Sede Amministrativa: Università degli Studi di Padova
Dipartimento di Scienze Sperimentali Veterinarie

SCUOLA DI DOTTORATO DI RICERCA IN: Scienze Veterinarie
INDIRIZZO: Scienze Biomediche Veterinarie e Comparate
CICLO XXIV

Welfare assessment in sea bass (*Dicentrarchus labrax*) reared under organic aquaculture

Direttore della Scuola: Ch.mo Prof. Massimo Morgante
Coordinatore d'indirizzo: Ch.mo Prof. Gianfranco Gabai

Supervisore: Dott.ssa Daniela Bertotto

Dottorando : Francesco Pascoli

INDEX

SUMMARY

During the last years, fish welfare is receiving increasing attention in public and therefore in the world of research, industry and governments. In aquaculture, fishes are often exposed to stress conditions, due to practices such as manipulation, grading, high stocking densities, transport, pre-slaughter conditions and slaughter methods. In order to reduce this stressors and to increase both fish welfare and production needs, it is seeking to introduce organic agriculture principles to the conventional aquaculture. Organic aquaculture is still relatively new in concept and development all over the World. Consumers expect organic producers to follow higher animal welfare standards. In this study, fish welfare was investigated in European sea bass (*Dicentrarchus labrax*) reared under organic aquaculture in a standard commercial farm, comparing it with a parallel conventional farming. At the beginning of the trial, the main difference between the two rearing systems was in the diet. Welfare was evaluated through different stress and immunological parameters, besides growing performances. Particularly, stress response was evaluated through the analysis of both serum and muscle cortisol, and oxidative stress was investigated with an histochemical (melanomacrophage centres count and relative pigments content) and an immunohistochemical approach, using an antibody anti-HNE (a lipid peroxidation marker), besides glutathione analysis. Hematological and innate immunity parameters was also carried out, such as hematocrit, leucocrit and serum lysozyme activity. The aims of the study were to investigate both differences in fish welfare between the two rearing systems and the seasonal variations of the parameters carried out. Concerning differences between rearing systems, this study highlighted a different growing trend, in which conventional fishes were longer and heavier than organic ones. On the other hand, stress and immunological parameters didn't show any significant differences, if not in some samplings, suggesting that more severe conditions may be required to affect these activities. Since this was a monitoring study on a commercial farming, potential differences in the investigated parameters may have been mitigated, as the two systems didn't differed so pronounced.

Concerning seasonality, all parameters investigated exhibited some seasonal trend, probably correlated to water temperature and photoperiod or to their combination.

RIASSUNTO

Negli ultimi anni, il benessere dei pesci o "fish welfare" sta ricevendo crescente attenzione nel pubblico e quindi nel mondo della ricerca, dell'industria e dei governi. In acquacoltura, i pesci sono spesso esposti a condizioni di stress, a causa di pratiche di allevamento come la manipolazione, la selezione, l'alta densità di allevamento, il trasporto, le condizioni di pre-macellazione e i metodi di macellazione. Al fine di ridurre questo stress e per aumentare sia il benessere dei pesci sia le esigenze di produzione, si sta cercando di introdurre i principi dell'agricoltura biologica nell'acquacoltura tradizionale. L'acquacoltura biologica è un concetto ancora relativamente nuovo nel mondo dell'acquacoltura. I consumatori si aspettano che i produttori biologici seguano standard più elevati per quanto riguarda il benessere animale.

In questo studio, il benessere dei pesci è stato valutato in branzini (*Dicentrarchus labrax*) allevati secondo disciplinare biologico in un allevamento commerciale. Contemporaneamente, gli stessi parametri sono stati studiati in branzini allevati in un parallelo allevamento convenzionale. All'inizio della sperimentazione, la differenza principale tra i due sistemi di allevamento riguardava il mangime utilizzato nella dieta. Il benessere è stato valutato attraverso parametri immunologici e di stress, oltre alle performance di crescita. In particolare, la risposta allo stress è stata valutata attraverso l'analisi di cortisolo sierico e muscolare e lo stress ossidativo è stato studiato con un approccio sia istochimico (conteggio dei centri melanomacrofagici e relativo contenuto in pigmenti) sia immunoistochimico, utilizzando un anticorpo anti-HNE (un marker della perossidazione lipidica), oltre all'analisi del glutatione totale. Inoltre si sono investigati parametri ematologici e immunitari, come l'ematocrito, il leucocrito e l'attività lisozimatica del siero. Gli obiettivi dello studio hanno riguardato sia la valutazione di eventuali differenze nel benessere dei pesci tra i due sistemi di allevamento sia lo studio delle variazioni stagionali dei parametri ricercati. Per quanto riguarda le differenze tra i sistemi di allevamento, questo studio ha evidenziato un diverso trend di crescita, per il quale i pesci allevati in modo convenzionale hanno mostrato un accrescimento maggiore sia in termini di peso

che di lunghezza rispetto a quelli biologici. D'altra parte, lo stress e i parametri immunologici non hanno evidenziato particolari differenze significative, se non in alcuni campionamenti, suggerendo che condizioni più severe sarebbero necessarie per incidere su questi indicatori. Dal momento che questo è stato uno studio di monitoraggio su una azienda ittica a fini commerciali e non un allevamento sperimentale di ricerca, eventuali differenze nei parametri indagati potrebbero essere state mitigate, considerando anche che i due sistemi non differivano in modo pronunciato.

Per quanto riguarda la stagionalità, tutti i parametri indagati hanno mostrato un trend stagionale, probabilmente correlato alla temperatura dell'acqua e al fotoperiodo o alla loro combinazione.

1. INTRODUCTION

1.1. Fish welfare

Fish welfare is receiving increasing attention in recent years in public and therefore in the world of research, industry and governments. The issue is controversial mainly because it has not yet reached a common definition of animal welfare because concept is complex and the word is used in many different ways (Dawkins 1998; Appleby 1999).

There are many reviews that have addressed this topic (Broom 2001; Désiré *et al*. 2002; Duncan 2002; Rose 2002; Chandroo *et al*. 2004a; Huntingford *et al*. 2006) and derive from these three broad categories in which the various definitions can be grouped (Huntingford *et al*. 2006). In the first, the "feelings-based definitions", the requirements for the welfare state that the animal feels well, is free from negative experiences such as fear and pain and who has access to positive experiences such as the company of conspecifics in case of social species. This definition of welfare is the most discussed because it implies that the animal has a subjective conscious experience and that man is able to interpret them.

In the second category, the "function-based definitions", the welfare is tied to the animal's ability to adapt to the environment and requires that the animal is in good health, be able to cope with the disruption of homeostasis and that is forced to respond to these beyond its capacity.

Finally, the third category, "nature-based definitions", derives from the view that each species has its own nature that must be expressed so that the animal must be able to lead a natural life that is free to express its natural behavior (Huntingford *et al*. 2006). The definition of welfare is important because it determines the manner in which this can be measured (Huntingford and Kadri 2009).

In fish, the capacity to experience subjective mental states such as pain or fear is still unclear and a point under discussion. Indeed, there are two schools of thought: one believes that the approach based on feelings is not applicable to fish because of the lack of neuro-anatomical structures that in humans are associated with subjective mental states (especially neo-cortex; Rose 2002), while the other

one assigns to fish mental capacities because believes that fish possess neurological and physiological systems that allow them to suffer for negative experiences (Chandroo *et al*. 2004a, b). Despite its less complex brain structure than human (small size, absence of neocortex), in favor of the theory that fish are able to experience suffering is that they show complex behavior away from stereotyped; recent studies have also shown that these animals are able to perceive painful stimuli and respond to these changes with physiological and behavioral characteristics which suggest an awareness of pain experienced (Sneddon 2003; Sneddon *et al*. 2003; Huntingford *et al*. 2006). Some species, for example, are able to make mental representations of the environment and use them to orient themselves (Reese 1989; Rodriguez *et al*. 1994). Others, that live in groups, are able to recognize companions (Swaney *et al*. 2001). Others have proved to be able to remember the negative experiences, as the Paradise fish, for example, which has been shown to avoid the place where he was attacked by a predator, showing the behavior for many months (Czanyi and Doka 1993), and carp, which showed the same behavior with the bait, after being hooked and released (Beukema 1970). Many species are also capable of learning complex spatial relationships and to form mental maps using a homologous to the part of the forebrain responsible for spatial memory in birds and mammals (Broglio *et al*. 2003). Moreover, many species have been shown capable of learning and integrating different information, skills that requires more complex processes of learning associations (Braithwaite 2006; Sovrano and Bisazza 2003). Huntingford and colleagues (2006), in their extensive review on this subject, concludes that in animals capable of such complex cognitive and behavioral processes the experience of suffering may be possible. Similarly, many authors conclude that animals that are under our influence or care have to be treated with respect by minimizing all those practices which can in some way undermining their welfare (Evans 2009; Volpato 2009; Iwama 2007).

Fish, in common with all other vertebrates, respond to environmental changes through a series of adaptative neuro-endocrine adjustments, that are defined as "stress response". These induce metabolic and behavioral changes that allow the

fish to cope with the stressful event and that, at least in the short term, are essential for the survival of the animal. By contrary, the prolonged activation of the stress response is damaging and leads to immunosuppression (Magnadóttir 2006), reduction in growth (Barton *et al*. 1987; Pickering 1993; Pankhurst and Van der Kraak 1997) and reproductive dysfunction (Contreras-Sanchez *et al*. 1998; Schreck *et al*. 2001). Fish exposure to stressful conditions, especially on a continuing basis, during human activities such as fishing and aquaculture undermines the animal welfare so that is not ethically acceptable, and also determines, in time, a reduction in productivity.

The stress response involves 3 stages (Barton 2002). The *primary response* involves the activation of two neuroendocrine axes. The hypothalamic-sympathetic-chromaffine axis produces catecholamines (adrenaline and noradrenaline) from chromaffin cells that represent the equivalent of the adrenal medulla of tetrapods. The second axis, the hypothalamic-pituitary-interrenal (HPI), produces corticosteroids (primarily cortisol in teleosts) from interrenal tissue which is the equivalent of the adrenal cortex of tetrapods. The sympatho-adrenergic response stimulates cardiovascular and respiratory functions and is involved in the mobilization of energy reserves necessary for the increased metabolic demand (Sumpter 1997). As in other vertebrates, the HPI axis is related to energy and hydrosaline metabolism (Mommsen *et al*. 1999).

The *secondary response* represents a physiological adjustment to stress conditions. It includes activation of several metabolic pathways that induce a wide range of changes in blood chemistry and hematology, breathing, balance in gill homeostasis, cellular response and immune functions (McDonald and Milligan 1997; Barton 2002; Iwama 2007). Enzymes and metabolic products, immune system, plasma glucose and heat shock proteins (HSPs) are used to measure this response (Broom and Johnson 1993; Mommsen *et al*. 1999; Iwama 2007).

The *tertiary response* is involved in chronic exposure to stress and includes changes in the whole organism, also behavioral, and in case on the population (Iwama 2007). Stress has an inhibitory effect on growth, due to its metabolic effects and interference with the endocrine pathways linked to it, and

reproduction (Pankhurst and Van Der Kraak 1997). Also the resistance to diseases can be deeply affected, since immunosuppression increases dramatically the incidence of disease and mortality rates (Broom and Johnson 1993; McDonald and Milligan 1997).

In general, stress could be defined as the response of the cell, or organism, to any demand placed on it such that it causes an extension of a physiological state beyond the normal resting state (Barton 1997).

In reference to fish species, but extendible to other vertebrates, "stress" means the condition in which the dynamic equilibrium of an organism, called homeostasis, is threatened or disturbed by the action of internal or external stimuli, commonly referred to stressful events (Colombo *et al*. 1990; Wendelaar Bonga 1997).

A wide range of stimuli will test the fish both in captivity and nature. Whatever is the stimulus (a threat to the survival of the individual, a new event or a source of illness), defined stressor, it requires a response of the animal. Normally, this response is essential for the survival of the organism and is the essence of adaptation to a new environment.

Measures of physiological stress response naturally feature prominently in studies of welfare. However, stress response is an adaptive function in the face of a perceived threat to homeostasis and, as suggested above, stress physiology does not necessarily equate to suffering and diminished welfare. In the short term, stress responses serve a very important function to preserve the individual. Welfare measures in aquaculture are, therefore, largely associated with tertiary effects of stress response that are generally indicative of prolonged, repeated or unavoidable stress (Barton 2002; FSBI 2002; Conte2004).

Physiological and behavioral stress responses are well studied in many species of teleosts and have close similarities with those of other vertebrates (Barton 1997; Sumpter 1997; Wendelaar Bonga 1997; Iwama 2007).

There is no single measure of welfare and although a wide range of physiological, biochemical and behavioral measures are used to assess welfare, none of these are considered reliable in isolation and multiple measures need to be taken

(Broom 1998). Indicators associated with chronic stress response provide a potential source of information concerning the welfare of the fish and are important because they allow the development of protocols that reduce stress. Behavioral and physiological measures are intrinsically linked and are dependent on one another for correct interpretation with regard to welfare (Dawkins 1998). The most realistic assessment of welfare, however, is obtained through a series of information measures combined together through an appropriate statistical approach.

1.2 Stress in aquaculture

Worldwide aquaculture has increased strongly over the past twenty years and it seems that this growth is likely to continue (FAO 2008-2011). The report of the FAO (Food and Agriculture Organization of the United Nations, 2008-2011) forecast an increase in global demand for fish and fishery products from 145 million tons in 2008 to 190 million tons in 2015, and it is expected that 73% of this increase will come from aquaculture, which represents 39% of global fish production (FAO 2008-2011). Public interest in fish welfare has increased in recent years, due also to the expansion of aquaculture.

Fish are exposed to stressors in nature, as well as in artificial conditions such as in aquaculture, or in the laboratory. Various stressors, such as grading, transportation and vaccination, are necessary components of modern intensive fish culture. Welfare of farmed fish depends on the use of husbandry practices appropriate to the species and their adaptability. Intrinsic and extrinsic stimuli may disturb the homeostatic balance of the animals, resulting in a coordinated series of behavioral and physiological responses whose purpose is to allow the animal to overcome the stress condition (Wendelaar Bonga 1997). Thus, stress is the not specific result to any abnormal request addressed to the fish.

In farming, fish are often subjected to very stressful conditions, under which they cannot escape because the confinement.

Stressors in aquaculture are unavoidable and reducing stress and its harmful effects is a fundamental goal for successful growth and production as well as

welfare. For this reason in recent years, thanks to the growing public concern for the welfare of farmed fish, the research has directed its efforts to identify which are the farming practices that are stressful for the various farmed fish species (Huntingford and Kadri 2009). The effects of a wide range of aquaculture practices on the stress physiology of fish are well documented (for review see Wedemeyer 1972; Pickering 1982; Conte 2004). Different species display a wide variation in physiological responses to stressors associated with aquaculture.

Among these practices, we can find manipulation, selection of sizes, storage density, fasting, transport, conditions of pre-slaughter and slaughter techniques (Poli 2009; Asley 2007; Conte 2004).

Water removal and air exposure, practices that occur in case of transfers, transportation and selection, produce in fish a high physiological response, and so they should be as small as possible. Sea bream exposed to air for 3 minutes, for example, produces, within 30 minutes, an increase in cortisol levels 50 times higher than basal levels (Arends *et al*. 1999). Also handling and netting are deleterious for the animal as they can produce abrasions and deprive the fish of the protective layer determined by the scales and mucus which is essential as a barrier against infection, for osmoregulation and locomotion.

Another highly stressful factor is crowding, or the high-density storage in the tank. This parameter, determined by the needs of industry to produce large quantities by reducing costs, more in some species than in others, can be a major factor of stress. In fact, it can cause stress due to social interactions or inability to exhibit normal behavior, for example in the species that are not gregarious, but is primarily related to water quality that can be compromised by excessive load capacity (that is the maximum number of fish that the system can support in terms of dissolved oxygen and removal of metabolic wastes such as carbon dioxide and nitrogen compounds; Ashley 2007).

Stocking density is a key factor that affects the fish welfare in aquaculture, especially where high densities are required in confined spaces for high productivity (Turnbull *et al*. 2005). Although rarely defined, stocking density is the term normally used to refer to the weight of fish per unit (usually, kg of fish/m^3;

Ellis 2001). The stocking density at any point in time will increase as fish grow or decrease following grading. Because fish are dependent on this medium for both physiological and behavioral needs the welfare concerns associated with stocking density should address both the carrying capacity of the holding environment and the spatial and behavioral needs of the species. Carrying capacity refers to the maximum number of fish that an environment can support through oxygen supply and removal of metabolic waste and will be determined by, amongst other things, the oxygen consumption rate of the fish and their response to metabolic waste products such as CO_2 and ammonia (Ellis 2001). Beyond providing for the physiological needs, the FAWC recommends that fish "need sufficient space to show most normal behavior with minimal pain, stress and fear" (FAWC, 1996).

For those species that live in large shoals, low density can be harmful, whereas for territorial species the opposite may be the case. Low stocking density can reduce the spread of diseases, but will also have an effect on many other aspects of welfare as the quality of water, particulates, and the interactions between individuals (Ellis *et al*. 2002).

Field studies show that the effect of density on welfare vary among species. For example, sea bass (*Dicentrarchus labrax*) showed higher levels of stress in high stocking densities, as indicated by cortisol, innate immune response and expression of genes related to stress (Vazzana *et al*. 2002; Gornati *et al*. 2004; Poltronieri *et al*. 2009).

High stocking densities in juvenile gilthead sea bream (*Sparus aurata*) also produce a chronic stress situation, reflected by high cortisol levels, immunosupression, altered metabolism (Montero *et al*. 1999). In contrast, Arctic charr (*Salvelinus alpinus*) feed and grow well when stocked at high densities but show depressed food intake and growth rates at low densities (Jorgensen *et al*. 1993).

Stocking density naturally has a great effect even on social interactions among the fish. In species where social hierarchies are formed, such as in salmonids, these can lead to chronic stress, social (Turnbull *et al*. 2005). For example, aggressive

interactions are a major cause of injury to the eyes, tail and pectoral fins, which cause secondary infections and mortality in breeding (Turnbull *et al*. 2005).

It is clearly very hard to propose within species maximum stocking densities for all situations. However expressed, stocking density alone will not directly predict welfare. A complex matrix of factors appears to influence the effect of stocking density on a range of welfare measures. It has been suggested that a more productive approach to ensure welfare is to study the specific and dynamic components of the overall effect such as behavior, water quality, health, stress physiology, and gene expression (Ellis 2002; Ellis *et al*. 2002; Turnbull *et al*. 2005).

Diet may also play an important role in stress sensitivity.

Inappropriate food composition and a not careful time distribution of the meals can be critical factors for fish welfare. Food providing in a small area of the entire available surface can cause competition and aggression among fishes and, therefore, may be responsible for variations in the growth of individuals, increasing dominance hierarchical phenomena (Stevenson 2007). The incidence of injuries due to aggression increases, especially in cases where competition for food is strong (Greaves *et al*. 2001).

Food composition can be an important factor in preserving welfare. Indeed, diets where some critical micronutrients are lacking have a severe effect on welfare. For example, Huntingford *et al*. (2006) observed small morphological and behavioral abnormalities, decreasing in growth rates and weakening of immune function. Insufficient levels of highly unsaturated fatty acids (HUFA), besides reducing the functionality of the immune system, can have negative effects on reproductive function (Poli 2009). African catfish (*Claria gariepinus*) receiving a diet with high supplementation of ascorbic acid (Vitamin C) during early development showed lower stress sensitivity (Merchie *et al*. 1997), although common carp (*Cyprinus carpio*) fed large doses of Vitamin C showed a more pronounced cortisol increase in response to stress when compared to fish fed recommended levels of the vitamin (Dabrowska *et al*. 1991). Juvenile gilthead sea bream (*Sparus aurata*) fed a Vitamin E deficient diet showed faster elevation of plasma cortisol levels in

response to stress and a lower survival rate than control fish (Montero *et al*., 2001). Feeding rainbow trout (*Oncorhynchus mykiss*) glucan in low doses, several weeks prior to a stressor, such as transport, shows potential for reducing the immunosuppressive effects of stress (Jeney *et al*. 1997; Volpatti *et al*. 1998).

Fish are often deprived of food before certain management procedures are carried out to reduce physiological stress during the procedure. Temporary starvation prior to transport, treatment of disease, and transfer of smolts from fresh water to seawater, serves to evacuate the fish's gut and to reduce metabolism, oxygen demand and waste production. As well as being beneficial to welfare, a reduction in metabolism prior to slaughter may also alter the qualities of the flesh (Johansson and Kiessling 1991; Einen *et al*. 1998; Gines *et al*. 2002).

There have been relatively few studies on the effects of starvation on stress physiology or behavior (Barton *et al*., 1988) and the majority of work in this area concerns the effect of prolonged starvation on growth, muscle protein and fat composition (Sumpter *et al*. 1991; Einen *et al*. 1998; Rios *et al*. 2002; Pirhonen *et al*. 2003; Lemieux *et al*. 2004). The impact of food depriving on fish welfare that has previously been fed regularly is not known. Therefore, food depriving farmed fish for short periods under appropriate conditions (e.g. temperature and season) may not cause welfare problems (FSBI 2002). However, it is important to consider other effects of starvation and reduced nutrition, such as changes in metabolic activity (Martinez *et al*. 2003) and changes in behavior related to competition, and the potential for increased aggression.

Among the main parameters affecting the fish welfare there are pre-slaughter (which include fishing, possible transportation, storage conditions, handling) and slaughter practices (Poli 2009; Terlouw *et al*. 2008). There is an increasing awareness in the aquaculture industry of the need to ensure that slaughter takes place under humane conditions and that fish should be stunned prior to slaughter by a method that causes immediate loss of consciousness that lasts until death (FAWC 1996). The development of new commercial technologies to produce humane slaughter techniques for aquaculture is an active area of research. Due to the diversity of species, also the EFSA (European Food Safety Authority), the

operational arm of the European Community in terms of food safety and animal welfare, has published in recent years several guidelines about the slaughter for many of the farmed fish species (http://www.efsa.europa.eu).

In fact, while some of qualitative and quantitative features of the species are already defined by death, such as the edible part yield and the distribution of fat deposits, many other qualitative factors change depending on the conditions of death (severity of stress during previous killing and death) and during storage (handling and storage temperatures) (Poli *et al*. 2005). Healthy animals have a higher food conversion efficiency, reduced mortality, good rates of growth. The sum of these factors results in a better quality of flesh. By contrast, the increase of muscle activity, generally associated with conditions of stress, causes the mobilization and use of energy and induces anaerobic glycolysis, resulting in the production of muscle lactate, decreased pH and increased lipidic oxidation (Poli *et al*. 2004).

The consequences of endocrine responses triggered by a condition of stress include increased heart rate, oxygen consumption, mobilization of energy reserves and of plasma glucose (Poli *et al*. 2004). The increase in heart rate and the need of increased oxygen consumption during stress causes an increase in the number of erythrocytes in blood flow and hematocrit value, which is a very simple parameter to be determined and used as an index of stress, although there are no standard values for all fish species (Reddy and Leatherland 1988).

1.3 Innate immunity

It is customary to divide the immune system into the innate (non-specific) and the acquired (specific) immune system. However, an increasing body of evidence, both from fish and mammalian immunology, shows that these are combinational systems. Innate response generally precedes the adaptive response, activates and determines the nature of the adaptive response and co-operates in the maintenance of homeostasis (Fearon and Locksley 1996; Fearon 1997).

There are several examples of the innate immune parameters of fish being more active and showing more diversity than comparable components of mammalian

species. An example of this is the diversity of certain complement components like C3 and Bf, and high spontaneous activity of the alternative pathway (Sunyer and Tort 1995; Sunyer *et al.* 1997; Zarkadis *et al.* 2001).

However, the innate immune system is far from redundant in mammalian species, as many studies of man and mice have shown. In fact the apparent down regulation of the activity of the innate system in mammals may be seen as an evolutionary shift in its function, in which communication with the acquired immune system and co-operation with its components in maintaining homeostasis become increasingly important (Fearon 1997; Lo *et al.* 1999; Marchalonis *et al.* 2002).

In recent years many review articles have been published about the innate immune system of fish (Alexander and Ingram 1992; Dalmo *et al* 1997; Du Pasquier 2001; Ellis 2001; Fletcher and Secombes 2002). Also a special issue of Developmental & Comparative Immunology (DCI) was devoted to the innate immune system (2001, volume 25). In addition, several papers have examined the function and modulation of different innate parameters with reference to disease resistance, prophylactic measures, environmental changes and genetic traits. Reflecting this renewed interest in the innate system of fish is the number of papers in the present issue that deal with both cellular and humoral innate components, like the activity of macrophages and cytotoxic cells, complement components, interferon and antibacterial peptides.

The innate immune system is also of primary importance in combating infections in fish (Magnadottir *et al*. 2006). The reason is basically the intrinsic inefficiency of the acquired immune response of fish due to its evolutionary status and poikilothermic nature. This results in a limited antibody repertoire, affinity maturation and memory and a slow lymphocyte proliferation. The acquired immune response of fish is therefore sluggish (up to 12weeks) compared to the instant and relatively temperature independent innate immune response (Du Pasquier 1982; Alexander and Ingram 1992; Ellis 2001).

The innate immune system is also important in activating an acquired immune response (Magnadottir *et al*. 2006). In recent years this communication between

the innate and the acquired system has received increased attention in mammalian research. Several studies, for example, of different "knock-out" mice and of acute phase response, have shown that the innate immune system is essential to the function of acquired immunity and determines the nature of the acquired response (Fearon and Locksley 1996; Fearon 1997; Carroll and Prodeus 1998; Vilmos and Kurucz 1998). The activation of innate recognition components, through the stimulation of phagocytes, production of cytokines and chemokines and activation of the complement system and various cell receptors, stimulates T- and B-cells and antigen presenting cells (Lo *et al.* 1999). Although less studied in fish a similar communication probably takes place between the innate and the acquired system in fish (Dixon and Stet 2001).

The innate immune parameters (PRRs and implementers of innate response) have been extensively studied in fish, both with respect to practical immunoprophylatic measures and in comparative or evolutionary immunology. Most of the parameters of the innate immune system of fish are shared by both invertebrates and higher vertebrates. The components of the innate immune system are commonly divided into physical parameters, cellular and humoral factors. The humoral parameters can be both cell associated receptors or soluble molecules of plasma and other body fluids.

The functions of fish macrophages as well as the activity of inflammatory cells and of cytotoxic cells is the subject of other papers in this issue. This subject will therefore not be addressed here except to mention the key cells of the innate immune system: the phagocytic cells (neutrophils and monocytes/macrophages) and the non-specific cytotoxic cells (Evans *et al.* 2001; Neumann *et al.* 2001). Epithelial and dendritic cells also participate in the innate defence in fish (Press 1994; Ganassin and Bols 1996; Dalmo *et al.* 1997).

Various lytic enzymes, acting either singly or in a cascade, are important defence elements especially against bacteria. These are hydrolases like lysozyme and chitinase, the cathepsins, the lytic pathway of the complement system and other bacteriolytic/haemolytic enzymes found in tissues and body fluids of fish (Alexander and Ingram 1992).

Lysozyme is an important parameter in the immune defence of both invertebrates and vertebrates. Lysozyme is bactericidal, hydrolysing b-[1,4] linked glycoside bonds of bacterial cell wall peptidoglycans resulting in lysis. Although primarily associated with defence against Gram positive bacteria, Gram negative bacteria can also be lysed by this enzyme. Lysozyme is also known to be an opsonin and activate the complement system and phagocytes (Jolles and Jolles 1984; Grinde 1989). It is present in mucus, lymphoid tissue, plasma and other body fluids of most fish species (Grinde *et al.* 1988; Grinde 1989; Lie *et al.* 1989; Yousif *et al.* 1994). Cod and several other marine species like haddock, pollack and wolf fish show very little or no lysozyme activity in their tissues or body fluids. These species on the other hand show high chitinase activity in their plasma and various organs (Fange *et al*. 1976). Chitinase is a hydrolase, which may be involved in the defence against bacterial and fungal pathogens but such a role in immune defence offish has still to be proven (Lindsay and Gooday 1985; Manson *et al.* 1992). Other natural lysins in fish serum, commonly detected by their spontaneous haemolytic effect on heterologous erythrocytes, are usually, but not always, attributed to the activation of the alternative pathway of the complement system (Alexander and Ingram 1992).

The analysis of lysozyme activity may be of diagnostic value for assessing the health of the fish (Saurabh and Sahoo 2008). In fact, the lysozyme molecule is an important defense of the innate immune system and has been shown that, due to stressful events, the concentration of lysozyme in the blood is altered (Moeck and Peters 1990; Røed *et al*. 1993; Demers and Bayne 1997). It is seen that after an acute stress, there is a temporary increase in the levels of lysozyme, followed by the well-known immunosuppressive effect of chronic stress (Nora *et al*. 1997).

In Teleosts, the analysis of lysozyme activity is usually carried out on serum, since the method is more practical and less variable (Lie *et al*. 1989; Moeck and Peters 1990).

The level of lysozyme produced is related to the seasonal period, sex, stage of sexual maturity, and stressful events to antigenic stimulation (Saurabh and Sahoo 2008).

1.4 Cortisol

Cortisol, or hydrocortisone, is the major circulating glucocorticoid in Teleosts. It is a fat-soluble hormone whose production is performed by steroidogenic interrenalic cells, which are localized in the anterior region of the kidney (head-kidney), mainly along the posterior cardinal veins and their branches (Mommsen *et al*. 1999). The biosynthesis of cortisol is made by various enzymes, which act in different cellular compartments. In mitochondria there is the conversion of cholesterol to pregnenolone, due to detachment of the side chain by cytochrome P450scc (side-chain cleavage); then pregnenolone passes into the smooth endoplasmic reticulum, where it is first converted to 17-hydrossipregnenolone by cytochrome P450c17, then to 17-hydroxyprogesterone with the 3βHSD (3β-hydroxysteroiddehydrogenase), and subsequently to 11-deoxycortisol by cytochrome P450c21. Finally, the 11-deoxycortisol switch back into mitochondria, where it is converted into cortisol through the action of cytochrome P450c11, which mediates the hydroxylation in position 11β (Hanukoglu 1992).

According to a study performed by Stocco and Clark (1996), it seems that the main limiting factor in steroidogenesis is the transport of cholesterol on the inner membrane of mitochondria, which is the first of these reactions.

Due to its fat-soluble nature, cortisol can not be accumulated within the cells that produce it, so it passes into the bloodstream, through which it reaches the target tissues upon which exerts its action (Hanukoglu 1992).

The release of cortisol is under the control of the hypothalamic-pituitary-interrenal axis (HPI) (Donaldson 1981). ACTH is the major corticotropic pituitary factor and its secretion is regulated by many factors, including hormones, stress and the negative feedback of cortisol in hypothalamus and pituitary (Mommsen *et al*. 1999). ACTH can also stimulate the release of catecholamines and chronic elevation of cortisol can affect their storage and secretion in trout. Because both the interrenal and chromaffine tissue, by which the catecholamines are secreted, are located in the front of the kidney, it is conceivable that in fish there is a paracrine control of the regulation of stress hormones (Reid *et al*., 1996). Exogenous ACTH in teleosts has been used to evaluate the functionality of the

interrenal tissue both *in vivo* and *in vitro* (Brodeur *et al*. 1997; Hontela 1998) and has been shown that this tissue in stressed fish is less sensitive to stimulation *in vitro* compared to that derived from animal stress (Mommsen *et al*. 1999).

The catecholamines are rapidly removed from the blood and therefore they are not suitable as stress indices (Wendelaar Bonga 1997). By contrast, cortisol, also called stress hormone, is widely used as stress indicator in both short and long term (Pickering and Pottinger 1995). Moreover, multiple-stress conditions amplify its production (Ortuño *et al*., 2002). However, there are variations due to differences between species (Vijayan and Moon 1994), to analytical methods used, sampling procedures, daily or seasonal changes (Thorpe *et al*. 1987), photoperiod (Audet *et al*. 1986), nutritional conditions (Reddy *et al*. 1995) and sexual maturity of animals (Pickering *et al*. 1987) that decrease the reliability of the cortisol analysis. Elevations in plasma cortisol levels can differ by as much as two orders of magnitude among different species of fish following identical stressors (Barton, 2000, 2002; Conte 2004). However, plasma cortisol is the primary indicator of stress in fish. The mean basal plasma cortisol concentrations in fish varies from very low values of 5 ng/ml, as in salmonids, with values between 10 and 50 ng/ml in many other teleosts (Davis *et al*. 1984), while it may increase 10-100 times as a result of an acute stress (Barton and Iwama 1991). The analysis of plasma cortisol, however, presents issues regarding the operational difficulties of bleeding the fishes: the events immediately prior to collection (capture, transport in the laboratory, anesthesia, waiting, difficulties in sampling) can lead a significant increase in acute stress and thus significantly alter the results of the analysis (Skjervold *et al*. 2001; Poli *et al*. 2005). To overcome this problem, recent studies have evaluated the possibility to quantify the cortisol in matrices other than blood. In particular, it was shown that muscle tissue, mucus, skin and fins, in some farmed species, can be considered valid substitutes for the quantification of the steroid (Simontacchi *et al*. 2008; Bertotto *et al*. 2010).

Gills, gut and liver are the main target organs of cortisol in fish, in which are explicated the two most important roles of the hormone: regulation of water and electrolyte balance and regulation of glucose and protein metabolism (Wendelaar

Bonga 1997). For this reason it has been stated that cortisol plays both a mineralocorticoid and glucocorticoid role. In addition there are other secondary activities of cortisol, such as the inhibitory effect on growth, reproductive success and effectiveness of the immune system since, as mentioned above, stress adaptation and causes a redistribution of energy for activities such as the restoration of homeostasis, respiration and movement (Gregory and Wood 1999).

1.5 Seasonality

Seasonality is a complex event made up of many potential cues, with the principle being changes in temperature and day length (Bowden *et al.* 2007). Many organisms respond to seasonal change physiologically, behaviorally or both (Bowden *et al.* 2007). Several studies concerning the effects of seasonality on the physiology of fishes have been carried out in the past. In fish, the blood levels of biochemical variables have turned out to be mainly affected by photoperiod, water temperature, dissolved oxygen, salinity, quality and rate of food consumed (Courtois 1976; Alliot *et al*. 1983; Audet *et al*. 1993; Vellas *et al*. 1994). It has been argued that fish responses to seasonal changes are controlled by one of the principal cues or by a combination of both (Bowden *et al.* 2007). Sea bass metabolic parameters show diurnal and seasonal changes (Carrillo *et al*. 1982; 1987; Pavlidis *et al*. 1997) and growth and feeding rates of this species are correlated with salinity, water temperature and photoperiod (Alliot *et al*. 1983; Castritsi-Catharios and Kavadias 1993; Begout-Anras 1995).

Temperature is a well-known principal environmental cue in fish and it has been extensively studied with regard to reproduction, animal behavior and immune response (Le Morvan *et al*. 1998; O'Steen and Bennet 2003; Pifferer *et al*. 2005). It co-ordinates their reproductive activity, affects body weight and condition, influences food intake and locomotor activity and is also believed to co-ordinate their immune response (Bromage *et al*. 2001). It is well documented that seasonality also affects the immune response of vertebrates (Zapata *et al*. 1992; Bowden *et al*. 2007). In general, parameters are suppressed during winter and raised in summer (Slater and Schreck 1998), and whilst the majority of research

has been carried out in mammals, the importance of seasonality on the immune response of fish is increasingly being recognized (Bowden *et al.* 2007).

Adaptive immunity in fish was shown to exhibit a seasonal cycle over a 12-month period, in particular, changes in resting antibody titre and response to antigenic challenge (Nakanishi *et al*. 1986). Other studies have evidenced seasonal changes also in the lymphoid system (Wojtowicz *et al*. 1997; Alvarez *et al*. 1998) and in the numbers of circulating lymphocytes (Slater and Schreck 1998). Innate immunological indicators have turned out to be strongly affected by low temperatures in gilthead sea bream (*Sparus aurata*). Affected fish showed severe immunosuppression involving significant decrease of serum complement activity, decrease of plasma lysozyme activity and reduction in circulating lymphocytes (Tort *et al*, 1998). Hematological and innate immune parameter seasonal variations were also investigated in rainbow trout (*Oncorhynchus mykiss*) (Morgan *et al*, 2008).

Water quality, available oxygen and food supply and quality are very important factors affecting fish health and welfare in aquaculture (Culberson and Piedrahita 1996; Thompson *et al*. 2008). When subjected to a stressor, fishes employ a host of physiological adjustments designed to overcome the perceived challenge (Wendelaar Bonga 1997). In fishes, stress causes both short- and long-term physiological changes mainly due to the action of hormones such as catecholamines and cortisol (Wendelaar Bonga 1997). Stimuli that activate the stress axis and affect corticosteroid levels in fish range from handling and crowding to temperature shock and social confrontation (Weyts *et al*. 1999). In fishes, an increase in plasma cortisol has been widely employed as a quantitative measure of stress (Pickering *et al*. 1982; Van Raaij *et al*. 1996; Simontacchi *et al*. 2008). As a result, physiological parameters can be used as tools to assess environmental impacts on fishes.

1.6 Oxidative stress

Oxidative stress is a condition due to the production of reactive oxygen species (ROS) (Ahmad *et al*. 2000; Barata *et al*. 2005). ROS, such as superoxide radical

($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\cdot}OH$) and the radical nitric oxide ($NO^{\cdot}$), are generally produced during normal metabolism. To minimize the toxic effect on cellular components, organisms have developed antioxidant defense mechanisms. Under conditions of oxidative stress, it is altered the balance between ROS production and availability of antioxidant defenses, so the defensive action become ineffective (Franzini *et al*. 2009). This imbalance results in enzyme inactivation, protein degradation, lipid peroxidation and severe damage to nucleic acids (Halliwell and Gutteridge 1999).

Oxidative stress is increasingly considered one of the major upstream components of the signaling cascade involved in many cellular functions, such as the inflammatory response, stimulating the adhesion molecules and the production of chemoattractive substances (Halliwell and Gutteridge 1999). Under conditions of oxidative stress, many effects of cellular dysfunction are mediated by products of non-enzymatic reactions, such as oxidation of proteins and polyunsaturated fatty acids. The lipids peroxidation derived from a reaction with free radicals and lipidic hydroperoxides, these are the major initial reaction products (Halliwell and Gutteridge 1999).

Subsequently, the lipidic hydroperoxides decomposition generates a series of degradation products that exhibits a variety of injurious actions. The aldehydic molecules, generated during lipid peroxidation, have been implicated in cytotoxic processes, initiated by exposure of biological systems to oxidizing agents (Esterbauer *et al*. 1991). Compared to free radicals, aldehydes are relatively stable and can diffuse within the cells. These aldehydes exhibit a marked reactivity towards biological molecules such as proteins, DNA and phospholipids, generating a variety of intra-and intermolecular covalent interactions (Esterbauer *et al*. 1991). It is evident that these aldehydes may also act as bioactive molecules in physiological and/or pathological conditions. These substances can affect and modulate the different cell functions at very low concentrations (and therefore non-toxic), including signal transduction, gene expression, cell proliferation and, more generally, the response of target cells.

Lipid peroxidation, specifically polyunsaturated fatty acid (PUFA) oxidation is acknowledged as being highly deleterious, resulting in damage to cellular biomembranes, particularly to those of subcellular organelles, which contain relatively large amounts of PUFA (Halliwell and Gutteridge 1999). Tissue lipid PUFA content and unsaturation index are critical factors in lipid peroxidation, and as fish, particularly marine fish, tissues contain large quantities of n-3 highly unsaturated fatty acids (HUFA) (Sargent *et al*. 2002), they may be more at risk from peroxidative attack than are mammals. However, marine fish are unable to synthesize HUFA due to a relative deficiency in the ω3 fatty acyldesaturase and/or the C18−20 elongase enzyme activities necessary for the desaturation and elongation of dietary PUFA and so marine fish must obtain preformed HUFA in their diet (Sargent *et al*. 2002). Therefore, although HUFA are essential for optimal growth and development of marine fish, they also impose a significant peroxidation burden. In fish, in vivo lipid peroxidation caused by oxygen radicals is a principal cause of several diseases such as jaundice (Sakai *et al*. 1989), nutritional muscular dystrophy (Watanabe *et al*. 1970; Murai and Andrews 1974) and haemolysis (Kawatsu 1969).To maintain health and prevent oxidation-induced lesions and mortalities, there must be effective antioxidant systems operating in fish. The components of these systems involve antioxidant compounds such as NADH/NADPH, glutathione (GSH), protein sulphydryl (-SH) groups and uric acid, and dietary micronutrients such as vitamins E and C, and carotenoids (Winston and Di Giulio 1991; Halliwell and Gutteridge 1999).

A widely used indicator of oxidative stress is the assessment of total glutathione (GSH), a tripeptide with antioxidant properties consisting of cysteine, glycine and glutamic acid. It is important its action against ROS or molecules such as benzoates and others. During its life, organism is affected by viruses, bacteria, fungi and toxins, inflammation processes and other events that cause stress to the immune system, which responds by activating lymphocytes. These cells, primarily involved in the control of viral or bacterial infections, while carrying out their activities, generate large amounts of ROS that will damage the same immune cells and other tissues. (Finkel and Holbrook 2000).

Food availability is well known to modify antioxidant levels as indicated in a previous study that reported disturbance of GSH redox status and increase of antioxidant activities in immature gilthead sea breams (*Sparus aurata*) maintained for 46 days under food restriction (Pascual *et al*. 2003). Diet composition appears also as an important confounding factor. Indeed, levels of lipids and vitamins influence oxidative status as pointed out by several studies that show a protective effect of lipid and vitamin rich diets (Mourente *et al*. 2000; Mourente *et al*. 2002). Dissolved oxygen concentration is also a parameter described as able to modulate antioxidant activities (Cooper *et al*. 2002; Lushchak *et al*. 2001).

The study of Esterbauer *et al*. (1991) on the production of cytotoxic molecules led to the discovery of a group of conjugated aldehydes with toxic potential. Within this group, the most abundant member was identified as 4-hydroxy-2-nonenal (HNE). HNE is also an endogenous lipid mediator (Diazani 1998) and can induce a variety of cellular processes that represent a program of cell response to oxidative stress conditions. Based on these results, it is believed that HNE may be involved in many of the pathophysiological effects associated with oxidative stress in cells and tissues (Uchida 2003). The breaking of the carbon chain of fatty acids produces a wide range of smaller fragments of different lengths (Esterbauer *et al*. 1991). Based on their structural characteristics, the short chain reactive aldehydes generated by lipid peroxidation can be mainly classified into three families: 2-alkenals, 4-hydroxy-2-alkenals and ketoaldehydes (Uchida 2003).

The 4-hydroxy-2-alkenals represent the most important and specific aldehydes in lipid peroxidation (Esterbauer *et al*. 1991). Of these, HNE is known to be the major aldehyde produced during peroxidation of polyunsaturated fatty acids n-6 series, such as linoleic acid and arachidonic acid. HNE is accumulated in membranes at concentrations from 10 μM to 5 mM, in response to oxidative stress. Among the negative effects at the cellular level after exposure to HNE, there are the inhibition of growth, alteration of the level of protein, inhibition of enzymes, inhibition of calcium sequestration in microsomes and the inhibition of the synthesis protein (Esterbauer *et al*. 1991). The peroxidation of polyunsaturated fatty acids n-3 series instead generates compounds closely

related to 4-hydroxy-2-Hexenal (HHE). Another important reactive aldehyde family arising from lipid peroxidation includes ketoaldehydes, such as malondialdehyde (MDA), glyoxal and 4-oxo-2-nonenal (ONE). MDA is the most abundant aldehyde produced in lipid peroxidation, it is formed in cells in various forms and it binds covalently to various compounds such as lysine residues or amines bound to phospholipids.

In a recently published study (Pascoli *et al*. 2010), the expression of various biomarkers of oxidative stress (MDA, ACR and HNE) has been evaluated in order to assess the degree of oxidative stress in *Zosterisessor ophiocephalus*, collected from different sites of the Venice Lagoon, affected by the effects of water pollution.

1.7 Melanomacrophage centres

Melanomacrophage centres (MMCs), also known as macrophage aggregates (MAs), are groups of pigments containing cells, located within the tissues of cold-blooded vertebrates (Roberts 1975). In fish, they are normally located in the stromaof spleen and kidney and, lesser, in the liver (Roberts 1975). MMCs can develop in association with chronic inflammatory lesions in various parts of the body and during ovarian atresia. In teleosts, they develop into complexes containing lymphocytes and macrophages, and they can be compared to the lymphonodi of mammals and birds (Ellis 1980).

MMCs were classified according to their structure and they are split up in three categories (Agius 1981): unstructured (consists of a group of at least two macrophages, non-aggregated, and not bounded by any capsule), partially structured (a group that includes a large number of pigmented macrophages, strongly thickened with rough edges) and fully structured (a group that includes a large number of pigmented macrophages, strongly thickened with smooth edges and bounded by a capsule composed of fibroblast-like cells and reticular fibers).

MMCs are generally located close to vessels (Agius 1981). Macrophages form dense aggregates of large size when there are events such as phagocytosis of heterogeneous materials (cellular debris, melanin pigments, granules of

haemosiderin and lipofuscin residues) as well as lipid droplets, protein aggregates and neutral mucopolysaccharides (Agius 1981).

The morphology of the MMCs may vary among fish species, but also among organs within a species, and also under physiological conditions such as advanced age, fasting, increasing in hemoglobin catabolism and pathological conditions, that cause an increase in the number of aggregates (Agius 1985). In addition, Peters and Schwarzen (1985) have suggested that stress may induce cellular changes in fish tissues, whose main effects are an increase in the number of macrophages and in a degradation of red blood cells.

MMCs ultrastructure is very complex (Roberts 1975). They have more nuclei and a large number of vacuoles containing a wide variety of materials phagocytosed, as pigment granules. Ferguson (1976) in his ultrastructural studies ofthe spleen of turbot, *Scophthalmus maximus*, described the relationship of the centres to the splenic ellipsoids. These are specialized arterioles or capillaries and comprise a flattened endothelium surrounded by a sheath of macrophages bound by a fibrous membrane. Ferguson (1976) also showed that in turbot spleens, the melanomacrophage centres have a definite capsule composed of both cellular and a cellular elements, separating them from the surrounding lymphoid elements. According to Ferguson, the centres themselves are composed of cells in varying degrees of degeneration, replete with dense osmiophilic debris. The associated lymphoid tissue comprises lymphocytic cells and typical plasma cells, which occasionally show cytoplasmic interdigitations with closely apposed dendritic fibres.

Meseguer *et al*. (1991) reported on the ultrastructure of the centres in sea bass, *Dicentrarchus labrax*, and sea bream, *Sparus aurata*, and concluded that they are essentially similar to those of turbot and other previously studied species. It has been suggested that the capsule, which in some cases is clearly evident, might represent a way of isolating melanomacrophage centres from the surrounding tissues.

As in higher vertebrates, stress in fish increases the risk of disease (Peters and Schwarzen 1985). This effect causes several changes in circulating white blood

cells, such as an increase of macrophages, a reduction in the number of lymphocytes and a higher hemocateresis (Peters and Schwarzen 1985). Fish living in polluted environments may alter their immune system activity or non-specific defense. For example Buke *et al*. (1992) used the MMCs as a biomarker for the measurement of the effects caused by exposure to chemical pollutants. Although environmental pollutants can directly affect mortality rates in fish, sublethal effects are more common (Buke *et al*. 1992). The MMCs can be used as indicators in various processes such as the presence of diseases (Roberts 1975; Agius 1981; Kranz 1989;), changes induced by starvation (Agius and Roberts 1981), exposure to chemical agents (Suresh 2009; Pascoli *et al*. 2010) and in particular heavy metals (Meilnet *et al*. 1997; Pascoli *et al*. 2010).

1.7.1 MMCs associated pigments

According to literature (Eldestein 1971; Zuasti *et al*. 1989; Pearse 1990; Wolke 1992) it is clear that the MMCs contain different types of pigments, frequently even within the same cell. These pigments are: melanin, lipofuscin and haemosiderin. Origin and nature of these pigments are clearly different. According to Pearse (1990) and Wolke *et al*. (1985), the origin and biochemical roles of these pigments are variable and not well known. Within the macrophages, lipofuscin appears to be the most abundant pigment, while melaninis often, but not always, the other major component. Haemosiderin can be present in considerable quantities under certain conditions such as haemolytic anaemia (Agius and Roberts 2003).

1.7.1.1 Melanin

Mammal melanin are classified in three groups according to their precursor and the molecular weight (Pearse 1990): eumelanin, pheomelanin and tricochromes.
Classically, melanin is produced by melanocytes derived embryologically from the neural crest and contained within spherical to ovoidal melanosomes. In fish, such melanogenesis takes place in the dermis and the pigmentary melanin-generating cells are the melanocytes. Melanocytes are immature melanophores actively

producing melanin but capable of becoming melanophores and moving up into the functional melanophore layer (Roberts 1975).

Ellis (1974) found that, unlike the melanocytes, the MMCs were not able to synthesize the melanin pigments. Agius and Agbede (1984) suggested that the melanin granules within melanomacrophage centres were remarkably similar to integumentary ones and this indicated that the melanin of melanomacrophage centres was probably simply derived from phagocytosis of melanosomes derived from normally occurring melanin-containing cells.

On the other hand, Gallone *et al*. (2002) showed that, in equivalent structures of an amphibian (*Rana esculenta*), melanogenesis within the MMCs is possible, but the biochemical pathway for such melanogenesis, however, differs from that of classical melanocyte melanogenesis, especially in relation to the nature of their dopa-oxidase, which has properties more akin to a peroxidase than atyrosinase.

Melanin are complex polymers that can absorb and neutralize free radicals, cations and other potentially toxic agents and they come from the degradation of phagocytosed cellular material (Zuasti *et al*. 1989). An important role for the polymer within MMCs would be to neutralize free radicals released by the catabolism of fatty acids derived from phagocytosis of cellular membranes at low temperatures. In addition, melanin may be important for the production of bactericidal compounds, particularly hydrogen peroxide (Wolke *et al*. 1985).

1.7.1.2 Haemosiderin

Haemosiderin are granular brown pigments relatively insoluble that contain iron-bound proteins (mainly Fe3+) (Agius, 1981). In higher animals, iron is normally stored in the body as ferritin. But when the organism, or a particular organ or tissue, is saturated with ferritin, iron continues to be accumulated within cells, but as haemosiderin rather than ferritin (Agius 1979). This pigment derives from hemoglobin breakdown of red blood cells and is an intermediate product of metabolism of recycling erythropoiesis compounds (Agius 1981). There are two possible mechanisms by which the content of haemosiderin increases: the increased catabolism of damaged erythrocytes and the increased retention of iron

within the MMCs as a protection mechanism. In teleosts, the distribution of haemosiderin was observed only in the spleen MMCs (Agius 1981). The amount of haemosiderin may increase significantly within the MMCs both after hemolytic anemia (Roberts 2001) and after prolonged fasting (Agius 1981). Usually these pigments, under normal conditions, are less abundant in these tissues.

1.7.1.3 Lipofuscins

Lipofuscin derive from peroxidation of polyunsaturated fatty acids of cell membranes (Agius 1981). Fish, with their high content of unsaturated fatty acids and low levels of vitamin E (natural antioxidant), are particularly liable to lipofuscin formation (Agius 1981). The deposition of lipofuscin was also observed in fish in various pathological conditions, including: nutritional deficiencies, bacterial and viral diseases and disorders caused by toxic substances (Agius 1981). Lipofuscin are accumulated by age and tissue destruction (Agius 1981). They are the most common pigments in MMCs in many fish species and they are widely distributed throughout the evolutionary scale, from agnates to primates, including human. Lipofuscin granules may also derive from mitochondria degeneration, through peroxidation of lipids associated with the double membrane (Agius 1981).

1.8 Organic aquaculture

Organic aquaculture is still relatively new in concept and development (Pelletier 2003).

According to the definition given by IFOAM (International Federation of Organic Agriculture Movement, 2005), organic agriculture is "a production system that sustains the health of soils, ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. Organic agriculture combines tradition, innovation and science to benefit the shared environment and promote fair relationships and a good quality of life for all involved".

The challenge for organic aquaculture is to follow the same general principles as terrestrial organic agriculture, given the basic differences between terrestrial and

aquatic animals, such as living conditions, treatment for sick animals and species themselves (Biao 2008).

There is intense debate within the organic and fish farming sectors as to how, or even if, organic standards for aquaculture can be developed. Extrapolating practices and standards originally developed for terrestrial species for aquatic species remains a major challenge. For example, although principles such as the banning of antibiotic and hormone use may be equally applicable to both land and aquatic animal production, the standard of feeding organically raised animals with organic feed poses a particular challenge for the production of piscivorous species of fish, as it is still questioned whether wild-caught fish and fish by-products can be used as organic feed (Boehmer *et al*. 2005).

Hence, standards defining organic aquaculture systems may change as principles incorporating environmental, food safety, social, and animal welfare objectives for aquaculture are refined. As such, those involved in developing organic aquaculture standards must allow for continuous review and adaptation to encompass advances in science and technology (Pelletier 2003).

Additionally, standard-setting should not only be a reflection of best practice and sound science, but should also address other relevant factors, such as consumer preference. In fact, due to increasing consumer concern about the sustainability, public health, and animal welfare issues associated with conventional farming (Biao *et al*. 2003; Lien and Anthony 2007), it has been suggested that animal agriculture industries are in need of ethical guidance (Apotheker 2000).

Consumers expect organic producers to follow higher animal welfare standards (Hermansen 2003). In recent years, a growing consumer awareness of food value, which is based not only on the request for a high degree of sanitary safety, but also for adequate nutritional value, sustainable production, eco-environmental attention, animal welfare and use of natural raw materials, has facilitated the spread of the "organic production" (Youssefi *et al*. 2002). The political process of preparing the implementing rules for organic aquaculture is resumed in Tab.1.

The European Community responded to the organic farming needs with the EC Regulation No.834/07 of June 28th 2007, and consequent EC Regulation

N.889/2008, in which are defined the first guidelines for control, labeling and rules of production of these systems.

The recent EC Regulation No. 710/09, which came into force last August 6th 2009, is the first for aquaculture; it establishes at Community level the procedures for starting a new production line and the process for the conversion from conventional to organic methods.

At the Article 10, it says: "The production of animals in aquaculture must ensure respect of specific requirements of each animal species. In this regard, farming practices, management systems and containment systems must meet the welfare requirements of animals. [...] To minimize pests and parasites and to ensure an optimal state of health and welfare of animals, maximum stocking densities should be fixed. [...]. "

In connection with the consideration that stocking densities are an important factor for fish welfare, the EC Regulation N.889/2008 argues that the stocking density should be assessed in relation to species-specific or species groups. Furthermore, the effects of density on the welfare of animals in the herd should be monitored, taking into account both the condition of the fish and water quality (Article 25f).

One of the main topic concerns the identification of raw materials and formulations that respond to the "principle of health", i.e. they are in agreement with the nutritional requirements of farmed fish species. Special attention deserve the micronutrients and/or antioxidants from natural origin that can positively influence nutritional and organoleptic qualities, morphological characters or livery, shelf-life and maintenance of quality after slaughter of farmed fish.

For this reason fish meal and oils are regarded as essential fish feed in aquaculture. The problem is that feed farmed fish with wild fish leads to further pressure, sometimes untenable, for fishing (Lymbery 2002). In order to mitigate this pressure vegetal protein and oil are used to partially replace components of animal origin. Unfortunately, over a certain level in the diet, vegetal components may exhibit anti-nutritional factors and unusable content of essential fatty acids and amino acids, resulting in a welfare reducing. Another crucial role for the

correct immune function and ability of fish to respond to stress events is represented by a proper intake of vitamins, minerals and antioxidants (Ashley 2007). Specific rules for carnivores animal feed in organic aquaculture prioritize sustainable exploitation of fisheries, promoting the use of ingredients from fishery discard (non-commercial species or processing waste of commercial species). Special attention is given to the nutrition components, which will ensure high quality of the final product with a low impact (EC Reg. N.889/2008, Article 25j). However, fishmeal derived only by commercial fishing discard appear to be rather low in amino acids and essential fatty acids and, moreover, produce a higher accumulation of catabolites. The most challenging problem is, therefore, identify the proportion of discard that represents the optimal compromise among the highest quality product for the consumer, the lowest environmental impact and sustainable exploitation of fisheries.

Concerning animal health management, the EC Reg. states that it should be based primarily on disease prevention. However, when there is a health problem it should be used an appropriate veterinary treatment, with a limit of two cycles of allopathic treatment per year. Moreover, it states that the slaughter techniques should make fish immediately unconscious and insensible to pain, but does not provide specific indications of the techniques to be used. This can lead to different applications of the Regulations in different countries.

1.8.1 Veneto Region project "Organic Aquaculture"

In 2007 Veneto Region entrusted to Veneto Agricoltura (Legnaro, PD) the development of 5 projects concerning the "Regional plan for confirmation and development of organic agriculture" (DGRV n° 4184 of 28/12/2006), with the aim of contributing to the analysis and the control of organic production in Veneto.

After a pilot project, performed in 2008, focused on the actualization of experimental activities to evaluate technical, environmental and economical sustainability of the sea bass (*Dicentrarchus labrax*) organic production, the "AquaBio" project started in 2009 performing a sea bass organic farming into outdoor ponds at Centro Ittico Valle Bonello (Porto Tolle, RO). Due to lacking of

accurate standards, Veneto Agricoltura decided to apply the AIAB (Italian Association for Organic Agriculture) PZ06 protocol for aquaculture (AIAB Ed. 01 Rev.00, 01.09.04), based on the IFOAM one. This protocol establishes several severe criteria, some of them are the following:

- maximum stocking density: 15 Kg/m^3
- use of antibiotics: ≤2 times/year
- monitoring of physico-chemical parameters in farming settings and in effluents
- qualitative and quantitative composition of the diet (see Tab. 2)

Tab.1 Political process of preparing the implementing rules for organic aquaculture. (Modified from IFOAM 2010 Dossier "Organic Aquaculture EU Regulations (EC) 834/2007, (EC) 889/2008, (EC) 710/2009 BACKGROUND, ASSESSMENT, INTERPRETATION")

June 2004	Regulation (EEC) No 2092/1991 still in effect; Commission launches the European Action Plan for Organic Food and Farming
12th–13th December 2005	DG Mare organises stakeholder conference on organic aquaculture in Brussels
21st December 2005	Commission publishes its proposal for revision of Regulation (EEC) No 2092/1991
May 2007	European Parliament adopts its report on the revision proposal
28th June 2007	Council adopts the new Organic Regulation (EC) No 834/2007 on organic production and labelling of organic products (published in the Official Journal of the EU on 20th July 2007)
18th September 2008	New organic implementing rules are published as Regulation (EC) No 889/2008 in the Official Journal of the EU following approval by the SCOF in July
October 2007–May 2008	DG Mare organises three series of experts' meetings as a preparation for the development of the organic aquaculture implementing rules: 22nd–23rd October 2007; 22nd–24th January 2008 and 28th–29th May 2008
25th June 2008	DG Mare issues its first working document on organic aquaculture implementing rules
1st January 2009	New Organic Regulation (EC) No 834/2007 comes into force together with the implementing rules
27th January 2009	Commission issues draft organic aquaculture implementing rules
6th August 2009	After being adopted by the SCOF in June, the organic aquaculture implementing rules are published in the Official Journal of the EU as Regulation (EC) No 710/2009 as amendments to the new organic implementing rules (Regulation (EC) No 889/2009)
1st July 2010	The organic aquaculture implementing rules are applicable.

Tab. 2 Qualitative and quantitative composition of the diet. Modified from AIAB; Ed. 01 Rev.00, 01.09.04

Fattening	
PD/ED (g of PD/Mj of ED)	21-23 g/Mj
Rawprotein	≤ 45%
Rawlipids	≤ 18%
Rawenergy	≤ 20 Mj/Kg
BHT	≤ 0,02% oflipidcompart
BHA	≤ 0,02% oflipidcompart
Ethoxyquin	≤ 150 mg/Kg

2. AIM OF THE THESIS

The aim of this study was to evaluate fish welfare in sea bass (*Dicentrarchus labrax*) reared under organic aquaculture in pond in Italy. To assess fish welfare, several biometric and stress indicators were investigated, such as growing performances, cortisol, hematocrit, leucocrit, serum lysozyme activity and some oxidative stress indicators (HNE, MMCs, GSH). As comparison, the same parameters were evaluated also in a parallel semi-intensive conventional fish stock reared in the same period.

The other main objective of this study was to monitor the trend of these parameters through the seasonal changes, correlating them with two principal seasonal cues: temperature and photoperiod.

3. MATERIAL AND METHODS

3.1 Animal species

Sea bass is a marine species belonging to the family Moronidae (order Perciformes), spread throughout the Mediterranean Sea and the Eastern Atlantic, from southern Morocco to the Norwegian coast (Fritsch *et al*. 2007).

This species usually lives in coastal waters and exceptionally it can reach up to a hundred meters deep. Sea bass is an euryhaline and eurythermal species (5-28 ° C), it is therefore very tolerant of salinity changes; it is, in fact, able to track down the rivers for several kilometers searching its prey, mainly represented by small fish, shrimp, crabs and squid (Froese and Pauly 2010). Sea bass has a slim long body, slightly compressed and a rather high caudal peduncle. The head is robust and ends with a wide mouth with prominent lower jaw slightly protractile, equipped with tiny teeth. The anal fin has 3 spines and 10-12 soft rays, the operculum two flat spines and the posterior margin finely serrated (Froese and Pauly 2010).

It has dark back, silver sides and white belly, while fry have back and sides covered with black points, a feature that persists for several months after hatch (Froese and Pauly 2010).

The average size are around 45-60 cm, but an adult can reach and exceed 1,5 meters length and 15 kg, while the maximum age reported is 15 years (Froese and Pauly 2010).

Sexual maturity is reached between the second and third years of life (Prat *et al*. 1990; Carrillo *et al*. 1995). However, in farming, several fishes reach maturity during the first year of life (Zanuy *et al*. 2001).

Reproduction occurs during winter months between December and March, with external fertilization (Froese and Pauly 2010).

In farming, which can be either extensive (lagoons and valleys) that intensive (tanks and cages) in marine and brackish waters, players are selected and manipulated to induce gametes spawning; natural reproduction is not uncommon to be implemented in confined environments, but its unpredictability both in terms of timing and success represents a serious obstacle to proper planning of

commercial production (Moretti *et al*. 1999). Artificial reproduction is therefore the almost obvious choice of most farms and also it allows to perform chromosomal manipulations such as polyploidization or gynogenesis (Blázquez *et al*. 1995).
Embryonated eggs, about 1 mm in diameter, are selected through an egg-counter equipped with color discriminating photocells, in order to eliminate non-viable white ones (Moretti et al. 1999). Fertilized eggs, however, are transparent and are placed in hatcheries where hatching occurs about 48 hours after deposition at 16-17 ° C (Moretti et al. 1999). To ensure that larvae growth is normal, rearing starts in total obscurity without any food. Light is only turned on as of about day 10, when the swim bladder of the larvae inflates with air. Since the bass larva is sufficiently large, feeding can be done directly with Nauplius of *Artemia* or, recently, using micro-granulates especially developed for this species. Growout takes place either inside inland ponds (North of France, Languedoc) or inside floating cages (South-East of France). The "portion" size is reached after two years of farming at ambient temperature (Haffray *et al.* 2007).
From the age of forty days after, larvae are transferred to weaning tanks where they continue to be fed with artificial food, up to the sixtieth or seventieth day of life, at the completion of metamorphosis. Fry at this point are selected according to size and transferred into pre-fattening tanks in which feeding begins with fine-grained dry food; passed this stage, fry are again selected and transferred to fattening ponds, where they reach the minimum size required for sale (Moretti *et al*. 1999).

3.2 Animal sampling and sample preparation

Sea bass (*D. labrax*) coming from the fish farm "Impianto Ittico Sperimentale di Pellestrina" (Veneto Agricoltura, Legnaro-PD) have been transferred to the "Centro Ittico Valle Bonello" in April 2009 into two separated 300 m^3 outdoor ponds (50 x 6 x 1 m), one for organic and one for conventional farming. At the beginning of the trial, the two rearing methods differed for the feed (see Tab. 3) and the use of separated equipment and water intake, while the stocking densities was the same (initial stocking density: 2 kg/m^3). For each rearing system, fish were fed with two

Tab. 3 Proximate composition (% as-fed) and fatty acid profile (% of total fatty acid methyl-esters) of the diets (modified from Trocino *et al.* 2012)

Rearing system	Organic	Conventional
Proximate composition		
Water (%)	7.10	6.32
Ether extract (%)	20.3	17.2
Crude protein (%)	40.7	44.5
Crude fibre (%)	0.73	1.18
Ash (%)	12.3	7.73
Fatty acid profile		
C14:0	5.03	3.05
C15:0	0.37	0.23
C16:0	13.1	12.3
C17:0	0.35	0.32
C18:0	2.50	3.82
C20:0	0.21	0.28
Other SFAs	0.77	0.53
Total SFAs	22.4	20.6
C16:1 $n-7$	3.90	3.03
C18:1 $n-7$	3.93	3.99
C18:1 $n-9$	18.5	21.3
C20:1 $n-9$	2.76	0.84
C22:1 $n-11$	8.08	0.62
C24:1 $n-9$	0.46	0.19
Other MUFAs	1.52	0.48
Total MUFAs	39.0	30.4
C18:3 $n-3$	2.92	4.96
C18:4 $n-3$	5.82	0.79
C20:5 $n-3$	4.47	5.96
C22:5 $n-3$	0.91	0.74
C22:6 $n-3$	5.90	3.10
PUFAs $n-3$	20.0	15.6
C18:2 $n-6$	13.3	29.3
C20:4 $n-6$	0.24	0.31
PUFAs $n-6$	13.5	29.6
Ratio of $n-3$ to $n-6$ PUFAs	1.48	0.53
Other PUFAs	1.00	0.67
Total PUFAs	34.5	45.8
Unknown FAs	4.15	3.21

FAs: fatty acids; SFAs: saturated FAs; MUFAs: monounsaturated FAs; PUFAs: polyunsaturated FAs.

different type of food in relation to fish size (Tab. 3), one pre- and one post winter starvation (from half of October to half of March). Fish were monitored for 18 months, until they reached the commercial size. Water temperature was recorded twice a day (morning and afternoon) with a digital thermometer. From May 2009 to November 2010, twenty fishes per pond were netted every two months reaching a total of ten samplings. Immediately after capture, animals were put into iced brackish water and brought to the near laboratory facilities. During each sampling, biometric measures (weight, total and standard lengths) were recorded and condition factor (K), an index to evaluate the relative robustness of fish, was calculated as follows:

$$\text{Condition factor (K)} = \left[\frac{Weight(gr)}{Length(cm)^3} \right] \times 100$$

Then, animals were bled by collecting blood from caudal vein using sterile syringes and transferred to tubes with and without K3-EDTA. Serum was isolated by allowing the blood to clot overnight at +4 °C and standard centrifugation, then conserved at -20 °C until analysis.

After that, animals were sacrificed and several organs and tissues (lateral muscle, skin, gills, liver, spleen, stomach, gut, head kidney, gonads) specimens were taken for further analyses and processed as described below.

3.3 Cortisol extraction

Cortisol from serum (100 µl) was extracted with 8mL of diethyl ether. Dry extracts were dissolved in 1 ml of phosphate buffer (PBS, pH 7.2) and various aliquots, depending on the serum cortisol contents, were used for RIA. Muscle (100 mg) was frozen by immersion in liquid nitrogen, ground in a pestle, transferred to a tube containing 1ml of PBS and extracted as above.

To validate RIA cortisol in the various matrices, parallelism and intra-assay precision tests were performed. The tests of parallelism were performed in

triplicate by analysing the serially diluted extracts of various matrices with high cortisol concentrations. The intra-assay precision test was performed by analysing six repetitions of each matrix sample with two different levels of hormone concentrations.

3.3.1 RadioImmuno Assay

Cortisol was measured with a specific microtitre RIA, as described by Simontacchi *et al*. (1995). A 96-well microtitre plate (Optiplate, Perkin Elmer Life Sciences, Waltham, MA, USA) was coated with anti-rabbit Y-globulin serum raised in goat, and the antiserum, diluted 1:1000 in 0.15mM sodium acetate buffer, pH 9, at +4 °C, was incubated overnight. The plate was washed twice with PBS and incubated again overnight at +4 °C with the anti-cortisol serum solution. It was then carefully washed with PBS, standards, quality controls, unknown extracts and ^{3}H tracer were added and the plate was re-incubated overnight at +4 °C. Finally, it was washed with PBS, then a scintillation cocktail (Microscint 20, Perkin Elmer Life Sciences) was added and counted on a β-counter (Top-Count, Perkin Elmer Life Sciences). The sensitivity of the assay was 3.125 pg $well^{-1}$ and was defined as the dose of hormone at 90% binding (B/B0).

The anti-cortisol-3 carboxymethyloxime-BSA serum raised in rabbit showed the following crossreactions: cortisol 100%, prednisolone 44.3%, 11-deoxycortisol 13.9%, cortisone 4.95%, corticosterone 3.5%, prednisone 2.7%, 17-hydroxyprogesterone 1.0%, 11-deoxycorticosterone 0.3%, dexamethasone 0.1%, progesterone <0.01%, 17-hydroxypregnenolone <0.01% and pregnenolone <0.01%.

3.4 Histology, histochemistry and histometry

Samples were collected from ten animals per rearing system during each sampling (gills, liver, spleen, head kidney, lateral muscle, skin, ovary and testis) and small specimens were fixed in 4% paraformaldehyde prepared in phosphate-buffered saline (PBS, 0.1M, pH7.4) at +4 °C overnight, washed in PBS, dehydrated through

a graded series of ethanol and embedded in paraffin. Serial sections were cut at a thickness of 4 μm using a microtome.

3.4.1 Schmorl's reaction for melanin

Melanin has the ability to reduce ferricyanide to ferrocyanide, which in the presence of ferric ions forms Prussian Blue (Bancroft and Stevens 1996). Melanin is commonly present within the MMCs (Agius 1981).

Schmorl's reaction was performed both to evaluate MMCs for count as to highlight melanins for pigments area calculation as described in Bancroft and Stevens (1996). Deparaffinized and hydrated in distilled water sections were treated with Working Solution (30 ml of 1 % ferric chloride + 4 ml of 1 % potassium ferricyanide, then 6 ml of distilled water), then washed in tap water and counterstained with Neutral Red, rapidly dehydrated in alcohols, cleared in xylene and mounted in resinous mountant (Eukitt).

3.4.2 Turnbull staining for haemosiderin

Haemosiderin are insoluble in alcohols and soluble in acids and they are usually contained in MMCs (Agius 1981). The main feature is that they contain iron (Spandrio 1988). The staining is based on a reducing agent such as ammonium sulphide, which is left to act on tissues in order to transform the ions Fe 3+ of the pigments ions in Fe 2+, so that potassium ferricyanide, in presence of ferrous ions, produces ferrous ferricyanide which will stain hemosiderin in blue. The presence of acid promotes the reaction between FeII, contained in hemosiderin, and the dye (Spandrio 1988). After hydration in distilled water, slides were incubated in an aqueous solution of 20%ammonium sulfide for 4 hours. Later, they were washed several times in distilled water and left for 2 hours in coloring mixture (8% hydrochloric acid, 4% potassium ferricyanide and distilled water). Finally they were washed in tap water, rinsed in distilled water, dehydrated in alcohols, cleared in xylene and mounted in resinous mountant (Eukitt).

3.4.3 Nile Blue staining for lipofuscin

Lipofuscin are usually associated with melanin, often within the same macrophage (Agius and Roberts 2003). For this reason, it is necessary to perform melanin bleaching prior to staining, in order to display only the lipidic pigments.

Sections were brought to water and left to incubate in a solution containing 20% hydrogen peroxide for 4 hours, irradiated with a UV lamp. After that, slides were washed in distilled water, immersed in a saturated aqueous solution of Nile Blue sulphate for 15 minutes, left in water for 15 minutes and put in hydrogen peroxide water to remove excess dye. Finally, sections were brought to distilled water without dehydrating tissues (since the dye was soluble in xylene) and mounted in an aqueous solution of glycerine (Sigma Aldrich).

3.4.4 MMCs count

Serial sections of spleen were stained with haematoxylin and eosin (H & E) sequential stain to ascertain structural details, and with Schmorl's reaction (as described above) to detect the melanomacrophage centres (MMCs). Following visual examination of the sections with the aid of a light microscope (Olympus Vanox photomicroscope, Japan), a quantitative assessment of MMCs was made using a computerized image analyzer system (Olympus CellB, Japan) on sections of spleen and head kidney since they were the organs which exhibited the highest number of MMCs, as also reported in literature (for review, see Agius and Roberts 2003). This quantitative assessment proceeded as follows: (1) Each haul was represented by 3 sections from each spleen. (2) Three fields from each spleen section were analyzed and the amount of MMCs was recorded.

3.4.5 Pigments' area evaluation

For the evaluation of the different pigments, three spleen serial sections were used for each sea bass sampled. Photos of 38000 μm^2 were carried out to evaluate areas of 5 MMCs, using a computerized image analyzer system (Cell B Olympus, Japan) and an optical microscope with a camera (Olympus Vanox photomicroscope, Japan). For each staining method (described above), total and

stained area of each MMC were measured, and the percentage of the total stained surface was calculated.

3.4.6 Immunohistochemistry

Immunohistochemistry was performed using the Elite ABC KIT system (Vector Laboratories, Inc., California).

Before applying the primary antibody (monoclonal Anti- 4-hydroxy-2-nonenal (HNE), raised in mouse; Ab Cam, UK), endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 in PBS (phosphate buffered saline). Non-specific binding sites were blocked by incubating the sections in normal goat serum (Dakocytomation, Italy). Sections were then incubated with the primary antiserum overnight at +4 °C. After washing with PBS, sections were incubated with biotin-conjugated anti-mouse Ig antibodies (Dakocytomation), washed with PBS and reacted with peroxidase-labeled avidin-biotin complex (Vector Laboratories). The immunoreactive sites were visualized using a freshly prepared solution of 10 mg of 3.3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Aldrich) in 15ml of a 0.5M Tris buffer at pH 7.6, containing 1.5ml of 0.03% H_2O_2. To ascertain structural details, sections were counterstained with Harris haematoxylin.

The specificity of the immunostaining was verified by incubating sections with: (1) PBS instead of the specific primary antibody; (2) preimmune serum instead of the primary antiserum; (3) PBS instead of the secondary antibody. The results of these controls were negative (i.e. staining was abolished).

3.5 Hematocrit and leucocrit

Hematocrit and leucocrit, defined as the volume of packed erythrocytes and leucocytes, respectively, were obtained by microcentrifugation of whole blood with portable centrifuge (12500 rpm, 5min; MicroCL 17, Thermo Scientific, Germany). The heparinized microhematocrit tubes were observed under a binocular magnifying glass (×20).

3.6 Serum lysozyme activity

Lysozyme activity in serum was measured turbidimetrically according to literature (Parry *et al*, 1965; Hutchinson and Manning, 1996). Lyophilized *Micrococcus lysodeikticus* (Sigma Aldrich) was added at a concentration of 0.3 mg/ml to a 0.067 M Sorensen sodium phosphate buffer (pH 5.83). Forty microlitres of serum were added to two replicate wells of a 96 multiwell plate (Perkin-Elmer Life and Analytical Sciences, Shelton, CT, USA), apart from the control wells. Two hundred and thirty microlitres of the bacterial suspension were added to all wells. Absorbance was read at 490 nm, 1 min after adding the buffer and then again after 5 min using a microplate photometer (Spectracount microplate photometer; Packard-Instrument, Meriden, CT, USA). Lysozyme activity was expressed as the amount of sample causing a decrease in absorbance of 0.001 min-1 (Ellis 1990). Units used were Units min^{-1} ml^{-1}.

3.7 Glutathione (GSH)

This procedure involves the use of NADPH, which, reacting with glutathione reductase, develops a colorimetric reaction that is proportional to the amount of GSH in the samples and quantified using a microplate reader, as described in Refs. (Tietze 1969; Baker *et al*. 1990). Initially, a standard curve is prepared by diluting GSH Standard stock solution with TF-E (0.1 M phosphate buffer, 0.6 mM EDTA); then in decreasing concentrations of standard solution (SS) thus obtained, are added precise amount of TF-E as listed in Table 4.

Subsequently, two reagents are prepared, the Reaction Solution (RS) and the Reductase. Respectively, for the RS 8.3 mg of NADPH are dissolved in 1 ml of distilled water, to which are then added 0.04 TF-E and 600 µl 5.5 '-ditiobis-2-nitrobenzoic acid (DTNB). For Reductase preparation, 15µl of the commercial reductase solution (Glutathione Reductase, 205 units/mg protein, Sigma-Aldrich) is diluted with 53.4 µl of ammonium sulphate 3.6 M (Sigma a-4915, MW132.1), then 65 µl of the obtained solution is diluted with 3835 µl of TF-E. A 96 multiwell plate is loaded with 30µl of TF-E (blank), the standard curve (30µl of the various points, in descending order) and samples, 15µl of each in duplicate. The RS is then added

and the plate is read at 405 nm (Microplate Photometer Spectracount, Packard Instrument, Meriden, CT, USA) for about ten minutes, until no differences in absorbances were recorded, so the reductase activity is over. At this point, 25µl of reductase are added to all wells and read on for 20 minutes. Samples results are compared to standard and expressed in nmol ml^{-1}.

Tab. 4 Standard curve preparation for GSH assay

Point 1	200µl SS+100µl TF-E
Point 2	100µl SS+100µl TF-E
Point 3	50µl SS+100µl TF-E
Point 4	50µl SS+250µl TF-E
Point 5	50µl SS+400µl TF-E
Point 6	50µl SS+550µl TF-E
Point 7	50µl SS+1150µl TF-E
Point 8	50µl SS+1450µl TF-E
Point 9	25µl SS+1175µl TF-E
Point 10	12.5µl SS+1187.5 µl TF-E

3.8 Statistical analysis

Statistical analysis was carried out with STATISTICA 8.1 (StatSoft) software. All data are reported as mean±SEM. Data were checked for normality using a Shapiro–Wilk test and parametric tests were performed: t-test for intra-sampling comparison between rearing systems and one-way ANOVA for intra-system comparison among samplings. Where assumption was not respected, non-parametric tests were carried out (Mann-Whitney U test). In all analyses a $p<0.05$ value was accepted as significant.

4. RESULTS AND DISCUSSION

Since the aims of this study were to investigate the differences between the two rearing systems and to evaluate the seasonal effects on the carried out parameters, the results of these two topics will be discussed separately.

4.1 Growing performances

4.1.1 Organic *vs* conventional

Growing performances were evaluated using biometric measures and condition factor (K). Since standard and total length were correlated ($r=0.99$; $p<0.05$), total length was used for analysis. Fishes exhibited significant differences in October 2009 and November 2010 sampling for weight and total length (Figs. 1, 2). Conventional fishes were higher than organic ones both in weight (169.6±37.4 *vs* 146.9±40.8 and 561±22 *vs* 451±20 g; test U Mann-Whitney, $p<0.05$) and in total length (25.2±1.7 *vs* 23.9±2.0 and 35±0.4 *vs* 33±0.5 cm; test U Mann-Whitney, $p<0.05$). Concerning the condition factor (K) (Fig. 3), significant differences were found in September and November 2010, where conventional fishes exhibited higher values than organic (1.30±0.1 *vs* 1.25±0.3 and 1.33±0.1 *vs* 1.30±0.2; test U Mann-Whitney, $p<0.05$).

Differences were found in both cases at the end of the feeding period, as the winter starvation occurred from half of October 2009 to the half of March 2010, as well as it started again at the end of October 2010.

From fillets analysis (Tab. 5; Trocino *et al.* unpublished data), conventional fishes showed higher levels for most indexes, including condition factor.

The differences found in the growing performances indicate probably that conventional feed leads to greater productivity. To support this hypothesis we can refers to the results reported by Trocino *et al.* (2012). As shown in Tab. 3 in M&M of this thesis, the chemical composition of the experimental diets differed with the rearing system. The organic diets showed higher ether extract values (20.3% *vs* 17.2%), higher ash content (12.3% *vs* 7.7 %) and lower crude protein (40.7% *vs* 44.5%) compared to the conventional diets.

The primary differences between the diets, however, were found in the lipid profile, i.e., the proportions of FAs. The diets differed between rearing systems (organic *vs* conventional) (see Tab.3). The level of saturated fatty acids (SFA) was similar (22.4% *vs* 20.6% of total FAs) in all diets. The level of total mono-unsaturated fatty acids (MUFAs) was higher (39.0% *vs* 30.4%) in organic diets because of the higher proportion of C20:1 n-9 (2.76% *vs* 0.84%) and especially of C22:1 n-11 (8.08% *vs* 0.62%). Polyunsaturated fatty acids (PUFAs) of the n-3 series were higher in the organic diet (20.0% *vs* 15.6), because of the higher proportion of C18:4 n-3 (5.82% *vs* 0.79%). PUFAs of the n-6 series were lower in organic diet (13.5% *vs* 29.6%). The ratio of n-3 to n-6 PUFAs was higher in the organic than in the conventional diets (1.48 *vs* 0.53).

The type of fish oil used in the commercial diets was unknown. However, based on their FA profile, we can suppose that the organic diets were supplemented with a blend of fish oils that contained herring oil. Herring oil is characterised by a high level of eicosanoic acid (C20:1 n-9) and cetoleic acid (C22:1 n-11) and by a high content of n-3 PUFAs (Martinez *et al.* 2009; Menoyo *et al.* 2002). Similarly, the higher occurrence rate of C18:4 n-3 of the organic diet is consistent with a higher dietary level of fish oil (Benedito-Palos *et al.* 2011). In the conventional diets, the high occurrence rate of n-6 PUFAs was evidently the consequence of the inclusion of high levels of vegetable oils, probably soybean oil.

The FA profile of the conventional diets was consistent with the trend of the past decade in aquaculture towards the reduction of the use of fish meals and oils in favour of vegetable protein sources and oils (Roncarati *et al.*, 2010; Turchini *et al.,* 2009). In contrast, owing to the lower availability of certified organic vegetable raw materials, the organic diets for fish contained lower levels of these substitutes for fish meal and oils.

Concerning the fillets, FAs composition, based on preliminary analyses performed by Trocino *et al.* (unpublished data), highlighted similar results reported in previous study (Trocino *et al.* 2012).

Is to report that in August 2009 raised up a viral encephalitis that came over at the beginning of October 2009, causing high mortality rate in both ponds, respectively 13.13% for organic and 8.31% for conventional.

Basing on farmers estimate of the number of fishes in ponds at the end of the trial (personal communication), calculated relative density was different, respectively 12 kg/m^3 for organic and 16 kg/m^3 for conventional.

4.1.2 Seasonal trend

In both rearing systems, fish weight exhibited a significant slowdown during cold months (from October to March), as well as the condition factor that decreased in the same period (Figs. 1, 3). This trend is related to water temperature that can affect diet and metabolism (Person-Le Ruyet *et al.* 2004), and the consequent starvation period. Temperature regulates the feed intake, as sea bass stop growing at 10 °C and below 7 °C food intake ceases (Pastoureaud 1991).

Tab. 5 Sea bass biometrics and proximate composition (% as-fed) of intact fish and fillets (Trocino *et al.* unpublished data)

Rearing system	Organic	Conventional	
Condition factor	1.20	1.27	**
Visceral index	12.2	14.4	**
Viscel fat index	3.02	4.21	**
Hepato-somatic index	2.88	3.72	**
Carcass weight (g)	380	403	ns
Dressing percentage (% LW)	86.2	83.7	**
Fillet yield (%)	46.1	47.4	ns
Water (%)	72.0	70.6	**
Ash (%)	1.1	1.1	ns
Crude protein (%)	19.5	19.4	ns
Ether extract (%)	7.2	8.9	**
TVBN (mg/100 g)	27.6	27.7	**

** = $p<0.05$
ns = not significant

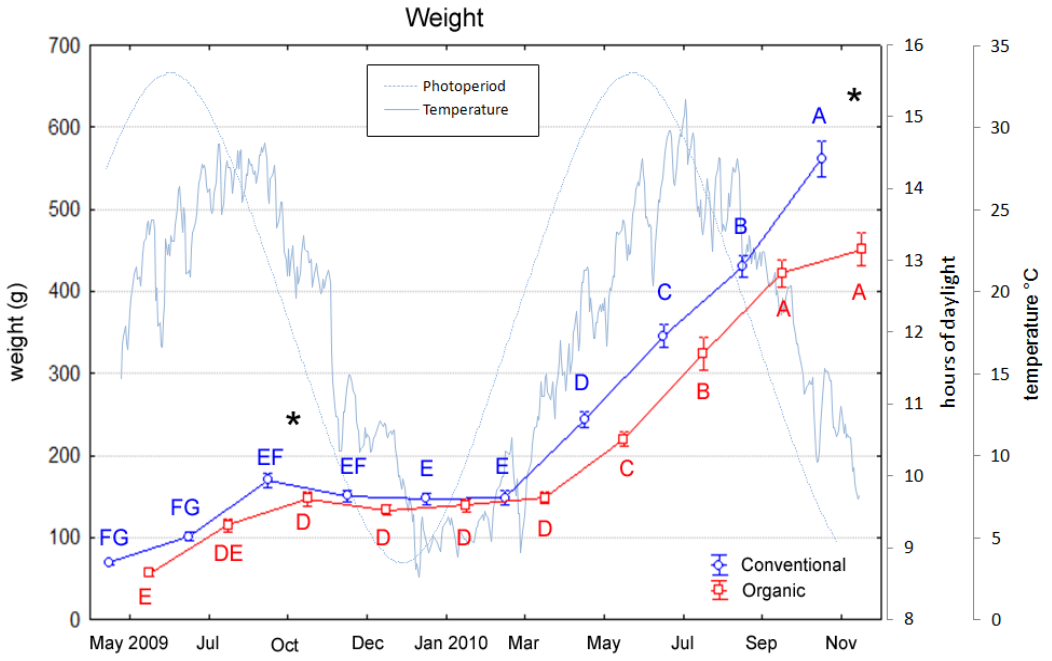

Fig. 1 Variations in weight of sea bass over a 18-months period (mean±SE) reared under conventional and organic aquaculture. Different letters indicate significant differences ($p<0.05$). *Asteriks* indicate significant differences between systems within the same sampling ($p<0.05$)

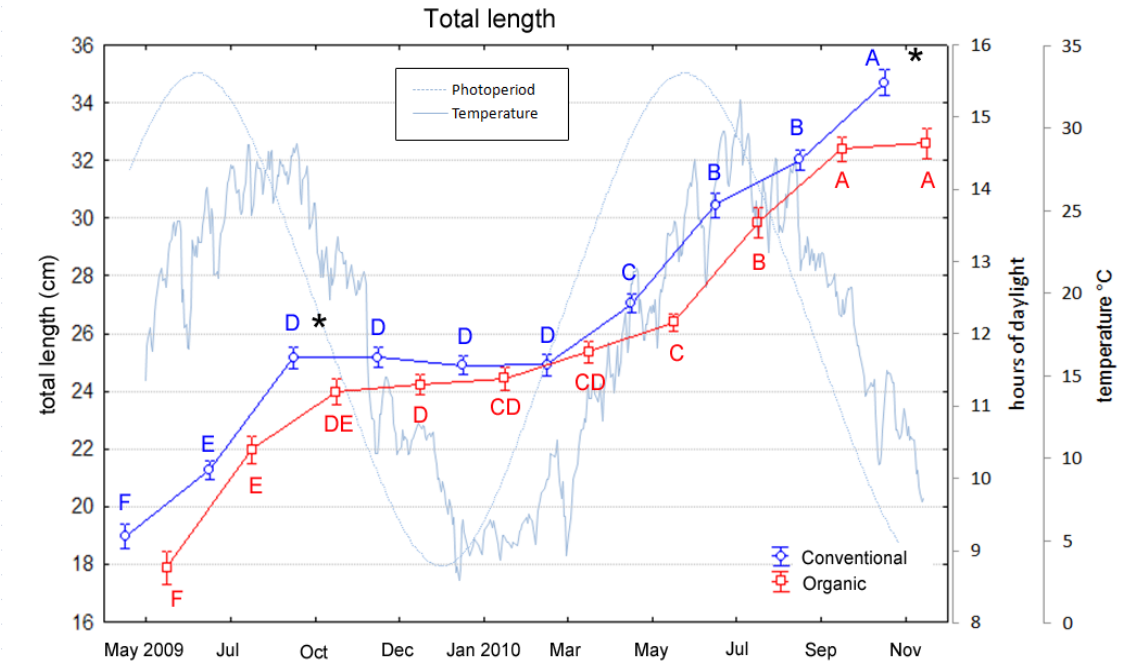

Fig. 2 Variations in total length of sea bass over a 18-months period (mean±SE) reared under conventional and organic aquaculture. Different letters indicate significant differences ($p<0.05$). *Asteriks* indicate significant differences between systems within the same sampling ($p<0.05$)

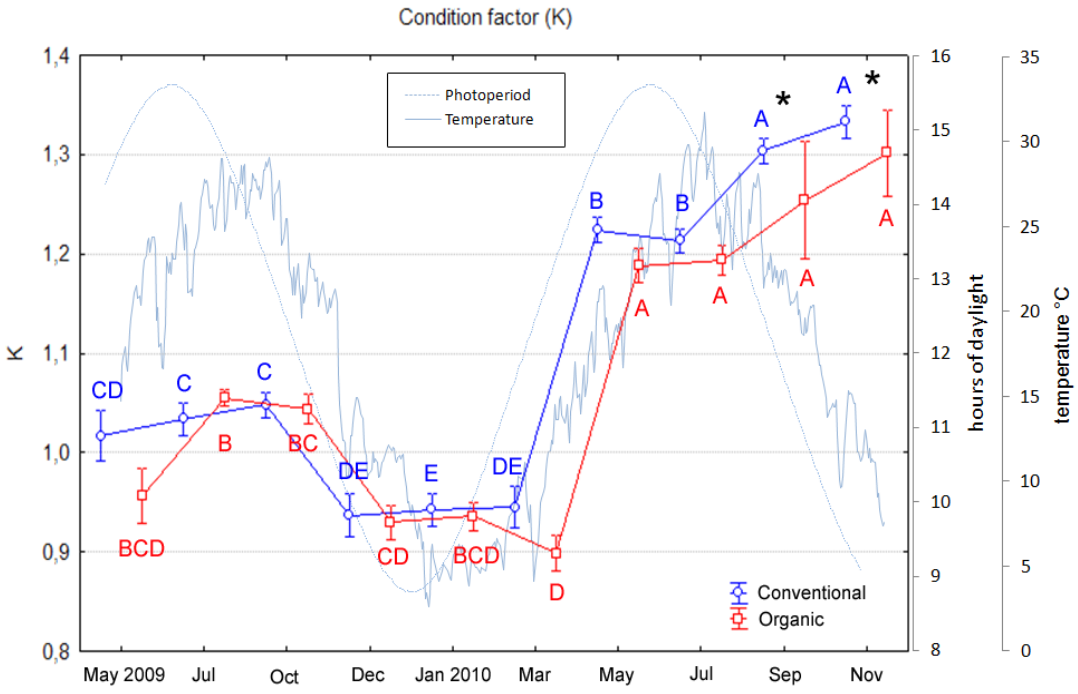

Fig. 3 Variations in condition factor of sea bass over a 18-months period (mean±SE) reared under conventional and organic aquaculture. Different letters indicate significant differences ($p<0.05$). *Asteriks* indicate significant differences between systems within the same sampling ($p<0.05$)

4.2 Cortisol

4.2.1 Organic *vs* conventional

Statistical analysis of serum cortisol data (Fig. 4) showed significant differences between rearing systems in three samples: May 2009 (t-test, $p<0.01$), July 2010 (t-test, $p<0.05$) and November 2010 (t-test, $p<0.01$). In May 2009 the highest values were recorded in sea bass reared under organic farming (393±31 *vs* 243±11 ng/ml), whereas cortisol levels were higher in conventional fishes in the samplings of July 2010 (189±20 *vs* 135±14 ng/ml) and November 2010 (181±21 *vs* 36±6 ng/ml).

Concerning muscle cortisol levels (Fig. 5), significant differences were found in the sampling of May 2009 (t-test, $p<0.01$), where organic fishes showed higher levels (10.7±0.8 *vs* 4.2±0.5 ng/g), whereas in October 2009, May 2010 and November 2010 (t-test, $p<0.01$) they were higher in the conventional ones (respectively 4.1±1.1 *vs* 1.1±0.4 ng/g; 5.1±0.6 *vs* 1.1±0.1 ng/g; 9.9±1.6 *vs* 1.4±0.3 ng/g).

With the exception of the organic samples of May 2009 (393±31 ng/ml), serum cortisol values were consistent with those reported for sea bass by Planas *et al.* (1990) and Hanke *et al.* (1991) and slightly higher than those reported by Roche *et al.* (1989) and Marino *et al.* (2001).

The few differences found between the two rearing systems could be explained by sampling procedure. The events preceding sample, such as capture, transport to the laboratory facilities, anesthesia in iced brackish water and pending in tank, can result in a significant acute stress increase and thus significantly alter the analysis results (Skjervold *et al.* 2001, Poli *et al.*, 2005). So, the very high levels found in May 2009 could be related to sampling procedure, as it was the first sampling, with the consequent difficulties.

With the aim to avoid this problem, the cortisol in muscle was also detected as this matrix is potentially less sensitive to acute stress (Simontacchi *et al.* 2008; Bertotto *et al.*, 2010). The very low levels of cortisol found by this analysis indicated that the fish were not in a state of prolonged stress (Simontacchi *et al.* 2008; Bertotto *et al.*, 2010) and, therefore, that both farming protocols have

proven effective in maintaining welfare of the animals. The point differences found in this analysis seems to be completely unrelated.

4.2.2 Seasonal trend

Concerning seasonal variations (see Fig.4), serum cortisol levels were higher during warm and hot months (May-September) respect to those colder (October-March) in both rearing systems, except for conventional samples of November 2010 that exihibited levels similar to those of summer (ANOVA, $p<0,05$ and HSD Tuckey *post hoc*). The high level of May 2009 could be explained as above.

Cortisol values fluctuations following water temperature and photoperiod has been already reported in literature (Planas *et al.*, 1990).

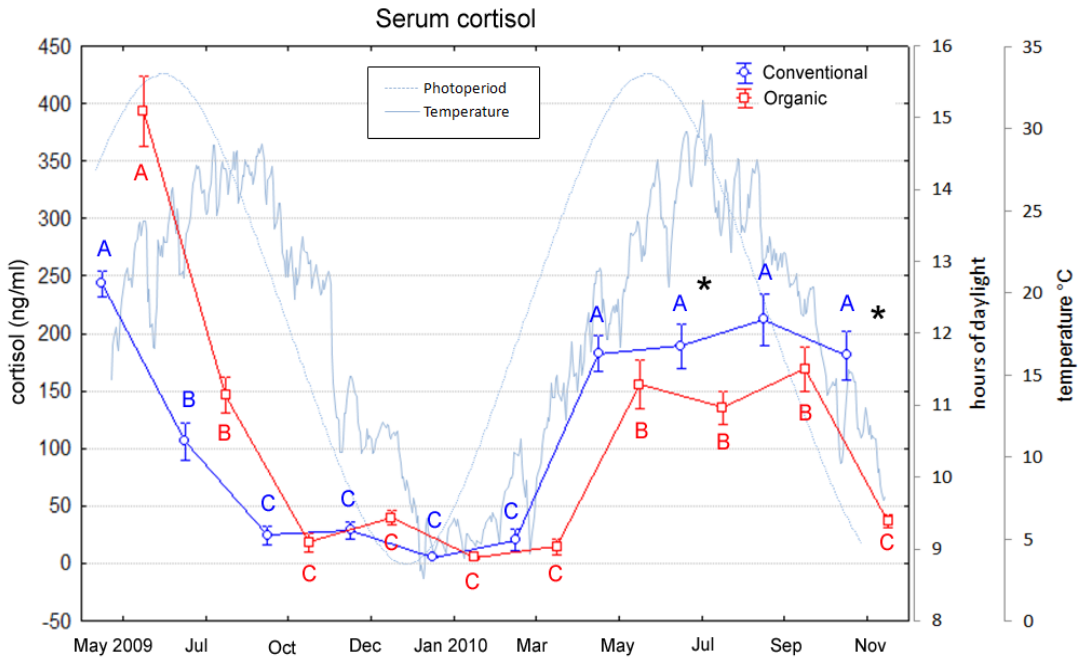

Fig. 4 Variations in serum cortisol of sea bass over a 18-months period (mean±SE) reared under conventional and organic aquaculture. Different letters indicate significant differences ($p<0.05$). *Asteriks* indicate significant differences between systems within the same sampling ($p<0.05$)

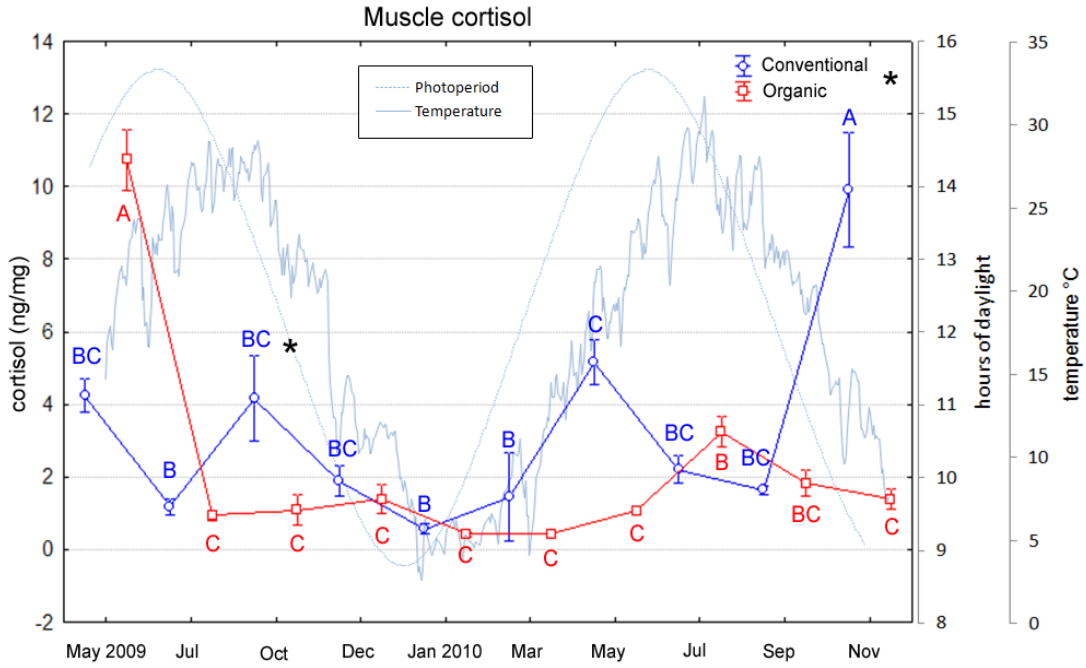

Fig. 5 Variations in muscle cortisol of sea bass over a 18-months period (mean±SE) reared under conventional and organic aquaculture. Different letters indicate significant differences ($p<0.05$). *Asteriks* indicate significant differences between systems within the same sampling ($p<0.05$)

4.3 Histochemistry and immunohistochemistry

Oxidative stress response was investigated through immunohistochemistry (IHC), with an antibody anti-lipid peroxidation marker (4-hydroxy-2-nonenal; HNE), and through the MMCs count and their pigments content.

First, Schmorl's reaction was performed to highlight MMCs and to permit their count. Then an immunohistochmical reaction was performed to localize the distribution of an antibody anti-HNE that showed a positive reaction in spleen, head kidney and liver mostly in the MMCs and spare macrophages (Fig. 6 A-D). Immunopositivity was found both in organic and conventional samples, and no differences in reaction intensity were found between the two rearing systems. The IHC results showed that a lipid peroxidation damage occurred in both rearing systems, but it was impossible to quantify the extent of the damage. To highlight any differences, a quantitative analysis shall be perform, such as Western Blot or enzymatic analysis, in order to investigate if the oxidative stress levels may indicate a serious injury.

Finally, Schmorl's reaction, Turnbull and Nile Blue stainings were performed to evaluate the pigments coverage area within the MMCs (Figs 7 A-C).

4.3.1 MMCs count

4.3.1.1 Organic *vs* conventional

MMCs count was performed on spleen sections as it is a common target organ for this analysis. MMCs count highlighted several differences between the two rearing systems (Fig. 8). In most samples, organic fishes exhibited a higher number of MMCs respect to conventional ones, except for March and May 2010, where no significant differences were found (ANOVA, $p<0.01$ and HSD Tuckey *post hoc*).

Pigments area evaluation doesn't highlighted relevant differences between the two rearing systems for all pigments type investigated, and the very few differences found are to be considered random.

Since the principle difference between the two rearing system was the feed composition, the differences in MMCs count found in the present study could be related to the feed. However, in a study by Montero *et al.* (1999) on the lack of

fatty acids with a high degree of unsaturation in the diet, such as EPA and DHA as part of the n-3 series, they reported an increase in the number of MMCs in *Sparus aurata* juveniles. Results reported here show an opposite trend. Fishes fed with diet containing a good n-3/n-6 ratio exhibited higher number of MMCs respect to those in which diet has an inverted ratio (see Tab. 3).

4.3.1.2 Seasonal trend

MMCs count showed a slight seasonal trend similar for both rearing systems, except for July and November 2010, where the trend was inverted (Fig. 8). The lowest values were found in January 2010 (ANOVA, $p<0.01$ and HSD Tuckey *post hoc*) that was the coldest month, whereas the other values followed the trend of temperature, although they were not significantly different from each other.

Literature on seasonal effects on MMCs is fairly scarce. Krüger *et al.* (1996) evidenced a correlation between the number of MMCs and seasonality, as found in our results. In particular, the average number decreases during the cold season and increase during summer where the temperature is favorable for higher metabolic activity (Krüger *et al.* 1996). Since sea bass are cold-blooded animals, at temperatures below 10 °C they are able to slow down their metabolism and stop eating (Pastoureaud, 1991), this would result into a reduction of cell (Clarke, 1993), so as circulating macrophages and MMCs.

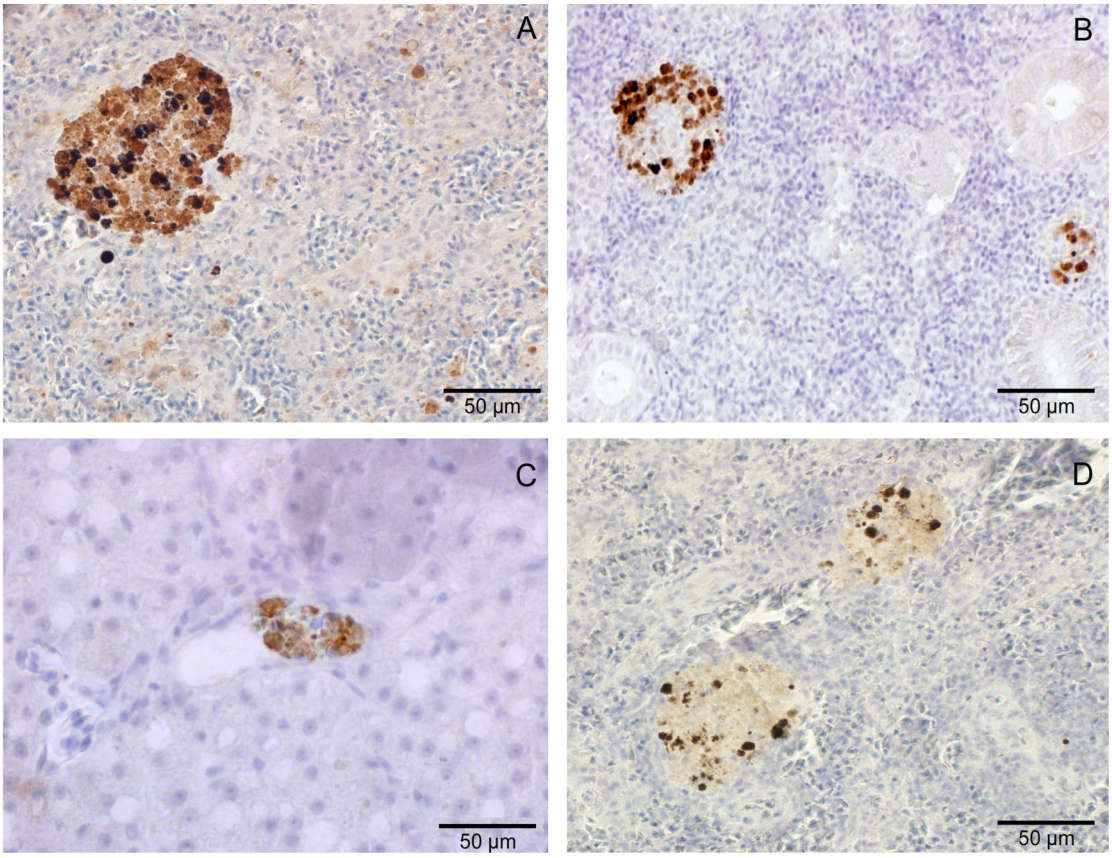

Fig. 6 Immunohistochemical localization of HNE in sea bass. All panels are counterstained with Harryr's haematoxylin. HNE-immunostaining is present in several melanomacrophage centers located in the parenchyma of A) spleen, B) kidney, C) liver. D) Negative control in which MMCs appear to be immunonegative.

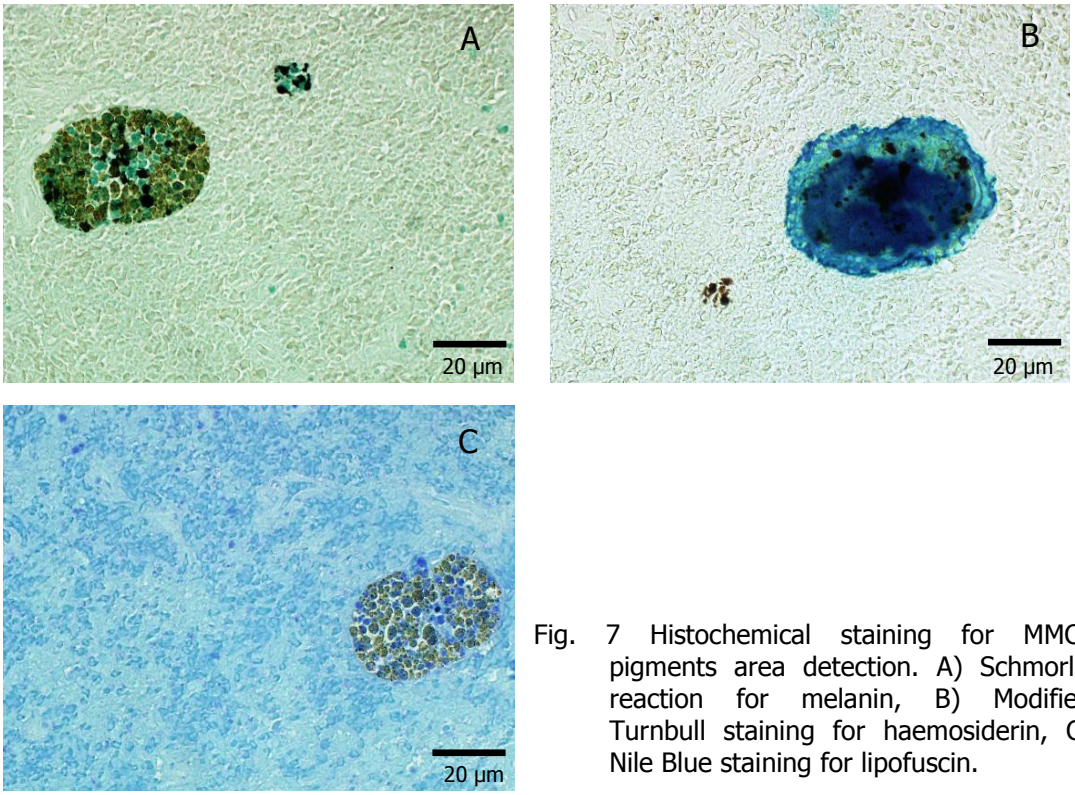

Fig. 7 Histochemical staining for MMCs pigments area detection. A) Schmorl's reaction for melanin, B) Modified Turnbull staining for haemosiderin, C) Nile Blue staining for lipofuscin.

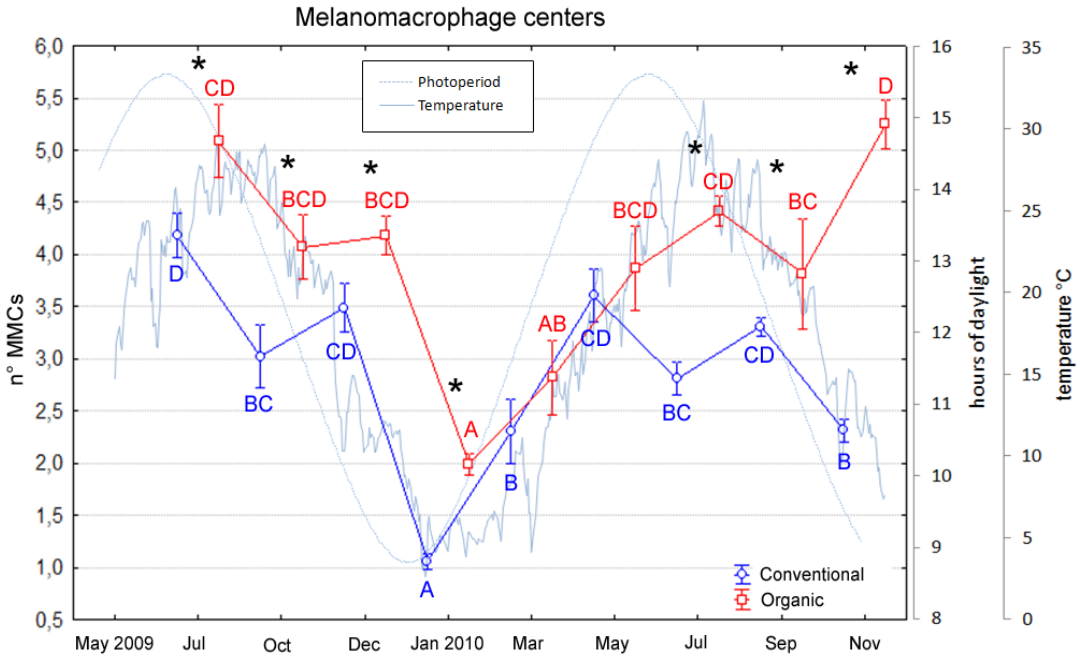

Fig. 8 Variations in melanomacrophage centers number of sea bass over a 18-months period (mean±SE) reared under conventional and organic aquaculture. Different letters indicate significant differences ($p<0.05$). *Asteriks* indicate significant differences between systems within the same sampling ($p<0.05$)

4.4 Hematocrit

4.4.1 Organic *vs* conventional

Statistical analyses on hematocrit values showed significant differences between rearing systems. Particularly, hematocrit was higher in conventional samples of January, May, July and September 2010 (t-test, $p<0.05$) respect to those of organic (see Fig. 9).

There is a strong relationship between hematocrit and dissolved oxygen in water (Gallaugher *et al.* 1995). Low levels of dissolved oxygen required an increase in red blood cell to ensure an optimal oxygen transportation around the organism. Comparing oxygen levels recorded during this trial (Fig. 10), it can be highlighted that significant differences found in hematocrit analyses could be explained by the levels of dissolved oxygen. In fact, statistical analysis showed lower levels of oxygen in the conventional pond in samples of January, May, July and September 2010, the same months in which hematocrit was higher. This could be explained with the increasing of stocking density in the conventional pond respect to the organic one, due both to the different mortality caused by the virosis and the different growing performances between the two rearing systems.

4.4.2 Seasonal trend

Except for the last sampling of November 2010, hematocrit values showed a seasonal trend in both rearing systems samples, probably related to water temperature (ANOVA, $p<0.05$ and HSD Tuckey *post hoc*). In fact, lowest levels were recorded in January and March 2010, whereas higher levels were found during summer (Fig. 9). It is known that at lower water temperature the solubility of oxygen is higher if compared to warm water. Consequently, fewer red blood cells are required to carry oxygen around the body of the fish in colder weather as the oxygen is more readily available (Stolen *et al.* 1984). This may explain the lower levels of hematocrit recorded in late winter in this study. Moreover, winter is a period of reduced activity and reduced metabolism, thus less oxygen is required. Results here reported are in accordance with those observed in other species, such as sea bream (*S. aurata*) (Tort *et al.* 1998) and rainbow trout (*O. mykiss*) (Morgan *et al.* 2008).

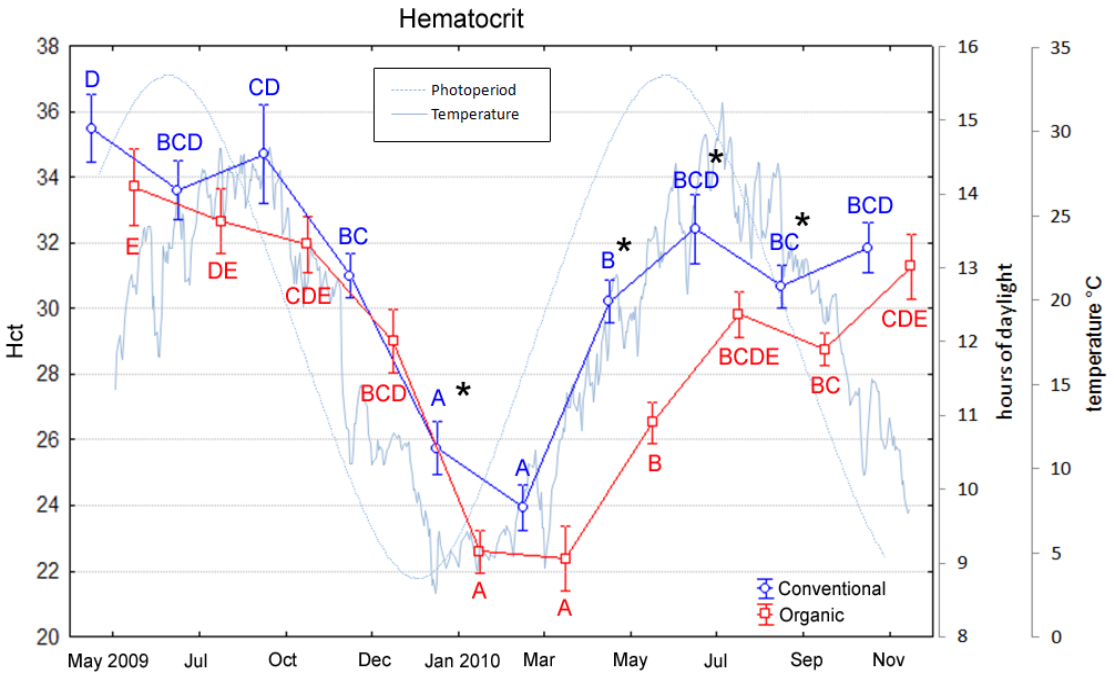

Fig. 9 Variations in hematocrit of sea bass over a 18-months period (mean±SE) reared under conventional and organic aquaculture. Different letters indicate significant differences ($p<0.05$). *Asteriks* indicate significant differences between systems within the same sampling ($p<0.05$)

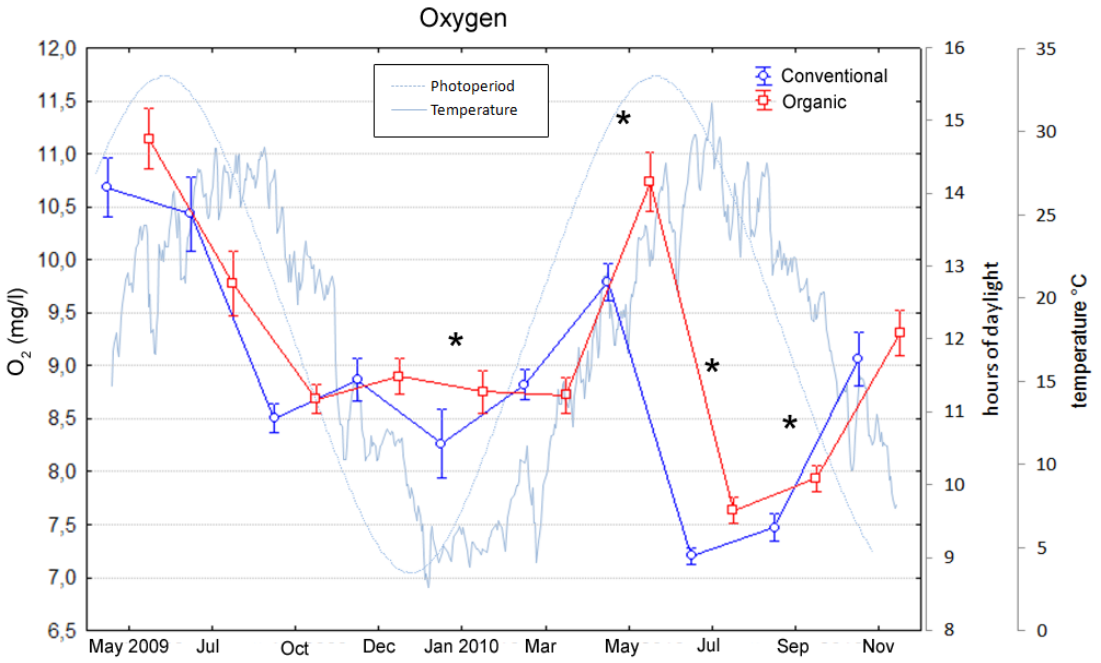

Fig. 10 Variations in dissolved oxygen in water over a 18-months period (mean±SE) Different letters indicate significant differences ($p<0.05$). *Asteriks* indicate significant differences between systems within the same sampling ($p<0.05$)

4.5 Leucocrit

4.5.1 Organic *vs* conventional

Leucocrit values showed differences between the two rearing systems only in the last two samplings, September and November 2010 (t-test, $p<0.01$; Fig. 11). In September organic samples showed higher values than conventional, whereas in November was the opposite.

4.5.2 Seasonal trend

Seasonal variations were found in leucocrit values, in both rearing systems (Fig. 11). The lowest levels were found during Winter (December-March), whereas higher values were found during Summer, and the highest during Autumn (October 2009 in both, then September 2010 for organic fish and November 2010 for conventional ones; ANOVA, $p<0.01$ and HSD Tuckey *post hoc*).

Seasonal variation in hematological and immune parameters has been found in tench (*Tinca tinca*) (Collazos *et al.* 1998), while seasonal trends in lysozyme activity have been observed in dab (*Limanda limanda*), halibut (*Hippoglossus hippoglossus*) and plaice (*Pleuronectes platessa*) (Hutchinson and Manning 1996; Bowden *et al.* 2004; Fletcher and White 1976). Leucocyte counts for tench were found to be significantly lower in winter and spring compared with those measured during summer and autumn (Collazos *et al.* 1998). It has been suggested that a shortening in day length may induce changes in the immune system in sight of winter, and this is corroborated by the highest white cell counts being recorded in tench during the autumn months (Collazos *et al.* 1998).

Inconsistent findings are reported concerning effects of elevated temperatures on the immune system of teleosts species. However, one should bear in mind that change in the proportion of a certain cell type can reflect different conditions, as already observed by other authors (Alcorn *et al.*, 2002; Köllner *et al.*, 2004). So, it could be interesting, as future perspective, to evaluate leucocyte formula from fish blood, in order to investigate if there are any changes in cell type proportion correlated to seasonality.

4.6 Serum lysozyme activity

Innate immunity was also investigated through the lysozyme activity analysis. Lysozyme is an important parameter in the immune defence of both invertebrates and vertebrates.

4.6.1 Organic *vs* conventional

The statistical analysis showed a significant difference in two samplings, May and July 2010 (t-test, $p<0.01$; Fig. 12). In these samples, the values of lysozyme activity were higher in sea bass reared under conventional method (respectively, 609.0±43.2 *vs* 556.4±103.2 and 668.5±39.5 *vs* 581.4±64.4). Generally, in hot water the bacterial charge is higher than in cold water. Moreover, high stocking density could affect water quality, behaviour and health of fish (Ellis, 2002; Ellis *et al.*, 2002; Turnbull *et al.*, 2005). Despite stocking density in this study was quite low if compare to those of literature, it was different between the two rearing systems since May 2010. So, combination of high water temperature and more crowding could be responsible of the differences found in this study concerning lysozyme activity. On the other hand, certain constituents of plants (mainly soybean) included in fish feed have also been shown to have immunological activity or to act as antigens (Baeverfjord and Krogdahl, 1996; Krogdahl *et al.*, 2003), as increasing in lysozyme in the intestinal mucosa. Although content in plant protein in the feed used in the farming here reported is very low if compared to those of cited studies, it could be interesting to investigate more this hypothesis by evaluation of histopatology of gastrointestinal mucosa and its content in lysozyme.

4.6.2 Seasonal trend

Lysozyme activity showed a seasonal effect in both rearing systems, with an increase in lysozyme activity from May to October, a decrease until January and a re-increase onwards (Fig. 12). The lowest values were found in January 2010 (194±17 and 163±13 U/min/ml, respectively conventional and organic), whereas higher levels were found in hotter months, with the exception of November 2010

for organic samples. These results are in according to previews studies for other species, as *Limanda limanda* (Hutchinson and Manning 1996) and *Pleuronectes platessa* (Fletcher and White 1976). As described for leucocrit, it has been suggested that a shortening in day length may induce changes in the immune system in sight of winter (Collazos *et al.* 1998). Moreover, lysozyme is an important anti-bacterial defence, and it is contained in lysosomes of macrophage and leucocytes, from which it is released in blood (Murray and Fletcher 1976). So, high values of leucocrit found in this study could explain the higher levels of lysozyme activity found in autumn months.

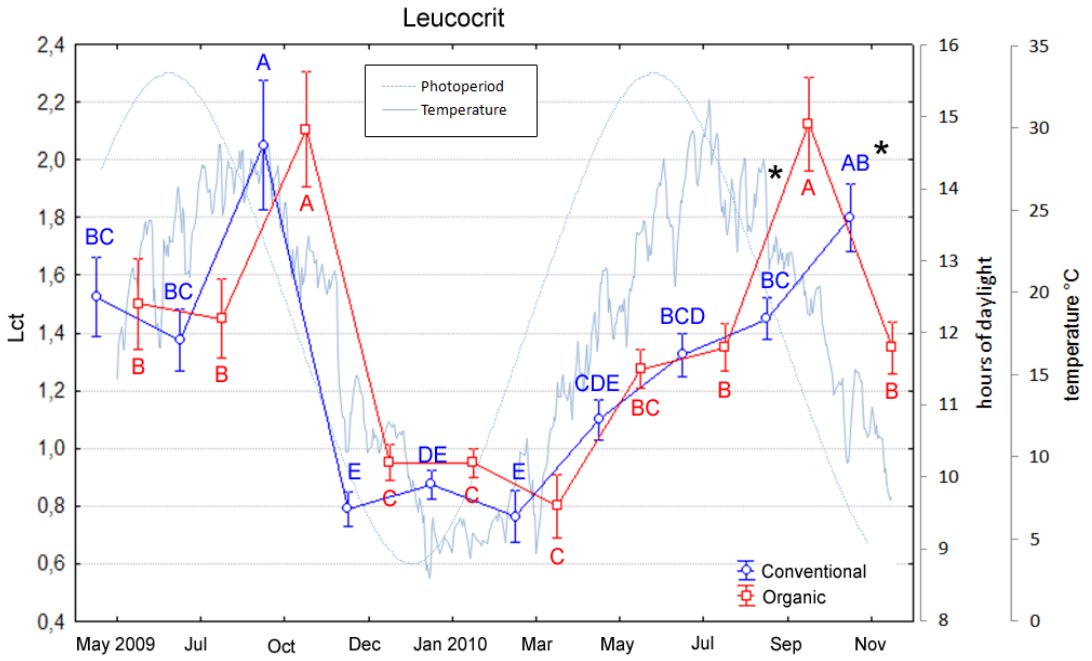

Fig. 11 Variations in leucocrit of sea bass over a 18-months period (mean±SE) reared under conventional and organic aquaculture. Different letters indicate significant differences ($p<0.05$). *Asteriks* indicate significant differences between systems within the same sampling ($p<0.05$)

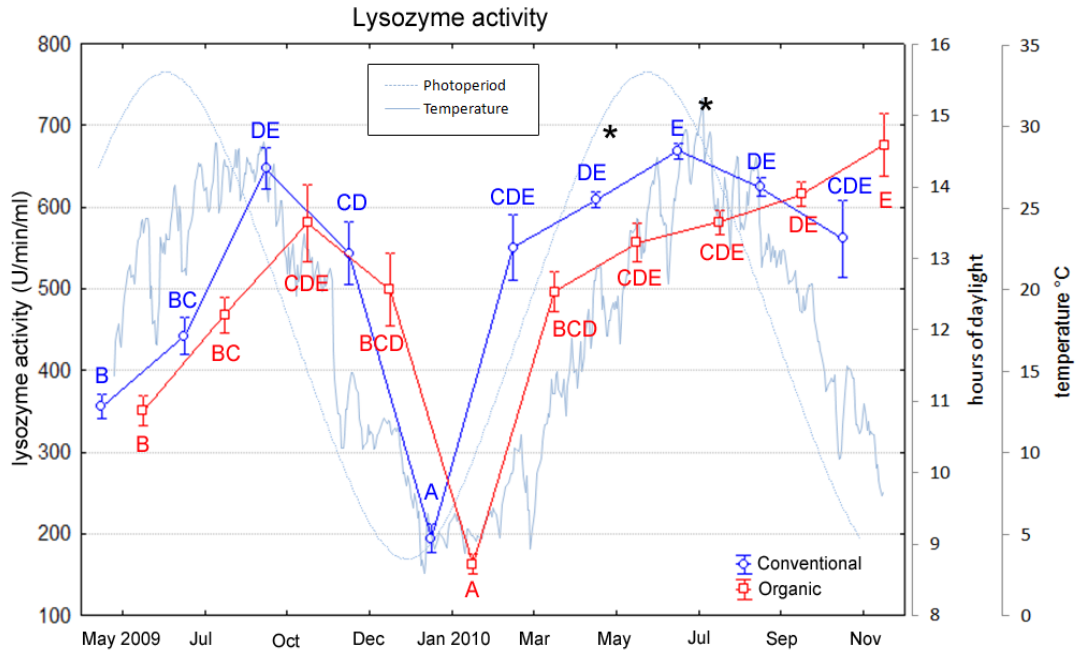

Fig. 12 Variations in serum lysozyme activity of sea bass over a 18-months period (mean±SE) reared under conventional and organic aquaculture. Different letters indicate significant differences ($p<0.05$). *Asteriks* indicate significant differences between systems within the same sampling ($p<0.05$)

4.7 Glutathione (GSH)

4.7.1 Organic *vs* conventional

Oxidative stress was investigated also by a spectrophotometric assay of total glutathione (GSH). Statistical analysis on GSH values highlighted significant differences between the two rearing systems only in two samplings, July and November 2010 (t-test, $p<0.05$; Fig. 13). In both samplings, conventional fishes showed higher values than organic ones (35.9±0.9 *vs* 34.0±1.8 and 36.2±0.5 e 29.0±2.2 nmol/ml).

Diet composition appears also as an important confounding factor. Indeed, levels of lipids and vitamins influence oxidative status as pointed out by several studies that showed a protective effect of lipid and vitamin rich diets (Mourente *et al.*, 2000; Mourente *et al.*, 2002). Dissolved oxygen concentration is also a parameter described as able to modulate antioxidant activities (Cooper *et al.*, 2002; Lushchak *et al.*, 2001). However, in this study slight differences were found between the two rearing systems, indicating that differences in both diet and farming conditions was not affecting GSH mediated antioxidant defences, although very scarce data are reported in literature for the reference values for this species.

4.7.2 Seasonal trend

Total glutathione levels showed some seasonal effects (Fig. 13). Glutathione levels decreased from July to December 2009, then increased to July 2010 and decreased onward. The lowest levels were found in Autumn-Winter months (October 2009-March 2010), whereas higher levels were found during Summer months. This seasonal variation is in agreement with previous studies reporting the influence of temperature on GSH levels in different organs in fish (Legatt *et al.* 2007; Pavlovic *et al.* 2010). In addition, the very low levels found in October 2009, if compared to those of September and November 2010, could be explained by the viral encephalitis that occurred in August and September 2009. In fact, in mammals, food restriction and inflammatory processes could affect the levels of glutathione, with a decrease in plasma levels (Malmezat *et al.* 2000). Moreover, food availability is well known to modify antioxidant levels as indicated in a

previous study that reported disturbance of GSH redox status and increase of antioxidant activities in immature gilthead sea breams (*Sparus aurata*) maintained for 46 days under food restriction (Pascual *et al.* 2003). So, low levels found in colder months could also be related to the stop of feed intake due to the winter starvation period (from half of October to half of March).

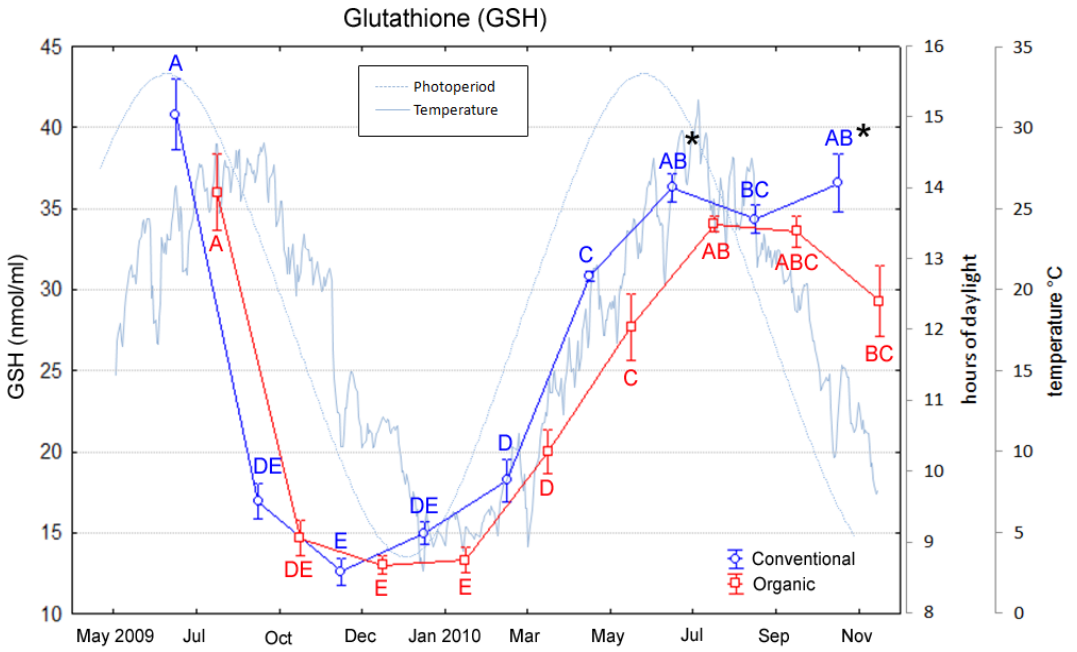

Fig. 13 Variations in glutathione of sea bass over a 18-months period (mean±SE) reared under conventional and organic aquaculture. Different letters indicate significant differences ($p<0.05$). *Asteriks* indicate significant differences between systems within the same sampling ($p<0.05$)

5. CONCLUSIONS

The aim of this study was to evaluate fish welfare in sea bass (*D. labrax*) reared under organic aquaculture in outdoor pond. Fish welfare was evaluated through several parameters, in order to investigate growing performances, stress response and innate immunity. Results were compared to those of a parallel sea bass farming stock reared under conventional aquaculture.

Growing performances and condition factor showed a positive growing trend for both rearing systems, but highlighted a quicker increase in conventional samples, suggesting that conventional feed leads to greater productivity. Moreover, the characteristics of the diets fed in the two rearing systems affected the nutritional value of the flesh in terms of the fatty acid profile, which was closer to that of wild fish and safer for human consumption in organic than in conventional sea bass. This is of great value if considering that the progressive substitution of cheaper vegetable oils for increasingly expensive fish ingredients in the conventional diets for farmed sea bass is surely decreasing the dietetic value of sea bass (and other farmed fish), measured in terms of the profile of FAs (Fuentes *et al*. 2010; Roncarati *et al*. 2010; Turchini *et al*. 2009). This decline in value is demonstrated by the 50% decrease of n-3 PUFAs from 26.8% in the study of Alasalvar *et al*. (2002) to 13.8% (Trocino *et al*. 2012) and the sharp decline of the ratio of n-3 to n-6 PUFAs ratio from 3 (Alasalvar *et al*. 2002) to 0.5 (Trocino *et al*. 2012). As a consequence of this change, the differences in nutritional value between conventional farmed sea bass and wild sea bass will receive emphasis, and wild products will be favoured (Alasalvar *et al*. 2002; Fasolato *et al*. 2010; Fuentes *et al*. 2010). However, this negative trend affecting farmed fish could work to favour organic sea bass because the organic aquaculture regulation (EC 2009) fixes a maximum inclusion level of vegetable feeds at 60% of the diet and indirectly imposes a 40% level of fish meal and oil as a minimum.

On the other hand, the standard of feeding organically raised animals with organic feed poses a particular challenge for the production of piscivorous species of fish, as it is still questioned whether wild-caught fish and fish by-products can be used as organic feed (Boehmer *et al*. 2005). Due to the exploitation of some fisheries

and to the unsustainability of several fishing methods, wild-caught fish could maybe not be the answer to the organic feed requirements, considering that organic production should be "a production system that sustains the health of soils, ecosystems and people" (IFOAM 2005).

Stress response, evaluated with serum and muscle cortisol levels, showed mean levels consistent with literature and no particular differences were recorded. Moreover, muscle cortisol values showed very low levels, suggesting that farming features were suitable for the species needs.

Oxidative stress expression was investigated both with histochemical and immunohistochemical approach and with spectrophotometric assay. Immunohistochemistry showed an immunopositivity at MMCs levels, indicating an oxidative stress occurred for both rearing systems, but no differences have been recorded. It could be interesting to quantify this reactivity, for examples using a Western Blot analysis, in order to asses any differences in the expression of oxidative stress markers. MMCs count highlighted higher values in organic samples in most samplings, indicating that the stress status is different between the two rearing systems, probably related to fish feed. However, GSH analysis showed differences in only two samplings in which conventional fish showed higher values. This suggests that the oxidative stress status was mostly not different between the two rearing systems and that the differences found in MMCs could be not fully related to oxidative stress. Unfortunately, standard GSH data for this species is very scarce, so it is difficult to note if fish exhibited a high or low oxidative stress status.

Hematocrit showed few differences that could be explained by the different dissolved oxygen levels found in the ponds. Higher values of hematocrit was recorded in conventional samples together with low levels of oxygen in water, maybe related to the different stocking density due to the mortality rate, consequent to the virosis, and the different growing trend. Hematocrit values were consistent to those of literature, suggesting that rearing conditions were suitable for the species.

Innate immunity was investigated both using leucocrit and serum lysozyme activity. Leucocrit didn't highlight many differences. However, several authors have shown that changes in the proportion of a certain cell type can reflect different conditions (Alcorn *et al.* 2002; Köllner *et al.* 2004). So, maybe any differences between the two rearing systems on the leucocyte content could be evaluated assessing the leucocyte formula from fish blood, in order to investigate if there are any changes in cell type proportion correlated to rearing conditions. Moreover, certain constituents of plants (mainly soybean) included in fish feed have also been shown to have immunological activity or to act as antigens (Baeverfjord and Krogdahl 1996; Krogdahl *et al.* 2003).

The serum lysozyme activity assay showed differences in two samplings, where conventional was higher than organic samples. As discussed in the previous chapter, combination of high water temperature and crowding could be responsible of the differences found. On the other hand, more analyses should be carry out to investigate the effect of the diet and the rearing conditions on immunological parameters, such as evaluation of gastrointestinal mucosa and GALT (gastrointestinal associated lymphoid tissue) and alternative pathways, i.e. serum complement hemolytic activity.

Concerning seasonality, all parameters investigated exhibited some seasonal trend, probably correlated to water temperature and photoperiod or to their combination. In fact, in natural environments changes are caused by a combination of features and it is difficult to assign the responsability to one or two factors. Different it is if the changes occurred in an artificial environment, were different parameters are modified in a targeted way. For further perspective, it could be interesting to investigate the effect of singular environmental factors (temperature, photoperiod, oxygen, pollution, etc.) on fish welfare.

Summarizing, this was the first study on organic aquaculture on sea bass reared in pond in Italy. Concerning fish welfare, no significant differences were observed between the two rearing systems, suggesting that more severe conditions may be required to affect these activities. Since this was a monitoring study on a

commercial farming, potential differences in the investigated parameters may have been mitigated, as the two systems didn't differed so pronounced.

On the other hand, main differences between the two rearing systems were found mostly in last samplings, particularly in November 2010. This could be interesting considering that at that moment fish reached the commercial size and were ready to be sold. Moreover, this suggests that in a research approach differences between farming conditions should be emphasized, in order to reduce time-depending changes and to highlight the main responsible that affect fish welfare.

6. REFERENCES

Agius C. (1981). Preliminary studies on the ontogeny of the melano-macrophages of teleost haemopoietic tissue and age-related changes. Develop Comp Immunol 5, 597–606.

Agius C. (1985) The melano-macrophage centres in fish: a review. In: Manning M.J. and Tatner M.F. (Eds). Fish Immunology, pp. 85–105. Academic Press, London.

Agius C. and Agbede S.A. (1984). Electron microscopical studies on the genesis of lipofuscin, melanin and haemosiderin in the haemopoietic tissues of fish. J Fish Biol 24, 471–488.

Agius C. and Roberts R.J. (2003). Melano-macrophage centers and their role in fish pathology. J Fish Dis 23, 499-509.

Ahmad I., Hamid T., Fatima M., Chand H.S., Jain S.K., Athar M., Raisuddin S. (2000). Induction of hepatic antioxidants in freshwater catfish (*Channa punctatus* Bloch) is a biomarker of paper mill effluent exposure. Biochim Biophys Acta 1523 (1), 37–48.

Alasalvar C., Taylor K.D.A., Zubcov E., Shahidi F., Alexis M. (2002). Differentiation of cultured and wild sea bass (*Dicentrarchus labrax*): total lipid content , fatty acid and trace mineral composition. Food Chem 79, 145-150.

Alcorn S. (2002). Effects of rearing temperature on immune functions in sockeye salmon (*Oncorhynchus nerka*). Fish Shellfish Immunol 12(4), 303-334.

Alexander J.B. and Ingram G.A. (1992) Noncellular nonspecific defence mechanisms of fish. Ann Rev Fish Dis 2, 249-279.

Alliot E., Pastoureaud A., Thebault H (1983). Influence de la temperature et de la salinite´ sur la croissance et la composition corporelle d'alevins de *Dicentrarchus labrax*. Aquaculture 31, 181–94.

Álvarez F., Razquin B.E., Villena A.J., Zapata A.G. (1998). Seasonal changes in the lymphoid organs of wild trout, *Salmo trutta* L.: a morphological study. Vet Immunol Immunopathol 64, 267-78.

Apotheker H. (2000). Is agriculture in need of ethics? J Agric Environ Ethics 12(1), 9–16.

Appleby M.C. (1999). Tower of Babel: Variation in ethical approaches, concepts of welfare and attitudes to genetic manipulations. Anim Welf 8, 381–390.

Arends R.J., Mancera J.M., Wendelaar Bonga S.E., Flik G. (1999). The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. J Endocrinol 163, 149–157.

Ashley P. (2007). Fish welfare: Current issues in aquaculture. Appl Anim Behav Sci 104(3-4), 199-235.

Audet C., Besner M., Munroe J., Dutil J.D. (1993). Seasonal and diel variations of various blood parameters in Atlantic cod (*Gadus morhua*) and American plaice (*Hippoglossoides platessoides*). Can J Zool 71, 611–8.

Audet C., FitzGerald G.J., Guderley H. (1986). Photoperiod effects on plasma cortisol levels in *Gasterosteus aculeatus*. Gen Comp Endocrinol 61, 76-81.

Baeverfjord G., Krogdahl A. (1996). Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: a comparison with the intestines of fasted fish. J Fish Dis 19, 375–387.

Baker M.A., Cerniglia G.J., Zaman A. (1990). Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large number of biological samples. Anal Biochem 190, 360-365.

Bancroft J.D. and Stevens A. (1996). Theory and practice of histological techniques. 4th edn, Churchill Livingstone, New York.

Barata C., Navarro J., Varo I., Riva M., Arun S., Porte C. (2005). Changes in antioxidant enzyme activities, fatty acid composition and lipid peroxidation in Daphnia magna during the aging process. Comp Biochem Physiol B 140, 81-90.

Barton B.A. (1997). Stress in sunfish: past, present and future – a historical perspective. In Fish Stress and Health in Aquaculture, Society for Experiment

Biology, Seminar Series 62 (Iwama G.K., Pickering A.D., Sumper J.P., Schreck C.B., eds), pp. 1–31. Cambridge University Press.

Barton B.A. (2002). Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. Integ Comp Biol 42, 517-525.

Barton B.A., Iwama G. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann Rev Fish Dis 1, 3–26.

Barton B.A., Schreck C.B., Barton L.D. (1987). Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. Dis Aquat Org 2, 173–185.

Barton B.A., Schreck C.B., Fowler L.G. (1988). Fasting and diet content affect stress induced changes in plasma glucose and cortisol in juvenile chinook salmon. Prog Fish Culturist 50, 16–22.

Begout-Anras M.L. (1995). Demand–feeding behaviour of sea bass kept in ponds: diel and seasonal patterns, and influences of environmental factors. Aquacult Int 3, 186–95.

Benedito-Palos L., Bermejo-Nogales A., Karampatos A.I., Ballester-Lozano G.F., Navarro J.C., Diez A., *et al*. (2011). Modelling the predictable effects of dietary lipid sources on the fillet fatty acid composition of one-year-old gilthead sea bream (*Sparus aurata* L.). Food Chem 124, 538–544.

Bertotto D., Poltronieri C., Negrato E., Majolini D., Radaelli G., Simontacchi C. (2010). Alternative matrices for cortisol measurement in fish. Aquacult Res 41 (8), 1261-1267.

Beukema J.J. (1970). Angling experiments with carp: decreased catchability through one trial learning. Neth J Zool 20, 81–92.

Biao X. (2008). The development of and prospects for organic aquaculture worldwide. Out Agric 37(4), 255-260.

Biao X., Xiaorong W., Zhuhong D., Yaping Y. (2003). Critical impact assessment of organic agriculture. J Agric Environ Ethics 16, 297–311.

Blazquez M., Piferrer F., Zanuy S., Carrillo M., Donaldson E.M. (1995). Development of sex control techniques for European sea bass (*Dicentrarchus labrax* L.) aquaculture: effect of dietary 17~methyltestosterone prior to sex differentiation. Aquaculture 135, 329-342.

Boehmer S., Gold M., Hauser S., Thomas B., Young A. (2005). Organic Aquaculture, Alternative Farming Systems Information Center, National Agricultural Library, US Department of Agriculture, Washington, DC.

Bowden T.J., Butler R., Bricknell I.R. (2004). Seasonal variation of serum lysozyme levels in Atlantic halibut (*Hippoglossus hippoglossus* L.). Fish Shellfish Immunol 17, 129-135.

Bowden T.J., Thompson K.D., Morgan A.L., Gratacap R.M.L. and Nikoskelainen S. (2007) Seasonal variation and the immune response: a fish perspective. Fish Shellfish Immunol 22, 695-706.

Braithwaite V.A. (2006). Cognitive ability in fish. Fish Physiol 24, 1-37.

Brodeur J.C., Sherwood J., Rasmussen J.B., Hontela A. (1997). Impaired cortisol secretion in yellow perch (Perca Flavescence) from lakes contaminated by heavy metals: in vivo and in vitro assessment. Can J Fish Aquat Sci 54, 2752-2758.

Broglio C., Rodriguez F., Salas C. (2003). Spacial cognition and its neural basis in teleost fishes. Fish Fish 4, 247–255.

Bromage N.R., Porter M.J.R., Randall C. (2001). The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. Aquaculture 197, 63-98.

Broom D.M, Johnson K.G. (1993). Stress and Animal Welfare. Chapman & Hall, London.

Broom D.M. (1998). Welfare, stress and the evolution of feelings. Advanc Study Behav 27, 371–403.

Broom D.M. (2001). Coping with challenge. Welfare in animals including humans. Dahlem University Press, Berlin.

Bucke D., Vethaak A.D., Lang T. (1992). Quantitative assessment of melanomacrophage centres (MMCs) in dab, *Limanda limanda* along a pollution transect in the German Bight. Mar Ecol Prog Series 91, 193–196.

Cahu C.L., Zambonino Infante J.L., Péres A., Quazuguel P., Le Gall M.M. (1998). Algal addition in sea bass (*Dicentrarchus labrax*) larvae rearing: effect on digestive enzymes. Unité Mixte de Nutrition des Poissons IFREMER– INRA, IFREMER, BP 70, 29280 Plouzané, France. Aquaculture 161, 479–489.

Carrillo M., Zanuy S., Herrera F. (1982). Growth and diurnal variations in metabolic parameters in the starved bass, *Dicentrarchus labrax*, after experimental feeding. Comp Biochem Physiol A 72, 11–6.

Carrillo M., Zanuy S., Prat F., Cerda J., Ramos J., Mananos E.,Bromage N. (1995). Sea bass (*Dicentrarchus labrax*). In: Bromage N.R. and Roberts R.J. (Eds.), Broodstock Management and Egg and Larval Quality. Blackwell, Oxford, pp. 138–168.

Carroll M.C. and Prodeus A.P. (1998) Linkages of innate and adaptive immunity. Curr Opin Immunol 10, 36-40.

Castritsi-Catharios J., Kavadias S. (1993). An experimental study of some haematological parameters of cage aquacultured sea bass *Dicentrarchus labrax* L. under extreme environmental conditions: preliminary results. Mar Life 3, 95–8.

Chandroo K.P., Duncan I.J.H., Moccia R.D. (2004a). Can fish suffer?: perspectives on sentience, pain, fear and stress. App Anim Behav Sci 86, 225-250.

Chandroo K.P., Yue S., Moccia R.D. (2004b). An evaluation of current perspectives on consciousness and pain in fishes. Fish Fish 5, 281-295.

Clarke A. (1993). Seasonal Acclimatization and Latitudinal Compensation in Metabolism: Do They Exist?. Func Ecol 7, 139-149.

Collazos M.E., Ortega E., Barriga C., Rodriguez B. (1998). Seasonal variation in haematological parameters in male and female tench Tinca tinca. Mol Cell Biochem 114, 432-435.

Colombo L., Pickering A.D., Belvedere P., Schreck C.B. (1990). Stress inducing factors and stress reaction in aquaculture. ln: Aquaculture Europe '89 - European Aquaculture Society. Special Publication No. 12, 93-121.

Conte F.S. (2004). Stress and the welfare of cultured fish. App Anim Behav Sci 86, 205-223.

Contreras-Sánchez W.M., Schreck C.B., Fitzpatrick M.S., Pereira C.B. (1998). Effects of stress on the reproductive performance of rainbow trout (*Oncorhynchus mykiss*). Biol Reprod 58(2), 439-447.

Cooper R.U., Clough L.M., Farwell M.A., West T.L. (2002). Hypoxia-induced metabolic and antioxidant enzymatic activities in the estuarine fish *Leiostomus xanthurus*. J Exp Mar Biol Ecol 279, 1–20.

Courtois L.A. (1976). Haematology of juvenile striped bass, *Morone saxatilis* (Walbaum), acclimated to different environmental conditions. Comp Biochem Physiol A 54, 221–223.

Culberson S.D. and Piedrahita R.H. (1996). Aquaculture pond ecosystem model: temperature and dissolved oxygen prediction mechanism and application. Ecol Model 89, 231-258.

Czanyi V. and Doka A. (1993). Learning interactions between prey and predator fish. Mar Behav Phy 23, 63-78.

Dabrowska H., Dabrowski K., Meyerburgdorff K., Hanke W., Gunther K.D. (1991). The effect of large doses of Vitamin-C and magnesium on stress responses in Common Carp, *Cyprinus carpio*. Comp Biochem Physiol A 99, 681–685.

Dalmo R.A., Ingebrigtsen K. and Bogwald J. (1997) Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES). J Fish Dis 20, 241-273.

Davis K.B., Suttle M.A., Parker N.C. (1984). Biotic and abiotic influences on corticosteroid hormone rhythms in channel catfish. Trans Am Fish Soc 113, 414-421.

Dawkins M.S. (1998). Evolution and animal welfare. Quart Rev Biol 73, 305-328.

Demers N.E. and Bayne C.J. (1997). The immediate effects of stress on hormones and lysozyme in Rainbow trout. Develop Comp Immunol 21(4), 363-373.

Demers N.E., Bayne C.J. (1997). The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. Develop Comp Immunol 21, 363– 373.

Désiré L., Boissy A., Veissier I. (2002). Emotion in farm animals: a new approach to animal welfare in applied ethology. Behav Proc 60, 165–180.

Dianzani M.U. (1998). 4-hydroxynonenal and cell signalling. Free Radical Res 28, 553–560.

Dixon B. and Stet R.J. (2001). The relationship between major histocompatibility receptors and innate immunity in teleost fish. Develop Comp Immunol 25, 683-699.

Donaldson E.M. (1981). The pituitary-interrenal axis as indicator of stress in fish. In Pickering A.D. (ed). Stress and Fish. Academic Press, London, pp. 11-47.

Du Pasquier L. (1982) Antibody diversity in lower vertebrates--why is it so restricted? Nature 296, 311-313.

Du Pasquier L. (2001) The immune system of invertebrates and vertebrates. Comp Biochem Physiol B 129, 1-15.

Duncan I.J.H. (2002). Poultry welfare: science or subjectivity? Br Poult Sci 43, 643-652.

EC (2008). Commission Regulation (EC) No 889/2008 of 5 September 2008 laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and control. Off J Eur Union 51, 1–84.

EC (2009). Commission Regulation No 710/2009 of 5 August 2009 amending regulation (EC) No 889/2008 laying down detailed rules for the implementation of Council Regulation (EC) No. 834/2007, as regards laying down detailed rules on organic aquaculture animal and seaweed production. Off J Eur Union 6.8.2009, L204/15.

Edelstein L.M. (1971). Melanin: a unique biopolymer. Pathobiol Ann pp. 309–324. Appleton-Century-Crofts, New York.

Einen O., Waagan B., Thomassen M.S. (1998). Starvation prior to slaughter in Atlantic salmon (*Salmo salar*). I. Effects on weight loss, body shape, slaughter- and fillet-yield, proximate and fatty acid composition. Aquaculture 166, 85–104.

Ellis A.E. (1974). Aspects of the lymphoid and reticulo-endothelial system in the plaice *Pleuronectes platessa* L.. Ph D Thesis, University of Aberdeen.

Ellis A.E. (1980). Antigen-trapping in the spleen and kidney of the plaice, *Pleuronectes platessa* (L.). J Fish Dis 3, 413–426.

Ellis A.E. (2001) Innate host defense mechanisms of fish against viruses and bacteria. Develop Comp Immunol 25, 827-39.

Ellis AE. (1990) Lysozyme assays. In: Stolen J.S., Fletcher T.C., Anderson D.P., Roberson B.S., van Muiswinkel W.B., editors. Techniques in fish immunology. New Jersey: SOS Publications pp. 101-103.

Ellis T. (2001). What is stocking density. Trout News, CEFAS 32, 35–37.

Ellis T. (2002). The effects of stocking density on the welfare of farmed rainbow trout. In: Report of the Workshop on Farmed Fish Welfare held on Monday 28th October 2002, DEFRA.

Ellis T., North B., Scott A.P., Bromage N.R., Porter M., Gadd D. (2002). The relationships between stocking density and welfare in farmed rainbow trout. J Fish Biol 61, 493–531.

Esterbauer H., Schaur R.J., Zollner H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radical Biol Med 11, 81–128.

Evans D.L., Leary J.H., Jaso-Friedmann L. (2001) Nonspecific cytotoxic cells and innate immunity: regulation by programmed cell death. Develop Comp Immunol 25, 791-805.

Evans J.C. (2009). The ethics of fish welfare. J Fish Biol 75, 2872–2874.

Fange R., Lundblad G., Lind J. (1976). Lysozyme and chitinase in blood and lymphomyeloid tissues of marine fish. Marine Biol 36, 277-282.

FAO, 2008-2011. Cultured Aquatic Species Information Programme. Dicentrarchus labrax. Text by Bagni, M. In: FAO Fisheries and Aquaculture Department. Rome.

Fasolato L., Novelli E., Salmaso L., Corain L., Camin F., Perini M. *et al*. (2010). Application of nonparametric multivariate analyses to the authentication of wild and farmed European sea bass (*Dicentrarchus labrax*). Results of a survey on fish sampled in the retail trade. J Agric Food Chem 58, 10979–10988.

FAWC (1996). Report on the welfare of farmed fish. http://www.fawc.org.uk/reports/fish/fishrtoc.htm.

Fearon D.T. (1997) Seeking wisdom in innate immunity. Nature 388, 323-324.

Fearon D.T. and Locksley R.M. (1996) The instructive role of innate immunity in the acquired immune response. Science 272, 50-53.

Ferguson H.W. (1976). The relationship between ellipsoids and melano-macrophage centres in the spleen of turbot (*Scophthalmus maximus*). J Comp Path 86, 377–380.

Finkel T. and Holbrook N.J. (2000). Oxidants, oxidative stress and the biology of ageing. Nature 408, 239–247.

Fletcher T. and White A. (1976). The lysozyme of the plaice *Pleuronectes platessa* L. Comp Biochem Physiol B 55, 207-210.

Fletcher T.C. and Secombes C.J. (2010). Immunology of Fish. eLS.

Franzini M., Fornaciari I., Fierabracci V. (2009). Indici di laboratorio di stress ossidativo (Rassegna). Ligand Assay 14, 41-50.

Fritsch M., Morizur Y., Lambert E., Bonhomme F., Guinand B. (2007). Assessment of sea bass (*Dicentrarchus labrax* L.) stock delimitation in the Bay of Biscay and the English Channel based on mark-recapture and genetic data. Fisher Res 83(2-3), 123-132.

Froese R. and Pauly D. Editors. 2010. FishBase. World Wide Web electronic publication. www.fishbase.org version (11/2010).

FSBI 2002. Fish Welfare. Briefing Paper 2, Fisheries Society of the British Isles, Cambridge.

Fuentes A., Fernández-Segovia I., Serra J. A., Barat J. M. (2010). Comparison of wild and cultured sea bass (*Dicentrarchus labrax*) quality. Food Chem 119, 1514–1518.

Gallaugher P., Thorarensen H., Farrell A.P. (1995). Hematocrit in oxygen transport and swimming in rainbow trout (*Oncorhynchus mykiss*). Resp Physiol 102, 279–292.

Gallone A., Guid G., Maida M., Cicero R. (2002). Spleen and liver pigmented macrophages of *Rana esculenta* L. A new melanogenic system?. Pigm Cell Res 15, 32–40.

Ganassin R.C. and Bols N.C. (1996). Development of long-term rainbow trout spleen cultures that are haemopoietic and produce dendritic cells. Fish Shellfish Immunol 6, 17-34.

Gatesoupe F.J. and Luquet P. (1981). Practical diet for mass culture of the rotifer *Brachionus plicatilis*: application to larval rearing of sea bass *Dicentrarchus labrax* L. Aquaculture 22, 149–163.

Gines R., Palicio M., Zamorano M.J., Arguello A., Lopez J.L., Afonso J.M. (2002). Starvation before slaughtering as a tool to keep freshness attributes in gilthead sea bream (*Sparus aurata*). Aquacult Int 10, 379–389.

Gornati R., Papis E., Rimoldi S.,Terova G., Saroglia M., Bernardini G. (2004). Rearing density influences the expression of stress-related genes in sea bass (*Dicentrarchus labrax* L.). Gene 341, 111-118

Greaves K. and Tuene S. (2001). The form and context of aggressive behaviour in farmed Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture 193, 139–147.

Gregory T.R. and Wood C.M. (1999). The effects of chronic plasma cortisol elevation on the feeding behaviour, growth, competitive ability, and swimming performance of juvenile rainbow trout. Physiol Biochem Zool 72, 286–295.

Grinde B. (1989) Lysozyme from Rainbow-Trout, *Salmo-Gairdneri* Richardson, As An Antibacterial Agent Against Fish Pathogens. J Fish Dis 12, 95-104.

Grinde B., Lie O., Poppe T., Salte R. (1988) Species and individual variation in lysozyme activity in fish of interest in aquaculture. Aquaculture 68, 299-304.

Haffray P., Tsigenopoulos C. S., Bonhomme F., Chatain B., Magoulas A., Rye M., Triantafyllidis A. and Triantaphyllidis C. (2007). Genetic effects of domestication, culture and breeding of fish and shellfish, and their impacts on wild populations. European sea bass-*Dicentrarchus labrax*. p 40-46, In: Svåsand T., Crosetti D., García-Vázquez E., Verspoor E. (eds). Genetic impact of aquaculture activities on native populations. Genimpact final scientific report (EU contract n. RICA-CT-2005-022802). http://genimpact.imr.no/

Halliwell B. and Gutteridge J.M.C. (1999). Free radicals in biology and medicine, 3rd ed. Oxford University Press Oxford.

Hanke W., Hegab S. A., Assem H., Berkowsky B., Gerhard A., Gupta O., Reiter S. (1991). Mechanisms of hormonal action on osmotic adaptation in teleost fish.

In: Fish Ecotoxicology and Ecophysiology. Eds: T. Braunbeck; W. Hanke; H. Segner, VCH, Weinheim. pp. 315-326.

Hanukoglu I. (1992). Steroidogenic enzymes: structure, function and role in regulation of steroid hormone biosynthesis. J Ster Biochem Mol Biol 43(8), 779-804.

Hermansen J.E. (2003). Organic livestock production systems and appropriate development in relation to public expectations. Livest Prod Sci 80, 3–15.

Hontela A. (1998). Interrenal dysfunction in fish from contaminated sites: in vivo and in vitro assessment. Environ Toxicol Chem 17, 44-48.

Huntingford F.A. and Kadri S. (2009). Taking account of fish welfare: lessons from aquaculture. J Fish Biol 75, 2862-2867.

Huntingford F.A., Adams C., Braithwaite V.A., Kadri S., Pottinger T.G., Sandøe P., Turnbull J.F. (2006). Current issues in fish welfare. J Fish Biol 68, 332-372.

Hutchinson T.H., Manning M.J. (1996). Seasonal trends in serum lysozyme activity and total protein concentration in dab (*Limanda limanda* L) sampled from Lyme Bay, UK. Fish Shellfish Immunol 6, 473-482.

IFOAM, 2005. Basic standards for organic production and processing. International Federation of organic Agricultural movements http://www.ifoam.org/about_ifoam/standards/norms/norm_documents_library/IBS_V3_20070817.pdf. Accessed 17 March 2011.

IFOAM, 2010. Organic Aquaculture. EU Regulations (EC) 834/2007, (EC) 889/2008, (EC) 710/2009. BACKGROUND, ASSESSMENT, INTERPRETATION. International Federa- tion of organic Agricultural movements http://www.ifoam.org/about_ifoam/around_world/eu_group-new/positions/publications/aquaculture/IFOAMEU_IAMB_organic_aquaculture_dossier.pdf. Accessed 17 March 2011.

Iwama G.K. (2007). The welfare of fish. Dis Aquat Org 75, 155-158.

Jeney G., Galeotti M., Volpatti D., Jeney Z., Anderson D.P. (1997). Prevention of stress in rainbow trout (*Oncorhynchus mykiss*) fed diets containing different doses of glucan. Aquaculture 154, 1–15.

Johansson L., Kiessling A. (1991). Effects of starvation on rainbow trout 2: eating and storage qualities of iced and frozen fish. Acta Agric Scand 41, 207–216.

Jolles P. and Jolles J. (1984) What is new in lysozyme research? Mol Cell Biochem 63, 165-189.

Jorgensen E.H., Christiansen J.S., Jobling M. (1993). Effects of stocking density on food intake, growth performance and oxygen consumption in Arctic charr (*Salvelinus alpinus*). Aquaculture 110, 191–204.

Kawatsu H. (1969). Studies on the anemia of fish-III. An example of macrcytic anemia found in brook trout, *Salvelinus fontinalis*. Bull Freshwater Res Lab 19, 161–167.

Köllner B., Fischer U., Rombout J.H.W.M., Taverne-Thiele J.J., Hansen J.D. (2004). Potential involvement of rainbow trout thrombocytes in immune functions: a study using a panel of monoclonal antibodies and RT-PCR. Develop Comp Immunol 28(10), 1049-1062.

Kranz H. (1989). Changes in splenic melano-macrophage centres of dab, *Limanda limanda* during and after infection with ulcer disease. Dis Aquatic Org 6, 167–173.

Krogdahl A., Bakke-Mckellep A.M., Baeverjord G. (2003). Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). Aquac Nutr 9, 361–371.

Krüger R., Pietrock M., Meinelt T., Yoshida T., Steffens W., Steinberg C. (1996). Distribution of Macrophage Centres in Bream (*Abramis brama* L.) Liver from the Oder River (Germany/Poland) within the Nature Reserve "Unteres Odertal" Near the Town of Schwedt. Intern Rev Hydrobiol 81, 635-644.

Le Morvan C., Troutaud D., Deschaux P. (1998). Differential effects of temperature on specific and non-specific immune defences in fish. J Exp Biol 201, 165-168.

Leggatt R.A., Brauner C.J., Iwama G.K., Devlin R.H. (2007). The glutathione antioxidant system is enhanced in growth hormone transgenic coho salmon (*Oncorhynchus kisutch*). J Comp Physiol B 177, 413-422.

Lemieux H., Dutil J.D., Guderley H., Larocque R. (2004). Growth, starvation and enzyme activity in white muscle of Atlantic cod: at what point do muscle metabolic capacities change? Mar Freshwater Behav Physiol 37, 287–294.

Lie Ø., Evensen Ø., Sørensen A., Frøysadal E. (1989) Study on lysozyme activity in some fish species. Dis Aquatic Org 6, 1-5.

Lien M.S. and Anthony R. (2007). Ethics and the politics of food. Journal of Agric Environ Ethics, 20, 413–417.

Lindsay G.J.H. and Gooday G.W. (1985). Chitinolytic enzymes and the bacterial microflora in the digestive tract of cod, *Gadus morhua*. J Fish Biol 26, 255-265.

Lo D., Feng L., Li L., Carson M.J., Crowley M., Pauza M., Nguyen A. *et al*. (1999). Integrating innate and adaptive immunity in the whole animal. Immunol Rev 169, 225-239.

Lushchak V.I., Lushchak L.P., Mota A.A., Hermes-Lima M. (2001). Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. Am J Physiol 280, 100-107.

Lymbery P. (2002). In Too Deep—the Welfare of Intensively Farmed Fish. Compassion in World farming, Petersfield Hampshire.

Magnadóttir B. (2006). Innate immunity of fish (overview). Fish Shellfish Immunol 20(2), 137-151.

Malmezat T., Breuille D., Capitan P., Minard P.P., Obled C. (2000). Glutathione turnover is increased during the acute phase of sepsis in rats. J Nutr 130, 1239-1246.

Manson F.D.C., Fletcher T.C., Gooday G.W. (1992). Localization of chitinolytic enzymes in blood of turbot, *Scophthalmus maximus*, and their possible roles in defence. J Fish Biol 40, 919-927.

Marchalonis J.J., Kaveri S., Lacroix-Desmazes S., Kazatchkine M.D. (2002) Natural recognition repertoire and the evolutionary emergence of the combinatorial immune system. The FASEB journal official publication of the Federation of American Societies for Experimental Biology 16, 842-848.

Marino G., Di Marco P., Mandich A., Finoia M. G., Cataudella, S. (2008). Changes in serum cortisol, metabolites, osmotic pressure and electrolytes in response to different blood sampling procedures in cultured sea bass (*Dicentrarchus labrax* L.). J Appl Ichthyol 17(3), 115-120.

Martinez I., Standal I.B., Axelson D.E., Finstad B., Aursand M. (2009). Identification of the farm origin of salmon by fatty acid and HR 13C NMR profiling. Food Chem 116, 766–773.

Martinez M., Guderley H., Dutil J.D., Winger P.D., He P., Walsh S.J. (2003). Condition, prolonged swimming performance and muscle metabolic capacities of cod *Gadus morhua*. J Exp Biol 206, 503–511.

McDonald G. and Milligan L. (1997). Ionic, osmotic and acid-base regulation in stress. In: Iwama G.K., Pickering A.D., Sumpter J.P., Schreck C.B. (editors). Fish Stress and Health in Aquaculture. Cambridge, UK: Cambridge University Press.

Meinelt T.R., Kruger M., Pietrock M.M., Osten R., Steinberg C. (1997). Mercury pollution and macrophage centres in pike (*Esox lucius*) tissues. Environ Sci Poll Res 4, 32–36.

Menoyo D., Lopez-Bote C.J., Bautista J.M., Obach A. (2002). Herring vs. anchovy oils in salmon feeding. Aquat Liv Res 15, 217–223.

Merchie G., Lavens P., Sorgeloos P. (1997). Optimization of dietary vitamin C in fish and crustacean larvae: a review. Aquaculture, 155(1-4), 165-181.

Meseguer J., Esteban M.A., Agulleiro B. (1991). Stromal cells, macrophages and lymphoid cells in the head-kidney of sea bass (*Dicentrarchus labrax* L.): an ultrastructural study Arch Histol Cytol 54, 299–309.

Moeck A. and Peters G. (1990). Lysozyme activity in rainbow trout, *Oncorhynchus mykiss* (Walbaum), stressed by handling, transport and water pollution. J Fish Biol 37, 873– 885.

Mommsen T.P., Vijayan M.M., Moon T.W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev Fish Biol Fish 9, 211-268.

Montero D., Izquierdo M.S., Tort L., Robaina L., Vergara J.M., (1999). High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. Fish Physiol Biochem 20, 53–60.

Montero D., Tort L., Robaina L., Vergara J.M., Izquierdo M.S. (2001). Low vitamin E in diet reduces stress resistance of gilthead seabream (*Sparus aurata*) juveniles. Fish Shellfish Immunol 11, 473–490.

Moretti A., Pedini Fernandez-Criado M., Cittolin G., Guidastri R. (1999). Manual on hatchery production of seabass and gilthead seabream – Volume 1. Food and Agriculture Organization of the United Nations, Rome, 194 pp.

Morgan A.L., Thompson K.D., Auchinachie N.A., Migaud H. (2008). The effect of seasonality on normal haematological and innate immune parameters of rainbow trout *Oncorhynchus mykiss* L. Fish Shellfish Immunol 25, 791–799.

Mourente G., Diaz-Salvago E., Bell J.G., Tocher D.R. (2002). Increased activities of hepatic antioxidant defence enzymes in juvenile gilthead sea bream (*Sparus aurata* L.) fed dietary oxidised oil: attenuation by dietary vitamin E. Aquaculture 214, 343–361.

Mourente G., Diaz-Salvago E., Tocher D.R., Bell J.G. (2000). Effects of dietary polyunsaturated fatty acid/vitamin E (PUFA/tocopherol ratio on antioxidant

defence mechanisms of juvenile gilthead sea bream (*Sparus aurata* L., Osteichthyes, Sparidae). Fish Physiol Biochem 23, 337–351.

Murai T. and Andrews J.W. (1974). Interaction of dietary α- tocopherol, oxidized menhaden oil and ethoxyquin on channel catfish (*Ictalurus punctatus*). J Nutr 104, 1416–1431.

Murray C.K. and Fletcher T.C. (1976). The immunohistochemical localisation of lysozyme in plaice (*Pleuronectes platessa*) tissues. J Fish Biol 9, 329-334.

Nakanishi T. (1986). Seasonal changes in the humoral immune response and the lymphoid tissues of the marine teleost, *Sebastiscus marmoratus*. Vet Immunol Immunopathol 12, 213-221.

Neumann N.F., Stafford J.L., Barreda D., Ainsworth A.J., Belosevic M. (2001) Antimicrobial mechanisms of fish phagocytes and their role in host defense. Develop Comp Immunol 25, 807-825.

O'Steen S., Bennett A.F. (2003). Thermal acclimation effects differ between voluntary, maximum, and critical swimming velocities in two cyprinid fishes. Physiol Biochem Zool 76, 484-496.

Ortuno J., Esteban M.A., Mesequer J. (2002) Lack of effect of combining different stressors on innate immune responses of sea bream (*Sparus auratus* L.). Vet Immunol Immunopathol 84, 17–27.

Pankhurst N.W. and Van Der Kraak G. (1997). Effects of stress on reproduction and growth. In Iwama G., Pickering A., Sumpter J., Schreck C. (Eds.), Fish Stress and Health in Aquaculture (pp. 73–94). Cambridge University Press.

Parry R.M., Chandan R.C., Shahani R.M. (1965). A rapid sensitive assay of muramidase. Proc Soc Exp Biol Med 119, 384-386.

Pascoli F., Negrato E., Di Giancamillo A., Bertotto D., Domeneghini C., Simontacchi C., Multinelli F., Radaelli G. (2010). Evaluation of oxidative stress biomarkers in *Zosterisessor ophiocephalus* from the Venice Lagoon, Italy. Aquat Toxicol 101, 512-520.

Pascual P. (2003). Effect of food deprivation on oxidative stress biomarkers in fish (*Sparus aurata*). Chem-Biol Inter 145(2), 191-199.

Pastoureaud A. (1991). Influence of starvation at low temperatures on utilization of energy reserves, appetite recovery and growth character in sea bass, *Dicentrarchus labrax*. Aquaculture 99, 167-178.

Pavlidis M., Berry M., Divanach P., Kentouri M. (1997). Diel pattern of haematocrit, serum metabolites, osmotic pressure, electrolytes and thyroid hormones in sea bass and sea bream. Aquacult Int 5, 237–247.

Pavlovic S.Z., Borkovic Mitic S.S., Radovanovic T.B., Perendija B.R., Despotovic S.G., Gavric J.P., *et al*. (2010). Seasonal variations of the activity of antioxidant defense enzymes in the red mullet (*Mullus barbatus* L.) from the Adriatic Sea. Mar Drugs 26, 413-428.

Pearse A.G.E. (1990). Trattato di Istochimica. Aggiornamenti ai volumi I - II, 3rd ed. Piccin, Padova.

Pelletier N. (2003). Understanding Proposed Organic Certification Standards for Farmed Salmon. BC Ministry of Agriculture, Food and Fisheries, Victoria, BC. http://www.fao.org/DOCREP/005/Y3781E/Y3781E00.htm (accessed 09/2005).

Person-Le Ruyet J., Mahe K., Le Bayon N., Delliou H. (2004). Effects of temperature on growth and metabolism in a Mediterranean population of European sea bass, *Dicentrarchus labrax*. Aquaculture 237, 269-280.

Peters G. and Schwarzen R. (1985). Changes in haemopoietic tissue of rainbow trout under influence of stress. Dis Aquat Org 1, 1–10.

Pickering A. D. (1993). Growth and stress in fish production. Aquaculture 111(1-4), 51-63.

Pickering A.D. and Pottinger T.G. (1995). Biochemical effects of stress. Biochem Mol Biol Fishes 5, 349-379.

Pickering A.D., Pottinger T.G., Carragen J.F., Sumpter J.P. (1987). The effects of acute and chronic stress and levels of reproductive hormones in the plasma of mature male browntrout, *Salmo trutta*. L. Gen Comp Endocrinol 68, 249-259.

Pickering A.D., Pottinger T.G., Christie P. (1982). Recovery of the brown trout, *Salmo trutta* L. from acute handling stress: a time-course study. J Fish Biol 20, 229-244.

Pickering A.D., Pottinger T.G., Christie P. (1982). Recovery of the brown trout, *Salmo trutta* L., from acute handling stress: a time-course study. J Fish Biol 20, 229–244

Piferrer F., Blásquez M., Navarro L., González A. (2005). Genetic, endocrine and environmental components of sex determination and differentiation in the European sea bass (*Dicentrarchus labrax* L.). Gen Comp Endocrinol 142, 102-110.

Pirhonen J., Schreck C.B., Reno P.W., Ogut H. (2003). Effect of fasting on feed intake, growth and mortality of chinook salmon, *Oncorhynchus tshawytscha*, during an induced *Aeromonas salmonicida* epizootic. Aquaculture 216, 31–38.

Planas J., Gutierrez J., Fernandez J., Carrillo M., Canals P. (1990). Annual and daily variations of serum cortisol in sea bass *Dicentrarchus labrax* L. Aquaculture 91, 171-178.

Poli B.M. (2009). Farmed fish welfare-suffering assessment and impact on product quality. It J Anim Sci 8, 139-160.

Poli B.M., Parisi G., Scappini F., Zampacavallo G. (2005). Fish welfare and quality as affected by pre-slaughter and slaughter management. Aquacult Int 13, 29-49.

Poli B.M., Scappini F., Parisi G., Zampacavallo G., Mecatti M., Lupi P., Mosconi G., Giorgi G., Vigiani V. (2004). Traditional and innovative stunning /slaughtering methods for European sea bass compared by the complex of the assessed behavioural, plasmatic and tissue stress and quality indexes at death and during shelf life. Proceedings of WEFTA Conference 2004, 12-15 september, 58-63.

Poltronieri C., Rosaria L., Bertotto D., Negrato E., Simontacchi C., Guerrera M. C., Radaelli G. (2009). Effects of exposure to overcrowding on rodlet cells of the teleost fish *Dicentrarchus labrax* (L.). Vet Res Comm 33, 619-629.

Prat F., Zanuy S., Carrillo M., De Mones A., Fostier A. (1990). Seasonal changes in plasma levels of gonadal steroids of sea bass, *Dicentrarchus labrax* L. Gen Comp Endocrinol 78, 361–373.

Press C. (1994) Immune and enzyme histochemical phenotypes of lymphoid and nonlymphoid cells within the spleen and head kidney of Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol 4, 79-93.

Reddy P.K. and Leatherland J.F. (1988). Stress Physiology. In: Leatherland J.F., Woo P.T.K. (Eds.) Fish Diseases, Volume III: Non-infectious disorders, C.A.B. International, pp.279-302.

Reddy P.K., Vijayan M.M., Leatherland J.F., Moon T.W. (1995). Does RU486 modify hormonal responses to handling stressor and cortisol treatment in fed and fasted rainbow trout? J Fish Biol 46, 341-359.

Reese E.S. (1989). Orientation behaviour of butterfly fishes (family Chaetodontidae) on coral reefs – spacial learning of route specific landmarks and cognitive maps. Environ Biol Fish 25, 79-86.

Reid S.G., Vijayan M.M., Perry S.F. (1996). Modulation of catecholamine storage and release by the pituitary-interrenal axis in the rainbow trout (*Oncorhynchus mykiss*). J Comp Physiol B 165, 665-676.

Rios F.S., Kalinin A.L., Rantin F.T. (2002). The effects of long-term food deprivation on respiration and haematology of the neotropical fish *Hoplias malabaricus*. J Fish Biol 61, 85–95.

Roberts R. J. (1975). Melanin-containing cells of the teleost fish and their relation to disease. The Pathology of Fishes, University of Wisconsin Press, Madison, WI.

Roberts R. J. (2001). Fish Pathology, 3rd ed. W.B. Saunders,Philadelphia, PA.

Robin J.H. (1989). The quality of living preys for fish larval culture: preliminary results on mineral supplementation. In: De Pauw N., Jaspers E., Ackefors H., Wilkins N. (Eds.), Aquaculture-A Biotechnology in Progress. European Aquaculture Society, Bredene, pp. 769–774.

Roche H., Chaar K., Peres G. (1989). The effect of a gradual decrease in salinity on the significant constituents of tissue in the sea bass (*Dicentrarchus labrax*, Pisces). Comp Biochem Physiol A 93, 785-789.

Rodriguez F., Duran E., Vargas J.P., Torres B., Salas C. (1994). Performance of goldfish trained in allocentric and egocentric maze procedures suggests the presence of a cognitive mapping system in fishes. Anim Learn Behav 22, 409-420.

Røed K.H., Larsen H.J., Linder R.D., Refstie T. (1993). Genetic variation in lysozyme activity in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 109, 237– 244.

Roncarati A., Sirri F., di Domenico A., Brambilla G., Iamiceli A. L., Melotti P., *et al*. (2010). Survey of qualitative traits of European sea bass cultivated in different rearing systems. Eur J Lipid Sci Technol 112, 770–779.

Rose J.D. (2002). The neurobehavioural nature of fishes and the question of awareness and pain. Rev Fisher Sci 10, 1-38.

Sakai T., Murata H., Endo M., Yamauchi K., Tabata N., Fukudome M. (1989). 2-Thiobarbituric acid values and contents of α-tocopherol and bile pigments in the liver and muscle of jaundiced yellowtail, *Seriola aquiqueradiata*. Agric Biol. Chem 53, 1739–1740.

Sargent J.R., Tocher D.R., Bell J.G. (2002). The lipids. In: Halver, J.E., Hardy, R.W. (Eds). Fish Nutrition, 3rd edn. Elsevier, USA. pp. 181–257.

Saurabh S. and Sahoo P.K. (2008). Lysozyme: an important defence molecule of fish innate immune system. Aquacult Res 39, 223-239.

Schreck C. (2001). Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture, 197(1-4), 3-24.

Simontacchi C., Bongioni G., Faresin L., Bono G. (1995). Messa a punto di un metodo RIA su micropiastra per il dosaggio diretto del progesterone ematico. XLIX Convegno Nazionale S:I.S.Vet., Salsomaggiore Terme, 27-30 settembre 1995, pp. 343-344.

Simontacchi C., Poltronieri C., Carraro C., Bertotto D., Xiccato G., Trocino A., Radaelli G. (2008). Alternative stress indicators in sea bass *Dicentrarchus labrax*, L. J Fish Biol 72, 747-752.

Skjervold P.O., Fjaera S.O., Oestby P.B., Einen O. (2001). Live-chilling and crowding stress before slaughter of Atlantic salmon (*Salmo salar*). Aquaculture 192, 265-280.

Slater C. and Schreck C. (1998). Season and physiological parameters modulate salmonid leucocyte androgen receptor affinity and abundance. Fish Shellfish Immunol 8, 379-391.

Sneddon L.U. (2003). The evidence for pain in fish: the use of morphine as an analgesic. Appl Anim Behav Sci 83, 153-162.

Sneddon L.U., Braithwaite V.A., Gentle M.J. (2003). Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system. Proc R Soc Lond Ser B-Biol Sci 270, 1115-1121.

Sovrano V.A. and Bisazza A. (2003). Modularity as a fish (*Zenotoca eisen*) views it: Conjoining and nongeometric information for spacial reorientation. J Exp Phychol 29, 199-210.

Spandrio L. (1988). Manuale di laboratorio. Vol. 3. Piccin, Padova.

Stevenson P. (2007). Closed waters: the welfare of farmed Atlantic salmon, rainbow trout, Atlantic cod & Atlantic halibut. CIWS, WSPA.

Stocco D.M. and Clark B.J. (1996). Regulation of the acute production of steroids in steroidogenic cells. Endocr Rev 17(3), 221-244.

Stolen J.S., Gahn T., Kasper V., Nagle J.J. (1984). The effect of environmental temperature on the immune response of a marine teleost (Faralichrhys denrufus). Develop Comp Immunol 8, 89-98.

Sumpter J.P., Lebail P.Y., Pickering A.D., Pottinger T.G., Carragher J.F. (1991). The effect of starvation on growth and plasma growth hormone concentrations of rainbow trout, *Oncorhynchus mykiss*. Gen Comp Endocrinol 83, 94–102.

Sumpter JP. (1997). The endocrinology of stress. In: Iwama G.K., Pickering A.D., Sumpter J.P., Schreck C.B. (Eds). Fish Stress and Health in Aquaculture. Cambridge University Press.

Sunyer J.O. and Tort L. (1995). Natural hemolytic and bactericidal activities of sea bream *Sparus aurata* serum are affected by the alternative complement pathway. Vet Immunol Immunopathol 45, 333-345.

Sunyer J.O., Tort L., Lambris J.D. (1997). Diversity of the third form of complement, C3, in fish: functional characterization of five forms of C3 in the diploid fish *Sparus aurata*. Biochem J 326, 877-881.

Suresh N. (2009). Effect of cadmium chloride on liver, spleen and kidney melano macrophage centres in *Tilapia mossambica*. Environ Biol 30, 505-508.

Swaney W., Kendal J., Capon H., Brown C., Laland K.N. (2001). Familiarity facilitates social learning of foraging behaviour in the guppy. Anim Behav 62, 591–598.

Terlouw E.M.C., Arnould C., Auperin B., Berri C., Le Bihan-Duval E., Deiss V., *et al*. (2008). Pre-slaughter conditions, animal stress and welfare: current status and possible future research. Animal 2, 1501-1517.

Thompson K.R., Rawles S.D., Metts L.S., Smith R., Wimsatt A., Gannam A.L. *et al*. (2008). Digestibility of dry matter, protein, lipid, and organic matter of two fish meals, two poultry by-product meals, soybean meal, and distiller's dried grains with solubles in practical diets for sunshine bass, *Morone chrysops* x *M. saxatilis*. J World Aquac Soc 39, 352–363.

Thorpe J.E., McConway M.G., Miles M.S., Muir J.S. (1987). Diel and seasonal changes in resting plasma cortisol levels in juvenile Atlantic salmon, *Salmo salar* L. Gen Comp Endocrinol 65, 19-22.

Tietze F. (1969). Enzymic method for quantitative determination of nanogram amount of total and oxidized glutathione: application to mammalian blood and other tissues. Anal Biochem 27, 502-522.

Tort L., Rotllant J., Rovira L. (1998). Immunological suppression in gilthead sea bream *Sparus aurata* of the North West Mediterranean at low temperature. Comp Biochem and Phys A 120, 175-179.

Trocino A., Xiccato G., Majolini D., Tazzoli M., Bertotto D., Pascoli F., Palazzi R. (2012). Assessing the quality of organic and conventionally-farmed European sea bass (*Dicentrarchus labrax*). Food Chem 131(2), 427-433.

Turchini G.M., Torstensen B.E., Ng W.K. (2009). Fish oil replacement in finfish nutrition. Rev Aquacult 1, 10–57.

Turnbull J.F., Bell A., Adams C., Bron J., Huntingford F.A. (2005). Stocking density and welfare of cage farmed Atlantic salmon: application of a multivariate analysis. Aquaculture 243, 121-132.

Uchida K. (2003). 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. Prog Lipid Res 42, 318-343.

Van Raaij M.T.M., Pit D.S.S., Balm P.H.M., Steffens A.B., Van den Thillart G.E.E.J.M. (1996). Behavioral strategy and the physiological stress response in rainbow trout exposed to severe hypoxia. Horm Behav 30, 85–92.

Vazzana M., Cammarata M., Cooper E.L., Parrinello N. (2002). Confinement stress in sea bass (*Dicentrarchus labrax*) depresses peritoneal leukocyte cytotoxicity. Aquaculture 210, 231-243.

Vellas F., Ferroni J.M., Bau F., Parent J.P. (1994). Recherches sur l'état physiologique des poissons de la retenue de Pareloup: données relatives a cinq années d'étude. Hydroecol Appl 6, 257–292.

Vijayan M.M. and Moon T.W. (1994). The stress response and the plasma disappearance of corticosteroid and glucose in a marine teleost, the sea raven. Can J Zool 72, 379-386.

Vilmos P. and Kurucz E. (1998) Insect immunity: evolutionary roots of the mammalian innate immune system. Immunol Lett 62, 59-66.

Volpato G.L. (2009). Challenges in assessing fish welfare. ILAR J 50(4), 329-337.

Volpatti D., D'Angelo L., Jeney G., Jeney Z., Anderson D.P., Galeotti M. (1998). Nonspecific immune response in fish fed glucan diets prior to induced transportation stress. J Appl Ichthyol 14, 201–206.

Watanabe T., Takashima F., Ogino C., Hibiya T. (1970). Effect of α-tocopherol on carp. Nippon Suisan Gakkaishi 36: 623–630.

Wedemeyer G. (1972). Some Physiological Consequences of Handling Stress in the Juvenile Coho Salmon and Steelhead Trout. J Fish Res 29(12), 178-1783.

Wendelaar Bonga S.E. (1997). The stress response in fish. Physiol Rev 77, 591-625.

Weyts F.A.A., Cohen N., Flik G., Verburg-van Kemenade B.M.L. (1999). Interactions between the immune system and the hypothalamopituitary-interrenal axis in fish. Fish Shellfish Immunol 9, 1-20.

Winston G.W. and Di Giulio R.T. (1991). Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat Toxicol 19, 137– 161.

Wojtowicz A. and Plytycz B. (1997). Seasonal changes of the gut associated lymphoid tissue in the common toad, *Bufo bufo*. J Nutr Immunol 5, 57-64.

Wolke R.E. (1992). Piscine macrophage aggregates: a review. Ann Rev Fish Dis 2, 91–108.

Wolke R.E., Murchelano R.A., Dickstein C., George C.J. (1985). Preliminary evaluation of the use of macrophage aggregates (MA) as fish health monitors. Bull Environ Contam Toxicol 35, 222–227.

Yousif A., Albright L., Evelyn T. (1994). In vitro evidence for the antibacterial role of lysozyme in salmonid eggs. Dis Aquat Org 19, 15-19.

Yussefi M. and Willer H. (2002). Organic Agriculture Worldwide 2002. Biofach 74, Nurnberg.

Zanuy S., Carrillo M., Felip A., Rodriguez L., Blazquez M., Ramos J., Piferrer F. (2001). Genetic, hormonal and environmental approaches for the control of reproduction in the European sea bass (*Dicentrarchus labrax* L). Aquaculture 202(3–4), 187–203.

Zapata A.G., Varas V., Torroba M. (1992). Seasonal variations in the immune system of lower vertebrates. Immunol Today 13, 142-7.

Zarkadis I.K., Mastellos D., Lambris J.D. (2001) Phylogenetic aspects of the complement system. Develop Comp Immunol 25, 745-62.

Zuasti A., Jara J.R., Ferrer C., Solano F. (1989). Occurrence of melanin granules and melano synthesis in the kidney of *Sparus auratus*. Pigm Cell Res 2, 93–99.

APPENDIX I

Published Journal Articles

Fish & Shellfish Immunology 31 (2011) 1081–1087

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Seasonal effects on hematological and innate immune parameters in sea bass *Dicentrarchus labrax*

F. Pascoli[a], G.S. Lanzano[a], E. Negrato[a], C. Poltronieri[a], A. Trocino[b], G. Radaelli[a], D. Bertotto[a,*]

[a] Department of Experimental Veterinary Sciences, Faculty of Veterinary Medicine, University of Padua, Italy
[b] Department of Public Health, Comparative Pathology and Veterinary Hygiene, Faculty of Veterinary Medicine, University of Padua, Italy

ARTICLE INFO

Article history:
Received 12 May 2011
Received in revised form
1 September 2011
Accepted 9 September 2011
Available online 18 September 2011

Keywords:
Innate immunity
Seasonality
Cortisol
Glutathione
Dicentrarchus labrax

ABSTRACT

The temperate aquatic environment is affected by two primary components of season, temperature and photoperiod, during the annual cycle. Many organisms respond to seasonal change physiologically, behaviorally or both. The aim of this study was to investigate the effect of seasonality on cortisol, hematological and innate immune parameters in European sea bass reared under traditional semi-intensive aquaculture. Sea bass (*Dicentrarchus labrax*) were reared in an outdoor pond and serum cortisol, hematocrit, leucocrit, serum lysozyme activity and total glutathione were bimonthly monitored over a 14-months period. The effect of seasonality was observed for all parameters carried out, with generally higher values in summer and lower in winter. These results could improve the understanding of the influence of seasonal cues on the immune system and hematological parameters in fish in order to optimize the husbandry practices.

1. Introduction

Seasonality is a complex event made up of many potential cues, with the principle being changes in temperature and day length [1]. Many organisms respond to seasonal change physiologically, behaviorally or both [1]. Several studies concerning the effects of seasonality on the physiology of fishes have been carried out in the past. In fish, the blood levels of biochemical variables have turned out to be mainly affected by photoperiod, water temperature, dissolved oxygen, salinity, quality and rate of food consumed [2–5]. It has been argued that fish responses to seasonal changes are controlled by one of the principal cues or by a combination of both [1]. Sea bass metabolic parameters show diurnal and seasonal changes [6,7] and growth and feeding rates of this species are correlated with salinity, water temperature and photoperiod [2,8,9].

Temperature is a well-known principal environmental cue in fish and it has been extensively studied with regard to reproduction, animal behavior and immune response [10–12]. It co-ordinates their reproductive activity, affects body weight and condition, influences food intake and locomotor activity and is also believed to co-ordinate their immune response [13]. It is well documented that seasonality also affects the immune response of vertebrates [1,14]. In general, parameters are suppressed during winter and raised in summer [15], and whilst the majority of research has been carried out in mammals, the importance of seasonality on the immune response of fish is increasingly being recognized [1].

Adaptive immunity in fish was shown to exhibit a seasonal cycle over a 12-month period, in particular, changes in resting antibody titer and response to antigenic challenge [16]. Other studies have evidenced seasonal changes also in the lymphoid system [17,18] and in the numbers of circulating lymphocytes [15]. Innate immunological indicators have turned out to be strongly affected by low temperatures in gilthead sea bream (*Sparus aurata* L.). Affected fish showed severe immunosuppression involving significant decrease of serum complement activity, decrease of plasma lysozyme activity and reduction in circulating lymphocytes [19]. Hematological and innate immune parameter seasonal variations were also investigated in rainbow trout (*Oncorhynchus mykiss*) [20].

Water quality, available oxygen and food supply and quality are very important factors affecting fish health and welfare in aquaculture [21,22]. When subjected to a stressor, fishes employ a host of physiological adjustments designed to overcome the perceived challenge [23]. In fishes, stress causes both short- and long-term physiological changes mainly due to the action of hormones such as catecholamines and cortisol [23]. Stimuli that activate the stress axis and affect corticosteroid levels in fish range from handling and crowding to

* Corresponding author. Tel.: +39 049 8272787; fax: +39 049 8272796.
E-mail address: daniela.bertotto@unipd.it (D. Bertotto).

1050-4648/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2011.09.014

temperature shock and social confrontation [24]. In fishes, an increase in plasma cortisol has been widely employed as a quantitative measure of stress [25–27]. As a result, physiological parameters can be used as tools to assess environmental impacts on fishes.

European sea bass (*Dicentrarchus labrax*) is a very important economical resource, as it represents the 46.2% of the total production of farmed fish in Mediterranean countries [28].

The aim of this study was to investigate the effect of seasonality on hematological parameters and innate immune system in European sea bass reared under traditional semi-intensive aquaculture in a farm located in Veneto (Italy). This was achieved by measuring serum cortisol (a stress indicator), serum lysozyme (an innate immune parameter), hematocrit and leucocrit (two hematological parameters) and total glutathione (an oxidative stress parameter) every two months over a 14-months period. The study was carried out under ambient temperature and photoperiod.

2. Materials and methods

2.1. Animal sampling and sample preparation

Sea bass (*D. labrax*) coming from the fish farm "Impianto Ittico Sperimentale di Pellestrina" (Veneto Agricoltura, Legnaro) have been transferred to the Centro Ittico Valle Bonello in May 2009 into an outdoor pond and monitored for 14 months, until sea bass reached the size of about 400 g (initial weight 60 g; tank density 2–12 kg/m^3). Water temperature was recorded twice a day (morning and afternoon) with a digital thermometer. From May 2009 to July 2010, twenty fishes were netted every two months reaching a total of eight samplings. Immediately after capture, animals were put into iced brackish water and brought to the near laboratory facilities. During each sampling, biometric measures (weight, total and standard lengths) were recorded and condition factor (*K*), an index to evaluate the relative robustness of fish, was obtained as described by Bagenal and Tesch [29]. Then, animals were bled by collecting blood from caudal vein using sterile syringes and transferred to tubes with and without K_3-EDTA.

2.2. Cortisol

Serum was isolated by allowing the blood to clot overnight at 4 °C and standard centrifugation and stored at −20 °C until analysis. Cortisol from serum was extracted with diethyl ether, diluted with phosphate buffered saline (PBS) solution and utilized for a specific microtitre radioimmunoassay (RIA) as described by Simontacchi et al. [30].

2.3. Hematology

Hematocrit and leucocrit, defined as the volume of packed erythrocytes and leucocytes, respectively, were obtained by microcentrifugation of whole blood with portable centrifuge (12,500 rpm, 5 min; MicroCL 17, Thermo Scientific, Germany). The heparinized microhematocrit tubes were observed under a binocular magnifying glass (×20).

2.4. Lysozyme

Lysozyme activity in serum was measured turbidimetrically according to Refs. [31, 32]. Lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich Co., St. Louis, MO, USA) was added to a 0.067 M Sorensen sodium phosphate buffer (pH 5.83) at a concentration of 0.3 mg/ml. Forty microliters of serum were added to two replicate wells of a 96 multiwell plate (Perkin–Elmer Life and Analytical Sciences, Shelton, CT, USA), apart from the control wells. Two hundred and thirty microliters of the bacterial suspension were added to all wells. Absorbance was read at 490 nm, 1 min after adding the buffer and then again after 5 min using a microplate photometer (Spectracount microplate photometer; Packard-Instrument, Meriden, CT, USA). Lysozyme activity was expressed as the amount of sample causing a decrease in absorbance of 0.001 min^{-1} [33]. Units used were min^{-1} ml^{-1}.

2.5. Glutathione (GSH)

Total GSH in blood serum was determined by an enzymatic recycling method originally described by Tietze [34] and adapted for microtitre plate reader [35].

2.6. Statistical analysis

Statistical analysis was carried out with STATISTICA 8.1 (StatSoft) software. All data are reported as mean ± SEM. Differences among sampling were tested with factorial analysis of variance and

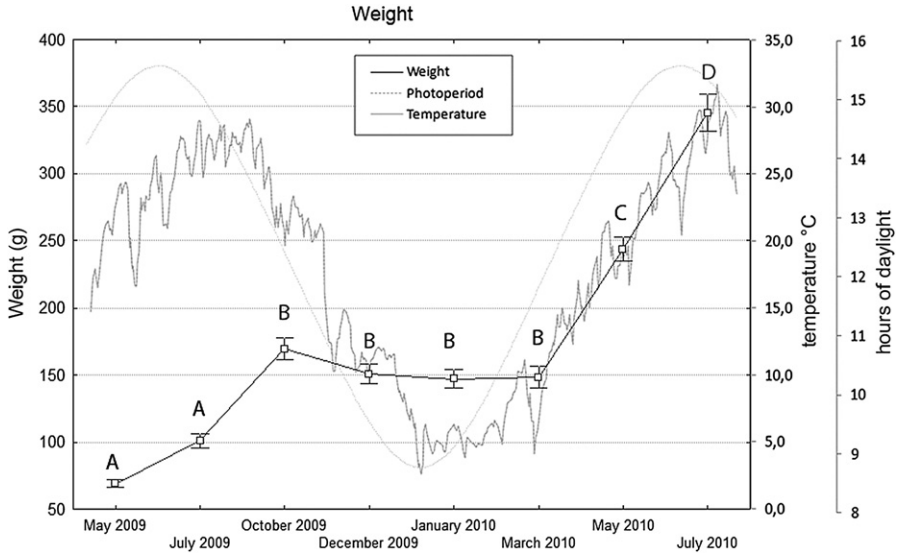

Fig. 1. Variations in weight of sea bass over a 14-months period (mean ± SE). Different letters indicate significant differences ($p < 0.05$).

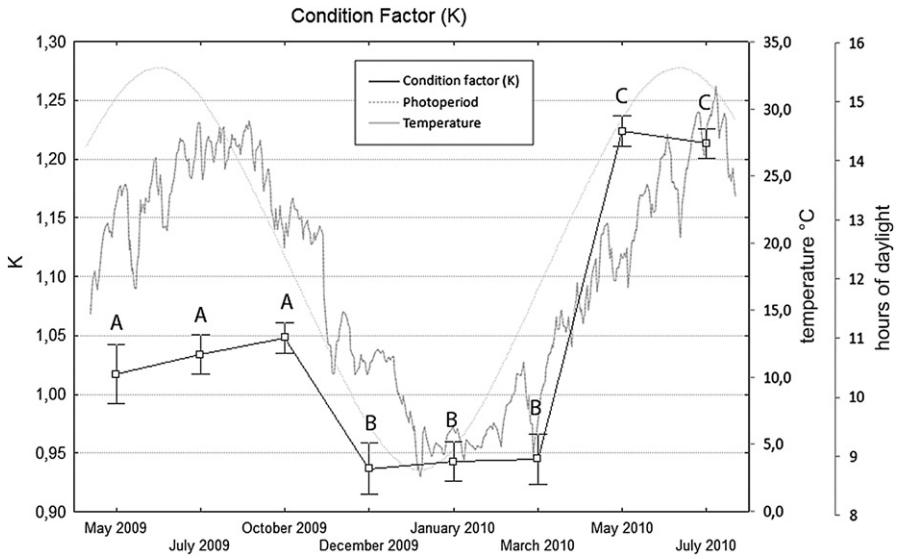

Fig. 2. Variations in condition factor (K) of sea bass over a 14-months period (mean ± SE). Different letters indicate significant differences ($p < 0.05$).

HSD-Tukey test. In all analyses a $p < 0.05$ value was accepted as significant.

3. Results

3.1. Growing performances

The average weight of fish at the start of the experiment was 69.1 ± 3.0 g and 345.5 ± 13.6 g at the end of the trial 14 months later (Fig. 1). The condition factor got worse from 1.02 to 1.21 during this time (Fig. 2). A significant effect on condition factor was found by month ($p < 0.01$). Condition factor was lower in December 2009, January and March 2010 than that in the other samplings, whereas it significantly increased in May and July 2010. From the middle of October to the middle of March there was the winter starvation period.

3.2. Cortisol

Cortisol level was significantly higher in May 2009, May and July 2010 than those in the other samplings ($p < 0.05$), indicating some seasonal influence. The lowest levels were found in October, December 2009, January and March 2010 ($p < 0.05$) (Fig. 3).

3.3. Hematocrit and leucocrit

Hematocrit was significantly lower in January and March 2010 than in the other samplings ($p < 0.05$) (Fig. 4).

Leucocrit showed significant low levels in December 2009, January and March 2010 ($p < 0.05$) (Fig. 5). The highest value was recorded in October ($p < 0.01$).

Both parameters exhibit a seasonal influence.

3.4. Lysozyme activity

Serum lysozyme activity increased from May to October 2009, then decreased to January 2010 and re-increased in the following months, indicating some seasonal influence (Fig. 6). The lowest levels of activity were recorded in January ($p < 0.01$). A significant effect on serum lysozyme activity was found by month ($p < 0.01$). The level of serum lysozyme activity was higher in July 2010 than in

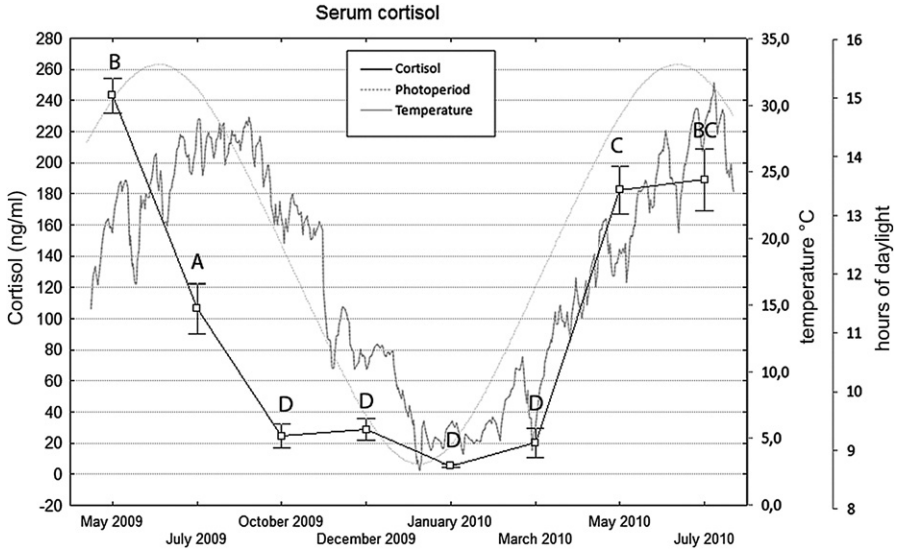

Fig. 3. Variations in serum cortisol of sea bass over a 14-months period (mean ± SE). Different letters indicate significant differences ($p < 0.05$).

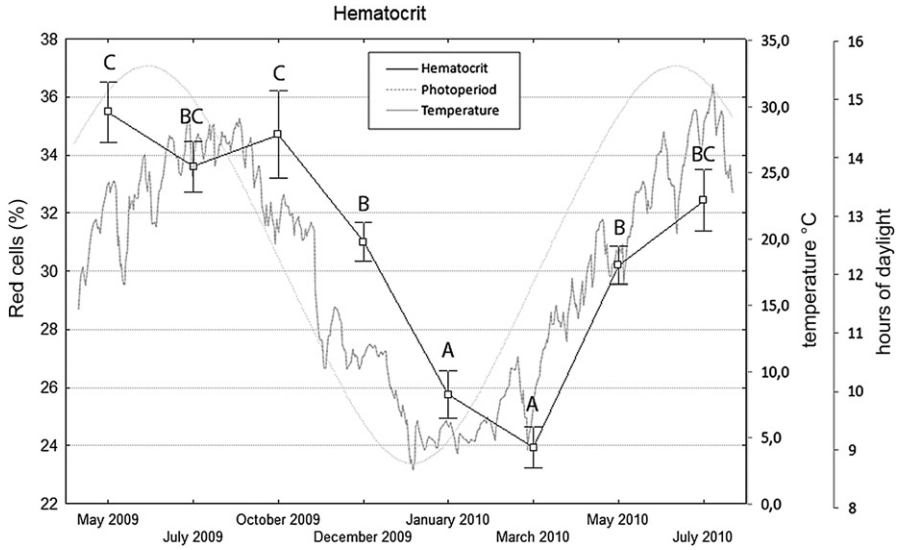

Fig. 4. Variations in hematocrit of sea bass over a 14-months period (mean ± SE). Different letters indicate significant differences ($p < 0.05$).

May, July and December 2009, but non significantly different from October 2009 and May 2010.

3.5. Glutathione

Total glutathione decreased from July to December 2009, than increased to July 2010, indicating some seasonal effect (Fig. 7). GSH was lower in October, December 2009, January and March 2010 than that in the other months, and higher both in July 2009 and 2010 ($p < 0.05$).

4. Discussion

Aquaculture fish production has increased significantly over the past few decades [28]. In order to mitigate disease outbreaks in aquaculture, that cause substantial economic loss, it is necessary to develop disease control strategies based on a better understanding of the effects of husbandry methods and environmental stressors on the health status of farmed fish [1].

In the present study, we investigated the effect of seasonal cues (temperature and photoperiod) on some stress and hematological parameters. The effect of seasonality was observed for all parameters carried out.

Fish weight exhibited a significant slowdown during cold months (from October to March), as well as the condition factor that decreased in the same period. This trend is possibly related to water temperature, that can affect diet and metabolism [36]. Temperature regulates also the feed intake, as sea bass stop growing at 10 °C and below 7 °C food intake ceases [37].

Cortisol mean levels are in accordance with those reported in other studies [38–41]. It is known that cortisol of sea bass is very sensible to acute stress, as during transport and handling [42, 43]. The high values found in May 2009 (243.3 ± 11.1 ng/ml) could be related to the short interval occurred between the transfer of the juveniles (April 2009) and the sampling. Serum cortisol exhibited

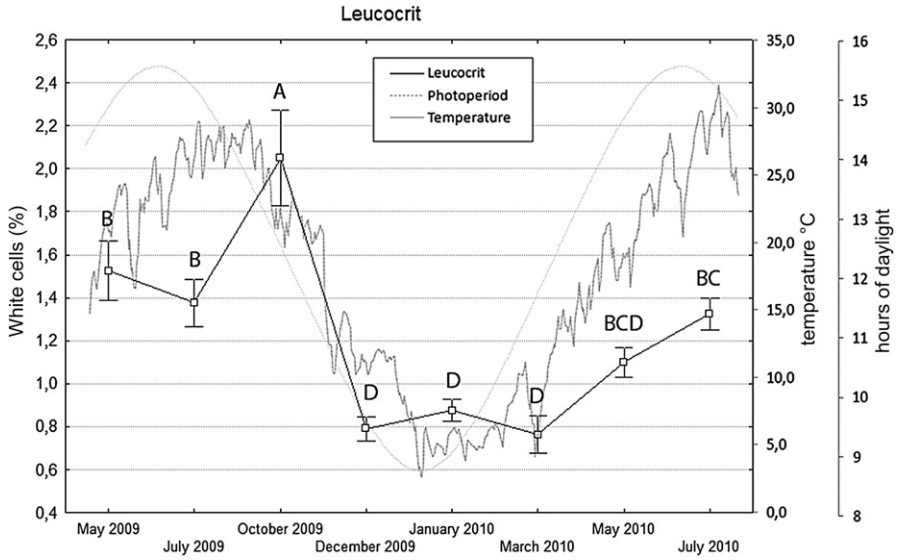

Fig. 5. Variations in leucocrit of sea bass over a 14-months period (mean ± SE). Different letters indicate significant differences ($p < 0.05$).

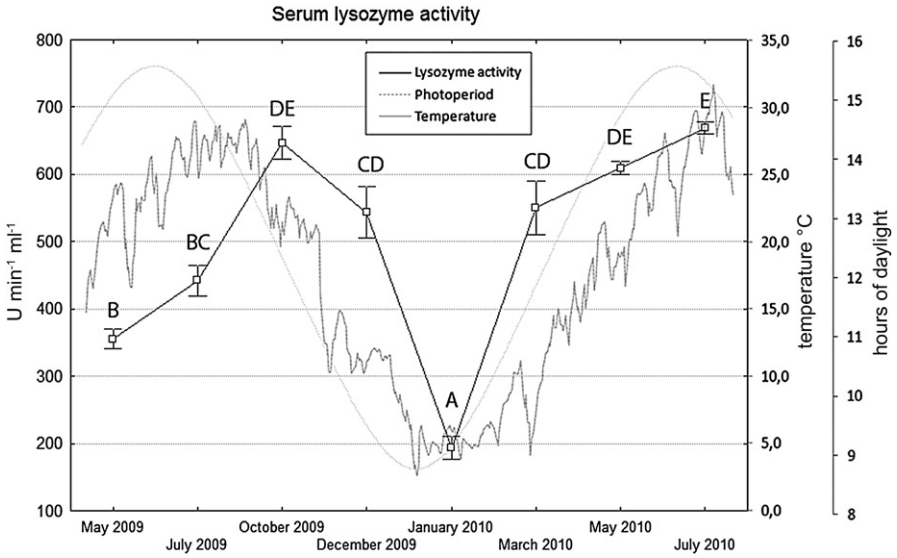

Fig. 6. Variations in serum lysozyme activity of sea bass over a 14-months period (mean ± SE). Different letters indicate significant differences ($p < 0.05$).

a seasonal variation, with the lowest levels in the coldest months, from October to March, and the highest values in hotter months. A possible correlation between cortisol levels and the fluctuation of temperature and photoperiod has been already reported [44].

It is known that at lower water temperature the solubility of oxygen is higher compared to warm water. Consequently, fewer red blood cells are required to carry oxygen around the body of the fish in colder weather as the oxygen is more readily available [45]. This may explain the lower levels of hematocrit recorded in January and March in our experimental protocol. Moreover, winter is a period of reduced activity and reduced metabolism, thus less oxygen is required. Our results are in accordance with those observed in other species, such as sea bream (*S. aurata*) [19] and rainbow trout (*O. mykiss*) [20].

Our results on leucocrit showed the highest level in October 2009, whereas lower levels were found during winter months (December–March). The high level of white blood cell found in October may be due also to a viral encephalitis which raised up in August 2009 and got over a couple of months causing the mortality of 8.31% of fishes. Seasonal variation in hematological and immune parameters has been found in tench (*Tinca tinca*) [46], while seasonal trends in lysozyme activity have been observed in dab (*Limanda limanda*), halibut (*Hippoglossus hippoglossus*) and plaice (*Pleuronectes platessa*) [32,47,48]. Leucocyte counts for tench were found to be significantly lower in winter and spring compared with those measured during summer and autumn [46]. It has been suggested that a shortening in day length may induce changes in the immune system in sight of winter, and this is corroborated by the highest white cell counts being recorded in tench during the autumn months [46].

In the present study, innate immunity was also investigated by serum lysozyme activity assay. Our results show seasonal variations, with an increase in lysozyme activity from May to October, a decrease until January and a re-increase onwards. Lower levels

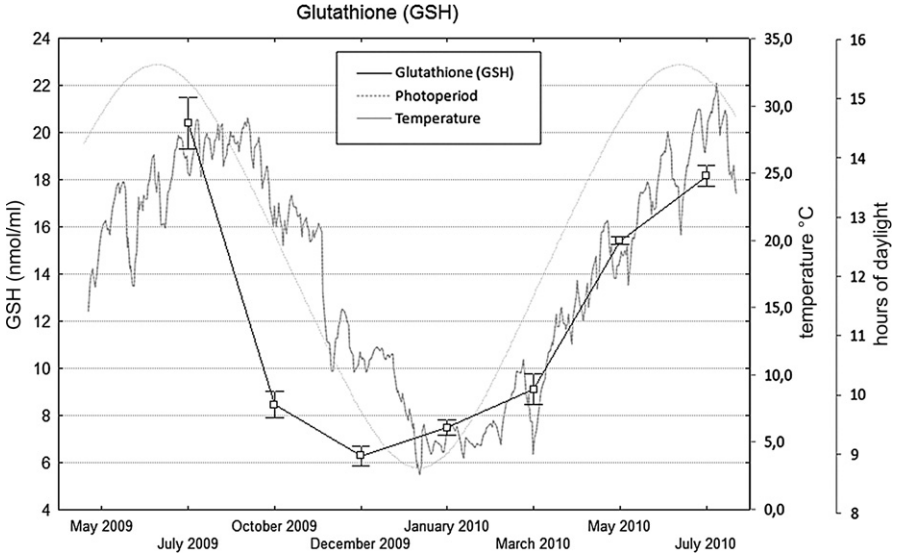

Fig. 7. Variations in glutathione (GSH) of sea bass over a 12-months period (mean ± SE). Evaluation of GSH on May 2009 wasn't possible because of a lack of serum. Different letters indicate significant differences ($p < 0.05$).

were recorded during cold months, according to preview studies for other species [32,48]. Particularly, in October, fish showed a high level of serum lysozyme activity, that could be related to the viral encephalitis. Although lysozyme is an important anti-bacterial defense, it is contained in lysosomes of macrophage and leucocytes, from which it is released in blood [49]. Therefore, the increased number of leucocytes detected with leucocrit could be responsible of the high level of serum lysozyme activity found in our study. Similar levels were also found in May and July 2010.

Glutathione (GSH) levels decreased from July to December 2009, then increased to July 2010. The hottest months (July 2009–2010) showed significantly higher levels of GSH than the colder ones, suggesting that the GSH exhibited a seasonal variation, according to previous studies reporting the influence of temperature on GSH levels in different organs in fish [50,51]. Low levels found in colder months could also be related to the stop of feed intake. In mammals, food restriction and inflammatory processes could affect the levels of glutathione, with a decrease in plasma levels [52]. The viral encephalitis in August and September and the starvation period (from half of October to half of March) could also explain the trend found in this study.

In conclusion, this is the first study on seasonal variations of serum cortisol, serum lysozyme, hematocrit, leucocrit and total glutathione in sea bass (*D. labrax*) reared in pond in Italy. All parameters investigated showed seasonal variation probably related to water temperature and photoperiod. Even though the mechanisms that regulate the influence of these seasonal components in fish have not yet been fully investigated, our results are in accordance with similar studies on different species and could represent a further support to better understand the influence of these seasonal cues on the immune system and stress response in fish. Such informations could be important in aquaculture in order to optimize husbandry practices (netting, vaccination, transfer) carrying out them when the animal immune system or the stress response are seasonally less efficient.

Acknowledgments

This research was supported by a grant of University of Padua to D. Bertotto (Progetto di Ateneo) and by European Social Fund grant to F. Pascoli (Cod. progetto 2105/101/1/722/2009). Special thanks to Veneto Agricoltura and the staff of Centro Ittico “Valle Bonello” for technical support.

References

[1] Bowden TJ, Thompson KD, Morgan AL, Gratacap RML, Nikoskelainen S. Seasonal variation and the immune response: a fish perspective. Fish Shellfish Immunol 2007;22:695–706.
[2] Alliot E, Pastoureaud A, Thebault H. Influence de la temperature et de la saliniteé sur la croissance et la composition corporelle d'alevins de *Dicentrarchus labrax*. Aquaculture 1983;31:181–94.
[3] Audet C, Besner M, Munroe J, Dutil JD. Seasonal and diel variations of various blood parameters in Atlantic cod (*Gadus morhua*) and American plaice (*Hippoglossoides platessoides*). Can J Zool 1993;71:611–8.
[4] Courtois LA. Haematology of juvenile striped bass, *Morone saxatilis* (Walbaum), acclimated to different environmental conditions. Comp Biochem Physiol A 1976;54:221–3.
[5] Vellas F, Ferroni JM, Bau F, Parent JP. Recherches sur l'état physiologique des poissons de la retenue de Pareloup: données relatives a cinq années d'étude. Hydroecol Appl 1994;6:257–92.
[6] Carrillo M, Zanuy S, Herrera F. Growth and diurnal variations in metabolic parameters in the starved bass, *Dicentrarchus labrax*, after experimental feeding. Comp Biochem Physiol 1982;72A:11–6.
[7] Pavlidis M, Berry M, Divanach P, Kentouri M. Diel pattern of hematocrit, serum metabolites, osmotic pressure, electrolytes and thyroid hormones in sea bass and sea bream. Aquacult Int 1997;5:237–47.
[8] Castritsi-Catharios J, Kavadias S. An experimental study of some hematological parameters of cage aquacultured sea bass *Dicentrarchus labrax* L. under extreme environmental conditions: preliminary results. Mar Life 1993;3:95–8.
[9] Begout-Anras ML. Demand-feeding behaviour of sea bass kept in ponds: diel and seasonal patterns, and influences of environmental factors. Aquacult Int 1995;3:186–95.
[10] Le Morvan C, Troutaud D, Deschaux P. Differential effects of temperature on specific and non-specific immune defences in fish. J Exp Biol 1998;201:165–8.
[11] O'Steen S, Bennett AF. Thermal acclimation effects differ between voluntary, maximum, and critical swimming velocities in two cyprinid fishes. Physiol Biochem Zool 2003;76:484–96.
[12] Piferrer F, Blásquez M, Navarro L, González A. Genetic, endocrine and environmental components of sex determination and differentiation in the European sea bass (*Dicentrarchus labrax* L.). Gen Comp Endocrinol 2005;142:102–10.
[13] Bromage NR, Porter MJR, Randall C. The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. Aquaculture 2001;197:63–98.
[14] Zapata AG, Varas V, Torroba M. Seasonal variations in the immune system of lower vertebrates. Immunol Today 1992;13:142–7.
[15] Slater C, Schreck C. Season and physiological parameters modulate salmonid leucocyte androgen receptor affinity and abundance. Fish Shellfish Immunol 1998;8:379–91.
[16] Nakanishi T. Seasonal changes in the humoral immune response and the lymphoid tissues of the marine teleost, *Sebastiscus marmoratus*. Vet Immunol Immunopathol 1986;12:213–21.
[17] Wojtowicz A, Plytycz B. Seasonal changes of the gut associated lymphoid tissue in the common toad, *Bufo bufo*. J Nutr Immunol 1997;5:57–64.
[18] Álvarez F, Razquin BE, Villena AJ, Zapata AG. Seasonal changes in the lymphoid organs of wild trout, *Salmo trutta* L.: a morphological study. Vet Immunol Immunopathol 1998;64:267–78.
[19] Tort L, Rotllant J, Rovira L. Immunological suppression in gilthead sea bream *Sparus aurata* of the North West Mediterranean at low temperature. Comp Biochem Physiol A 1998;120:175–9.
[20] Morgan AL, Thompson KD, Auchinachie NA, Migaud H. The effect of seasonality on normal haematological and innate immune parameters of rainbow trout *Oncorhynchus mykiss* L. Fish Shellfish Immunol 2008;25:791–9.
[21] Culberson SD, Piedrahita RH. Aquaculture pond ecosystem model: temperature and dissolved oxygen prediction mechanism and application. Ecol Model 1996;89:231–58.
[22] Thompson KR, Rawles SD, Metts LS, Smith R, Wimsatt A, Gannam AL, et al. Digestibility of dry matter, protein, lipid, and organic matter of two fish meals, two poultry by-product meals, soybean meal, and distiller's dried grains with solubles in practical diets for sunshine bass, *Morone chrysops* x *M. saxatilis*. J World Aquac Soc 2008;39:352–63.
[23] Wendelaar Bonga SE. The stress response in fish. Physiol Rev 1997;77:591–625.
[24] Weyts FAA, Cohen N, Flik G, Verburg-Van Kemenade BML. Interactions between the immune system and the hypothalamo-pituitary-interrenal axis in fish. Fish Shellfish Immunol 1999;9:1–20.
[25] Pickering AD, Pottinger TG, Christie P. Recovery of the brown trout, *Salmo trutta* L., from acute handling stress: a time-course study. J Fish Biol 1982;20:229–44.
[26] Van Raaij MTM, Pit DSS, Balm PHM, Steffens AB, Van den Thillart GEEJM. Behavioral strategy and the physiological stress response in rainbow trout exposed to severe hypoxia. Horm Behav 1996;30:85–92.
[27] Simontacchi C, Poltronieri C, Carraro C, Bertotto D, Xiccato G, Trocino A, et al. Alternative stress indicators in sea bass *Dicentrarchus labrax*, L. J Fish Biol 2008;72:747–52.
[28] FAO. The State of World Fisheries and aquaculture (SOFIA), http://www.fao.org/fishery/sofia/en; 2008–2011.
[29] Bagenal TB, Tesch FW. Age and growth. In: Begenal T, editor. Methods for assessment of fish production in fresh waters. 3rd ed. IBP Handbook No.3, Oxford: Blackwell Science Publications; 1978. p. 101–36.
[30] Simontacchi C, Marinelli L, Gabai G, Bono G, Angeletti R. Accuracy in naturally occurring anabolic steroid assays in cattle and first approach to quality control in Italy. Analyst 1999;124:307–12.
[31] Parry RM, Chandan RC, Shahani RM. A rapid sensitive assay of muramidase. Proc Soc Exp Biol Med 1965;119:384–6.
[32] Hutchinson TH, Manning MJ. Seasonal trends in serum lysozyme activity and total protein concentration in dab (*Limanda limanda* L) sampled from Lyme Bay, UK. Fish Shellfish Immunol 1996;6:473–82.
[33] Ellis AE. Lysozyme assays. In: Stolen JS, Fletcher TC, Anderson DP, Roberson BS, van Muiswinkel WB, editors. Techniques in fish immunology. New Jersey: SOS Publications; 1990. p. 101–3.
[34] Tietze F. Enzymic method for quantitative determination of nanogram amount of total and oxidized glutathione: application to mammalian blood and other tissues. Anal Biochem 1969;27:502–22.
[35] Baker MA, Cerniglia GJ, Zaman A. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large number of biological samples. Anal Biochem 1990;190:360–5.
[36] Person-Le Ruyet J, Mahe K, Le Bayon N, Delliou H. Effects of temperature on growth and metabolism in a Mediterranean population of European sea bass, *Dicentrarchus labrax*. Aquaculture 2004;237:269–80.
[37] Pastoureaud A. Influence of starvation at low temperatures on utilization of energy reserves, appetite recovery and growth character in sea bass, *Dicentrarchus labrax*. Aquaculture 1991;99:167–78.

[38] Roche H, Chaar K, Peres G. The effect of a gradual decrease in salinity on the significant constituents of tissue in the sea bass (*Dicentrarchus labrax*, Pisces). Comp Biochem Physiol A 1989;93:785–9.
[39] Roche H, Bogé G. Fish blood parameters as a potential tool for identification of stress caused by environmental factors and chemical intoxication. Mar Environ Res 1996;41:27–43.
[40] Marino G, Di Marco P, Mandich A, Finoia MG, Cataudella S. Changes in serum cortisol, metabolites, osmotic pressure and electrolytes in response to different blood sampling procedures in cultured sea bass (*Dicentrarchus labrax* L.). J Appl Ichthyol 2001;17:115–20.
[41] Rotllant J, Tort L, Montero D, Pavlidis M, Martinez M, Wendelaar Bonga SE, et al. Background colour influence on the stress response in cultured red porgy *Pagrus pagrus*. Aquaculture 2003;223:129–39.
[42] Skjervold PO, Fjaera SO, Oestby PB, Einen O. Live-chilling and crowding stress before slaughter of Atlantic salmon (*Salmo salar*). Aquaculture 2001;192: 265–80.
[43] Poli BM, Parisi G, Scappini F, Zampacavallo G. Fish welfare and quality as affected by pre-slaughter and slaughter management. Aquacult Int 2005;13: 29–49.
[44] Planas J, Gutierrez J, Fernandez J, Carrillo M, Canals P. Annual and daily variations of serum cortisol in sea bass *Dicentrarchus labrax* L. Aquaculture 1990;91:171–8.
[45] Stolen JS, Gahn T, Kasper V, Nagle JJ. The effect of environmental temperature on the immune response of a marine teleost (*Faralichrhys denrufus*). Dev Comp Immunol 1984;8:89–98.
[46] Collazos ME, Ortega E, Barriga C, Rodriguez B. Seasonal variation in haematological parameters in male and female tench *Tinca tinca*. Mol Cell Biochem 1998;114:432–5.
[47] Bowden TJ, Butler R, Bricknell IR. Seasonal variation of serum lysozyme levels in Atlantic halibut (*Hippoglossus hippoglossus* L.). Fish Shellfish Immunol 2004; 17:129–35.
[48] Fletcher T, White A. The lysozyme of the plaice *Pleuronectes platessa* L. Comp Biochem Physiol B 1976;55:207–10.
[49] Murray CK, Fletcher TC. The immunohistochemical localisation of lysozyme in plaice (*Pleuronectes platessa*) tissues. J Fish Biol 1976;9:329–34.
[50] Leggatt RA, Brauner CJ, Iwama GK, Devlin RH. The glutathione antioxidant system is enhanced in growth hormone transgenic coho salmon (*Oncorhynchus kisutch*). J Comp Physiol B 2007;177:413–22.
[51] Pavlović SZ, Borković Mitić SS, Radovanović TB, Perendija BR, Despotović SG, Gavrić JP, et al. Seasonal variations of the activity of antioxidant defense enzymes in the red mullet (*Mullus barbatus* L.) from the Adriatic Sea. Mar Drugs 2010;26:413–28.
[52] Malmezat T, Breuille D, Capitan P, Minard PP, Obled C. Glutathione turnover is increased during the acute phase of sepsis in rats. J Nutr 2000;130:1239–46.

Food Chemistry 131 (2012) 427–433

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Assessing the quality of organic and conventionally-farmed European sea bass (*Dicentrarchus labrax*)

Angela Trocino [a,*], Gerolamo Xiccato [b], Duilio Majolini [b], Marco Tazzoli [b], Daniela Bertotto [c], Francesco Pascoli [c], Renato Palazzi [d]

[a] Department of Public Health, Comparative Pathology and Veterinary Hygiene, University of Padova, Viale dell'Università 16, I-35020 Legnaro, Padova, Italy
[b] Department of Animal Science, University of Padova, Viale dell'Università 16, I-35020 Legnaro, Padova, Italy
[c] Department of Veterinary Experimental Sciences, University of Padova, Viale dell'Università 16, I-35020 Legnaro, Padova, Italy
[d] Veneto Agricoltura, Innovation and Development Section, Viale dell'Università 14, I-35020 Legnaro, Padova, Italy

ARTICLE INFO

Article history:
Received 13 July 2011
Received in revised form 26 August 2011
Accepted 31 August 2011
Available online 5 September 2011

Keywords:
Sea bass (*Dicentrarchus labrax*)
Organic aquaculture
Biometric traits
Proximate composition
Fatty acid profile
NIRS analysis

ABSTRACT

The biometric and nutritional traits of European sea bass from organic or semi-intensive conventional production systems at two commercial sizes (small and medium) were compared. The analysis included a total of 80 specimens. The biometric traits and the texture were not affected by the rearing system, whereas they changed significantly with fish size. The fillet fatty acid profile varied significantly, both with rearing system and sea bass size, depending on the fatty acid profile of the diets. The ratio of $n-3$ to $n-6$ polyunsaturated fatty acids was higher ($p < 0.001$) in organic than in conventional fish (1.60 vs. 0.54) and in small than in medium-sized sea bass (1.15 vs. 0.98). Near infrared reflectance spectroscopy (NIRS) successfully classified fillets according to sea bass size in both fresh-minced and freeze-dried samples (90% correct classification), whereas it only classified organic vs. conventional sea bass fairly well (65–75% correct classification) for freeze-dried fillets.

1. Introduction

Consumers display a positive attitude towards organically produced foods, to which they also assign additional ethical attributes (Zander & Hamm, 2010). In aquaculture, organic production systems are gaining a market share and enjoying success. Following an initial period of uncertainty, specific rules now regulate this sector (EC, 2009). Because the regulations are relatively recent, data on the quality of organically reared fish are not yet available. However, these product quality data are necessary to increase and sustain consumer confidence, as is the case for other organically-produced foods (Siderer, Maquet, & Anklam, 2005).

Among the farmed species, European sea bass (*Dicentrarchus labrax*) continues to be the leading cultured fish product in the Mediterranean area, but data on the quality of organically-produced sea bass are not yet available. However, several previous studies have compared wild and cultured sea bass (Alasalvar, Taylor, Zubcov, Shahidi, & Alexis, 2002; Fasolato et al., 2010; Fuentes, Fernández-Segovia, Serra, & Barat, 2010). In fact, different rearing systems and feeding regimes used for sea bass production may affect the flesh quality of the fish, especially in terms of fat concentration (Xiccato, Trocino, Tulli, & Tibaldi, 2004) and the fatty-acid profile (Grigorakis, 2007; Poli et al., 2001; Roncarati et al., 2010).

As an additional method of physical and chemical analysis, near infrared reflectance spectroscopy (NIRS) may provide comprehensive and rapidly generated information on fish quality (Weeranantanaphan, Downey, Allen, & Sun, 2011) and has been successfully used to predict the chemical composition of sea bass and to classify specimens according to the rearing system (Majolini, Trocino, Xiccato, & Santulli, 2009; Xiccato et al., 2004).

The goal of the present study was to compare the biometric and physicochemical traits, the proximate composition and the fatty acid (FA) profile of sea bass reared in organic or semi-intensive conventional production systems at two commercial sizes. NIRS analysis was also used to predict the proximate chemical composition of the flesh and to classify sea bass according to the rearing system (organic vs. conventional) and the fish size (small vs. medium).

2. Materials and methods

2.1. The set of samples

Over an 18-month period from May 2008 through November 2009, 1400 European sea bass (1 year old, initial weight 63 g) were

* Corresponding author. Tel./fax: +39 049 8272639.
E-mail address: angela.trocino@unipd.it (A. Trocino).

0308-8146/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2011.08.082

reared on an Italian experimental fish farm owned by the Veneto Region (Veneto Agricoltura, Centro Ittico Bonello, Sacca di Scardovari, Rovigo, Italy). Half of the animals were assigned to the organic rearing system, conducted according to the rules established by the Italian Association for Organic Agriculture (AIAB; Ed. 01 Rev.00, 01.09.04). The remaining animals were reared under the semi-intensive system currently used on the farm. The primary difference between the two systems was the type of feed, organic vs. conventional. All of the sea bass were reared in eight circular tanks of 4 m^3 capacity (four tanks per rearing system) with the same water conditions: temperature, 18.4 ± 6.9 °C; dissolved O_2, 10.2 ± 2.1 mg/L; pH, 8.2 ± 0.4. At the end of the trial, the stock density averaged approximately 15 kg/m^3 in both rearing systems.

A total of 80 sea bass (20 specimens of each size per rearing system) were collected during two sampling periods, the first after 12 months of rearing (in May 2009) and the second after 18 months (in November 2009) to obtain two commercial sizes, small (S, weight at slaughter 259 ± 54 g) and medium (M, weight at slaughter 434 ± 65 g). All fish were slaughtered by immersion in ice slurry; five specimens per tank per period were immediately transported to the laboratory in thermally insulated boxes and stored on ice in a refrigerated room (2 °C) for subsequent analysis on the day following collection. Samples of the organic and conventional diets were taken on the same day that the fish were collected.

2.2. Biometric measurements and physicochemical traits of sea bass

On the day after collection, the intact fish were weighed to determine the slaughter weight (SW). The colour of the skin was determined at three points on the dorsal side and at two points on the ventral side with a Minolta spectrophotometer CM-508 C (Minolta, Milano, Italy) according to the CIE $L^*a^*b^*$ method (1976) and using the D_{65} light source and a 10° observer.

A texture profile analysis (TPA) was performed at a central position on the lateral side under the first dorsal fin, using a TA.HDI dynamometer (Stable Micro Systems Ltd., Godalming, UK) with a 20-mm diameter cylindrical probe moving with 5 mm compression at a constant speed of 2 mm/s for two consecutive cycles separated by a 5-s interval.

The following biometric measurements were taken on the intact fish (Poli et al., 2001): total length, standard length, head length and maximum height. The fish were then dissected, and the carcass, viscera, liver and fillets (with skin) were weighed. The condition factor (100 × live weight/total length^3), visceral index (100 × viscera weight/slaughter weight), hepatosomatic index (100 × liver weight/slaughter weight), dressing percentage (100 × carcass weight/slaughter weight) and fillet yield (100 × fillets weight/slaughter weight) were calculated.

The colour indexes, as described above for skin, and the final pH were measured at three points on the dorsal side of the fillets taken from the right side of the fish (the right fillets).

2.3. Proximate composition and FA analysis

The fillets were separated from the skin and minced. The right fillets were freeze-dried and stored under vacuum at 4 °C for subsequent analysis of their proximate composition. The left fillets were stored under vacuum at −18 °C for subsequent analysis of their fatty acid profile.

Both diets and the freeze-dried fillets were analysed with AOAC (2000) methods, to determine the concentrations of dry matter (934.01), ash (967.05) and crude protein (2001.11). Ether extracts of the fillets were analysed with AOAC method 991.36. Ether extracts of the diets were analysed after acid-hydrolysis treatment (EC, 1998). The crude fibre content of the diets was analysed with AOAC method 962.09.

Both diets and fresh minced fillets were analysed for FA composition. For this purpose, fat was extracted from the samples with accelerated solvent extraction (ASE®, Dionex, Sunnyvale, CA, Application Note 334). The procedure consisted of two extraction cycles with petroleum ether as a solvent at a temperature of 125 °C and a pressure of 10.3 MPa, with a heating phase of 6 min and an extraction phase of 2 min.

First, transmethylation was performed on the extracted lipids to determine fatty acid methyl esters (FAMEs) using a solution of 1 M sodium methoxide in methanol (1 vol.) and a solution of oxalic acid in diethyl ether (Christie, 1982). An internal standard (19:0 methyl ester) was added to the extracts prior to methylation. After centrifugation, the supernatant was injected into the split/splitless system of an 8000 Top CE gas chromatograph (ThermoQuest Italia S.p.A., Milan, Italy) with a Restek (Bellefonte, PA) Rtx-2330 capillary column (70 m × 0.18 mm internal diameter, 0.10 μm film thickness). Hydrogen at 1.55 mL/min was used as the carrier. An oven temperature of 50 °C was held 1 min, raised to 100 °C at the rate of 50 °C/min, held 1 min, raised to 150 °C at the rate of 3 °C/min and raised to 220 °C at 2 °C/min. The injector and the detector temperatures were both set at 250 °C. The FAs were identified by comparing their retention times with a standard mixture of 37 FAMEs (F.A.M.E. Mix C_4–C_{24}, Supelco, Bellefonte, PA). The concentration of individual FAMEs was expressed as a percentage of the total area of eluted FAMEs (known plus unknown).

2.4. NIRS analysis

NIRS analysis was performed with a monochromator spectrometer (InfraAlyser 500, Bran + Luebbe GmbH, Norderstedt, Germany) in the 1100–2500 nm range with a 2-nm step. Each fish was analysed both as fresh minced meat (left fillet) and freeze-dried minced meat (right fillet).

Spectra were collected as absorbance values using Sesame software (Sesame version 3.00, Bran + Luebbe). Each fillet sample was scanned twice. The averaged spectra were exported into Unscrambler software (Unscrambler version 7.0, CAMO ASA, Trondheim, Norway) using the JDX format and then transformed to the second derivative, calculated over a 20-point smoothing segment (CAMO ASA, 1998).

2.5. Statistical analyses

Biometric measurements and physicochemical traits of sea bass and fillets were analysed with the GLM procedure of SAS (version 9.1). The rearing system and sea bass size were used as the experimental factors, and the tank was used as a block.

The NIRS calibration equations were calculated on the transformed spectra with partial least squares regression (PLSR) to predict the contents of water (%), crude protein (%) and ether extract (%). The number of factors used as independent variables in the prediction equations was fixed at less than 10% of the number of samples used in the calibration to avoid overfitting (Shenk & Westerhaus, 1994). The optimal number of factors was chosen as a function of the first local minimum in the validation residual–variance plot. Full cross-validation was used (Martens, 1999). Outliers were identified and excluded on the basis of the Mahalanobis value ($H > 3$) (CAMO ASA, 1998).

The prediction equations were evaluated in terms of the coefficients of determination for calibration (r^2c) and cross-validation (r^2cv) and the standard errors of calibration (SEC) and of cross-validation (SECV).

Principal component analysis (PCA) was used to calculate models for each cluster of NIR spectra on the basis of the rearing system

or the sea bass size using full cross-validation. A soft independent modelling class analogy (SIMCA) method was used to measure the model-to-model distance and classify samples by rearing system and sea bass size. The cluster membership in SIMCA was tested at a $p < 0.05$ level. Both PCA and SIMCA were performed using Unscrambler software.

3. Results and discussion

3.1. Biometric measurements and physicochemical traits of sea bass

The rearing system did not affect ($p > 0.05$) the biometric traits or the texture measured on the intact fish (Table 1). Other authors observed that the visceral and hepatosomatic indexes were different in sea bass from inshore cages, offshore cages, in-land basins or extensive systems in which fish had different opportunities to swim (Roncarati et al., 2010; Tulli, Balenovic, Messina, & Tibaldi, 2009). Fuentes et al. (2010) found no differences between the body shapes of farmed and wild sea bass, whereas Fasolato et al. (2010) reported a lower condition factor in wild sea bass. The different nutritional status of farmed sea bass compared to wild sea bass partly accounted for these results (Grigorakis, 2007). In the present study, neither swimming possibilities nor nutritional status of sea bass were different between the organic and conventional rearing systems.

The yellow index measured on the skin was significantly lower in organic sea bass than in conventional fish ($p < 0.01$). The yellow index measured on the fillet showed an opposite trend ($p < 0.001$) (Table 1). In another study, the muscle lightness and pH were lower in farmed sea bass than in wild fish (Fuentes et al., 2010). Differences in fish colour may depend on several factors, e.g., rearing environment, swimming possibilities, feeding regime (Fuentes et al., 2010), but in the present study they were likely to be conditioned primarily by the diet.

The size of the sea bass affected the quality traits of the intact fish and fillets (Table 1). In addition to the obvious increase in body length, the increase in size produced lower values of the condition factor (1.14 and 1.22 in small-sized organic and conventional sea bass vs. 1.11 and 1.10 in medium-sized organic and conventional sea bass; $p = 0.02$) and higher visceral ($p = 0.04$) and hepatosomatic ($p < 0.001$) indexes. The dressing percentage (by 2.5% on average) and fillet yield (by 4.9% on average) also increased with size ($p < 0.001$). The analysis of fish texture showed that firmness increased as the sea bass size increased (38.6 and 36.8 N in small-sized organic and conventional sea bass vs. 54.0 and 54.2 N in medium-sized organic and conventional sea bass; $p < 0.01$).

The skin and fillets were darker, the red index of the skin had higher values ($p = 0.01$) and the yellow index of fillet increased ($p < 0.001$) in medium-sized sea bass. The fillet pH also increased ($p = 0.03$) in heavier sea bass. Differences in moisture content between small- and medium-sized sea bass may explain the changes in colour between fish, as reported by Fuentes et al. (2010).

3.2. Proximate composition and FA composition of diets

The chemical composition of the experimental diets differed with the rearing system. The organic diets showed higher ether extract values (19.2% vs. 16.6%, averaged data from the first and second samples), higher ash content and lower crude protein (41.0% vs. 45.0%, averaged data from the first and second samples) compared with the conventional diets (Table 2). Regardless of the type of diet, in the last rearing period the fish were fed diets that were higher in ether extract and lower in crude protein compared with the diets fed in the first period.

The primary differences between the diets, however, were found in the lipid profile, i.e., the proportions of FAs. The diets differed between rearing systems (organic vs. conventional) but also between the sampling periods within each system. The level of saturated fatty acids (SFA) was similar (20.6–22.4% of total FAs) in all diets. The level of total mono-unsaturated fatty acids (MUFAs) was higher (39.4% and 39.0% in organic diets from the first and second sampling, 25.5% and 30.4% in conventional diets from the first and second sampling) in organic diets because of the higher proportion of C20:1 $n-9$ (5.51% and 2.76% in organic diets from the first and second sampling, 0.66% and 0.84% in conventional diets from the first and second sampling) and especially of C22:1 $n-11$ (7.83% and 8.08% in organic diets from the first and second sampling, 0.58% and 0.62% in conventional diets from the first and second sampling). In addition, the MUFA concentration increased in the

Table 1
Sea bass biometrics, texture and colour of intact fish and fillets.

Sea bass size (S)	Small		Medium		Probability			RSD
Rearing system (R)	Organic	Conventional	Organic	Conventional	R	S	$R \times S$	
Sea bass (No.)	20	20	20	20				
Slaughter weight (SW) (g)	259	259	447	421	0.50	<0.001	0.51	58
Total length (cm)	28.2	28.4	34.2	33.6	0.72	<0.001	0.43	1.70
Standard length (cm)	24.0	24.1	29.6	28.9	0.51	<0.001	0.39	1.48
Head length (cm)	7.04	7.01	8.77	8.36	0.14	<0.001	0.21	0.51
Maximum height (cm)	5.81	5.87	7.30	7.02	0.29	<0.001	0.11	0.01
Condition factor	1.14	1.22	1.11	1.10	0.18	0.02	0.40	0.04
Visceral index (% SW)	8.58	8.75	9.36	9.46	0.66	0.04	0.91	0.91
Hepatosomatic index (% SW)	1.07	1.08	1.75	1.64	0.49	<0.001	0.40	0.23
Carcass weight (g)	226	226	401	377	0.50	<0.001	0.52	52
Dressing percentage (% SW)	87.2	87.1	89.7	89.6	0.74	<0.001	0.97	0.98
Fillet yield (% SW)	41.6	41.7	46.2	46.9	0.50	<0.001	0.59	1.44
Body firmness (N)	38.6	36.8	54.0	54.2	0.65	<0.01	0.58	5.78
Skin								
L^*	48.4	47.9	43.8	44.2	0.99	<0.001	0.49	3.06
a^*	−0.91	−0.77	−0.22	−0.74	0.11	0.01	0.02	0.35
b^*	6.70	9.73	6.16	8.18	<0.01	0.08	0.34	2.00
Fillet								
pH	6.22	6.22	6.23	6.26	0.12	0.03	0.18	0.06
L^*	39.2	38.8	37.5	36.8	0.23	<0.01	0.73	2.25
a^*	−2.31	−2.20	−2.39	−2.70	0.64	0.21	0.34	0.44
b^*	−1.42	−1.78	1.39	0.54	0.01	<0.001	0.21	0.85

Table 2
Proximate composition (% as-fed) and fatty acid profile (% of total fatty acid methyl esters) of the diets.

Sampling[a]	1		2	
Rearing system	Organic	Conventional	Organic	Conventional
Proximate composition				
Water (%)	8.43	6.86	7.10	6.32
Ether extract (%)	18.1	16.0	20.3	17.2
Crude protein (%)	41.4	45.5	40.7	44.5
Crude fibre (%)	1.01	1.25	0.73	1.18
Ash (%)	11.4	8.02	12.3	7.73
Fatty acid profile				
C14:0	4.22	2.72	5.03	3.05
C15:0	0.47	0.26	0.37	0.23
C16:0	12.4	12.9	13.1	12.3
C17:0	0.48	0.36	0.35	0.32
C18:0	2.61	3.52	2.50	3.82
C20:0	0.33	0.37	0.21	0.28
Other SFAs	0.36	0.49	0.77	0.53
Total SFAs	20.9	20.6	22.4	20.6
C16:1 $n-7$	3.42	2.96	3.90	3.03
C18:1 $n-7$	3.59	3.77	3.93	3.99
C18:1 $n-9$	17.4	17.0	18.5	21.3
C20:1 $n-9$	5.51	0.66	2.76	0.84
C22:1 $n-11$	7.83	0.58	8.08	0.62
C24:1 $n-9$	0.57	0.26	0.46	0.19
Other MUFAs	1.27	0.34	1.52	0.48
Total MUFAs	39.4	25.5	39.0	30.4
C18:3 $n-3$	3.51	4.08	2.92	4.96
C18:4 $n-3$	1.98	0.94	5.82	0.79
C20:5 $n-3$	4.29	6.15	4.47	5.96
C22:5 $n-3$	0.93	0.90	0.91	0.74
C22:6 $n-3$	6.05	3.25	5.90	3.10
PUFAs $n-3$	16.7	15.3	20.0	15.6
C18:2 $n-6$	18.1	35.7	13.3	29.3
C20:4 $n-6$	0.41	0.39	0.24	0.31
PUFAs $n-6$	18.5	36.0	13.5	29.6
Ratio of $n-3$ to $n-6$ PUFAs	0.91	0.42	1.48	0.53
Other PUFAs	1.60	0.81	1.00	0.67
Total PUFAs	36.8	52.2	34.5	45.8
Unknown FAs	2.96	1.71	4.15	3.21

FAs: fatty acids; SFAs: saturated FAs; MUFAs: monounsaturated FAs; PUFAs: polyunsaturated FAs.
[a] Sampling period 1: sample of small-sized fish; sampling period 2: sample of medium-sized fish.

conventional diet from the first to the second sampling period, owing to a 25% increase of oleic acid (C18:1 $n-9$). Polyunsaturated fatty acids (PUFAs) of the $n-3$ series were higher (20.0%) in the organic diet used in the last period of growth (sampling period 2) than in other diets (15.3–16.7%) because of the higher proportion of C18:4 $n-3$ (5.82% vs. 0.79–1.98%). PUFAs of the $n-6$ series were lower in organic diets (18.5% and 13.5% in organic diets from the first and second sampling, 36.0% and 29.6% in conventional diets from the first and second sampling). Oleic acid also decreased by 25–30% in both diets in the second sampling period. The ratio of $n-3$ to $n-6$ PUFAs was higher in the organic than in the conventional diets and increased substantially from the first to the second sampling period in the organic diet (from 0.91 to 1.48).

The type of fish oil used in the commercial diets was unknown. However, based on their FA profile, we can suppose that the organic diets were supplemented with a blend of fish oils that contained herring oil. Herring oil is characterised by a high level of eicosanoic acid (C20:1 $n-9$) and cetoleic acid (C22:1 $n-11$) and by a high content of $n-3$ PUFAs (Martinez, Standal, Axelson, Finstad, & Aursand, 2009; Menoyo, Lopez-Bote, Bautista, & Obach, 2002). Similarly, the higher occurrence rate of C18:4 $n-3$ in the second sample of the organic diet is consistent with a higher dietary level of fish oil (Benedito-Palos et al., 2011). In the conventional diets, the high occurrence rate of $n-6$ PUFAs was evidently the consequence of the inclusion of high levels of vegetable oils, probably soybean oil.

The FA profile of the conventional diets was consistent with the trend of the past decade in aquaculture towards the reduction of the use of fish meals and oils in favour of vegetable protein sources and oils (Roncarati et al., 2010; Turchini, Torstensen, & Ng, 2009). In contrast, owing to the lower availability of certified organic vegetable raw materials, the organic diets for fish contained lower levels of these substitutes for fish meal and oils.

3.3. Proximate composition and FA profile of fillets

The proximate composition of sea bass fillets was not affected by the rearing system, whereas fillets were fatter, richer in protein and lower in water as the size increased (Table 3).

The FA profile of the fillets changed greatly between organic and conventional sea bass, whereas in a narrow range between small and medium-sized sea bass (Table 3). In most cases, the interaction between the rearing system and the sea bass size also had a significant effect on the contents of the FAs in the fillets.

The proportion of total SFAs was higher in organic than conventional sea bass ($p < 0.001$), primarily as a consequence of the higher proportion of C14:0, and decreased with increasing fish size ($p < 0.001$).

Among MUFAs, the proportions of C16:1 $n-7$ and C18:1 $n-7$ were higher in organic fish, whereas that of the most-frequently represented acid, C18:1 $n-9$, was lower. C16:1 $n-7$ decreased, C18:1 $n-9$ increased and C20:1 $n-9$ decreased with fish size. C22:1 $n-11$ increased, with the highest value in medium-sized organically-reared sea bass (3.23%).

The proportion of $n-3$ PUFAs was significantly higher in organic sea bass (23.3% and 21.3% in small and medium-sized organic sea bass, 13.8% and 14.4% in small and medium-sized conventional sea bass; $p < 0.001$) owing to the higher proportions of both eicosapentaenoic acid (C20:5 $n-3$) and docosahexaenoic acid (C22:6 $n-3$). Both C20:5 $n-3$ and C22:6 $n-3$ decreased significantly in heavier fish, whereas the proportion of C18:3 $n-3$ increased. The proportion of C18:4 $n-3$ was exceptionally high in medium-sized organically reared sea bass (3.77%; rearing system $\times$ sea bass size interaction: $p < 0.001$): in the last period of growth, these fish received the diet with the highest amount of stearidonic acid.

The proportion of $n-6$ PUFAs was halved in organically-reared sea bass, compared with conventional fish, owing to the lower proportion of dietary C18:2 $n-6$, and increased from small to medium-sized fish (13.4% and 14.8% in small and medium-sized organic sea bass, 24.8% and 27.6% in small and medium-sized conventional sea bass). As a consequence, the ratio of $n-3$ to $n-6$ PUFAs was three times higher in organic fish than in conventional fish (1.60 vs. 0.54, average of small and medium-sized sea bass), whereas this ratio decreased by 17% from small to medium-sized fish (1.15–0.98, average of organic and conventional sea bass).

On average, our results confirm that C16:0 is the most abundant SFA in sea bass muscle and C18:1 $n-9$ is dominant among the MUFAs (Alasalvar et al., 2002; Fasolato et al., 2010; Testi, Bonaldo, Gatta, & Badiani, 2006). The level of $n-3$ PUFAs is substantial, even if it depends primarily on the dietary level and the types of supplemented fish meal and oils. However, higher levels of $n-6$ PUFAs are the result of the supplementation of the diet with vegetable oil (Benedito-Palos et al., 2011; Grigorakis, 2007; Turchini et al., 2009).

The progressive substitution of cheaper vegetable oils for increasingly expensive fish ingredients in the conventional diets for farmed sea bass is surely decreasing the dietetic value of sea

Table 3
Proximate composition (% as-fed) and fatty acid profile (% of total fatty acid methyl esters) of sea bass fillets.

Sea bass size (*S*)	Small		Medium		Probability			RSD
Rearing system (*R*)	Organic	Conventional	Organic	Conventional	*R*	*S*	*R* × *S*	
Proximate composition								
Water (%)	74.9	74.8	72.9	72.4	0.36	<0.001	0.62	1.07
Ether extract (%)	4.02	4.42	5.40	5.86	0.18	<0.01	0.92	1.12
Crude protein (%)	19.9	19.6	20.5	20.4	0.07	<0.001	0.08	0.25
Ash (%)	1.19	1.20	1.29	1.25	0.33	<0.001	0.08	0.02
Fatty acid profile								
C14:0	4.16	2.94	4.19	2.59	<0.001	0.10	0.05	0.17
C15:0	0.48	0.33	0.35	0.24	<0.001	<0.001	0.07	0.02
C16:0	15.8	15.3	15.4	14.8	0.02	0.01	0.90	0.34
C17:0	0.70	0.48	0.46	0.37	<0.001	<0.001	<0.001	0.02
C18:0	3.82	3.70	2.85	3.62	<0.01	<0.001	<0.001	0.13
Other SFAs	0.98	0.83	0.94	0.82	<0.001	0.12	0.31	0.03
Total SFAs	25.5	23.3	23.8	22.2	<0.001	<0.001	0.25	0.48
C16:1 $n-7$	5.52	3.70	4.69	3.37	<0.001	<0.001	<0.001	0.10
C18:1 $n-7$	4.82	3.51	4.55	3.57	<0.001	0.37	0.16	0.22
C18:1 $n-9$	17.1	21.6	18.8	21.8	<0.001	<0.01	<0.001	0.43
C20:1 $n-9$	2.18	2.20	1.92	0.75	<0.001	<0.001	<0.001	0.08
C22:1 $n-11$	1.74	1.67	3.23	0.83	<0.001	<0.001	<0.001	0.11
C24:1 $n-9$	0.55	0.34	0.32	0.17	<0.001	<0.001	0.04	0.03
Other MUFAs	0.91	0.81	1.27	0.79	<0.001	<0.001	<0.001	0.04
Total MUFAs	32.9	33.8	34.8	31.3	<0.001	0.16	<0.001	0.38
C18:3 $n-3$	1.75	2.53	2.43	3.31	<0.001	<0.001	0.20	0.07
C18:4 $n-3$	1.37	0.82	3.77	1.58	<0.001	<0.001	<0.001	0.07
C20:5 $n-3$	9.42	4.22	5.56	4.12	<0.001	<0.001	<0.001	0.18
C22:5 $n-3$	1.92	1.02	1.43	0.97	0.001	<0.001	<0.001	0.06
C22:6 $n-3$	8.86	5.26	7.97	4.35	<0.001	<0.001	0.96	0.31
PUFAs $n-3$	23.3	13.8	21.3	14.4	<0.001	0.03	<0.001	0.56
C18:2 $n-6$	12.6	24.4	14.4	27.3	<0.001	<0.001	0.03	0.43
C20:4 $n-6$	0.75	0.38	0.41	0.28	<0.001	<0.001	<0.001	0.02
PUFAs $n-6$	13.4	24.8	14.8	27.6	<0.001	<0.001	0.01	0.44
Ratio of $n-3$ to $n-6$ PUFAs	1.75	0.56	1.44	0.52	<0.001	<0.001	<0.001	0.04
Other PUFAs	1.31	1.34	1.01	1.41	0.07	0.38	0.08	0.20
Total PUFAs	38.0	39.9	37.0	43.4	<0.001	<0.01	<0.001	0.70
Unknown FAs	3.63	2.91	4.50	3.09	0.01	0.18	0.37	0.70

FAs: fatty acids; SFAs: saturated FAs; MUFAs: monounsaturated FAs; PUFAs: polyunsaturated FAs.

bass (and other farmed fish), measured in terms of the profile of FAs (Fuentes et al., 2010; Roncarati et al., 2010; Turchini et al., 2009). This decline in value is demonstrated by the 50% decrease of $n-3$ PUFAs from 26.8% in the study of Alasalvar et al. (2002) to 13.8% in our results and the sharp decline of the ratio of $n-3$ to $n-6$ PUFAs ratio from 3 (Alasalvar et al., 2002) to 0.5 (our results). As a consequence of this change, the differences in nutritional value between conventional farmed sea bass and wild sea bass will receive emphasis, and wild products will be favoured (Alasalvar et al., 2002; Fasolato et al., 2010; Fuentes et al., 2010). However, this negative trend affecting farmed fish could work to favour organic sea bass because the organic aquaculture regulation (EC, 2009) fixes a maximum inclusion level of vegetable feeds at 60% of the diet and indirectly imposes a 40% level of fish meal and oil as a minimum.

3.4. NIRS prediction of chemical composition of sea bass fillets

The proximate composition of the sea bass fillets varied substantially for both water and ether extract. The water content averaged 73.7 ± 1.54% and ranged from a minimum of 70.8 to a maximum of 76.6%. The ether extract content was 4.92 ± 1.33% and varied from 2.41% to 7.83%. In contrast, crude protein, on average 20.1 ± 0.45%, varied only from 19.1% to 21.0%.

The calibration and validation results for the prediction of the chemical composition are reported in Table 4. The NIRS predictions of the water and ether extract content were accurate and satisfactory ($r^2_{cv} > 0.90$), even on fresh minced fillets (SECV: 0.387% and 0.154% for water and ether extract, respectively). For these parameters, freeze-drying did not improve the coefficients of determination in cross-validations or reduce the standard error of prediction (SECV: 0.328% and 0.175%, respectively). The NIRS prediction of crude protein content was generally acceptable (r^2_{cv} equal to 0.587 and 0.640 and SECV equal to 0.290% and 0.274% for fresh minced and freeze-dried fillets, respectively). The estimate calculated on the spectra obtained from freeze-dried samples was more robust because of the lower number of factors selected in calibration (3 vs. 6).

For the NIRS analysis of fresh minced and freeze-dried sea bass fillets, Xiccato et al. (2004) calculated higher coefficients of determination for water and ether extract predictions ($r^2_{cv} > 0.90$) but also found higher standard errors of prediction (0.87–0.70% for water and 0.70–0.62% for ether extract) because the range of variation in chemical composition in the sea bass samples was wider than that found by the present study. However, in agreement with our results, freeze-drying did not substantially increase the performance of NIRS in predicting the chemical composition of sea bass except in the case of crude protein.

In several species other than sea bass, the ability of NIRS to predict the water, fat and protein contents of fish has been extensively demonstrated both on fresh minced fillets and freeze-dried fillets (Weeranantanaphan et al., 2011).

3.5. NIRS classification of sea bass according to rearing system and commercial size

If SIMCA was used to classify sea bass according to their origin (organic vs. conventional sea bass), freeze-drying of the fillets was necessary to achieve acceptable results. In fact, the model-to-model

Table 4
NIRS prediction of sea bass chemical composition based on spectra recorded for fresh minced and freeze-dried fillets.

	N of outliers	N of factors	r^2_c	r^2_{cv}	SEC	SECV
Fresh minced fillets						
Water (%)	0	3	0.957	0.935	0.343	0.387
Ether extract (%)	0	3	0.988	0.984	0.120	0.154
Crude protein (%)	0	6	0.925	0.587	0.117	0.290
Freeze-dried fillets						
Water (%)	1	3	0.960	0.953	0.291	0.328
Ether extract (%)	1	3	0.986	0.982	0.148	0.175
Crude protein (%)	1	3	0.681	0.640	0.251	0.274

r^2c, coefficient of determination in calibration; r^2_{cv}, coefficient of determination in cross-validation; SEC, standard error of calibration; SECV, standard error of cross-validation.

Table 5
Performance of two-cluster SIMCA analysis of NIRS spectra recorded for fresh minced and freeze-dried fillets.

	Correctly classified (%)	Classified in two clusters (%)	Wrongly classified (%)	Not classified (%)
Fresh minced fillets				
Organic	25.0	62.5	2.5	10.0
Conventional	20.0	75.0	–	5.0
Freeze-dried fillets				
Organic	65.0	12.5	–	22.5
Conventional	72.5	5.0	–	22.5
Fresh minced fillets				
Small size	90.0	2.5	–	7.5
Medium size	92.5	2.5	–	5.0
Freeze-dried fillets				
Small size	90.0	–	–	10.0
Medium size	92.5	–	–	7.5

distance of clusters was not sufficient to discriminate between organic and conventional sea bass if fresh minced fillets were analysed (distance 1.39), whereas an acceptable value for classification (3.10) was reached if freeze-dried samples were used. Consequently, only 20–25% of the spectra of fresh fillets were correctly classified, and the largest proportion of the set was classified in both clusters (Table 5). If the clusters were based on the spectra of freeze-dried fillets, the proportion of correctly classified samples reached 65.0% for organic sea bass and 72.5% for conventional sea bass. However, almost one-quarter of the samples (22.5% from both rearing systems) were not classified at all.

In contrast, the model-to-model distance for clusters according to sea bass size was sufficient (>3) to discriminate between small-sized and medium-sized sea bass, both on the spectra of fresh minced fillets (distance 3.48) and freeze-dried samples (23.8). Therefore, if the spectra of fresh minced sea bass were used, 90% of the small sea bass were correctly classified, 2.5% were assigned to both clusters and 7.5% were not classified at all (Table 5). Among medium-sized sea bass, 92.5% were correctly assigned, 2.5% were assigned to both clusters and 5.0% were not classified at all. If the spectra of freeze-dried sea bass were used, the percentage of spectra correctly assigned to small-sized or medium-sized clusters did not increase, no spectra were assigned to both clusters and the amount of unclassified samples increased.

In a previous study of ours (Xiccato et al., 2004), a satisfactory classification of sea bass according to origin was achieved only on freeze-dried fillets. The distances between clusters of sea bass from extensive, intensive and semi-intensive systems and sea cages were, however, higher (from 8.4 to 30.4) than the distance we measured between organic and conventional sea bass. Furthermore, classification was more successful: the percentage of spectra correctly classified increased from 74% to 83%. Similarly, on a larger set of freeze-dried sea bass fillets (340 specimens from 11 Italian fish farms characterised by different rearing systems: extensive lagoons, intensive land-based basins and sea cages), the extensively-reared sea bass were separated from the intensively-reared ones on the PCA score plot and the fish reared in sea cages were partially confused with the extensively-reared fish (Majolini et al., 2009). In these two studies, the spectral variations among clusters probably were not exclusively dependent on the feeding regime but were magnified by a combination of other factors (e.g., water quality, growth pattern, muscular activity and competition).

Among other applications of NIRS to fish authentication, Solberg (1996) successfully classified the minced fresh fillets of *Gadus morhua*, *Mallotus villosus* and *Salmo salar* by species using PCA. More recently, NIRS has been used with some success to assess the degree of fish freshness and to distinguish between fresh and thawed fish (Lin, Mousavi, Al-Holy, Cavinato, & Rasco, 2006; Uddin et al., 2005).

4. Conclusions

The physicochemical traits of the organic and conventional sea bass were similar and were not substantially affected by the difference in production systems. However, the characteristics of the diets fed in the two rearing systems affected the nutritional value of the flesh in terms of the fatty acid profile, which was closer to that of wild fish and safer for human consumption in organic than in conventional sea bass. The commercial size of the sea bass had a greater effect on the biometric traits of the fish than the production system, but it had a relatively small effect on the dietary value of the flesh.

Near-infrared reflectance spectroscopy was confirmed to be a powerful instrument for the rapid evaluation of fish chemical composition and to offer interesting possibilities of applications for discriminating sea bass according to their size and rearing system.

Acknowledgements

This study was funded by the University of Padova (CPDA082999/08). The authors thank Dr. Valerio Bondesan (Veneto Agricoltura, Veneto Region, Italy) for his technical collaboration.

References

Alasalvar, C., Taylor, K. D. A., Zubcov, E., Shahidi, F., & Alexis, M. (2002). Differentiation of cultured and wild sea bass (*Dicentrarchus labrax*): Total lipid content, fatty acid and trace mineral composition. *Food Chemistry, 79*, 145–150.

Association of Official Analytical Chemists (2000). *Official Methods of Analysis* (17th ed.). Arlington, VA, USA: AOAC.

Benedito-Palos, L., Bermejo-Nogales, A., Karampatos, A. I., Ballester-Lozano, G. F., Navarro, J. C., Diez, A., et al. (2011). Modelling the predictable effects of dietary lipid sources on the fillet fatty acid composition of one-year-old gilthead sea bream (*Sparus aurata* L.). *Food Chemistry, 124*, 538–544.

CAMO ASA (1998). *The Unscrumbler. USER manual, CAMO ASA*. Norway: Oslo.

Christie, W. W. (1982). A simple procedure for rapid transmethylation of glycerolipids and cholesterol ester. *Journal of Lipid Research, 23*, 1072–1075.

CIE, Commission International de l'Eclairage (1976). Official recommendations on uniform colours spaces, colour differences equations and metric colours terms. *Supplement no. 2, Publication no. 15, Colorimetry*, Paris, France

EC (1998). Commission Directive 98/64/EC of 3 September 1998 establishing community methods of analysis for the determination of aminoacids, crude oils and fats, and olaquindox in feedingstuffs and amending Directive 71/393/EEC. *Official Journal of the European Union 19.9.1998*, L257/14.

EC (2009). Commission Regulation No 710/2009 of 5 August 2009 amending regulation (EC) No 889/2008 laying down detailed rules for the implementation of Council Regulation (EC) No. 834/2007, as regards laying down detailed rules on organic aquaculture animal and seaweed production. *Official Journal of the European Union 6.8.2009*, L204/15.

Fasolato, L., Novelli, E., Salmaso, L., Corain, L., Camin, F., Perini, M., et al. (2010). Application of nonparametric multivariate analyses to the authentication of wild and farmed European sea bass (*Dicentrarchus labrax*). Results of a survey on fish sampled in the retail trade. *Journal of Agricultural and Food Chemistry, 58*, 10979–10988.

Fuentes, A., Fernández-Segovia, I., Serra, J. A., & Barat, J. M. (2010). Comparison of wild and cultured sea bass (*Dicentrarchus labrax*) quality. *Food Chemistry, 119*, 1514–1518.

Grigorakis, K. (2007). Compositional and organoleptic quality of farmed and wild gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) and factors affecting it: A review. *Aquaculture, 272*, 55–75.

Lin, M., Mousavi, M., Al-Holy, M., Cavinato, G., & Rasco, B. A. (2006). Rapid Near Infrared Spectroscopic method for the detection of spoilage in rainbow trout (*Oncorhynchus mykiss*) fillet. *Journal of Food Science, 71*, S18–S23.

Majolini, M., Trocino, A., Xiccato, G., & Santulli, A. (2009). Near infrared reflectance spectroscopy (NIRS) characterization of European sea bass (*Dicentrarchus labrax*) from different rearing systems. *Italian Journal of Animal Science, 8*(Suppl. 2), 860–862.

Martens, H. (1999). Multivariate calibration and chemometrics for NIR: Why and how it started. In *Abstract book, Proceedings of the ninth international conference on near-infrared spectroscopy*, O. 3–1.

Martinez, I., Standal, I. B., Axelson, D. E., Finstad, B., & Aursand, M. (2009). Identification of the farm origin of salmon by fatty acid and HR ^{13}C NMR profiling. *Food Chemistry, 116*, 766–773.

Menoyo, D., Lopez-Bote, C. J., Bautista, J. M., & Obach, A. (2002). Herring vs. anchovy oils in salmon feeding. *Aquatic Living Resources, 15*, 217–223.

Poli, B. M., Parisi, G., Zampacavallo, G., Mecatti, M., Lupi, P., Gualtieri, M., et al. (2001). Quality outline of European sea bass (*Dicentrarchus labrax*) reared in Italy: Shelf life, edible yield, nutritional and dietetic traits. *Aquaculture, 202*, 303–315.

Roncarati, A., Sirri, F., di Domenico, A., Brambilla, G., Iamiceli, A. L., Melotti, P., et al. (2010). Survey of qualitative traits of European sea bass cultivated in different rearing systems. *European Journal Lipid Science and Technology, 112*, 770–779.

Shenk, J. S., & Westerhaus, M. O. (1994). The application of near infrared reflectance spectroscopy (NIRS) to forage analysis. In G. C. Fahey, M. Collins, D. R. Mertens, & L. E. Moser (Eds.), *Forage quality, evaluation and utilization* (pp. 406–449). Lincoln, Nebraska, USA: Crop. Science Society of America.

Siderer, Y., Maquet, A., & Anklam, E. (2005). Need for research to support consumer confidence in the growing organic food market. *Trends in Food Science & Technology, 16*, 332–343.

Solberg, C. (1996). Use of near infrared transmittance in quality analysis of fish. In *Proceedings of the seventh international conference on near infrared spectroscopy* (pp. 591–595). Montreal, Canada.

Testi, S., Bonaldo, A., Gatta, P. P., & Badiani, A. (2006). Nutritional traits of dorsal and ventral fillets from three farmed fish species. *Food Chemistry, 98*, 104–111.

Tulli, F., Balenovic, I., Messina, M., & Tibaldi, E. (2009). Biometry traits and geometric morphometrics in sea bass (*Dicentrarchus labrax*) from different rearing systems. *Italian Journal of Animal Science, 8*(Suppl. 2), 881–883.

Turchini, G. M., Torstensen, B. E., & Ng, W. K. (2009). Fish oil replacement in finfish nutrition. *Reviews in Aquaculture, 1*, 10–57.

Uddin, M., Okazaki, E., Turza, S., Yumiko, Y., Tanaka, M., & Fukuda, Y. (2005). Non-destructive visible/NIR spectroscopy for differentiation of fresh and frozen-thawed fish. *Journal of Food Science, 70*, C506–C510.

Weeranantanaphan, J., Downey, G., Allen, P., & Sun, D. (2011). A review of near infrared spectroscopy in muscle food analysis: 2005–2010. *Journal of Near Infrared Spectroscopy, 19*, 61–104.

Xiccato, G., Trocino, A., Tulli, F., & Tibaldi, E. (2004). Prediction of chemical composition and origin identification of European sea bass (*Dicentrarchus labrax* L.) by near infrared reflectance spectroscopy (NIRS). *Food Chemistry, 86*, 275–281.

Zander, K., & Hamm, U. (2010). Consumer preferences for additional ethical attributes of organic food. *Food Quality and Preference, 21*, 495–503.