PhD School in Molecular and Cellular Biology

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Molecular biomarkers for fish welfare and species authentication

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Abstract

Aquaculture is currently contributing almost half of fish consumed by the human population and it keeps growing more rapidly than other animal food production sectors. The introduction of molecular techniques, such as various genome projects, gene expression analysis and functional genomics, and the monitoring of stress levels through very early indicators such as molecular biomarkers, can bring considerable benefits to the quantity and quality of production and improve welfare of reared animals. Moreover in recent years, some teleost species have become model organisms, such as zebrafish and pufferfish, and the knowledge about them could be transferred to further improve reared animals and husbandry. Therefore, species of interest in aquaculture could, in turn, become new animal models. In this context, we looked for a new molecular biomarker for stress in *Dicentrarchus labrax*.

Stress could involve alterations of brain functioning that may precipitate to mood disorders. The neurotrophin Brain Derived Neurotrophic Factor (BDNF) has recently been involved in stress-induced adaptation. BDNF is a key regulator of neuronal plasticity and adaptive processes. Regulation of BDNF is complex and may reflect not only stress-specific mechanisms, but also hormonal and emotional responses. For this reason, we used, as an animal model of stress, a fish, *D. labrax*, whose brain organization is very similar to that of higher vertebrates, but is generally considered free of emotional reactions. We provide, for the first time in a species of great interest in aquaculture, a comprehensive characterization of BDNF gene and its transcriptional, translational and post-translational regulation following acute stress. While total BDNF mRNA levels are unchanged, BDNF splicing variants 1c and 1d resulted down regulated after acute stress. Acute stress induces also a significant increase in proBDNF levels and reduction in mature BDNF suggesting altered regulation of proBDNF proteolytic processing. Notably, we provide here the first evidence that fishes possess a simplified proteolytic regulation of BDNF since the pro28kDa form, generated by the SKI-1 protease in mammals, is absent in fishes. The cleavage site, in fact, has first emerged in reptilians. Finally, we show that the proBDNF/totBDNF ratio is a highly predictive novel quantitative biomarker to detect

stress in fishes with sensitivity = 100%, specificity = 87%, and Negative Predictive Value = 100%.

The high predictivity of proBDNF/totBDNF ratio for stress in lower vertebrates indicates that processing of BDNF is a central mechanism in adaptation to stress and predicts that a similar regulation of pro/mature BDNF has likely been conserved throughout evolution of vertebrates from fish to man.

The second part of this thesis is focused on the problem of seafood and fish species authentication. This is an important issue within the seafood industry to protect consumers from fraudulent practices, like species substitution, resulting from the increasingly wide diversification of species and globalization of fish trade. DNA-based methods for species identification are by far the best: they are generally based on PCR amplification of a target sequence, followed by a post-PCR analysis of amplified products, which could consist in sequencing or obtaining species-specific patterns of restriction fragments.

The gene coding for the 5S ribosomal RNA is a suitable target for fish species identification because, for its particular sequence features, it does not require any further treatment after PCR. This gene consists of a small coding conserved region and a variable region of noncoding DNA, which is termed not transcribed spacer (NTS). Both regions are tandem repeated in the genome. The NTS, which is species-specific for length and sequence, has been used, here, to discriminate species subjected to substitution in the Italian fish market. Although preliminary, our results have demonstrated the value of this approach.

1. Introduction

1.1 Fish aquaculture and modern biotechnology

Aquaculture is currently contributing almost half of the fish consumed by the human population. This sector has been growing extensively in the last 50 years and it keeps growing more rapidly than other animal food producing sectors (FAO, **The State of World Fisheries and Aquaculture 2008**. 2009). The introduction of molecular techniques in addition to the more traditional method of biotechnology has supplied the resources to significantly increase the production in world aquaculture. The new approaches include the various genome projects, cDNA microarray/expression analysis, functional genomics, transgenic technologies (based on microinjection or retroviruses), and proteome analysis. All these approaches could be apply for improving growth and cost effectiveness, increasing resistance to environment and pathogens, improving broodstock quality and control reproduction, creating new and/or better products (Melamed *et al.*, 2002). Some examples of the relevance of these innovations are reported below.

1.1.1 Growth enhancement

Growth enhancement is an important aim for aquaculture especially if it can be obtained through more economic and faster manipulations than selective breeding methods. One of the ways to increase growth rates in animals, originally demonstrated in mice (Palmiter *et al.*, 1982), is the introduction of additional growth hormone (GH). Alternatively, transgenic salmonids possessing an "all-fish" gene construct consisting of antifreeze protein promoter and GH cDNA show a dramatic growth enhancement (Du *et al.*, 1992; Devlin *et al.*, 1994), they appear healthy and some produce second and third generation transgenic offsprings which keep the phenotype (Saunders *et al.*, 1998).

An interesting phenomenon studied in intensive aquaculture as a means of enhancing growth rates is the "compensatory growth", an exceptionally fast growth, which occurs in fish after periods of fasting. The mechanisms by which food intake activates an increase in somatic growth, especially in muscle growth, are complex and not yet fully understood. Terova *et al.* (2006; 2007a) identified three genes involved in compensatory growth in sea bass (*Dicentrarchus labrax*): the insulin-like growth factor I and II, which are potent mitogens, and the myostatin, an important factor for skeletal muscle growth.

1.1.2 Increase resistance

The molecular approaches to increase the resistance to viral and bacterial pathogen are based on DNA vaccines and antimicrobial agents. The firsts consist in the injection of naked DNA encoding part of the antigen that induced production of antibodies and it has already been successfully used in fish in a number of studies (Traxler *et al.*, 1999; Lorenzen *et al.*, 1999). Antimicrobial proteins, like lysozyme, can be used, conversely, to target the non-specific immune response (Andreu and Rivas, 1999) or transgenic fish carrying genes encoding a number of antimicrobial peptides could be created.

Molecular techniques can be used also to increase resistance of fish to adverse environmental condition, like excessive cold. Some marine teleosts have high levels of serum antifreeze protein (AFP) or glycoprotein (AFGP) which effectively reduce the freezing temperature by preventing ice-crystal growth. The gene encoding the liver AFP from winter flounder was successfully introduced into the genome of Atlantic salmon (Hew *et al.*, 1999). The developing of stocks harbouring this gene would clearly be a major benefit in commercial aquaculture countries where winter temperatures often border the physiological limits of these species.

1.2 Fish aquaculture and model organisms

In recent years, it is rising the use of some teleost species as vertebrate model organisms used to study the genetics underlying development, normal body function and disease, with a parallel increase of new tools and methods available for this purpose. Transferring knowledge from models to the species of interest for aquaculture can achieve a further improvement of reared animals and husbandry, especially for what concerns the growth, stress and disease resistance (Dahm and Geisler, 2006).

The most popular fish model is the freshwater teleost zebrafish, *Danio rerio*, which has become an experimental model for embryogenesis, organogenesis, general development in vertebrate (Love *et al.*, 2004) and human diseases like cancer or neurodegenerative disorders (Amatruda and Patton, 2008). Medaka (*Oryzias latipes*) is another model organism employs to study aspects of embryonic development (Wittbrodt *et al.*, 2002);

studies about behaviour and molecular evolutionary-developmental biology have been performed on stickleback, *Gasterosteus aculeatus* (Tickle and Cole, 2004); the two pufferfish, *Takifugu rubripes* and *Tetraodon nigroviridis*, have become important organisms to understand genome architecture, organization and function, because of the relatively small size and simple organization of their genomes.

Other aquatic animal models have been used in biomedical research and many of them are directly relevant to understand physiology, genetics, anatomy and pathology of human disease processes, like specific cancers, toxicological responses and infectious diseases (Schmale, 2004). In this regard it can be cited the green swordtail (*Xiphophorus* sp.) as a well established model for human melanoma (Meierjohann *et al.*, 2004); another study used the adult brain of the Atlantic salmon (*Salmo salar*) as an experimental model for neuronal tissue regeneration after injury and, although this is not an evident model for human diseases, the authors suggested that some of the involved proteins may play a role in homolog processes occurring in mammals (Zupanc *et al.*, 2006). Some teleost species are also suitable for environmental monitoring: besides zebrafish, the rare minnow (*Gobiocypris rarus*) has recently been selected as a model for aquatic toxicological studies (Zhong *et al.*, 2008); exposures to heavy metals like zinc and cadmium, biological toxins or more undefined pollutant mixtures have been monitored through proteomic studies in different species (Forné *et al.*, 2010).

1.3 Genomic resources for fish

Often, the major drawback for the application of molecular techniques is the lack of genetic information about species of interest in aquaculture compared to the model organisms, although the resources are being expanded. A recent review summarized the present-day nucleotide information about fish (Oleksiak, 2010). Currently there are five fish genome projects that comprise the aforementioned zebrafish *(D. rerio)*, medaka (*O. latipes*), *T. rubripes*, *T. nigroviridis* and *G. aculeatus*. Atlantic salmon (*S. salar*) and rainbow trout (*Oncorhyncus mykiss*) genome project are underway and to be completed by 2011.

Although nuclear genome projects are lacking for most fish species, a wealth of sequence data exists for many more fish species, largely based on expressed sequence tags (EST) projects. ESTs represent a partial sequence of much longer RNA expressed in a cell. Being encoding genes that are actively transcribed, without intron sequences, they can be more informative about the ultimate function of the gene.

ESTs resources represent one of the efforts that are reducing the gap of knowledge between farming species from model organisms. With this aim our laboratory realized, in recent years, eight cDNA libraries obtained from different tissues of three teleosts: *Dicentrarchus labrax, Perca fluviatilis* and *Thunnus thynnus* (Chini *et al.*, 2006 and 2008; Rossi *et al.*, 2007).

1.4 Molecular biomarkers for animal welfare

Protecting the welfare of farmed animals is a central requirement of any animal-rearing system, fish included. Animal welfare involves the subjective feelings of animals and the experience of pleasure, pain, frustration, hunger or other states and it is difficult to define and to measure (Dawkins, 1990). Recently Korte *et al.* (2007) have proposed a new animal welfare concept based on allostasis, which means stability through change and it has the potential to replace homeostasis as the core model of physiological regulation. Not constancy or freedoms, but capacity to change is crucial to good physical and mental health and good animal welfare. Therefore, this new animal welfare concept has to be taken into account.

Health and welfare of farmed animals are both influenced by husbandry practices, which are the major cause of stress. Knowledge of these stress levels represents a fundamental parameter to achieve and maintain high standards of animal welfare. This point is particularly important when welfare of animals reared for commercial interest is to monitor. In fact, production and quality have to be equally improved with benefits on the public perception of the products and consequent positive repercussions on marketing aspects.

A biomarker is defined as any biological response to a stress factor measured inside the organism indicating a deviation from the normal state. This response can range from molecular through cellular and physiological to behavioural changes. The generalized stress response in fish has been broadly characterized into primary, secondary and tertiary response. The primary response starts with a blood increase of neuroendocrine/endocrine factors such as cortisol. The secondary response comprises various hormone induced biochemical and physiological effects, that results in the alteration of haematological parameters such as glucose concentration. The tertiary response is associated to the involvement of "fish social life" that may result in appetite loss, compromised anabolic processes, reduced reproductive capability and frequent occurrence of infective pathologies (Terova *et al*., 2009b).

Therefore, stress conditions were traditionally evaluated by monitoring blood levels of cortisol, haemoglobin, and glucose (Roche and Bogè, 1996), but these descriptors may not be sufficiently reliable when chronic stresses are applied and animal welfare is concerning. It is therefore necessary to search for further parameters, which are capable to describe, taking into account the "allostatic concept" (Korte *et al*., 2007) biological stress and animal emotional responses. Molecular biomarkers directly indicate a gene activity and for this reason they have the characteristics for being useful early indicator.

The search for molecular markers can be approached looking for them among those genes whose expression could reasonably be modified by the different farming conditions or, alternatively, following a strategy addressed at searching for any gene whose expression can be modified by changes, for example, in rearing densities, through techniques like differential display. With this technique Gornati *et al*. (2004a) obtained six bands differentially expressed, comparing gene expression of sea bass (*D. labrax*) farmed at different population densities. One of these bands resulted to be coding for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), a key enzyme of the cholesterol synthesis. Quantitative evaluation of HMGCR expression by real-time PCR confirmed an up-regulation of transcription at higher population densities (Gornati *et al*., 2005).

Other classical stress-related genes were evaluated such as metallothioneins (MT), heat shock proteins (HSP) (Gornati *et al*., 2004b; 2005), enolase, Na +/H+ exchanger (NHE)-1 c-

Fos, glucocorticoid receptor (GR), glucose transporter (GLUT2) (Rimoldi *et al.*, 2009; Terova *et al.*, 2005; 2009a). Moreover genes related to specific stress conditions, as oxygen fluctuation (HIF-1a) and food deprivation have been considered (Terova *et al.*, 2007b; 2008a; 2008b).

In this thesis project, we decided to consider, for the first time in sea bass (*D.labrax*), a neurobiological marker such as brain derived neurotrophic factor.

1.4.1 Brain derived neurotrophic factor

Brain Derived Neurotrophic Factor (BDNF) is the most abundant and widely expressed neurotrophin, a family of structurally related proteins required for the development and function of the vertebrate nervous system (Casaccia-Bonnefil *et al.*, 1999; Huang and Reichardt, 2001; Poo, 2001). In the vertebrate brain, BDNF also governs long lasting changes in synaptic efficacy and morphology (McAllister *et al.*, 1999; Thoenen, 2000; Braham and Massaoudi, 2005; Lu *et al.*, 2008; Braham, 2007). Recent studies have suggested that BDNF may be involved in stress-induced adaptation in adult (Marini *et al.*, 2008). Indeed, several types of injury and cell stress affect the expression of BDNF in the mammalian brain; in particular, chronic stress decreases the synthesis of hippocampal BDNF (Smith *et al.*, 1995a; 1995b; 1995c; Nibuya *et al.*, 1999) while acute stress induces complex alterations in the expression of BDNF, including a decrease in the hippocampus and an increase in the prefrontal cortex (Marmigère *et al.*, 2003; Nair *et al.*, 2007; Lee *et al.*, 2008; Fuchikami *et al.*, 2009). "Stress" is a biological term which refers to the consequences of the failure of a human or animal body to adequately answer to environmental stimuli. Stress induction is also used to study alterations of brain functioning leading to mood disorders which are often precipitated or exacerbated by acute or chronic stressful life events (Gold and Chrousos, 2002; Brown *et al.*, 2003; Duman and Monteggia, 2006). Stress involves also subjective feelings that are particularly complicated in mammals and primates in which the emotional components may have a dominant effect. Since alterations in BDNF expression were also found in response to emotions such as anxiety or fear in rodents (Rasmusson *et al.*, 2002) and BDNF affects emotional preferences in humans (Gasic *et al.*, 2009), it remains to be determined how

the stress itself or the associated behavioural responses contribute in mediating these changes. In this view, it is interesting to use as an animal model of stress, a fish whose brain organization is very similar to that of higher vertebrates, but is generally considered free of emotional reactions.

1.4.2 Neurotrophin family members and their receptors

Neurotrophin family, besides BDNF, includes Nerve Growth Factor (NGF), Neurotrophin-3 (NT-3) and Neurotrophin-4/5 (NT-4/5); other members are Neurotrophin-6 (NT-6) and Neurotrophin-7 (NT-7), both found only in fish.

All known neurotrophin genes share a common organization: they encode for a pre-pro-protein that is translocated to the endoplasmic reticulum and proteolitically processed to yeld the mature protein. While all mature neurotrophins bind to the p75 receptor with similar affinity, the specificity of their activity is dependent upon the activation of three different tyrosine kinase (Trk) receptors and their downstream signalling cascades: NGF specifically activates TrkA, BDNF and NT-4/5 interact with TrkB, whereas NT-3 preferentially binds to Trk-C. NT-6 and NT-7 do not have orthologues in mammals or birds and appear to interact with the same receptors as the mammalian proteins.

The mature neurotrophin proteins are non-covalently associated homodimers. Although some neurotrophin monomers are able to form heterodimers with other neurotrophin monomers *in vitro*, there is no evidence that these heterodimers exist at significant concentrations *in vivo*. The structures of NGF, NT-3 and NT-4 homodimers and of the BDNF-NT-3/4 heterodimers have been solved. Each of these four proteins shares a highly homologous structure with features, such as tertiary folds and cystine knots, which are present in several other growth factors.

The first receptor to be discovered, p75 neurotrophin receptor (p75NTR), was identified as a low-affinity receptor for NGF, but was subsequently shown to bind each of the neurotrophins with a similar affinity. p75NTR is a member of the tumour necrosis receptor superfamily with an extracellular domain that includes four cysteine-rich motifs, a single transmembrane domain and a cytoplasmic domain that includes a 'death' domain. p75NTR inhibits activation of Trk receptors by non-preferred neurotrophins both

in vitro and *in vivo*. Pro-neurotrophins bind with high affinity to p75NTR, which can cooperate with many different protein partners and form multimeric receptor complexes to produce a number of cellular responses, including apoptosis.

In mammals, the three members of the Trk subfamily of receptor tyrosine kinases constitute the second major class of neurotrophin receptors. The extracellular domain of each of the Trk receptors consists of a cysteine-rich cluster followed by three leucine-rich repeats, another cysteine-rich cluster and two immunoglobulin-like domains. Each receptor spans the membrane once and is terminated with a cytoplasmic domain consisting of a tyrosine kinase domain surrounded by several tyrosines that serve as phosphorylation-dependent docking sites for cytoplasmic adaptors and enzymes. The neurotrophins dimerize the Trk receptors, resulting in activation through transphosphorylation of the kinases present in their cytoplasmic domains.

Each Trk receptor controls three major signalling pathways, which promote neuronal differentiation including neurite outgrowth, survival and growth of neurons and other cells, synaptic plasticity and gene transcription.

Neurotrophins features and their signalling pathways have been reviewed by Reichardt (2006), Lu et al. (2005) and Lanave et al. (2007).

1.5 *Dicentrarchus labrax* Linnaeus, 1758 (Moronidae)

[From: FAO. © 2005-2010. - . Cultured Aquatic Species Information Programme. See: References]

The European Sea bass, *Dicentrarchus labrax*, is of great interest for Mediterranean aquaculture as it is an excellent food fish, with high commercial value; it is often marketed as Mediterranean seabass, branzino, in Northern Italy, or spigola in other parts of Italy.

Figure 1.1 *Dicentrarchus labrax*

1.5.1 Features, habitat and biology

Its body is rather elongate; the opercle has two flat spines and the preopercle has large, forward-directed spines on its lower margin. The mouth is terminal and moderately protractile with vomerine teeth in a crescentic band, without a backward extension on midline of roof of mouth. Sea bass has two separate dorsal fins; the first with 8 to 10 spines; the second with 1 spine and 12 or 13 soft rays. The anal fin has 3 spines and 10 to 12 soft rays. The scales are small; the lateral line is complete, but not extending onto caudal fin, that is moderately forked. The colour is silvery grey to bluish on the back, silvery on the sides, sometimes tinged with yellow on the belly. Young specimens have some dark spots on upper part of body.

The European sea bass are eurythermic (5-28 °C) and euryhaline (3‰ to full strength sea water); thus they are able to frequent coastal inshore waters, and occur in estuaries and brackish water lagoons. Sometimes they venture upstream into freshwater. There is only one breeding season per year, which takes place in winter in the Mediterranean population (December to March), and up to June in Atlantic populations. Sea bass spawn small (1.02-1.39 mm) pelagic eggs in water with salinities lower than 35‰, near to river

mouths and estuaries or in littoral areas where the salinity is high (≥30‰). Being not particularly sensitive to low temperature some fish may over-winter in coastal lagoons instead of returning to the open sea. Sea bass are predators and their feeding range includes small fish, prawns, crabs and cuttlefish.

1.5.2 Historical background

Sea bass were historically cultured in coastal lagoons and tidal reservoirs before the race to develop the mass-production of juveniles started in the late 1960s. During that time, France and Italy competed to develop reliable mass-production techniques for juvenile sea bass and, by the late 1970s, these techniques were well enough developed in most Mediterranean countries to provide hundreds of thousands of larvae. The European sea bass was the first marine non-salmonid species to be commercially cultured in Europe and at present is the most important commercial fish widely cultured in Mediterranean areas. Greece, Turkey, Italy, Spain, Croatia and Egypt are the biggest producers.

1.5.3 Production cycle in intensive system

The bulk of sea bass aquaculture production comes from sea cage farming. To secure a reliable and sufficient supply of good quality fish eggs, most hatcheries have established their own broodstock units, where breeders of different age groups are maintained long-term. Parents may come either from a farm or from the wild. The management of captive broodstock in the breeding stations includes natural maturation, the induction of ovulation by photoperiod manipulation or hormonal treatments, fertilisation in spawning tanks and incubation in an open-water circulation system. At the onset of the spawning season it is necessary to move selected batches of breeders from their long term holding facilities to the spawning tanks, where they can be better treated and their performance can be easily monitored. When fertilised eggs are required outside the natural spawning period, out-of-season sexual maturation is obtained by promoting gametogenesis by manipulating the photoperiod and temperature. The hatchery management decides on the periods of egg production according to its marketing and/or farm needs. Hormonal

with human chorionic gonadotropin treatment is used to trigger the last phase of egg maturation.
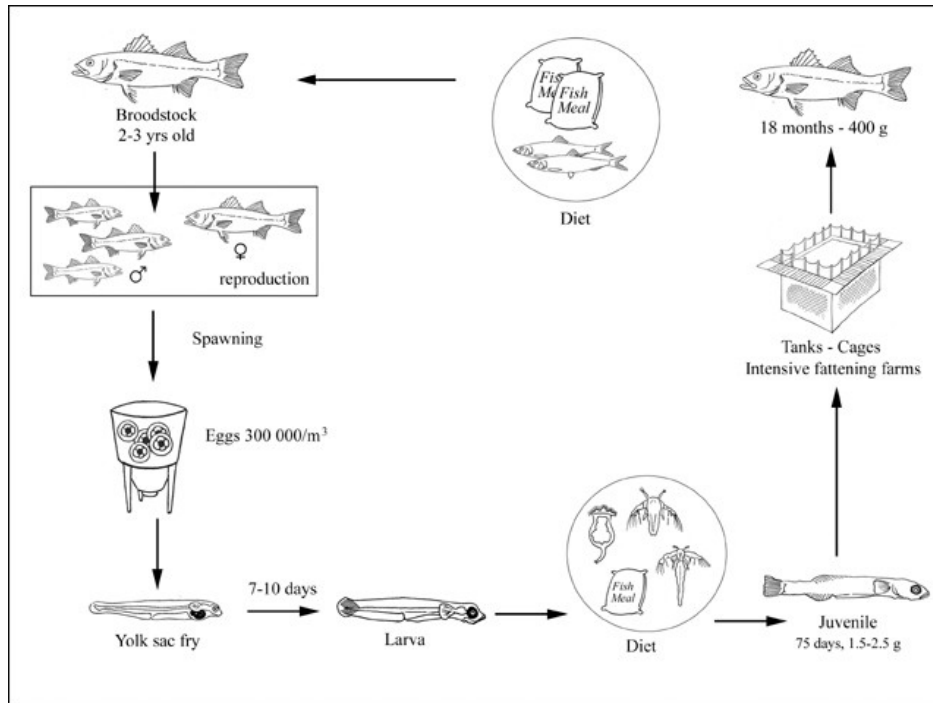
Figure 1.2 Production cycle of *D. labrax* - intensive system.

In intensive production, on growing units are supplied with fry from hatcheries and controlled diet is provided. Juveniles are sold to farmers as on growing stock at a size of 1.5-2.5 g. The on growing juveniles reach 400-450 g in 18-24 months. Cages can be of different kinds but the principle is the same; all types are based on a natural exchange of water through pens. The quality of sites is therefore highly variable, according to local conditions such as tide and current.

Tanks are usually supplied with seawater (38‰) maintained in a continuous flow-through system under ambient temperature. High stocking densities are applied (20-35 kg/m³); this means that accurate control of water quality and careful observations of fish health are essential. A recirculation system, to control water temperature (between 13-18 °C) is used during autumn/winter, frequently full-time in hatchery and the pre-fattening phase of the production cycle; this system is also used for fattening in high technology farms. This practice improves growth but can be highly expensive due to the required

technology for water quality control (filtering, air stripping, UV treatment, catabolite removal).

Killing methods should result in rapid and irreversible loss of consciousness. Methods that kill fish rapidly result in a reduction of stress, thus an improvement in welfare and in quality. Prolonged crowding before harvesting is avoided, to ensure high product quality and fish welfare. Greater muscle activity at slaughter leads to a rapid decrease in energy reserves (i.e. adenosine triphosphate, ATP), and to the build up of lactic acid and consequently a drop in post-mortem pH. An animal that struggles at slaughter goes into rigor very rapidly, adversely affecting the quality of fish fillets by softening the muscle texture.

Although a sturdy species, sea bass are subject to a wide range of diseases under rearing conditions. These outbreaks have important effects on commercial production and could prevent the expansion of the industry in some countries. Stress is considered an important factor co-responsible for disease outbreaks; thus improved husbandry is generally suggested to reduce stress. Another problem is the lack of authorized effective therapeutants, particularly for parasites, in most European countries.

1.5.4 Market and trade

One of the largest success stories in European aquaculture has been the Mediterranean sea bass industry, which in less than 15 years grew from a few thousand tonnes to 57 000 tonnes today, having peaked at nearly 71 000 tonnes in 2000. When farmed bass started getting to market in the late 1980s and early 1990s, the farmed quality was seen to complement the wild species and prices were very high. Prices of the wild product may have suffered initially, as the volumes from aquaculture continued growing, but today there is clear distinction in the market between wild and farmed product, with the prices for wild bass several times higher than those of the farmed fish.

1.6 Molecular techniques for food authentication

1.6.1 The problem

Development in food preservation, processing technologies and liberalization of trade have contributed significantly to the globalization of fish trade and to the diversification of seafood, both in terms of species and products. It is currently estimated that more than 800 fish species are traded internationally under many different forms, shapes, brands and preparations. For this reason authentication of fish and seafood species has become an important issue within the seafood industry to protect the consumers from fraudulent and deceptive practices whereby low value species are substituted for high value similar species. At national level, food legislation generally indicates that the label must not mislead consumers, but international trade and the use of similar terms for different products makes it complicated when a product from one country is introduced to another.

In Italy food imports from Far East is increasingly easy with competitive prices, often at the expense of food quality and safety. This makes the market more vulnerable to cases of species substitution. The most common substitutions in Italian fish market are reported in Table 1.1.

Fish species identification is traditionally based on external morphological features, including body shape, pattern of colours, scale size and count, number and type of fin and rays and various relative measurements of body parts. Yet, in some cases morphological features are of limited value for identification and differentiation purposes, even with whole specimens, because they can show either considerable intraspecific variations or small differences between species (Teletchea, 2009). Moreover, the respect for labelling regulations becomes complicated in processed food such as frozen fillets and precooked seafood because the original identifying morphological characteristics are absent. Therefore, the development of protocols for assurance and control of seafood safety is currently a major challenge. To improve detection of commercial seafood fraud, a variety of protein and DNA-based technique have been developed (Rasmussen and Morissey, 2008).

High commercial value species	Replaced with…
Solea vulgaris	*Pleuronectes platessa, Pangasius* sp.*, Oreochromis* sp.*, Hippoglossus hippoglossus*
Pleuronectes platessa	*Limanda* sp.
Perca fluviatilis	*Lates niloticus*
Dentex dentex	*Pagrus* sp.*, Pagellus* sp.
Epinephelus sp.	*Pangasius* sp.*, Oreochromis* sp.
Dicentrarchus labrax	*Pomatomus saltatrix*
Mustelus sp.	*Heptranchias perlo*
Xiphias gladius	*Mustelus* sp.*, Prionace glauca, Squalus* sp.
Sardina pilchardus	*Neosalanx tangkahkei*

Table 1.1 Common substitutions in Italian fish market

1.6.2 Protein-based methods for seafood and fish species identification

Analytical diagnosis of fish and seafood using proteins has traditionally been based on species-specific electrophoresis, chromatography, or immunological assays.

Chromatographic approach comprises techniques such as HPLC (High Performance Liquid Chromatography) and gas-chromatography, which require, however, expensive analysis instruments. The identification is based on the different sarcoplasmic proteins or amino acids profiles between meats from different species (Knuutinen and Harjula, 1998; Armstrong *et al*., 1992).

Isoelectric focusing (IEF) technique has been used, for example, to identify four freshwater fish commercially labelled "perch" (Berrini *et al*., 2005) and puffer fish species (Chen *et al*., 2003). Puffer fish proteome was also analyzed by two-dimensional electrophoresis technique (2DE), which resulted useful to discriminate harmless species from the ones that accumulates lethal level of tetrodotoxin in their muscle (Chen *et al*., 2004). In general 2DE resulted powerful to distinguish among closely related species, as it is reported by Pineiro *et al*. (1998) that characterized the water-soluble protein profiles of eight gadoid species.

At present there are only few examples of rapid immunoassay, such as a strip test or enzyme-linked immunosorbent assay (ELISA) kit, capable of identifying species of fish, even if this technique could be easy and fast to apply. The issue is obtaining species-specific antibodies for each fish product liable to fraud and it is essential that antibodies don't cross-react with non-target species. Gajewski *et al*. (2009) realized monoclonal antibodies for rapid identification of two species of Pangasius, both commercially labelled as catfish, but with considerable differences in their meat quality. Asensio *et al*. (2003a and b; 2008) developed ELISA and strip immunoassay with polyclonal and monoclonal antibodies to identify grouper (*Epinephelus guaza*), Nile perch (*Lates niloticus*) and wreck fish (*Polyprion americanus*) fillets and they demonstrated the effectiveness of the method directly in fish markets.

Protein-based methods are generally reliable only on fresh or frozen tissue because intense heat processing or drying can destroy the biochemical properties and structural integrity of proteins, make the analysis impractical. Also other characteristics, reported in the following paragraph, make DNA the most suitable molecule for fish species identification.

1.6.3 DNA-based methods for seafood and fish species identification

DNA-based identification methods present several advantages over protein analysis (Teletchea, 2009):

   I. DNA is more resistant and thermostable than proteins and it is possible to amplify by PCR (Polimerase Chain Reaction) very small fragment with sufficient information to allow identification;

  II. DNA is present in almost all cells of an organism, so it can be recovered from any substrate;

 III. because of the degeneracy of the genetic code and the presence of many non-coding regions, DNA provides more information than proteins do;

 IV. DNA is always the same, regardless tissue type, age, nutritional and physiological state.

Genetic species identification is based on the principle of DNA polymorphism, or genetic variations that take place as a result of naturally occurring mutations in the genetic code. Determination of fish and seafood can be carried out using either nuclear DNA (nDNA) or mitochondrial DNA (mtDNA). Some major advantages of mtDNA over nDNA are that it is relatively small compared to nDNA because it lacks features such as large noncoding sequences (introns), pseudogenes, repetitive DNA and transposable elements; it is relatively easy to extract; it does not undergo genetic rearrangements such as recombination and sequence ambiguities resulting from heterozygous genotypes avoided. Moreover, mtDNA presents a higher copy number and a faster rate of mutation, making it generally more appropriate in the study of evolutionary genetics and inter-intraspecies variability. However, high intraspecies variation can become a disadvantage to species diagnostic methods and the maternal inheritance pattern of mtDNA may produce misleading results in the event of species hybridization. The most common mtDNA genes exploited in species identification research have been cytocrome b, 12S and 16S rRNA (Rasmussen and Morrisey, 2008).

Despite the advantages of mtDNA, a number of nDNA targets have proven to be successful in the differentiation of fish and seafood species, such as nuclear 5S rRNA, p53 gene, nuclear ribosomal internal transcribed spacer locus, 18S rRNA gene and major histocompatibility complex class II gene. In addition, nDNA also contains tandem repeated segments of DNA that occur throughout the genome and exhibit a high degree of polymorphism called satellite, minisatellite and microsatellite (Rasmussen and Morrisey, 2008).

The first step of fish identification is always based on PCR carried out with universal primer or species specific primers designed, for example, on the basis of single nucleotide polymorphisms. Following PCR amplification, the resulting DNA fragments must be properly analyzed to verify the presence or absence of species-specific genetic markers. Some methods include restriction fragment length polymorphism (RFLP), forensically informative nucleotide sequencing (FINS), amplified fragment length polymorphism (AFLP) or single strand conformational polymorphism (SSCP). All these technique are reviewed with many literature examples by Rasmussen and Morrisey (2008). In recent

years, several online resources have been developed for specific use in the field of DNA-based identification of fish and seafood species, like the FISH-BOL (http://www.fishbol.org/), which is part of the Consortium for the Barcoding of Life (CBOL; http://www.barcoding.si.edu/). The purpose of CBOL is sequencing the mt COI gene in all biological species.

1.6.4 5S rRNA gene: special features for fish species identification

Among the aforementioned genes, 5S rRNA is a very suitable target for easy, rapid and cheap fish species identification, because it does not require post-PCR analysis methods. This gene, represented in Fig. 1.3, consists of a small 120 bp conserved region coding for 5S rRNA and a variable region of noncoding DNA termed nontranscribed spacer (NTS) that has a species-specific length and sequence (Aranishi, 2005). Conserved region and NTS are repeated several times in the genome. Due to the rapid mutation rate of the NTS region, 5S rRNA amplicons can often be differentiated for the species simply by visualizing the fragment length using gel electrophoresis, without the need for further analysis such as sequencing or RFLP (Moran and Garcia-Vazquez, 2006).
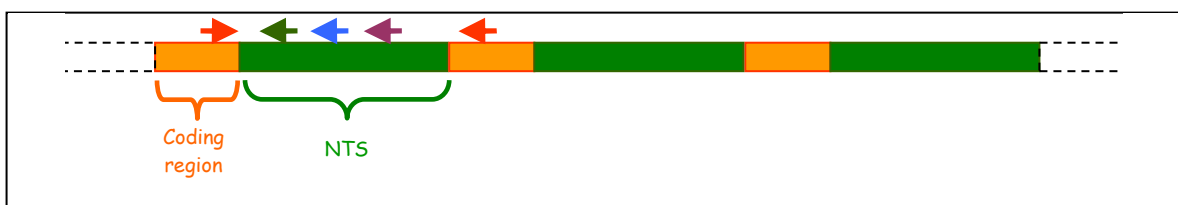
Figure 1.3 Schematic representation of 5S rRNA gene: the coding region and the NTS are represented in orange and green respectively. Orange arrows represent universal primer designed on conserved sequence. The other arrows represent species-specific primers, which could be designed on NTS of different species.

Nuclear 5S rRNA has been used to identify mackerel, gadoids, salmonids and others species (Karaiskou *et al.*, 2003; Aranishi and Okimoto, 2004; Moran and Garcia-Vazquez, 2006; Carrera *et al.*, 2000; Lockley *et al.*, 2000; Cespedes *et al.*, 1999) and it has been effective in recognition of a variety of samples including larvae, eggs, frozen and canned food (DeSalle and Birstein, 1996).

In this thesis we decided to use 5S rRNA as target for identification of fish species commonly found in Italian fish market. We performed PCR with species-specific primers designed on NTS sequences, capable to give the amplicon only when the DNA of corresponding species was used as template. Therefore, after the initial effort of cloning and sequencing the NTS of the species of interest, we have demonstrated the value of this approach.

The analyzed species in this work are: sea bass (*D. labrax*), Nile perch (*Lates niloticus*), perch (*Perca fluviatilis*), swordfish (*Xiphias gladius*), bluefin tuna (*Thunnus thynnus*), plaice (*Pleuronectes platessa*), sole (*Solea vulgaris*), Atlantic salmon (*Salmo salar*), trout (*Salmo trutta*) and pangasius (*Pangasius hypophthalmus*).

1.6.5 5S rRNA gene: genome organization, structure, interactions and biological functions

Small non-coding RNAs are a topic of great interest for molecular biologists because they can be regarded as relics of a hypothetical "RNA world" which preceded the modern stage of organic evolution on Earth.

Ribosomes are large ribonucleoprotein (RNP) particles consisting of two unequally sized subunits that associate upon the initiation of translation. Their role is to provide an appropriate environment for the correct positioning of mRNA, tRNAs and translation factors during the decoding process, as well as catalytic activity for peptide bond formation. In bacteria, the large ribosomal subunit (50S) is composed of two rRNA molecules (5S and 23S rRNAs) and 34 proteins. The eukaryotic 60S subunit contains three rRNAs (5S, 28S and 5.8S) and 50 proteins. The small subunit in bacteria (30S) and eukaryotes (40S) contains a single rRNA, of 16S and 18S respectively. The number of proteins in the small subunit varies from 21 in bacteria to over 30 in eukaryotes. The small ribosomal subunit is responsible for decoding, whereas the large subunit performs catalytic functions.

In higher eukaryotes, tandem arrays of ribosomal RNA genes are organized in two distinct multigene families composed of hundreds of thousands of copies. One class is

represented by the 45S rDNA that codes for the 18S, 5.8S and 26S/28S rRNAs and the other one, represented by the 5S rDNA, codes for the 5S rRNA, an element of the largest subunit of ribosomes. The 5S rDNA repeats consist of 120 base pairs coding sequences, which are separated from each other by a non transcribed spacer (NTS) that shows an accentuated length variation. The copy number of the 5S rRNA genes is highly variable among vertebrates.

The genomic organization of the 5S rRNA genes is known on several eukaryote organisms. The accumulating data demonstrate that 5S RNA genes are highly conserved, even among non related taxa, both with respect to length and nucleotide sequence, whereas the NTS evolves more rapidly. Different 5S rDNA classes, which differs each other for the NTS sequence, have been observed in mammals and fish species (Hallemberg *et al.*, 1994; Martins and Galletti, 2001); in the bony fish, two distinct 5S rDNA classes were characterized by distinct NTSs and base substitution in the 5S rRNA gene; thus, possession of two 5S rDNA classes seems to be a general trend for the organization of these sequences in the genome of fish, even with some exceptions due to polyploidy (Qin *et al.*, 2010).

The transcription of 5S rRNA gene is performed by non-nucleolar RNA polymerase III and requires transcription factors like TFIIIA, B, C; an internal control region (ICR) functions as promoter for the gene and a TATA sequence, located in the NTS, plays an important role in regulation of 5S rRNA gene expression in mammals (Hallemberg *et al.*, 1994) and it has been also observed in fish (Qin *et al.*, 2010).

In eukaryotes, the 5S rRNA molecule binds ribosomal protein L5 and 28S rRNA, whereas in bacteria it interacts with three different ribosomal proteins and 23S rRNA. The interactions with ribosomal proteins and transcription factors determine the stability of 5S rRNA and its transfer inside the cell to the place of ribosomal subunit assembly. 5S rRNA was found to be absent from the mitochondrial ribosomes of some fungi, vertebrates and most protists. Thus, 5S rRNA requires also mechanism of import into mitochondria (Entelis *et al.*, 2001; Smirnov *et al.*, 2008).

5S rRNA is supposed to play an important role during protein synthesis on ribosomes, but its function is still not clearly elucidated. Based on the results of cross-linking

experiments, it was suggested that it may serve as a signal transducer between the peptidyltransferase centre and domain II, responsible for translocation, or as a determinant of large-subunit stability. Its importance for the protein biosynthesis machinery was demonstrated in *Escherichia coli*, in which deletion of more than one 5S rRNA gene greatly impairs the growth rate (Ammons *et al.*, 1999).

The genome organization, structure, interactions and biological functions of 5SrRNA have been reviewed by Smirnov *et al.* (2008), Szymanski *et al.* (2003) and Barciszewska *et al.* (2001).

2. Materials and Methods

2.1 BDNF: gene structure characterization; mRNA and protein quantification after stress

2.1.1 Animals

European seabass eggs, at stage of somites formation, were obtained from a commercial hatchery in Crete. The eggs were incubated in three 500 l-cylindriconical polyester tanks (~50,000 eggs/tank) at the Institute of Aquaculture of the Hellenic Center for Marine Research (Heraklion, Crete). During the autotrophic stages (complete absorption of lecith reserves) larvae were kept in darkness. The oxygen level was maintained at about 7 mg/l throughout the experimental period. Following mouth opening and eye development, the larvae, under intensive conditions, are exposed to low light intensity (5-10 lux) without food for a period of 2-4 days, until swim bladder is fully inflated. Only when inflated swim bladder is observed in more than 80% of the population, larvae were fed using an automatic feeding system. Ten larvae were taken every day for determining the morphological characteristic and total length. The general conditions of rearing are presented in the Table 2.1, while the modality of the sampling, carried out in November 2008, is reported in Table 2.2. Three pools of larvae for each developmental stage were randomly sampled and weighted. The samples were stored in RNAlater (Ambion, Austin, TX, USA) and kept at -20°C until the molecular biology analysis.

Seasons of rearing	Winter – Spring
Density of eggs	100 egg l^{-1} (INTENSIVE REARING)
Water quality	Tank filled with filtered sea water from deep drill; renewal from biological filter ; pseudogreen water method (Papandroulakis *et al.*, 2001); closed recirculation system; controlled temperature and light
Temperature range	Constant 17,5±1°C
Water renewal rate	Initially 10% h^{-1}, gradual increase to 40% h^{-1} at 35 dph
Photoperiod	12light:12dark
Larval food	Enriched rotifers (5 ind ml^{-1}); enriched *Artemia* Instar II (0.5-1.0 ind ml^{-1}); artificial feed. Daily addition of phytoplankton (in order to maintain a concentration of ca. 650±300x10^3 cells ml^{-1}) for 15 days after hatching

Table 2.1 Conditions of rearing

Fingerling sea bass were obtained from NuovaAzzurro® hatchery in Civitavecchia (RM, Italy), and reared into three fiberglass raceway tanks with 2.5 m^3 water each, with inconsistent mortality, at low biomass density (<10 Kg/m^3). The tanks were connected to a water recirculation system where salinity (obtained adding salt Oceanfish 600 LT from Prodac Int® to dechlorinated tap water) was 20 g/l. Other water conditions were: temperature 21 ± 1°C, pH 8.2, total ammonia <0.2 mg/l; dissolved oxygen was maintained over 99% of the saturation, by insufflating pure O$_2$ to the system. At average weight of 450 g (adult animals), two groups of fifteen animals were randomly sampled. The first group (control) was rapidly killed by severing the cervical column; brain, liver, kidney and muscle were removed, frozen in liquid N$_2$ and stored at -80°C for molecular biology analysis. The second group (stressed) was kept for 30 minutes in a water deprivation condition (water volume of 20 l in a bucket 50 × 50 × 50 cm), then tissues were removed as described above. The experimental protocol of this study was approved by the Ethics Committee of the University of Insubria.

SAMPLES	Dph	stage	total weight
1a; 1b; 1c	6	Mouth opened-black eyes	404 mg (wet)
2a; 2b; 2c	16	Lipid droplet absorption	300 mg (wet)
3a; 3b; 3c	27	Nothocord flexion	961 mg (wet)
4a; 4b;4c	33	Post-flexion	949 mg (wet)
5a; 5b; 5c	44	Dorsal and anal fins	1 g (wet)

Table 2.2 Sample timing; dph=days post hatching

2.1.2 Isolation and amplification of genomic DNA

Genomic DNA was extracted from 25 mg of liver with DNeasy Blood & Tissue Kit (Qiagen, Milan, Italy) according to the manufacturer's procedure. Introns were amplified with primers designed in proximity of putative exon/intron junctions, inferred comparing ortologue sequences of BDNF (Genomic PCR section). Primers used for BDNF

amplification are reported in Table 2.3. The PCRs were set using 500 ng of genomic DNA, Herculase Enhanced DNA Polymerase 5U/µl (Stratagene, La Jolla, CA, USA) in its own buffer. The couple of primers used were Dl_ex1beta_fw and Dl_ex1a_rev, Dl_ex1a_fw and Dl_ex1c_rev, Dl_ex1c_fw and Dl_P2_ant_BDNF. The reactions were incubated in a thermal cycler at the conditions suggested in the manufacturer's procedure. PCR fragments were run on a 0,7% agarose gel, stained with ethidium bromide and run in TAE 1× buffer at 100 mV for 30 min. Single bands were gel-purified and sequenced. Another set of PCRs on genomic DNA were performed with 80 nM solutions of specific primers deduced on obtained sequences, 250 ng of genomic DNA, 2 U of PCR Extender Polymerase Mix (5PRIME,Gaithersburg, MD, USA), 5 µl 10× Tuning Buffer, 0,5mM dNTPs mix. The PCR was performed with the following conditions: 93°C for 3 min and 10 cycles at 93°Cfor 15 s, annealing at 65°C for 30 s elongation at 68°Cfor 8 min, plus 20 cycles with elongation time increased of 20 s each cycle. After gel electrophoresis single bands were gel-purified, cloned into pGEM-T Easy Vector (Promega, Milan, Italy), and sequenced.

2.1.3 5' Genome walking

To clone 5' flanking sequence of the gene, genome walking was carried out with the Genome Walker Universal Kit (Clontech, Saint-Germain-en-Laye, France) according to the manufacturer's procedure. Briefly, aliquots of genomic DNA (2.5 µg) were separately digested overnight with the following blunt-end restriction endonucleases: DraI, EcoRV, PvuII and StuI. After inactivation, the four digested DNA preparations were ligated to the Genomic Walker adaptors. Two rounds of PCR were performed with the BD Advantage 2 PCR kit (Clontech, Saint-Germain-en-Laye, France). Adaptor-ligated DNA fragments were used as template for primary PCR amplification, with the outer adaptor primer (AP1) and a gene specific 5'-outer primer (Table 2.3, Genome Walking section). Reactions were run using 0.2 µM solution of specific primers, 1 µl of template, 1 µl of 50× Advantage 2 Polymerase Mix, 5 µl 10× Advantage2 PCR buffer, 0.2 mM dNTPs mix. The amplification protocol consisted of two-step cycle parameters: 7 cycles at 95°C for 25 s and 72°C for 3 min, 37 cycles at 94°C for 25 s and 67°C for 3 min plus a final extension at 67°C for 7 min. Aliquots (1 µl) of 50-fold diluted primary PCR products were used as template in the

secondary PCR amplification, with the nested adaptor primer (AP2) and a nested gene-specific primer (Table 2.3, Genome Walking section) with the same reactions mix described above. The amplification protocol consisted of two-step cycle parameters: 5 cycles at 95°C for 25 s and 72°C for 3 min, 24 cycles at 94°C for 25 s and 67°C for 3 min plus a final extension at 67°C for 7 min. Amplified products were analyzed in 1% agarose gel and sequenced as above reported.

2.1.4 Endonucleases digestion

The 3 Kb PCR product obtained with the primers Int1a/1c_Fwnew and Int1a/1c_REV_5 was very tricky in cloning and sequencing steps because of the presence of highly repeated region. For these reasons a blunt digestion, with 1 U of HaeIII/1 µg of PCR product, was performed in order to obtain smaller fragments. The reaction was incubated at 37°C for 2 h. The four bands obtained from the digestion, of 1.5 Kb, 1 Kb, 0.4 Kb, 0.1Kb respectively, were gel purified, A-tailed with DNA Polymerase, ligated into pGEM-T Easy Vector (Promega, Milan, Italy), and sequenced.

2.1.5 RNA extraction, mRNA retro transcription and amplification

Total RNA was extracted with TRIzol Reagent (Invitrogen, S. Giuliano Milanese, MI, Italy) from about 100 mg of each pool of larvae and tissue following the manufacture's instruction, then treated with DNase (DNA free, Ambion, Austin, TX, USA). The first strand cDNA was synthesized using 2 µg of total RNA, 150 pmol random primers (for larvae's RNA) and dT16 primer (for tissues' RNA), 1 µl dNTPs mix 10 mM, in a volume of 12 µl. The mix was heated at 65°C for 15 min, chilled on ice and then 4 µl 5× retrotranscription buffer, 2 µl of 0.1 M DTT, 1 µl RNaseOUT and 200 U M-MLV retrotranscriptase (Invitrogen, S. Giuliano Milanese, MI, Italy) were added to a final volume of 20 µl. After incubation at 37°C for 50 min, the reaction was stopped at 75°C for 15 min. The generated cDNA was stored at -20°C.

The open reading frame was obtained by RT-PCR performed with specific primers Dl_BDNF_up and Dl_BDNF_down designed within conserved regions of BDNF coding sequence belonging to other species. The bipartite BDNF transcripts were evaluated,

when necessary, by two rounds of PCR with primers deduced on the obtained exon sequences. Reactions were run using 1 µM solution of specific primers (Table 2.3, Qualitative PCR section), 1 µl of cDNA, 0.75 U of GoTaq DNA Polymerase (Promega, Milan, Italy), 5 µl 5× Green GoTaq Reaction buffer, 0.2 mM dNTPs mix. The first round PCR was performed with the following conditions: 94°C for 3 min and 34 cycles at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 50 s and final extension at 72°C for 4 min. The second round PCR was performed on 1 µl of first round PCR product for 30 cycles at the same conditions. The PCR products were loaded into 1% agarose gel stained with ethidium bromide and run in TAE 1× buffer at 100 mV for 30 min; β-actin and GAPDH were used as housekeeping genes. For each sample a set of PCR has been run without retrotranscription to exclude any genomic contamination.

2.1.6 5' and 3' Rapid Amplification of cDNA Ends (RACE)

The 5'-RACE was performed according to the method published by Semple-Rowland *et al.*, with slight modifications. Briefly, 1 µg poly-A+ RNA, extracted from sea bass brain, was reversed transcribed using 200 U M-MLV reverse transcriptase (Invitrogen, S. Giuliano Milanese, MI, Italy) following the manufactured instruction and using 20 pmol of sequence-specific antisense primer RACE_BDNF_GSP1. The reaction was incubated at 42°C for 50 min and stopped placing the tube on ice; excess primers, dNTPs and buffer were removed using a QIAquick PCR purification kit (Qiagen, Milan, Italy). In the final step of the procedure the DNA was eluted in 30 µl of water. A poly dCTP tail was added to the single-stranded cDNA present using terminal deoxynucleotidyltransferase (Promega, Milan, italy). The mixture was denatured at 94°C for 3 min, chilled on ice, incubated at 37°C for 10 min and stopped at 70°C for 10min; excess of dCTP and buffer was removed as reported above. Second strand cDNA synthesis was carried out using 5 U Taq Polymerase (Qiagen, Milan, Italy), 0.2 µM of a poly d(G) anchor primer (RACE_AAP), 200 mM dNTPs mix and 10× PCR buffer. The reaction was incubated in a thermo cycler at the following conditions: 40°C for 5 min, 72°C for 2 min, than the temperature was increased at 80°C. At this point 0.2mM of the nested sequence-specific primer RACE_BDNF_GSP2 and a nested anchor primer RACE_AUAP were added for the amplification at the

following conditions: 94°C for 1 min, 54°C for 1 min, 72°C for 1 min (30 cycles), last extension time 72°C for 10 min; kept at 4°C. 1 µl of a 1:10 dilution of the PCR products is re-amplified using the nested anchor primer RACE_AUAP and the nested sequence-specific primerRACE_BDNF_GSP3. The PCR cycle parameters were as follow: 8 touchdown cycles with annealing temperature from 58 to 54°C, than 94°C for 1 min, 54°C for 1 min, 72°C for 1 min (27 cycles), last extension time 72°C for 10 min; kept at 4°C. The resulting products were run on a 1% agarose gel, purified, cloned into pGEM-T Easy Vector (Promega, Milan, Italy) and sequenced. The 3' race was performed with the following protocol: 4 µg of total RNA and 10 pmol of Adapter Primer (AP) in a volume of 10 µl were incubated at 70°C for 10min. The mix was chilled on ice and then 4 µl of 5× reverse transcription buffer, 2 µl of 25 mM $MgCl_2$ solution, 1 µl of 10 mM dNTPs mix and 2 µl of 0.1 M DTT were added. The mix was incubated at 42°C for 5 min and then 200 U of SuperScript III reverse transcriptase (Invitrogen, S. Giuliano Milanese, Italy) were added. After incubation at 42°C for 50 min, the reaction was stopped at 70°C for 15 min. The generated cDNA (2 µl) was used as template for PCR. The reactions were run using 1 µM solution of Universal Amplification Primer (UAP) and gene specific 3'-outer primer (SP1FW_3'), 0.75 U of GoTaq DNA Polymerase (Promega, Milan, Italy), 10 µl 5× Green GoTaq Reaction buffer, 0.2 mM dNTPs mix. The reaction was incubated in a thermo cycler at the following conditions: 95°C for 2' and 30 cycles at 95°C for 30 s, annealing depending on the melting temperature of the primers for 30 min, elongation at 72°C for 2 min and final extension at 72°C for 6 min. A second round PCR was performed at the same conditions using 1 µl of first PCR product, 1 µM solution of UAP and a nested gene-specific primer (SP2FW_3', BDNF_3'race). The resulting products were run on a 1% agarose gel, purified, cloned into pGEM-TEasy Vector (Promega, Milan, italy) and sequenced.

	NAME	SEQUENCE 5' → 3'	Tm °C	Notes
Genome Walking	1beta_SP1	CCACTGAGTCCAACCCTTCCAGCAATGC	72.2	3
	1beta_SP2	ACCATTTTCCCCTACGCTGTCCTGGAGATAG	70.2	3
	1beta_SP3	ACTTCTGCTGTGCTCAGTAGATCGCCCACC	71.9	3
	1beta_SP4	GGCAAATATCAACAAGCCCGGGTTGTCAG	72.0	3
	1beta_SP5	GGCAATCCAAGTTTGTGGGGGTACTAGTTC	68.3	3
	1beta_SP6_A	GAGTGTTAACTCCCTCTTTGGCGAGGGG	69.7	3
	1beta_SP6_B	GGCCTATTACGCATACGCACAAACTGGTC	69.0	3
	AP1	GTAATACGACTCACTATAGGGC	59.0	4
	AP2	ACTATAGGGCACAGCGTGGT	71.0	4
Genomic PCR	Dl_ex1beta_fw	CCAAGTGGTGGGCGATC	57.6	2
	Dl_ex1c_fw	CCATGCAATTTCCACCATC	54.5	2
	Dl_ex1a_fw	GTTAACTTTGGGAAATGCAAG	54.0	2
	Dl_P1_ant_BDNF	CCATAGTAACGAACAGGATG	55.3	2
	Dl_P2_ant_BDNF	GTCATCACTCTTCTAACCTGTTG	58.9	2
	Dl_ex1a_rev	CACTTGCATTTCCCAAAGTTAAC	54.0	2
	Dl_ex1c_rev	GATGGTGGAAATTGCATGG	54.5	2
	Int1c/2_FW	CCAGACAGTTTCTGTATTGTTGTTTTGGAGGGG	70.2	3
	Int1c/2_REV	TCCAGCCATGTGAGGATCAATTGTGAACGG	73.6	3
	Int1c/2_up	GTATTTTCTTAATTGCACACAGCGTGGGTGGG	71.4	3
	Int1c/2_low	CATTCTTAATTGGTATCTGGGGCCGTGGC	71.0	3
	Int1c/2_FWnew	CTCTAGGTGCGTTGTCATGCACAAAGGC	69.8	3
	Int1c/2_REVnew	AGGGGTAATATTGCAGTAGCAGGGGGTGG	70.0	3
	Int1a/1c_FW	ATGCTCCCAATATGGGACCTTAAGACGCTGC	72.2	3
	Int1a/1c_FWnew	GTAATCGTTGCGTTGTGCTTAATCATGCTCC	66.8	3
	Int1a/1c_FW1	GGTCTGCTGCATTCATGTTTTGTCTTGATG	68.7	3
	Int1a/1c_FW2	GCCCTACTCTTTACCCCCCCCCACCC	70.2	3
	1crev_SP1	TCTCCCGACAAGCTTCAGGATATCTCTTCAGC	70.8	3
	Int1a/1c_REV_1	TTTTGCGTAACIGCGCGTCTCCACCAIGTC	74.5	3
	Int1a/1c_REV_2	CAAACTCCTGGATATGAGCTTAAAGGAGGC	66.4	3
	Int1a/1c_REV_3	CGTTTGGCATGTAGCAGTATGGGAGTGG	69.3	3
	Int1a/1c_REV_4	CCTTTCAAGGCTTCTCTTGCCAAATGC	68.2	3
	Int1a/1c_REV_5	CATCCTGCCAGCATGTGCAACTGC	69.6	3
	Int1a/1c_REV_6	CCTGACTCACTTTTAGCCTATCTGACATGCAGG	69.3	3
	Int1a/1c_REV_7	CACACACACACACACACACACACTGTG	68.3	3
	Int1beta_FW	GACCAGTTTGTGCGTATGCGTAATAGGCC	69.0	3
3'-RACE	SP1FW_3'	GGCTGCAGAGGAATAGACAAGCGGCAT	70.4	3
	SP2FW_3'	CCAATGCAGGACAACCCAGTCCTACGT	69.3	3
	BDNF_3'race	GACCATTAAGAGGGGCAGATAG	60.3	3
	AP	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTTT	71.1	4
	UAP	CUACUACUACUAGGCCACGCGTCGACTAGTAC	64.3	4
5'-RACE	RACE_BDNF_GSP1	CTTGGTTGCTGATCATC	50.4	3
	RACE_BDNF_GSP2	CTGTGAGTGAGGGCAGTTC	58.8	3
	RACE_BDNF_GSP3	CGAACAGGATGGTCATCACTC	59.8	3
	AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	>75.0	4
	AUAP	GGCCACGCGTCGACTAGTAC	66.0	4
Qualitative and semiquantitative PCR	Dl_BDNF_up	ATGACCATCCTGTTCGTTAC	55.3	2
	Dl_BDNF_down	CTATCTGCCCCTCTTAATG	54.5	2
	1beta_FW_nested	GCGAGGGTGTTACGTATATCTG	58.7	3
	1beta_Rev_new	CCACTCACTCCAACAGATGC	59.3	3
	1a_FW_new	GCTTATTCTGAGGGAGCCTG	59.0	3
	1a_Rev_new	CCCAAAGTTAACGCAGTGTG	59.2	3
	1bFW	CTCAGCTCTGCAGAGTTGGGGT	61.8	3
	1c_FW_new	CGTTTCACCATGCGACAAC	61.1	3

	1c_Rev_new	GCCCAGTCGTAAAACAGACC	59.6	3
	1dFW	GTCCTGATGGAAACAGGAAATCAC	63.1	3
	1dRev	CACAGATGACGTCTCTTCCAGGT	62.9	3
	Ex2_FW_new	CTTCAGTTGCATGAGAGCTGC	61.3	3
	Ex2_Rev_new	ACCCTCATGCACATATTAGCG	60.0	3
	Dl_BDNFreal_low	TTGCTTCAGTTGGCCATTGG	57.3	3
	Dl_Act_FW_RT	GGTATTGTCATGGACTCCGGTGAT	61.9	1
	Dl_Act_Right	TTAGAAGCATTTGCGGTGGA	58.0	1
	D.l._GAPDH_FW	GAGGGTGACAAGCTGGTCGT	58.8	1
	D.l._GAPDH_Rev	CAAAGATGGAGGAGTGAGTGTCAC	58.8	1
Universal Primers	T7	TAATACGACTCACTATAGGG	53.2	
	SP6	CATTTAGGTGACACTATAG	50.2	

Table 2.3 Primers used for BDNF amplification: 1 - primers deduced on sequences available in public databases (Actin: AY148350; GAPDH: AY863148). 2 - primers deduced on ortologue sequences (BDNF: *Danio rerio* AL935207 clone CH211-251J8; *Fugu rubripes* http://www.fugu-sg.org/ scaffold_1; *Paralichthys olivaceus* AY074888). 3 - primers deduced on obtained sequences. 4 - primers included in the kits used during the experiments.

2.1.7 Semiquantitative analysis

The bipartite BDNF transcripts expression, in control and stressed brain samples, were evaluated by semiquantitative PCR. The reactions were performed with the same specific primers and conditions of qualitative PCR, and normalization was carried out using cytoplasmatic β-actin (cDNA 1:50 diluted). The PCR products were loaded into 1% agarose gel and run in TAE 1× buffer at 100 mV for 30 min. The semiquantitative analysis was carefully performed by BIO-RAD Gel Doc 2000 connected to the software Quantity one™ that allowed to determine, in arbitrary units, the fluorescence value of the area of each considered band. After having obtained all the values, we have normalized them with those of β-actin; than we evaluated the ratio of the "stressed" samples compared to the control ones. In this way, we have avoided differences due to template concentration in the PCR tube. The data were statistically compared using the two tail omoschedastic Student's t-test. The significance level was set at $p < 0.05$.

2.1.8 Bioinformatic analysis

BDNF gene exon-intron boundaries were determined by Blast, ClustalW analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi;http://www.ebi.ac.uk/Tools/clustalw2/index.htm) and by direct comparison of PCR-amplified sequences with genomic pufferfish (http://www.fugu-sg.org/BLAST/Export.htm), zebrafish, human and rodent DNA from the NCBI database (GeneBank accession numbers: AL935207 clone CH211-251J8; AF411339; AY057907, respectively).

2.1.9 Western-blot analysis

Brain and liver proteins were extracted from control (N = 15) or stressed animals (N = 15) and immediately frozen in liquid nitrogen. The tissues were mechanically homogenized at 4°C using an extraction buffer solution containing 25 mM Tris HCl pH 7.5, EDTA 1 mM, Spermidin 1mM, PMSF 1 mM, IAA 1 mM, Soy Bean Trypsin Inhibitor (SBTI), 10 μg/ml Turkey Egg White inhibitor (TEWI). After homogenization 0.1% Triton X-100 was added and samples were incubated in agitation for 1 hour at 4°C. Samples were centrifuged for 5 min, at 4°C (10.000×g) and the soluble fraction (supernatant) of the lysate was collected for Western blot analysis. Total protein content in lysate tissue samples was determined using Bradford assay (Sigma-Aldrich). Samples (10 μg) were run in 15% SDS-PAGE and proteins were transferred onto a nitrocellulose membrane (Protran Nitrocellulose Transfer Membrane, Whatman) using transfer buffer solution [39 mM Glycine, 48 mM Tris-HCl, 0,037% (v/v) SDS, 20% (v/v) methanol]. Subsequently, the membrane was stained using Ponceau dye (Sigma-Aldrich) to check for the complete protein transfer. Membranes were cut at the level of 44 kDa according to protein markers. The two membranes were incubated for 1 hour at room temperature in blocking solution [4% (v/v) non fat milk powder, 0.05% Tween-20 in phosphate buffer saline solution]. The upper part of the membrane (>44 kDa) was incubated over night (O/N) at 4°C with anti-α-tubulin antibody (Sigma-Aldrich, mAB diluited 1:10.000). The lower part of the membrane (<44kDa) was incubated with anti-BDNF antibody (N-20, pAB, Santa Cruz Biotechnology, diluted 1:500). The anti-BDNF antibody recognizes the first 20 N-terminal aminoacids of mature BDNF and therefore is able to detect both the mature and the precursor form of

BDNF. Moreover, as the human and seabass mature BDNF are highly homolog (more than 90%) we have used human BDNF as positive control (Michalski *et al.*, 2003; Peng *et al.*, 2005). After O/N hybridization with the specific antibody, membranes were incubated with secondary antibodies for 1 hour at room temperature, we used goat anti-mouse HRP (Sigma-Aldrich, dil. 1:20.000) for α-tubulin, and goat anti rabbit HRP (Dako Cytomation, dil. 1:10.000) for BDNF. Finally, membranes were washed with blocking solution and immuno reactive bands were detected using a chemiluminescence system (ECL-advance, Amersham Biosciences).

2.1.10 Densitometry and statistical analysis

Densitometric analysis of immunoreactive bands was obtained by scanning films at 16-bit level and applying Quantity One software procedures (Biorad). Data were normalized using as internal control the Western blot for the housekeeping gene α-tubulin. The ratio ProBDNF vs total-BDNF or matBDNF vs total-BDNF was expressed as % and obtained with the formula: proBDNF/(proBDNF+matureBDNF)×100. Each set of data was statistically analyzed using Student's t-test and one-way ANOVA (Holm-Sidak). The statistical analysis was performed using Sigma Stat 3.1 software. A p value of 0.05 was set as the minimal level for statistical significance.

2.1.11 Calculation of test performance

We considered positive to the proBDNF/totalBDNF test, individuals whose score was >1SD with respect to the average value in the normal, non-stressed population. Stressed animals positive to test are true positive (= a), non-stressed animals which tested positive are false positive (= b), stressed animals that tested negative are false negative (= c) while non-stressed animals that tested negative are true negatives (= d). The sensitivity, calculated as a/(a+c), measures the proportion of actual positives which are correctly identified as such; and the specificity, calculated as d/(d+b), measures the proportion of negatives which are correctly identified. The positive predictive value is the probability that a test positive is a true positive: a/(a+b) and it is the most important measure of a diagnostic method as it reflects the probability that a positive test reflects the underlying

condition being tested for. Its value does however depend on the prevalence of the disease, which may vary. The negative predictive value is the probability that a test negative is a true negative: d/(c+d). The negative predictive value is the proportion of individuals with negative test results who are correctly identified.

2.2 5S rRNA gene: PCR-based method for fish species identification

2.2.1 Analyzed species

Most of fish were purchased at the local supermarket; *D. labrax* were reared at the DBSM animal facility (University of Insubria, Varese, Italy) and *P. fluviatilis* were provided by local farmer (Cooperativa Tinella, Varese)

Fillets, from three different fishes for each analyzed species were immediately frozen and stored at -20°C until the molecular biology analysis.

All the fish species analyzed are reported in Table 2.4.

Linnaeus classification	Common name	Processing
Dicentrarchus labrax	Seabass	The fishes have been bought as fresh fillets and immediately frozen and conserved at -20°C until the molecular biology analysis.
Lates niloticus	Nile perch	
Pangasius hypophthalmus	Pangasius	
Perca fluviatilis	Perch	
Pleuronectes platessa	Plaice	
Salmo salar	Atlantic salmon	
Salmo trutta	Trout	
Solea vulgaris	Sole	
Thunnus thynnus	Bluefin tuna	
Xiphias gladius	Swordfish	

Table 2.4 Fish species analyzed in this work

2.2.2 Isolation of genomic DNA

Total genomic DNA was prepared from frozen samples by the urea-SDS-Proteinase K method (Aranishi and Okimoto 2004). Briefly, about 100 mg of tissue from fillet were immersed in 500 µl of extraction buffer (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 2% SDS, 4M urea, 4,12 µl Proteinase K 800 U/ml), and incubated at 55°C for 1 h with shaking. One-tenth volume of 5 M NaCl was added and mixed; then an equal volume of phenol-chloroform-isoamyl alcohol was added and mixed. Following centrifugation at 10,000xg for 5 min, the upper aqueous phase containing DNA was collected and subjected to

ethanol precipitation. DNA pellet was washed twice with 70% ethanol, dried, and resuspended in 50 µl of 10T0.1E (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0).

The genomic DNA was quantified measuring the absorbance at the wave length of 260 nm and applying the following formula: (Abs260*ε*dilution factor)/1000= sample concentration (µg/µl), where ε is the molar extinction coefficient, which value for double strand DNA is 50.

2.2.3 PCR and DNA sequencing

All the primers used in this work are reported in Table 2.5.

20 ng of genomic DNA were amplified using 1 µM solution of 5s rRNA "universal" primers with 0.75 U of GoTaq DNA Polymerase (Promega, Milan, Italy), 5 µl 5× Green GoTaq Reaction buffer, 0.2 mM dNTPs mix in a final volume of 25 µl. The PCR was performed at the following conditions: 95°C for 2 min and 24 cycles at 95°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 30 s and final extension at 72°C for 4 min.

The products resulting from the PCR were run on a 1% agarose gel, purified, cloned into pGEM-TEasy Vector (Promega, Milan, Italy) and sequenced (BMR Genomics, Padova, Italy).

2.2.4 Sequences analysis and PCR amplification

The obtained sequences were aligned by ClustalW (www.ebi.ac.uk/Tools/clustalW2/index.html; Appendix 2) to design species-specific primers along not conserved region of the NTS (Table 2.5).

20 ng of genomic DNA were then amplified with the species-specific primers in the same described conditions.

	Name	Sequence (5' → 3')	Tm (°C)	Notes
Universal primers	Fish5S_FW	TACGCCCGATCTCGTCCGATC	60,05	1
	Fish5S_REV	CAGGCTGGTATGGCCGTAAGC	60.21	1
Species-specific primers	D.labrax_5SFW	GTTATCACTTGTCCACTGCAGG	59.66	2
	D.labrax_5SREV	TGAGCTTCACCTGCAAATGG	62.31	2
	L.niloticus_5SFW	GTAGTCGAGACAGGGCACGT	60.33	2
	L.niloticus_5SREV	TCATTCATTCGGCAGCAGT	60.37	2
	Pangasius_5S_FW	TGGGAGGAAGATTGACTAGAAAC	58.77	2
	Pangasius_5S_REV	CTAATTCAAATGAAGGCCTCAC	57.89	2
	Perca_5S_up	TCCCATTGTACAACAGCAG	55.50	2
	Perca_5S_low	CTTTAGAGTAGGTGAACACAAGG	55.04	2
	P.platessa_5SFW	TCAGTGGGTGGCTTCATTC	59.62	2
	P.platessa_5SREV	GTAAATACGTCAGGTAAAAGCGG	59.16	2
	Salmo_5S_up	GTGTGCCCCTGCACTATTTC	60.53	2
	Salmo_5S_low	GGCAGATGCTAATTGAAAACAC	58.77	2
	S.vulgaris_5SFW	GCACACGTTAACGTCCTTCT	57.88	2
	S.vulgaris_5SREV	GGGGACACAGACTCCTTTTATAG	58.22	2
	T.thynnus_5SFW	TGCGTGCGCTTTACAACTA	59.22	2
	T.thynnus_5SREV	CTATCCTTAATAACTTCCGTGTGC	58.37	2
	Trota_5S_up	GGAATTTCAACTCTTTGTCCG	58.68	2
	Trota_5S_low	CTTGCTTACTTATTTCAAAGCGAG	58.88	2
	X.gladius_5SFW	CTTACTCATTGGAAAAAAAGAGA	56.17	2
	X.gladius_5SREV	ACAGTGTCTGCTTCTGGATG	56.31	2

Table 2.5 Primers used for 5s rRNA amplification: 1 – primers deduced from literature (Pendas *et al.*, 1994); 2 – primers deduced on obtained sequences.

3. Results

3.1 BDNF: gene structure characterization; mRNA and protein quantification after stress

3.1.1 Genomic organization of BDNF

As the gene encoding BDNF in *Dicentrarchus labrax* (*D. labrax*) was not described before, we first cloned the entire gene and determined its genomic organization. We used a strategy of cloning each exon separately using PCR primers designed on a consensus sequence inferred from the ortologue sequences of BDNF in *Danio rerio* (zebrafish), and *Fugu rubripes* (pufferfish). Zebrafish, pufferfish, and seabass are all teleosts and therefore we expected a similar exon/intron organization of their BDNF gene and closely related sequences. To clone the 5' flanking sequence of the *D. labrax* BDNF gene, we carried out a genome walking. Finally, to determine the D. labrax BDNF gene exon/intron boundaries and identify the mRNAs transcribed from the gene, we performed a combination of 5' and 3' rapid amplification of cDNA ends (5' and 3' RACE), RT-PCR and bioinformatic analysis.

The gene spans about 15 Kb and it is organized in 6 exons and 5 introns as reported in Fig. 3.1A (GeneBank accession number FJ711591). Exons were identified by ClustalW analysis (see methods) as the most highly conserved segments and were all found to be flanked by the typical consensus splice donor (GT) site in eukaryotes. The exons length and position, and their exon/intron junctions are summarized in Table 3.1. In analogy with zebrafish and pufferfish, also in *D. labrax* the BDNF coding sequence is contained in the exon 2 and this tract resulted highly conserved with respect to other vertebrate species (*D. rerio* 84%, *F. rubripes* 91%, *H. sapiens* 77%, *M. musculus* 78%, *R. norvegicus* 78%,).

Upstream to the coding exon we have found other five untranslated exons: 1b, 1a, 1b, 1c and 1d. By aligning these exon sequences with those of the corresponding zebrafish exons, we found an identity of 85%, 43%; 82%; 74% and 82%, respectively. *D. labrax* BDNF transcripts analysis indicated that upstream untranslated exons can be spliced independently to the major coding exon to form distinct bipartite BDNF transcripts with different 5' UTR lengths and a common coding region (Gene-Bank accession number

DQ915807). Interestingly, in the exons 1β, 1b and 1d we have identified in-frame ATG codons that could be used as translation start sites leading to the prepro-BDNF proteins with longer N-termini (Fig. 3.1B).

The five exons located upstream to the coding region did not show any significant identity when aligned with mammalian BDNF genes (rat, mouse and human) with the exception of a 75% identity between *D. labrax* exon 1b and mammalian exon 1, and for the presence of the highly conserved segments HCS1, HCS2 and HCS3. HCS2 is located in *D. labrax* BDNF exon 1a and mammalian exon IIC and showed 96% identity; HCS1 in D. labrax BDNF exon 1c showed 38-41% identity with a similar sequence in mouse, rat and human exon IV while the HCS3 is localized in the 3'UTR of *D. labrax*, mouse, rat and human BDNF and was 97% identical (39 of 41 nucleotides are identical in fish and man) between these species (Fig. 3.2 and 3.3).

The coding region encoded a protein precursor (Fig. 3.4A) with a signal peptide at the N-terminus, the propeptide of 150 amino acids (AA) in the center and the mature BDNF of 129 amino acids at the C-terminus. This organization is similar to that of zebrafish (Hashimoto and Heinrich, 1997) avian (Maisonpierre *et al.*, 1992) and mammalian BDNF (Hofer *et al.*, 1990; Jones *et al.*, 1990; Maisonpierre *et al.*, 1991). The proBDNF resulted only 87% identical to zebrafish BDNF and 74-75% to the mammalian counterparts. However, two regions were >95% identical (Fig. 3.4B): the first 20 N-terminus AA, comprising the signal peptide, and 35 AA just upstream of the cleavage site which also encoded for the glycosilation consensus site (Fig. 3.4A). Analysis of the extended N-terminal sequences with the prediction programme SignalP 3.0 (Bendtsen *et al.*, 2004) showed that the N-termini produced by exons 1b and 1b have poor scores as signal peptides because of the presence of a putative signal anchor, while the very long sequence produced by exon 1d does not encode for a signal peptide.

A

15 Kb

ATG — ex1*f*A

HCS2 → ATG — ex1a

ATG — ex1b

HCS1 → ATG — ex1c

ATG — ex1d

ATG

HCS3 →

EX2

TAG

B

= Higly Conserved Sequence

= coding region

Prepro-BDNF N-termini	BDNF transcripts
MFHQVRRV**MTILFLTMVI**	1*f*A
MFHQVRRV**MTILFLTMVI**	1b
MFHQVRRV**MTILFLTMVI**	1d
MTILFLTMVI	all others

METGNHRDRNKIICCSRCEDTQGNGTQLHTVEWHYPQQPPPLHSLPYGDLEETSSVVFHQVRRV**MTILFLTMVI**
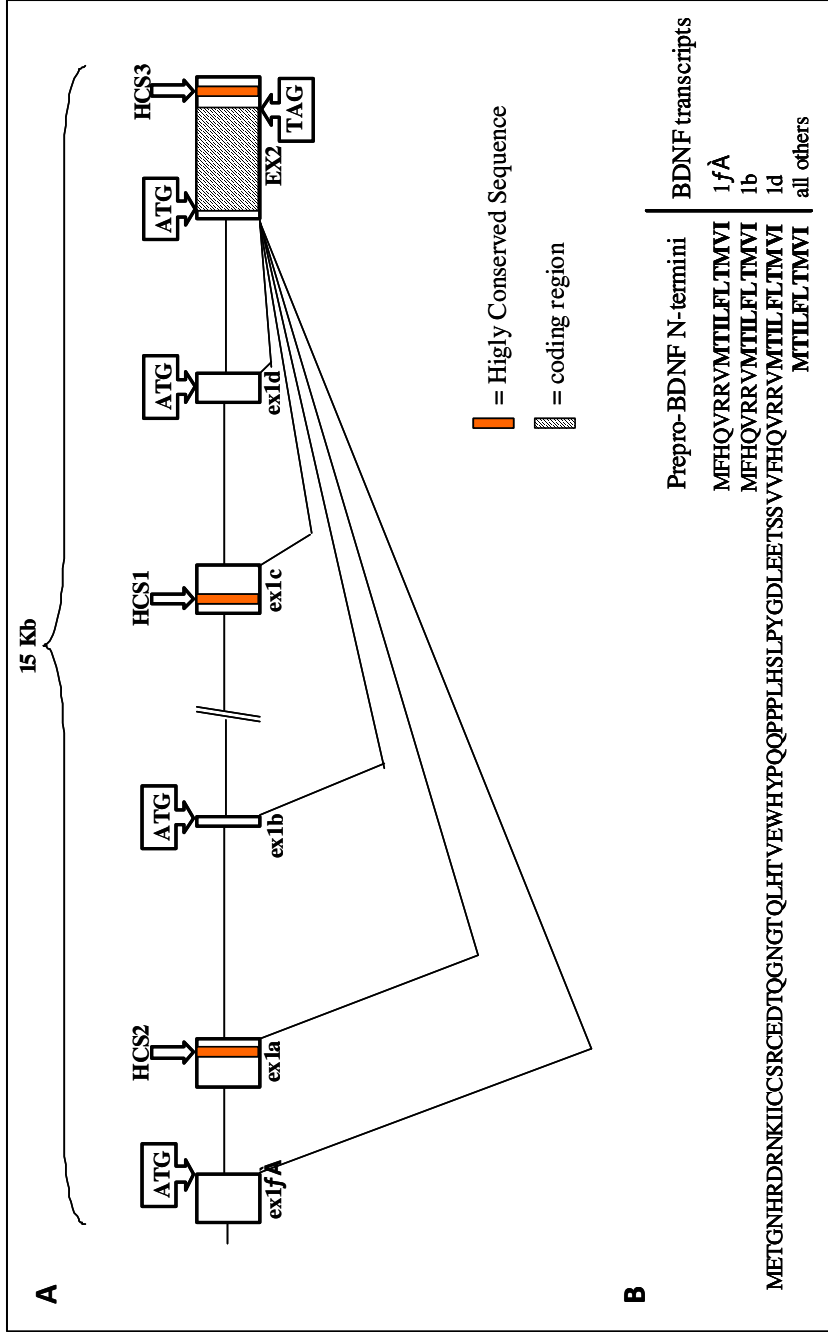
Figure 3.1 Gene organization and amino acid sequence of BDNF. Panel A. Organization of BDNF gene in *Dicentrarchus labrax*. Exons are shown as boxes and introns as lines. Alternative start codons (ATG) as well as stop codon (TAG) were reported. Panel B. Amino acid sequences of different potential prepro-BDNF N-termini. Amino acids encoded by exon 2 are in bold and sequences encoded by alternative in frame ATG are in normal style. The transcripts encoding the respective N-termini of BDNF (i.e., 1β, 1b, 1d) are listed adjacent to the N-terminal sequences.

Exon	Start	Splice acceptor	End	Splice donor	Length (bp)
1β	nt203	-	nt602	GGAAAATGgtaagtag	282
1a	nt1367	-	nt1818	TTGTAAAGgtaagagc	452
1b	nt4270	-	nt4315	ACCTGATGgtaggttt	46
1c	nt8347	-	nt8646	AGTAAAAGgtatgtgt	300
1d	nt11540	-	nt11797	CTGTGGTTgttatgct	258
2	nt14063	ccctccagTTCCACCA	nt15130	-	1068

Table 3.1 Structure of *Dicentrarchus labrax* gene. **Position numbering is based on gene sequence (FJ711591).**

HCS1 (38-41%)
```
R.n./M.m.    ATTACCTCCGCCATGCAATTTCC---ACTATCAATAATTTAA 39
H.s.         ATTACCTCCGCCATGCAATTTCC---ACTATCAATAATTTAA 39
D.r.         ATTACCTCAACCATGCAATTTCC---ACCATCAATAATTTAA 39
D.l.         GCAGCCATGGGAGTGCATTACCTCATACCATCAATAATTTAA 42
                 **       **** *  *    ** *************
```
HCS2 (96%)
```
R.n./M.m     GTTAACTTTGGGAAATGCAAGTGTT 25
H.s.         GTTAACTTTGGGAAATGCAAGTGTT 25
D.r.         GTTAACTTTGGGAAATGCAAGTGTT 25
D.l.         GTTAACTTTGGGAAATGCAAGTCTT 25
             ********************** **
```
HCS3 (97%)
```
D.r.         TATCTATTTGTATAT--ACATAACAGGGTAAATTATTCAGT 39
H.s.         TATCTATTTGTATATATACATAACAGGGTAAATTATTCAGT 41
R.n./M.m     TATCTATTTGTATATATACATAACAGGGTAAATTATTCAGT 41
D.l.         TATCTATTTGTATATATACATAACAGGGTAAATTATTCCGT 41
             **************   ******************** **
```

Figure 3.2 Alignment of Highly Conserved Sequence in man, rodent and fish. (H.s) *Homo sapiens*, (R.n.) *Rattus norvegicus,* (D.r.) *Danio rerio*, (D.l.) *Dicentrarchus labrax.*

Figure 3.3 Organization of BDNF gene in man, rodent and fish. Exons are shown as boxes and introns as lines. D.l.: sea bass BDNF; D.r.: zebrafish BDNF; M.m.: rodent BDNF; H.s.: human BDNF.

47

A

```
           aggaacagaatgattacagtttgcaaacaatttgaaaacactgatgacacagcgccaata
 61        tcagtttaataaaaaaacattctgtgattattccaaagctactgactcccttttttagtgt
121        gcaaagaaagagtggagggtgtaaataagaggaaaggcttacattgtgcattatggatca
181        agaataagctaacttcattgaaaatgctctttgactaaccgtcttgctctctctccccccc
241        tccttccctcttgttttctctctttctctcccccctccagttccaccaggttagaagagtg

301        atgaccatcctgttccttactatggttatttcatacttcagttgcatgagagctgcgccc
  1          M   T   I   L   F   L   T   M   V   I   S   Y   F   S   C   M   R   A   A   P
361        ctgagagacgcccccgggcatgcgggggccatcggacggaaggctacttgggcgccgctgcg
 21          L   R   D   A   P   G   M   R   G   H   R   T   E   G   Y   L   G   A   A   A
421        acggccgcgcgaggccatgggactccacagagtggtggtgggccaggccagcgcggggaa
 41          T   A   A   R   G   H   G   T   P   Q   S   G   G   G   P   G   Q   R   G   E
481        ctgccctcactcacagacacgtttgagcaggtgatagaggagctgctggaggtggaggga
 61          L   P   S   L   T   D   T   F   E   Q   V   I   E   E   L   L   E   V   E   G
541        gaggcggcacagctgggacaggggggctgataagagccaggagggtggggggcccatcctct
 81          E   A   A   Q   L   G   Q   G   A   D   K   S   Q   G   G   G   G   P   S   S
601        gtggtcaccacagaggccaaggatgtcgacctgtacgactcgcgggtgatgatcagcaac
101          V   V   T   T   E   A   K   D   V   D   L   Y   D   S   R   V   M   I   S   N
661        caagtgcctttggagccgccgttgctctttcttctggaggaatacaaaaactatctggat
121          Q   V   P   L   E   P   P   L   L   F   L   L   E   E   Y   K   N   Y   L   D
721        gccgctaatatgtgcatgagggtgcggcgacactccgatccctcacggcgtggagagctc
141          A   A   [N   M   C]  M   R   V   R   R   H   S   D   P   S   R   R   G   E   L
781        agcgtgtgtgacagtattagccagtgggtgacagctgtggataaaaagacggcaatagac
161          S   V   C   D   S   I   S   Q   W   V   T   A   V   D   K   K   T   A   I   D
841        atgtctgggcagacagttaccgtcatggaaaaggtccctgtccccaatggccaactgaag
181          M   S   G   Q   T   V   T   V   M   E   K   V   P   V   P   N   G   Q   L   K
901        caatactttatgagaccaaatgcaaccccatggggtacacaaaggagggctgcagagga
201          Q   Y   F   E   T   K   C   N   P   M   G   Y   T   K   E   G   C   R   G
961        atagacaagcggcattataattcccaatgcaggacaacccagtcctacgtgcgagcgctt
221          I   D   K   R   H   Y   N   S   Q   C   R   T   T   Q   S   Y   V   R   A   L
1021       accatggatagcaaaaagaagattggctggcggtttataaggatagacacttcatgtgta
241          T   M   D   S   K   K   K   I   G   W   R   F   I   R   I   D   T   S   C   V
1081       tgcacattgaccattaagagggggcagatagtgtataaaatgtatagatttttattgaagag
261          C   T   L   T   I   K   R   G   R   –
1141       tttaaaaaagagaataaagagaaaatatctatttgtatatatacataacagggtaaatta
1201       ttccgtcaaatgaaaatttttatggactgcatgtaaaaaaagatgaagtttatacagtaaa
1261       agtgatactacagtctatttattgaacatattcatgaccttgtaaacaattaaaaaaaat
1281       ctgatcagtcaaaaaaaaaaaaaaaaa     1308
```

[N M C]: consensus sequence for N-glycosilation

B

Figure B alignment:

```
            1                        20              115                                                      150
D.l.   MTILFLTMVISYFSCMRAAP LRDAP... ...YDSRVMISNQVPLEPPLLFLLEEYKNYLDAANMCMRVRRHSDPSRR
D.r.   MTILFVTMVISYFSCMRAAP MREIP... ...YASRVMISNQVPLEPPLLFLLEEYKNYLDAANMSMRVRRHSDPARR
M.m.   MTILFLTMVISYFGCMKAAP MKEVN... ...YTSRVMLSSQVPLEPPLLFLLEEYKNYLDAANMSMRVRRHSDPARR
H.s.   MTILFLTMVISYFGCMKAAP MKEAN... ...YTSRVMLSSQVPLEPPLLFLLEEYKNYLDAANMSMRVRRHSDPARR
```
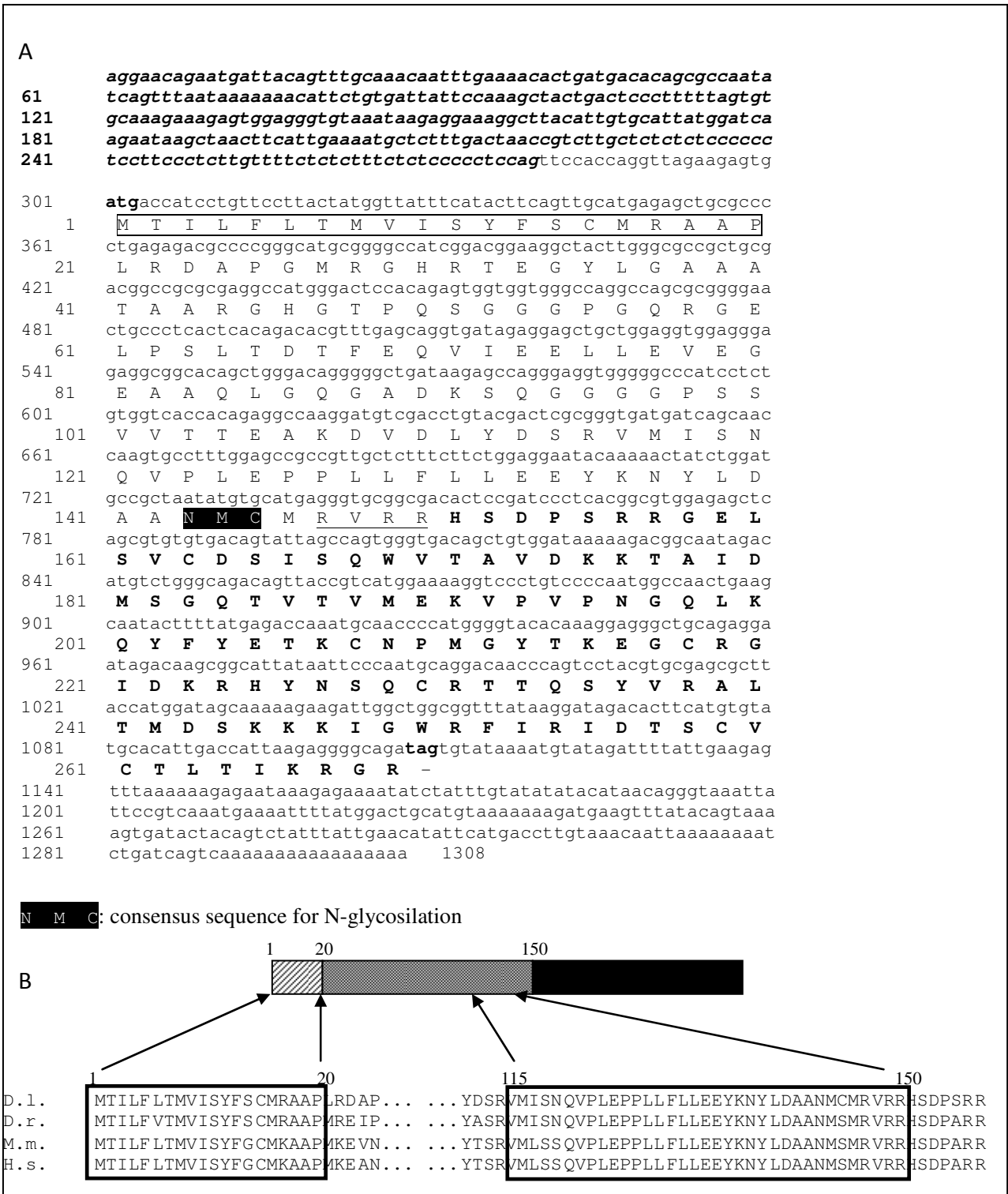
Figure 3.4 Protein and cDNA sequence of BDNF. Panel A. *Dicentrarchus labrax* cDNA sequence and deduced amino acid sequence (GeneBank accession number FJ711591). AA sequence of signal peptide is boxed in white; AA sequence for N-glycosilation is boxed in black; the cleavage sequence is underlined; start codon and the mature BDNF are shown in bold. Panel B. Representation of the entire BDNF protein and alignment, among different species, of AA sequence of two conserved regions in the prepro protein: *Dicentrarchus labrax* (D.l.); *Danio rerio* (D.r.); *Mus musculus* (M.m.); *Homo sapiens* (H.s). The dashed, the stippled and the black areas correspond to the signal peptide, the propeptide and mature secreted protein respectively.

3.1.2 Developmental and tissue-specific expression of BDNF splice variants

To learn more about the possible role of BDNF transcripts in the seabass, we analyzed their expression during post-hatching development and their tissue distribution in the adult. The different transcripts were amplified using 5' exon forward specific primers in combination with a reverse primer located on the exon 2. Expression of the coding exon 2 was determined using internal primers. When no amplicon was detectable after the first PCR reaction, a second round of PCR was carried out to increase sensitivity.

Analysis of BDNF expression at 6, 16, 27, 33 and 44 days post-hatching (dph) showed that, besides variant 1d/2, all BDNF variants were expressed during the entire larval maturation. Of note, variant 1d/2 transcript was undetectable at all stages even after the second round PCR. Although this analysis cannot be considered quantitative, it is clear that the generated bipartite transcripts showed striking differences in their expression with 1c/2 splice variant showing the highest expression throughout all post hatching development stages (Fig. 3.5).
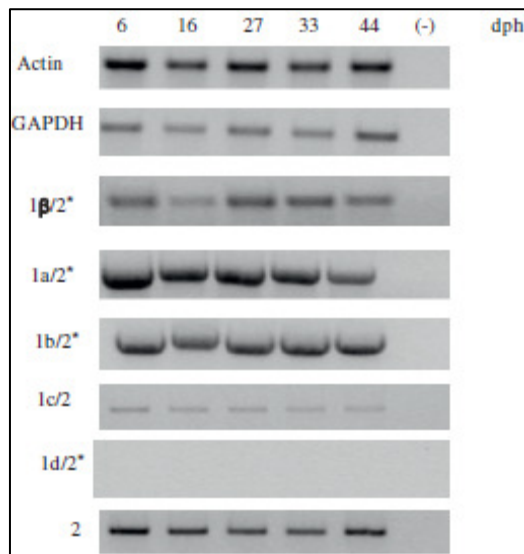

Figure 3.5 Example of expression of _Dicentrarchus labrax_ alternative mRNAs during larva development obtained by RT-PCR. *: Aliquots of first PCR products were amplified in 2nd round of PCR. For details see Material and Methods.

Expression of splice variants was also determined in brain, liver, kidney and muscle of adult animals. An example of the PCR analysis, after gel electrophoresis, is shown in Fig. 3.6.
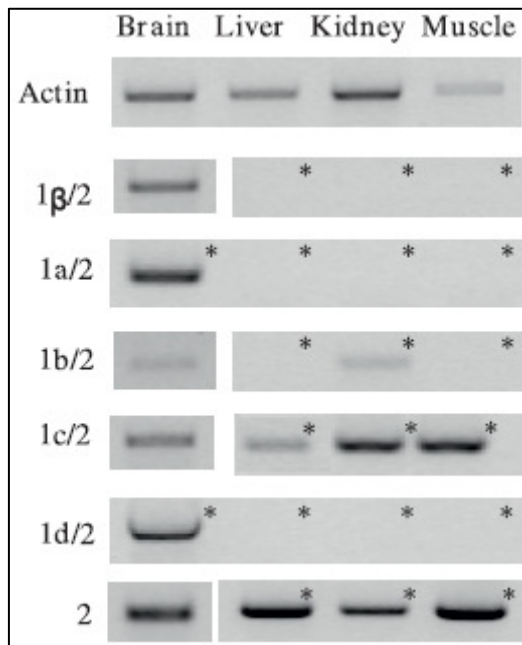
Figure 3.6 Example of expression of _Dicentrarchus labrax_ alternative mRNAs in different tissues of adult animals obtained by RT-PCR. *: Aliquots of first PCR products were amplified in 2nd round of PCR. For details see Material and Methods.

EXON	BRAIN	LIVER*	KIDNEY*	MUSCLE*
1β/2	+	n.d.	n.d.	n.d.
1a/2	+*	n.d.	n.d.	n.d.
1b/2	+/-	n.d.	+/-	n.d.
1c/2	+	+/-	+	+
1d/2	+*	n.d.	n.d.	n.d.
2	++	+	+	+

Table 3.2 Expression of 5' non coding and coding exons in different tissues of adult _D. labrax._ * An aliquot of first PCR product was amplified in a 2nd round of PCR. n.d.: not detectable

The highest expression levels of the D. labrax BDNF transcripts were observed in the brain even though some variants, such as 1b/2 and 1c/2, were detected also in non neuronal tissues even if only after a second round of PCR. A semi-quantitative evaluation of the tissue-specific expression of D. labrax BDNF alternative transcripts is reported in Table 3.2. Bioinformatic promoter analysis using Transfac® 6.0 and public version of Match™ software (Matys _et al._, 2003; Gößling _et al._, 2001) highlighted two regions (TGACGTCA and TGAAGTCA), upstream to the exon 1c with highly conserved consensus for the cAMP/calcium responsive element binding protein (CRE) which are also found in mammals upstream to the exon IV (Liu _et al._, 2006; Chen _et al._, 2003; Martinowich _et al._,

2003; Tao *et al*., 2002). The presence of the HCS1 in fish exon 1c and mammalian exon IV supports the likelihood that these two exons are true orthologs.

3.1.3 Effects of acute stress on the expression of BDNF splice variants

Since acute stress induces variations in BDNF transcripts expression in the brain of rodents (Marmigère *et al*., 2003; Fuchikami *et al*., 2009; Nair *et al*.,2007) we investigated if any changes occurs when fishes underwent to a brief stressful event consisting in 30 minutes of controlled water deprivation condition (see methods). Semi-quantitative PCR analysis of BDNF transcripts expression in the sea bass brain revealed that in the stressed group there were no significant differences in the expression of coding exon 2, and in the upstream exons 1b, 1a, 1b compared to the control group (Fig. 3.7). In contrast, we found a significant decrease in the expression of the exons 1c and 1d (p < 0.05; Fig. 3.7). Thus, acute stress in the sea bass, in absence of an emotional component, induces a rapid down regulation of the exons belonging to the second exon cluster.
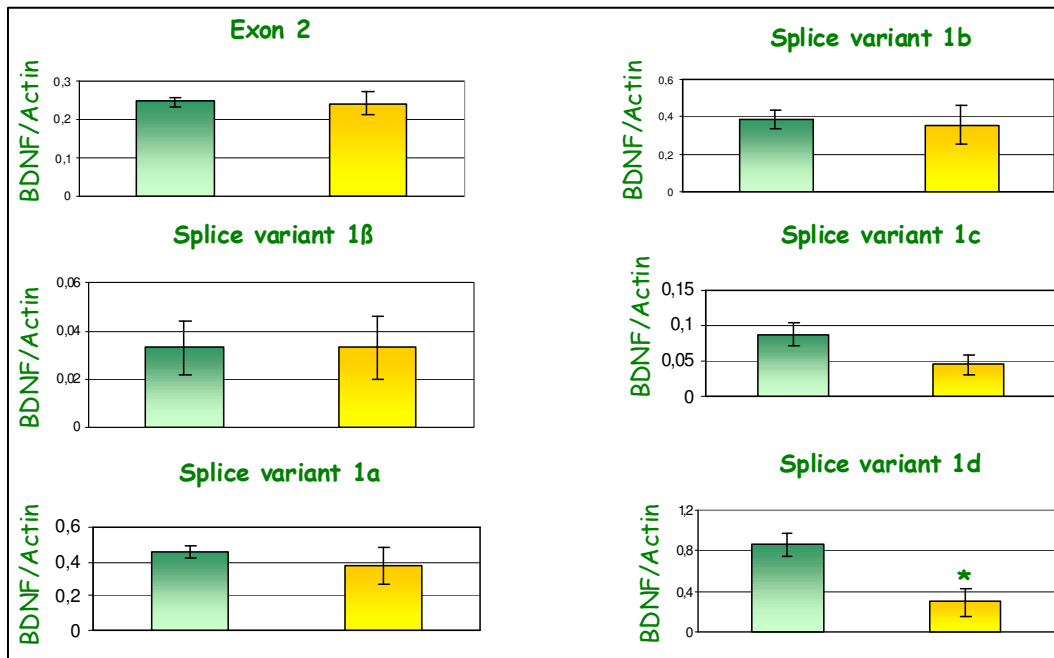

Figure 3.7 Histogram of semiquantitative-PCR of BDNF. Variants and coding exon in control are reported as grey bars, stressed samples are reported as white bars. Values are means ± SD; *p < 0.05. n = 5.

3.1.4 BDNF protein processing in sea bass after acute stress

To understand if acute stress can also alter BDNF protein levels and/or processing, we analysed by Western blot the liver and the brain from 15 normally reared controls and 15 animals that underwent acute stress. In both liver and brain, anti-BDNF antibodies recognized two bands with apparent molecular weight of 27 KDa and 18 KDa which correspond to proBDNF (calculated Mw = 28,7 KDa) and mature BDNF (calculated Mw = 13,3 KDa), respectively (Fig. 3.8A and 3.9A). Remarkably, sea bass BDNF does not contain the cleavage site (RGLT) that in mammals is recognized by the Membrane-bound transcription factor site-1 protease (MBTPS1 also known as SKI-1 protease) to generate the pro28KDa-BDNF isoforms after a cleavage at Threonin 57 (Seidah *et al.*, 1999). Thus, in fishes, there is only a proBDNF (equivalent to mammalian pro32KDa) and a mature BDNF.

Preincubation of the anti-BDNF antibody with the corresponding immunizing peptide, abolished staining of both bands indicating that they represent the seabass BDNF (data not shown). In the liver, BDNF was mostly in the mature form (58% of total BDNF) nevertheless, there was also a large amount of proBDNF (42% of total BDNF; Fig. 3.8A). No statistically significant difference was observed in proBDNF and matBDNF in stressed animal (Fig. 3.8A, B).

Similarly, in the brain, the mature form consisted in 60% of total BDNF and the proBDNF in 40% of total BDNF (Fig. 3.9B). 30 min of acute stress had no effects on the total amount of BDNF in the brain but induced a highly significant increase in the proBDNF levels and a corresponding significant reduction in mature BDNF (p <0.01 vs. control, Fig. 3.9B). The scatter-plot distribution analysis of the two populations showed that in the brain of every animal of the stressed group, the percentage of proBDNF is at least 1 standard deviation (SD) above the mean value of the control group (Fig. 3.9C). Analogous distribution, but towards lower levels, was also found for mature BDNF (not shown). We further calculated if the measurement of the percentage of proBDNF on total BDNF in the brain could represent a predictive test to identify stressed animals. Therefore, we calculated both sensitivity and specificity which are statistical measures of the performance of a binary classification test (see methods).
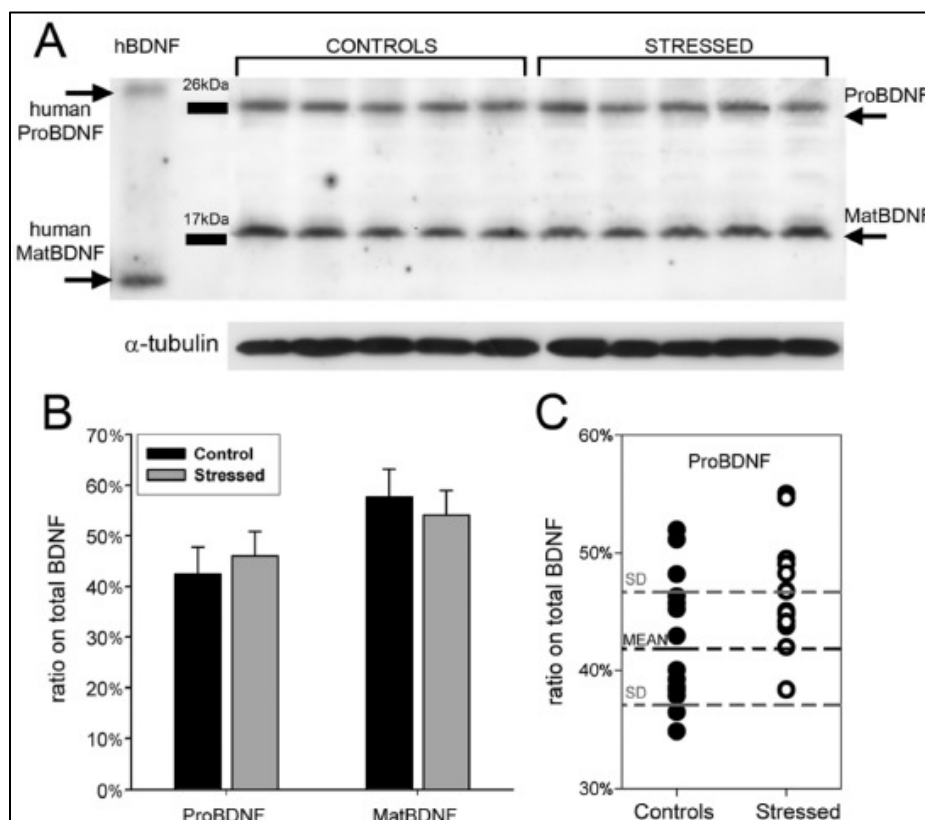
Figure 3.8 Stress does not alter the ratio of pro/matBDNF in the sea bass liver. A) A representative Western blot of proBDNF and matBDNF expression in the sea bass liver. α-tubulin (MW 55 KDa) is used for normalization. Human recombinant proBDNF and matBDNF shows reactivity of the anti-BDNF antibody. The bands corresponding to the sea bass proBDNF and matBDNF are indicated on the right side. B) Mean percentage of proBDNF vs. total BDNF (totalBDNF = proBDNF+matBDNF) of 10 control and 10 stressed animals. Error bars represent SE. No significant difference between control and stressed animals was found. C) Scatter-plot showing the large overlap of the percentage of proBDNF on total BDNF in individual animals from the control and the stressed populations. The F values, obtained by One way Anova analysis, for proBDNF and mature BDNF were 4.154 and 0.055, respectively.

All stressed animals had ratio of proBDNF/totBDNF above 1 SD from the mean value of controls (15/15 stressed are true positive = a, and 0/15 are false negative = c; see methods), hence the sensitivity of the test is 100% while the specificity is 87% due to the presence of two false positives (2/15 controls are false positive = b, thus 13/15 are true negative = d; see methods). The test also had a Positive Predictive Value of 88% and a Negative Predictive Value of 100% (see methods). In sum, this test is a perfect exclusion test, which means that all specimen with proBDNF/totBDNF values below 43% are certainly not stressed. Thus, the brain proBDNF/totBDNF ratio (or its counterpart matBDNF/totBDNF) owing to its remarkable difference between stressed and non-

stressed animals represents a highly reliable neurological biomarker capable to detect biological stress in sea bass.
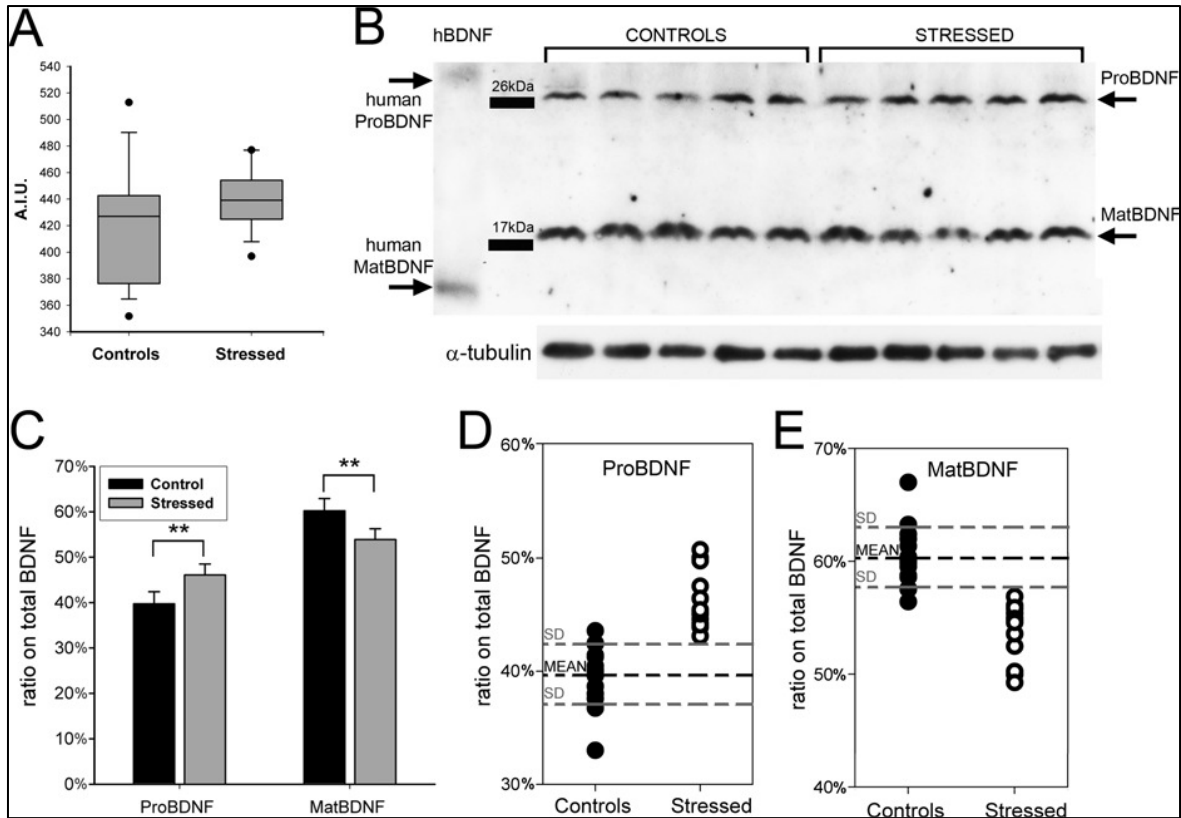

Figure 3.9 Stress increases significantly the percentage of proBDNF while decreasing matBDNF in the brain of stressed sea bass. A) The quantification of total BDNF in the brain of sea bass using an ELISA assay, showed no difference between control and stressed group (A.I.U. = arbitrary intensity units). B) A representative Western blot of proBDNF and mature BDNF expression in the seabass brain. Human proBDNF and mature BDNF is shown in the first lane from left. α-tubulin used for normalization is shown at the bottom. C) ProBDNF on total BDNF is significantly increased (** = p < 0.01) in stressed animals with respect to control animals and mature BDNF percentage is increased (** = p <0.01; n = 15 animals, each in duplicate. Error bars are SE). D) The scatter plot shows that in the brain of all stressed animals proBDNF are above the mean of controls+1SD. E) Similarly, in all stressed animals mature BDNF values are below the mean of controls -1SD. Only 2 control animals have a proBDNF percentage above the controls' mean+1SD for proBDNF or below the mean-1SD. The F values, obtained by One way Anova analysis, for proBDNF and mature BDNF were 19.028 and 6.225, respectively.

3.2 5S rRNA: PCR based method for fish species identification

In order to obtain a reliable genetic marker for fish species identification, we conducted a bibliographic research and we found that the nuclear 5S rRNA gene is a good candidate for food authentication. This gene has 120 nucleotides coding sequence, highly conserved between species, tandem repeated in the genome. The repeats are separated by a sequence named not transcribed spacer (NTS), which is, on the contrary, species-specific in length and sequence.

For our purpose we chosen ten species of fish of high commercial interest, some of which are subjected to substitution. For each species we have sequenced the 5S rRNA gene performing a PCR using universal primers designed on the conserved sequence (Table 2.5). The results of the obtained sequences together with their length and accession numbers are summarized in Table 3.3. At this point, by ClustalW, we performed an alignment, among all the species (Appendix 2), in order to design species-specific primers within the not conserved region of NTS.

Specific primers generate amplicons, of well defined length (Table 3.4), only when primers match with template genomic DNA (Fig. 3.10), allowed discriminating the tested species. Noteworthy, it is possible identifying species belonging to the same genus, like *S. salar* (Fig. 3.10 A) and *S. trutta* (Fig. 3.10 B), and species that are subject to substitution each other like *P. Fluviatilis* (Fig. 3.10 F) and *L. niloticus* (Fig. 3.10 G), or *S. vulgaris* (Fig. 3.10 H) and *P. platessa* (Fig. 3.10 I) or *P. hypophthalmus* (Fig. 3.10 J and K).

The presence of multiple bands, detected in some amplified products (see Fig. 3.10 B, F, H and I), is probably due to the repetition of gene sequence in the genome and does not influence the evaluation of the results.

On the other hand, a problem might be caused by the presence of aspecific amplified; from our results emerged that, in spite of species-specific primers designed on *T. thynnus* and *X. gladius* NTS, it wasn't possible differentiate, by PCR reaction, these two species (Fig. 3.10 C and D), that showed amplicons not only of the same sizes but also equally expressed. This, maybe, it was due to the presence of various sequences of NTS in the

same species. In this particular case, it is useful to point out that tuna and swordfish are not replaceable each other for the unique characteristics of their meat, but this is a problem that has to be take into account when, on the contrary, the two species are affected by fraud.

Analyzed species	Sequence length	ACCESSION NUMBER
S. salar	528 bp	S73106.1*
D. labrax	546 bp	HM014363*
T. thynnus	351 bp	Submission in progress
X. gladius	316 bp	
S. trutta	379 bp	
P. fluviatilis	222 bp	
L. niloticus	577+258 bp	
S. vulgaris	415 bp	
P. platessa	181 bp	
P. hypophthalmus	351 bp	

Table 3.3 List of the submitted sequence and accession number (submission in progress). *Sequences already present in NCBI database.

	EXPECTED AMPLICONS SIZE WITH…	
	…5S rRNA universal primers	…5S rRNA species -pecific primers
S. salar	256 bp	128 bp
S. trutta	377+294 bp	74 bp
T. thynnus	349 bp	127 bp
X. gladius	350 bp	275 bp
D. labrax	544 bp	269 bp
P. fluviatilis	222 bp	98 bp
L. niloticus	550+250 bp	157 bp
S. vulgaris	413 bp	231 bp
P. platessa	179 bp	166 bp
P. hypophthalmus	350 bp	319 bp

Table 3.4 Expected amplicons size with 5S rRNA universal primers and species-specific primers.
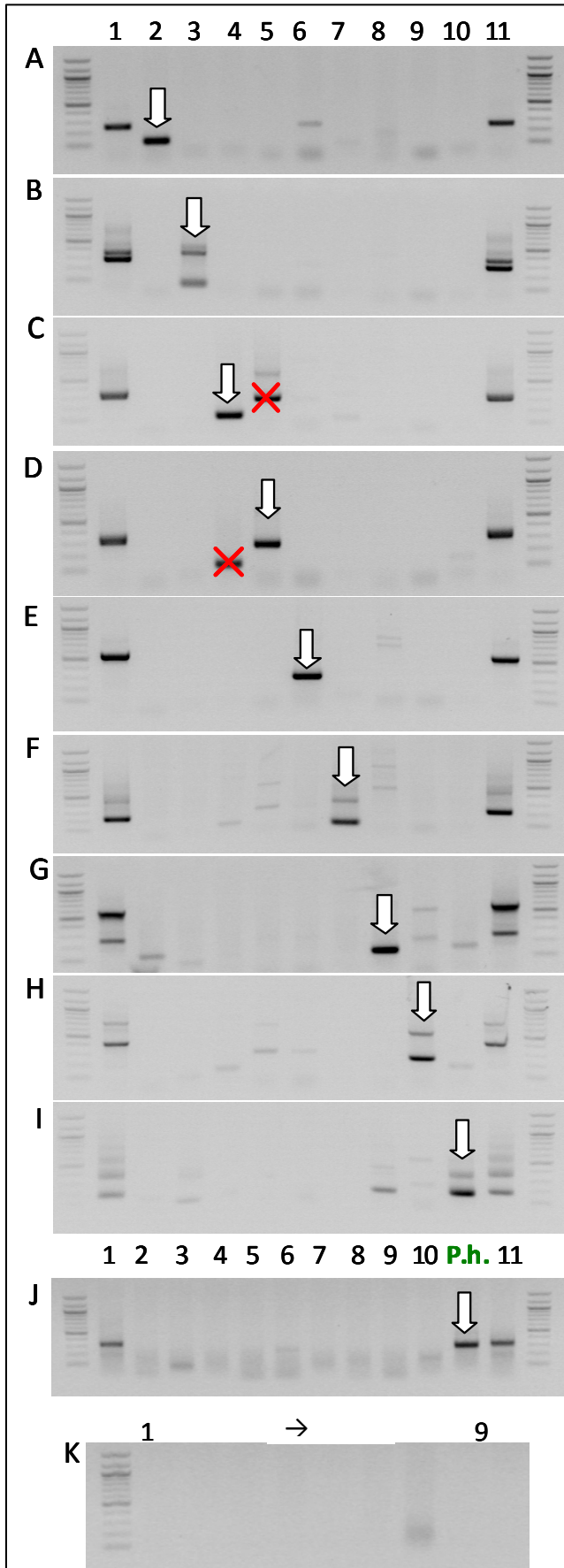
Figure 3.10 Species-specific amplification of 5S rRNA gene. Each panel has been obtained by amplification of genomic DNA belonging to a single species: A) *Salmo salar* (S. s); B) *Salmo trutta* (S.t.); C) *Thunnus thynnus* (T. t.); D) *Xiphias gladius* (X. g.); E) *Dicentrarchus labrax* (D. l.); F) *Perca fluviatilis* (P.f.); G) *Lates niloticus* (L. n.); H) *Solea vulgaris* (S. v.); I) *Pleuronectes platessa* (P. p.); J) *Pangasius hypophthalmus* (P. h.). In each lane has been used a couple of universal (lane 1 and 11) or species-specific primers designed on 5S rRNA sequnces: lane 2) S. s.; 3) S. t; 4) T. t.; 5) X. g.; 6) D. l..; 7) P. f.; 8) L. n.; 9) S. v.; 10) P. p.; in panel J, the lane P. h. represents the primers of *P. hypophthalmus*, wich are the only ones that gave amplicon with corresponding genomic DNA, and didn't give any product with genomic DNA from other species (panel K lane, 1-9).

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4. Discussion and Conclusions

4.1 BDNF: gene structure characterization; mRNA and protein quantification after stress

The first part of the study concerns the characterization of the BDNF gene in *Dicentrarchus labrax* and its transcriptional, translational and post-translational regulation following acute stress.

We found that in the sea bass, BDNF is highly expressed in the brain and that the transcripts 1c and 1d from the second promoters cluster are down regulated after acute stress. In addition, we show that acute stress induces a significant increase in the proBDNF levels and a corresponding reduction in mature BDNF suggesting altered regulation of proBDNF proteolytic processing. Finally we show that the proBDNF/totBDNF ratio (or its counterpart matBDNF/totBDNF) is a highly reliable novel quantitative neurological biomarker capable to detect biological stress in fishes with sensitivity 100%, specificity 87%, Positive Predictive Value of 88% and Negative Predictive Value of 100%.

All known vertebrate BDNF genes share a similar multiple exons organization and encode for a pre-proprotein that is translocated to the endoplasmic reticulum and proteolitically processed to yield the mature protein (Aid *et al*., 2007; Pruunsild *et al*., 2007; Tettamenti *et al*., 2010). The *D. labrax* BDNF gene consists of at least five alternative 5'-exons and one 3'-coding exon. For what concern the nomenclature, we have referred to previous studies on zebrafish (Heinrich and Pagtakhan, 2004) because mammalian, avian and amphibians BDNF follow a different nomenclature (Kidane *et al*., 2009). Analysis of *D. labrax* BDNF transcripts, carried out both in the early developmental stages and in adult tissues, shows that all alternative upstream exons (1b, 1a-1d) are spliced to the protein-coding exon 2. This indicates that *D. labrax* BDNF transcripts structure is similar to other vertebrates. In fact, multiple transcriptional initiation sites and splicing into two-part transcripts can also be found in humans, chimpanzees, dogs, pigs, cats, cows, chicken, frogs, lampreys, zebrafish, and pufferfish (Heinrich and Pagtakhan, 2004). With the exception of the exon 1a (43%), we found strong sequence homology with zebrafish and pufferfish genes for most exons. Furthermore, exon 1b contains 170 nt segment that is highly similar to human exon 1 (75% identity). Three highly conserved segments were

found in the sea bass BDNF, HCS2 in exon 1a which is present also in exon IIc of mammalian BDNF; HCS1 in exon 1c also found in mammalian exon IV while in the 3'UTR encoded by exon 2, we have found the HCS3 which is also present in the 3'UTR in mammals. These results suggest that mammalian exons I, II, and IV (coding for the 5' untranslated region) evolved early in the vertebrate radiation and may play a major role in BDNF action, while more recently evolved splice variants including other 5' exons may participate in more specialized functions of BDNF such as, for example, synaptic plasticity. Further research in this direction may allow testing this hypothesis.

The post-hatching developmental analysis indicates that although in different amounts, all *D. labrax* BDNF transcripts, except 1d/2, are well represented at all stages analyzed (day post-hatching 6, 16, 27, 33, 44).

On the other hand, the distribution of various BDNF transcripts in adult sea bass is tissue specific with all transcripts being most expressed in the brain. The splice form 1c/2 was also expressed, even though at low levels, in all the examined extra nervous tissues (liver, kidney, muscle), while exon 1b/2 transcript was found only in the kidney. Organ-specific expression also holds for most BDNF exons in zebrafish and mammals suggesting conserved transcriptional regulation among the vertebrates (Liu *et al*., 2006; Aid *et al*., 2007; Pruunsild *et al*., 2007; Tettamanti *et al*., 2010; Heinrich and Pagtakhan, 2004). According to this view, our bioinformatic analysis of *D. labrax* BDNF gene suggests that the region upstream to exon 1c contains two potential responsive elements, belonging to the CRE family. These elements function as responsive elements also in BDNF exon IV of rat cortical neurons (Liu *et al*., 2006; Heinrich and Pagtakhan, 2004) and may be responsible of the higher expression of the isoform 1c/2 in adult sea bass tissue. Of note, a previous study on transcriptional analysis of Zebrafish HCS1 reported that this highly conserved sequence in the 5' exon 1c (and vertebrate exon IV) has properties of a dehancer and, depending on the sequence context, as an enhancer (Heinrich and Pagtakhan, 2004).

In the second part of our study we have examined the expression of BDNF transcripts after acute stress caused by water deprivation for 30 minutes. Although no significant difference was found in the total BDNF mRNA levels between stressed and control groups

(measured by analysis of the protein-coding exon 2, common to all transcripts), we found a significant decrease in exons 1c and 1d. This finding is consistent with data in rodents in which single immobilization stress induces down regulation of exon IV, homologous to fish exons 1c (they both contain HCS1), due to decreased histone acetylation at this promoter immediately after acute stress (Fuchikami *et al.*, 2009). Of note, in a recent study we showed that exons of the second promoter clusters (mammalian exons IV-VII, fish 1c-d), are particularly important for cell survival in response to cellular cytotoxic stress in human neuroblastoma cells (Baj and Tongiorgi, 2009). Thus, activation of promoters upstream to these exons might relate to a rapid adaptative response to various types of stress.

Western blot analysis showed that in brain, but not in liver, proBDNF content is significantly increased in the stressed samples. Mammalian BDNF transcripts produce the well-known 32 kDa propeptide precursor that is cleaved either to pro28kDa or to the mature 14 kDa BDNF forms by two different proteases (Mowla *et al.*, 2001). Pro28kDa BDNF peptide is not further processed into the mature 14 kDa BDNF form but it represents a true final proteolytic product generated by a specific Ca^{2+}-dependent serine proteinase known as Membrane-Bound Transcription Factor Site-1 protease (MBTFS-1; EC= 3.4.21.112, alternative names: S1P endopeptidase, Site-1 protease), also known as Subtilisin/kexin-isozyme 1 (SKI-1) (Seidah *et al.*, 1999); mature 14 kDa BDNF is generated intracellularly by furin (Mowla *et al.*, 2001), or extracellularly by plasmin and matrix metalloprotease-7 (Lee *et al.*, 2001). In contrast, in the sea bass we only found two BDNF forms, a proBDNF form corresponding to mammalian pro32KDa precursor and a mature BDNF, while the pro28KDa peptide was absent. Comparison of *D. labrax* BDNF protein with that of rodents and human BDNF, revealed that the mammalian SKI-1 cleavage site at Threonine 57 (Arg-Gly-Leu-Thr↓) is absent in fishes and amphibians and has first emerged in reptilians during vertebrates' evolution (Tettamanti *et al.*, 2010; see Appendix 1). Limited proteolysis of one inactive precursor to produce active peptides and proteins is a general mechanism to generate biologically diverse products from a single gene. Here, we provide the first evidence that fishes possess a simplified proteolytic regulation

of BDNF and that the pro28KDa proteolytic product, whose function remains yet to be determined, is absent at this stage of vertebrates evolution.

We found that acute stress profoundly alters the relative amount of proBDNF and mature BDNF. Our data are suggestive of a lower proteolytic activity to generate mature BDNF and thus, the uncleaved product is accumulated in the sea bass brain, but not in liver, immediately after an acute stress. Although, the mechanisms by which stress can prevent efficient conversion of proBDNF into mature BDNF are presently unknown, several recent studies have pointed out that pro32KDa BDNF has a biological function distinct from that of mature BDNF. Both proBDNF precursor and mature BDNF can be released from neurons (Lee *et al.*, 2001; Yang *et al.*, 2009).

While proBDNF binds only to p75 receptor, mature BDNF displays high affinity to TrkB and lower affinity to p75 (Chao, 2003). Binding of proBDNF to p75 promotes cell death and attenuates synaptic transmission by inducing long term depression (Teng *et al.*, 2005; Woo *et al.*, 2005), while mature BDNF sustains long term potentiation and cell survival (Lee *et al.*, 2001, Patterson *et al.*, 1996; Pang *et al.*, 2004). It is therefore conceivable that the shift towards higher proBDNF and lower BDNF level observed after acute stress may have the biological role of attenuating proactive behaviour inducing reduced activity in stressed animals.

Stress affects the hormonal response in fish in much the same way it does in higher animals. Stress stimulates the hypothalamus, one of the oldest parts of the brain (in evolutionary terms) and is responsible for controlling the most basic functions such as hunger, thirst, sex drive and, in mammals, body temperature; all functions that are mediated also by BDNF. A reduced behavioural activity may thus represent an adaptive response to dangerous situations represented here by shallow waters, to allow for an immediate energy saving and recovery in preparation for future actions. In this context, it is striking that 100% of animals in our experimental stress group showed >1SD increase in proBDNF levels (and corresponding decrease in mature BDNF). A theoretical, optimal prediction test can achieve 100% sensitivity (i.e. predict all people from the sick group as sick) and 100% specificity (i.e. not predict anyone from the healthy group). Thus, our test

performances will make it feasible to screen for stress even in low prevalence populations, particularly where samples are first pooled before testing.

In conclusion, we have determined the structure of *Dicentrarchus labrax* BDNF gene, its expression in neuronal and non neuronal tissues, and we have demonstrated that the proBDNF/totBDNF ratio (or its counterpart matBDNF/totBDNF) is a novel quantitative neurological biomarker capable to detect biological stress in fishes with sensitivity 100%, specificity 87%, Positive Predictive Value of 88% and Negative Predictive Value of 100%.

The high predictivity of proBDNF/totBDNF ratio for stress in lower vertebrates indicates that processing of BDNF is a central mechanism in adaptation to stress and predicts that a similar regulation of pro/mature BDNF has likely been conserved throughout evolution of vertebrates from fish to man.

4.2 5S rRNA: PCR based method for fish species identification

The detection of species substitution has become an important topic within the food industry and there is growing need for rapid, reliable and reproducible tests to verify species in commercial fish and seafood products.

The predominant recent approaches to vertebrate species identification generally involve PCR amplification of a genomic target followed by post-PCR analysis, like RFLP or sequencing, of obtained fragments. These approaches, although robust and effective for species identification, are relatively time consuming and expensive, requiring downstream analysis that are not always possible and reproducible. For example, sequencing, which has proven to be the most direct and reliable way to obtain information from PCR fragments, it is not appropriate for the analysis of samples containing multiple species. On the other hand, restriction endonuclease analysis can lead to errors in case of intraspecies variation in which individuals from the same species exhibit different restriction patterns; therefore numerous individuals from the species must be analyzed to verify a lack of intraspecies polymorphism at the target sites. Moreover it is not guarantee that restriction pattern is unique for each species.

In this paper we report an alternative and efficient approach for species identification which requires only a PCR without additional downstream manipulation of the amplified product. The method, based on 5S rRNA gene amplification, resulted effective for a rapid and cheap identification of fish species subjected to substitution (*D. labrax, P. fluviatilis, X. gladius, T. thynnus, S. vulgaris, S. salar, S. trutta*) or sold in place of more valuable ones (*Lates niloticus, P. platessa, P. Hypophthalmus*) in European market. Indeed, among nuclear markers, the 5S rRNA gene has a special interest in species identification because of its noteworthy structure that makes it a species-specific gene in higher eukariotes. As already reported this gene comprises a 120 nt highly conserved coding sequence and a NTS which is species specific; the coding sequence and the NTS are tandemly repeated a variable number of times on the chromosome depending on the species. The oligonucleotides Fish5S_FW and Fish5S_REV (Table 2.5), based on conserved region of this gene in *S. salar* (Pendas *et al.*, 1994), have already been used to amplify a whole unit

of the 5S rRNA gene (coding sequence and NTS) from different fish templates (Moran and Garcia-Vazquez, 2006; Karaiskou et al., 2003; Aranishi, 2005; Aranishi and Okimoto, 2004; Carrera et al., 2000; Lockley and Bardsley, 2000). These primers have also been used by us to amplify 5S rRNA gene in all analyzed species reported in this work. In some cases, the amplification with universal primers, gave more than one product (Fig. 3.10 B, F, G, H, I). As reported by the literature, and as already described in the introduction, this is probably due to the particular gene organization, that posses different 5S rDNA classes, which differs each other for the NTS sequence, in mammal (Hallenberg *et al*., 1994) as well as in fish (Carrera *et al.*, 2000; Martins and Galetti, 2001). Moreover, Qin *et al*. (2010) reported that in fish different classes of 5S rDNA gene, characterized by slightly differences in NTS sequences, could be present simultaneously (class I: 203 bp; class II: 340 bp; and class III: 477 bp; class IV: 188) and this is the result of the influence of polyploidy on the organization and evolution of the multigene family of 5S rDNA in fish. In our experiments these characteristics influence also the amplification of the gene with species specific primers (Fig. 3.10 B, F, H, I).

Although the length of the NTS, even when present in multiple bands, appears to be species-specific in fish, to make the identification certain for each fish studied, we decided to design forward and reverse species-specific primers, on the NTS sequences (Table 2.5). To confirm the effectiveness of the designed primers we tested each couple on all the considered species. The results, showed in Fig. 3.10, confirmed that the genomic DNA of a particular species was amplified only with the its own oligonucleotides, allowing to discriminate different fish. In some cases it can happen that nonspecific PCR products have been amplified (Fig. 3.10 A, F, G, H, I); being the expression of the these bands much less than the specific ones, and the expected sizes different, these products do not cause problems with interpretation of the results. In the case of *T. thynnus*, its genome is amplified both with the specific its own primers than *X. gladius* primers giving an aspecific amplicon expressed as well as the specific ones even though of different size (Fig. 3.10 C); viceversa, the same problem has been detected also for *X. gladius* genome with *T. thynnus* specific nucleotides (Fig. 3.10 D). Although these two species are not replaceable each other, the impossibility of discrimination highlights maybe the unique

limit of this approach: increasing the number of the analyzed species, there are growing chances of finding two species with the same PCR amplification pattern; in this case following sequence analysis may be necessary.

In conclusion, the PCR amplification of selected NTS fragments by species-specific primers is a powerful technique for the identification of the considered species, because of its simplicity, specificity, sensitivity and cheapness. With this method the identification relies not only on the different size of the amplicons obtained, but also on the presence of the target sequences specific of each species studied.

This simple PCR method could provide a powerful approach to detect mislabelling or fraudulent substitution of fish species, particularly in the market of processed food such as frozen fillets and precooked seafood. Moreover, it could be apply not only as routinely laboratory practice in qualified organizations but also in mobile laboratories, normally equipped for molecular biology investigation, allowing on-site (restaurant, fish market, supermarket) analysis in a relatively short time.

6. Appendix 1: BDNF pre-pro-protein alignment between different species.

1-*Pan troglodytes* (common chimpanzee); 2-*Homo sapiens* (human); 3-*Mus musculus* (mouse); 4-*Rattus Norvegicus* (rat); 5-*Japalura splendida* (Japalura tree dragon); 6-*Cyclophios major* (greater green snake); 7-*Gallus gallus* (chicken); 8-*Xenopus laevis* (African clawed frog); 9-*Dicentrarchus labrax* (sea bass); 10-*Danio rerio* (zebrafish).

Amino acid sequence of signal peptide, with the cleavage site "Ala-Ala", is boxed in red and it is highly conserved from mammals to fish.

The blue box contains the subtilisin/kexin-isozyme 1 cleavage site (blue arrowhead), absent in fish and amphibians and first emerged in reptilians during vertebrate evolution. In mammals this cleavage site gives Pro28kDa BDNF form of the protein.

A highly conserved, multifunctional decapeptide with the site of sulfoglycation (yellow circle) and serine-protease site of action (green arrowhead). The decapeptide is partially superimposed (His-Ser) to the last part of the sequence (pink box), that represents 14 kDa BDNF mature form.

```
1    MTILFLTMVISYFGCMKAAPMKEAN-IRG-QGGLAYPGVRTHGT------LESVNGPKAG 52
2    MTILFLTMVISYFGCMKAAPMKEAN-IRG-QGGLAYPGVRTHGT------LESVNGPKAG 52
3    MTILFLTMVISYFGCMKAAPMKEVN-VHG-QGNLAYPGVRTHGT------LESVNGPRAG 52
4    MTILFLTMVISYFGCMKAAPMKEAN-VHG-QGNLAYPAVRTHGT------LESVNGPRAG 52
5    MTILFLTMVISYFSCMKAAPMKEVS-FRG-QGSLAYPGLRTQGH------LEDLGGPNDA 52
6    MTILFLTMVISYLSCMKATPMKEVS-IRG-QGSLAYPGLRTQGN------LETLSGPNDA 52
7    MTILFLTMVISYFSCMKAAPMKEAS-VRG-HGSLAYPGLRTHGT------LESLTGPNAG 52
8    MTILFLTMVISYFSCMKAAPMKEAS-VRG-QNGLAYPGLRTHGT------LESIGGP-SG 51
9    MTILFLTMVISYFSCMRAAPLRDAPGMRG-HRTEGYLGAAATAA-------RGHGTPQSG 52
10   MTILFVTMVISYFSCMRAAPMREIPGVQGGHRAEGYLGAAAAAAAAVTSGSRGHGTPQSG 60
     *****:******:.**:*:*::: .:* : .*.  :.     .   *   .

1    SRGLT-----SLADTFEHVIEELLDED----QKVRPNEENN--------------KDADL 89
2    SRGLT-----SLADTFEHVIEELLDED----QKVRPNEENN--------------KDADL 89
3    SRGLTT---TSLADTFEHVIEELLDED----QKVRPNEENH--------------KDADL 91
4    SRGLTT---TSLADTFEHVIEELLDED----QKVRPNEENH--------------KDADL 91
5    TRGQT-----SLADTFEHVLEELLDEQ----QAIQPSKKN--------------KDADL 88
6    TRGLT-----SLADTFEHVIEELLDER----QVIQPSKEN--------------KDADL 88
7    SRGLT-----SLADTFEHVIEELLDED----QDIQPSEEN--------------KDADL 88
8    SRGGGL---PSLTDTFEQVIEELLEEE----QSIR-SEES--------------KDSDL 88
9    GGPGQRGELPSLTDTFEQVIEELLEVEGEA-AQLGQGADKSQGGGGPSSVVTTEAKDVDL 111
10   GG------LPSLTDTFEQVIEELLEVEGEATQQLGPGADQGQGGGGP--IDAADSKDVDL 112
     **:****:*:****:          :  . ..           ** **

1    YTSRVMLSSQVPLEPPLLFLLEEYKNYLDAANMSMRVRHSDPARRGELSVCDSISEWVT 149
2    YTSRVMLSSQVPLEPPLLFLLEEYKNYLDAANMSMRVRHSDPARRGELSVCDSISEWVT 149
3    YTSRVMLSSQVPLEPPLLFLLEEYKNYLDAANMSMRVRHSDPARRGELSVCDSISEWVT 151
4    YTSRVMLSSQVPLEPPLLFLLEEYKNYLDAANMSMRVRHSDPARRGELSVCDSISEWVT 151
5    YSSRVMLSSQVPLEPPLLFLLEEYKNYLDAANMSMRVRHSDPARRGEFSVCDSTSEWVT 148
6    YSSRVMLSSQVPLEPPLLFLLEEYKNYLDAANMSMRVRHSDPARRGELSVCDSTSEWVT 148
7    YTSRVMLSSQVPLEPPLLFLLEEYKNYLDAANMSMRVRHSDPARRGELSVCDSTSEWVT 148
8    YSSRVMLSSQVPLEPPLLFLLEEYKNYLDAANMSMRVRHSDPARRGELSVCDSISEWVT 148
9    YDSRVMISNQVPLEPPLLFLLEEYKNYLDAANMCMRVRHSDPSRRGELSVCDSISQWVT 171
10   YASRVMISNQVPLEPPLLFLLEEYKNYLDAANMSMRVRHSDPARRGELSVCDSISQWVT 172
     * ****:*.*********************:** .*****:**:****:***** *:***
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1    AADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFYETKCNPMGYTKEGCRGIDKRHWNSQCR  209
2    AADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFYETKCNPMGYTKEGCRGIDKRHWNSQCR  209
3    AADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFYETKCNPMGYTKEGCRGIDKRHWNSQCR  211
4    AADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFYETKCNPMGYTKEGCRGIDKRHWNSQCR  211
5    AAEKKTAVDMSGATVTVLEKVPVPKGQLKQYFYETKCNSKGYTKEGCRGIDKRYWNSQCR  208
6    AAEKKTAVDMSGATVTVLEKVPVPKGQLKQYFYETKCSTKGYAKEGCRGIDKRYWNSQCR  208
7    AAEKKTAVDMSGATVTVLEKVPVPKGQLKQYFYETKCNPKGYTKEGCRGIDKRHWNSQCR  208
8    AANKKTAVDMSGQTVTVLEKVPVSKGQLKQYFYETKCNPMGYMKEGCRGIDKRYWNSQCR  208
9    AVDKKTAIDMSGQTVTVMEKVPVPNGQLKQYFYETKCNPMGYTKEGCRGIDKRHYNSQCR  231
10   AVDKKTAIDMSGQTVTVLEKVPVTNGQLKQYFYETKCNPLGYTKEGCRGIDKRHYNSQCR  232
     *.:****:**** ****:*****.:*************.. ** **********::*****

1    TTQSYVRALTMDSKKRIGWRFIRIDTSCVCTLTIKRGR  247
2    TTQSYVRALTMDSKKRIGWRFIRIDTSCVCTLTIKRGR  247
3    TTQSYVRALTMDSKKRIGWRFIRIDTSCVCTLTIKRGR  249
4    TTQSYVRALTMDSKKRIGWRFIRIDTSCVCTLTIKRGR  249
5    TTQSYVRALTMDNKKRVGWRFIRIDTSCVCTLTIKRGR  246
6    TTQSYVRALTIDNKKRVGWRFIRIDTSCVCTLTIKRGR  246
7    TTQSYVRALTMDNKKRVGWRFIRIDTSCVCTLTIKRGR  246
8    TTQSYVRAFTMDSKKKVGWRFIRIDTSCVCTLTIKRGR  246
9    TTQSYVRALTMDSKKKIGWRFIRIDTSCVCTLTIKRGR  269
10   TTQSYVRALTMDSKRKIGWRFIRIDTSCVCTLTIKRGR  270
     ********:* *.*::.*********************
```

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7. Appendix 2: 5S rRNA gene alignment between analyzed species.

The universal primers Fish5S_FW and Fish5S_REV have been highlighted in yellow and green respectively. The species-specific primers (see Table 2.5, Materials and Methods), highlighted in different colours, have been designed in not conserved regions of NTS for each species.

```
D.labrax        GCTTACGGCCATACCAGCCTGAATACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGG 60
P.fluviatilis   GCTTACGGCCATACCAGCCTGAATACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAAG 60
L.niloticus     GCTTACGGCCATACCAGCCTGAATACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGG 60
T.thunnus       GCTTACGGCCATACCAGCCTGAATACGCCCGATCTCGTCCGATCTAGGAAGCTAAGCAGG 60
X.gladius       GCTTACGGCCATACCAGCCTGAATACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGG 60
S.salar         GCTTACGGCCATACCAGCCTGAATACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGG 60
S.trutta        GCTTACGGCCATACCAGCCTGAATACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGG 60
P.hypophthalmus GCTTACGGCCATACCAGCCTGAATACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGG 60
P.platessa      GCTTACGGCCATACCAGCCTGAATACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGG 60
S.vulgaris      GCTTACGGCCATACCAGCCTGAATACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGG 60
                ************************************************ *********** *

D.labrax        GTCGGGCCTGGGTAGTACTTGGATGGGAGACCGCCTGGGAATACCAGGTGCTGTAAGCC- 119
P.fluviatilis   GTAGGGCCGGGTCAGTACTTGGATGGGAGACTGCCTGGGAATACCCG-TGCTCTAAGA-- 117
L.niloticus     GTCGGGCCTGGTTAGTACTTGGATGGGAGACCGCCTGGGAATACCAGGTGCTGTAAGCTT 120
T.thunnus       ATAGGGCCTGGTTAGTACTTAGATGGGAGACCGCCTAGGAATACCAGGTGCTGTAGGCAT 120
X.gladius       GTCGGGCCTGGTTAGTACTTGGATGGGAGACCGCCTGGGAATACCAGGTGCTGTAAGC-- 118
S.salar         GTCGGGCCTGGTTAGTACTTGGATGGGAGACCGCCTGGGAATACCAGGTGCTGTAAGCAT 120
S.trutta        GTCGGGCCTGGTTAGTACTTGGATGGGAGACCGCCTGGGAATACCAGGTGCTGTAAGCTT 120
P.hypophthalmus GTCGGGCCTGGTTAGTACTTGGATGGGAGACCGCCTGGGAATACCAGGTGCTGTAAGCTT 120
P.platessa      GTCGGGCCTGGTTAGTACTTGGATGGGAGACTGCCTGGGAATACCAGGTGCTGTAAGCTT 120
S.vulgaris      GTCGGGCCTGGTTAGTACTTGGATGGGAGACCGCCTGGGAATACCAGGTGCTGTAAGCTT 120
                * ***** **  ******* ********** **** ******** * **** ** *

D.labrax        ------------TTTTCATTTCTCTTAGCAAACAGTAGAG--GCTGCTGCTTCTTCACTG 165
P.fluviatilis   ------------TTTTTATTCCCATTGTACAACAGCAGGG--GCTA-TGCTCCTTCTATA 162
L.niloticus     TTTGTCCCCATCTTTTCCTGCCTATCTTACAACAGCAGAAT-GCTGCTTCCTCTGTAGTC 179
T.thunnus       TTTTCAACTGCG----------------------------TGCGCTTTACAACTAG-- 148
X.gladius       TTTTTACTTG------------------------------ACTTGGCATCCAG-- 141
S.salar         TTTGTCCACGAGGGTGTGCCCCTGCACTATTTCATCAGCAACACTGCCTTGTAGTAAGCA 180
S.trutta        TTTGTC------------------------------ACTGCCTTGTGG------ 138
P.hypophthalmus TTTAGT------------------------------GAGGCCTTCATT------ 138
P.platessa      TTTTCCGCTTT-----------------------TACCTGACGTATTTACATGT 151
S.vulgaris      TTACAC---------TGCTGCTTCCTTACAAGAAACATGGGCTTGCAATTACGTTGACG 170

D.labrax        TACACAGAACGTT-----GTTCAGATATTTTTTCCACTGTTGTTGTATAATATTTAAGCT 220
P.fluviatilis   TACCACAAGTATA-----CTT---GCATTTTACCATGTATAAATACCTTGTGTTCA--CC 212
L.niloticus     GAGACAGGGCAC------GTCAAAGCACTTTTATTTTGCAGCCTGTATTTCGCGC---TC 230
T.thunnus       -----------------CGGAGAAAAGTGCTGCTCCTTCTGAGGAGACAGATTCTA-CG 189
X.gladius       ------------------AAGCAGACACTGTTGCTACTTCTGCGGAGACAT-TTTGA-AA 181
S.salar         TTTAATGATGAATTGACTAAATGCAGCTTCCTGCCTTTTTAATAGGATCAAATTTCC-TT 239
S.trutta        -----------------AATTTCAACTCTTTGTCCGTTT------------TTTCC-CT 167
P.hypophthalmus ------------------TGAATTAGGGTTTTAATTCTCTCAGGAT-ATCATACTGTT-TT 179
P.platessa      ATAAATAAGGCT------CAGTGGGTGGCTTCATTC----------------------- 181
S.vulgaris      CACGGGCACACGTTAACGTCCTTCTAGTTTCAGCCTTGGATGTCGCTCTGCAAGCATGTG 230

D.labrax        TTCCGTCTAGATGGAATTCCTTTGATTCTACTATTGTTATTATCATTGTTGTTGTTGTT- 279
P.fluviatilis   TACTCTAAAG-------------------------------------------------- 222
L.niloticus     TGTTTTCTACTTCTGTTTTATGTCATGTCACTTTCAGCAATCGGATATTAGGCGCTGGA- 289
T.thunnus       TAGCACGGAGTCTACTTATTCACCA-------GTAAATACGGAGAAGGTGCACACGGAA- 241
X.gladius       AAGCAGGAAGTCTTCTTACTCATTG-------GAAAAAAAGAGA--------------- 219
S.salar         GTGTTTTCAATTAGCATCTGCCTTGTATTTATAAGACAAACAAGAATATTGTTGCTGAAT 299
S.trutta        ------------------------------------CAAG---------GCTGAA- 177
P.hypophthalmus GGG--------------------------------AGGAAG---------ATTGAC- 194
P.platessa      ----------------------------------------------------------
S.vulgaris      AGAAGCTTGGAGATGGGGAGCAGCTTAC--CCATCTCGGTGTAGCTTCCTAATACTGGAA 288
```

```
D.labrax          ---------ATCACTTGTCCACTGCAGGATAATATCTAAACTTCATTCATGAGGACATGT 330
P.fluviatilis     ------------------------------------------------------------
L.niloticus       ---------A--AGTTACCTCTGTAAAGTCTGCACTGCTGCCGAATGAATGAGCACAGTC 338
T.thunnus         ---GTTATTAAGGATA--------GTCAGTCAACACACATTTATTTAAAGGATAATCTTC 290
X.gladius         ---------------------GTCAGTCAAAACACATTTATTTAAATGAAAATCTTC 255
S.salar           GCAGCTTGTGTGTGCTTTGTGCTCCTTTGCCCTGTTCAAATGATGAAAGGAAATAAAGTT 359
S.trutta          -------ATATGTGC---------CCTCGCTTTG---AAATAA-GTAAGCAAGGTTAGTT 217
P.hypophthalmus   --------TAGAAAC-----------TTGAAAAGGAGAGAGGAATCAAATAAATAAA-TA 234
P.platessa        ------------------------------------------------------------
S.vulgaris        TAGAGTGTAGCAGGTAGTGGAGATAAAGGCTCAAGTGCAGTGTGTTCGAAAGGCTGACTT 348

D.labrax          TGAATTTTTGTCCTGCACACCCTGTTAATGAATTGATTTCGTGGGACCTGATTTTGGGAG 390
P.fluviatilis     ------------------------------------------------------------
L.niloticus       TGGGTTTTGCGCAAAATGTCCCCAA----------------------------------- 363
T.thunnus         TACATATCCACTTTCAAATCCCTACTCCGCTATAAAACCTATGTTATTGAGCAGTGTGTT 350
X.gladius         TACATATCCACCTTCAAATCCCTACTCCGCTATAAAACCTATGTTATTGAAAATTGTGTT 315
S.salar           TGTATTTTATCCAACTA-CCTGTGCTAAAAAGTGATGGGAACAGTGTTTGTAATTCTTC 418
S.trutta          TGTATTTCATCCAACAA-TCTGTGCTACAAATT-ATGGCTACAGTATATGCAATTCTTC 275
P.hypophthalmus   AATAAATAAACAAACAA-ACAAACAAACAAACAAACCAACCAACCAATCA---------- 283
P.platessa        ------------------------------------------------------------
S.vulgaris        TTGAGCTCCTCCACGAGCAGGGGGCCTTGCCTAGTCTATAAAAGGAGTCTGTGTCCCCAG 408

D.labrax          AGTTCAAGTCAGTAAATGCATGAATTAATACAGAACTAAGTTGGTCATCAAATCGTTAGT 450
P.fluviatilis     ------------------------------------------------------------
L.niloticus       ------------------------------------------------------------
T.thunnus         T----------------------------------------------------------- 351
X.gladius         T----------------------------------------------------------- 316
S.salar           TACTTTTTCAGTGACACAAAGTTCAAGCTGCTTATCTAATTGGTGAAAATGCAATGTCTG 478
S.trutta          CACTTTTTGAGTGACACAAAG------ATGCTTATCTAAATGGTGAAAATGCAACAGCTG 329
P.hypophthalmus   ------------------------------------------------------------
P.platessa        ------------------------------------------------------------
S.vulgaris        CCCGTCG----------------------------------------------------- 415

D.labrax          ACATAAATAAACGTCTTGAATGGACTCTAATCCCACAGACAAACAGAACATGGTGTAACA 510
P.fluviatilis     ------------------------------------------------------------
L.niloticus       ------------------------------------------------------------
T.thunnus         ------------------------------------------------------------
X.gladius         ------------------------------------------------------------
S.salar           TTTATCACACTCACTTTGACTATATATGTTGTAGCAAGAGATTGTTTCTC---------- 528
S.trutta          TTAATCAGGCTCACTTTGACTTTACATAGTGCACCAAGAGATTGTTTCTC--------- 379
P.hypophthalmus   ------------------------------------------------------------
P.platessa        ------------------------------------------------------------
S.vulgaris        ------------------------------------------------------------

D.labrax          ACGTCTTTAAAAGGTCCATTTGCAGGTGAAGCTCAC 546
P.fluviatilis     ------------------------------------
L.niloticus       ------------------------------------
T.thunnus         ------------------------------------
X.gladius         ------------------------------------
S.salar           ------------------------------------
S.trutta          ------------------------------------
P.hypophthalmus   ------------------------------------
P.platessa        ------------------------------------
S.vulgaris        ------------------------------------
```

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