Accepted Manuscript

A multidisciplinary study of the extracutaneous pigment system of European sea bass (*Dicentrarchus labrax* L.). A possible relationship between kidney disease and dopa oxidase activity level

Marcella Arciuli, Adalberto Brunetti, Daniela Fiocco, Valentina Zacchino, Gerardo Centoducati, Antonio Aloi, Raffaele Tommasi, Arcangela Santeramo, Emanuele De Nitto, Anna Gallone, Professor

PII: S1050-4648(14)00405-7

DOI: 10.1016/j.fsi.2014.10.031

Reference: YFSIM 3187

To appear in: *Fish and Shellfish Immunology*

Received Date: 2 September 2014

Revised Date: 27 October 2014

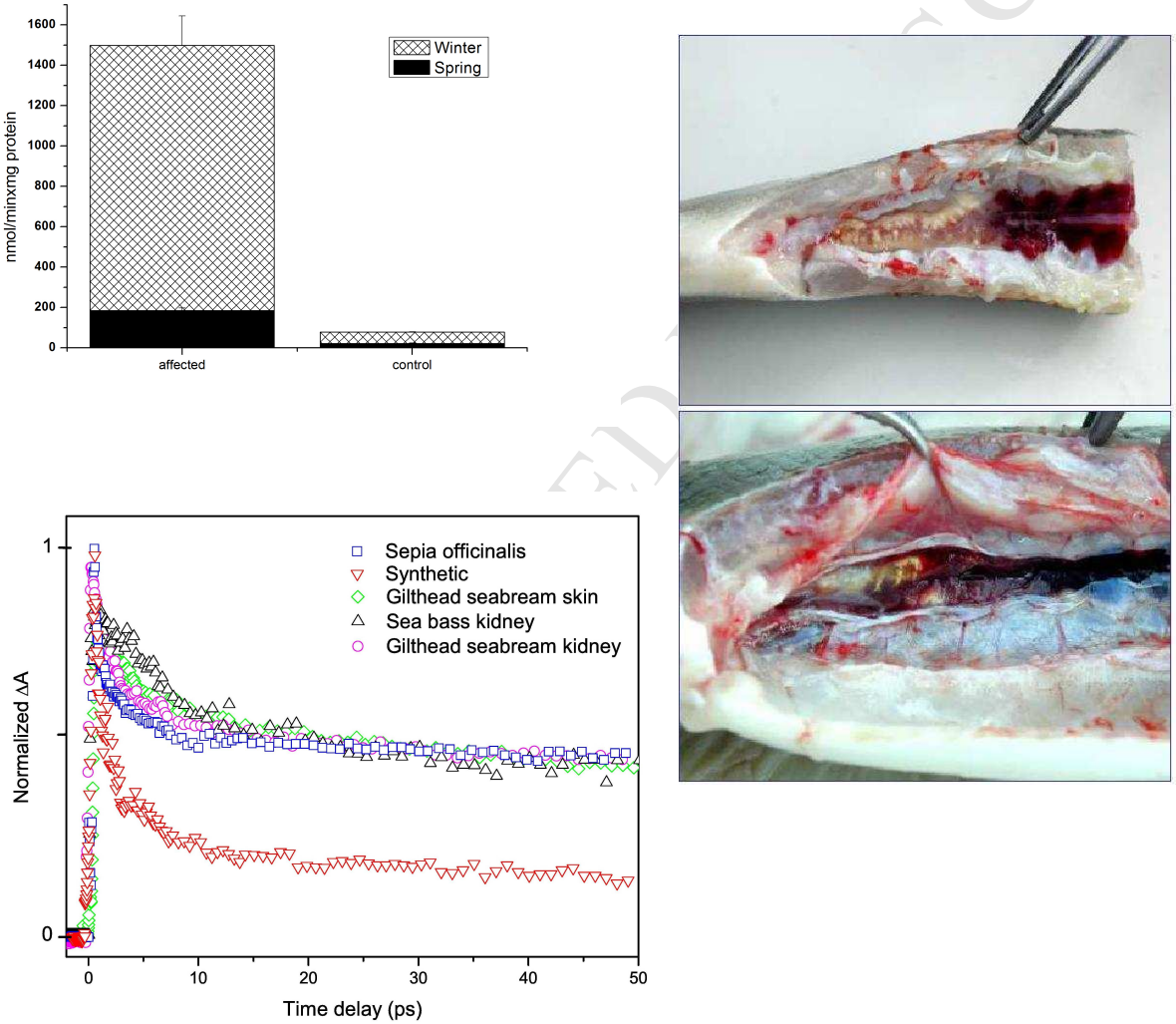
Accepted Date: 28 October 2014

Please cite this article as: Arciuli M, Brunetti A, Fiocco D, Zacchino V, Centoducati G, Aloi A, Tommasi R, Santeramo A, De Nitto E, Gallone A, A multidisciplinary study of the extracutaneous pigment system of European sea bass (*Dicentrarchus labrax* L.). A possible relationship between kidney disease and dopa oxidase activity level, *Fish and Shellfish Immunology* (2014), doi: 10.1016/j.fsi.2014.10.031.

A multidisciplinary study of the extracutaneous pigment system of European sea bass (*Dicentrarchus labrax* L.). A possible relationship between kidney disease and dopa oxidase activity level.

Marcella Arciuli[a], Adalberto Brunetti[a], Daniela Fiocco[b], Valentina Zacchino[c], Gerardo Centoducati[c], Antonio Aloi[a], Raffaele Tommasi[a], Arcangela Santeramo[a], Emanuele De Nitto[a] and Anna Gallone[a]

Graphical Abstract



It was found a relationship between kidney disease of farmed European sea bass (*Dicentrarchus labrax* L.) and dopa oxidase activity level, demonstrating the DO activity as a possible indicator for fish kidney nephrocalcinosis whose diagnosis can be, simply, highlighted by a gross examination of the farmed fishes during the autopsy. Moreover variations of dopa oxidase activity in extracutaneous pigment system have been observed with respect to environmental temperature.

The comparative Femto-TA analysis of pigments isolated from sea bass kidney tissue, fish skin melanin, natural melanin from *S. officinalis* and synthetic melanin, by pointing out that fish melano-macrophages centres do contain melanins.

A multidisciplinary study of the extracutaneous pigment system of European sea bass (*Dicentrarchus labrax* L.). A possible relationship between kidney disease and dopa oxidase activity level.

Marcella Arciuli[a], Adalberto Brunetti[a], Daniela Fiocco[b], Valentina Zacchino[c], Gerardo Centoducati[c], Antonio Aloi[a], Raffaele Tommasi[a], Arcangela Santeramo[a], Emanuele De Nitto[a] and Anna Gallone[a]

[a] Department of Basic Medical Sciences, Neuroscience and Sense Organs, University of Bari Aldo Moro, Bari, 70124, Italy.

[b] Department of Medical and Experimental Medicine, University of Foggia, 71122, Foggia, Italy.

[c] Department of Soil, Plant and Food Science, University of Bari Aldo Moro, Bari, 70126,Italy.

Please address all correspondence to the corresponding author:

Professor Anna Gallone,

Department of Basic Medical Sciences, Neuroscience and Sense Organs,

University of Bari Aldo Moro,

Policlinico-Piazza G. Cesare

70124 Bari, ITALY

Tel: +39/080/5448555

e-mail: anna.gallone@uniba.it; marcella.arciuli@uniba.it

ABSTRACT

Infectious diseases and breeding conditions can influence fish health status. Furthermore it is well known that human and animal health are strongly correlated. In lower vertebrates melano-macrophage centres, clusters of pigment-containing cells forming the extracutaneous pigment system, are widespread in the stroma of the haemopoietic tissue, mainly in kidney and spleen. In fishes, melano-macrophage centres play an important role in the immune response against antigenic stimulants and pathogens. Hence, they are employed as biomarker of fish health status. We have investigated this cell system in the European sea bass (*Dicentrarchus labrax* L.) following the enzyme activities involved in melanin biosynthesis. We have found a possible relationship between kidney disease of farmed fishes and dopa oxidase activity level, suggesting it as an indicator of kidney disease. Moreover variations of dopa oxidase activity in extracutaneous pigment system have been observed with respect to environmental temperature. At last, for the first time, using femtosecond transient absorption spectroscopy (Femto-TA), we pointed out that pigment-containing cells of fish kidney tissue present melanin pigments.

Key words: melano-macrophages; dopa oxidase; peroxidase; extracutaneous melanogenesis; melanins.

1. INTRODUCTION

Lower vertebrates present a visceral pigment cell system that is not derived from the neural tube and is localized in the liver, spleen, kidney and other body districts [56]. In the liver and spleen of reptiles [55,52], amphibians [14,57] and fishes [67,3,46] pigment-containing cells occur in melano-macrophage centres. In teleosts, melanin-containing cells are copious and dispersed in the reticulo-endothelial system, which forms a supporting matrix of haemopoietic tissues, particularly in the kidney and spleen [10,33,58]. It was demonstrated that these cells express not only the tyrosinase gene [22,23] and tyrosinase-related proteins (TRP1 and TRP2) genes [58], but also exhibit tyrosinase activity [19,20,5, 25]. Tyrosinase (EC 1.14.18.1) is the main enzyme involved in melanin biosynthesis. This copper-containing enzyme catalyzes the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa) (monophenolase or cresolase activity, EC 1.14.18.1) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenolase or catechol oxidase activity, EC 1.10.3.1) [48,35,21]. DHICA oxidase (TRP1) and dopachrome tautomerase (TRP2) are involved in the melanogenic pathway [43,27]. Moreover, data in literature report peroxidase involvement in very different melanin-synthesizing systems, unlike the melanocytes, such as in neuromelanin synthesis system or in ink gland of *Sepia officinalis* [44,42]. A peroxidase–H_2O_2 system has been demonstrated to be involved during the terminal stages of melanogenesis, acting solely or collaboratively with tyrosinase in the oxidative polymerizations of pigment precursors [29]. A peroxidase activity had been evidenced in frog spleen and liver melanosome proteins [19].

In vertebrates, the pigment content may increase in tissues and organs in different physiological states as a response to pathological and inflammatory conditions. In particular in fishes, the presence of melanin pigment has been associated to protective functions against pathological and stress conditions [3]. In Atlantic salmons affected by

granulomatous myopathy, an inflammatory condition, abnormal pigmented changes in muscle and in other visceral organs were observed, resulting from aggregates of melano-macrophages [30,31]. Pigment accumulations are caused by nonspecific immune mechanisms [30,40,5]. In the immune response of fish against antigenic stimulants and pathogens, melano-macrophage centres play an important role [3,30,31].

Moreover, in the liver of *Rana esculenta* L. and other amphibians, melanin biosynthesis and tyrosinase activity vary greatly according to the season, with maximum levels in winter and minimum levels in warmer seasons [14]. The quantitative variations in melanins are correlated with metabolic changes in the liver in different seasonal hibernation-activity phases. During natural hibernation, melanin may play a vicarious role respect to decreased hepatocyte defence functions [6].

The European sea bass (*Dicentrarchus labrax* L.) is the most important commercial fish widely farmed in the Mediterranean Sea. Sea bass habitats include estuaries, lagoons, coastal waters and rivers. Due to its importance for aquaculture, European sea bass rearing is rising along with the main pathologies affecting this species. Many studies have reported that the occurrence of a fish disease depends on the balance between host, pathogen and the environment, three components that continuously interact at several levels. Additionally, intensive farming systems have caused the development of husbandry diseases [53]. In fact, oxygen availability, extreme temperature conditions and animal density can affect farmed fish and make them more susceptible to diseases [47].

Nephrocalcinosis seems to be correlated with water parameter alterations and nutritional factors and is characterized by mineral deposits in renal tubules, mainly composed by insoluble precipitates of calcium and magnesium. Nephrocalcinosis diagnosis can be simply highlighted by a gross examination of the farmed fishes during the autopsy, as previously demonstrated by Bruno [11]. This is a degenerative chronic process that does not elicit high level of mortality in fish and sometimes it can go unobserved [39,16]. As

described in literature [7,11], grossly the affected kidneys appear swollen, white-grey in colour with an irregular surface. As the condition develops, a characteristic whitish chalky deposit is observed, ascribable to the insoluble precipitate deposits, mostly made of calcium and magnesium. Nephrocalcinosis may also be associated with the presence of large urinary cysts which contribute to a swelling of the abdominal cavity.

Because human and animal health are strongly correlated, it may be helpful to identify biomarkers for verifying the health status of farmed fish. Strong evidences suggest melano-macrophages centres of liver, kidney and spleen as bio-indicators of fish health status [3,46]. Several authors have suggested the involvement of melano-macrophage centres of kidney, liver and spleen as a possible monitor of the health of fish populations [3]. As well as changes brought about in melano-macrophage centres are caused by many factors such as stress and pathological conditions [2]. Here we investigated for a possible relationship between fish disease and levels of dopa oxidase (DO) activities detected in the melanosomal protein fractions of these organs. To this aim, we studied wild sea bass control samples collected in the sea of Taranto (Apulia, Italy), and farmed samples of the same species showing symptoms of nephrocalcinosis.

In melano-macrophages, melanins can absorb and neutralize free radicals and other potentially toxic agents, carrying out a cytoprotective functions [5,46]. Besides Wolke et al. [62] suggested that in macrophages of heterotherms, melanin might possess bactericidal properties. These important melanin functions deserve a detailed study. The spectroscopic investigation of the biopolymer results very complex for the high percentage of proteins tied to natural melanin structure and for the difficulty to separate them [12]. In order to get an insight into this issue, we characterized isolated pigments from fish kidney tissue by femtosecond transient absorption (Femto-TA) spectroscopy and compared them, for the first time, with fish skin melanin, natural melanin from *Sepia officinalis* and synthetic melanin.

2. MATERIALS AND METHODS

Chemicals were purchased from Sigma–Aldrich Co. (St. Louis, Mo.) including 3-methyl-2-benzothiazolinonehydrazone hydrochloride (MBTH), catalase, L-3-(3,4-dihydroxyphenyl) alanine (L-dopa), PMSF (phenylmethylsulfonyl fluoride), kojic acid, melanin from *Sepia officinalis* and synthetic melanin. All other chemicals were of analytical grade.

2.1. Source and preparation of samples

Each experiment was performed using about sixty sexually mature samples. Farmed European sea bass (*Dicentrarchus labrax*) were randomly collected from the aquaculture industry “Panittica Pugliese Azienda Agricola S.p.A.”. Samples used as control were randomly collected in the sea of Taranto (Apulia, Italy) because all farmed samples were affected by nephrocalcinosis. When the farmed fish had reached the required commercial size (351-600 g), appropriate slaughter procedures were applied with the respect of fish welfare and EU legislation (EFSA 2009). Particularly, fishes were sacrificed by immersion in ice to simultaneously chill and kill them and then by an incision on the abdominal wall. Kidney, liver, spleen and skin were removed and pooled in 0.05 M sodium phosphate buffer, pH 6.8, supplemented with 0.1 mM PMSF. The melanosomal protein fractions were prepared according to Cicero et al. [14]. All processes were carried out at 4℃. Briefly, tissues were minced and homogenized with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 700 x *g* for 5 min. The pellet was discarded and the supernatant was collected and centrifuged at 22,000 x *g* for 15 min. The supernatant was discarded, and the pellet, containing the melanosomes, was washed twice with the sodium phosphate buffer, layered twice in 30% sucrose and centrifuged at 22,000 x *g* for 15 min.

The pellet was incubated overnight under mild stirring, in 0.1 M sodium phosphate buffer, pH 7.2, with 1% Brij 35. After a further centrifugation at 22,000 x *g* for 60 min, the supernatant containing the crude extract of protein (melanosomal protein fraction) and the pellet (melanin/pigment pellet) were recovered. The melanosomal protein fraction of kidney, liver and spleen was used to assay enzyme activities. The protein content of samples was determined by the Bio-Rad protein assay kit, in accordance with Bradford [9]. The melanin/pigment pellet of kidney and skin was used for optical measurements.

2.2. Nephrocalcinosis detection

As described in literature [7,11] nephrocalcinosis diagnosis was based on gross anatomopathological examination during the autopsy of both control fishes, collected in the sea of Taranto, and farmed fishes. In particular, to verify whether control and farmed fishes were affected by the disease, kidneys were isolated and examined for the presence of typical pathological lesions such as white-grey mineral deposits and irregularities on the surface of the tissue according to Barni et al. [7] and Bruno [11].

2.3. Enzyme activities measurements

Tyrosine hydroylase (TH) and dopa oxidase (DO) activities. TH and DO activities of the melanosomal protein fractions isolated from kidney, liver and spleen tissues were determined using the Winder and Harris spectrophotometric method according respectively to Maida et al. [35] and Gallone et al. [19]. The TH activity was determined using 5–15 µg of proteins, in the presence of 4 mM MBTH, 1 mM L-tyrosine and 10 µM catalytic L-dopa in a 0.1 M sodium phosphate buffer, pH 6.8, at 25℃. DO activity was

determined using 5–15 µg of proteins, in the presence of 4 mM MBTH and 1.8 mM L-dopa in a 0.1 M sodium phosphate buffer, pH 6.8 at 25℃. The measurements were made at 505 nm (ε_{505}=8100 $M^{-1} \cdot cm^{-1}$).

Peroxidase (POD) activity. POD activity was assayed on melanosome proteins from kidney, spleen and liver tissues according to Gallone et al. [19]. The enzymatic activity was spectrophotometrically detected using

a) *Guaiacol*. The reaction mixture contained 0.1 M Na-phosphate buffer pH 6.8, 2 mM Guaiacol, 40 µg of melanosome proteins. The reaction was started by adding 0.01% H_2O_2. The absorbance increase was measured at 470 nm.
b) *p-phenylenediamine-pyrocatechol* (PPD-PC). The reaction mixture contained 40 µg of melanosome proteins in a 0.1 M K-phosphate buffer pH 6.5, plus 0.17 mM PPD and 4.5 mM PC. The reaction was started by adding 250 µM H_2O_2. The absorbance increase was measured at 575 nm.

Inhibition experiments. KCN and Kojic acid inhibitors were used at a final concentration of 4mM.

Catalase treatment. Melanosome proteins were incubated with 2000 E.U. catalase for 60 min at 4℃ before the assay.

2.4. Dopa oxidase activity on SDS-PAGE

DO activity of the melanosomal protein fraction isolated from kidney tissue was revealed on non-reducing SDS-PAGE using the method described by Jimenez-Cervantes et al. [28] with minor modifications. 60 µg of protein were subjected, after electrophoretic separation, to the dopa reaction. The reaction mixture contained 2.5 mM L-dopa supplemented with 4 mM MBTH in a 10 mM sodium phosphate buffer, pH 6.8. The reaction was carried out at room temperature for 15-30 min.

2.6. Sample preparation and femtosecond transient absorption (Femto-TA) spectroscopy .

Pigments isolated from kidney tissue, melanin isolated from skin tissue, natural *Sepia officinalis* melanin and synthetic melanin were dispersed in a 0.1 M aqueous suspension of NaOH at 100℃ for 1h according to Meredith and Sarna [38]. These samples were used for time-resolved optical measurements.

Femtosecond pump and probe experiments were carried out in noncollinear configuration at room temperature. A commercial diode-pumped Ti:Sapphire femtosecond oscillator (Mai Tai, Spectra Physics), produced 100 fs pulses at a 78 MHz repetition rate. These pulses were stretched and then amplified by a regenerative Ti:Sapphire amplifier (Spitfire Pro, Spectra Physics), pumped by a Q-switched Nd^{3+}:YLF laser (Empower, Spectra Physics) at a 1 kHz repetition rate, and finally compressed to produce 90 fs pulses of up to 3.4 mJ at 799 nm. The pulse duration was measured by an intensity autocorrelator (Pulse Scout, Spectra Physics). The amplified laser beam was divided by a beam splitter into two parts (90% transmitted and 10% reflected). The transmitted pulses were sent to an OPA (optical parametric amplifier, TOPAS, Spectra Physics) based on collinear phase matching in a sapphire plate. The OPA, in turn, provided 120 fs pulses tunable in a broad spectral range (290 – 2600 nm). The output of the OPA constituted the pump beam that was sent into a broadband ultrafast transient absorption spectrometer (Helios, Ultrafast Systems). The pump beam passed through a depolarizer to avoid orientational effects in the measured decay curves. Then, after crossing an optical chopper, the pump beam was focused onto the sample. The pump energy was typically 5 mJ $pulse^{-1}$, yielding excitation densities of $\approx 5 \times 10^{14}$ photon $pulse^{-1}$ cm^{-2} when the excitation beam was focused onto a spot of ≈1 mm in diameter The samples were stirred using a magnetic microstirrer to

protect them from photodegradation. The weaker beam reflected by the beam splitter after the output of the Ti:Sapphire regenerative amplifier entered the transient absorption spectrometer where it was time delayed thanks to a variable optical delay line. Then it was focused onto a CaF_2 crystal to generate a white light continuum (WLC) in a broad spectral range (450-800 nm). WLC was used as a probe and focused within the pump spot at the sample. After passing the sample, the WLC was detected by using a fiber-coupled CCD spectrometer. The time resolution of the experiment was determined by the Full Width at Half Maximum (FWHM) of the temporal cross correlation between pump and probe pulses (≈200 fs). Surface Xplorer (Ultrafast Systems) software was used to correct for dispersion in the transient absorption spectra due to chirp in the WLC.

3. RESULTS AND DISCUSSION

3.1. European sea bass analyses.

Anatomopathological analysis (general analyses).

The anatomopathological examination of farmed fishes and control fishes, collected in the sea of Taranto, was carried out. The typical kidney lesions caused by nephrocalcinosis were well grossly observed as described in literature [7,11]. They were only observed in farmed European sea bass collected in winter and spring, compared to control. As shown in fig. 1a, an increased renal volume, the presence of a lighter colour and areas or whitish streaks of chalky appearance were observed. In fig. 1b, the posterior kidney was more affected as the caudal part abounded of mineral deposits of considerable size and calcareous aspect, sign of chronic development of the disease. Our results are in agreement with Beraldo and Galeotti [7] that found similar lesions by grossly observation

in farmed sea bass after examination with a prevalence of 100% of nephrocalcinosis in the investigated sample.

Tyrosine hydroylase (TH) and dopa oxidase (DO) activities (general analyses).

To verify if the extracutaneous pigment system is related to protective functions against stress/temperature conditions and/or pathological states, we searched for tyrosinase activity in visceral organs of extracutaneous pigment systems of sea bass collected in periods in which environmental temperatures are different, such as spring and winter. Analyses were carried out on protein melanosomal extracts of kidney, liver and spleen tissues. We highlighted a DO activity in control samples and farmed affected samples. The DO reaction was inhibited by the commonly used KCN, generally demonstrating an oxidase activity. Using Kojic acid, a proper tyrosinase inhibitor [10], the enzyme reaction was fully inhibited, moreover H_2O_2 did not affect DO activity similarly to what shown by Gallone et al. [20], thus demonstrating a tyrosinase reaction. TH activity was not detected, pointing out that fish pigmented macrophages contain a tyrosinase with typical features, similarly to what indicated by Gallone et al. [19].

Peroxidase(POD) activity (general analyses).

The use of crude melanosomal extracts poses the problem of the coexistence of enzyme activities that may interfere with tyrosinase, including peroxidase [19]. Peroxidases have been shown to be able to catalyze the tyrosinase activity in various systems, such as those of the *Saepia officinalis* ink sac [44], human eosinophils and neurons, as well as murine melanoma cells [42]. We seeked for enzymatic activity of peroxidase, being a likely candidate of the phenolic compounds' metabolism as suggested by d'Ischia et al. [15].

We analysed the POD activity in visceral organs of extracutaneous pigment systems of control wild and breading sea basses collected in spring and winter. Interestingly, POD activity was evinced only by the PPD-PC substrate in accordance to what previously demonstrated in extracutaneous frog system [19,20]. Moreover, POD activity was not

evinced by guajacol (data not shown) as previously shown in frog extracutaneous system [19,20]. The effect of catalase, which is known to negatively affect POD activity, was studied, and such treatment was found to totally inhibit POD activity. As expected, POD reaction resulted inhibited by KCN. This form of peroxidase, revealed with a particular substrate (PPD-PC), was also highlighted in the melano-macrophages of *Rana esculenta* in previous studies [20].

Control healthy European sea bass extracts.

The macroscopical analysis of control samples collected in the sea of Taranto (Apulia, Italy) did not evidence any nephrocalcinosis anatomopathological lesions.

DO activity. Fig. 2 shows the level of DO activity of the melanosomal protein fractions of kidney, spleen and liver tissues of sea bass control samples collected in winter and spring. Data show a higher activity in kidney and spleen tissues and a negligible level of DO activity in liver tissue. Moreover differences between winter and spring samples were evidenced in healthy fishes. Particularly, a higher DO activity of winter melanosomal protein fractions respect to the spring protein extract was observed in kidney and spleen. No substantial differences of the DO activity of the liver melanosomal protein fractions was revealed during the year differently to what shown in *Rana esculenta*, hibernating vertebrates.

POD activity: In Fig. 3 the levels of POD activity of the melanosomal protein fractions of kidney, spleen and liver tissues of sea bass control samples collected in winter and spring are represented. The histogram shows no significant level of POD activities in liver tissue during the year and no substantial differences between the levels of POD activity in winter and spring kidney protein fractions. Slight differences between winter and spring samples were evidenced in spleen tissue, being the POD activity higher in winter season.

Farmed European sea bass extracts.

The macroscopical analysis of farmed sample evidences the typical kidney nephrocalcinosis anatomopathological lesions. Farmed fishes showed in fact white-grey mineral deposits and irregularities on the surface of the tissue, in accordance with Beraldo and Galeotti [7].

DO activity: Fig. 4 shows DO activity recorded in melanosomal protein fraction of farmed sea bass samples collected in winter and spring. A higher enzymatic activity in kidney tissue with respect to other organs was observed, similarly to what was revealed by previous studies on the Atlantic salmon [5]. In detail, in our system, DO activity was much higher in winter kidney melanosomal fraction in comparison to that observed in winter liver and spleen extracts. As regards the spring season, no activity seemed to be present in liver and spleen tissue. These results highlight some features that are in common with the visceral pigment cell system of cold-blooded vertebrates. It has been suggested that the melano-macrophage centres may represent primitive analogues of germinal centres as seen in mammalian lymph nodes [60]. In teleosts, kidney plays a dual function as a lymph node and as a haematopoietic organ [66]. Previous studies in salmon suggest that melanin synthesis may be associated to nonspecific immune mechanisms [30,5].

POD activity: A POD activity in spring and winter melanosomal protein fractions of kidney, liver and spleen tissues was detected (Fig. 5). Data showed the presence of a significant POD activity mainly in kidney and spleen tissues, while no activity was detected in the liver. A significant difference was observed in activity levels between the winter and spring kidney fractions, with a higher POD activity in winter season. Conversely, in spleen fractions, no difference between the activity levels of winter and spring spleen fractions was observed. As revealed in control samples, a much higher enzymatic activity was detected using PPD-PC as substrate with respect to guaiacol. This form of peroxidase,

revealed with a particular substrate (PPD-PC), was also highlighted in melano-macrophages of *Rana esculenta* [20].

Similarly to what shown in the extracutaneous pigment system of amphibians [14,19,20], seasonal variations were observed in our control system. The higher DO activity level in kidney from winter control samples, compared to spring samples, leads us to think that this variation might depend on the different season temperature conditions that can influence the immune response. Generally, in marine fish species, metabolic changes are directly correlated with environmental temperature, and sea bass individuals become less metabolically active at low temperatures, showing an optimum of metabolism activity at 20-25℃ [47]. Moreover in previous study it was demon strated a temperature-dependent expression of tyrosinase gene family in cultured pigment-producing leukocyte-like cell-line (SHK-1 cells) of Atlantic salmon cells [32]. Esteban et al. [18] have indicated in both *Dicentrarchus labrax* and *Sparus aurata* a clear circadian rhythm of the immune parameters, for example lysozyme activity follows an evident daily rhythm. Yıldız et al. [65] have demonstrated that seasonal changes could affect the metabolism of farmed sea bass. Interestingly Wolke et al. [62] suggested that in macrophages of heterotherms, melanin might possess bactericidal properties and thus it might represent a particular benefit to these vertebrates in which enzymatic activities may be severely restricted at low temperatures. According to this, analysis of our data shows that in the kidney of nephrocalcinosis-affected samples the DO activity is significantly higher with regard to the control samples, both in winter and in spring season. Fig. 6 shows an increased tyrosinase activity in winter season and even greater differences in the absolute values of the DO activity in affected samples with respect to melanosomal protein fraction from control individuals. Such data point out a possible relationship between kidney disease and dopa oxidase activity according to Agius & Roberts [2] who have described changes in melano-

macrophage centres caused by factors such as stress/temperature conditions and/or pathological states.

Fig.7 shows the POD activity of kidney extracts. There are no differences in the activity level between winter and spring control systems. Moreover in farmed affected fish kidney extracts, winter samples exhibit higher POD activity respect to spring extract, thus pointing out a correlation of POD activity with temperature variations rather than with kidney disease being both samples affected.

These results together with the observation that tyrosinase activity is not affected by either catalase nor H_2O_2, suggest that: 1) the POD activity and the DO activity are supported by different enzymes; 2) the DO activity is catalysed by a tyrosinase-like enzyme present in the pigment containing cell. Schraermeyer and Stieve [54] showed that tyrosinase and peroxidase co-exist in secondary lysosomes of the retinal pigmented epithelium (RPE) degrading the shedded photoreceptors membranes. As is known, melanosomes are thought to be lysosome derivatives. Though the melanomacrophages are ontogenetically distant from the RPE, both cell types carry out phagocytosis and exert protective functions towards generated stress conditions. The differences between a tyrosinase-like and a typical tyrosinase may be the result of tissue-specific differentiation events taking place in the various cells with melanogenic ability, as previously suggested in the extracutaneous pigment system of *R. esculenta* [19,20]. Regarding the TH activity, more investigations are necessary for a better understanding of this melanogenic system.

*Dopa oxidase activity on SDS-PAGE***.** The DO activity of kidney melanosomal protein extracts of sea bass was revealed on SDS-PAGE. For brevity, only the analysis of spring affected animals that present even a lower DO activity is shown (fig.8). The DO activity was confined to a high MW band, over 200 kDa, that barely entered the separating gel thus making an estimation of its precise size very difficult. Similarly, in Gallone et al. [20] the in-gel DO activity of liver melanosomal extract from amphibians was confined to a high

MW band over 200 kDa. These findings suggest that the active form of the tyrosinase from extracutaneous pigment systems could be differently aggregated, as previously described in other melanogenic systems [21]. Indeed, Gallone et al. [20] and Arciuli et al. [5] hypothesized that tyrosinase and tyrosinase-related proteins could aggregate in a multi-enzymatic complex of high molecular mass. Moreover, post-translational modifications, such as glycosylation, are crucial for the correct folding and full activity of tyrosinase [21] and may account for the difference between the observed protein bands and the expected molecular mass.

*Femtosecond transient absorption spectroscopy***.**

As tyrosinase is responsible for melanin synthesis, we isolated pigments from kidney tissue of sea bass and gilthead sea bream and compared them with natural fish skin melanin, natural *Sepia officinalis* melanin and synthetic melanin, using femtosecond transient absorption (Femto-TA) spectroscopy. First Femto-TA measurements were carried out on synthetic melanin [41]. Further studies reported a positive absorption signal characterized by a rapid rise within the instrumental response, followed by a multi-exponential decay, underlying a real photo-induced absorption process [63].

In this work we investigated the VIS transient absorption changes of our samples, induced by ultrafast UV photoexcitation. In particular, we used an ultrashort (~100 fs) quasi-monochromatic pump pulse to excite the melanin suspensions at 350 nm and investigated the temporal evolution of photoinduced absorption changes with sub-picosecond time resolution, using white light supercontinuum (450−800 nm) probe pulses [50]. In Femto-TA experiments, the measured differential absorption ΔA is a function of both probing wavelength (λ) and time delay (τ) between the pump and probe laser pulses [1]; at each time delay the transmitted intensity of the probe pulse both with and without excitation is recorded, and the signal is evaluated as:

$$\Delta A(\lambda,\tau) = -log_{10}\frac{I_{TP\&p}(\lambda,\tau)}{I_{Tp}(\lambda)}$$

where $I_{TP\&p}(\lambda,\tau)$ and $I_{Tp}(\lambda)$ are the transmitted intensities of the probe pulse in presence and in absence of the pump pulse, respectively [59]. A positive $\Delta A(\lambda,\tau)$ is obtained when the transmitted intensity of the probe pulse with excitation is smaller than the transmitted intensity without the pump pulse, which means that a photoinduced absorption process occurs; on the contrary, a negative $\Delta A(\lambda,\tau)$ is related to photobleaching and/or emission processes [1]. This kind of technique gives the possibility to investigate spectral changes due to electronic transitions, which occur in the sample after photoexcitation [51] and compare the ultrafast de-excitation dynamics between synthetic and natural melanin/pigments in suspension. In this respect, it is worth noting that a precise evaluation of suspensions concentration was difficult because of the many heating procedures carried out for each natural melanin sample. For this reason, to better compare the experimental results data normalization was carried out using Sepia melanin as standard [45]. Fig. 9 reports the ultrafast normalized transient absorption dynamics of fish melanin/pigments samples compared with both natural *Sepia officinalis* melanin and synthetic melanin. Clearly, in our experiments the TA signals were always positive demonstrating that after melanin/pigments are excited by UV pump pulses (λ_{Pump}=350 nm), the VIS probe pulses (λ_{probe}=650 nm) are absorbed by excited states [64,8]. Similar results were obtained when probing in the whole VIS range (not reported here). For each investigated sample, the decay of the signal was not monoexponential, and the experimental data were fitted using a biexponential response function R(t):

$$R(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}$$

where τ_i are the decay times and A_i are the corresponding pre-exponential factors.

The negligible luminescence quantum yield of melanin [34] explains the absence of fluorescence signals, thus a complete non-radiative relaxation process occurs in these pigments [41] proving the blocking of light-induced damage through the effective conversion of radiant energy into thermal energy [37].

Even if natural pigments are extracted from different fish tissues and species, the transient absorption spectra show comparable non-radiative deactivation processes, demonstrating similar response to ultrafast UV excitation. As it is clearly depicted in fig. 9, the temporal dynamics of natural fish pigments are very close to those of natural *S. officinalis* melanin. On the contrary, the dynamics of synthetic melanin is characterized by different decay times ($\tau_1 \approx 1.0 \pm 0.2$ ps, $\tau_2 \approx 6 \pm 1$ ps) both shorter than those measured in the natural melanin samples ($\tau_2 \approx 2.3 \pm 0.5$ ps, $\tau_2 \approx 16 \pm 3$ ps) (fit curves not shown here). We attribute the difference in the dynamics to the proteinaceous components occurring in natural melanin samples. In fact, the isolation of *Sepia* melanin involves mainly repeated centrifugation steps, leading to the native melanin containing 5 to 7.6% of proteinaceous components [48,17] while synthetic melanin (down pointed triangles in fig. 8) is characterized by the absence of proteinaceous components. This difference in ultrafast dynamics represents clear, additional evidence that the investigated samples have a standard melanin molecular structure.

4. CONCLUSIONS

The extracutaneous pigment system of sea bass was studied, and found to share some interesting features with the visceral pigment cell system of other cold-blooded vertebrates. Our results indicate, for the first time, variations of dopa oxidase activity in fish extracutaneous pigment system with respect to environmental temperature. Moreover a

relationship between kidney disease and the level of DO activity in melanosomal protein extracts has been highlighted, indicating the DO activity as a possible indicator for fish kidney disease. As teleosts lack lymph nodes and Peyer's patches, head-kidney and spleen are regarded as the most important secondary lymphoid organs. Hence our results indicate that the pigment system plays an important role in kidney tissue, similarly to what suggested for *Salmo salar* [5]. More investigations are necessary to explain the differences observed in liver and spleen tissues. In addition the comparative Femto-TA analysis of pigments isolated from sea bass kidney tissue, fish skin melanin, natural melanin from *S. officinalis* and synthetic melanin, by pointing out that fish melano-macrophages centres do contain melanins, supports our conclusions.

DISCLOSURES

No conflicts of interest are declared by the authors.

ACKNOLEDGEMENTS

This research was supported by grants from the University of Bari "Aldo Moro" Italy, and by Regione Puglia-project "VIS MARIS" (Bando FESR 2007-13 linea 1.2 - Azione 1.2.4 Bando "Aiuti a Sostegno dei Partenariati Regionali per l'Innovazione").

We thank Regione Puglia, project "Reti di Laboratori Pubblici di Ricerca" prog. cod. 20 for the instrumental facilities.

We would like to thank Dr. Antonia Mallardi (CNR-IPCF, Istituto per i Processi Chimico-Fisici, UOS di Bari, Italy) for her helpful discussion and observations.

REFERENCES

[1] Abramczyk H. Introduction to Laser Spectroscopy. Elsevier; 2005.

[2] Agius C. Preliminary studies on the ontogeny of the melano-macrophages of teleost haemopoietic tissues and age-related changes. Dev Comp Immunol. 1981 Fall;5(4):597-606.

[3] Agius C, Roberts RJ. Melano-macrophage centres and their role in fish pathology. J Fish Dis 2003; 26(9):499-509.

[4] Alonso MC, Cano I, Garcia-Rosado E, Castro D, Lamas J, Barja JL, Borrego JJ. Isolation of lymphocystis disease virus from sole, *Solea senegalensis* Kaup, and blackspot sea bream, Pagellus bogaraveo (Brunnich). J Fish Dis 2005; 28(4):221-228.

[5] Arciuli M, Fiocco D, Cicero R, Maida I, Zanna PT, Guida G, Horsberg TE, Koppang EO, Gallone A. Melanogenesis in visceral tissues of *Salmo salar*. A link between immunity and pigment production? Biochem Cell Biol 2012; 90(6):769-778.

[6] Barni S, Bertone V, Croce AC, Bottiroli G, Bernini F, Gerzeli G. Increase in liver pigmentation during natural hibernation in some amphibians. J Anat 1999; 195:19-25.

[7] Beraldo P, Galeotti M. Nephrocalcinosis in sea bass (Dicentrarchus labrax, L. 1758): anatomopathological findings. Ittiopatologia 2006; 3:179-188.

[8] Berera R, van Grondelle R, Kennis JTM. Ultrafast transient absorption spectroscopy: principles and application to photosynthetic systems. Photosynth Res 2009; 101:105-118.

[9] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976; 72: 248-254.

[10] Brattgjerd S, Evensen Ø. A Sequential light microscopic and ultrastructural study on the uptake and handling of *Vibrio salmonicida* in phagocytes of the head kidney in experimentally infected Atlantic salmon (*Salmo salar* L.). Vet Pathol 1996; 33:55-65.

[11] Bruno D W. Nephrocalcinosis. Aquaculture information series. 1996; Vol 16: 1-8.

[12] Capozzi V, Perna G, Carmone P, Gallone A, Lastella M, Mezzenga E, Quartucci G, Ambrico M, Augelli V, Biagi PF, Ligonzo T, Minafra A, Schiavulli L, Pallara M, Cicero R. Optical and photoelectronic properties of melanin. Thin Solid Films 2006; 511-512:362-366.

[13] Chang TS. An Updated Review of Tyrosinase Inhibitors. Int J Mol Sci 2009; 10:2440-2475.

[14] Cicero R, Mallardi A, Maida I, Gallone A, Pintucci G. Melanogenesis in the pigment cells of *Rana esculenta* L. liver: evidence for tyrosinase–like activity in the melanosome protein fraction. Pigment Cell Res 1989; 2(2):100-108.

[15] d'Ischia M, Napolitano A, Prota G. Peroxidase as an alternative to tyrosinase in the oxidative polymerization of 5,6-dihydroxyindoles to melanin(s). Biochim Biophys Acta 1991;1073(2):423-430.

[16] Diamant, A., Harpaz, S., Traub, W.: Nephrocalcinosis and urolithiasis in cultured gilthead sea bream. Diseases of fish and shellfish. Fish Culture Research Institute, Szarvas, Hungary, 1991

[17] Duchon J, Borovansky J, Hack P. Chemical composition of ten kinds of various melanosomes. Pigment Cell Res 1973; 1:165-170.

[18] Esteban MA, Cuesta A, Rodrıguez A, Meseguer J. Effect of photoperiod on the fish innate immune system: a link between fish pineal gland and the immune system. J Pineal Res 2006; 41(3):261-266.

[19] Gallone A, Guida G, Maida I, Cicero R. Spleen and liver pigmented macrophages of Rana esculenta L. A new melanogenic system? Pigment Cell Res 2002; 15(1):32-40.

[20] Gallone A, Sagliano A, Guida G, Ito S, Wakamatsu K, Capozzi V, Perna G, Zanna P, Cicero R. The melanogenic system of the liver pigmented macrophages of *Rana esculenta* L.–tyrosinase activity. Histol Histopathol 2007; 22(10):1065-1075.

[21] García-Borrón JC, Solano F. Molecular anatomy of tyrosinase and its related proteins: beyond the histidine-bound metal catalytic center. Pigment Cell Res 2002; 15(3): 162-173.

[22] Guida G, Maida I, Gallone A, Boffoli D, Cicero R. Ultrastructural and funtional study of the liver pigment cells from *Rana esculenta* L. In Vitro Cell Dev Biol Anim 1998; 34(5): 393-400.

[23] Guida G, Gallone A, Maida I, Boffoli D, Cicero R. Tyrosinase gene expression in the Kupffer cells of Rana esculenta L. Pigment Cell Res 2000; 13(6):431-435.

[24] Guida G, Zanna P, Gallone A, Argenzio E, Cicero R. Melanogenic response of the Kupffer cells of *Rana esculenta* L. to melanocyte stimulating hormone. Pigment Cell Res 2004; 17(2):128-134.

[25] Haugarvoll E, Thorsen J, Laane M, Huang Q, Koppang EO. Melanogenesis and evidence for melanosome transport to the plasma membrane in a CD83 teleost leukocyte cell line. Pigment Cell Res 1990; 19(3):214-225.

[26] Hossain M, Myung-Joo O. Histopathology of Marine and Fresh Lymphocystis Disease Virus (LCVD). Sains Malaysiana 2011; 40(10):1049-1052.

[27] Ito S. The IFPCS presidential lecture: a chemist's view of melanogenesis. Pigment Cell Res 2003; 16(3):230-236.

[28] Jimenez-Cervantes C, Valverde P, Garcia-Borron JC, Solano F, Lozano J. Improved tyrosinase activity stains in polyacrylamide electrophoresis gels. Pigment Cell Res 1993; 6(6): 394-399.

[29] Kasraee B. Peroxidase-Mediated Mechanisms Are Involved in the Melanocytotoxic and Melanogenesis-inhibiting Effects of Chemical Agents. Dermatology 2002; 205(4):329-339.

[30] Koppang EO, Haugarvoll E, Hordvik I, Aune L, Poppe T. Vaccine-associated granulomatous inflammation and melanin accumulation in Atlantic salmon, *Salmo salar* L., white muscle. Fish Dis 2005; 28(1):13-22.

[31] Larsen HA, Austbø L, Mørkøre T, Thorsen J, Hordvik I, Fisher U, Jirillo E, Rimstad E, Koppang, EO. Pigment-producing granulomatous myopathy in Atlantic salmon: a novel inflammatory response. Fish Shellfish Immunol 2012; 33:277-285.

[32] Larsen HA, Austbø L , König M , Sørum H, Rimstad E, Koppang EO. Transcription of the tyrosinase gene family in an Atlantic salmon leukocyte cell line (SHK-1) is influenced by temperature, but not by virus infection or bacterin stimulation. Developmental and Comparative Immunology 2013; 41:50-58.

[33] Leknes IL. Melano-macrophage centres and endocytic cells in kidney and spleen of pearl gouramy and platyfish (Anabantidae, Poeciliidae: Teleostei). Acta Histochem 2007; 109(2):164-168.

[34] Liu Y, Simon JD. Isolation and Biophysical Studies of Natural Eumelanins: Applications of Imaging Technologies and Ultrafast Spectroscopy. Pigment Cell Res 2003 16(6):606-618.

[35] Maida I, Arciuli M, Guida G, Zanna PT, Cicero R. Seasonal variations of *Rana esculenta* L. skin tyrosinase. Comp. Biochem. Physiol. B Biochem Mol Biol 2009; 152(1): 79-84.

[36] Mason HS. The chemistry of melanin: mechanism of the oxidation of dihydroxyphenylalanine by tyrosinase. J Biol Chem 1948; 172(1):83-99.

[37] Meng S, Kaxiras E. Mechanisms for Ultrafast Nonradiative Relaxation in Electronically Excited Eumelanin Constituents. Biophys J 2008; 95(9):4396-4402.

[38] Meredith P, Sarna T. The physical and chemical properties of eumelanin. Pigment Cell Res 2006; 19(6):572-594.

[39] Mulchachy, M.F., Collins, N., Mcauliffe, T.: Nephrocalcinosis in freshwater and saltwater farmed rainbow trout in Ireland. In: Acuigroup (Eds). Fish Diseases, Fourth CORPAQ Session, ATP, Madrid, Spain, 1984; 53-158.

[40] Mutoloki S, Alexandersen S, Evensen Ø. Sequential study of antigen persistence and concomitant inflammatory reactions relative to side-effects and growth of Atlantic salmon (*Salmo salar* L.) following intraperitoneal injection with oil-adjuvanted vaccines. Fish Shellfish Immunol 2004; 16(5):633-644.

[41] Nofsinger JB, Ye T, Simon JD. Ultrafast Nonradiative Relaxation dynamics of Eumelanin. J Phys Chem B 2001; 105:2864-2866.

[42] Okun MR. The role of peroxidase in neuromelanin synthesis: a review. Physiol Chem Phys Med NMR 1997; 29(1):15-22.

[43] Olivares C, García-Borrón JC, Solano F. Identification of active site residues involved in metal cofactor binding and stereospecific substrate recognition in mammalian tyrosinase. Implications to the catalytic cycle. Biochemistry 2002; 41(2):679-686.

[44] Palumbo A, Jackson IJ. Peroxidase activity in the ink gland of *Sepia officinalis* and partial nucleotide sequence of a candidate cDNA encoding the enzyme. Biochim Biophys Acta 1995; 1247(2):173-178.

[45] Palumbo A. Melanogenesis in the Ink Gland of *Sepia officinalis*. Pigment cell Res 2003; 16:517-522.

[46] Passantino L, Santamaria N, Zupa R, Pousis C, Garofalo R, Cianciotta A, Jirillo E, Acone F, Corriero A. Liver melanomacrophage centres as indicators of Atlantic bluefin tuna, *Thunnus thynnus* L. well-being. J. Fish Dis. 2014; 37(3), 241-250. doi: 10.1111/jfd.12102.

[47] Person-Le Ruyet J, Mahé K, Le Bayon N, Le Delliou H. Effects of temperature on growth and metabolism in a Mediterranean population of European sea bass, *Dicentrarchus labrax*. Aquaculture 2004; 237(1-4):269-280.

[48] Pezzella A, d'Ischia M, Napolitano A, Palumbo A, Prota G. An integrated approach to the structure of *Sepia* melanin. Evidence for a high proportion of degraded 5,6-dihydroxyindole-2-carboxylic acid units in the pigment backbone. Tetrahedron 1997; 53: *8281-8286.*

[49] Raper HS. The tyrosinase-tyrosine Reaction: Production from Tyrosine of 5,6-Dihydroxyindole and 5,6-Dihydroxyindole-2-carboxylic Acid-the Precursors of Melanin. Biochem J 1927; 21(1):89-96.

[50] Ruckebusch C, Sliwa M, Pernot P, de Juan A, Tauler R. Comprehensive data analysis of femtosecond transient absorption spectra: A review. Journal of Photochemistry and Photobiology C: Photochemistry Reviews 2012; 13**:**1-27.

[51] Rullière C, Amand T, Marie X. Femtosecond Laser Pulses, principles and experiments. 2th ed. Springer 2004.

[52] Scalia M, Geremia E, Corsare C, Scuito S, Sichel G. The extracutaneous pigmentary sistem: evidence for the melanosynthesis in amphibia and reptilia liver. Comp Biochem Physiol 1988; 89B:715-717.

[53] Scapigliati G, Romano N, Buonocore F, Picchietti S, Baldassini MR, Prugnoli D, Galice A, Meloni S, Secombes CJ, Mazzini M, Abelli L. The immune system of sea bass, *Dicentrarchus labrax*, reared in aquaculture. Dev Comp Immunol 2002; 26(2):151-160.

[54] Schraermeyer U. and Stieve H. A newly discovered pathway of melanin formation in cultured retinal pigment epithelium of cattle. Cell Tissue Res. 1994; 276, 273-279.

[55] Sichel G. Qualche osservazione sulle cellule pigmentate del fegato di rettili. Mon Zoo Italy 1984; 57:3-7.

[56] Sichel G. Biosynthesis and function of melanins in hepatic pigmentary system. Pigment Cell Res 1988; 1(4):250-258.

[57] Sichel G, Scalia M, Corsaro C. Amphibian Kupffer cells. Microsc Res Tech 2002; 57(6):477-490.

[58] Thorsen J, Høyheim B, Koppang EO. Isolation of the Atlantic salmon tyrosinase gene family reveals heterogeneous transcripts in a leukocyte cell line. Pigment Cell Res 2006; 19(4):327–336.

[59] Tkachenko NV. Optical spectroscopy: measurements and instrumentations. Elsevier, Amsterdam 2006.

[60] Vigliano FC, Bermúdez R, Quiroga MI, Nieto JM. Evidence for melano-marophage centres of teleost as evolutionary precursors of germinal centres of higher vertebrates: an immunohistochemical study. Fish Shellfish Immunol 2006; 21(4):467-471.

[61] Wang S, Wang Y, Zhang Z, Xie F, Lin P, Tai Z. Tyrosinase, a new innate humoral immune parameter in large yellow croaker (*Pseudosciaena crocea* R)*. Chinese Journal of Oceanology and Limnology 2009; 27(3):527-535.

[62] Wolke RE, Murchelano RA, Dickstein CD, George CJ. Preliminary evaluation of the use of macrophage aggregates (MA) as fish health monitors. Bull Environ Contam Toxicol. 1985; 35(2):222-227.

[63] Ye T, Simon JD. Comparison of the Ultrafast Absorption dynamics of Eumelanin and Pheomelanin. J Phys Chem B 2003; 107:11240-11244.

[64] Ye T, Fu D, Warren WS. Nonlinear Absorption Microscopy. Photochemistry and Photobiology 2009; 85:631-645.

[65] Yıldız M, Erdal Ş, Metin T. Effects of Variations in Feed and Seasonal Changes on Body Proximate Composition of Wild and Cultured Sea Bass (*Dicentrarchus labrax* L.). Turkish Journal of Fisheries and Aquatic Sciences 2007; 7:45-51.

[66] Zapata A, Amemiya CT. Phylogeny of lower vertebrates and their immunological structures. Curr Top Microbiol Immunol 2000; 248:67-107.

[67] Zuasti A, Ferrer C, Aroca P, Solano F. Distribution of extracutaneous melanin pigment in *Sparus auratus*, *Mugil cephalus*, and *Dicertranchus labrax* (Pisces, Teleostei). Pigment Cell Res 1990; 3(3):126-131.

FIGURE LEGENDS

Fig. 1 Farmed European sea bass posterior kidney, clearly affected by nephrocalcinosis. Presence of a lighter color and areas or whitish streaks of chalky appearance (A) and presence of abundant mineral deposits in the caudal part (B) are shown.

Fig. 2 DO activity in control European sea bass spring and winter extracts. DO activity levels in kidney, liver and spleen melanosomal protein fractions of sea bass control samples are shown. Values are reported as means ± SEM for at least three independent experiments.

Fig. 3 POD activity in control European sea bass spring and winter extracts. POD activity levels in kidney, liver and spleen melanosomal protein fractions of sea bass control samples are shown. Values are reported as means ± SEM for at least three independent experiments.

Fig. 4 DO activity in farmed European sea bass spring and winter extracts. DO activity levels in kidney, liver and spleen melanosomal protein fractions of sea bass affected by nephrocalcinosis are shown. Values are reported as means ± SEM for at least three independent experiments.

Fig. 5 POD activity in farmed European sea bass spring and winter extracts. POD activity levels in kidney, liver and spleen melanosomal protein fractions of sea bass affected by nephrocalcinosis are shown. Values are reported as means ± SEM for at least three independent experiments.

Fig. 6 DO activity in control and farmed European sea bass spring and winter extracts. DO activity levels in kidney melanosomal protein fractions of control and affected sea bass samples are shown. Values are reported as means ± SEM for at least three independent experiments.

Fig. 7 POD activity in control and farmed European sea bass spring and winter extracts. POD activity levels in kidney melanosomal protein fractions of control and affected sea bass samples are shown. Values are reported as means ± SEM for at least three independent experiments.

Fig. 8 In-gel DO activity in European sea bass spring extracts. In-gel DO activity of mushroom tyrosinase control sample (A) and spring melanosomal protein fraction of kidney sea bass affected by nephrocalcinosis (B) are shown.

Fig. 9 Normalized femtosecond transient absorption of natural melanin/pigments and synthetic melanin suspensions, obtained by photoexciting the suspensions at 350 nm and probing the induced absorption changes at 650 nm.