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**Evaluation of the growth performance and the welfare status in sea bass
(*Dicentrarchus labrax*) reared under organic and traditional aquaculture
by immunohistochemical and biomolecular approach**

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Summary

A growing consumer awareness of food quality, based on high degree of safety, adequate nutritional value, sustainable production, eco-environmental attention, animal welfare and use of raw materials, has facilitated the spread of the "organic production". Organic aquaculture has lagged behind the organic agriculture sector in terms of quantities and diversity of certified organic products because of the absence of detailed accepted standards and criteria until recently.

The main challenges for organic aquaculture are to improve the coordination between production and market and to achieve an appropriate framework to drive further development. In organically cultured fish, differences in feeds and nutrition compared to conventional systems are likely to result in differences in the quality of the flesh, and this is a significant factor in consumer choice.

The present study aims at evaluating the growing performances, IGF-I and IGF-II levels, oxidative stress and contaminant markers in European sea bass (*Dicentrarchus labrax*) fed with organic and conventional diet in field conditions. Growing performances and condition factors showed a positive growing trend in both groups, as evidenced by molecular analyses. IGF-I and IGF-II showed similar levels throughout the whole experimental period confirming their role during growth.

A greater productivity in conventional fed fish compared to the organic ones was observed. The higher productivity was likely due to diet composition, since differences were significantly mitigated during starvation period. Fillet analysis of organic sea bass showed a higher content in MUFA and lower in PUFAs n-6 indicating that diets with a content in fatty acids closer to that of wild fish reflect the same fatty acid profile of the

flesh. On the other hand, the considered oxidative stress and contaminant markers did not show any significant differences among groups.

Riassunto

La crescente domanda di prodotti alimentari ottenuti da agricoltura biologica in questi ultimi anni è verosimilmente l'espressione di una aumentata consapevolezza del consumatore che sempre più spesso ricerca alimenti di qualità. La qualità del prodotto alimentare derivante da produzioni biologiche non si basa soltanto sul concetto tradizionale di salubrità e di valore nutrizionale del prodotto stesso, ma anche sul concetto globale di sostenibilità e salvaguardia ambientale. L'agricoltura biologica infatti è un metodo di produzione che rispetta il benessere umano e animale e difende la biodiversità ambientale e culturale dei territori proponendo un modello di sviluppo sostenibile volto a salvaguardare ambiente e territorio e valorizzare la qualità delle risorse delle comunità locali.

Questo studio di campo si prefigge di confrontare le performances di crescita e il benessere in branzini (*Dicentrarchus labrax*) alimentati con dieta convenzionale rispetto a branzini alimentati con dieta certificata biologica attraverso un approccio immunoistochimico e biomolecolare valutando i fattori di crescita (IGF-I e IGF-II), parametri dello stress ossidativo e di contaminazione ambientale. Le performances di crescita e i parametri biometrici hanno dimostrato un trend di crescita positivo in entrambi i sistemi di allevamento, come confermato anche dalla quantificazione dei fattori di crescita. I fattori di crescita hanno evidenziato livelli simili in entrambi i gruppi e durante tutto il periodo sperimentale confermando il loro ruolo determinante nella crescita corporea. Il gruppo alimentato con la dieta convenzionale ha mostrato una produttività più

elevata rispetto al gruppo biologico. La più elevata produttività registrata a carico del gruppo convenzionale è verosimilmente determinata dalle differenze nella composizione della dieta, considerato che tali differenze venivano mitigate durante i periodi di digiuno. L'analisi del filetto ha evidenziato un più alto contenuto in acidi grassi monoinsaturi e un più basso contenuto in acidi grassi polinsaturi (n- 6) a carico dei filetti biologici. Questo dato sta ad indicare che la dieta biologica caratterizzata da un contenuto in acidi grassi più vicino a quello del pesce pescato probabilmente influisce sul profilo degli acidi grassi delle carni di pesce. Per contro, i markers di stress ossidativo e contaminazione ambientali considerati in questo studio di campo non hanno dimostrato alcuna differenza significativa tra i due gruppi.

1. Introduction

1.1 Organic aquaculture

Many fish stocks on which capture fisheries depend are overexploited or severely depleted because of the high demand for fish production. In this context of overall resource scarcity and decreasing trend on catches, aquaculture production assumes a key importance (Tidwell and Allan 2001). Aquaculture reduces the dependency on fisheries and alleviates the economic impact of fisheries decline on coastal communities, namely by creating new jobs (Fernandes et al. 2000).

Organic aquaculture could represent an attractive choice for consumers as an alternative to fishery exploitation and traditional aquaculture. In fact, organic aquaculture has gained importance in major organic food markets over the last decade as a result of consumer and market reaction to concerns about poor taste and texture, contamination, animal welfare, sustainability and adverse environmental impacts of traditional aquaculture. More broadly, the development of organic production should respond to a growing consumer request for a high degree of food safety, high nutritional value, sustainable production and eco-environmental attention (Yussefi and Willer 2002).

Consumers expect organic producers to follow higher animal welfare standards (Hermansen 2003). In the current marketing of seafood products, eco-labelling and organic certifications are two common concepts (Ward and Phillips 2008; Perdikaris and Paschos 2010). Organic certification is focused on aquaculture, while eco-labelling is more oriented towards sustainability of capture fisheries and their impact on the ecosystem (FAO 2005). These eco-schemes are basically "seals of approval" that transmit the information that the product meets ethical, environmental and good governance criteria,

thus allowing consumers to make informed choices about fish products (Mente et al. 2011).

1.2 Regulation in organic aquaculture

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.

Organic agriculture matches tradition, innovation and science to benefit the shared environment and promote fair relationships and a good quality of life. The challenge for organic aquaculture is to follow the same general principles as the more developed terrestrial organic agriculture, given the basic differences primarily identified as living conditions, treatment of diseases and species themselves (Biao 2008).

The European Community responded to the organic aquaculture needs with the EC Regulation No.834/07 of June 28th 2007, and consequent EC Regulation N.889/2008, defining the first guidelines for control, labeling and rules of production of these systems. The EC Regulation No. 710/09, which came into force on August 6th 2009 establishes the procedures for starting a new production line and the process for the conversion from conventional to organic methods.

However, for the further growth of the organic aquaculture sector in Europe and globally, specific standards need to be set and there are a number of issues that should be further addressed by detailed rules and guidelines. Specifically, the stocking density shall provide for the comfort and well-being of the animals which, in particular, shall depend on the species, the age of the animals and their behavioral needs. This could affect other

parameters including water quality and the impact on the environment, and consequently the animals' well-being and growth (Cottee and Petersan 2009). The low stocking densities are desirable in organic aquaculture because, as with many other disease or parasitic infections, infestation levels are host-density dependent. Stocking densities should be as close as possible to natural conditions.

1.3 Feeds and feeding in organic aquaculture

In aquaculture, the nutrition highly determines the economic viability and sustainability of the business. In many conventional aquaculture operations, feed accounts for over 50% of the variable operating cost (Rana et al 2009), while in organic operations a 50% surcharge is assumed for organic certified feeds, although lowering feed conversion ratios can compensate their costs (Bergleiter et al 2009).

Nutrition, as a broader term, involves the whole series of actions that ensure the provision of the nutrients required for vital processes to an organism (Guillaume et al 1999) and includes both external and internal nutrient sources, as well as the action of feeding and the applied feeding methods.

As a general principle in nutrition of organically farmed aquatic animals, feeding shall be performed in a way that allows natural food intake and ensure that the developmental, physiological and behavioral needs of animals are met. Feeds must be balanced according to the nutritional requirements of the organisms, promote the growth and health, ensure high quality of the final edible product and cause low environmental impact. However, in nutrition of aquaculture animals, differences exist according to the intensity of culture (e.g. extensive, semi-intensive, intensive, super intensive) and the intensity of feeding (e.g. low–high feeding rations), the culture media (e.g. earthen ponds, integrated

aquaculture systems, concrete tanks, closed recirculation systems in tanks/race-ways, sea cages, net pens) and the feeding habits of farmed species (e.g. herbivorous, omnivorous, carnivorous), the feed formulations (e.g. diets for carnivorous species require specific amounts of marine resources such as fishmeal and fish oil) and the type of manufactured feed (e.g. pelleted or extruded feed) among others.

At intensive systems, where very high levels of production are required and/or clear water systems (tanks, cages etc.) are used, all nutrient requirements of cultured species must be met by exogenous complete feeds (Jauncey 1998).

In the case of carnivorous species ss in the case of the sea bass, organic aquafeeds shall consist of fishmeal and fish oil to maintain animal's health, satisfy their nutritional requirements for specific amino acids and fatty acids and to suit to their carnivorous feeding habits. The use of fishmeal and fish oil contradicts the organic principle of sustainability owing to the decline of fisheries and overexploitation of wild stocks. Organic feeds may consist of marine ingredients coming from sustainable use of fisheries and of plant feed ingredients originated from organic production (EU 2008, 2009).

Regulation on organic fish certification permits fishmeal and oil from certified sustainable fishery areas or from by-products and trimmings from seafood-processing fishery or from certified organic aquaculture, but of a different species or from by-catches from food fisheries. The feed ration may comprise a maximum of 60% of organic plant products (EU 2009).

The availability of fishmeal will likely decline in the future, such that fishmeal no longer can be considered a sustainable protein source for aquafeeds (Craig and McLean 2006). Accordingly, alternate proteins are needed to replace fishmeal, especially for diets of carnivorous species. Plant proteins can replace fishmeal up to 25–35% (Tidwell and Allan

2001; Pereira and Oliva-Teles 2003). Research has not determined clearly the proportions of aquatic and plant origin that should be used in organic feed. This aspect is of great importance especially for the culture of carnivorous species that dominate European aquaculture. The use of organically produced plant oils to substitute fish oil in aquafeeds could be challenging and should also be examined, given especially the effects that such feed formulations may have on fish muscle fatty acid compositions, including reductions in long chain fatty acids such as EPA and DHA, and ultimately on the quality of the final product.

1.4 Fish welfare and growth

Fish respond to environmental changes through a series of adaptive neuro-endocrine adjustments, which are defined as "stress response". This physiological response is adaptive in the short term but a prolonged activation of the stress response leads to immunosuppression (Magnadóttir 2006), reduction in growth (Barton et al. 1987; Pickering 1993; Pankhurst and Van der Kraak 1997) and reproductive dysfunction (Schreck et al. 2001). Fish exposure to stressful conditions during fishing and aquaculture procedures damages the animal welfare resulting in negative impact in productivity and raising ethical concerns.

The stress response includes three stages (Barton 2002). The primary response of the fish versus a condition of stress involves the activation of two neuroendocrine axes: the hypothalamic-sympathetic-chromaffine axis that produces catecholamines (adrenaline and noradrenaline) from chromaffin cells and the hypothalamic-pituitary-interrenal axis with the release of corticosteroids (primarily cortisol in teleost) from interrenal tissue. The

sympatho-adrenergic response stimulates cardiovascular and respiratory functions responsible for the increasing metabolic demand (Sumpter 1997).

As in other vertebrates, the hypothalamic-pituitary-interrenal axis controls the energy and hydrosaline metabolism (Mommsen et al. 1999). The secondary response represents a physiological adjustment to stress conditions (e.g. the stress hormone effects on target organs). It includes activation of several metabolic pathways that induce 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 (reproductive performances, immune status, growth and behavior) and consequently in the population (Iwama 2007).

Stress has a negative effect on growth (Pankhurst and Van Der Kraak 1997) and on the immune system dramatically increasing the incidence of diseases and mortality rates (Broom and Johnson 1993; McDonald and Milligan 1997). A wide range of stimuli will rouse the animal stress response which is considered essential for the adaptation to a new environment and survival of the organism. Fish are exposed to stressors in nature, as well as in artificial conditions such as in aquaculture. In modern intensive aquaculture, many stressors such as grading, transportation and vaccination result unavoidable.

Stocking density is a key factor that affects fish welfare in aquaculture, especially where high densities are required for high productivity (Turnbull et al. 2005). 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).

Diet also play an important role in stress sensitivity. In particular, a wrong food composition and/or meal administration can negatively affect fish welfare. Diets lacking in critical micronutrients severely affect fish welfare. For example, Huntingford et al. (2006) observed small morphological and behavioral abnormalities, decreasing in growth rates and weakening of immune function. Low levels of highly unsaturated fatty acids (HUFA), besides decreasing the immune system, can negatively affect the reproductive function (Poli 2009). African catfish (*Claria gariepinus*) receiving a diet high in ascorbic acid (Vitamin C) during early development showed lower stress sensitivity (Merchie et al. 1997). On the contrary, common carp (*Cyprinus carpio*) fed with a diet rich in Vitamin C showed an increased cortisol release in response to stress when compared to fish fed with recommended levels of the same vitamin (Dabrowska et al. 1991). Juvenile gilthead sea bream (*Sparus aurata*) fed with a diet poor in Vitamin E showed faster elevation of plasma cortisol in response to stress and a lower survival rate than control fish (Montero et al. 2001). Healthy animals likely express a higher food conversion efficiency, lower mortality and a higher growth rate. In fact, the increased muscular activity, generally associated with stress condition, causes energy mobilization, resulting in the production of lactate, decreased pH and increased lipid oxidation (Poli et al. 2004).

Growth in vertebrates depends on a regulatory network in which the growth hormone (GH)-insulin-like growth factor (IGF)-I axis plays a key role in growth regulation together with insulin, thyroid hormones and sex steroids (Jones and Clemmons 1995). While IGF-I mRNA is expressed mainly in liver of adult fish, as in mammals and non-mammalian vertebrates, (Shamblott and Chen 1993; Duguay et al. 1996; Funkenstein et al. 1997;

Radaelli et al. 2003; Patruno et al. 2008), IGF-II is proved to be ubiquitously expressed, working essentially as a growth factor (Radaelli et al. 2003; Vong et al. 2003; Caelers et al. 2004; Carnevali et al. 2005; Wood et al. 2005; Funes et al. 2006; Patruno et al. 2006; Radaelli et al. 2008).

Food composition is also considered an important factor in preserving welfare, growth, development and reproduction of the fish. An important role among all the nutrients is certainly played by the Highly Unsaturated Fatty Acids (HUFAs): docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (AA, 20:4n-6) (Sargent et al. 1993; Sargent et al. 1995; Sargent et al. 1997). HUFAs must be provided by the diet because, in case of deficiency, a decreased growth rate and a weakening of immune function were observed.

Under farming conditions, the fish are often subjected to unavoidable stressors such as manipulation, size grading, stocking density, fasting, transport, conditions of pre-slaughter and slaughter techniques that could affect health status (Ashley 2007; Conte 2004; Poli 2009).

1.5 Oxidative stress

The oxidative stress is an inescapable component of aerobic life. In the healthy aerobic organism, a balance between the reactive oxygen species (ROS) production and the system to protect cells from ROS exists. Ascendancy of the ROS production results in defects that may cause cell or organism damages or death. This imbalance is referred to as oxidative stress.

Oxidative stress is 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). Oxidative stress is a condition that leads to the production of reactive oxygen species (ROS) (Ahmad et al. 2000; Barata et al. 2005). ROS, such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH) and the radical nitric oxide (NO), 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).

The lipid peroxidation derived from a reaction with free radicals (Halliwell and Gutteridge 1999) leads to formation of ROS (Winston and Di Giulio 1991).

When the production of ROS is greater than the ability of cells to remove them, the formation of protein carbonyl groups and DNA damage can develop (Halliwell and Gutteridge 1999). 4-hydroxy-2-nonenal (HNE) is the most abundant and toxic α,β -unsaturated aldehyde, which originates from lipid peroxidation and mediated by the β -cleavage of hydroperoxides from ω -6 PUFAs. HNE is mainly involved in the inhibition of protein and DNA synthesis and is also considered a potent mutagenic agent (Aldini et al. 2007). High dietary levels of lipids and vitamins likely influence the oxidative status as pointed out by several studies that show a protective effect of such diets (Mourente et al. 2000; Mourente et al. 2002).

In order to prevent oxidation-induced lesions and mortalities, there must be effective antioxidant systems involving compounds such as glutathione (GSH). Total glutathione is

used as an indicator of oxidative stress due to its action against ROS or molecules such as benzoates and others. GSH is 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 can be affected by viruses, bacteria, fungi and toxins, and other events that cause stress to the immune system, which is stimulated to activate lymphoid cells. These cells, primarily involved in the control of viral or bacterial infections generate large amounts of ROS that will damage the same immune cells and other tissues. (Finkel and Holbrook 2000).

One of the most important biomarkers of *in vivo* oxidative damage to DNA are products of the specific modifications and hydroxylations of purine and pyrimidine bases and products of damage to the deoxyribosephosphate backbone and protein-DNA cross-links (Valavanidis et al. 2006). DNA damage includes the modification of bases, such as the oxidation of deoxyguanosine to form 8-hydroxy-2'- deoxyguanosine (8-OHdG) (Ploch et al. 1999). The amount of 8-OHdG in aquatic organisms is considered as a solid biomarker of oxidative stress in relation to environmental pollutants (Jebali et al. 2007). Due to the presence of fish meal and fish oil, commercially marine feed could represent a source of persistent organic pollutants and heavy metals such as mercury, cadmium and arsenic (Berntssen et al. 2010).

1.6 Biomarkers

By definition, a biomarker is a biological response that can be related to an exposure to, or a toxic effect of an environmental chemical or chemicals. Generally, biomarker responses provide qualitative and semi-quantitative information on the nature of the chemical insult

and information on the relationship between the biological effects and levels of environmental contamination (Henderson et al. 1989).

It must be said that no single biomarker sensitive and specific enough for oxidative stress has been identified (Di Giulio and Meyer 2008) and not all biomarkers are good biomarkers and that it is necessary to be aware of potential confounding factors. Moreover, and that it is necessary to be aware of potential confounding factors. The confounding factors can be both such as biological (e.g., species, age, sex, genetic population, feeding status, reproductive phase) and environmental ones (e.g., temperature, oxygen concentration, pH, salinity). Because of these factors, it is necessary to standardize monitoring and only use well studied species in well studied environments. For example, Traven et al. (2013) did not see an induction in ethoxyresorufin O-deethylase (EROD) activity in adult sea bass (*Dicentrarchus labrax*) at a site that was highly contaminated with PAHs. Numerous other studies have shown that EROD is very sensitive to PAH contamination in a number of species (Van der Oost et al., 2003).

The cytochromes P450 are a diverse multigene family of heme-containing proteins that oxidize, hydrolyze, or reduce compounds through the insertion of an atom of atmospheric oxygen to the substrate during the reaction cycle (Nelson et al. 1993). The assessment of the Cytochrome P4501A (CYP1A) in fish represents an environmental biomarker since CYP1A is involved in the biotransformation of a variety of aquatic contaminants such as oil compounds, dioxins, polychlorinated biphenyl (PCBs), polycyclic aromatic hydrocarbons (PAHs), etc. (Nebert and Gonzalez 1987; Sarasquete and Segner 2000; Ribocco et al. 2012). Beyond the liver, which represents the main site of CYP1A expression in fish, the epithelia of organs in direct contact with the environment (gills, intestine and kidney) and the vascular endothelia exhibit a remarkable CYP1A expression as well. Embedded in the

smooth endoplasmic reticulum, they metabolize both endogenous and exogenous compounds (phase I reactions), generally increasing the water solubility of substrates, thereby enhancing their elimination. In this way, cytochromes P450 such as CYP1A tend to detoxify xenobiotic chemicals; however, the phase I metabolites of some PAH and other contaminants may be more toxic than the parent compound (Guengerich et al. 1986). The most useful aspect of CYP1A for biomonitoring purposes is the enzyme's tendency to increase in concentration upon chemical exposure. Induction of CYP1A is mediated through the binding of xenobiotics to a cytosolic aryl hydrocarbon receptor (AhR). AhR ligands generally have isoteric configurations and are similar in structure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD), a model CYP1A inducer. In fish, early life stages appear to be particularly sensitive to AhR ligands, and recent evidence indicates the involvement of CYP1A enzymes specifically in this toxic response (Zeldin and Seubert 2008).

The use of CYP1A induction as an assessment technique has increased in recent years. This is due mainly to the optimization of protocols for the rapid and relatively inexpensive measurement of its catalytic activity as EROD (Celandier 2011).

Melanomacrophage Centres (MMCs) evaluation is often used as a reliable indicator of pollutant exposure, in particular heavy metals (Meilnet et al. 1997; Pascoli et al. 2011; Passantino et al. 2014) indicating sub-lethal effects (Bucke et al. 1992). 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 stroma of 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 lymph nodes 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 under physiological conditions that cause an increase in the number of aggregates such as advanced age, fasting, increasing in hemoglobin catabolism and pathological conditions (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 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 phagocytosed materials, as pigment granules. Ferguson (1976) in his ultrastructural studies of the 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 2003), exposure to chemical agents and in particular heavy metals (Pascoli et al. 2011). According to

literature (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 (1992), 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 melanin is 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).

2. Aim of the thesis

The present study aims at evaluating the growing performances, IGF-I and IGF-II levels, oxidative stress and contaminant markers in European sea bass (*Dicentrarchus labrax*) fed with organic and conventional diet in field conditions. The main object of the research program is to compare the effect of organic diet versus a conventional one on animal welfare and growth performance in sea bass investigating the oxidative stress and several contaminant markers.

Moreover the fillets were analyzed for proximate and FA composition.

Although a preliminary study demonstrated that a colorimetric analysis is able to distinguish sea bass produced under organic *vs.* conventional protocols (Costa et al. 2013), this is the first study in field conditions aimed to evaluate the effects of organic feeding on production, oxidative stress and contaminant markers in this species.

Attention was also given to the relationship among these parameters and the water temperature and photoperiod throughout the trial period.

3. Materials and methods

3.1 Rearing conditions

European sea bass (*D. labrax*) from the fish farm "Impianto Ittico Sperimentale di Pellestrina" (Pellestrina, VE, Italy) were transferred to the "Centro Ittico Valle Bonello" (Porto Tolle, RO, Italy) in April 2009 into two separated 300 m³ outdoor ponds (50 x 6 x 1 m), one for organic and one for conventional feeding trial. In both ponds, stocking density was the same (initial stocking density: 2 kg/m³), as all the other parameters (water temperature and photoperiod, both environmental).

Fish of the two ponds (initial weight ~80 grams) were fed with different types of diet, one certified organic and one conventional (Tab. 1).

Table 1

Proximate composition (% as-fed) of the diets.

Rearing system (R)	Organic	Conventional
<i>Proximate composition</i>		
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
<i>Fatty acid profile</i>		
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
C16:1n-7	3.90	3.03
C18:1n-7	3.93	3.99
C18:1n-9	18.5	21.3
C20:1n-9	2.76	0.84
C22:1n-9	0.71	0.13

Rearing system (R)	Organic	Conventional
C22:1n-11	8.08	0.62
C24:1n-9	0.46	0.19
Other MUFAs	0.81	0.35
Total MUFAs	39.0	30.4
C18:3n-3	2.92	4.96
C18:4n-3	5.82	0.79
C20:5n-3	4.47	5.96
C22:5n-3	0.91	0.74
C22:6n-3	5.90	3.10
PUFAs n-3	20.0	15.6
C18: 2n-6	13.3	29.3
C20:4n-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.

3.2 *Animal sampling and samples preparation*

Fish were monitored for 18 months, from May 2009 to November 2010, until they reached the commercial size. Bimonthly, twenty fishes per pond were netted 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 Fulton's condition factor (K) was calculated ($K = \text{fish weight} / \text{fish total length}^3$, Fulton, 1902). Animals were then euthanized with overdose of anesthetic (MS222) and the blood and the following organs were collected: liver, spleen, gut, head kidney. Specimens for histology were immediately fixed in 4% paraformaldehyde prepared in phosphate-buffered saline while liver tissue sampled also for molecular analyses, was immediately fixed in trizol and processed as described below. Blood was allowed to clot at +4°C for 2 hours and then centrifuged for 5 minutes (12,500 rpm; MicroCL 17, Thermo Scientific, Germany). Serum was separated and stored at -20°C. All animals were treated as requested by EFSA (European Food Safety Authority) guidelines. Pond water temperature was recorded twice a day (morning and afternoon) with a digital thermometer. The dissolved oxygen was kept at normoxic conditions for European sea bass (>7.5 mg/l) as recommended by EFSA (2008) and ranged from 8 to 12 mg/l in both the tanks.

3.3 *Histochemistry*

Tissue samples were washed in PBS, dehydrated through a graded series of ethanol and embedded in paraffin. Serial sections were cut at a thickness of 4 µm. Haematoxylin and eosin (HandE) staining and Schmorl's reaction, used to evaluate MMCs for count (see below), were performed as described in Bancroft and Gamble (2008).

3.4 *Tissue microarray*

The array was built using paraffin liver and gut donor blocks of the 200 sampled fishes. Using the Beecher TMA instrument (Beecher Instruments, Sun Prairie, WI) one core of 0.6 mm per donor blocks was removed and transferred into the recipient block. Cores were arranged in sectors, each containing 4 rows with 10 cores per row following a previously drawn, preset unequivocal plot. Positive and negative control samples were included in each array. Serial 4 µm thick sections were cut.

3.5 *Immunohistochemistry*

Immunohistochemical stainings for 8-OHdG, HNE e CYP1A in liver and gut sections were performed using the automated immunostainer Benchmark Ultra Ventana as described in Pascoli et al. (2011b). Immunoreactive scoring for 8-OHdG and HNE was done by counting positive nuclei in 100 cells by two independent operators. Samples stained with CYP1A were evaluated for the presence and distribution of immunopositivity, and a score from negative (-) to strong (++) was assigned to the intensity of the reaction.

3.6 *Melanomacrophage centre (MMCs) count*

Serial sections of spleen were stained with H and E sequential stain to ascertain structural details, and with Schmorl's reaction (as described above) to detect the melanomacrophage centres (MMCs). Microscopic quantitative assessment of MMCs was made through a computerized image analyzer system (Olympus CellB, Japan) on sections of spleen since it is the organ which exhibited the highest number of MMCs, as also reported in literature (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.

3.7 *Glutathione (GSH)*

Total GSH in blood serum was determined by an enzymatic recycling method adapted for microtitre plate reader (Baker et al. 1990). 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. 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 μ l 5.5'-ditiobis-2-nitrobenzoic acid (DTNB). For Reductase preparation, 15 μ l of the commercial reductase solution (Glutathione Reductase, 205 units/mg protein, Sigma-Aldrich) is diluted with 53.4 μ l of ammonium sulphate 3.6 M (Sigma a-4915, MW132.1), then 65 μ l of the obtained solution is diluted with 3835 μ l of TF-E. A 96 multiwell plate is loaded with 30 μ l of TF-E (blank), the standard curve (30 μ l of the various points, in descending order) and samples, 15 μ l 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, 25 μ l 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.

3.8 Qualitative reverse transcription/PCR and quantitative real-time PCR for IGF-I and IGF-II

Qualitative reverse transcription/PCR and quantitative real-time PCR were performed by following the methods already used with this study species by Bertotto et al. (2011). Briefly, RNAs were extracted from the liver tissue (50 mg sample) of 12 individuals (6 conventional, 6 organic) per sampling period (4 periods: May 2009, December 2009, May 2010, November 2010), using TRIZOL Reagent (Gibco-BRL, Gaithersburg, Md., USA).

Total RNA (1.5 µg) was retrotranscribed into cDNA. First-strand cDNAs were synthesized by using Superscript II RNase reverse transcriptase protocols (Invitrogen, Life technologies, UK) and a mixture of random hexamers as primer (synthesized by MWG-Biotech, Ebersberg, Germany). The obtained cDNAs were used as templates for PCR expression analysis. We refer the reader to Bertotto et al., (2011) for details on IGF-I primer design and efficiency. The primer for IGF-II was designed by using Primer Express 3.0 software (Applied Biosystems) (forward, 5'-AGTGTTGTTTCCGTAGCTGTGA-3', reverse 5'-ATCCTGAGGGCCAAAAAGTATCG-3') and its specificity checked by PCRs. Data were normalized to the housekeeping gene β -actin.

Quantification assays to detect the relative expression of IGF-I and IGF-II mRNA were carried out by using the ABI 7500 Real-Time PCR System (Applied Biosystems) as described by Bertotto et al. (2011). Data from SYBR Green I PCR amplicons were collected with ABI 7500 System SDS Software. The $\Delta\Delta C_t$ method was used for relative quantification (comparative method) using a calibrator sample as basis for comparative results (see Chemistry Guide, Applied Biosystem, 2003). Dissociation melting curves confirmed the specific amplification of the cDNA target and the absence of nonspecific products.

3.9 *Proximate composition and FA analysis*

A total of 16 sea bass (8 specimens per rearing system) were collected in January 2011. All fish were slaughtered by immersion in ice slurry and 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. Fresh minced fillets were analyzed for FA composition. As detailed by Trocino et al. (2012), for this purpose, fat was extracted from the samples with accelerated solvent extraction (ASE®, Dionex, Sunnyvale, CA, USA, Application Note 334); transmethylation was performed on the extracted lipids after adding an internal standard (19:0 methyl ester) to the extracts; after centrifugation, the supernatant was injected into the split/splitless system Chromatograph 8000 Top CE (Thermo Quest, Italy) with a Restek (Bellefonte, PA, USA) Rtx-2330 capillary column (70 m x 0.18 mm internal diameter, 0.10 µm film thickness). The FAs were identified by comparing their retention times with a standard mixture of 37 FAMES (F.A.M.E. Mix C4-C24, cod. 18919-1AMP, SUPELCO, Bellefonte, PA, USA). The concentration of individual FAMES was expressed as a percentage of the total area of eluted FAMES (known plus unknown).

3.10 *Statistical analysis*

Statistical analysis was carried out with STATISTICA 9 (StatSoft) and SAS (Version 9.1) softwares. All data are reported as mean±SEM. Data of growth and biomarkers were checked for normality using a Kolmogorov-Smirnov test and log-transformed when necessary. The effect of feeding condition (organic *vs* conventional) and sampling period (10 sampling periods from May 2009 to November 2010) on growing performances (weight, length, condition index), IGF-I and IGF-II expression levels, MMCs count and GSH

levels were analyzed by means of univariate two-way factorial ANOVAs (GLM). Feeding condition and sampling periods were included in the model as independent fixed factors; growing and oxidative stress parameters as dependent variables. HSD-Tukey's post-hoc tests were performed when identifying a significant effect.

The fillet data collected in the study were analysed with the GLM procedure of SAS. The diet was used as the experimental factor. Published data (Pascoli et al., 2011b) were also included for comparison.

4. Results

4.1 Growing performances

Growing performances were evaluated using biometric measures and condition factor (K). Since standard and total length were highly correlated ($r=0.99$; $p<0.001$), total length (TL) is considered hereinafter. It 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% for organic and 8% for conventional.

At the end of the trial calculated relative density was respectively 12 kg/m^3 for organic and 16 kg/m^3 for conventional group.

The two-way ANOVA evidenced a significant effect of both the groups and the sampling period on weight (diet: $F_{1,9}= 16.78$, $p<0.001$; period: $F_{1,9}=331.36$, $p<0.001$) and TL (diet: $F_{1,9}= 8.99$, $p<0.001$; period: $F_{1,9}=258.01$, $p<0.001$). The interaction between the two factors (diet x period) was also significant for both variables (weight: $F_{1,9}= 4.25$, $p<0.001$; TL: $F_{1,9}= 2.20$, $p=0.02$). Indeed, conventional fed fishes show generally higher weight and length than organic ones (Fig. 1-2).

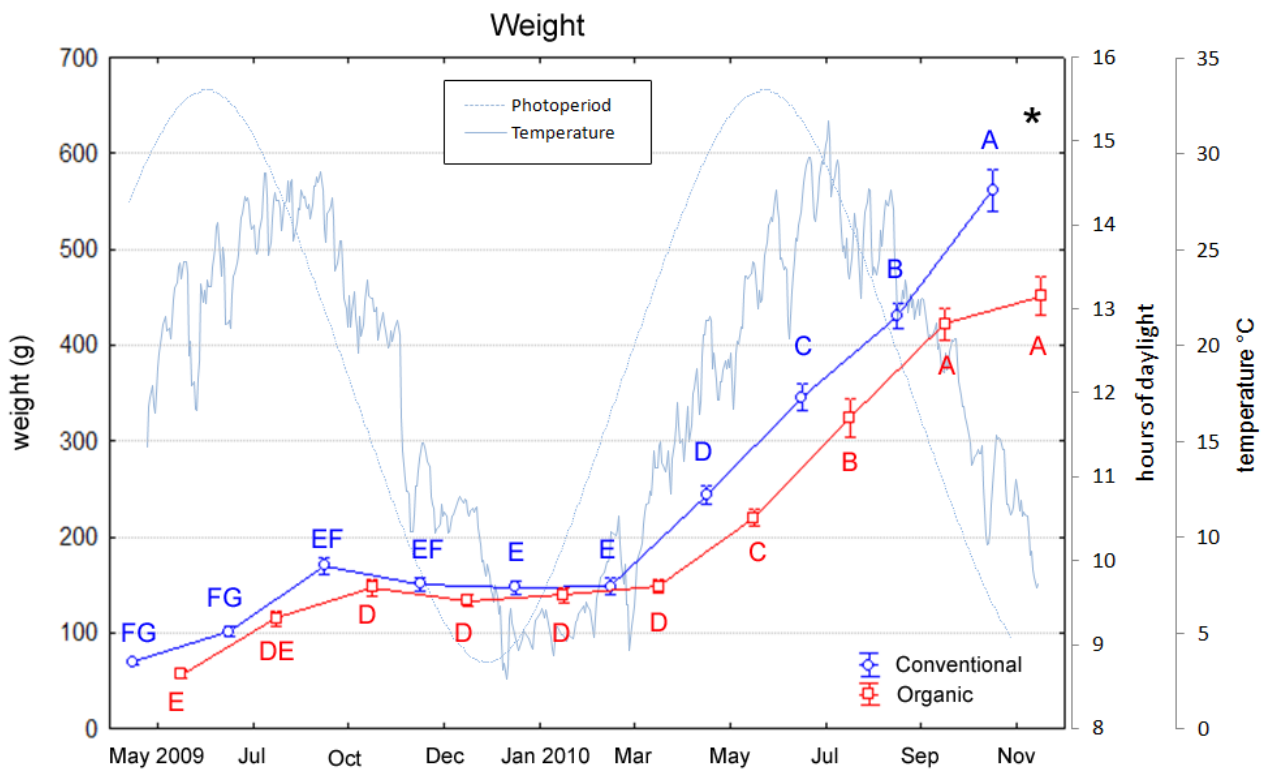


Fig. 1 Variations in weight of sea bass over a 18-months period (mean±SE) fed conventional and organic diets. Different letters indicate significant differences ($p < 0.05$) between means. Asterisks indicate significant differences between diets within the same sampling ($p < 0.05$).

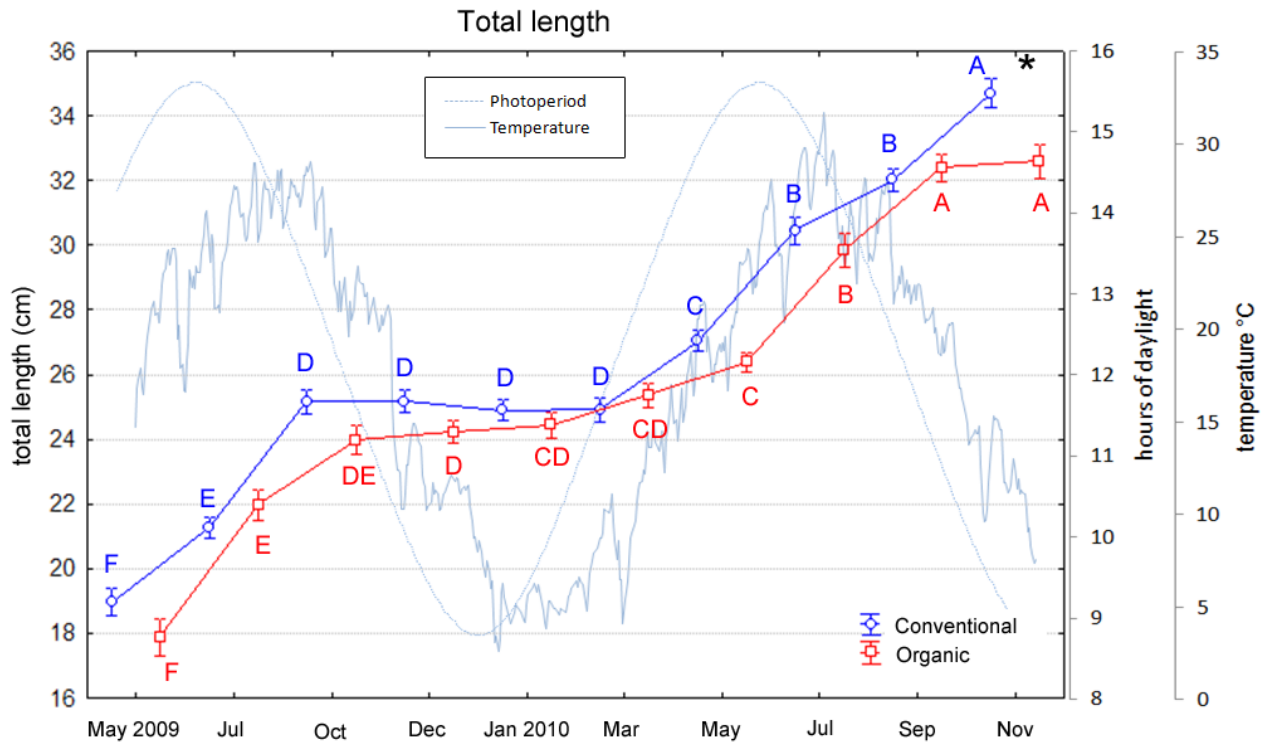


Fig. 2 Variations in total length of sea bass over a 18-months period (mean±SE) fed conventional and organic diets. Different letters indicate significant differences ($p < 0.05$) between means. Asterisks indicate significant differences between diets within the same sampling ($p < 0.05$).

Moreover, in both groups, fish exhibited a clear seasonal trend with an interruption of growth during cold months (from October 2009 to March 2010) and a recovery of growth from May to the end of the sampling period (Tukey's tests: March 2010 significantly differed from the following months, all $p < 0.05$. Figs. 1-2).

The interaction between factors (diet x period) was evident at the end of the feeding period, when conventional fishes showed a higher increase in weight and TL than organic ones. Indeed, at the end of the trial (November 2010) weight and TL of conventional fishes significantly differed from those of organic ones (Tukey's test: weight, $p < 0.001$; TL, $p = 0.038$).

With regard to the condition factor "K", the analyses evidenced a significant effect of both the groups and the sampling period (diet: $F_{1,9} = 5.44$, $p = 0.02$; period: $F_{1,9} = 84.58$, $p < 0.001$), but no interaction between the two factors (sampling x period).

Indeed, in both groups, as observed for growth and TL, fish exhibited a seasonal trend in K values that were lower during cold months (from October 2009 to March 2010, Fig. 3) and significantly increased from May to the end of the feeding period (Tukey's tests: March 2010 significantly differed from the following months, all $p < 0.001$. Fig. 3).

However, this trend was similar for conventional and organic fed fishes and no difference between the two groups was observed at the end of the feeding period.

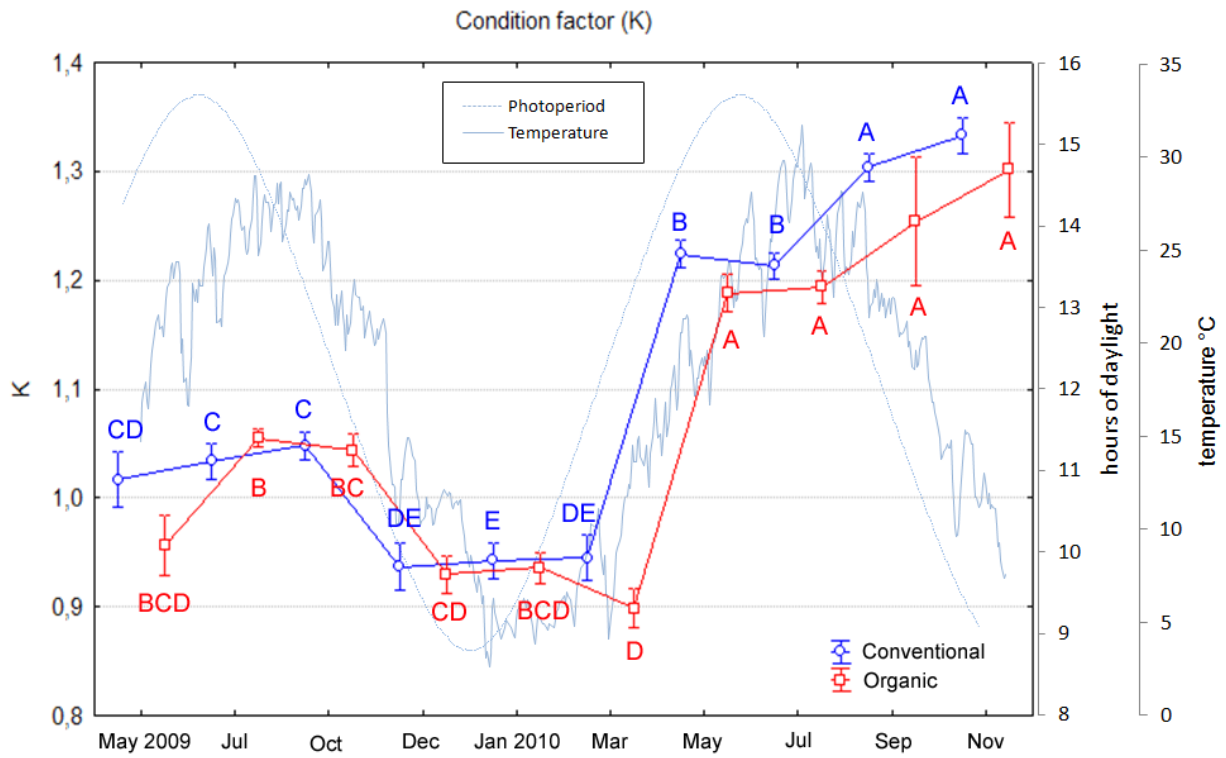


Fig. 3 Variations in condition factor (K) of sea bass over a 18-months period (mean±SE) fed conventional and organic diets. Different letters indicate significant differences ($p < 0.05$) between means. Asterisks indicate significant differences between systems within the same sampling ($p < 0.05$).

4.2 Immunohistochemistry

Immunohistochemistry for 8-OHdG and CYP1A performed in liver and gut did not show any statistically difference between the two groups (ANOVA, $p > 0.05$; Fig. 4).

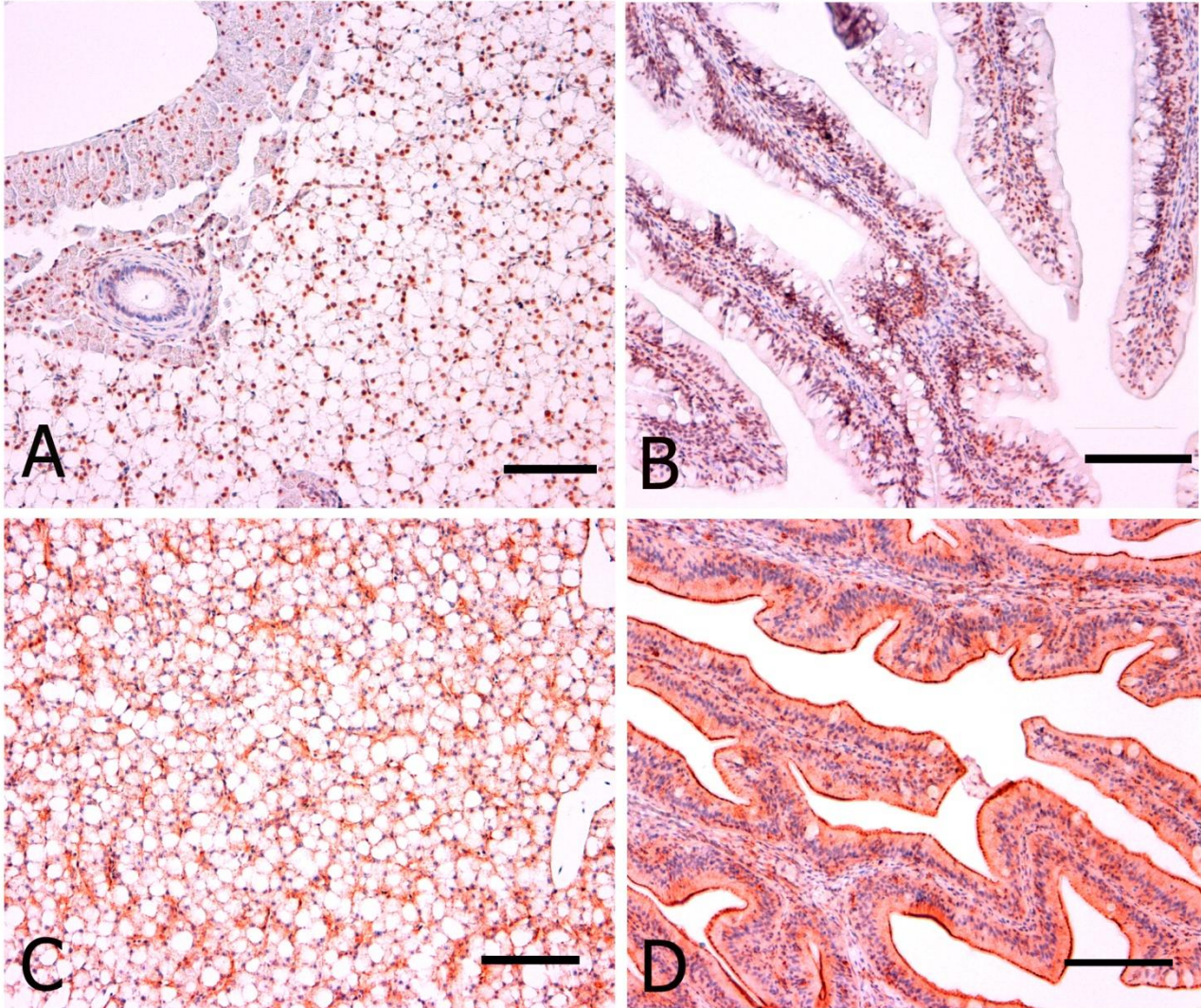


Fig. 4 Immunohistochemical localization of CYP1A and 8-OHdG in sea bass. All panels are counterstained with Harryr's haematoxylin. Bar size = 50 μm . A) Liver, strong and diffuse nuclear immunoreactivity of hepatocytes to anti-8-OHDG antibody. B) Liver, endothelial and sinusoidal immunoreactivity of hepatocytes to anti-CYP-1A. C) Gut, nuclear immunoreactivity of most enterocytes to anti-8-OHDG antibody. D) Gut, diffuse strong immunoreactivity of the intestinal mucosa to anti-CYP-1A

The anti-HNE staining was detected in the spleen, head kidney and liver mostly in the MMCs and spare macrophages (Fig. 5). Immunopositivity was found both in organic and conventional samples with no differences in intensity between the two groups.

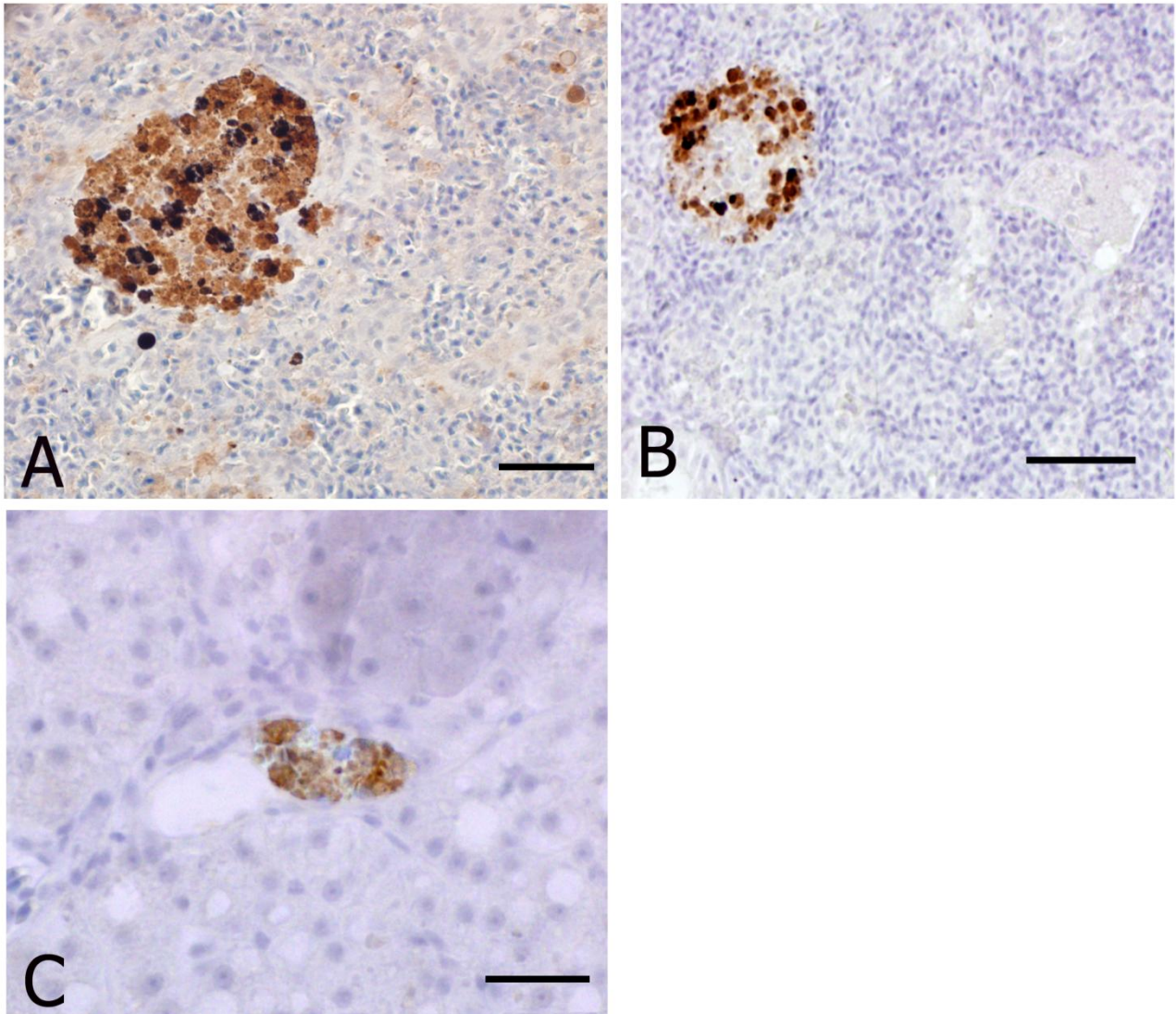


Fig. 5 Immunohistochemical localization of HNE in sea bass. All panels are counterstained with Harry's haematoxylin. Bar size: 50 µm. HNE-immunostaining is present in several melanomacrophage centers located in the parenchyma of A) spleen, B) kidney, C) liver.

4.3 MMCs count

MMCs counts were performed on spleen sections and revealed several differences between the two groups (Fig. 6). In most samples, organic fed fishes exhibited a higher number of MMCs respect to conventional fed ones, except for March and May 2010, where no significant differences were found (Tukey's test, $p < 0.01$).

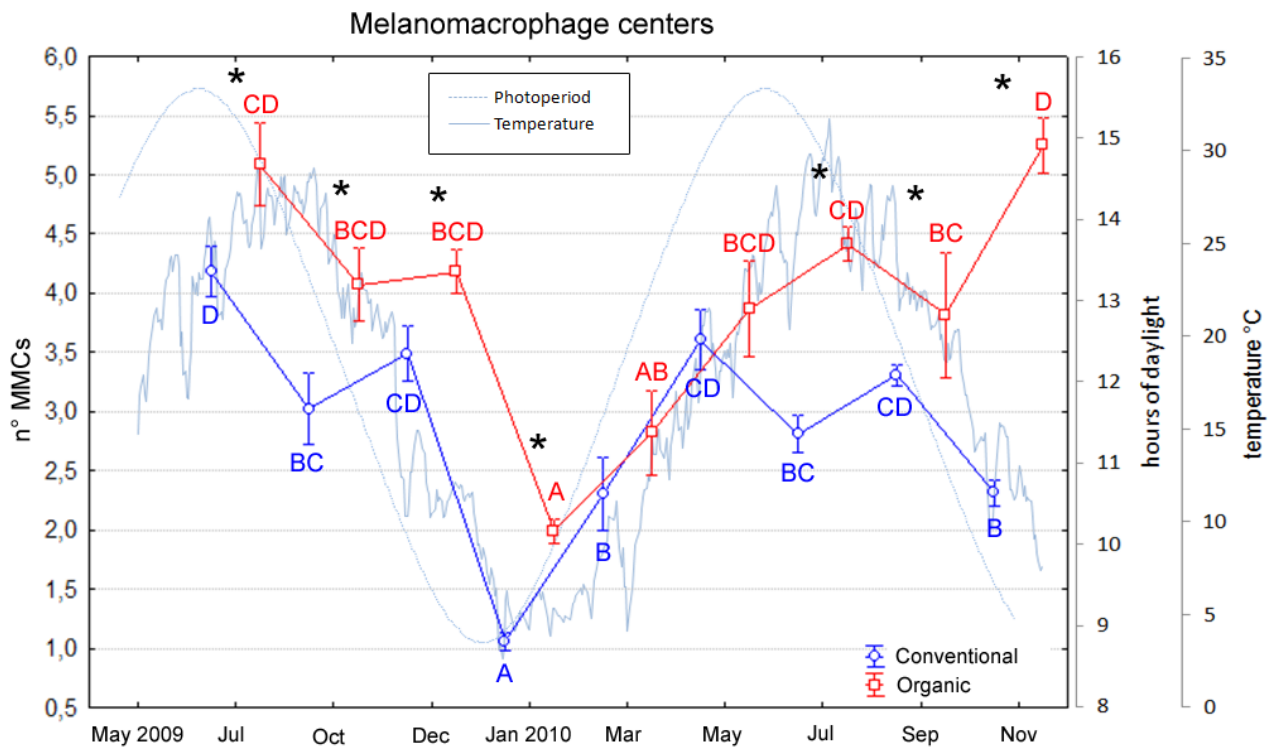


Fig. 6 Variations in melanomacrophage centers number of sea bass over a 18-months period (mean \pm SE) reared under conventional and organic aquaculture. Different letters indicate significant differences ($p < 0.05$) between means. Asteriks indicate significant differences between diets within the same sampling ($p < 0.05$).

4.4 Total glutathione (GSH)

Statistical analysis of data obtained by the spectrophotometric assay of total glutathione (GSH) showed significant differences between the two groups only in July and November 2010 (Tukey's tests, $p < 0.05$; Fig. 7). In both samplings, conventional fed fishes showed higher values than organic ones (35.9 ± 0.9 vs 34.0 ± 1.8 and 36.2 ± 0.5 vs 29.0 ± 2.2 nmol/ml).

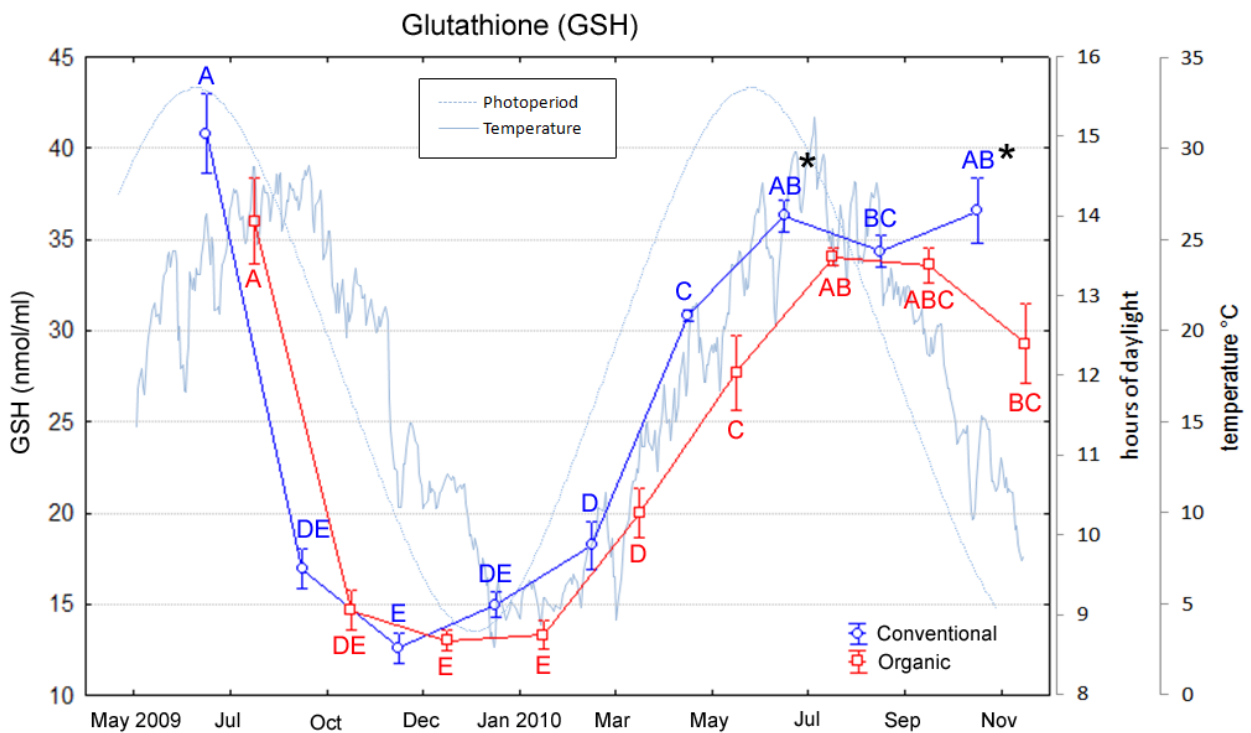


Fig. 7 Variations in glutathione of sea bass over a 18-months period (mean \pm SE) fed conventional and organic diets. Different letters indicate significant differences ($p < 0.05$) between means. Asterisks indicate significant differences between diets within the same sampling ($p < 0.05$).

4.5 *IGF-I and IGF-II expression*

Neither the feeding condition nor the sampling period had a significant effect on IGF-I and IGF-II expression, and no significant interaction between the two factors was observed (IGF-I: diet: $F_{1,3} = 0.13$, $p=0.72$; period: $F_{1,3}=0.27$, $p=0.85$; diet x period: $F_{1,3} = 0.20$, $p=0.89$. IGF-II: diet: $F_{1,3} = 0.004$, $p=0.95$; period: $F_{1,3}=0.78$, $p=0.51$; diet x period: $F_{1,3} = 0.30$, $p=0.82$).

4.6 *Proximate composition and FA analysis*

The chemical composition of the experimental diets fed during the last period of growth of sea bass differed showing lower ether extract values (18.6 and 19.9% in the organic and in the conventional diet), higher ash content (13.5 and 7.96%) and crude protein content (46.7% and 43.8%) compared with the conventional diet (Table 1).

The proximate composition and fatty acid profile of sea bass fillets are reported in Table 2. Despite the starvation period prior to slaughtering, the ether extract content tended to be higher in sea bass that were fed with the diet containing the highest ether extract ($P=0.06$), i.e. the conventional diet, in the last period of feeding compared to those fed the organic diet.

Some differences were found in the fatty acid profile of sea bass fed the two diets. In fact, a higher MUFA content was recorded in fillets of sea bass fed the organic diets (37.4% vs. 33.8%; $P=0.02$), especially due to the higher content of C20:1n-9 and C22:1n-11, even if C18:1n-9 content was lower in the same sea bass fillet compared to those fed the conventional diet. Besides, the PUFAs n-6 content was lower in the former fillets (12.0% vs. 15.8%; $P=0.04$) due to the lower content of C18: 2n-6 (11.0% vs. 14.3%; $P=0.04$).

Table 2

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

Rearing system (R)	Organic	Conventional	Probability	RSD
Live weight (g)	432	473	0.18	57
<i>Proximate composition</i>				
Water (%)	71.3	69.3	0.08	0.60
Ether extract (%)	7.93	9.72	0.06	0.47
Crude protein (%)	19.5	19.5	0.92	0.37
Ash (%)	1.14	1.16	0.41	0.01
<i>Fatty acid profile</i>				
C14:0	4.32	3.30	0.10	0.34
C15:0	0.53	0.40	0.15	0.06
C16:0	16.6	17.1	0.66	0.94
C17:0	0.54	0.49	0.32	0.04
C18:0	7.45	7.00	0.84	2.10
Other SFAs	4.18	2.90	0.22	0.72
Total SFAs	33.6	31.2	0.62	4.16
<i>Fatty acid profile</i>				
C16:1n-7	4.50	4.52	0.59	0.03
C18:1n-9	19.6	22.8	0.05	0.77
C18:1n-7	2.24	2.44	0.04	0.04

Cont'd table 2				
Rearing system (R)	Organic	Conventional	Probability	RSD
C20:1n-9	4.24	1.75	<0.001	6.15
C22:1n-11	3.41	0.69	<0.001	7.40
Other MUFAs	3.41	1.54	0.03	0.34
Total MUFAs	37.4	33.8	0.02	0.53
C18:3n-3	1.71	1.77	0.75	0.00
C18:4n-3	1.04	0.73	0.09	0.10
C20:5n-3	2.72	4.41	0.10	0.56
C22:5n-3	0.70	0.96	0.29	0.18
C22:6n-3	3.36	3.67	0.79	1.06
PUFAs n-3	10.1	12.0	0.45	2.10
C18: 2n-6	11.0	14.3	0.04	0.69
C20:4n-6	0.34	0.46	0.08	0.04
PUFAs n-6	12.0	15.8	0.04	0.79
Ratio of n-3 to n-6 PUFAs	0.83	0.76	0.64	0.12
Other PUFAs	3.03	3.63	0.10	0.20
Total PUFAs	23.8	29.9	0.18	2.96
Unknown FAs	5.14	5.13	0.99	0.00

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

5. Discussion

The present study compared an organic feed with a conventional one, in order to investigate any differences in the productivity, oxidative stress status, possible contaminant response and fillet composition. As regards growth, the expression of IGF-I and IGF-II mRNA was similar throughout the whole experimental period confirming their role during the growth. Nevertheless, neither the feeding condition nor the sampling period had a significant effect on mRNA expression.

As expected, the feed intake was affected by temperature, since both groups of fish experienced reduction of food intake during the cold season recording a temporary growth arrest (Pastoureaud 1991). At the end of the trial, conventional fed fishes showed a significant increase in growth, suggesting that conventional feeding leads to a greater productivity compared to the organic feeding administered in this field study. The lower productivity registered in the organic fed group could reflect not just the origin of the administered raw materials but the imbalance of the organic diet.

Specifically, organic feeding can replace fishmeal up to 25–35% with the use of plant proteins (Tidwell and Allan 2001; Pereira and Oliva-Teles 2003; Chou et al. 2004; Hardy 2010; Enami 2011). Plant proteins are probably the most widely used alternative to fishmeal, but they pose problems, including lower crude protein levels, palatability issues, amino acid deficiencies and the occurrence of antinutritional factors such as trypsin inhibitors and phosphorus and nitrogen release to the environment (Francis et al. 2001).

There are a limited number of studies investigating partial replacement of fishmeal with organic diets (Craig and McLean 2005, 2006; Li et al. 2006; Lunger et al. 2007). Lunger et al. (2006) fed cobia fish (*Rachycentron canadum*) for 6 weeks, an organically certifiable yeast-based protein source diet as a fishmeal replacement and showed that up to 25%

fishmeal could be replaced without affecting growth rates, feed efficiency or biological indices. Substitution levels above this resulted in decreased performance in all measured parameters. Further dietary inclusion rates for this protein might be problematic, however, because of amino acid imbalances and limitations by added amino acids (Craig and McLean 2006). Craig and McLean (2005) replaced fishmeal and soybean meal with an organic diet (yeast), and there was no difference in growth rates in tilapia. Browdy et al. (2006) conducted a nutritional study by replacing fishmeal with a plant-based diet (algal fermentation), in shrimps *Litopenaeus vannamei*, and showed that there were no significant difference in final production, survival and FCR.

In the present study, the chemical composition of the organic and conventional diets administered during the last period of growth to the sea bass differed in terms of ether extract values, lower in the organic diet, and ash content and crude protein content higher compared with the conventional diet.

Since the fillet FA profile is known to be strictly dependent on the diet, the level of n-3 PUFAs largely depends on the dietary level and the types of supplemented fish meal and oils, specially vegetable oil (Grigorakis 2007; Turchini et al. 2009; Benedito-Palos et al. 2011). Even if the precise source of fish oil used in the two diets of the present study was not declared by the producer, the high level of eicosenoic acid and cetoleic acid makes to presuppose that the organic diet was likely supplemented with a blend of fish oils containing herring oil. 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 and this should be reflected in the FA fillet composition of organic vs conventional fed fishes as previously found by Trocino et al. (2013).

Fish oil provides the fish with essential fatty acids while it is also a very significant energy source. In general, the replacement of fish oil with plant oils causes no negative effects on growth, while the fatty acid composition of the fish tissues is affected, reflecting the fatty acid composition of the diet (Turchini et al. 2009). Hence, the use of organically produced plant oils to substitute fish oil in aquafeeds could be challenging and should also be examined, given especially the effects that such feed formulations may have on fish muscle fatty acid compositions, including reductions in long chain fatty acids such as EPA and DHA, and ultimately on the quality of the final product.

The development of nutritionally efficient diets using organic sources of ingredients in organic aquaculture diets is a challenge. Research is still needed to evaluate the biophysical and biochemical characteristics of new alternative sustainable proteins and lipids as replacements for fishmeal and fish oil, to determine their nutrient availability, to assess their efficiency for various life stages of organic aquaculture species, to reduce their environmental impacts and to supply them with low cost.

In the present study, the very similar formulation between conventional and organic diets and the starvation period (Delgado et al. 1994) could have likely reduced the differences observed in the FA profile of the two groups.

Anyway, the diet composition did not seem to affect neither the oxidative stress parameters (GSH, 8-OHdG, HNE) nor the CYP1A expression, although several studies demonstrated the anti-oxidative effect of lipid and vitamin-rich diets (Mourente et al. 2000; Mourente et al. 2002). It is likely that a more pronounced variation in the composition of such nutrients would need to affect antioxidant defenses. Conversely, a significant higher expression of MMCs was observed in organic vs conventional diet fed fishes. This preliminary result, to be confirmed by a greater number of data, is discordant

with Montero et al. (1999) that reported an increased number of MMCs in *Sparus aurata* juveniles fed with a diet, low in EPA and DHA but could be related to a more efficient system of detoxification in the organic fishes or to the source of proteins and lipids within the organic diet. Organic diet could have developed a more reactive system of detoxification in fishes e.g. a higher MMCs rate leading to a better ability to respond versus potential stressors. Otherwise, piscivorous fish represent an important accumulator of heavy metals and/or other pollutants since fish meal and oil used in organic aqua-feed are notoriously a source of contaminants (Berntssen et al. 2010a). As reported in salmon feeds (Jacobs et al. 2002; Hites et al. 2004), the fish oils obtained from feral pelagic fish species are considered the main source of persistent organic pollutants (POPs). In order to contain this problem, decontamination techniques have recently been developed to effectively remove persistent organic contaminants from fish oils (Berntssen 2010b; Olli et al. 2010).

Moreover, wild foods are notoriously highly nutritious but restricted in quantity and the use of fish-derived meal and oil, if on the one hand is closer to the natural life of the fish, on the other hand it does not match the principle of sustainability, causing overexploitation of fisheries and wild stocks (Crampton et al. 2010) and should be reduced. Organic aqua-feeds may consist of marine feed ingredients coming from sustainable use of fisheries and of plant feed ingredients originated from organic production (EU 2008, 2009).

Regulation on organic fish certification permits fishmeal and oil from certified sustainable fishery areas or from by-products and trimmings, from seafood-processing fishery or from certified organic aquaculture, but of a different species or from by-catches from food fisheries. The feed ration may comprise a maximum of 60% of organic plant products (EU

2009). The production of fishmeal has remained relatively stable over the past 15 years, and this situation is unlikely to improve (Tacon and Metian 2008). Indeed, it has been suggested that the availability of fishmeal will decline in the future, such that fishmeal no longer can be considered a sustainable protein source for aquafeeds (Craig and McLean 2006). Anyway, oily fish is also an important source of health promoting nutrients such as the very long chain omega-3 (VLC-n3) fatty acids since the Joint FAO and WHO expert consultation still recommends to consume fish as the potential risks are considered to be lower than the benefits (Joint FAO/WHO 2011).

In conclusion, this was the first field study aimed to evaluate the growth performances as well as the expression of IGF-I and IGF-II mRNA, the oxidative stress, the contaminant response and the fillet composition in European sea bass fed with organic diet. Our results highlighted a greater productivity in conventional fed fish comparing to the organic ones. The higher productivity was likely due to diet composition, since differences were significantly mitigated during starvation period. The fact of feeding fish with diets with a content in fatty acids closer to that of wild fish definitely affect the nutritional value of the flesh in terms of the fatty acid profile.

The consumption of the derived flesh could be considered more appropriate and healthier than the conventional one. These data are of great interest if considering the raising awareness of customers on fish welfare and food quality and value. Due to the exploitation of some fisheries, wild-caught fish could not be the answer to the organic feed requirements, considering that organic production should be by definition “a production system that sustains the health of soils, ecosystems and people” (IFOAM 2005).

Research on organic nutrition and aquafeeds needs to focus on feeds that have a better utilization of dietary nutrients to improve the efficiency of nutrient utilization or optimizing gut health, fish health, optimize growth and performance and disease resistance of the fish. The findings and limitations raised in this study could stimulate a challenging debate in the field of organic fish nutrition in order to think a new concept of organic fish nutrition and consequently to better address required future researches in the same field.

References

- Agius C (1981) Preliminary studies on the ontogeny of the melano-macrophages of teleost haemopoietic tissue and age-related changes. *Devel and 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, Roberts RJ (2003) Melano-macrophage centers and their role in fish pathology. *J Fish Dis* 23:499-509.
- Ahmad I, Hamid T, Fatima M et al. (2000) Induction of hepatic antioxidants in freshwater catfish (*Channa punctatus* Bloch) is a biomarker of paper mill effluent exposure. *Biochim Biophys Acta* 1523:37–48.
- Albrektsen S, Mundheim H, Aksnes A (2006) Growth, feed efficiency, digestibility and nutrient distribution in Atlantic cod (*Gadus morhua*) fed two different fish meal qualities at three dietary levels of vegetable protein sources. *Aquaculture* 261:626-640.
- Aldini G, Dalle-Donne I, Facino RM et al. (2007) Intervention strategies to inhibit protein carbonylation by lipoxidation-derived reactive carbonyls. *Med Res Rev* 27:817–868.
- Ashley P (2007) Fish welfare: Current issues in aquaculture. *Appl Anim Behav Sci* 104:199-235.

- Baker MA, Cerniglia GJ, Zaman A (1990) Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Anal Biochem* 190:360-365.
- Bancroft JD and Gamble M (eds) (2008) *Theory and Practice of Histological Techniques*. 6th ed. Edinburgh: Churchill Livingstone.
- Barata C, Navarro J, Varo I et al. (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 BA (2002) Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integ Comp Biol* 42:517- 525.
- Barton BA, Schreck CB, Barton L. (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.
- Benedito-Palos L, Bermejo-Nogales A, Karampatos AI 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.
- Bergleiter S, Berner U, Censkowsky U et al. (2009) *Organic aquaculture 2009. Production and markets*. Naturland e.V. and Organic Services GmbH. Report, 120 p.

- Berntssen MH, Julshamn K, Lundebye AK (2010a) Chemical contaminants in aquafeeds and Atlantic salmon (*Salmo salar*) following the use of traditional- versus alternative feed ingredients. *Chemosphere* 78:637-646.
- Berntssen MH, Olsvik PA, Torstensen BE et al. (2010b) Reducing persistent organic pollutants while maintaining long chain omega-3 fatty acid in farmed Atlantic salmon using decontaminated fish oils for an entire production cycle. *Chemosphere* 81:242-252.
- Bertotto D, Poltronieri C, Negrato E et al. (2011) Whole body cortisol and expression of HSP70, IGF-I and MSTN in early development of sea bass subjected to heat shock. *Gen Comp Endocrinol* 174:44-50.
- Biao X (2008) The development of and prospects for organic aquaculture worldwide. *Outlook Agric* 37:255–260.
- Broom DM, Johnson KG (1993) *Stress and Animal Welfare*. Chapman and Hall, London.
- Browdy C, Seaborn G, Atwood H et al. (2006) Comparison of Pond Production Efficiency, Fatty Acid Profiles, and Contaminants in *Litopenaeus vannamei* Fed Organic Plant-based and Fish-meal-based Diets. *J World Aquac Soc* 37:437–451.
- Bucke D, Vethaak AD, 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.

- Caelers A, Berishvili G, Meli ML et al. (2004) Establishment of a real-time RT-PCR for the determination of absolute amounts of IGF-I and IGF-II gene expression in liver and extrahepatic sites of the tilapia. *Gen Comp Endocrinol* 137:196- 204.
- Carnevali O, Cardinali M, Maradonna F et al. (2005) Hormonal regulation of hepatic IGF-I and IGF-II gene expression in the marine teleost *Sparus aurata*. *Mol Reprod Develop* 71:12-18.
- Celander MC, (2011) Cocktail effects on biomarker responses in fish. *Aquatic Toxicol* 105:72–77.
- Chatzifotis S, Polemitou I, Divanach P et al. (2008) Effect of dietary taurine supplementation on growth performance and bile salt activated lipase activity of common dentex, *Dentex dentex*, fed a fish meal/soy protein concentrate-based diet. *Aquaculture* 275:201–208.
- Chou RL, Her BY, Su MS et al. (2004) Substituting fishmeal with soybean meal in diets of juvenile cobia, *Rachycentron canadum*. *Aquaculture* 229:325– 333.
- Conte FS (2004) Stress and the welfare of cultured fish. *Appl Anim Behav Sci* 86:205-223.
- Costa C, Menesatti P, Rambaldi E et al. (2013) Preliminary evidences of colour differences in European sea bass reared under organic protocols. *Aquacult Engin* 57:82– 88.

Cottee SY and Petersan P (2009) Animal welfare and organic aquaculture in open systems. *J Agric Environ Ethics* 22:437–461.

Craig SR and McLean E (2005) The organic aquaculture movement a role for NuPro as an alternative protein source. In: *Nutritional Biotechnology in the Food and Feed Industry* (Jacques, K. and Lyons, P. eds.), pp. 285–293. Nottingham University Press, UK.

Craig SR and McLean E (2006) Nutrigenomics in aquaculture research: a key in the 'Aquanomic' revolution. In: *Nutritional Biotechnology in the Food and Feed Industry* (Jacques, K. and Lyons, P. eds.), pp. 433–444. Nottingham University Press, UK.

Crampton VO, Nanton DA, Ruohonen K et al. (2010) Demonstration of salmon farming as a net producer of fish protein and oil. *Aquacult Nutr* 16:437–446.

Dabrowska H, Dabrowski K, Meyerburgdorff K et al. (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.

Delgado A, Estevez A, Hortelano P et al. (1994) Analyses of fatty acids from different lipids in liver and muscle of sea bass (*Dicentrarchus labrax* L.). Influence of temperature and fasting. *Comp Biochem Physiol* 108:673-680.

Di Giulio RT and Meyer JN (2008) Reactive Oxygen Species and Oxidative Stress. In: The Toxicology of Fishes CRC Press pp. 273–32

Duguay SJ, Lai-Zhang J, Steiner DF et al. (1996) Developmental and tissue-regulated expression of IGF-I and IGF-II mRNAs in *Sparus aurata*. J Mol Endocrinol 16:123-132.

EFSA (2008) Scientific Opinion of the Panel on Animal Health and Welfare on a request from the European Commission on animal welfare aspects of husbandry systems for farmed European seabass and Gilthead seabream. EFSA J 844:1-21

EFSA, European Food Safety Authority, <http://www.efsa.europa.eu>.

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

Ellsaesser CF and Clem LW (1986) Hematological and immunological changes in channel catfish stressed by handling and transport. J Fish Biol 28:511-521.

Enami HR (2011) A review of using canola/rapeseed meal in aquaculture feeding. J Fish Aquat Sci 6: 22–36.

FAO (2005) Guidelines for the ecolabelling of fish and fishery products from marine capture fisheries. Food and Agriculture Organization of the United Nations, Rome.

- Ferguson HW (1976) The relationship between ellipsoids and melano- macrophage centres in the spleen of turbot (*Scophthalmus maximus*). J Comp Path 86:377–380.
- Fernandes T, Miller K, Read P (2000) Monitoring and regulation of marine aquaculture in Europe. J Appl Ichthyol, 16:138–143.
- Finkel T and Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408:239–247.
- Francis G, Makkar HPS and Becker K (2001) Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199:197–227.
- Franzini M, Fornaciari I, Fierabracci V (2009) Indici di laboratorio di stress ossidativo (Rassegna). Ligand Assay 14:41-50.
- Fulton TW (1902) Rate of growth of sea fishes. Sci Invest Fish Div Scotl Reprod 20:1-22.
- Funes V, Asensio E, Ponce M et al. (2006) Insulin-like growth factor I and II in the sole *Solea senegalensis*: cDNA cloning and quantitation of gene expression in tissues and during larval development. Gen Comp Endocrinol 149:166-172.
- Funkenstein B, Almuly R, Chan SJ (1997) Localization of IGF-I and IGF-I receptor mRNA in *Sparus aurata* larvae. Gen Comp Endocrinol 107:291–303.

- Gornati R, Papis E, Rimoldi S et al. (2004) Rearing density influences the expression of stress-related genes in sea bass (*Dicentrarchus labrax L.*). *Gene* 341:111-118.
- 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.
- Guengerich FP, Shimada T, Umbenhauer DR et al. (1986) Structure and function of cytochrome P-450. *Adv Exp Med Biol* 197:83-94.
- Guillaume J, Kaushik S, Bergot P et al. (1999) Nutrition and Feeding of Fish and Crustaceans, INRA- IFREMER and Springer-Praxis Publishing, UK.
- Halliwell B, Gutteridge JMC (1999) Free radicals in biology and medicine, 3rd ed. Oxford University Press Oxford, New York, 888 pp.
- Hardy RW (2010) Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Review article. *Aquacult Res* 41:770–776.
- Henderson RF, Bechtold WE, Bond JA et al. (1989) The use of biological markers in toxicology. *Toxicology* 20:65-82.
- Henderson RF, Bechtold WE, Bondghi JA et al. (1989) The Use of Biological Markers in Toxicology. *Critical Rev Toxicol* 20:65-82.

Hermansen JE (2003) Organic livestock production systems and appropriate development in relation to public expectations. *Livest Product Sci* 80:3–15.

Hites RA, Foran JA, Carpenter DO et al. (2004) Global assessment of organic contaminants in farmed salmon. *Science* 303:226–229.

Huntingford FA, Adams C, Braithwaite VA, et al.(2006). Current issues in fish welfare. *J Fish Biol* 68:332-372.

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.

Iwama GK (2007) The welfare of fish. *Dis Aquat Org* 75:155-158.

Jacobs MN, Covaci A, Schepens P (2002) Investigation of selected persistent organic pollutants in farmed Atlantic salmon (*Salmo salar*), salmon aquaculture feed, and fish oil components of the feed. *Environ Sci Technol* 36:2797– 2805.

Jauncey K (1998) *Tilapia Feeds and Feeding*. Pisces Press Ltd., Bridge of Allan, Scotland

- Jebali J, Banni M, DE Almeida EA et al. (2007) Oxidative DNA damage levels and catalase activity in the clam *Ruditapes decussatus* as pollution biomarkers of Tunisian marine environment. *Environm Monitor Assessment* 124:195-200.
- Joint FAO/WHO Expert Consultation on the risks and benefits of fish consumption (2011) FAO Fisheries and Aquaculture Report 978, pp 50.
- Jones JI, Clemmons DR (1995) Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev* 16:3–34.
- Kranz H (1989) Changes in splenic melano-macrophage centres of dab, *Limanda limanda* during and after infection with ulcer disease. *Dis Aquat Organ* 6:167–173.
- Li MH, Robinson EH, Mischke CC et al. (2006) Effects of Organic Fertilization and Organic Diets on Production of Channel Catfish in Earthen Ponds. *N Am J Aquac* 68:53–62.
- Lunger AN, McLean E and Craig SR (2007) The effects of organic protein supplementation upon growth feed conversion and texture quality parameters in juvenile cobia (*Rachycentron canad- um*). *Aquaculture* 264:342–352.
- Magnadóttir B (2006) Innate immunity of fish (overview). *Fish Shellfish Immunol* 20:137-151.

- McDonald G and Milligan L (1997) Ionic, osmotic and acid-base regulation in stress. In: Iwama GK, Pickering AD, Sumpter JP, Schreck CB (editors). Fish Stress and Health in Aquaculture. Cambridge, UK: Cambridge University Press.
- Meinelt TR, Kruger M, Pietrock MM et al. (1997) Mercury pollution and macrophage centres in pike (*Esox lucius*) tissues. Environ Sci Poll Res 4:32–36.
- Mente E, Karalazos V, Karapanagiotidis IT et al. (2011) Nutrition in organic aquaculture: an inquiry and a discourse. Aquac Nut 17:798–817.
- Merchie G, Lavens P, Sorgeloos P (1997) Optimization of dietary vitamin C in fish and crustacean larvae: a review. Aquaculture 155:165-181.
- Meseguer J, Esteban MA, 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.
- Mommsen TP, Vijayan MM, Moon TW (1999) Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev Fish Biol Fish 9:211- 268.
- Montero D, Izquierdo MS, Tort L et al. (1999) High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. Fish Physiol Biochem 20:53–56.

- Montero D, Tort L, Robaina L et al. (2001) Low vitamin E in diet reduces stress resistance of gilthead seabream (*Sparus aurata*) juveniles. *Fish Shellfish Immunol* 11:473–490.
- Mourente G, Diaz-Salvago E, Bell JG et al. (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 DR et al. (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.
- Nebert DW, Gonzalez FJ (1987) P450 genes: structure, evolution and regulation. *Ann Rev Biochem* 56:945-93.
- Nelson DR, Kamataki T, Waxman DJ et al. (1993) The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol* 12:1-51.
- Olli JJ, Breivik H, Mørkøre T, Ruyter B et al. (2010) Removal of persistent organic pollutants from Atlantic salmon (*Salmo salar* L.) diets: Influence on growth, feed utilization efficiency and product quality. *Aquaculture* 310:145-155.

- Pankhurst NW 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.
- Pascoli F, Lanzano GS, Negrato E, Poltronieri C et al. (2011) Seasonal effects on hematological and innate immune parameters in sea bass *Dicentrarchus labrax*. *Fish Shell Immunol* 31:1081-1087.
- Pascoli F, Negrato E, Di Giancamillo A et al. (2011) Evaluation of oxidative stress biomarkers in *Zosterisessor ophiocephalus* from the Venice Lagoon, Italy. *Aquat Toxicol* 101:512-520.
- Passantino L, Santamaria N, Zupa R et al. (2014) Liver melanomacrophage centres as indicators of Atlantic bluefin tuna, *Thunnus thynnus* L. well-being. *J Fish Dis* 37:241-250.
- 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.
- Patrino M, Maccatrozzo L, Funkenstein B et al. (2006) Cloning and expression of insulin-like growth factors I and II in the shi drum (*Umbrina cirrosa*). *Comp Biochem Physiol Part B* 137:137-151.

- Patruno M, Sivieri S, Poltronieri C et al. (2008) Real-time polymerase chain reaction, in situ hybridization and immunohistochemical localization of insulin-like growth factor-I and myostatin during development of *Dicentrarchus labrax* (Pisces: Osteichthyes). *Cell Tissue Res* 331:643-658.
- Pearse AGE (1990). *Trattato di Istochimica. Aggiornamenti ai volumi I - II*, 3rd ed. Piccin, Padova.
- Perdikaris C and Paschos I (2010) Organic aquaculture in Greece: a brief review. *Rev Aquac* 2:102–105.
- Pereira TG and Oliva-Teles A (2003) Evaluation of corn gluten meal as a protein source in diets for gilthead sea bream (*Sparus aurata* L.) juveniles. *Aquac Res* 34:1111–1117.
- Peters G and Schwarzen R (1985) Changes in haemopoietic tissue of rainbow trout under influence of stress. *Dis Aquat Org* 1:1–10.
- Pickering AD (1993) Growth and stress in fish production. *Aquaculture* 111:51-63.
- Ploch SA, Lee Y, MacLean E et al. (1999) Oxidative stress in liver of brown bullhead and channel catfish following exposure to tert-butyl hydroperoxide. *Aquatic Toxicol* 46:231–240.
- Poli BM (2009) Farmed fish welfare-suffering assessment and impact on product quality. *Ital J Anim Scie* 8:139-160.

Poli BM, Scappini F, Parisi G et al.(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 et al. (2009) Effects of exposure to overcrowding on rodlet cells of the teleost fish *Dicentrarchus labrax* (L.). Vet Res Comm 33:619-629.

Radaelli G, Domeneghini C, Arrighi S et al. (2003) Localization of IGF-I, IGF-I receptor and IGFBP-2 in developing *Umbrina cirrosa* (Pisces: Osteichthyes). Gen Comp Endocrinol 130:232-244.

Radaelli G, Poltronieri C, Bertotto D et al. (2008) Cellular localization of insulin-like growth factor-II protein in the sea bass (*Dicentrarchus labrax*) from hatching to adult. Histol Histopathol 23:523-530.

Rana KJ, Siriwardena S, Hasan MR (2009) Impact of rising feed ingredient prices on aquafeeds and aquaculture production. FAO Fisheries and Aquaculture Technical Food and Agriculture Organization of the United Nations, Rome.

Ribecco C, Hardiman G, Sasik R et al. (2012) Teleost fish (*Solea solea*): a novel model for ecotoxicological assay of contaminated sediments. Aquatic Toxicol 109:133-142.

- 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. pp. 399-428.
- Sarasquete C, Segner H (2000) Cytochrome P4501A (CYP1A) in teleostean fishes. A review of immunohistochemical studies. *Sci Total Environ* 247:313-32.
- Sargent JR, Bell JG, Bell MV et al. (1993) The metabolism of phospholipids and polyunsaturated fatty acids in fish, in: Lahlou, B., Vitiello, P. (Eds.), *Aquaculture: Fundamental and Applied Research. Coastal and Estuarine Studies 43*. American Geophysical Union, Washington, DC, 103–124.
- Sargent JR, Bell JG, Bell MV et al. (1995) Requirement criteria for essential fatty acids. *Symposium of European Inland Fisheries Advisory Commission. J Appl Ichthyol* 11:183–198
- Sargent JR, McEvoy LA, Bell JG (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture* 155:117–127.
- Satoh S, Hernández A, Tokoro T et al. (2003) Comparison of phosphorus retention efficiency between rainbow trout (*Oncorhynchus mykiss*) fed a commercial diet and a low fish meal based diet. *Aquaculture* 224:271–282.
- Schreck C (2001) Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture* 197:3-24.

Shamblott MJ, Chen TT (1993) Age related and tissue-specific levels of five forms of insulin-like growth factor mRNA in a teleost. *Mol Mar Biol Biotechnol* 2:351–361.

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.

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

Tidwell JH, Allan GL (2001) Fish as food: aquaculture's contribution. Ecological and economic impacts and contributions of fish farming and capture fisheries. *EMBO Rep* 2:958-63.

Traven L, Mićović V, Lušić D, Smital T (2013) The responses of the hepatosomatic index (HSI), 7-ethoxyresorufin-O-deethylase (EROD) activity and glutathione-S-transferase (GST) activity in sea bass (*Dicentrarchus labrax*, Linnaeus 1758) caged at a polluted site: implications for their use in environmental risk assessment. *Environ Monitor Assessment* 85:9009–9018.

Traven L, Mićović V, Vukić Lušić D et al. (2013) The responses of the hepatosomatic index (HSI), 7-ethoxyresorufin-O-deethylase (EROD) activity and glutathione-S-transferase (GST) activity in sea bass (*Dicentrarchus labrax*, Linnaeus 1758) caged at a polluted

site: implications for their use in environmental risk assessment. *Environ Monit Assess* 185:9009-18.

Trocino A, Xiccato G, Majolini D et al. (2012) Assessing the quality of organic and conventionally-farmed European sea bass (*Dicentrarchus labrax*). *Food Chem* 131:427-433.

Turchini GM, Torstensen BE and Ng WK (2009) Fish oil replacement in finfish nutrition. *Rev Aquac* 1:10–57.

Turchini GM, Torstensen BE, Ng WK (2009) Fish oil replacement in finfish nutrition. *Rev Aquaculture* 1:10–57.

Turnbull JF, Bell A, Adams C, et al. (2005) Stocking density and welfare of cage farmed Atlantic salmon: application of a multivariate analysis. *Aquaculture* 243:121-132.

Valavanidis A, Vlahogiannia T, Dassenakis M et al. (2006) Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotox Environm Safety* 64:178–189.

Van der Oost R, Beyer J, Vermeulen N P E (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: *Environ Toxicol Pharmacol* 13:57–149.

- Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ Toxicol Pharmacol* 13:57-149.
- Vazzana M, Cammarata M, Cooper EL et al. (2002) Confinement stress in sea bass (*Dicentrarchus labrax*) depresses peritoneal leukocyte cytotoxicity. *Aquaculture* 210:231-243.
- Vong QP, Chan KM, Cheng CH (2003) Quantification of common carp (*Cyprinus carpio*) IGF-I and IGF-II mRNA by real-time PCR: differential regulation of expression by GH. *J Endocrinol* 178:513-521.
- Wang XJ, Kim K W, Bai SC et al. (2003) Effects of the different levels of dietary vitamin C on growth and tissue ascorbic acid changes in parrot fish (*Oplegnathus fasciatus*). *Aquaculture* 215:203–211.
- Ward T and Phillips B (2008) *Seafood Ecolabelling*. Wiley-Blackwell Publishing, Oxford, UK.
- Winston GW, Di Giulio RT (1991) Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquat Toxicol* 19:137– 161.
- Wolke RE (1992) Piscine macrophage aggregates: a review. *Ann Rev Fish Dis* 2:91–108.
- Wood AW, Duan C, Bern HA (2005) Insulin-like growth factor signaling in fish. *Intern Rev Cytol* 243:215-285.

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

Zeldin DC, Seubert JM (2008) Structure, mechanism and regulation of cytochromes P450
R.C. Smart, E. Hodgson (Eds.) In: Molecular and Biochemical Toxicology, John Wiley
and Sons, Hoboken, NJ, USA, pp. 147–172.