculture. The different diets did not seem to affect seabass health and welfare since 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 to aerobic and anaerobic metabolism. Real-time monitoring of fish behaviour and physiological state offers new possibilities for welfare monitoring in the aquaculture sector [221,250,251,267], especially with recent advances in data transmission through acoustic instead of radio channels, which provides greater applicability on production scales [252]. Second, based on all the indicators considered, a well-balanced organic diet does not seem to negatively affect the health and welfare of the European seabass, which suggests that organic aquaculture may address challenges of the sector without compromising fish welfare [32,33].

2.3. Multi-Parametric Approach Applied to Organic Aquaculture for the Evaluation of the Effects of an Enriched-Organic Diet Composition

As mentioned above, organic aquaculture is an alternative mode of production that combines environmentally friendly practices, the maintenance of biodiversity, preservation of natural resources, high animal welfare standards and production methods in line with defined standards of quality, using natural substances and processes [268]. Organic feed is composed of a greater percentage of proteins and oils from land-based agriculture, combined with natural antioxidants [233,234]. The partial substitution of fishmeal and fish oil with plant proteins and oils was found to be a promising alternative to the fish protein (and oil) already adopted for commercial diets [234,269,270], while total substitution was found to disrupt physiological processes and growth [235,236]. Currently, soybean meal is the predominant choice in terms of a vegetable protein source, considering its relatively high protein content and suitable amino acid profile [271]. Nevertheless, some limitations still exist regarding the soybean meal percentage that can be tolerated in fish feed formulations, especially for carnivorous fishes. For European seabass, a key species of European marine aquaculture [272,273], it has been shown that a partial substitution of fishmeal with raw plant material can be used, showing interesting results concerning physiological and growth performance, along with improved flesh quality [274,275]. To do so, a holistic approach [33] 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 7-ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferase (GST) enzymatic activity as an index of the functionality of the hepatic microsomal mixed-function oxygenase (MFO) system [276,277] in order 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 in quantifying welfare in organic aquaculture. In conclusion, both in terms of growth performance and physiological welfare status, this study supports the transition towards organic aquaculture for European seabass, 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 [278]. The multi-parametric approach has enabled us to outline a comprehensive picture of the physiological state of seabass fed with three different diets. Even though not all of the sixteen parameters gave globally consistent responses, 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 assessing welfare conditions [88,178]. Other physiological indicators, such as cortisol concentration, glucose or lysozyme, are important for welfare assessment, even though these parameters are highly variable (e.g., for cortisol) [173]. Finally, the PCA and MCDA methods appear to be powerful tools for assessing welfare in aquaculture using a multi-parametric approach, as recommended by Huntingford et al. [6].

2.4. Social and Spatial Stress Effects on Sea Bream Welfare

In the aquaculture framework, the environment available to reared fish is very different from the environment in which their wild counterparts live [212]. 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 living environment is much simpler, and fish may be disturbed by rearing activities and being restricted at high densities to limited and crowded spaces, with the consequent risk of disease spreading 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 a matter of increasing public concern [210]. The increasing importance of fish welfare in aquaculture comes from ethical considerations as well as from the perspective of improving standards from an economic point of view and the quality of fish production technologies and aquaculture products. One further aspect to consider when evaluating aquaculture for creating optimal conditions is the behavioural profile of the reared species and the interaction between animals in artificial environments [279]. In gregarious species, the social organization is retained a considerable source of stress [177] especially in the species where the individuals are organized into territories, with an established hierarchical ranked organization. In such cases, social stress can be considered the result of the physical contact between animals (high density and agonistic interaction) and psychological components, such as hierarchical instability and submission. This stress can affect several aspects of vertebrate physiology, including allostatic response in terms of the body expenses during stressful situations (chronic stress) [280]. Studies on fish have obtained similar results to those produced in other vertebrates [281,282]. Indeed, studies have been carried out on primates and small laboratory mammals under acute stress conditions, e.g., animals subjected to strong stimuli for a short period, and the behavioural and physiological responses were monitored during and shortly after the removal of the stressor [283].

Social interactions are structured around relationships of dominance and hierarchies in which the individual at the top occupy the most profitable positions [284] governing for the access to resources such as water, food, space and, individual fitness and/or reproductive success [183]. Dominant-subordinate relationships can have consequences on the physiological status and responsiveness of an animal [285]. Responses to social stress depend on the characteristics of the species, such as sex and age [286]. During social interactions, individuals receive multiple forms of sensory information and use these signals to establish and maintain dominance hierarchies [184,287]. Social interactions between conspecifics are, at least for some fish species, dynamic processes where subordinates frequently try to revert the role and dominants try to maintain their status. These interactions usually take place by using direct attack or displaying signs to the others [288]. The "winner effect" of dominant individuals underlies a significant probability of winning ulterior encounters [289]. On the contrary, the defeats experienced by socially subordinate fish could determine the activation of secondary and tertiary stress responses [87,290]. In addition, alterations in subordinate fish might affect appetite inhibition, reducing food intake, and consequently, limit the energy available for biological processes, like growth [67], aggression reduction [291,292] and decreased reproductive behaviour, impairing fitness [42]. Moreover, contact between conspecific fish does not promote habituation [176,284,293,294]. Variations in aggressive behaviour and stress physiology [238], and the formation of a social hierarchies associated with widespread physiological differences between individuals, are used as welfare indicators [295]. Dominance and social rank are inextricably linked to the regulation of testosterone and cortisol hormones [296]. Plasma cortisol levels appeared to be a heritable trait and could exert permissive, suppressive or stimulatory effects in vertebrates [297–299].

In our research [34,35,87], social stress and its effects on reared animals has been investigated using *Spaus aurata* as a model of study. In this species, the overall dominant fish carry out more aggressive acts and bite at food more often than the subordinates (Figure 9), resulting in a higher relative specific growth rate. Direct competition for food is supposed to be the the major social mechanism which regulate individuals growth in limited groups composed by small number of juveniles of this species, when food is scarce and defendable [300]. Recently, Ref. [301] showed that the effects of social stress could be limited aged by aquaculture management. Therefore, comprehending the connection that exists between aggression—feeding behaviours—social hierarchy—stress physiology of sea bream is of great importance for the aquaculture industry, in order to finding and optimize the best farming practices for the species [302]. In Cammarata et al. (2012), social stress was investigated using paired fish and indicating the established hierarchy between two specimens of gilt-head bream, showing 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. In Dara et al. (2022), the introduction of one extra specimen into the experimental design, with respect to the previous work, increased the complexity of the interactions among the individuals, but it confirmed the establishment of a hierarchy obtained through aggression and feeding priority, confirming the stress pattern previously observed: in small groups of fish, stress is related to social position, as schematically represented in Figure 9, and the fish at the bottom of the hierarchy are affected by high stress levels, whereas the specimens at the top experience low stress levels.

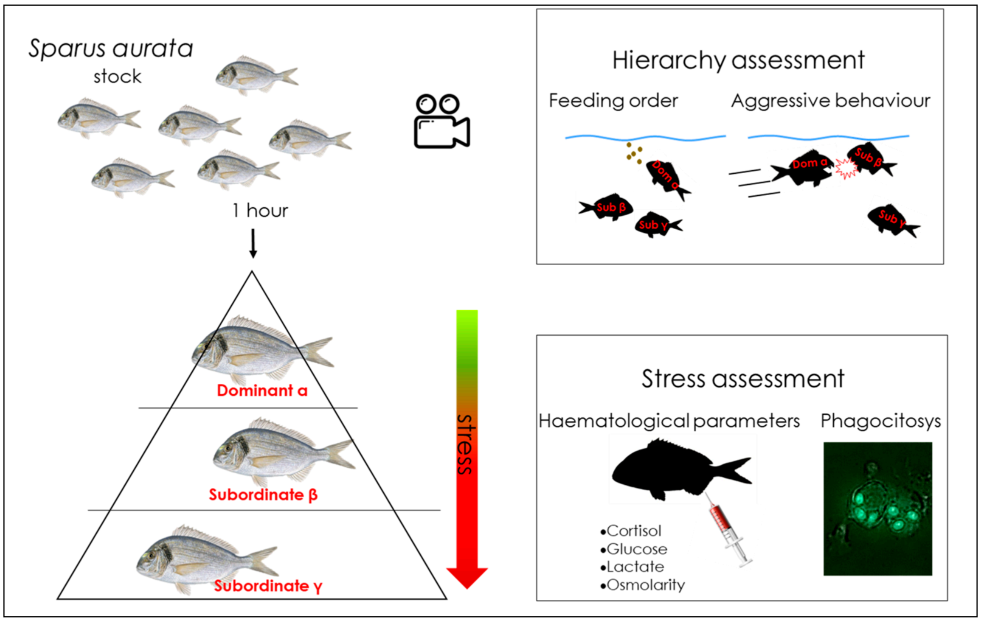

Figure 9. Schematic representation of hierarchy establishment, the assessment methods of social rank and stress markers used for welfare assessment.

In Dara et al. (2023), the role of environmental sensibilization on the determination of hierarchy was explored using two experimental models in which the fish are either inserted in a time-sequential manner (sequential model) for territorial evaluation or simultaneously (simultaneous model) in an aquarium divided by a divisor panel. In the former, the fish were placed sequentially in the same aquarium, whereas in the second model, called the "confusion model", two fish were placed contemporarily in the same aquarium which was divided into two spaces by a curved plexiglass panel.

The sequential model demonstrated the importance of the time expended in territorial exploration for the establishment of a dominance hierarchy. The status of the animals was evaluated after 15 days. This endpoint was shown to be a critical time-point for the physiological status in paired specimens [87]. At this stage, have been observed increased levels of cortisol in the plasma of subordinate specimens (αand β) with respect to the dominant. Also, have been observed a similar level of phagocytic activities among dominant and subordinate fish, with a slight increase in the β specimens. These results are consistent with Cammarata et al. (2012) which showed the same modulation response of peritoneal exudate cells (PECs) in subordinate individuals in the paired experimental model. The sequential model also allowed us to highlight the importance of the acquisition of territoriality in the establishment of a dominance hierarchy. In addition, it was shown that the fish that was placed in the tank first became the dominant, demonstrating that the time expended for space exploration is crucial in the formation of the social hierarchy as an example of sensitization, a non-associative learning in which the progressive amplification of a response follows repeated administrations of a stimulus [303]. The more time spent exploring corresponds to a greater sense of territoriality, which results in the dominance hierarchy as an example of environmental influences, spatial memory and the manipulation of the environment [304].

Through the second model, named the "confusion model", the role of exploration was highlighted, underlining the importance of time expended in the exploration of the territory and confirming the theory formulated through the results obtained by sequential model experiments. Indeed, in this case, the two fish were placed in the same aquarium separated physically by a panel, and the fish had the possibility and the time to explore the territory, acquiring the dominance of their space. Once the panel was removed, the two fish competed to defend "their" territory and always tried to feed first. In practice, both fish acted as dominant, confirming the theory of the importance of exploration time in order to assess dominance in the group. The situation was different in the control condition tank, where the two fish were placed at the same time in the same space, and according to Cammarata et al. (2012) and Dara et al. (2022), after a short period the onset of a hierarchy was observed that was maintained during the experimentation.

Through these two experimental expedients coupled with an integrative approach, the process of hierarchy establishment was examined through behavioural observation (using "aggressiveness" and "feeding priority" as parameters) and the evaluation of biomarkers of stress and immune-cell response. The results showed that social stress mainly affects the immunity cells (mediated by PEC response) in subordinate individuals, as revealed by phagocytosis and respiratory burst activity modulation [34,87]. Prolonged stress, considered deleterious, plays an adaptive role, which temporarily allows fish to cope with environmental changes, safeguarding single specimens and populations [242].

Furthermore, since it has been demonstrated that hierarchy is a cause of chronic stress, these results support the need to find solutions to mitigate its effects on the conditions of the fish, preserving aquaculture's final product from its consequent negative effects. Solutions could be to improve the rearing conditions of fish, implementing rearing methods, as well as the structural complexity of rearing tanks, which are under-implemented in fish farming [305]. For instance, self-feeding reinforces the social hierarchy, which might lead to higher competitiveness for resources among fishes, increasing the social hierarchy and, therefore, stress, when compared to hand feeding. Thus, hand feeding could reduce the deleterious effects 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 [306]. Indeed, an articulated space, introducing the possibility of avoiding direct interaction, defending territory, escaping during social conflict, investigating and interacting with enrichment, could reduce stress. Studies have reported the relevant importance of environment on gilt-head sea bream behaviour and aggressivity; for example, the presence of a blue or red-brown substrate on the tank bottom resulted in the suppression of aggressive behaviour, compared to green substrate and no-substrate tanks [307]. This hypothesis is supported by Arechavala-Lopez et al. (2020), who demonstrated the influence of environmental enrichment on the enhancement of cognition, exploratory behaviour and brain physiological functions of sea bream [307].

3. Conclusions and Perspectives

The results of the studies presented here, schematized in Table 1, are aimed at understanding how animal welfare touches and impacts on different aspects of zoology and animal husbandry as an interdisciplinary topic. One of the first difficulties to tackle is the acquisition of information and data about the animals. To date, our knowledge of animal farming and captivity has been obtained mainly from the direct observation of animals which share the same terrestrial environment as human. Difficulties arise when studying underwater animals [215], and to overcome these difficulties technologists have developed identification systems that facilitate the collection of necessary data through acoustic telemetry. This approach was first utilized for terrestrial animals before being recently applied to underwater environments thanks to the miniaturization of the device used. Currently, acoustic telemetry is the only method which allows the continuous collection of data on fish raised 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 [308]. Despite these advantages, the implantation of transmitters requires handling and surgical insertion, creating the risk of influencing the conditions of the fish and thus altering the collected information. However, the results presented in these studies investigating the effects of the surgical implantation of tags and manipulation of farmed sea bream and seabass exemplars, demonstrate that, using appropriate procedures in a controlled environment, they do not affect the animals, confirming the validity of using accelerometer tags. Indeed, acoustic telemetry may have many different applications for monitoring fish, and this has encouraged the expansion of the use of this technology in a wide number of studies, for example, to monitor different experimental groups reared with different conditions and or under different diets. Organic aquaculture is a method for farming fish based on organic principles and has become popular recently thanks to consumer concerns about the harmful impact of aquaculture on both themselves and the environment. The effects of different conventional or organic methodologies of rearing conditions were studied by Carbonara [32,33], who evaluated fish conditions, growth performance and physiology after an experimental period with different diets. The results related to the application of radiotelemetry discussed in this review support the utilization of this technology in aquaculture contexts for welfare evaluation in relation to different conditions, when coupled with other welfare indicators. From findings presented by Alfonso and Carbonara [31–33], it is possible to reach two main conclusions. First, the use of acoustic transmitters opportunely calibrated for 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 certain parameters, such as oxygen consumption, muscle activity and metabolic and immunological indicators, with swimming activity. Second, 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 seabass, suggesting that organic aquaculture may be able to deal with the challenges of maintaining high standards for fish welfare.

Other relevant aspects for the aquaculture covered in this review is related to the effects of cohabitation and sociality on the welfare of individuals of gregarious species. Dara and colleagues [34] studied the implications of hierarchical organization and the mechanisms that lead to its establishment through an analysis of physiological stress markers coupled with behavioural observation. Indeed, it has been demonstrated that hierarchy is a cause of chronic stress, affecting animal welfare by impacting their homeostasis and causing serious deleterious mid-long term effects on different biological functions (e.g., feeding, mating, fitness, immune response) [34] presented results clarifying the links between behaviour, physiological stress profile and immunity, in relation to social hierarchy. This connection, previously investigated in paired fish, has been here investigated in gilt-head bream triads. Subordinate sea bream appeared to be more stressed; indeed, they displayed greater stress levels (indicated by higher plasma cortisol, glucose and lactate levels), as well lower immunity (lower percentage of phagocytosis) than dominant fish [87]. Subsequently, Dara et al. (2023) presented results of a study aimed at investigating the processes involved in the social organization of gilt-head sea bream and the role of territoriality in its formation. They used the two experimental approaches: in the first, so-called "sequential model", and the second, so-called "simultaneous model".

To study the effects of social stress and territorial acquisition in the two models, behavioural observation was used, 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, cortisol and other biochemical stress marker levels were higher in subordinate individuals than in dominant ones. Further, a different modulation of phagocytic activity of the peritoneal cavity cells was evidenced, demonstrating the effects of social stress on immune response. Differently from the first model, no differences were found between the two dominant fish involved in the simultaneous model, where both "confused" fish acted as dominant, defending the territory perceived as their "own" in the same manner and monopolizing food access. In this study, underlying the importance of the time dedicated to the exploration of territory and territoriality, has been provided insight on the hierarchy-formation process, linking it to the physiological stress profile (cortisol, glucose and osmolarity) and immunity effectors (PECs). Results here obtained confirm data obtained in previous papers, showing that social stress exerts effects on subordinate sea bream.

In conclusion, from the new insights presented in this review, the validity of using the methodologies presented to assess the welfare of farmed fish is evident, thus contributing to the improvement and development of the F&A sector and pointing to the need to analyse certain, sometimes underrated, aspects that can influence fish production. Examples of these benefits are: individuating critical aspects for rearing the selected species, contributing to finding the best rearing conditions; looking at the influence of welfare on fish health in terms of preventing disease, pathogen outbreaks, zoonosis and reducing the use of drugs and antibiotics, contributing to guaranteeing a good final product, respecting the trade-off between farmer, fish welfare, customers and all the other stakeholders. From this point of view, the physiology and telemetry approaches may contribute to increasing the sustainability of F&A, contributing to the achievement of the blue economy pillars and of the recently adopted the United Nations SDG14 "Life below water" objective: Conserve and sustainably use the oceans, seas and marine resources for sustainable development" [205]. Making these sectors more sustainable guarantees the marine ecosystem services that have, among the others, ecological and economic value. These ecosystem services, indeed, offer a renewable opportunity to meet basic human needs, support a healthy and sustainable economy, and provide jobs for a growing global population. Indeed, all these results confirms that the physiological approach is an effective investigation toolbox, rich in tools with different applications, and that its efficacy is empowered when coupled with integrative methods that allow us to obtain a global overview on the status of fish and their welfare in different conditions.

One of the future perspectives for application of the approaches presented along this review is the investigation on fish of different sex, age, sexual maturity, in order to individuate different physiological pattern along the fish life history individuating different and appropriate farming condition and improve the current farming methodologies. In the studies aimed to investigate the social stress, for example, the testosterone level which has an important role in the regulation of cellular immune response [75], has not been assessed. This because sea bream is a proterandrous hermaphrodite species and at this stage, fish were males not sexual mature. Further studies could also addressed to investigate changes in testosterone levels during the life stage, studying the establishment of social hierarchy in sea bream, and the equilibrium in fish relationship during and after sexual inversion, when males become mature and after when they turn female.

The Annual report 2022 of Federation of European aquaculture producer [209] for the year 2020 reports that the main farmed species along the European countries are Trout (farmed on land) with the 77.6% of the total freshwater production, European seabass sea and gilt-head sea bream (with pagrus) respectively with 49.8% and 44.7% of the total marine Mediterranean production and Atlantic salmon with 92.38% of the total production of the Marine cold water. We retain that one of the reasons for the monopolizing production of only this few species often is caused by gap in the knowledge of other species farm procedures. We can assume that the utilization of the approaches here presented may be a good starting point for to fill the gap of knowledge understanding the farming needs for other species expanding the market availability of farmed fish and hence reducing the fishing load on the marine wild fish stock from the fisheries industries.

In the papers discussed along this review have been considered interaction between specimen in monospecific groups. We retain that all the approaches presented in this review can be applied in order to understand the interaction among different specimens belonging at different species in polyculture farms. Indeed, polyculture is even more seen as alternative to monoculture and one of the solutions that could improve aquaculture sustainability. This practice encompasses all farming practices in which different species are reared together in a same space contemporarily [309,310]. Differently from monoculture, polyculture can improve farming methodologies efficiency by enhancing the use of resources and/or by recycling nutrients [311–314] contributing to decrease environmental impacts of current F&A industries [314]. If on one the one hand polycultures seems the solution at different problems, on the other hand, these are complex farming systems, in which different species have to share resources potentially resulting in interspecific competition and animal welfare issues [310]. Potential polyculture benefits came from the idea to put together species that can live in the same production system minimising detrimental interactions or competition for resources or, even better, species complementarity (i.e., co-farmed species can use different portions of available resources or display commensal/mutualistic interactions) occurring among co-farmed taxa. For to realize this it is fundamental know the complementarity of the different species and their welfare when farmed together.

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