Functional genomics in fish:

towards understanding stress and immune responses at a molecular level

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Les ciències tenen les arrels amargues,
però molt dolços els fruits.

Aristòtil (384 a.c.- 322 a.c.)

Un científic pot prendre's la
llibertat de plantejar-se qualsevol
qüestió, de dubtar de qualsevol
afirmació, de corregir errors.

Julius Robert Oppenheimer (1904-1967)

AGRAÏMENTS

Després de dies de clausura tancada a casa davant l'ordinador redactant tot el treball recollit durant la tesi doctoral, per fi arriba la redacció de l'apartat dels agraïments!. Però ben pensat, agrair a tothom a qui en certa manera ha tingut a veure amb els meus últims quatre anys de vida, no és una tasca fàcil. Molta gent voluntària o involuntàriament ha format part de la tesi doctoral aquí exposada, i avui, a través d'aquestes paraules els hi dono les Gràcies!!

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Lo difícil se consigue, lo imposible se intenta. No sé de quién es, lo oí una vez en la tele y se me quedó grabado (de la meva amiga Lupe)

Moltes gràcies a tots, us tindré sempre a dins meu!

Laia Ribas

ABBREVIATIONS

aa: aminoacids
ACTH: adrenocorticotropic Hormone
ANOVA: one-way analysis of variance
APS: acute phase serum amyloid A
ATU: urbic antrosol
BACH1: BTB and CNC homolog 1
BLAST: Basic Local Alignment Search Tool
bp: base pairs
BrdU: 5-bromouracil deoxyriboside
BTG1: B-cell translocation gene 1 protein
C/ebp: CCAAT/enhancer binding protein alpha
C: cerebellum
C24: control animals at 24 hours after PBS IP injection
C72: control animals at 72 hours after PBS IP injection
CCL4/MIP.1β: macrophage inflammatory protein 1β
CD209/DC-SIGN: dendritic cell-specific ICAM-grabbing non-integrin,
CDS: coding sequence region
CNS: central nervous system
COP: coatomer protein
COX: cytochrome oxidase
CRH: corticotropin Releasing Hormone
d: days
DC: dendritic cell
DD: differential display
DDBJ: DNA Databank of Japan
DEPC: diethyl pyrocarbonate
DNA: deoxyribonucleic acid
dNTP: nucleotide triphosphate
dsRNA: double-stranded RNA
E.coli: *Escherichia coli*
EIF-2-beta: eukaryotic translation initiation factor 2 subunit 2
EMBL: European Molecular Biology Laboratory
eno: enolase
EST: expressed sequence tag
Ets: E26-transformation–specific
FAO: Food and Agriculture Organization of the United Nations

FEAP: Federation of European Aquaculture Producers
FL: fasciculus longitudinalis
FLL: fasciculus longitudinalis lateralis
FLM: nucleus fasciculi longitudinalis medialis
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GATA-1: GATA binding protein 1 (globin transcription factor 1)
GATA-2: GATA binding protein 2 (globin transcription factor 2)
G-CSF: growth factor receptor
GL: granular layer
GM-CSF: granulocyte macrophage colony stimulating factor
GO: Gene Ontology
GRAP-2: GRB2 (growth factor receptor-bound)-related protein 2
GTP: guanidin trifosfato
h: hours
HD: high-density
HIF-1: hypoxia-inducible transcription factor
HK: head kidney
HK-2: hexokinase-2
HL: hipotalamic lobe
HPI: hypothalamic-pituitary-interrenal
HSP: heat shock protein
ICAM: intercellular adhesion molecule
IFN: interferon
Ig: immunoglobulins
IHC: immunohistochemistry
IHNV: infectious Hematopoietic Necrosis Virus
IL: interleukine
ILR: interleukin receptor
IMP synthase: phosphoribosylaminoimidazolecarboxamide formyltransferase
IP: intraperitoneal
ISH: *in situ* hybridization
LD: low-density
l-plastin: lymphocyte cytosolic protein 1 (L-plastin)
LPS: lipopolysaccharide
LPS24: LPS treated animals 24 hours after LPS IP injection
LPS72: LPS treated animals 72 hours after LPS IP injection
LY: lymphotoxin
Lys: lysine

MBP-1: myc-binding protein
M-CSF: macrophage colony stimulating factor
MDP: muramyl dipeptide
MERP-1: mammalian ependymin related protein-1
mEST: macrophage cell culture stimulated 12 hours with LPS 10 μg/ml
MHC: major histocompatibility complex
MHCII Ii: MHC class II invariant chain
MIP-1β: macrophage inflammatory protein 1β
ML: maximum likelihood
ML: molecular layer
MLF: nucleus fasciculi longitudunalis medialis
MMCs: melanomacrophage centers
MMP : matrix metaloproteinase
MO: medulla Oblongata
MPO: myeloperoxidase precursor
mRNA: messenger ribonucleic acid
MSE: muscle specific enolase
MT: tegmentum of the midbrain
NADH: nicotinamide adenine dinucleotide
NADPH: nicotinamide adenine dinucleotide phosphate
NF-E2: nuclear factor erythroid-derived 2
NFκB: nuclear factor κB
NNE: non-neural enolase
NOS: nitrous oxide synthase
NOS2: nitric oxide synthase 2
NSE: neural-specific enolase
OB: olfactory bulb
OBF-1: B-cell-specific transcriptional coactivator
OT: optic tectum
PAF: paraformaldheid
PAMPs: pathogen-associated molecular patterns
PBS: phosphate saline buffer
PCR: polymerase chain reaction
PEMT2: phosphatidylethanolamine N-methyltransferase
PEST: proline–glutamic acid–serine–threonine domain
PGK: phosphoglycerate kinase
PGS-2: prostaglandin endoperoxide synthase
PHAPI2: putative HLA class II-associated protein

PRRs: pathogen recognition receptors

PU.1: purine-rich sequence 1

RL: lateral Recess

RM: red muscle

RT-PCR: revers transcriptase Polymerase chain reaction

RTVP-1: glioma pathogenesis-related protein

SEM: standard error of the mean

S-enolase: *Sparus aurata* enolase

SFFV: spleen focus-forming virus

SNK: Student-Neuman-Keuls

Spi-1: SFFV provirus integration site-1

SUMO-1: anthracycline-associated resistance

SV: saccus vasculosus

T: telencephal

TIGR: The Institute for Genomic Research

TLR: toll-like receptor

TNF: tumor necrosis factor

TNF-DR: tumor necrosis factor Decoy receptor

TNFR: tumor necrosis factor receptor

TNFRSF: tumor necrosis factor receptor super family

TNFRV: tumor necrosis factor receptor virtual

TRAF: tumor necrosis receptor associated factors

TSC: torus semicircularis pars centralis

TSL: torus semicircularis pars lateralis

TTRAF: TRAF and TNFR associated

V: VentricleVC: valvula of cerebellum

Vd: ventral area, pars dorsalis of the telencephal

Vv: ventral area, pars ventralis of the telencephal

WM: white muscle

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Chapter 1

General Introduction

1. Aquaculture

Aquaculture can be defined as the science, art, and business of cultivating marine or freshwater food fish or shellfish under controlled conditions. The first fish species cultured in Europe were carps, imported from China, to use in pond culture during the Middle Ages by monks. Better understanding of the husbandry requirements, allowed the activity to expand considerably, spreading throughout Europe into every state. Worldwide growth in aquaculture production in the last 30 years has rapidly increased (Van Biesen, G. et al. 2005) due to worldwide food demand and a significant reduction of wild fish stocks (Deviller, G. et al. 2005). The European leaders in aquaculture production are Norway, France, Spain and Italy whereas outside Europe the world leaders are China, Thailand, Indonesia, and South American (Fig.1.1) (Spanish Ministry and Food and Agriculture Organization of the United Nations, FAO). The total world production of fish, shellfish and other aquatic animals was 125 million tones in 1999. In 2000, EU production from aquaculture of the main fin fish species (salmon, trout, sea bass and bream, carp, eels, turbot and cod) was 876.265 tones worth 2,959 million euros (Federation of European Aquaculture Producers, FEAP). Fish health is a prerequisite of all aquaculture operations, not only due to financial loses caused by fish disease, but also disease control and basic hygiene are an obligatory aspect of all farming operations (González Laxe, F. et al. 2004). Undoubtedly the research, development and regulatory measures needed for an integrated health management programme in aquaculture involve considerable expertise, organization and expense. Timely information on the health status of aquaculture stocks is essential for an effective disease control programmes, enabling a quick response to disease outbreaks and a reduced mortality rate in infected stock (Pillay, T.V.R. 2000).

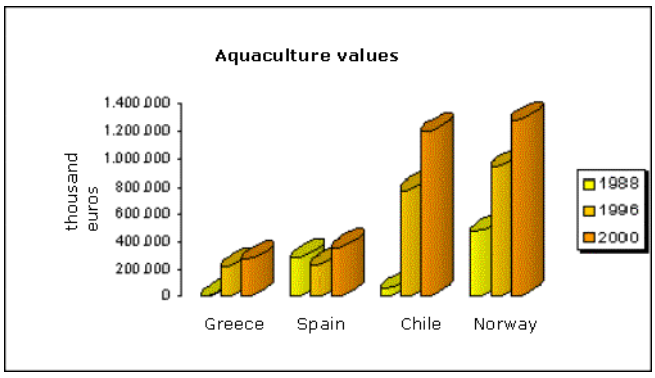


Fig.1.1. Aquaculture values of different countries in terms of thousand euros through 1988,1996,2000 years, Spanish Ministry.

2. Molecular gene markers for aquaculture

The rationale of aquaculture is not limited merely to socio-economic and marketing advantages; there are also scientific principles that weigh very much in favour of aquatic farming of fish and shellfish. Aquaculture is interested in modern technologies that may improve fish production and quality and, at the same time, reduce environmental impact with benefits in the public perception of the industry (Gornati, R. et al. 2005). Beside the traditional markers (biochemical, histological, morphological and physiological), it is important to look for alternative parameters such as molecular biomarkers. 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. Knowledge of fish genome coupled with reproducible challenge models for selected pathogens of importance to commercial fish, would open the way for a new generation of research tools and studies leading to improvements in the health of many cultured aquatic animal species and lowered disease losses by the commercial aquaculture sector (Thorgaard, G.H. et al. 2002). Expression studies of certain genes which play roles in immune function are increasing interest in aquaculture. Such tools will also permit comparative genomic studies yielding information on how the presence or expression of various genes can affect the innate resistance of fish to certain pathogens or fish welfare conditions. Finding good biomolecular markers will provide critical information that can be used by the commercial industry to assist in marker-assisted selection of brood stocks.

3. Fish evolution

Fish are a heterogeneous non-taxonomic assemblage of more than 28,000 identified species, more species that for all other vertebrate groups combined (Nelson, J.S. 1994). In fish, the strengths and limitations of classificatory approaches are described in terms of their ability to reflect the extremely diverse body forms, sizes, lifestyles and physiological responses to the enormous range of environments they inhabit. Recent phylogenetic approaches are arguably limited to the evolutionary in a morphological sense, by the growing fossil evidence, and in the so-called post genomic era, by the compromise between the sampling of evolutionary processes and the choice of appropriate genetic markers that may account for the phylogenetic relationships in such a highly diverse group (Fig.1.2) (Cummings, M.P. et al. 2005). Although precious little is known about the genomic features of several high order taxonomic groups of fish, from the species analysed to date it has become widely assumed that, in fish, the ecological diversification that extends over 550 million years of history parallels the genomic diversity. Fish

genome sizes vary between 0.32 and 133 billion base pairs, and exhibit a variable species-specific genomic scaffolding that owes its plasticity to the frequent changes: polyploidy, gene and chromosomal duplications, gains and losses of introns and exons, genome compaction and abundance of transposable elements (Crollius, H.R. et al. 2005; Krasnov, A. et al. 2005a). Notwithstanding a feasible model organism as demonstrated by their genomic plasticity, fish show a great complexity in terms of phylogenetic relationships. The phylogeny of basal and higher fish taxa has been recently reviewed using an extensive dataset of complete mitochondrial DNA sequences from more than 50 representative teleosts (Inoue, J.G. et al. 2003; Miya, M. et al. 2003). Despite the intensive taxonomic sampling and the accuracy of numerous comparative anatomical and morphological studies, still remains strong controversy over several groups. Such incongruities highlights the uncertainties in the knowledge of the hidden influences of evolutionary relationships in Actinopterygii, by far the most diversified group of fish, which includes several "model" species used in comparative genomic analysis. The fish inter-specific relationships can undoubtedly be considered a remarkable effort towards an understanding of the fish phylogeny and a conciliation between the phylogenies constructed from molecular and morphological data (Miya, M. et al. 2003).

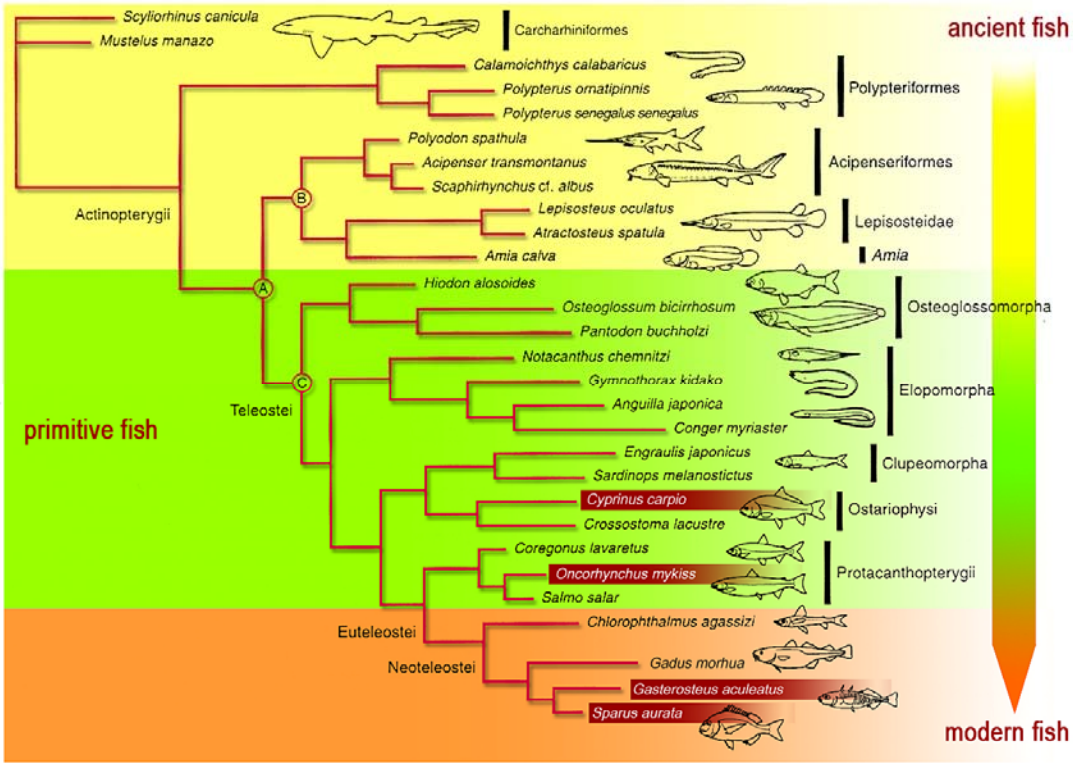


Fig.1.2. Phylogenetic relationships of selected Actinopterygian fish derived from analysis of mitogenomic data, adapted from (Inoue, J.G. et al. 2003). Encased species refer to fish species used for this thesis: *Sparus aurata*, *Oncorhynchus mykiss, Gasterosteus aculeatus*, and *Cyprinus carpio* sp.

4. Stress in fish

The definition of stress is subject to many controversies. Throughout the years, scientists have tried to find a definition of stress that would be suitable under any circumstance. The concept of stress encompasses many different phenomena, observed at the organizational levels of cells, organisms, populations and ecosystems (Wendelaar Bonga, S.E. 1997). The best definition would be that stress is a condition in which the dynamic equilibrium of a organism is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimulus, commonly defined as stressors (Chrousos, G.P. et al. 1992). The main neuro-immune-endocrine control mechanisms of the stress response in fishes are comparable to those of mammals and other terrestrial animals and thus conform to a general vertebrate pattern (Donaldson, E.M. 1981). There exists an extensive bibliography on the effects of stressors on the immune system of fish, considering all types of stressors, from natural environmental changes; reproductive status and water characteristics (Hontela, A. et al. 1992), to chemical products in water and husbandry procedures (Pickering, A.D. et al. 1987; Jensen, F.B. 2003). In terms of aquaculture, several stressor models have been applied in the laboratory to mimic fish farm conditions; such as handling (Barton, B.A. et al. 1980; Molinero, A. et al. 1997; Tort, L. et al. 2001), confinement (Rotllant, J. et al. 2001; Barton, B. et al. 2005) or transport (Acerete, L. et al. 2004). Although stress and its effects are commonly discussed, many of the mechanisms by which stress affects the individual are still poorly understood.

The stressors are primarily perceived by the sensors of the nervous system and the first response is induced in integrating brain centres, specifically in the hypothalamus where the two major regulatory axes are stimulated: the sympathico-chromaffin axis, via nervous fibres innervating chromaffin cells of the head kidney region and the hypothalamic-pituitary-interrenal (HPI) axis, via an endocrine cascade. The first hormone in the HPI axis is the CRH (Corticotropin Releasing Hormone) released by hypothalamic neurons of the preoptic region. CRH stimulates the release of the Adrenocorticotropic Hormone (ACTH) from the pituitary which in turn induces production and releases of the major stress steroid, cortisol, by the interrenal cells of the head kidney. Glucocorticoids may modulate immune responses in numerous ways, including though gene expression, transcription, translation, post-translation processing, protein secretion, and cell progenitor proliferation and differentiation (O'connor, T.M. et al. 2000). Connections between nervous-immune-endocrine systems have received particular attention because they may help to understand the biological response of fish under stress conditions (Fig.1.3) (Engelsma, M.Y. et al. 2002; Holland, J.W. et al. 2002).

The cross talk between these systems and resultant secretion of regulatory molecules modulate the physiological response at target tissues acting at a transcriptional level causing changes in gene expression.

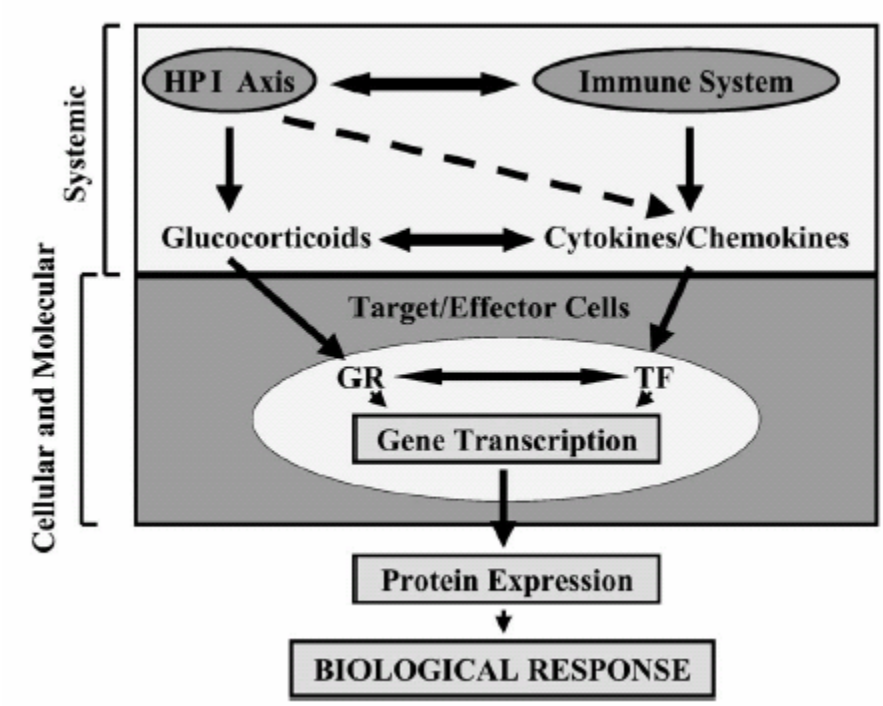


Fig.1.3. Scheme depicting systemic and cellular/molecular interplay between the HPA axis and the immune system in the regulation of glucocorticoid/cytokine secretion and gene expression. Abbreviations: GR, glucocorticoid receptor; TF, transcription factors. Figure adapted from (Haddad, J.J. et al. 2002).

5. Fish immunology

The ability of the host to discriminate self from potentially harmful non-self and act appropriately is a central feature of immune defence. Over 98% of pluricellular organisms are able to maintain their integrity thanks to an innate immune system based on cell phagocytosis and secretion of soluble antimicrobial molecules. The innate response is the basis of immune defence of invertebrates and lower vertebrates. It is the earliest immune mechanism characterised by being non-specific and therefore not depending upon previous recognition of the surface structures of the invader. The innate immune response has also the advantage of being inducible by external molecules but at the same time is constitutive and reacts within a very short time scale. The response of fish to immune challenge is mainly based on the innate immune response (Tort, L. et al. 2003a). Fish are a heterogeneous group composed for 28.000 different species which include the agnathans (lampreys and myxines), condryctians (sharks and rays) and teleosteans

(bony fish) (Nelson, J.S. 1994). As in all vertebrates, fish have cellular and humoral immune responses, and central organs whose main function is involved in immune defence (Zapata, A.G. et al. 1996) (Fig.1.4). Taking into account differences due to body compartments and cell organization, most of the generative and secondary lymphoid organs present in mammals are also found in fish, except for the lymphatic nodules and the bone marrow (Press, C.M. et al. 1999). Instead, the head kidney tissue is the principal immune organ that assumes hematopoietic functions (Meseguer, J. et al. 1995; Zapata, A.G. et al. 1996) which phagocytosis (Danneving, B.H. et al. 1994), antigen processing (Kaattari, S.L. et al. 1985; Brattgjerd, S. et al. 1996) and formation of IgM and immune memory (Herraez, M.P. et al. 1986; Tsujii, T. et al. 1990) are proposed. The head kidney is also an important endocrine organ, homologous to mammalian adrenal glands, releasing corticosteroids and other hormones. In addition, it is a well innervated organ and received sympathic nervous fibers from brain. Thus, the head kidney is an important organ with key regulatory functions providing a good model for studying the effects of regulatory stimulus (nervous and endocrine system) on the immune system (Tort, L. et al. 2003b).

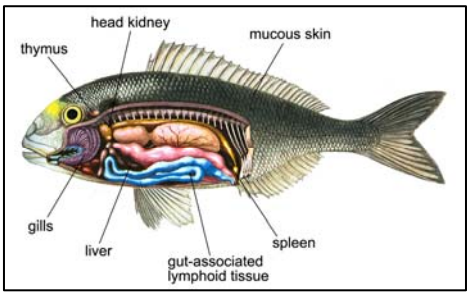


Fig.1.4. Immune structures in teleost fish.

The absence of the adaptive immune response, such as immunoglobulins (Ig), T cell receptors and products of the major histocompatibility complex (MHC) components, in invertebrates and agnathans suggest the “explosion” of adaptive immunity 450 millions of years ago (Secombes, C.J. et al. 1983). Teleost fish (including sea bream *Sparus aurata* and rainbow trout *Oncorhynchus mykiss*) are the first animal group in the phylogenesis that shows antibody activity in which a simple profile of Ig appears in this group (Andersson, E. et al. 1995; Magnadottir, B. et al. 2005). In fact, Ig are mainly limited to an IgM tetramer of approximately 800 kD (Castillo, A. et al. 1993; Evans, D.A. et al. 1998) and no definitive evidence of Ig diversity has been demonstrated (Kaattari, S. et al. 1998). Fish immune cells show the same main features as those of other vertebrates, and lymphoid and myeloid cell families have been determined. The lymphoid system is a relatively

recent evolutionary development since most animals prior to vertebrates rely on non-lymphoid cells or serum molecules. The existing functional analysis together with the reactivity of monoclonal antibodies suggest the presence of helper, cytotoxic T lymphocytes and subpopulations of B cells in teleost fish (Scapigliati, G. et al. 1999; Köllner, B. et al. 2001). The monocyte/macrophage cell lineage is the most studied in fish and from where the majority of cytokines identified to date and data concerning cytokine regulation has been obtained (Secombes, C.J. et al. 2001).

6. Hematopoiesis

The hematopoiesis is a highly orchestrated developmental process which comprises the proliferation, differentiation, and maturation of a very small population of self-renewing, pluripotent hematopoietic stem cells (HSC) into distinct blood cell types (Gangenahalli, G.U. et al. 2005). A hematopoietic stem cell is a cell isolated from the bone marrow that can renew itself, can differentiate to a variety of specialized cells, can mobilize out of the bone marrow into circulating blood, and can undergo programmed cell death. In mammals, hematopoiesis is found in the bone marrow whereas in fish, the head kidney tissue has been described as the analogous organ with the hematopoietic function (Meseguer, J. et al. 1995; Zapata, A.G. et al. 1996) (Fig.1.5).

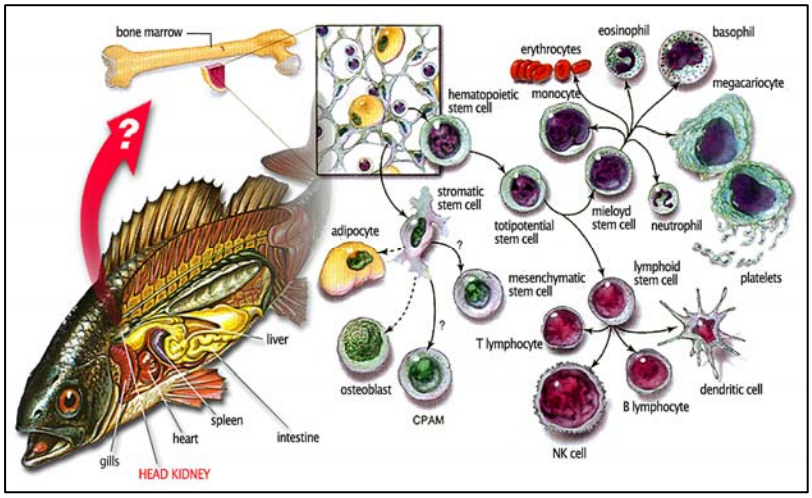


Fig. 1.5. Schematic hematopoiesis process in fish: hematopoietic stem cells are capable of differentiating into different hematopoietic lineages.

About 1 in every 10,000 to 15,000 bone marrow cells is thought to be a stem cell, whereas in the blood stream the proportion falls to 1 in 100,000 blood cells (Mackey, M.C. 2001). Hematopoietic stem cells in the bone marrow can either self-renew or give rise to progenitor cells that generate precursors of the erythroid,

myeloid or lymphoid lineage. Myeloid progenitor is the precursor of granulocytes, macrophages, dendritic cells and mastocytes of the immune system, and lymphoid progenitor is the precursor of lymphocytes B and T (Janeway, C. et al. 2000). In fish, not all the immune cell types already identified and characterized in mammals have been described. Figure 1.6 shows hematopoietic lineage which dendritic cells and thymic cortical epithelial cells have never been described in fish.

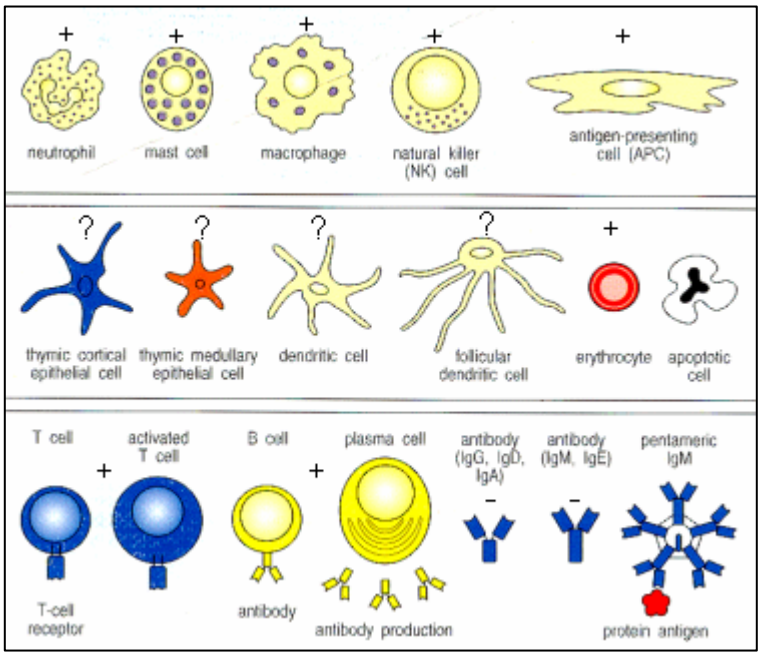


Fig.1.6. Immune cell types in fish (+ present; - not present; ? not characterized).

Hematopoiesis process is controlled by the cumulative effects of growth factors that are responsible for cellular proliferation and the hierarchical combination of transcription factors that activate lineage-specific genes and determine the blood cell phenotype. Various lineage-restricted transcription factors are potential candidates for regulation lineage commitment and development of hematopoietic stem cells (Krause, D.S. et al. 2001). These factors are finely controlled by endogenous and exogenous signals during hematopoietic development (Shivdasani, R.A. et al. 1996; Huo, X. et al. 2005). Some hematopoietic transcription factors include GATA-1, GATA-2, NF-E2, C-myb, PU.1, c/ebp-α. GATA-1 protein is a zinc finger transcription factor required for the survival and maturation of erythroid precursor cells (Yamamoto, M. et al. 1997). GATA-2 is abundantly expressed in immature erythroid progenitors playing an important role during the early stages of erythropoiesis (Dorfman, D.M. et al. 1992; Nagai, T. et al. 1994). NF-E2 plays a critical role in erythroid and megakaryocytic gene expression (Andrews, N.C. et al. 1993a; Andrews, N.C. et al. 1993b). c-Myb is expressed at high levels in immature hematopoietic cells and regulates their

proliferation and differentiation forcing the expression to inhibit erythroid differentiation (Mucenski, M.L. et al. 1991). PU.1 is a transcription factor essential for myeloid and lymphoid cell development (Scott, E.W. et al. 1994). In fish, hematopoiesis is far to be understood. C/ebp-α is early expressed in myeloid precursors (Hohaus, S. et al. 1995). In fish, some hematopoietic transcription factors have been mostly described in zebrafish, which provides a powerful model organism for studying the molecular control of normal and abnormal myelopoiesis (Berman, J.N. et al. 2005). Hematopoietic transcription factors described in fish include PU.1 (Anderson, M.K. et al. 2001; Lieschke, G.J. et al. 2002; Ward, A.C. et al. 2003), GATA-1 and GATA-2 (Galloway, J.L. et al. 2005; Rhodes, J. et al. 2005), c/ebp-α (Lyons, S.E. et al. 2001), mpo (Lieschke, G.J. et al. 2001) and l-plastin (Herbomel, P. et al. 1999). However, hematopoiesis development in fish remains an area with major unknown and uncertainies.

7. LPS and immune system

The endotoxin lipopolysaccharide (LPS), a major component of the outer membranes of Gram-negative bacteria, is the best-known target of innate recognition and induces a robust inflammatory response by phagocytic cells (Wright, S.D. 1999). Fish, as other vertebrates and invertebrates, activate their immune system after recognition of pathogen-associated molecular patterns (PAMPs) by pathogen recognition receptors (PRRs), that include most notably members of the toll-like receptor (TLR) family (Iliev, D.B. et al. 2005a). Apart form LPS, typical PAMPS are peptidoglycans which structural component is muramyl dipeptide (MDP) of Gram-positive bacteria, fungal beta-glucans and double-stranded RNA (dsRNA). The initial recognition of pathogens and substances containing PAMPs involves activation of non-clonotypic PRRs. This results in the stimulation of a complex process of the innate immune system and leads to an inflammatory reaction and the commencement of the proper adaptive immune response (Aderem, A. et al. 2000; Schnare, M. et al. 2001; Janeway, C.A., Jr. et al. 2002). It is well known that fish possess the major types of myeloid cells involved in the innate immune response including different types of polymorphonuclear granulocytes and mononuclear phagocytes (Ainsworth, A.J. 1992; Watts, M. et al. 2001). The manner in which antigen presenting cells (macrophages, dendritic cells and B-cells) recognise foreign structures and internalise them for presentation to cells of the adaptive immune system will determine the overall quality and effectiveness of immune responses (Mcgreal, E.P. et al. 2004). These cells produce a dynamic and complex pattern of gene expression, such inflammatory cytokines, reactive oxygen and nitrogen species and activate the adaptive immune system

through antigen presentation (Ellis, A.E. 2001; Neumann, N.F. et al. 2001). The functional capacity of fish myeloid cells to phagocytose antigen throughout differentiation has been demonstrated where the phagocytic capacity of trout macrophages is clearly much higher than that of the freshly isolated monocytes macrophages cells (Mackenzie, S. et al. 2003). In *in vivo* LPS, such changes in cellular activity could result from changes in the transcriptional profile of the cell possibly as a consequence of migration into other body compartments in which exogenous factors dictate the cellular response (Mackenzie, S. et al. 2003).

Intraperitoneal administration of lipopolysaccharides (LPS) has been extensively used in mammals as a model to mimic bacterial infection and to stimulated defense system. It has been known for a long time that lower vertebrates, most notably fish and amphibians, are likely to be resistant to the toxic effects of LPS (Berczi, I. et al. 1966) because an *in vivo* challenge to high concentrations of LPS in fish does not result in endotoxin-mediated mortality (Iliev, D.B. et al. 2005a). It is also remarkable that in many *in vitro* studies on leukocytes from different fish species, high concentrations of LPS have been used to induce immune responses in comparison to studies in mammals (Zou, J. et al. 2003; Hirono, I. et al. 2004). Recently it has been postulated that fish tolerance to LPS may differ to mammals in receptor-mediated recognition of LPS. TLR4-associated molecules that participate in the TLR4-mediated response to the endotoxic moiety of LPS in mammals, may be absent or may perform different functions in fish (Iliev, D.B. et al. 2005b). However, in rainbow trout cultured macrophages LPS provokes a TNF-α secretion similar to those observed in mammals indicating a conservation of LPS activation pathways and a conservation of main inflammatory response pathway (Mackenzie, S. et al. 2003; Iliev, D.B. et al. 2005a).

Interactions between the immune and nervous systems play an important role in modulating host susceptibility and resistance to inflammatory and infectious diseases (Fig.1.3) (Sternberg, E.M. 1997). Several studies give evidence of the activation of the hypothalamic-pituitary-adrenal (the analogous to HPI in fish) by LPS which in turn releases several cytokines (IL-1, IL-6 and TNF-α) (Perlstein, R.S. et al. 1993; Schobitz, B. et al. 1994; Beishuizen, A. et al. 2003). Experiments over the last two decades give evidence of the existence of an innate immune system in the brain that is under control of a wide variety of molecules. In fact, LPS is used as a good model to activate systemic immune cells, which in turn produces cytokines in brain (Wrona, D. 2006). Recent findings (Rivest, S. 2003) indicate that central nervous system responds to systemic bacterial infection with innate immune reaction without pathogen's direct access to the brain. This interaction between LPS and immune receptors, takes place in the circumventricular organs, leptomeninges

and choroid plexus and allows intracellular signalling and then rapid transcription of pro-inflammatory cytokines to cope with the challenge.

In general, bacterial lipopolysaccharide is a strong activator of various immune responses and stimulates monocytes/macrophages to release a variety of inflammatory cytokines (Brubacher, J.L. et al. 2000). Inflammation is not typically found with apoptosis, buy may be extremely important for activation of the immune system when TNF-induced necrosis is part of the anti-pathogen response (Locksley, R.M. et al. 2001). Conversely, purely apoptotic pathways may be involved in tissue remodelling during development, where inflammation could be detrimental (Locksley, R.M. et al. 2001). The result is a complex biologic cascade, involving chemokines, cytokines, and the induction of endothelial adhesions, that recruits and activates granulocytes, monocyte/macrophages, and lymphocytes at the damaged or infected tissue sites (Idriss, H.T. et al. 2000).

8. Genomics in fish

In the last decade there has been an explosion in the amount of DNA sequence data available, due to the very rapid progress of genome sequencing projects. There are three principal comprehensive databases of nucleic acid sequence in the world today: the EMBL (European Molecular Biology Laboratory) maintained at the European Bioinformatics Institute in Cambridge, UK (Stoesser, G. et al. 2003); the Genebank maintained at the National Center for Biotehcnology Information in Maryland, USA (Benson, D.A. et al. 2003) and the DDBJ (DNA Databank of Japan) (Miyazaki, S. et al. 2003). These three databases share information and hence contain almost identical sets of sequences.

The objective of these databases is to ensure that DNA sequence information is stored in a way that is publicly, and freely, accessible and that it can be retrieved and used by other researchers in the future. This policy has proved tremendously successful for the progress of science, and has led to a rapid increase in the size and usage of sequence databases. The amount of sequence data information is exponentially increasing with time; in 1982, 606 sequences were available whereas in 2006 this number increases to 69.456.332 sequences. Beside the data explosion in the past decade, fish databases are still relatively shallow. Currently, the number of ESTs (Expressed Sequence Tag) from zebrafish, trout and salmon taken together is 10 times lower than the number of EST sequences present in the human and the murine EST databases. In addition, the sequences of the fish genomes are still not completely assembled and contain numerous gaps which most of the times is an impediment for genomic fish researchers. In the future, the availability of the complete sequences of the trout genome will greatly enhance the search for these

and other important features of innate disease resistance and provide new approaches to enhance the health of fish in culture systems (Thorgaard, G.H. et al. 2002).

The 'omics' revolution in the biological sciences, has opened up many new fields of research and has allowed for innovative strategies in experimentation leading to increased understanding of basic biological processes. Merge of high-throughput analytic technologies with bioinformatic and data mining is referred to as a novel scientific discipline; Functional Genomics (Fig.1.7) (Higgs, P.G. et al. 2005). Genomics refer to scientific studies dealing with whole sets of genes rather than single genes. Methods of functional genomics are rapidly expanding towards new species, which are important to gain better understanding of fish physiology and response to immune challenge (Koskinen, H. et al. 2004). Elucidation of the changes in gene expression associated with biological processes is a central problem in biology. Advances in molecular and computational biology have led to the development of powerful, high-throughput methods for the analysis of differential gene expression (Carulli, J.P. et al. 1998). The possibility of applying transcritpomic technologies to the fish research field is rapidly becoming a reality with the development of specific-species microarrays generated from extensive efforts in EST analysis and annotation of genes. These microarray chips includes: zebrafish (Linney, E. et al. 2004), medaka (Kimura, T. et al. 2004), salmo salar (Rise, M.L. et al. 2004), rainbow trout (Krasnov, A. et al. 2005b), flounder (Byon, J.Y. et al. 2005) and sea bream (Sarropoulou, E. et al. 2005). Microarray analysis, generate large amounts of data that must be carefully analyzed to look for trends and statistically significant features. The most fundamental question to ask of microarray data is which genes have been significantly up or down regulated in the sample relative to the reference sample (Higgs, P.G. et al. 2005). High-throughput experiments are closely linked to bioinformatics because they raise question both of data interpretation and modelling. Bioinformatics is the development of computational methods for studying the structure, function, and evolution of genes, proteins, and whole genomes. Overall, genomic strategies are revolutioning scientific research also in the understanding of fish physiology and gene evolution. One challenge for bioinformatics in the post-genome age is to integrate data from many different sources. This requires biologists in different areas of expertise to use a common language so that terms used in different databases really mean the same thing, and that entries in different databases can be systematically cross-linked. A step towards doing this is to define ontologies, which are specification of the meanings of concepts used in a given field and the relation ships between those concepts. The Gene Ontology (GO) has been developed as a tool to help

unify the annotation of genome databases from a range of different species. It describes concepts related to molecular functions, cellular components, and biological processes (Ashburner, M. et al. 2000).

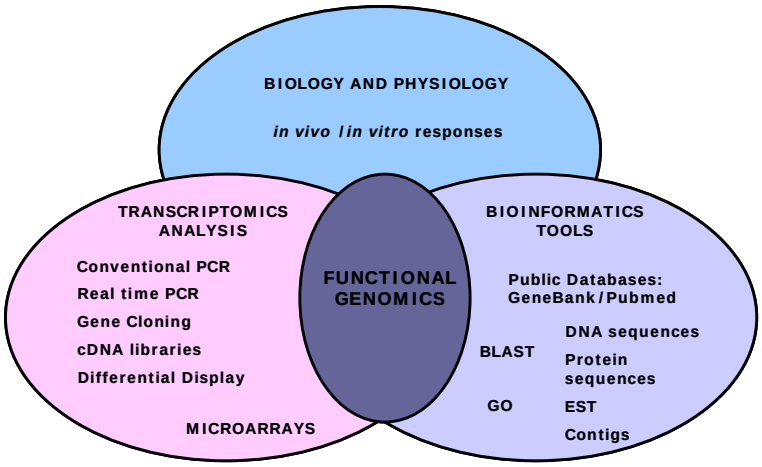


Fig.1.7. Network of functional genomics. Abbreviations: BLAST (Basic Local Alignment Search Tool), EST (Expressed Sequence Tag), GO (Gene Ontology).

9. Aim and outline of this thesis

The research carried out in this thesis was to study gene expression profiles for understanding fish responses under stress and immune challenges by using molecular, bioinformatics and genomics tools. C*hapter 2* refers to high density confinement in sea bream (*Sparus aurata*) as a model for isolation of genes differentially expressed in brain tissue as well as studying physiological parameters responses to an acute stressor. From this experiment, enolase gene was isolated and considered as a possible fish marker in aquaculture. Further analysis and studies over enolase gene, like gene cloning in several fish species, tissue distributions, phylogenetic studies and microarray analysis are developed in *chapter 3*. In *chapter 4*, selection of genes obtained from a macrophage cell culture EST (mEST) database in rainbow trout (*Oncorhynchus mykiss*), was carried out by bioinformatic analysis and gene expression patterns in order to determine candidate genes for studying the immune response in fish. From this selection, the transcription factor PU.1 was considered as a good candidate immune gene due to its key role in the hematopoiesis development. In *chapter 5*, PU.1 gene was characterized and its expression levels in the head kidney and brain by *in situ* hybridization technique were developed. Further analysis studying cell proliferation in the head kidney after an immune challenge was also assisted in this chapter. Microarray analyses in the head kidney and in brain were developed in *chapter 6.* A set of differentially expressed genes gave some answers about the immune

response in fish for coping an LPS immune challenge. The implication of results as well as general discussion and conclusions are reported in *chapter 7*.

10. References

Acerete, L., Balasch, J.C., Espinosa, E., Josa, A. and Tort, L. (2004). Physiological responses in Eurasian perch (Perca fluviatilis, L.) subjected to stress by transport and handling. *Aquaculture*. 237: 167-178.

Aderem, A. and Ulevitch, R.J. (2000). Toll-like receptors in the induction of the innate immune response. *Nature*. 406(6797): 782-7.

Ainsworth, A.J. (1992). Fish granulocytes: morphology, distribution, and function. *Annu. Rev. Fish Dis*. 123–148.

Anderson, M.K., Sun, X., Miracle, A.L., Litman, G.W. and Rothenberg, E.V. (2001). Evolution of hematopoiesis: Three members of the PU.1 transcription factor family in a cartilaginous fish, Raja eglanteria. *Proc Natl Acad Sci U S A*. 98(2): 553-8.

Andersson, E., Peixoto, B., Tormanen, V. and Matsunaga, T. (1995). Evolution of the immunoglobulin M constant region genes of salmonid fish, rainbow trout (Oncorhynchus mykiss) and Arctic charr (Salvelinus alpinus): implications concerning divergence time of species. *Immunogenetics*. 41(5): 312-5.

Andrews, N.C., Erdjument-Bromage, H., Davidson, M.B., Tempst, P. and Orkin, S.H. (1993a). Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein. *Nature*. 362(6422): 722-8.

Andrews, N.C., Kotkow, K.J., Ney, P.A., Erdjument-Bromage, H., Tempst, P. and Orkin, S.H. (1993b). The ubiquitous subunit of erythroid transcription factor NF-E2 is a small basic-leucine zipper protein related to the v-maf oncogene. *Proc Natl Acad Sci U S A*. 90(24): 11488-92.

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M. and Sherlock, G. (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet*. 25(1): 25-9.

Barton, B., Ribas, L., Acerete, L. and Tort, L. (2005). Effects of chronic confinement on physiological responses of juvenile gilthead sea bream, *Sparus aurata* L., to acute handling. *Aquaculture Research*. 36: 172-179.

Barton, B.A., Peter, R.E. and Paulencu, C.R. (1980). plasma cortisol levels of fingerling rainbow trout (*Salmo gairdneri*) at rest, and subjected to handling, confinement, transport and stocking. *Canadian Journal of Fisheries and Aquatic Science*. 37: 805-811.

Beishuizen, A. and Thijs, L.G. (2003). Endotoxin and the hypothalamo-pituitary-adrenal (HPA) axis. *J Endotoxin Res*. 9(1): 3-24.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J. and Wheeler, D.L. (2003). GenBank. *Nucleic Acids Res*. 31(1): 23-7.

Berczi, I., Bertok, L. and Bereznai, T. (1966). Comparative studies on the toxicity of Escherichia coli lipopolysaccharide endotoxin in various animal species. *Can. J. Microbiol*. 12: 1070–1071.

Berman, J.N., Kanki, J.P. and Look, A.T. (2005). Zebrafish as a model for myelopoiesis during embryogenesis. *Exp Hematol*. 33(9): 997-1006.

Brattgjerd, S. and Evensen, O. (1996). A sequential light microscopic and ultrastructural study on the uptake and handling of Vibrio salmonicida inthe head kidney phagocytes of experimentally infected Atlantic salmon, Salmo salar L. *Vet Pathol*. 33: 55-65.

Brubacher, J.L., Secombes, C.J., Zou, J. and Bols, N.C. (2000). Constitutive and LPS-induced gene expression in a macrophage-like cell line from the rainbow trout (Oncorhynchus mykiss). *Dev Comp Immunol*. 24(6-7): 565-74.

Byon, J.Y., Ohira, T., Hirono, I. and Aoki, T. (2005). Use of a cDNA microarray to study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder (Paralichthys olivaceus) following DNA vaccination. *Fish Shellfish Immunol*. 18(2): 135-47.

Carulli, J.P., Artinger, M., Swain, P.M., Root, C.D., Chee, L., Tulig, C., Guerin, J., Osborne, M., Stein, G., Lian, J. and Lomedico, P.T. (1998). High throughput analysis of differential gene expression. *J Cell Biochem Suppl*. 30-31: 286-96.

Castillo, A., Sanchez, C., Dominguez, J., Kaattari, S.L. and Villena, A.J. (1993). Ontogeny of IgM and IgM-bearing cells in rainbow trout. *Dev Comp Immunol*. 17(5): 419-24.

Chrousos, G.P. and Gold, P.W. (1992). The concept of stress and stress system disorders. Overview of physical and behaviour homeostasis. *Journal of American Medical Associations*. 267: 1244-1252.

Crollius, H.R. and Weissenbach, J. (2005). Fish genomics and biology. *Genome Res*. 15(12): 1675-82.

Cummings, M.P. and Meyer, A. (2005). Magic bullets and golden rules: data sampling in molecular phylogenetics. *Zoology (Jena)*. 108(4): 329-36.

Danneving, B.H., Lauve, A., Press, C. and T., L. (1994). Receptor-mediated endocytosis and phagocytosis by rainbow trout head kidney sinusoidal cells. *Fish Shellfish Immunol*. 4: 3-18.

Deviller, G., Palluel, O., Aliaume, C., Asanthi, H., Sanchez, W., Franco Nava, M.A., Blancheton, J.P. and Casellas, C. (2005). Impact assessment of various rearing systems on fish health using multibiomarker response and metal accumulation. *Ecotoxicol Environ Saf*. 61(1): 89-97.

Donaldson, E.M. (1981). The pituitary-interrenal axis as an indicator of stress in fish. Stress and Fish. A. D. Pickering. London, Academic Press. 77-102.

Dorfman, D.M., Wilson, D.B., Bruns, G.A. and Orkin, S.H. (1992). Human transcription factor GATA-2. Evidence for regulation of preproendothelin-1 gene expression in endothelial cells. *J Biol Chem*. 267(2): 1279-85.

Ellis, A.E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Dev Comp Immunol*. 25(8-9): 827-39.

Engelsma, M.Y., Huising, M.O., Van Muiswinkel, W.B., Flik, G., Kwang, J., Savelkoul, H.F. and Verburg-Van Kemenade, B.M. (2002). Neuroendocrine-immune interactions in fish: a role for interleukin-1. *Vet Immunol Immunopathol*. 87(3-4): 467-79.

Evans, D.A., Klemer, J. and Kaattari, S.L. (1998). Heuristic models of the intermonomeric disulfide bonding process. *J Theor Biol*. 195(4): 505-24.

Galloway, J.L., Wingert, R.A., Thisse, C., Thisse, B. and Zon, L.I. (2005). Loss of gata1 but not gata2 converts erythropoiesis to myelopoiesis in zebrafish embryos. *Dev Cell*. 8(1): 109-16.

Gangenahalli, G.U., Gupta, P., Saluja, D., Verma, Y.K., Kishore, V., Chandra, R., Sharma, R.K. and Ravindranath, T. (2005). Stem cell fate specification: role of master regulatory switch transcription factor PU.1 in differential hematopoiesis. *Stem Cells Dev*. 14(2): 140-52.

González Laxe, F., Lupin, H.M. and Cretón De La Cal, J.A. (2004). Acuicultura: producción, comercio y trazabilidad. A Coruña, Netbiblo.

Gornati, R., Papis, E., Rimoldi, S., Chini, V., Terova, G., Prati, M., Saroglia, M. and Bernardini, G. (2005). Molecular markers for animal biotechnology: sea bass (Dicentrarchus labrax, L.) HMG-CoA reductase mRNA. *Gene*. 344: 299-305.

Haddad, J.J., Saade, N.E. and Safieh-Garabedian, B. (2002). Cytokines and neuro-immune-endocrine interactions: a role for the hypothalamic-pituitary-adrenal revolving axis. *J Neuroimmunol*. 133(1-2): 1-19.

Herbomel, P., Thisse, B. and Thisse, C. (1999). Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development*. 126(17): 3735-45.

Herraez, M.P. and Zapata, A.G. (1986). Structure and function of the melanomacrophage centres of the goldfish Carassius auratus. *Vet Immunol Immunopathol*. 12: 117-126.

Higgs, P.G. and Attwood, T.K. (2005). Bioinformatics and Molecular Evolution. Oxford, Blackwell Publishing.

Hirono, I., Takami, M., Miyata, M., Miyazaki, T., Han, H.J., Takano, T., Endo, M. and Aoki, T. (2004). Characterization of gene structure and expression of two toll-like receptors from Japanese flounder, Paralichthys olivaceus. *Immunogenetics*. 56(1): 38-46.

Hohaus, S., Petrovick, M.S., Voso, M.T., Sun, Z., Zhang, D.E. and Tenen, D.G. (1995). PU.1 (Spi-1) and C/EBP alpha regulate expression of the granulocyte-macrophage colony-stimulating factor receptor alpha gene. *Mol Cell Biol*. 15(10): 5830-45.

Holland, J.W., Pottinger, T.G. and Secombes, C.J. (2002). Recombinant interleukin-1β activates the hypothalamic-pituitary-interrenal axis in rainbow trout, *Oncorhynchus mykiss*. *Journal of Endocrinology*. 175: 261-267.

Hontela, A., Rasmussen, J.B., Audet, C. and Chevalier, G. (1992). Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. *Arch Environ Contam Toxicol*. 22(3): 278-83.

Huo, X. and Zhang, J. (2005). Important roles of reversible acetylation in the function of hematopoietic transcription factors. *J Cell Mol Med*. 9(1): 103-12.

Idriss, H.T. and Naismith, J.H. (2000). TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc Res Tech*. 50(3): 184-95.

Iliev, D.B., Liarte, C.Q., Mackenzie, S. and Goetz, F.W. (2005a). Activation of rainbow trout (Oncorhynchus mykiss) mononuclear phagocytes by different pathogen associated molecular pattern (PAMP) bearing agents. *Mol Immunol*. 42(10): 1215-23.

Iliev, D.B., Roach, J.C., Mackenzie, S., Planas, J.V. and Goetz, F.W. (2005b). Endotoxin recognition: in fish or not in fish? *FEBS Lett*. 579(29): 6519-28.

Inoue, J.G., Miya, M., Tsukamoto, K. and Nishida, M. (2003). Basal actinopterygian relationships: a mitogenomic perspective on the phylogeny of the "ancient fish". *Mol Phylogenet Evol*. 26(1): 110-20.

Janeway, C., Travers, P., Walport, M. and Shlomchik, M. (2000). Immunobiology: The immune system in health and disease, Masson.

Janeway, C.A., Jr. and Medzhitov, R. (2002). Innate immune recognition. *Annu Rev Immunol*. 20: 197-216.

Jensen, F.B. (2003). Nitrite disrupts multiple physiological functions in aquatic animals. *Comp Biochem Physiol A Mol Integr Physiol*. 135(1): 9-24.

Kaattari, S., Evans, D. and Klemer, J. (1998). Varied redox forms of teleost IgM: an alternative to isotypic diversity? *Immunol Rev*. 166: 133-42.

Kaattari, S.L. and Irwin, M.J. (1985). Salmonid spleen and anterior kidney harbor populations of lymphocytes with different B cell repertories. *Dev Comp Immunol*. 9: 433-444.

Kimura, T., Jindo, T., Narita, T., Naruse, K., Kobayashi, D., Shin, I.T., Kitagawa, T., Sakaguchi, T., Mitani, H., Shima, A., Kohara, Y. and Takeda, H. (2004). Large-scale isolation of ESTs from medaka embryos and its application to medaka developmental genetics. *Mech Dev*. 121(7-8): 915-32.

Köllner, B., Blohm, U., Kotterba, G. and Fischer, U. (2001). A monoclonal antibody recognising a surface marker on rainbow trout (Oncorhynchus mykiss) monocytes. *Fish Shellfish Immunol*. 11: 127-142.

Koskinen, H., Pehkonen, P., Vehniainen, E., Krasnov, A., Rexroad, C., Afanasyev, S., Molsa, H. and Oikari, A. (2004). Response of rainbow trout transcriptome to model chemical contaminants. *Biochem Biophys Res Commun*. 320(3): 745-53.

Krasnov, A., Koskinen, H., Afanasyev, S. and Molsa, H. (2005a). Transcribed Tc1-like transposons in salmonid fish. *BMC Genomics*. 6: 107.

Krasnov, A., Koskinen, H., Pehkonen, P., Rexroad, C.E., 3rd, Afanasyev, S. and Molsa, H. (2005b). Gene expression in the brain and kidney of rainbow trout in response to handling stress. *BMC Genomics*. 6(1): 3.

Krause, D.S., Theise, N.D., Collector, M.I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S. and Sharkis, S.J. (2001). Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell*. 105(3): 369-77.

Lieschke, G.J., Oates, A.C., Crowhurst, M.O., Ward, A.C. and Layton, J.E. (2001). Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. *Blood*. 98(10): 3087-96.

Lieschke, G.J., Oates, A.C., Paw, B.H., Thompson, M.A., Hall, N.E., Ward, A.C., Ho, R.K., Zon, L.I. and Layton, J.E. (2002). Zebrafish SPI-1 (PU.1) marks a site of myeloid development independent of primitive erythropoiesis: implications for axial patterning. *Dev Biol*. 246(2): 274-95.

Linney, E., Dobbs-Mcauliffe, B., Sajadi, H. and Malek, R.L. (2004). Microarray gene expression profiling during the segmentation phase of zebrafish development. *Comp Biochem Physiol C Toxicol Pharmacol*. 138(3): 351-62.

Locksley, R.M., Killeen, N. and Lenardo, M.J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell*. 104(4): 487-501.

Lyons, S.E., Shue, B.C., Oates, A.C., Zon, L.I. and Liu, P.P. (2001). A novel myeloid-restricted zebrafish CCAAT/enhancer-binding protein with a potent transcriptional activation domain. *Blood*. 97(9): 2611-7.

Mackenzie, S., Planas, J.V. and Goetz, F.W. (2003). LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and in vitro differentiated macrophages. *Dev Comp Immunol*. 27(5): 393-400.

Mackey, M.C. (2001). Cell kinetic status of haematopoietic stem cells. *Cell Prolif*. 34(2): 71-83.

Magnadottir, B., Lange, S., Gudmundsdottir, S., Bogwald, J. and Dalmo, R.A. (2005). Ontogeny of humoral immune parameters in fish. *Fish Shellfish Immunol*. 19(5): 429-39.

Mcgreal, E.P., Martinez-Pomares, L. and Gordon, S. (2004). Divergent roles for C-type lectins expressed by cells of the innate immune system. *Mol Immunol*. 41(11): 1109-21.

Meseguer, J., López-Ruiz, A. and García-Ayala, A. (1995). Reticulo-endothelial stroma of the head-kidney from the seawater teleost gilthead seabream (Sparus aurata L): an ultrastructural and cytochemical study. *Anat Rec*. 241: 303-309.

Miya, M., Takeshima, H., Endo, H., Ishiguro, N.B., Inoue, J.G., Mukai, T., Satoh, T.P., Yamaguchi, M., Kawaguchi, A., Mabuchi, K., Shirai, S.M. and Nishida, M. (2003). Major patterns of higher teleostean phylogenies: a new perspective based on 100 complete mitochondrial DNA sequences. *Mol Phylogenet Evol*. 26(1): 121-38.

Miyazaki, S., Sugawara, H., Gojobori, T. and Tateno, Y. (2003). DNA Data Bank of Japan (DDBJ) in XML. *Nucleic Acids Res*. 31(1): 13-6.

Molinero, A., Gómez, E., Balasch, J. and Tort, L. (1997). Stress by fish removal in the gilthead sea bream , Sparus aurata: A time course study on the remaining fish in the same tank. *J. Appl. Aquacult.* 7(2): 1-12.

Mucenski, M.L., Mclain, K., Kier, A.B., Swerdlow, S.H., Schreiner, C.M., Miller, T.A., Pietryga, D.W., Scott, W.J., Jr. and Potter, S.S. (1991). A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell*. 65(4): 677-89.

Nagai, T., Harigae, H., Ishihara, H., Motohashi, H., Minegishi, N., Tsuchiya, S., Hayashi, N., Gu, L., Andres, B., Engel, J.D. and Et Al. (1994). Transcription factor GATA-2 is expressed in erythroid, early myeloid, and CD34+ human leukemia-derived cell lines. *Blood*. 84(4): 1074-84.

Nelson, J.S. (1994). Fishes of the world. New York, Wiley & Sons.

Neumann, N.F., Stafford, J.L., Barreda, D., Ainsworth, A.J. and Belosevic, M. (2001). Antimicrobial mechanisms of fish phagocytes and their role in host defense. *Dev Comp Immunol*. 25(8-9): 807-25.

O'connor, T.M., O'halloran, D.J. and Shanahan, F. (2000). The stress response and the hypothalamic-pituitary-adrenal axis: from molecule to melancholia. *Qjm*. 93(6): 323-33.

Perlstein, R.S., Whitnall, M.H., Abrams, J.S., Mougey, E.H. and Neta, R. (1993). Synergistic roles of interleukin-6, interleukin-1, and tumor necrosis factor in the adrenocorticotropin response to bacterial lipopolysaccharide in vivo. *Endocrinology*. 132(3): 946-52.

Pickering, A.D. and Pottinger, T.G. (1987). Poor water quality suppresse the cortisol response of salmonid fish to handling and confinement. *Journal of Fish Biology*. 30: 363-374.

Pillay, T.V.R. (2000). Aquaculture: Principles and Practices. Oxford, Blackwell Science.

Press, C.M. and O., E. (1999). The morphology of the immune system in teleost fishes. *Fish Shellfish Immunol*. 9: 309-318.

Rhodes, J., Hagen, A., Hsu, K., Deng, M., Liu, T.X., Look, A.T. and Kanki, J.P. (2005). Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *Dev Cell*. 8(1): 97-108.

Rise, M.L., Von Schalburg, K.R., Brown, G.D., Mawer, M.A., Devlin, R.H., Kuipers, N., Busby, M., Beetz-Sargent, M., Alberto, R., Gibbs, A.R., Hunt, P., Shukin, R., Zeznik, J.A., Nelson, C., Jones, S.R., Smailus, D.E., Jones, S.J., Schein, J.E., Marra, M.A., Butterfield, Y.S., Stott, J.M., Ng, S.H., Davidson, W.S. and Koop, B.F. (2004). Development and application of a salmonid EST database and cDNA microarray: data mining and interspecific hybridization characteristics. *Genome Res*. 14(3): 478-90.

Rivest, S. (2003). Molecular insights on the cerebral innate immune system. *Brain Behav Immun*. 17(1): 13-9.

Rotllant, J., Balm, P.H., Perez-Sanchez, J., Wendelaar-Bonga, S.E. and Tort, L. (2001). Pituitary and interrenal function in gilthead sea bream (Sparus aurata L., Teleostei) after handling and confinement stress. *Gen Comp Endocrinol*. 121(3): 333-42.

Sarropoulou, E., Kotoulas, G., Power, D.M. and Geisler, R. (2005). Gene expression profiling of gilthead sea bream during early development and detection of stress-related genes by the application of cDNA microarray technology. *Physiol Genomics*. 23(2): 182-91.

Scapigliati, G., Romano, N. and Abelli, L. (1999). Monoclonal antibodies in fish immunology: identification, ontogeny and activity of T and B-lymphocytes. *Aquaculture*. 172: 3-28.

Schnare, M., Barton, G.M., Holt, A.C., Takeda, K., Akira, S. and Medzhitov, R. (2001). Toll-like receptors control activation of adaptive immune responses. *Nat Immunol*. 2(10): 947-50.

Schobitz, B., Sutanto, W., Carey, M.P., Holsboer, F. and De Kloet, E.R. (1994). Endotoxin and interleukin 1 decrease the affinity of hippocampal mineralocorticoid (type I) receptor in parallel to activation of the hypothalamic-pituitary-adrenal axis. *Neuroendocrinology*. 60(2): 124-33.

Scott, E.W., Simon, M.C., Anastasi, J. and Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science*. 265(5178): 1573-7.

Secombes, C.J., Van Groningen, J.J. and Egberts, E. (1983). Separation of lymphocyte subpopulations in carp Cyprinus carpio L. by monoclonal antibodies: immunohistochemical studies. *Immunology*. 48(1): 165-75.

Secombes, C.J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K.J., Cunningham, C. and Zou, J. (2001). Cytokines and innate immunity of fish. *Dev Comp Immunol*. 25(8-9): 713-23.

Shivdasani, R.A. and Orkin, S.H. (1996). The transcriptional control of hematopoiesis. *Blood*. 87(10): 4025-39.

Sternberg, E.M. (1997). Neural-immune interactions in health and disease. *J Clin Invest*. 100(11): 2641-7.

Stoesser, G., Baker, W., Van Den Broek, A., Garcia-Pastor, M., Kanz, C., Kulikova, T., Leinonen, R., Lin, Q., Lombard, V., Lopez, R., Mancuso, R., Nardone, F., Stoehr, P., Tuli, M.A., Tzouvara, K. and Vaughan, R. (2003). The EMBL Nucleotide Sequence Database: major new developments. *Nucleic Acids Res*. 31(1): 17-22.

Thorgaard, G.H., Bailey, G.S., Williams, D., Buhler, D.R., Kaattari, S.L., Ristow, S.S., Hansen, J.D., Winton, J.R., Bartholomew, J.L., Nagler, J.J., Walsh, P.J., Vijayan, M.M., Devlin, R.H., Hardy, R.W., Overturf, K.E., Young, W.P., Robison, B.D., Rexroad, C. and Palti, Y. (2002). Status and opportunities for genomics research with rainbow trout. *Comp Biochem Physiol B Biochem Mol Biol*. 133(4): 609-46.

Tort, L., Balasch, J.C. and Mackenzie, S. (2003a). Fish immune system. A crossroads between innate and adaptive responses. *Inmunología*. 23: 277-286.

Tort, L. and Mackenzie, S. (2003b). Fish health challenge after stress. Indicators of immunocompetence. *in press*.

Tort, L., Montero, D., Rubaina, L., Fernández-Palacios, H. and Izquierdo, M.S. (2001). Consistency of stress response to repeated handling in the gilthead sea bream *Sparus aurata* Linnaeus, 1758. *Aquaculture Research*. 32 (7): 593-598.

Tsujii, T. and Seno, S. (1990). Melano-macrophage centers in the aglomerular kidney of the sea horse (Teleost): morphologic studies on its formation and possible function. *Anat Rec*. 226: 460-470.

Van Biesen, G. and Parrish, C.C. (2005). Long-chain monounsaturated fatty acids as biomarkers for the dispersal of organic waste from a fish enclosure. *Mar Environ Res*. 60(3): 375-88.

Ward, A.C., Mcphee, D.O., Condron, M.M., Varma, S., Cody, S.H., Onnebo, S.M., Paw, B.H., Zon, L.I. and Lieschke, G.J. (2003). The zebrafish spi1 promoter drives myeloid-specific expression in stable transgenic fish. *Blood*. 102(9): 3238-40.

Watts, M., Munday, B.L. and Burke, C.M. (2001). Immune responses of teleost fish. *Aust Vet J*. 79(8): 570-4.

Wendelaar Bonga, S.E. (1997). The Stress Response in Fish. *Physiological Reviews*. 77: 591-623.

Wright, S.D. (1999). Toll, a new piece in the puzzle of innate immunity. *J Exp Med*. 189(4): 605-9.

Wrona, D. (2006). Neural-immune interactions: An integrative view of the bidirectional relationship between the brain and immune systems. *J Neuroimmunol*. 172(1-2): 38-58.

Yamamoto, M., Takahashi, S., Onodera, K., Muraosa, Y. and Engel, J.D. (1997). Upstream and downstream of erythroid transcription factor GATA-1. *Genes Cells*. 2(2): 107-15.

Zapata, A.G., Chibá, A. and A., V. (1996). Cells and tissues of the immune system of fish. The fish immune system. Organism, pathogen and environment. G. Iwama and T. Nakanishi, Academic Press.

Zou, J., Secombes, C.J., Long, S., Miller, N., Clem, L.W. and Chinchar, V.G. (2003). Molecular identification and expression analysis of tumor necrosis factor in channel catfish (Ictalurus punctatus). *Dev Comp Immunol*. 27(10): 845-58.

Chapter 2

Physiological responses and differential gene expression in sea bream (*Sparus aurata)* under different stress situations

1. Introduction

1.1. Physiological Parameters

Characteristic endocrine, immune, and other physiological responses of teleostean fish to stressors typically encountered in aquaculture have been studied thoroughly during the last two decades (Barton, B.A. et al. 1991; Iwama, G.K. et al. 1997; Wendelaar Bonga, S.E. 1997). Such responses include elevations in plasma concentrations of cortisol, glucose, lactate and in marine species, osmolality, all of them well established as useful indicators of the degree of acute stress experienced by fish (Barton, B.A. et al. 2002). These stress responses, particularly changes in circulating cortisol, however, can be modified extensively by genetic, development and internal and external environmental factors (Barton, B.A. 2002). For example, prior exposure to other stressors can appreciably alter the response of cortisol to acute stress. The presence of pollutants or contaminants in the water sufficient to chronically elevate plasma levels of cortisol in fish have been shown to attenuate the response to an acute stressor (Hontela, A. et al. 1992; Norris, D.O. et al. 1999; Laflamme, J.S. et al. 2002). This effect possibly occurs as a result of either down-regulation of the hypothalamic-pituitary-interrenal (HPI) axis from continuous negative feedback of cortisol or direct toxic action of the chemical stressor on the functional integrity of the axis (Hontela, A. 1997). A reduced corticosteroid response following an acute stressor has been mimicked by prior continuous treatment with cortisol-impregnated feed, demonstrating the negative-feedback effect of elevated circulating cortisol on the HPI axis (Barton, B.A. et al. 1987; Rotllant, J. et al. 2000) or on related cellular mechanisms (Basu, N. et al. 2002). An altered interrenal res(Barton, B.A. *et al.* 1987)ponse to an acute stressor resulting from chronic stress has been shown in fish exposed to chemical stressors but few studies have demonstrated this phenomenon in fish subjected to a chronic physical stressor such as the continuous crowding or confinement experienced in intensive aquaculture. Continuous crowding suppressed the subsequent cortisol response to an acute stressor in brown trout caused by water quality (Pickering, A.D. et al. 1987). Recently, results showed that confining yellow perch, *Perca flavescens,* reduced their cortisol response to an acute lipopolysaccharide (LPS) challenge (Haukenes, A.H. et al. 2004).

In this study, we compared the physiological responses of juvenile gilthead sea bream, *Sparus aurata*, with an acute handling stressor after holding them at low and high tank densities. Previous studies have documented various responses of these species to handling and confinement stressors (Rotllant, J. et al. 2001; Tort, L. et al. 2001; Rotllant, J. et al. 2003). Further understanding of the nature of these species responses to aquaculture-related stressors are practically important

as the gilthead sea bream is one of the most commonly used fish for commercial marine aquaculture in the Mediterranean Sea (Gasca-Leyva, E. et al. 2003). The persistence of a chronic stressor, such as high population density, may result in decrease in energetic resources, susceptibility to diseases and an overall reduction of performance (Tort, L. et al. 1998). Stress in fish caused by physical disturbances encountered in aquaculture induced changes grouped as primary, secondary, and tertiary responses (Barton, B.A. et al. 1991). Current measured physiological parameters including plasma cortisol, glucose, may not be adequate for a complete biological interpretation when chronic stresses in fish culture are studied (Gornati, R. et al. 2003). Multidirectional immuno–neuro-endocrine interactions from the central focus for the stress response (Besedovsky, H.O. et al. 1996). The cross talk between these systems and resultant secretion of regulatory molecules modulate the physiological response at target tissues acting at a transcriptional level causing changes in gene expression. Therefore, the identification of such target genes for use as molecular markers capable of describing fish welfare is clearly an important aim in the development of diagnostic technologies for increased efficiency in fish farm management and fish welfare. We have identified a differentially expressed gene named *enolase* as a possible candidate for aquaculture as a biomolecular marker from sea bream brain subjected to at confinement different densities.

1.2. The Enolase Enzyme

The Enolase Enzyme Superfamily Members contain ligands for the essential Mg^{2+} ion located a the ends of the third, fourth, and fifth β-strands; the conservation of homologues of these at appropriate locations in the sequences of proteins with more than 300 residues in length, is the primary criterion for identifying new members of the superfamily in the sequence database (Gerlt, J.A. et al. 2005). Enolase Superfamily is composed for a large number of members, to date recognized hundreds of them into database, can be partitioned into four subgroups (enolase, muconate lactonizing, mandelate racemase, 3-methylaspartate ammonia lyase) based on variation of the active site residues but with divergent evolution and functional diversity because catalyse a common partial reaction, Mg^{2+}assisted enolization of the carbon acid, but the enolate anion intermediates are directed to different products by different partial reaction (Babbitt, P.C. et al. 1996). The orthologous members of the enolase subgroup contain a conserved Lysine (Lys) at the end of the sixth β-strand and are thought to be isofunctional catalysing the conversion of 2-phosphoglycerate to phosphoenolpyruvate by hydrolysis (Day, I.N. et al. 1993).

Enolase enzyme (2-phospo-D-glycerate hydrolase) was discovered in 1934 by Lohman and Mayerhof (Lohman, K. et al. 1934) while they were studying the conversion of 3- phosphoglycerate to pyruvate in muscle extracts. Enolase enzyme was further studied during the fifties (Malmstrom, B.G. 1951, 1953, 1955; Malmstrom, B.G. et al. 1959) and since that, intensive and large number of investigations have been carried on from different science fields. Enolase enzyme performs a key role in glycolysis (Babbitt, P.C. et al. 1996) and forms a group of highly conserved molecules which are found in all organisms (Tracy, M.R. et al. 2000) so far that it is used for extensive phylogeny studies (Vidal, N. et al. 2005).

In avian and mammalian tissues three types of enolase isoenzymes (α, β, γ) have been described. The active form of the enzyme is a dimmer compose of, α-subunits in immature organs and in adults liver, β-subunits in adult skeletal muscle and γ-subunits in adult neurons (Rider, C.C. et al. 1975; Fletcher, L. et al. 1976). In developing muscle and in heart muscle the $\alpha\beta$-heterodimer (Rider, C.C. et al. 1975) and in developing nervous system the $\alpha\gamma$-heterodimer (Fletcher, L. et al. 1976; Jorgensen, O.S. et al. 1982) were also detected. In general terms, it is considered that vertebrates express three tissue-specific isoforms non-neural enolase (NNE) or alpha enolase, muscle specific enolase (MSE) or beta enolase and neural-specific enolase (NSE) or gamma enolase (Rider, C. et al. 1975; Segil, N. et al. 1988; Bishop, J.G. et al. 1990) encoded by three different genes (Tracy, M.R. et al. 2000).

Glycolysis has been highly conserved among species, even after the emergence of atmospheric oxygen, which has provided a mean to further oxidize pyruvate by oxidative phosphorylation, resulting in a high yield of energy. Although ambient expression of glycolytic enzymes is necessary for steady-state glucose metabolism, the coordinated increased expression of genes encoding glycolytic enzymes is particularly important for the adaptation to hypoxia (Kim, J.W. et al. 2005). The hypoxia-inducible transcription factor (HIF-1) induce almost all the genes encoding glycolytic enzymes (Poellinger, L. et al. 2004; Schofield, C.J. et al. 2004). Some studies revealed that during hypoxia enolase expression levels are over expressed (Aaronson, R.M. et al. 1995; Discher, D.J. et al. 1998; Roland, I. et al. 2000), as well in fish (Gracery, A.Y. et al. 2001). Enolase detection, has been used as a marker for studying different illness in humans, not only detected directly on tissues (Kinloch, A. et al. 2005; Kuroda, N. et al. 2006; Santini, D. et al. 2006) but also analysing enolase serum protein levels in human patients (Cunningham, R.T. et al. 1991; Lins, H. et al. 2005; Stalnacke, B.M. et al. 2005).

Recent findings have suggested that glycolytic enzymes are more complex and multifaceted proteins rather than simple components of the glycolytic pathway. This suggest links between metabolic sensors and transcription processes are

established directly through enzymes that participated in metabolism (Kim, J.W. et al. 2005). These new functions include transcriptional regulation [hexokinase-2 (HK-2), lactate dehydrogenase-A, glyceraldehyde-3-phosphate dehydrogenase (GADH) and α-enolase], apoptosis (HK-2 and GAPD) and cell motility (glucose-6-phosphate isomerase). α-Enolase when acting as a transcriptional regulator in terms of biological functions has been described as a alternative splicing form of Myc-binding protein (MBP-1) (Feo, S. et al. 2000; Subramanian, A. et al. 2000). Several studies have identified MBP-1 as a transcriptional suppressor of c-Myc via binding to the Myc promoter (Ray, R.B. et al. 1995). Ectopic MBP-1 expression induces cell death and growth suppression, and its N terminal is essential for DNA binding and transcriptional repressor activity (Ray, R.B. et al. 1995; Ghosh, A.K. et al. 1999; Subramanian, A. et al. 2000). Recent studies have revealed that the coding sequence of MBP-1 is an internally initiated translational product of α-enolase mRNA, sharing 95% sequence identity with that of α–enolase, and both genes are located in chromosome 1 (Feo, S. et al. 2000; Subramanian, A. et al. 2000). Interestingly, α-enolase is down-regulated in lung cancer, and reduced expression is associated with poor survival of patients (Chang, Y.S. et al. 2003). Other studies of enolase functions involved in immune system as an immunosuppressive protein have been reported (Veiga-Malta, I. et al. 2004; Chandran, V. et al. 2006). The multifunctional role of enolase in mammalian cells is further supported by the identification of the Arabidopsis LOS2 as a locus responsible for cold-responsive gene transcription where enolase was encoded as modulating gene promoter (Lee, H. et al. 2002). Enolase is expressed on the surface of a variety of eukaryotic cells as a strong plasminogen-binding receptor (Miles, L.A. et al. 1991; Nakajima, K. et al. 1994) however, it still remains to be clarified how the glycolytic enzyme translocates the membranes (Ueta, H. et al. 2004). The neurotrophic and neuroprotective properties of γγ enolase have been also been reported (Hattori, T. et al. 1995). Enolase has also been identified as a heat shock protein, involved in both thermal tolerance and growth control (Iida, H. et al. 1985). Other functions has been identified enolase as eye τ-crystallin protein in fish, reptiles and birds (Wistow, G.J. et al. 1988).

Objectives

1. Determine from a physiological point of view, whether sea bream subjected to a high-density (HD) confinement stressor would show reduced responses, particularly in cortisol, to a subsequent acute stressor compared with those in fish held at a low density (LD).

2. Identify by developing molecular technologies, gene transcriptional levels in sea bream brain subjected to chronic confinement.

3. Isolate and determine such gene/s as a possible gene/s aquaculture fish markers as candidates for determine of fish welfare and measuring relative responses to stress.

2. Materials and Methods

2.1. Animals

Sea bream *Sparus aurata* of 50 g approximately were obtained from Aquadelt Fish Farm, Sant Carles de la Ràpita, Spain. The fish were transported to aquarium facilities at the Universitat Autònoma de Barcelona where they were divided equally into eight rectangular fibreglass tanks. About 260 fish were held in 200 L of 33–36% seawater at 19ºC and a density of 6 kg/m^3 for 2 weeks acclimation before the experiments. Water was recirculated by a small submersible aquarium pump (Eheim, Berlin, Germany) in each tank that passed the water through an individual biofilter unit containing nitrifying bacteria to remove ammonia wastes. During acclimation and experiments, un-ionized ammonia levels ranged from 0.08 to 2.85 mg/L, pH ranged from 6.35 to 8.55, and nitrite levels ranged from 0.01 to 2.61 mg/L. Water was vigorously aerated continuously with compressed air supplied by a common diaphragm pump and delivered in each tank through an airstone. Tanks were kept partially covered under a photoperiod of 12h light/12h dark with the light period commencing at 7:00 h. The fish were fed at maintenance ration of about 0.3–0.5% body weight per day with a commercial sea bream growth diet (INVE Technologies, Baasrode, Belgium). Water level was checked each time experiment in order to keep a correct confinement density.

2.2. Chronic confinement

For the chronic high density (HD) confinement treatment, four tanks containing 33 juvenile sea bream per tank were held at a density of 26 kg/m^3 for 14 days. The remaining four tanks containing 33 fish per tank and more water at the acclimation density of 6 kg/m^3 served as low density (LD) control groups. Five fish were removed carefully to avoid unduly disturbing remaining fish from each of two of the four tanks (N=10) for both LD and HD treatments at the onset of the experiment (day 0). Five fish per tank were then removed from the same two tanks on day 2 and from the other two tanks on days 1 and 7 for each treatment. This approach was taken to keep any acute disturbance to remaining fish from fish removal as low as possible; both density groups were treated in the same manner. On day 14, two to three fish per tank were taken from all four tanks (N=10) for

each treatment just before commencing the acute handling experiment, these samples also served as hour 0 samples for acute handling.

All fish were sampled by first placing them into a lethal concentration of 2-phenoxy-ethanol (500 µl/L) after being removed from the tank with a hand-net; fish were immobilized in less than 1 min. Blood was obtained from the caudal vasculature and transferred to a 1.5 ml microcentrifuge tube containing 5% sodium heparin. Plasma was separated by 5 min centrifugation and stored at –20°C for subsequent analysis of cortisol, glucose, lactate, osmolality. Moreover, brain tissues were removed after 14 days of experiment to both HD and LD groups and directly frozen in liquid nitrogen. Brains were stored at -80ºC for future RNA extraction, Differentially display and Northern-Blot analysis.

2.3. Acute handling stress

After 14 days of HD or LD confinement, fish in all tanks were subjected to an acute handling stressor by holding them in the air in a net for 30 s. Fish from each treatment group were then divided equally into eight tanks per treatment in order to provide separate replicate sample groups at post-handling times without disturbing remaining fish. Water volumes in tanks were kept similarly to those used for the LD and HD chronic treatments accordingly. Five fish from each of two tanks (N=10) were bled at hours 1, 2, 4 and 8 after handling.

2.4. LPS intraperitoneal injection

Twenty fish, *S.aurata*, were used for this experiment. Animal conditions were the same as said above. All fish, previously anaesthetised with a low concentration of phenoxy-ethanol (500 µl/L), were injected intraperitoneally (IP) with 8 mg/kg of lipopolysaccharide Escherichia coli (LPS) (LPS IP), except control fish in which saline buffer was used. LPS and saline were administrated via intra-peritoneal (IP) injection. Time 0 h of this experiment was IP administration using five fish per group. Samples from five fish per group were taken 12, 48 and 72 h post-injection. Brain tissues were removed from all fish and directly frozen in liquid nitrogen and stored at –80ºC for future RNA extraction and Northern-Blot analysis.

2.5. Physiological parameters

Plasma cortisol levels were measured by radioimmunoassay (R.I.A) following the procedure described by (Rotllant, J. et al. 2001). The antibody used for the assay was purchased from Biolink, S.L.. Serum glucose and lactate levels were determined by enzymatic colorimetric analysis in ELISA plate using

commercial kits (Biomerieux, 61270 and 61192). Osmolality in plasma was assessed by measuring freezing point in an Osmomat osmometer.

2.6. RNA extractions

Total RNA was extracted from *S.aurata* brain tissues using an established RNA purification method with minor modifications (Mackenzie, S. et al. 2002). In brief, tissues were homogenized with guanidinium thiocyanate homogenisation buffer. Nucleic acids were extracted in phenol/chloroform/isoamyalcohol with 2M Sodium Acetate, and precipitated overnight at –20ºC with ethanol 100%. The precipitate was washed in ethanol (70%). Samples were dissolved at required concentrations in DEPC water.

2.7. Differential display

Differential display (DD) was performed with RNAimage® kit (GenHunter Corporation, U.S.). Brain samples from fish subjected to confinement stress at 0 and 14 days were analysed by DD. Three reverse transcription reactions (RT-PCR) for each RNA sample (0.1 μg/μL) were done. Each reaction contained; 2 μM of one of the three different one-base-anchored H-T_{11}M primers (where M was G, A or C), 5X RT buffer (125mM Tris-Cl, ph 8.3, 188 mM KCl, 7.5 mM $MgCl_2$ [Cat- Nº. S401] and 25 mM DTT), dNTP Mix 1, 1 μL MMLV (Moloney murine leukemia virus) reverse transcriptase and DEPC water in a final volume of 20 μL. The thermocycler was programmed to 65°C for 5 min, 37°C for 60 min, 75°C for 5 min and 4°C. Amplification of cDNA products was performed in 20 μL reaction for each primer pair combination. Each PCR mixture contained 2 μL of cDNA and 18 μL of a solution containing 10X PCR buffer, 1 mM dNTPs Mix 2, 2 μM H-T_{11}M primers, 2 μM concentration of one of three 5'-arbitrary primer (AP49, AP53 or AP55), 1 unit of Taq DNA polymerase (Qiagen), 10 μCi $_{35}$S-ATP, DEPC water was added to adjust final volume. The sequences of different primers are the following: AP49 (5'-aagctttagtcca-3'), AP53 (5'-aagcttcctctat-3'), AP55 (5'-aagcttacgttag-3'). Reaction mixtures were subjected to a PCR using the following parameters: 94°C: 30 s, 42°C: 2 min, 72°C: 30 s 40 cycles followed by 72°C: 5 min and 4°C. All PCR were performed in duplicate and a negative control was checked. The amplified cDNAs produced from duplicate reactions of RNA isolated from HD time 0 days and 14 days of experiment were size-fractionated in parallel by 6% polyacrylamide 8M urea electrophoresis gels. Following electrophoresis, gels were dried onto a Whatman 3MM paper (Whatman, Maidstone, Kent, UK) on a gel dryer at -80°C for 2 hours and exposed to Kodak X-AR film (Eastman Kodak, Rochester, NY, USA) for 48h. Differentially expressed cDNAs were visualized by autoradiography. To isolate

differentially expressed cDNA fragments, regions were cut from the gel, and DNA was extracted by incubating the gel slices with 100 μL of MQ water for 15 min. at 100°C. The fragments were then reamplified as above but using dNTP Mix 1 and with a final volume of 40 μL. PCR products were run in 1% agarose gel stained with ethidium bromide. The reamplified cDNA were extracted from agarose gel by using a Nucleotrap® kit (Macherey-Nagel) and cloned into a bacterial plasmid vector (pGEM-T Easy, Promega). For screening recombinant bacterial colonies for the presence of target plasmid DNA with DNA inserts, CloneChecker™ Kit (GibcoBRL®) were used. DNA inserts were subsequently sequenced using T7 and SP6 primers by a dye terminator cycle sequencing kit (Thermo Sequenase II, Amersham). Analysis of cDNA sequence data including comparisons, alignments and translations to protein, were performed using a BioEdit software (Internet downloaded). In order to find homology with other gene sequences, cDNA sequence were analysed by Basic Local Alignment Search Tool (BLAST) through the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Significant homology was considered when more than 50% of nucleotide acid compositions of bands was found.

2.8. Northern blot analysis

Approximately 10 μg of total RNA from brain tissues at HD time 0 days and 14 days from chronic confinement experiment (N=3) and 10 μg of total RNA from brain tissues from LPS injection experiment (N=2), was loaded onto a formaldehyde-agarose gel, transferred onto a nylon membrane (Nytran® Super Charge, Schleicher & Shuell) and cross-linked. The membrane was hybridised overnight at 42°C with a ^{32}P-labelled 1.1 kb cDNA Enolase fragment ($1.1x10^6$ cpm/μL) obtained as a probe by described above. The membrane was washed two times with 2XSSC/0.1% SDS at room temperature, once with 1XSSC/0.1%SDS at 42°C and once with 0.1XSSC/0.1% SDS at 42°C. Subsequently, the membrane was exposed to Kodak X-AR film at –80°C. After stripping, the same blot was rehybridized using S18 ^{32}P-labelled cDNA fragment from *Oncorhyncus mykiss* muscle as a housekeeping gene (rRNA). Bands obtained from developed film of Northern Blot analysis, were measured by using Bio-Rad Multi-Analyst™/PC Version 1.1 software. The data were analysed statistically as explained next.

2.9. Data analysis

Values were calculated as the mean ± SEM for each experimental group (N=10). Results of physiological parameters were analysed with the SPSS 10.0 (Statistical Package for Social Sciences) statistical package. A one-way analysis of

variance (ANOVA) was applied to detect significant differences in physiological parameters. For plasma cortisol and glucose analysis of chronic stress experiment, a Duncan post-hoc test and a Tukey post-hoc test was used respectively. The rest of the parameters and groups were analysed with Student-Neuman-Keuls (SNK) as a post-hoc test. The level for accepted statistical significance was $P \leq 0.05$. For molecular biology results, a significant homology of bands with some gene from BLAST was considered when more than 50% identities of nucleotides composition were found. In order to calculate identities and similarities for sequence alignments, a PAM250 Similarity Matrix was used. Values from RNA quantification of Northern Blot were calculated as the mean ± SEM for each experimental group. Data from density experiment was analysed by T-Student (N=3) and for LPS experiment by ANOVA with SNK as a post-hoc test (N=2). Significant difference was considered when $P \leq 0.05$.

3. Results

3.1. Physiological parameters

3.1.a. Cortisol analysis

Density experiments revealed that fish subjected to a confinement stress for 14 days at 26 kg/m^3, showed significant increase of plasma cortisol levels (Fig.2.1.A). Plasma cortisol levels increased up to 22,17 ng/ml (7 day) and 22.78 ng/ml (14 day) in fish held at HD; this value was 12,6 (7 day) 13.0 (14 day) fold higher than in control group 1,75 ng/ml. In addition, after 1 and 2 hours of acute handling stress, lower plasma cortisol levels were observed in the HD compared to the LD group (Fig.2.1.B). Moreover, significant increases were detected between LD groups at time 1 and 2 hours post acute stress.

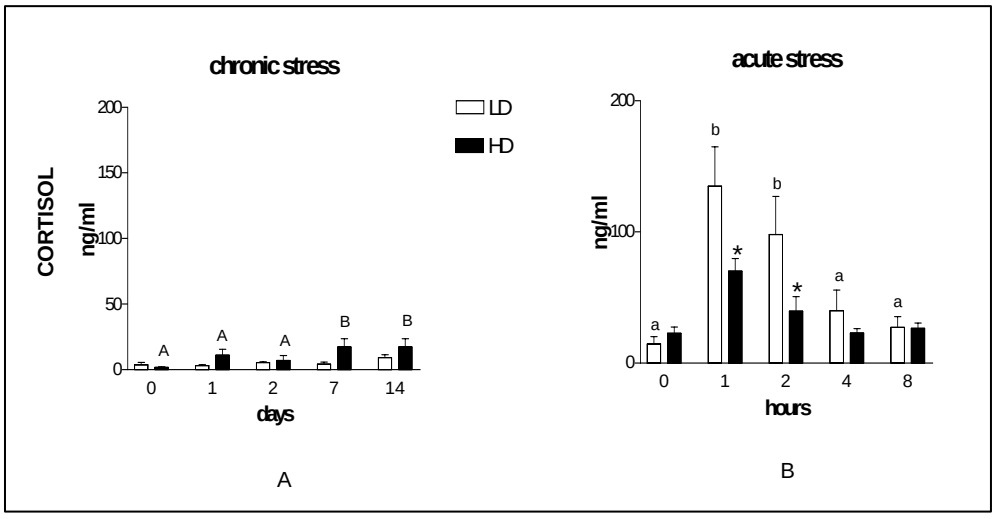


Fig.2.1. Physiological responses of sea bream *Sparus aurata* to acute handling following chronic confinement: plasma cortisol levels. A) Plasma cortisol levels of fish subjected to chronic confinement treatment during 14 days. B) Plasma cortisol levels of fish subjected to an acute handling stress (30 s) after being confined for 14 days. Values are represented as the mean ± SEM (N=10). Significant differences were considered when P≤0.05. Asterisk represents significant difference between HD and LD at a same time. Lower letters represent significant difference between LD group and higher letter between HD groups. LD= low density group; HD= high density group.

3.1.b. Glucose analysis

No significant differences were found for glucose levels in plasma neither for HD and LD groups during 14 days of confinement (Fig.2.2). However, plasma glucose levels were significantly higher after 4 hours of acute handling stress in the HD group compared to LD (Fig.2.2). The peak of plasma glucose levels shows a temporal difference. Thus, HD glucose levels were highest 4 hours after handling stress, whereas in the LD group this peak was reached only 2 hours after acute handling stress.

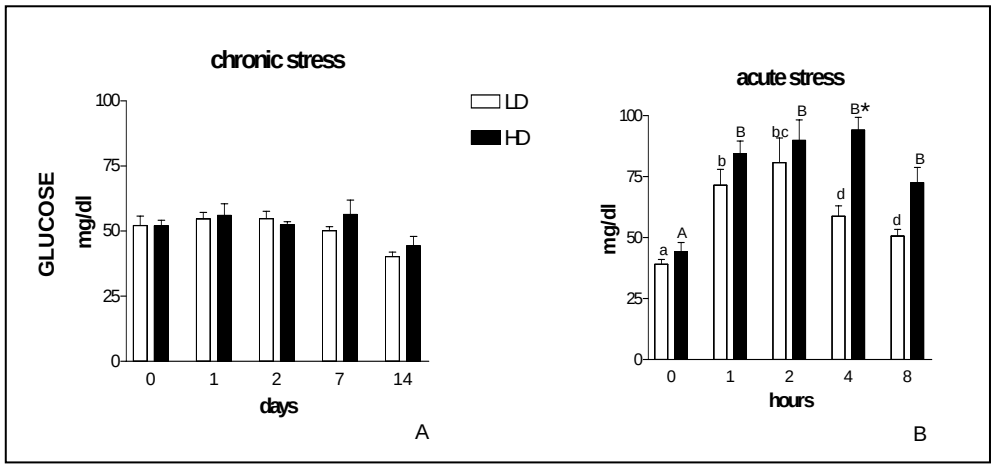


Fig.2.2. Physiological responses of sea bream *Sparus aurata* to acute handling following chronic confinement: plasma glucose levels. A) Plasma glucose levels of fish subjected to chronic confinement treatment during 14 days. B) Plasma glucose levels of fish subjected to an acute handling stress (30s) after being confined for 14 days. Values are represented as the mean ± SEM (N=10). Significant differences were considered when P≤0.05. Asterisk represents significant difference between HD and LD at a same time. Lower letters represent significant difference between LD group and higher letter between HD group. LD= low density group; HD= high density group.

3.1.c. Lactate analysis

Plasma lactate levels did not show any significant difference between fish subjected to chronic confinement stressor at any sampling time (Fig.2.3). However, after 2 hours of acute handling, significant differences were found between LD and HD groups. Within the LD group lactate levels increased significantly after 1 and 2

hours of acute handling stress whereas for the HD group this increase was only observed 1 hour after acute handling stress (Fig.2.3).

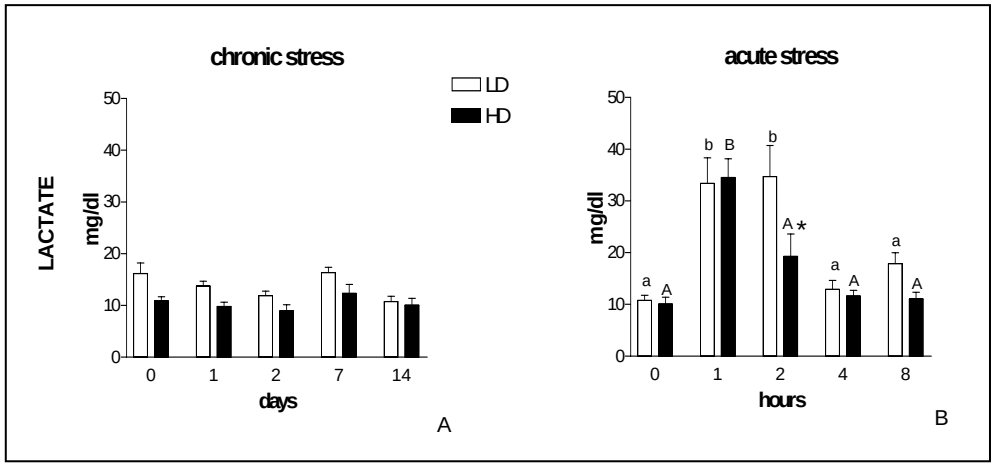


Fig.2.3. Physiological responses of sea bream *Sparus aurata* to acute handling following chronic confinement: plasma lactate levels. A) Plasma lactate levels of fish subjected to chronic confinement treatment during 14 days. B) Plasma lactate levels of fish subjected to an acute handling stress (30 s) after being confined for 14 days. Values are represented as the mean ± SEM (N=10). Significant differences were considered when P≤0.05. Asterisk represents significant difference between HD and LD at a same time. Lower letters represent significant difference between LD group and higher letter between HD groups. LD= low density group; HD= high density group.

3.1.d. Osmolality analysis

Significant differences were found at days 2 (decrease of osmolality) and 14 (increase of osmolality) in confinement experiments, both for LD and HD group (Fig.2.4). The LD group showed a significant difference after 1 and 2 hours of acute handling stress, whereas in the HD group this significant difference in osmolality was observed 1 hour after (Fig.2.4).

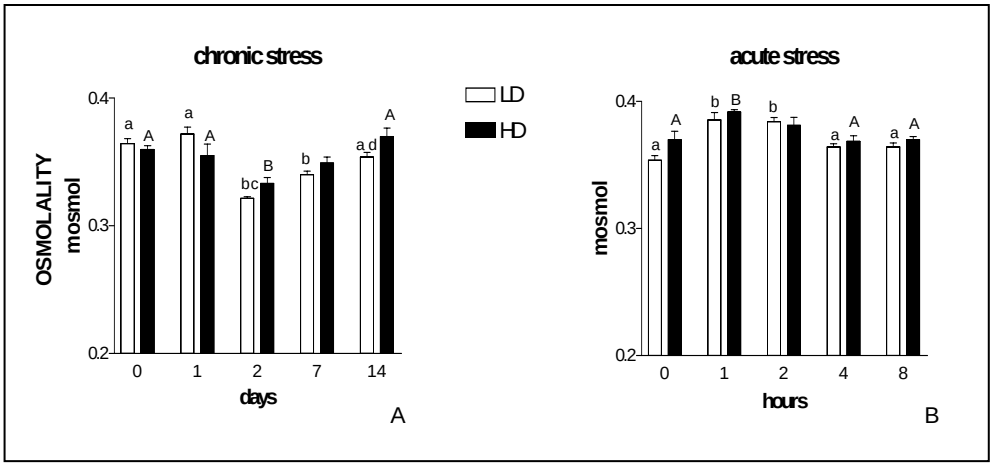


Fig.2.4. Physiological responses of sea bream *Sparus aurata* to acute handling following chronic confinement: plasma osmolality levels. A) Plasma osmolality levels of fish subjected to chronic

confinement treatment during 14 days. B) Plasma osmolality levels of fish subjected to an acute handling stress (30 s) after being confined for 14 days. Values are represented as the mean ± SEM (N=10). Significant differences were considered when P≤0.05. Asterisk represents significant difference between HD and LD at a same time. Lower letters represent significant difference between LD group and higher letter between HD groups. LD= low density group; HD= high density group.

3.2. Differential display (DD)

In order to check the differential gene expression in brain tissues from fish subjected to 14 days confinement stress compared to control group (HD 0 confinement days), the DD molecular technique was performed. Seven cDNA bands were obtained by using primer pairs (see Materials and Methods section 2.5) which showed differentially gene expression. The size of these bands were between 210 to 1106 nucleotides, but only two of them showed significant homology to some gene of the Gene Bank public database. For this reason, we could consider those five sequences as novel genes or probably to unidentifiable coding sequences in sea bream. One sequence with 181 bp resulted with a significant homology (more than 50% of nucleotide composition) by blasting it against the EST database to a cloned EST sequence named Mochii (Accession Number: BJ079382) which was obtained from the tailbud of *Xenopus laevis*. More interestingly, we found significant and high homology of the Enolase gene nucleotide sequence with one of those sequences obtained by DD. Concretely percentages of protein identities, 74% homology to human enolase-1, 74% to rat neuron-specific enolase, 73% to mice enolase-3 beta muscle, 75% to frog enolase-1, 65% to zebrafish enolase-3 and 90% to salmo trutta enolase alpha-2. The enolase sequence cloned was composed of 1106 base pairs (bp), where 777 bp were homologous to the coding sequence region (CDS) to other enolase already cloned. This CDS contents 55.6% of A+T, and 44.4% of G+C. Translation of the *Sparus aurata* enolase (S-enolase) fragment to protein resulted in a 259 amino acid sequence. Protein alignment (Fig.2.5) was carried out to check conservation of this gene between different species throughout evolution, and to compare the homology of our sequence with other published sequences of different species: *Homo sapiens* enolase-1; NM001428, *Rattus norvegicus* neuron-specific enolase; AF019973, *Mus musculus* enolase 3 beta muscle; NM007933 *Xenopus laevis* enolase-1 similar to enolase-3 beta muscle; BC045082; *Danio rerio* enolase-3; AY130387 *Salmo trutta* enolase alpha-2; AY005162. The novel S-enolase gene of protein containing sequences for both nucleotide and amino acid composition, were submitted to the Gene Bank database with accession number AY263379.

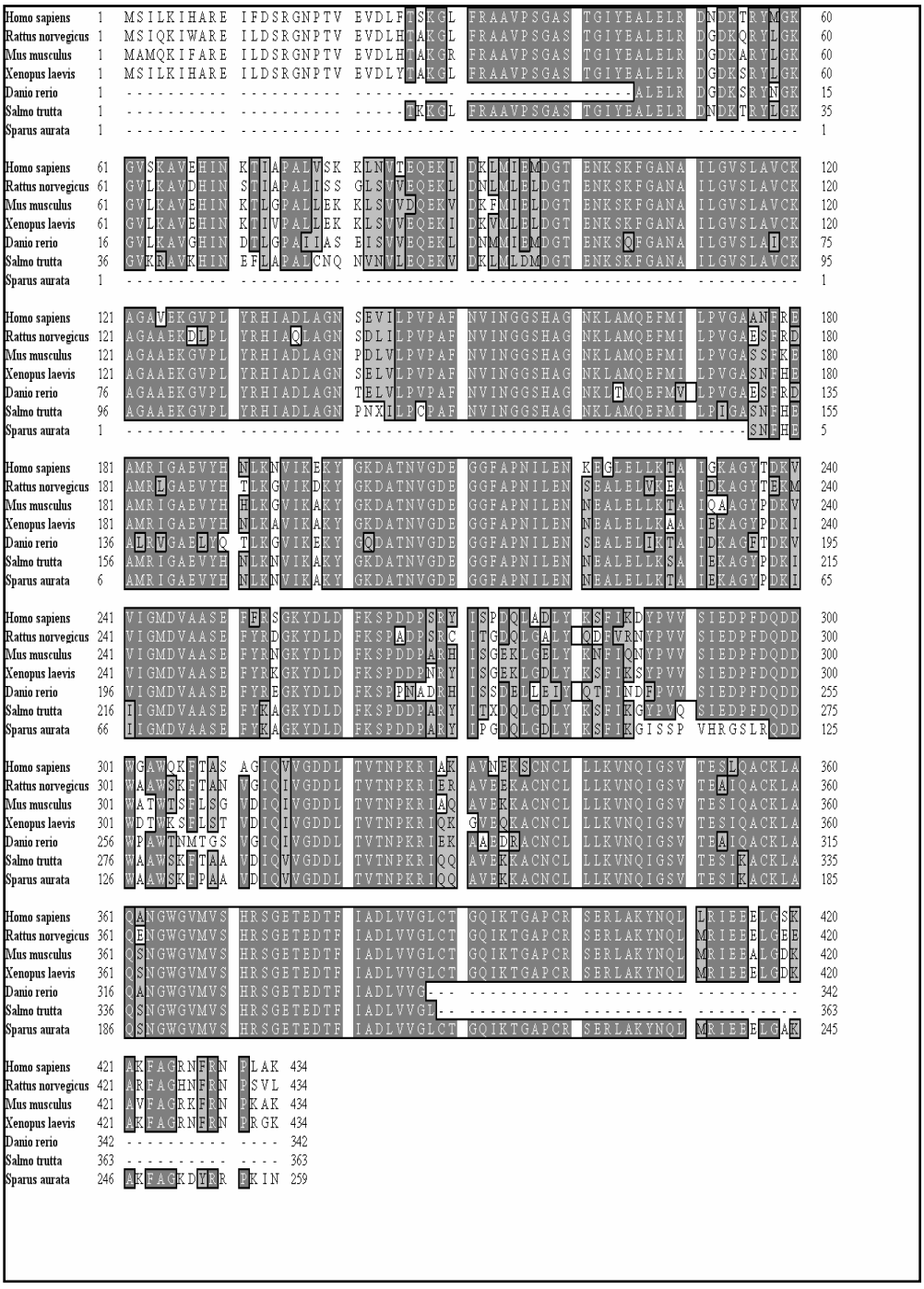


Fig.2.5. Protein alignment of different enolase sequences of different species. Accession numbers to Gene Bank data base are the followings: *Homo sapiens* enolase-1; NM001428, *Rattus norvegicus* neuron-specific enolase; AF019973, *Mus musculus* enolase 3 beta muscle; NM007933 *Xenopus laevis* enolase-1 similar to enolase-3 beta muscle; BC045082; *Danio rerio* enolase-3; AY130387 *Salmo trutta* enolase alpha-2; AY005162, *Sparus aurata* enolase; AY 263379.

3.3. Northern blot analysis

A specific Enolase mRNA with a size of 1.9 kb was identified by Northern Blot Analysis. From the density experiment blot, an up regulation of enolase enzyme was seen after 14 days of confinement. In addition, a significant up-regulation of S-enolase (3.95 fold higher than control) was also detected after 48 hours of LPS IP administration. Bands were compared to housekeeping gene rRNA S18 (1.8 kb). These results indicate that the S-enolase sequence obtained by DD is detectable in fish brain mRNAs population. Bands from both experiments obtained from Northern Blot are shown in the following figures (Fig.2.6 and Fig.2.7).

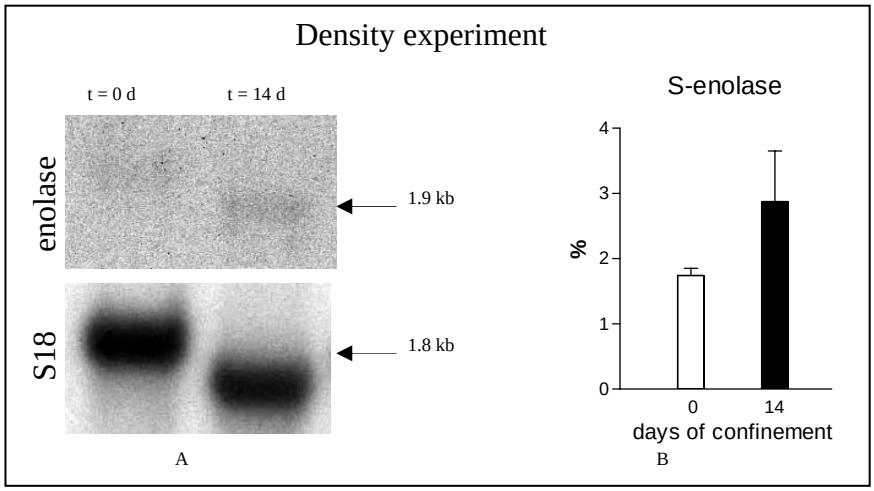


Fig.2.6. Northern Blot Analysis results from fish brain confined 0-14 days (26 kg/m^3). A: mRNAs S-enolase bands from confined fish brain and S18 bands from confined fish brains. B: Results obtained after the quantification of mRNA bands. T-Student was used as a statistical test. No significant differences (P≤0.05) were found. Values are represented as the mean ± SEM (N=3).

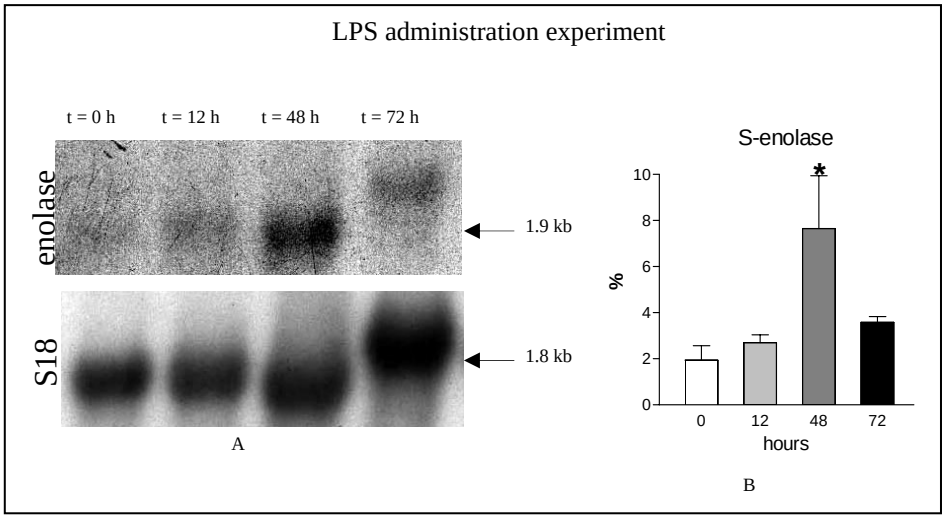


Fig.2.7. Northern Blot Analysis results of fish brain after LPS administration. A: Brain mRNAs S-enolase bands at several sampling times. B: Brain mRNAs S18 bands at several sampling times. C: Results obtained after the quantification of mRNA bands. ANOVA was used as a statistical test. * indicates

significant difference (P≤0.05). Values are represented as the mean ± SEM (N=2). For Northern Analysis, one representative experiment is shown.

4. Discussion

When water quality criteria are preserved and feeding restrictions are avoided, fish density may become one of the most relevant factors expected to potentially affect fish welfare. In our experiments, chronic exposure to confinement stress induced a significant elevation in plasma cortisol levels at 7 days, and it was maintained at least until 14 days of exposure. The duration of such elevation depends on the species studied and the nature of the stressor (Barton, B.A. et al. 1987). When an acute handling stress was applied after confinement, cortisol response was significantly lower in HD group, which were previously exposed to high density situation, than in LD at time 1 and 2 h. These results suggest that there is a limit to the maximum cortisol levels which can be reached, and therefore a limit to the capacity of the interrenal tissue to be stimulated and/or release cortisol (Rotllant, J. et al. 1997). It is well documented in mammals that corticosteroids, acting by negative feedback at the levels of both the hypothalamus and pituitary gland, inhibit the response to stress, this is also true in fish (Rotllant, J. 1999). Cortisol may exert ultra-short-loop negative feedback directly at the level of the interrenal gland to effect self-suppression (Bradford, C.S. et al. 1992). Fish treated with exogenous cortisol prior to confinement have lower plasma ACTH levels than control fish, thus showing that cortisol acts in sea bream by suppressing the release of ACTH from corticotropic cells in the pituitary (Rotllant, J. et al. 2000). The reason for the observed lower cortisol response to the acute stressor in the HD group may be a result of down-regulation of HPI axis from chronic stress. This would have been caused by the higher level of cortisol observed during 14 days of the chronic stressor. Results suggest that the fishes ability to display normal acute cortisol response is reduced after being confined.

Glucose levels did not show significant differences between HD and LD groups during confinement. Although the results obtained are not conclusive, this response has been observed in the sparid red porgy *Pagrus pagrus* (Rotllant, J. et al. 1997). However, glucose levels between LD and HD groups did differ after acute handling stress. Glucose levels in the HD group peaked at 4 h whereas the LD peak was at 2 h. Furthermore, glucose levels were higher in the HD group in all samplings. It has been demonstrated that the first plasma glucose increases results from a consequence of catecholamine release (Andersen, D.E. et al. 1991). This phenomenon is adaptive, that is, it may be amplified after each stress event (Barton, B.A. et al. 1986). Our results suggest that the HD group may produce

more glucose as a consequence of the acute handling stress situation. High stocking density has been shown to produce a wide variety of effects such as increased mobilization of energy sources in fish (Montero, D. et al. 1999). Fish struggling and muscle contraction may produce the observed increase in plasma lactate. Nevertheless, chronic and low density stressors may not induce significant changes in lactate (Tort, L. et al. 1998). Although lactate levels during the density experiment did not show any significant difference in both groups, these levels were significantly lower in the HD group 2 h after acute handling. In view of these results, it may appear that the HD group did not use a prolonged anaerobic muscular response to acute stress as LD did. As previously mentioned, glucose levels were higher in HD after acute handling stress, so it may be hypothesized that HD fish used more aerobic metabolism sources. There is a shift from catabolism to anabolism and an important imbalance in the ionic and osmotic regulation, mostly due to the cortisol action in gills (Wendelaar Bonga, S.E. 1997). Due to lack of stable values in the LD group, osmolality results in the chronic experiment were not conclusive. Since, after acute handling stress higher levels of osmolality are observed in both groups, osmolality was not a sensitive indicator to detect differences between HD and LD in our experiment.

The Differential Display technique allows the study of differential gene expression after experimental variations. By using DD brain samples from sea bream subjected to high density confinement during a period of 14 days, showed seven different bands that were isolated, cloned and sequenced. Five of them did not have significant homologies with any sequences found within the Gene Bank database at the moment. However, with the large increase in ESTs availability for sea bream and better annotation software this may be resolved in the near future. Until that, considering them as novel genes or unidentifiable coding sequences in *Sparus aurata* is legitimate. Furthermore, one sequence of 181 bp had a significant homology (more than 50% of nucleotide composition) with an EST sequence named Mochii (Accession Number: BJ079382) which was obtained from the tailbud of *Xenopus laevis*.

In addition to this, a specific band was obtained by DD. This sequence showed high homology to the enolase glycolytic enzyme. The enzyme enolase (2-phospo-D-glycerate hydrolase) is found in all organisms (Tracy, M.R. et al. 2000). Enolase is responsible to catalyse the conversion of 2-phosphoglycerate to phosphoenolpyruvate (Day, I.N. et al. 1993) by hydrolysis. Vertebrates exhibit three, tissue-specific enolase isozymes: non-neural enolase (NNE), muscle specific enolase (MSE) and neural-specific enolase (NSE) (Rider, C. et al. 1975; Segil, N. et al. 1988; Bishop, J.G. et al. 1990). NNE is a homodimer of α-enolase subunits and

is found in liver tissue, MSE is a homodimer of β-enolase muscle cells, and NSE is a homodimer of γ-enolase, are localized in neuronal and neuroendocrine cells (Oliva, D. et al. 1989). An α heterodimer from neuronal tissue has also been reported (Schmechel, D.E. et al. 1980). Enolase is a member of the Enolase Superfamily consisting in a large number of enzymes which retain the ability to catalyse the thermodynamically to step proton abstraction (Babbitt, P.C. et al. 1996). Analysis of sequence and structural similarities among these proteins suggests that all of their chemical reactions are mediated by a common active site architecture (Babbitt, P.C. et al. 1996; Gerlt, J.A. et al. 2005). The enolase enzyme belongs to a conserved gene family which allows to undertake an evolutionary study of gene duplication in animals (Tracy, M.R. et al. 2000), where a single ancestor for the members of this gene family has been proposed (Oliva, D. et al. 1991).

A cDNA enolase sequence, S-enolase, was partially cloned for the first time in the sparid fish *Sparus aurata*. Eventually, the sequence database were sufficiently populated to allow that the homologous relationship of enolases that S-enolase could be recognized by sequence alignment to other species. To date, the current databases contain sequencer for more than 600 enolases. Results from Gene Bank database analysis revealed high homology of S-enolase to enolases of other species (e.g.: 74% to *Homo sapiens* enolase-1). Studies in fish are often complicated due to the large amount of existing species and the scarcity of the genomic resources for most species as the majority of all genes databases are belonging to zebrafish (*Danio rerio*) and fugu (*Fugu rubripes*). For these reasons, the cloned S-enolase sequence is an important contribution to the knowledge of fish gene database. S-enolase nucleotide and amino acid sequences isolated from brain have been submitted to the Gene Bank database (Accession number: AY263379). Differences in gene expression primarily identified by DD were certified by Northern analysis where, S-enolase was up-regulated during both chronic confinement stressor for 14 days and after LPS challenge. As previously mentioned, enolase is a glycolytic enzyme responsible to catalyse the conversion of 2-phosphoglycerate catabolism to phosphoenol pyruvate by hydrolysis. Enolase enzyme has a central role related to metabolic sources in the animal, as it is necessary to convert the glucose reservoir to functional energy for the organism (Voet, D. et al. 1990). The activities of glycolytic enzymes in the brain of teleost species such as rainbow trout support the contention of a high glycolytic potential (Soengas, J.L. et al. 1998a). The absence of food in teleosts elicits profound changes in brain energy metabolism (increase glycogenolysis and use of ketones) and when glucose is under limited supply, teleost brains utilize other fuels such as lactate or ketones (Soengas, J.L. et al. 2002). In a study of gene expression in a euryoxic fish (G*illichthys mirabilis*) in

which hypoxia was induced, the enolase gene in liver was up regulated after 72 and 144 hours of hypoxia (Gracery, A.Y. et al. 2001). In fact, coordinating increased expression of genes encoding glycolytic enzymes is particularly important for the adaptation to hypoxia (Kim, J.W. et al. 2005). The effects of this hypoxia varied substantially among tissues and notably, very few genes exhibited differential activity in brain. This is likely, as glucose levels in the brain must be maintained as brain is a privileged organ that has to support essential coordinating functions (Pertea, G. et al. 2003). As previously mentioned, we have identified enolase enzyme in the brain at a transcriptional level from fish subjected a chronic stress confinement. Therefore, threatened fish cope with an stress situation by regulating their metabolism in brain tissue, at least from transcriptional level.

In the density experiment, we did not find any significant difference in the HD group enolase mRNA expression, although a trend towards up-regulation is observed (Fig.2.6). This may be explained as the population density used may not represent an intense crowding situation (26 kg/m^3) to be detected by Northern analysis. Recently, in a study comparing gene expression in seabass (*Dicentrarchus labrax*, L.) at different population densities (80, 100 kg/m^3), six bands, not identified, were found to be differentially expressed (repressed or enhanced) in liver and brain, giving evidence that high densities may be responsible for changes in gene expression in specific fish organs (Gornati, R. et al. 2003). Northern results obtained after an IP LPS administration to sea bream, displayed a significantly up-regulated brain enolase mRNA expression after 48 hours and, at 72 hours, a down-regulation is detected (Fig.2.7). Administration of LPS results in the activation of the HPA axis (Grinevich, V. et al. 2001) where the interaction between the immune-nervous-endocrine systems plays an important role in modulating host susceptibility and resistance to inflammatory disease (Sternberg, E.M. 2001; Haddad, J.J. et al. 2002). There is a direct evidence that implicates fish cytokines as the effectors of the immune system signalling to the neuro-endocrine system and activate the HPI stress axis (Holland, J.W. et al. 2002). Although less data is available than in higher vertebrates, cytokines act as modulators of the immune response (Secombes, C.J. 1996). LPS injection induces synthesis of various cytokines, especially interleukin IL-1β, IL-6 and tumour necrosis factor by macrophages and lymphocytes (Grinevich, V. et al. 2001) and elevates plasma cortisol levels significantly in rainbow trout (Holland, J.W. et al. 2002) and in sea bream (Acerete, L. unpublished data). Studies have demonstrated that LPS treatment develops an inflammatory stimulation in fish by TNF-mRNA induction from monocytes and macrophages cell cultures (Mackenzie, S. et al. 2003). *In vitro* analyses of hematopoietic cells (neutrophils, B cells, T cells, monocytes stimulated

with LPS express significantly higher amounts of α-enolase on their surface (Arza, B. et al. 1997; Fontan, P.A. et al. 2000). Interestingly, our results derived from LPS Northern analysis, give new evidence toward the existence of an interconnection between the neuro-immuno-endocrine network. Brain S-enolase enzyme was identified after stress confinement situation and was further up-regulated after LPS challenge. As S-enolase is a glycolytic enzyme, this is the first evidence in fish of the presence of metabolism regulation in brain as a consequence of immune system activation. Therefore, this reinforces current evidence that interconnections between these systems in fish exist.

Interestingly, the enolase enzyme have additional functions aside from those described for glucose catabolism including: a Myc-binding protein (MBP-1) acting in transcriptional regulation (Subramanian, A. et al. 2000; Kim, J.W. et al. 2005), a cell associated stress protein involved in cellular protection during hypoxia (Aaronson, R.M. et al. 1995; Roland, I. et al. 2000), or a potential plasminogen receptor (Miles, L.A. et al. 1991). Enolase analysis are further used as markers for detecting several illness in different tissues, most of them are related to carcinomas (Ejeskar, K. et al. 2005; Santini, D. et al. 2006). To date, the possibility of enolase as a candidate gene molecular marker in fish had never been explored. The identification of enolase as a target gene for using as a molecular marker capable to describe fish welfare and with further increased efficiency in fish farm management and fish welfare, not only in *Sparus aurata* but also in other commercial interesting species, such as rainbow trout or carps, is a very interesting issue. Further data analysis data and experimental results about enolase gene, are studied, exposed and discussed in the next thesis chapter, reinforcing the evidence that the enolase gene may be a potential molecular marker for fish welfare.

Conclusions

Physiological parameters of sea bream subjected to a high-density confinement stress would show reduced responses, particularly in cortisol levels, to a subsequent acute stressor compared with those in fish held at a low density. Studying transcriptional level of brain high density confinement we identified a glycolytic enzyme, S-enolase, which not only fundamentally functions within metabolism but also plays a role in the response to inflammation and confinement exposure. Therefore, enolase represents an interesting potential marker gene for the stress response in fish under diverse conditions including population density and inflammation.

5. References

Aaronson, R.M., Graven, K.K., Tucci, M., Mcdonald, R.J. and Farber, H.W. (1995). Non-neuronal enolase is an endothelial hypoxic stress protein. *J Biol Chem*. 270(46): 27752-7.

Andersen, D.E., Reid, S.D., Moon, T.W. and Perry, S.F. (1991). Metabolic effects associated with chronically elevated cortisol in rainbow trout (*Oncorhyncus mykiss*). *Can. J. Fish Aquatic Sci.* 48: 1811-1817.

Arza, B., Felez, J., Lopez-Alemany, R., Miles, L.A. and Munoz-Canoves, P. (1997). Identification of an epitope of alpha-enolase (a candidate plasminogen receptor) by phage display. *Thromb Haemost*. 78(3): 1097-103.

Babbitt, P.C., Hasson, M.S., Wedekind, J.E., Palmer, D.R., Barret, W.C., Reed, G.H., Rayment, I., Ringe, D., Kenyon, G.L. and Gerelt, J.A. (1996). The enolase superfamily: a general strategy for enzyme catalyzed abstraction of the alpha-protons of carboxylic acids. *Biochemistry*. 35(enolasa): 16489-16501.

Barton, B.A. (2002). Stress in Fishes: A diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. *Integ. and Comp. Biol.* 42(stress in fishes): 517-525.

Barton, B.A. and Iwama, G.K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Disease*. 1: 3-26.

Barton, B.A., Morgan, J.D. and Vijayan, M.M. (2002). Physiological and condition-related indicators of environmental stress in fish. Biological Indicators of Aquatic Ecosystem Stress. S. M. Adams. Bethesda. MD. USA, American Fisheries Society**:** 111-148.

Barton, B.A., Scherck, C.B. and Sigismondi, L.A. (1986). Physiological changes in fish from stress in aquaculture with emphasis on the resposes and effects of corticosteroids. *Trans. Am. Fish. Soc.* 115: 254-251.

Barton, B.A., Sherck, C.B. and Barton, L. (1987). Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions and stress responses in juvenile chinook salmon. *Transactions of the American Fisheries Society*. 115: 245-251.

Basu, N., Kennedy, C.J., Hodson, P.V. and Iwama, G.K. (2002). Altered stress responses in rainbow trout following a dietary administration of cortisol and β-napthoflavone. *Fish Fisiology and Biochemistry*. 25: 131-140.

Besedovsky, H.O. and Del Rey, A. (1996). Immune-neuro-endocrine interactions: facts and hypotheses. *Endocr Rev*. 17(1): 64-102.

Bishop, J.G. and Corces, V.G. (1990). The nucleotide sequence of a *Drosophila melanogaster* enolase gene. *Nucleic Acids Res.* 18: 191.

Bradford, C.S., Fitzpatrick, M.S. and Schreck, C.B. (1992). Evidence for ultra-short-loop feedback in ACTH-induced interrenal steroidogenesis in coho salmon: Acute self-suppression of cortisol secretion in vitro. *General and Comparative Endocrinology*. 87: 292-299.

Chandran, V. and Luisi, B.F. (2006). Recognition of Enolase in the Escherichia coli RNA Degradosome. *J Mol Biol*.

Chang, Y.S., Wu, W., Walsh, G., Hong, W.K. and Mao, L. (2003). Enolase-alpha is frequently down-regulated in non-small cell lung cancer and predicts aggressive biological behavior. *Clin Cancer Res*. 9(10 Pt 1): 3641-4.

Cunningham, R.T., Young, I.S., Winder, J., O'kane, M.J., Mckinstry, S., Johnston, C.F., Dolan, O.M., Hawkins, S.A. and Buchanan, K.D. (1991). Serum neurone specific enolase (NSE) levels as an indicator of neuronal damage in patients with cerebral infarction. *Eur J Clin Invest*. 21(5): 497-500.

Day, I.N., Peshavaria, M. and Quinn, G.B. (1993). A differential molecular clock in enolase isoprotein evolution. *J. Mol. Evol.* 36: 599-601.

Discher, D.J., Bishopric, N.H., Wu, X., Peterson, C.A. and Webster, K.A. (1998). Hypoxia regulates beta-enolase and pyruvate kinase-M promoters by modulating Sp1/Sp3 binding to a conserved GC element. *J Biol Chem*. 273(40): 26087-93.

Ejeskar, K., Krona, C., Caren, H., Zaibak, F., Li, L., Martinsson, T. and Ioannou, P.A. (2005). Introduction of in vitro transcribed ENO1 mRNA into neuroblastoma cells induces massive cell death. *BMC Cancer*. 5(1): 161.

Feo, S., Arcuri, D., Piddini, E., Passantino, R. and Giallongo, A. (2000). ENO1 gene product binds to the c-myc promoter and acts as a transcriptional repressor: relationship with Myc promoter-binding protein 1 (MBP-1). *FEBS Lett*. 473(1): 47-52.

Fletcher, L., Rider, C.C. and Taylor, C.B. (1976). Enolase isoenzymes. III. Chromatographic and immunological characteristics of rat brain enolase. *Biochim Biophys Acta*. 452(1): 245-52.

Fontan, P.A., Pancholi, V., Nociari, M.M. and Fischetti, V.A. (2000). Antibodies to streptococcal surface enolase react with human alpha-enolase: implications in poststreptococcal sequelae. *J Infect Dis*. 182(6): 1712-21.

Gasca-Leyva, E., León, C.J. and J.M., H. (2003). Management strategies for seabream *Sparus aurata* cultivation in floating cages in the Mediterranean Sea and Atlantic Ocean. *Journal of World Aquaculture Society*. 34: 29-39.

Gerlt, J.A., Babbitt, P.C. and Rayment, I. (2005). Divergent evolution in the enolase superfamily: the interplay of mechanism and specificity. *Arch Biochem Biophys*. 433(1): 59-70.

Ghosh, A.K., Steele, R. and Ray, R.B. (1999). Functional domains of c-myc promoter binding protein 1 involved in transcriptional repression and cell growth regulation. *Mol Cell Biol*. 19(4): 2880-6.

Gornati, R., Terova, G., Vigetti, D., Mariangela, P., Saroglia, M. and Bernardini, G. (2003). Effects of population density on seabass (*Dientrarchus labrax*, L.) gene expression. *Aquaculture*.

Gracery, A.Y., Troll, J.V. and Somero, G.N. (2001). Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *PNAS*. 98 (4): 1993-1998.

Grinevich, V., Ma, X.M., Herman, J.P., Jezova, D., Akmayev, I. and Aguilera, G. (2001). Effect of repeated lipopolysaccharide administration on tissue cytokine expression and hypothalamic-pituitary-adrenal axis activity in rats. *J Neuroendocrinol*. 13(8): 711-23.

Haddad, J.J., Saade, N.E. and Safieh-Garabedian, B. (2002). Cytokines and neuro-immune-endocrine interactions: a role for the hypothalamic-pituitary-adrenal revolving axis. *J Neuroimmunol*. 133(1-2): 1-19.

Hattori, T., Takei, N., Mizuno, Y., Kato, K. and Kohsaka, S. (1995). Neurotrophic and neuroprotective effects of neuron-specific enolase on cultured neurons from embryonic rat brain. *Neurosci Res*. 21(3): 191-8.

Haukenes, A.H. and Barton, B.A. (2004). Characteritzation of the cortisol response following and acute challenge with lipopolysaccharide in yellow perch and the influence of rearing density. *Journal of Fish Biology*. 64: 851-862.

Holland, J.W., Pottinger, T.G. and Secombes, C.J. (2002). Recombinant interleukin-1β activates the hypothalamic-pituitary-interrenal axis in rainbow trout, *Oncorhynchus mykiss*. *Journal of Endocrinology*. 175: 261-267.

Hontela, A. (1997). Endocrine and physiological response of fish to xenobiotics: role of glucocorticosteroid hormones. *Reviews in Toxicology*. 1: 1-46.

Hontela, A., Rasmussen, J.B., Audet, C. and Chevalier, G. (1992). Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. *Arch Environ Contam Toxicol*. 22(3): 278-83.

Iida, H. and Yahara, I. (1985). Yeast heat-shock protein of Mr
48,000 is an isoprotein of enolase. *Nature*. 315: 688–690.

Iwama, G.K., Pickering, A.D., Sumpter, J.P. and Scherck, C.B. (1997). Fish Stress and Health in Aquaculture. Society for Experimental Biology Seminar Series. 62.

Jorgensen, O.S. and Centervall, G. (1982). Alpha gamma-enolase in the rat: ontogeny and tissue distribution. *J Neurochem*. 39(2): 537-42.

Kim, J.W. and Dang, C.V. (2005). Multifaceted roles of glycolytic enzymes. *Trends Biochem Sci*. 30(3): 142-50.

Kinloch, A., Tatzer, V., Wait, R., Peston, D., Lundberg, K., Donatien, P., Moyes, D., Taylor, P.C. and Venables, P.J. (2005). Identification of citrullinated alpha-enolase as a candidate autoantigen in rheumatoid arthritis. *Arthritis Res Ther*. 7(6): R1421-9.

Kuroda, N., Hes, O., Miyazaki, E., Shuin, T. and Enzan, H. (2006). Frequent expression of neuroendocrine markers in mucinous tubular and spindle cell carcinoma of the kidney. *Histol Histopathol*. 21(1): 7-10.

Laflamme, J.S., Couillard, Y., Campbell, P.G. and Hontela, A. (2002). Interrenal metallothionein and cortisol secretion in relation to Cd, Cu, and Zn exposure in yellow perch, *Perca flavescens*, from Abitibi lakes. *Canadian Journal of Fisheries and Aquatic Science*. 57: 1692-1700.

Lee, H., Guo, Y., Ohta, M., Xiong, L., Stevenson, B. and Zhu, J.K. (2002). LOS2, a genetic locus required for cold-responsive gene transcription encodes a bi-functional enolase. *Embo J*. 21(11): 2692-702.

Lins, H., Wallesch, C.W. and Wunderlich, M.T. (2005). Sequential analyses of neurobiochemical markers of cerebral damage in cerebrospinal fluid and serum in CNS infections. *Acta Neurol Scand*. 112(5): 303-8.

Lohman, K. and Meyerhof, O. (1934). Über die enzymatische Umwandlung von Phosphoglyzerinsäure in Brenztraubensäure und Phosphorsäure Enzymatic transformation of phosphoglyceric acid into pyruvic and phosphoric acid. *Biochem. Z*. 273: 60–72.

Mackenzie, S., Fernandez-Troy, N. and Espel, E. (2002). Post-transcriptional regulation of TNF-alpha during in vitro differentiation of human monocytes/macrophages in primary culture. *J Leukoc Biol*. 71(6): 1026-32.

Mackenzie, S., Planas, V.J. and Goetz Frederick, W. (2003). LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and in vitro differentiated macrophages. *Dev Comp Immunol*. 27: 393-400.

Malmstrom, B.G. (1951). Interaction of manganous ions with enolase. *Nature*. 171(4348): 392-3.

Malmstrom, B.G. (1953). The interaction of purified enolase with its activating metal-ions. *Archives of biochemistry and biophysics.* 46(2): 345-363.

Malmstrom, B.G. (1955). Metal-ion specificity in the activation of enolase. *Archives of biochemistry and biophysics.* 58(2): 381-397.

Malmstrom, B.G., Vanngard, T. and Larsson, M. (1959). An electron-spin-resonance study of the interaction of manganous ions with enolase and its substrate. *Biochimica et biophysica acta*. 30(1): 1-5.

Miles, L.A., Dahlberg, C.M., Plescia, J., Felez, J., Kato, K. and Plow, E.F. (1991). Role of cell-surface lysines in plasminogen binding to cells: identification of alpha-enolase as a candidate plasminogen receptor. *Biochemistry*. 30(6): 1682-91.

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

Nakajima, K., Hamanoue, M., Takemoto, N., Hattori, T., Kato, K. and Kohsaka, S. (1994). Plasminogen binds specifically to alpha-enolase on rat neuronal plasma membrane. *J Neurochem*. 63(6): 2048-57.

Norris, D.O., Donahue, S., Dores, R.M., Lee, J.K., Maldonado, T.A., Ruth, T. and Woodling, J.D. (1999). Impaired adrenocortical response to stress by brown trout, Salmo trutta, living in metal-contaminated waters of the Eagle River, Colorado. *Gen Comp Endocrinol*. 113(1): 1-8.

Oliva, D., Barba, G., Barbieri, G., Giallongo, A. and Feo, S. (1989). Cloning expression and sequence homologies of cDNA from human gamma enolase. *Gene*. 79: 355-360.

Oliva, D., Cali, L., Feo, S. and Giallongo, A. (1991). Complete structure of the human gene encoding neuron-specific enolase. *Genomics*. 10 (1): 157-65.

Pertea, G., Huang, X., Liang, F., Antonescu, V., Sultana, R., Karamycheva, S., Lee, Y., White, J., Cheung, F., Parvizi, B., Tsai, J. and Quackenbush, J. (2003). TIGR Gene Indices clustering tools (TGICL): a software system for fast clustering of large EST datasets. *Bioinformatics*. 19(5): 651-2.

Pickering, A.D. and Pottinger, T.G. (1987). Poor water quality suppresse the cortisol response of salmonid fish to handling and confinement. *Journal of Fish Biology*. 30: 363-374.

Poellinger, L. and Johnson, R.S. (2004). HIF-1 and hypoxic response: the plot thickens. *Curr Opin Genet Dev*. 14(1): 81-5.

Ray, R.B., Steele, R., Seftor, E. and Hendrix, M. (1995). Human breast carcinoma cells transfected with the gene encoding a c-myc promoter-binding protein (MBP-1) inhibits tumors in nude mice. *Cancer Res*. 55(17): 3747-51.

Rider, C. and Taylor, C.B. (1975). Enolase isoenzymes. II. Hibriditzation studies, development and phylogenetic aspects. *Biochim. Biophysys*. 405: 175-187.

Rider, C.C. and Taylor, C.B. (1975). Enolase isoenzymes. II. Hybridization studies, developmental and phylogenetic aspects. *Biochim Biophys Acta*. 405(1): 175-87.

Roland, I., Minet, E., Ernest, I., Pascal, T., Michel, G., Remacle, J. and Michiels, C. (2000). Identification of hypoxia-responsive messengers expressed in human microvascular endothelial cells using differential display RT-PCR. *Eur J Biochem*. 267(12): 3567-74.

Rotllant, J. (1999). Endocrine Stress Response of Gilthead sea bream (Sparus aurata). Effects of temperature and culture procedure-related stressors. *Doctoral thesis*.

Rotllant, J., Arends, R.J., Mancera, J.M., Flik, G., Wendelaar Bonga, S.E. and Tort, L. (2000). Inhibition of HPI axis response to stress in gilthead sea bream (Sparus aurata) with physiological plasma levels of cortisol. *Fish Physiology and Biochemistry*. 23: 13-22.

Rotllant, J., Balm, P.H., Perez-Sanchez, J., Wendelaar-Bonga, S.E. and Tort, L. (2001). Pituitary and interrenal function in gilthead sea bream (Sparus aurata L., Teleostei) after handling and confinement stress. *Gen Comp Endocrinol*. 121(3): 333-42.

Rotllant, J., Montero, D., Caballero, M.J., Robaina, L., Izquierdo, M.S. and Tort, L. (2003). Differences in interrenal tissue, biosynthetic capacity and ACTH sensitivity in progeny of sea bream from parents selected for high or low cortisol response. *Journal of Fish Biology*. 62: 744-748.

Rotllant, J. and Tort, L. (1997). Cortisol and glucose response after acute stress by net handling in the sparid red porgy previously subjected to crowding stress. *Fish Biology*. 51: 21-28.

Santini, D., Poli, F. and Lega, S. (2006). Solid-papillary tumors of the pancreas: histopathology. *Jop*. 7(1): 131-6.

Schmechel, D.E., Brightman, M.W. and Marangos, P.J. (1980). Neurons switch from non-neuronal enolase to neuron-specific enolase during differentiation. *Brain Res*. 190(1): 195-214.

Schofield, C.J. and Ratcliffe, P.J. (2004). Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol*. 5(5): 343-54.

Secombes, C.J. (1996). Cytokines in fish. An update. *Fish Shellfish Immunol.* 6: 291-304.

Segil, N., Shrutkowski, A., Dworkin, M.B. and Dworkin-Rastl, E. (1988). Enolase isoenzymes in adult and developing Xenopus laevis and characterization of a cloned enolase sequence. *Biochem J.* 251(1): 31-9.

Soengas, J.L. and Aldegunde, M. (2002). Energy metabolism of fish brain. *Comp Biochem Physiol B Biochem Mol Biol*. 131(3): 271-96.

Soengas, J.L., Strong, E.F. and Andrés, M.D. (1998a). Glucose, lactate, and beta-hydorxybutyrate utilitzation by rainbow trout brain: changes during food deprimvation. *Physiol. Zool.* 71: 285-293.

Stalnacke, B.M., Bjornstig, U., Karlsson, K. and Sojka, P. (2005). One-year follow-up of mild traumatic brain injury: post-concussion symptoms, disabilities and life satisfaction in relation to serum levels of S-100B and neurone-specific enolase in acute phase. *J Rehabil Med*. 37(5): 300-5.

Sternberg, E.M. (2001). Neuroendocrine regulation of autoimmune/inflammatory disease. *Journal of Endocrinology*. 169: 429-435.

Subramanian, A. and Miller, D.M. (2000). Structural analysis of alpha-enolase. Mapping the functional domains involved in down-regulation of the c-myc protooncogene. *J Biol Chem*. 275(8): 5958-65.

Tort, L., Gómez, E., Montero, D. and Sunyer, O. (1998). Stress and immunosuppression in fish. *Comp. Biochem. & Physiol.* 5.

Tort, L., Montero, D., Rubaina, L., Fernández-Palacios, H. and Izquierdo, M.S. (2001). Consistency of stress response to repeated handling in the gilthead sea bream *Sparus aurata* Linnaeus, 1758. *Aquaculture Research*. 32 (7): 593-598.

Tracy, M.R. and Hedges, B.S. (2000). Evolutionary history of the enolase gene family. *Gene*. 259(enolasa): 129-138.

Ueta, H., Nagasawa, H., Oyabu-Manabe, Y., Toida, K., Ishimura, K. and Hori, H. (2004). Localization of enolase in synaptic plasma membrane as an alphagamma heterodimer in rat brain. *Neurosci Res*. 48(4): 379-86.

Veiga-Malta, I., Duarte, M., Dinis, M., Tavares, D., Videira, A. and Ferreira, P. (2004). Enolase from Streptococcus sobrinus is an immunosuppressive protein. *Cell Microbiol*. 6(1): 79-88.

Vidal, N. and Hedges, S.B. (2005). The phylogeny of squamate reptiles (lizards, snakes, and amphisbaenians) inferred from nine nuclear protein-coding genes. *C R Biol*. 328(10-11): 1000-8.

Voet, D. and Voet, J.G. (1990). Bioquímica. P. Lane. Barcelona, Omega. 6**:** 456-494.

Wendelaar Bonga, S.E. (1997). The Stress Response in Fish. *Physiological Reviews*. 77: 591-623.

Wistow, G.J., Lietman, T., Williams, L.A., Stapel, S.O., De Jong, W.W., Horwitz, J. and Piatigorsky, J. (1988). Tau-crystallin/alpha-enolase: one gene encodes both an enzyme and a lens structural protein. *J Cell Biol*. 107(6 Pt 2): 2729-36.

Chapter 3

Enolase, a possible biomolecular welfare fish marker

1. Introduction

1.1. Aquaculture and biotechnology

Clinical parameters and zootechnical indexes may be not sufficient to monitor fish welfare when chronic stresses are applied (Roche, H. et al. 1996). Therefore, beside the traditional markers (biochemical, histological, morphological and physiological), it may be important to look for alternative parameters such as molecular biomarkers (Ryan, J.A. et al. 1996). A biomarker is a biological response that gives a measure of a pollutant exposure or toxic effect, and a biomarker approach may employ molecular through population level response (Peakall, D.B. 1994). Progress in genetic improvement of cultured fish and shellfish has been reviewed and discussed on several occasions during the last decade (Gjedrem, T. 1997; Benzie, J.A.H. 1998). One growing problem in fish farming is the necessity to develop functional genomic models for the aquaculture industry (Alestrom, P. et al. 2006). Modern technologies: sex and chromosome manipulations, transgenesis and genome mapping, have matured from the experimental phase and reached a stage where they can be incorporated into aquacultural breeding programs (Hulata, G. 2001). In fact, genomic strategies are revolutioning scientific research also in the understanding of fish physiology and gene evolution; in part by the isolation of novel and homologous genes in public databases (Parrington, J. et al. 2002). The research of those biomolecular fish markers which have the potential capacity to indicate and measure animal welfare, will be undoubtedly well considered for fish farms due to its important relevance in aquaculture production.

1.2. Enolase in fish

As exposed in chapter 2 the enolase gene was identified as a possible biomolecular marker for determining fish welfare. One of the key points in aquaculture when designing a biomolecular marker is its suitability among different fish species. What is described in this thesis chapter is the cloning of the enolase gene from different fish species: rainbow trout (*Onchoryncus mykiss*), stickleback (*Culaea inconstans*), leather carp (*Cyprinus carpio*) and mirror carp (*Cyprinus carpio carpio*) and study of the tissue distribution of enolase expression in fish. The enolase gene has been conserved during evolution as its sequence shows high homology between species (Tracy, M.R. et al. 2000). Analyses of amino acid and nucleotide sequences from different species, have suggested that enolase rapidly arose from single ancestral gene through a ‘burst’ event early in vertebrate evolution (Day, I.N. et al. 1993). All

enolases are made up of two identical subunits, and have a molecular weight in the range of 82,000–100,000 Da (Wold, F. 1971). In vertebrates, the enolase enzyme occurs as three isoenzymes: α-enolase, also named as non neuronal specific (NNS), found in a variety of tissues including liver, β-enolase also named as muscle specific enolase (MSE) almost exclusively found in muscle tissues and γ-enolase also called as neuron specific enolase (NSE) found in neuron and neuroendocrine tissues (Marangos, P.J. et al. 1978; Tracy, M.R. et al. 2000; Harper, J.T. et al. 2004). In higher vertebrates, there are three independent genetic loci, α, β and γ, that encode for the three isozymes (Marangos, P.J. et al. 1978; Kato, K. et al. 1983; Babbitt, P.C. et al. 1996). Several earlier studies have determined from their chromatographic and immunological analysis that neuron/brain specific enolase can be found in homodimeric and heterodimeric forms such as $\alpha\alpha$, $\alpha\beta$, $\beta\beta$, $\alpha\gamma$ and $\gamma\gamma$ (Fletcher, L. et al. 1976; Royds, J.A. et al. 1982). The $\alpha\alpha$ isoenzyme predominates in the rat fetus: however, as development progresses, this isoenzyme is replaced by $\alpha\beta$ and $\beta\beta$ types in adult heart and by $\beta\beta$ type in adult muscle whereas the $\alpha\alpha$ isoform occurs in liver, brain, spleen, adipose tissue and kidney in rat (Rider, C.C. et al. 1975; Merkulova, T. et al. 1997). In fish, several enolase genes (α,β,γ isoforms) have been cloned in fugu (*Takifugu rubripes*), zebrafish (*Danio rerio*), bowfin (Amia calva), shark (*Chiloscyllium punctatum*) and in some salmonids such as the brown trout (*Salmo trutta*) and the rainbow trout (*Oncorhynchus mykiss*). However, there are few reports concerning enolase phylogeny throughout evolution (Tracy, M.R. et al. 2000; Harper, J.T. et al. 2004), the branching order of the three genes has not been definitively resolved. If any, there is a lack of enolase expression and tissue distribution data in fish. In this chapter, enolase expression levels in fish tissues will be further studied, not only in sea bream, but also in rainbow trout, leather and mirror carp and stickleback species. In addition, we have studied differentialy enolase expression in rainbow trout treated with a lipopolysaccharide *E.coli* (6mg/kg) intraperitoneal administration (LPS IP). Enolase expression was further studied by using microarray analysis. A large number of microarray data from different fish salmonid samples were analysed to determine enolase expression patterns through fish sample experiments. This type of analysis is an important subject to determine whether enolase mRNA expression is regulated in fish in order to cope a stress situation for fish surveillance. This kind of analysis is an important subject to determine if enolase mRNA expression is regulated when fish health is threatened. This thesis chapter is also focused on the analysis of a large number of microarray data from different fish samples to determine enolase

expression patterns through all fish sample experiments. Microarray analysis will provide valuable and reliable information about enolase regulation when fish are subjected to different kind of stressors, reinforcing the evidence of considering enolase gene as a potential biomolecular marker in the fish aquaculture industry and fish research.

Objectives

1. Clone enolase genes from different fish species and identify these cloned sequences to their corresponding enolase isoform by phylogenetic studies.

2. Study tissue distribution of enolase in sea bream and rainbow trout. Study expression levels in rainbow trout tissues after LPS immune challenge.

3. Use microarray platform for understanding the expression patterns of enolase when fish are subjected to different stress challenges.

2. Material and methods

2.1. Animals and experimental proceedings

In order to clone enolase gene across species, the following fish species were used for this study: *Sparus aurata* (sea bream), *Oncorhynchus mykiss* (rainbow trout), *Cyprinus carpio* (mirror carp), *Cyprinus carpio carpio* (leather carp), and *Culaea inconstans* (stickleback). Animal conditions were followed as reported in chapter 2 section 2.1. For sea bream expression level studies, tissues from control fish (N=5) were dissected and frozen with liquid nitrogen, and kept in –80ºC freezer for further RNA extractions. Rainbow trout enolase expression studies after an immune challenge 24 fish were required. Half of them were intraperitoneal injected with lipopolysaccharide Escherichia coli (LPS IP) (6 mg/kg dose) (Sigma, L8274, 026:B6) and the other half with phosphate saline buffer (PBS) and kept them at different tanks. After 24 and 72 hours, six fish saline group and six fish LPS group were sampled by first placing them into a lethal concentration of 2-phenoxy-ethanol in less than 1 min, and bled. Rainbow trout tissues were carefully dissected and quickly frozen in liquid nitrogen and stored at –80ºC for avoiding RNA degradation.

2.2. Enolase cloning from different tissues and fish species

For cloning the enolase gene from different tissues and different fish species, mRNA tissues from sea bream and rainbow trout species and RNA brains from stickleback, leather carp and mirror carp species, were extracted by TriReagent

(Molecular Research Center, Inc.). Total RNA was extracted from all tissues according to the manufacturer's protocol (Chomczynski, P. et al. 1987; Chomczynski, P. 1993) and continuously, 4 μg of that RNA were converted to the first-strand cDNA synthesis by using Super-Script™ II (Invitrogen). For product amplification, a pair of primers were carefully designed against sea bream enolase already cloned by Differently Display (chapter 2) and β-actin or S18 amplification were used as control products (Table 1, Annex). For conventional PCR the following protocol was used (Amplitaq DNA Polymerase; Biotols): 5 min initial denaturation at 95ºC, followed by 30 cycles of 45 s denaturation at 95ºC, 45 s annealing at 55ºC, and 45 s extension at 72ºC. Samples were run on ethidium bromide stained 1% agarose gels. The amplified cDNA products were extracted from agarose gel by using a Nucleotrap® kit (Macherey-Nagel) and cloned into a bacterial plasmid vector (pGEM-T Easy Vector, Promega) and transfected to DH5-α bacterial cells (Invitrogen). After overnight incubations, bacterial cultures were filtered by mini-columns (Machery-Nagel) and DNA vectors were digested with EcoRI enzyme in order to screen recombinant bacterial colonies for the presence of target plasmid with DNA inserts. DNA inserts were subsequently sequenced using T7 and SP6 primers by a manufactured company. For homologies and similarities results, sequences cloned were briefly blasted against public databases.

2.3. Phylogenetic analysis

For enolase protein phylogenetic analysis, phylogenetic trees were constructed by an exhaustive search method, maximum likelihood (ML), based on the evaluation of changes in character states in the population of theoretically possible tree topologies for a given number of taxa (Higgs, P.G. et al. 2005). Although the ML gives some statistical support for the entire tree topology, the reliability of the clusters obtained was assessed by the bootstrapping resampling method, with 1000 iterations (Felsenstein, J. 1989). Phylogenetic analyses were performed using the Phylip software package (Felsenstein, J. 1989). A part from non completed enolase sequence cloned, other completed sequence from public database were added to enolase phylogenetic analysis. For rainbow trout, two enolase contigs were identified from TIGR database (Pertea, G. et al. 2003), five sequences from fugu genome, four from zebrafish genome and fourteen other more complete sequences from non fish vertebrate species. *Drosophila* enolase protein sequence was taken as an outgroup in all phylogenetic trees. Accessions numbers of the sequences used for phylogenetic analysis are the followings: *H.sapiens* alpha/beta/gamma: CAA47179, CAA40163,

AAP36047, *G.gallus* alpha/beta/gamma: NP990451, P07322, NP990207, *M.musculus*: alpha/beta/gamma AAH39179, NP031959, AAH31739, *P.regius* alpha: AAD41646, *A.mississippiensis* alpha: AAD41643, *S.undulates* alpha: AAD41644, *X.laevis* alpha: CAA68706, O.cuniculus: AAF71925, *D.rerio*: AAQ97775, AAH92869, AAH59434, AAH72713, *T.rubripes*: CAF89801, CAG06916, CAF90638, BAE27335, CAA34513, *O.mykiss*: TC79739, TC78568, *S.aurata* brain: AY263379, *D.melanogaster*: AAT47775.

2.4. Enolase and microarray chip

The use of microarray bioinformatic tools for understanding fish behaviour to immune challenge caused by LPS IP inflammation will be extensively discussed in chapter 6 (see this chapter for further methodology information). Interestingly, enolase genes were represented within four clones in the cDNA microarray platform. More than 160 different experiment samples have been run and analysed through the microarray chip. From all of this data analysis, 29 different experiment samples were selected in concordance to significance of enolase expression level ($P<0.01$). These experiments differed from species, tissue tested and treatments and therefore enolase expression levels have been extensively studied in different physiological situations. These experiments involved different experimental approaches such as stress, toxicity and pollution, feeding and disease. Thus, handling stress for 1, 3 and 5 days of exposure with netting for 2 min (brain and kidney rainbow trout) (Krasnov, A. et al. 2005a); immune challenge; 24 and 72 hours post injection of LPS IP (6mg/kg) (red muscle and white muscle rainbow trout); toxicity combinations (cadmium, tetrachloride and pyrene) for 4 days in brown trout liver (unpublished results); feeding with soya rainbow trout (intestine and liver) (unpublished results); m74 disease in Baltic salmon fry (Vuori, K.A. et al. 2006). Further microarray bioinformatic analysis were run to identify gene partners in which expression levels correlated to enolase gene in all experiments tested. Differential expression level ($P<0.01$) of enolase clones were considered as log(expression ratio).

3. Results

3.1. Enolase cloning from different species

Conventional RT-PCR resulted in the amplification of several nucleotide bands of approximately 430 bp depending on fish species and tissue (Fig.3.1). The putative aminoacid sequences obtained from sea bream muscle differed (81% homology) to that cloned from brain by Differential Display (see chapter 2). BLAST results for this

enolase muscle sequence showed a higher homology to beta-enolase isoform. From rainbow trout, enolase sequences amplified and cloned by RT-PCR, showed no differences regardless the tissue tested. The enolase sequence cloned from sea bream brain and rainbow trout brain had 90% homology and 89% homology to those cloned in muscle. The putative enolase protein sequences cloned in brain from stickleback, leather carp and rainbow trout were practically the same sequence although differed in three aminoacids to mirror carp enolase sequence (data not shown).

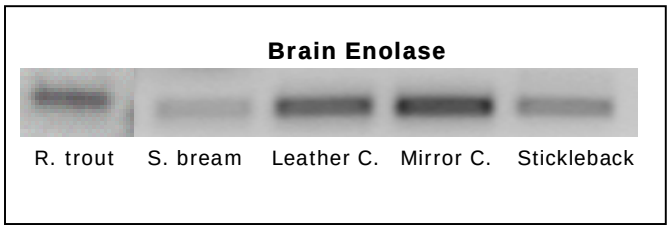


Fig.3.1. Enolase PCR cloned fragment from brain control tissue in different species. R. trout= rainbow trout, S. bream= sea bream, Leather/Mirror C.= Leather Carp. Results of RT-PCR (30 cycles, 430 bp PCR product length) on total RNA (4 µg) extracted from brain tissue. Samples were separated on ethidium bromide-agarose gels.

3.2. Phylogenetic analysis

In order to identify and annotate the fish enolase cloned sequences, phylogenetic trees were constructed from enolase aminoacid sequences of different vertebrates. Enolase phylogenetic analysis of some vertebrate species shows a clear classification of the three enolase paralogs to each corresponding cluster: α-enolase (89%), β-enolase (90%), and γ-enolase (90%) (Fig.3.2). The inferred phylogenetic tree constructed from partial sequences obtained from cloned enolase forms in four species of representative fish (*Oncorhynchus mykiss, Cyprinus carpio, Gasterosteus aculeatus,* and *Sparus aurata*) is shown in figure 3.3. The classification of the tree enolase paralogs: α-enolase (100%), β-enolase (69%), and γ-enolase (100%) were also observed in the enolase fish phylogenetic tree, confirming that in fish the three monophyletic enolase isoforms exist. Independently to the tissue (brain or muscle) from where enolase sequences were cloned, all new sequences were classified as beta type enolase in the phylogenetic analysis.

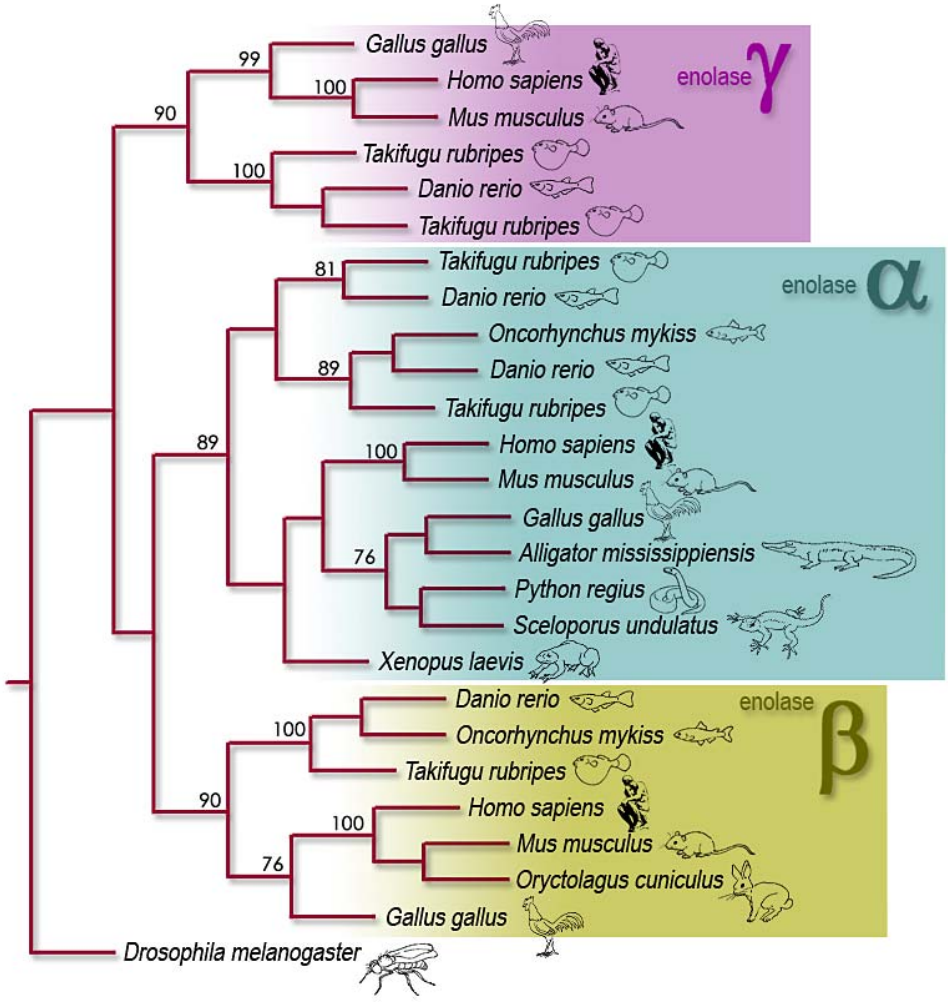


Fig.3.2. Enolase phylogenetic tree from several species. Unrooted tree was performed by using aminoacid complete enolase sequences from several species. Tree was produced using Maximum Likelihood method (Phylip package) (Felsenstein, J. 1989), nodes with 1000 bootstrap values greater than 70. Accession numbers: *H.sapiens* alpha/beta/gamma: CAA47179, CAA40163, AAP36047, *G.gallus* alpha/beta/gamma: NP990451, P07322, NP990207, *M.musculus*: alpha/beta/gamma AAH39179, NP031959, AAH31739, *P.regius* alpha: AAD41646, *A.mississippiensis* alpha: AAD41643, *S.undulates* alpha: AAD41644, *X.laevis* alpha: CAA68706, O.cuniculus: AAF71925, *D.rerio*: AAQ97775, AAH92869, AAH59434, AAH72713, *T.Rubripes*: CAF89801, CAG06916, CAF90638, BAE27335, CAA34513, *O. mykiss*: TC79739, TC78568, *D.melanogaster*: AAT47775.

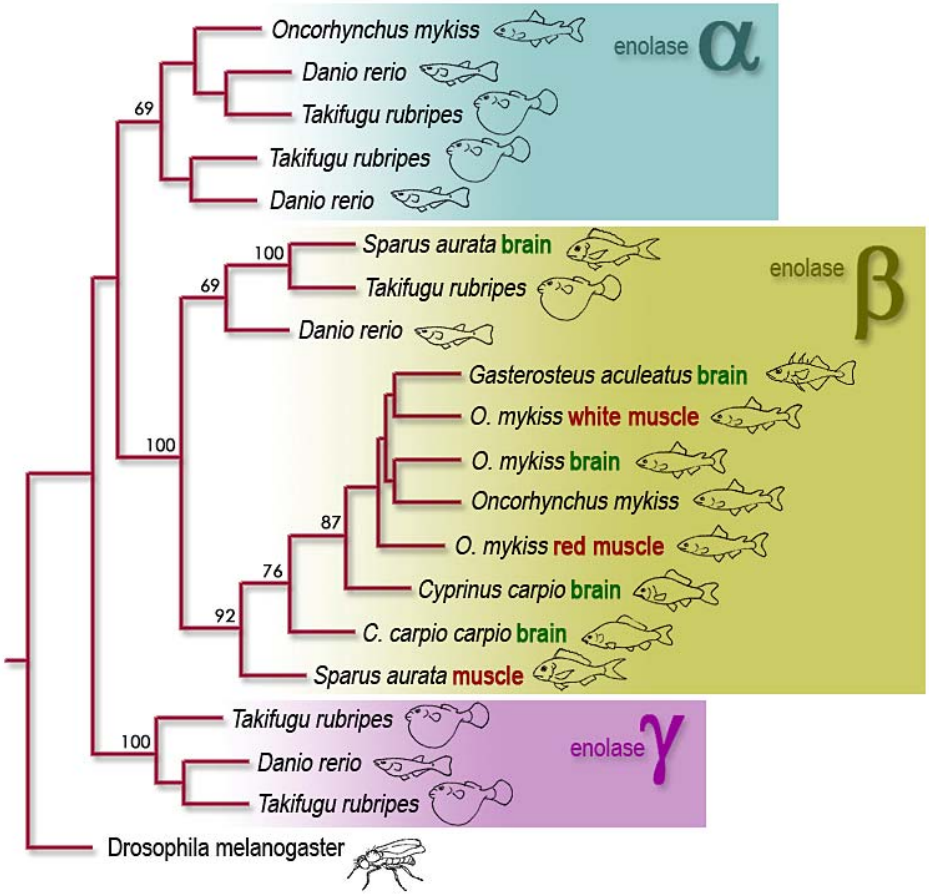


Fig.3.3. Fish enolase phylogenetic tree. Unrooted tree was performed by using aminoacid non-completed enolase sequences from several fish species. Tree was produced using Maximum Likelihood method (Phylip package) (Felsenstein, J. 1989), nodes with 1000 bootstrap values greater than 70. Accession numbers: *S.aurata* brain: AY263379, *D.rerio*: AAQ97775, *D.rerio*: AAH92869, *D.rerio*: AAH59434. *D.rerio*: AAH72713, *T.rubripes*: CAF89801, *T.Rubripes*: CAG06916, *T.rubripes*: CAF90638, *T.rubripes*: BAE27335, *T.rubripes*: CAA34513, *O. mykiss*: TC79739, *O.mykiss*: TC78568, *D.melanogaster*: AAT47775.

3.3. Enolase expression levels in sea bream tissues

Using conventional RT-PCR analysis in sea bream, enolase expression was detected in heart, liver, head kidney and muscle albeit at very low levels (35 PCR cycles) (Fig.3.4). To confirm the low levels of expression observed, we further designed more primer pairs and repeated RT-PCR analysis with more PCR cycles (35-40), concurring with those obtained in the first experimental series (data not shown). Therefore, S-enolase appears to have a very low basal expression levels in sea bream in all tissues although we were able to identify expression in the above mentioned tissue samples.

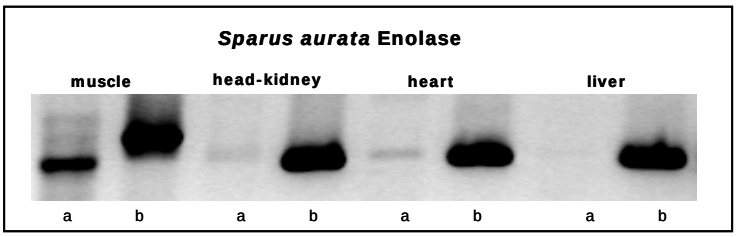


Fig.3.4. Results of RT-PCR on total RNA extracted from tissue samples taken from non-stimulated sea bream. For conventional RT-PCR, polymerase chain reactions were initiated and at 35 cycles samples were taken and separated on ethidium bromide-agarose gels and visualized under UV light. a; enolase PCR product: b; β-actin PCR.

3.4. Enolase expression levels in rainbow trout tissues

In rainbow trout, enolase expression levels were found in all tissue tested (Fig.3.5). As expected, mRNA enolase was highly expressed in muscle, both white and red muscles. Liver, heart and head kidney mRNA enolase levels were also high. In the head kidney, enolase expression decreased after an LPS IP treatment 24 hours stimulation and control levels are not recovered at 72 hours postinjection.

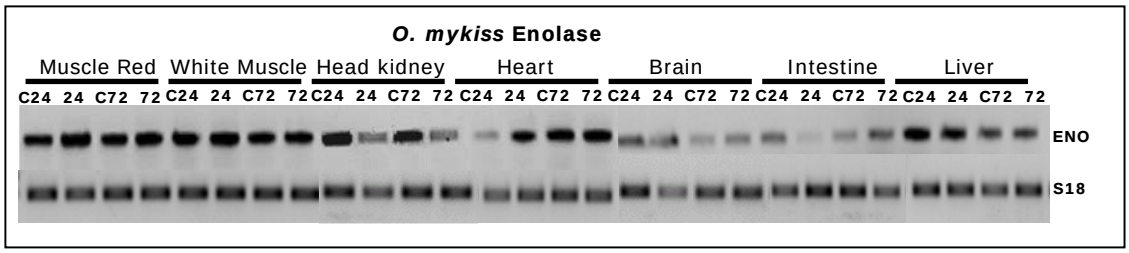


Fig.3.5. Results of Enolase expression in rainbow trout by RT-PCR. Tissue distribution of PU.1 gene to LPS IP timecourse analysis. PCR product length was 437 bp, 30 cycles. Each time point six animals per group (N=6) were analysed.

3.5. Enolase and microarray analysis

Analysis of enolase gene expression levels of different experiment samples analysed in the microarray chip were performed (Fig.3.6). Enolase mRNA levels were significantly regulated throughout all different experiments. Enolase mRNA was up regulated in 84% of the total number of experimental samples analysed, and in 16% of enolase expression levels were down regulated. Experiments in which an up regulation of enolase mRNA detected were related to physical stress, toxicity and fish

diseases. Interestingly, mRNA enolase levels increased in rainbow trout brain after one day of handling exposure (rainbow trout) and its levels continued high 2 days after exposure to return to basal levels 5 days with handling exposure. Head kidney was also analysed in the same experiment, but enolase levels in this tissue were not as high as in brain one day after handling exposure. LPS decreased enolase mRNA levels in rainbow trout red and white muscle 24 hours post treatment. Interestingly, LPS affected differently the red and white muscle 72 hours after injection by increasing enolase expression in white muscle. An up regulation of enolase transcriptional levels of m74 experiment was showed by microarray analysis. As already reported (Vuori, K.A. et al. 2006), the increase of anaerobic metabolism in m74 compared to healthy fry can be seen in several glycolytic enzymes: enolase, GAPDH and 6-phosphofructokinase. Enolase mRNA expression in fish (brown and rainbow trout) fed with soya was smoothly affected downregulating its mRNA expression levels in liver whereas up regulated in intestine. Most of the brown trout subjected to different toxicity metal combinations (cadmium, tetrachloride and pyrene) for 4 days resulted in an up regulation of enolase mRNA expression level.

Gene correlation studies with enolase gene, revealed that glyceraldehyde-3-phosphate dehydrogenase-2 (GAPDH) gene was the best gene partner for enolase. Expression levels of both enolases and GAPDH behave in a similar fashion throughout all experiments tested. Two other unknown genes also clustered to enolase gene expression. Myosin heavy chain gene presented a similar correlation pattern to that for enolase gene, although without statistical significance.

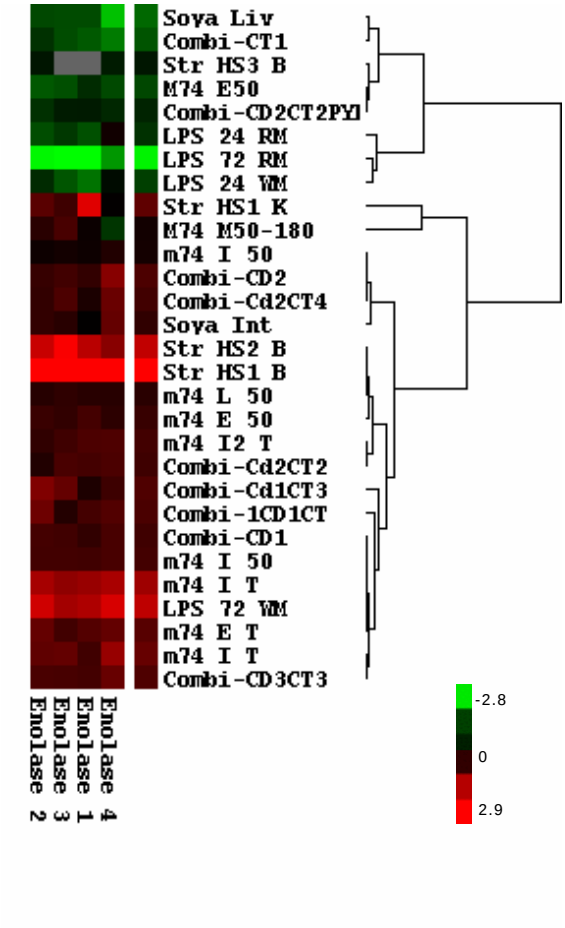


Fig.3.6. Enolase gene expression levels on different experiments analysed by microarray. Four enolase clones exists in the microarray platform. Last column is the mean expression of enolase genes to each experiment tested. Red colour indicates up-regulated genes, green colour indicates down regulated genes and black indicates no change. Data not available is represented in grey colour. m74 disease (early death of Baltic salmon, respiration failure) E=early disease, I=intermediate disease, 50-180= ATU (urbic antrosol) ; LPS24/72= LPS IP injection to rainbow trout 24/72 hours post injection, WM/M=white muscle, RM=red muscle; Combi= Toxicity combination 4 days (brown trout, liver), CD= cadmium, CT= tetrachloride, PYR= pyrene; Soya liv/Int = rainbow trout feed with soya; Liv= liver, Int= intestine; Str HSX B= Brain samples of brain subjected to Handling stress (2 min each day) (X=1/3/5 days after exposure); Str HS1 K = Handling stress (2 min) 1 day of exposure kidney.

4. Discussion

4.1. Cloning results

After considering that the enolase gene could be a possible biomolecular marker capable to describe fish welfare, we proceeded to clone enolase gene fragments from different species. Although primers were designed against S-enolase from sea bream brain, cloning enolase sequences from different tissues and species was successful, emphasising the evidence that the enolase enzyme belongs to a highly conserved gene family (Babbitt, P.C. et al. 1996). The homology percentage between the enolase sequences obtained from sea bream muscle respect to S-enolase (S-enolase, AY263379) obtained from brain tissue was 81%. In rainbow trout, no differences between amplified muscle and brain enolase sequences were detected, suggesting that primers did not work properly for annealing to beta-like isoform, or the lack of an additional isoform in this tissue. From brain tissue, the rainbow trout enolase sequence was homologous by 90% to that cloned in sea bream, whereas from stickleback and leather carp sequences only differed in three aminoacids to that sequenced for mirror carp. Results confirmed what was previously reported about high conservation of enolase family (Babbitt, P.C. et al. 1996; Tracy, M.R. et al. 2000) but also give evidence of the necessity to perform phylogenetic analysis to distinguish among enolase isoforms, and possibly need more in depth cloning or basically more sequencing.

4.2. Enolase expression studies

Enolase mRNA expression in different tissues; liver, head kidney and muscle of unstimulated sea bream (*Sparus aurata*) were detectable by both conventional RT-PCR and in brain by Northern blot analysis (Chapter 2, Fig.2.6 and 2.7). However, expression control levels observed were very low and required an elevated number of PCR cycles (35-40). The higher signal obtained in muscle tissue is likely due to the co-amplification of the muscle-specific beta isoform of enolase. Sequencing the PCR product from sea bream muscle, another enolase sequence identified as a beta-like enolase was obtained. Low levels of alpha enolase protein expression have been reported in bovine endothelial cells (Aaronson, R.M. et al. 1995) and in rat, avian and xenopus tissues (Segil, N. et al. 1988). Enolase alpha is absent in the liver of the long jaw mudsucker under control conditions (*G.mirabilis*) (Gracey, A.Y. et al. 2001). Contrary of results observed in the sea bream, where enolase expression was low/undetectable under basal conditions, in the rainbow trout, the enolase gene was

detected in all tissue tested in the control group. Muscle, head kidney, liver and heart provided the highest mRNA enolase expression. From PCR LPS IP results, there is no clear evidence of LPS effects on enolase expression in tissues studied in rainbow trout, although by Northern blot analysis in sea bream, enolase expression was increased after 48 hours of LPS IP administration (chapter 2). Interestingly, primers used for RT-PCR designed for sea bream enolase result in a better amplification of enolase mRNA in rainbow trout tissues. Enolase sequences cloned from brain and muscle were different in sea bream, but not in rainbow trout, probably because beta-like type in rainbow trout is ubiquitously tissue distributed, and/or the similarity in nucleotide sequences between enolase isoforms may result in cross-hybridisation in rainbow trout, as already reported to each species (Oliva, D. et al. 1989). Differences in expression levels between salmonid and sparid fish may result from different enolase expression to each specie which may be influenced throughout evolution. In conclusion, enolase expression in fish seems to be ubiquitous with high expression levels in muscle tissue. Enolase expression levels in each tissues depends upon fish species with a marked difference between sparids and salmonids.

4.3. Phylogenetic analysis

The inferred phylogenetic tree derived from the new enolase sequences cloned from brain tissue of several fish species (sea bream, rainbow trout, leather and mirror carp and stickleback) and from muscle tissue (sea bream and rainbow trout) clusters all the partial cloned sequences as beta type enolases. As described below, it is reported that vertebrates express three tissue-specific isoforms non-neural enolase (NNE) or alpha enolase, muscle specific enolase (MSE) or beta enolase and neural-specific enolase (NSE) or gamma enolase (Rider, C. et al. 1975; Segil, N. et al. 1988; Bishop, J.G. et al. 1990). In addition, β-enolase has also been identified in brain tissue as homodimeric and heterodimeric forms such as $\alpha\alpha$, $\alpha\beta$, $\beta\beta$, $\alpha\gamma$ and $\gamma\gamma$ (Fletcher, L. et al. 1976; Royds, J.A. et al. 1982). However, our results suggest a different scenario for tissue distribution in the species of fish selected. Irrespectively of origin (brain or muscle), the widespread expression of beta type enolases reinforces, in a sense, the multifunctional and ubiquitous role of this conserved enzyme as a unique multifunctional protein with unrestricted location diversity (Babbitt, P.C. et al. 1996; Pancholi, V. 2001; Gerlt, J.A. et al. 2005). Thus, enolase isoforms in fish may not present a direct isoform tissue specificity. It must be considered, however, the incomplete character of the sequences evaluated and the reduced number of species

included in the phylogenetic analysis. It is noteworthy the scarcity of completed genome projects in fish and the extreme diversity of fish genomes in terms of structure, size and complexity (Furutani-Seiki, M. et al. 2004), which preclude accurate resolution in phylogenetic evaluations. Although the branching order of the three genes is not significantly resolved, our results support the monophily of the enolase isozymes in accordance with previous broad-scale studies (Tracy, M.R. et al. 2000; Harper, J.T. et al. 2004). Despite the intensive sampling efforts of some phylogenetic studies, which also suffer from sequence incompleteness and ambiguous relationships between different taxa. In consideration, our samplings include two species considered "primitive" (*O.mykiss* and *C.carpio sp*), one of which seems to be undergoing a deconstructive process of diploidization from a tetraploid ancestral form (Moghadam, H.K. et al. 2005) and two more advanced fish (*S.aurata* and *G.aculeatus*) (see Fig.1.2, chapter 1). In conclusion, we have contributed to increase the number of enolase sequences in different fish species, although additional genomic data is necessary to provide a better resolution of enolase phylogeny in fish.

4.4. Enolase in microarray

The 'omics' revolution in the biological sciences has opened up many new fields of research and has allowed for innovative strategies in experimentation leading to increased understanding of basic biological processes (Higgs, P.G. et al. 2005). Aquaculture invests in new technology approaches with the capacity to improve fish production. At this moment the possibility of applying transcriptomic technologies in aquaculture is rapidly becoming a reality with the development of specific-species microarrays generated from extensive efforts in EST analysis and annotation of genes (Krasnov, A. et al. 2005b). The microarray technique allow the study of enolase gene expression in different fish conditions, not only in different fish treatments and fish tissues, but also in a variety of fish species, mainly salmonids. Enolase expression patterns among several fish species have been intensively studied using a microarray platform, designed specifically for salmonid fish (Krasnov, A. et al. 2005a). Enolase expression levels through a large number of experiments run on the microarray chip analysis were compared and studied in order to understand enolase expression levels in the animal as a response to a stressor. Enolase mRNA levels were regulated significantly throughout all different experiments. Experiments in which an up regulation of enolase mRNA was detected were related to physical stress, toxicity and fish diseases. Chemicals, changes in physiological and pathological conditions, or

environmental modifications are capable of eliciting responses at the transcriptomic level (Gornati, R. et al. 2005). It is noteworthy that the enolase gene was significantly up regulated in rainbow trout brain one day after net handling exposure, and its expression levels were high within five days of the stress exposure. The mRNA enolase levels in kidney were also enhanced after 1 day of handling exposure. However, enolase mRNA expression decreased in the head kidney 24 and 72 hours after LPS IP treatment (see RT-PCR results in section 3.4. and microarray results in section 3.1. in chapter 6). The response to a physical stressor with an up regulation of enolase gene in brain was already observed from our results in sea bream, and further confirmed after a LPS IP injection (chapter 2). These results give evidence of the transcriptional activity in the brain for enolase gene to cope with the stressors, and the activation of its glycolytic pathway to cope with the challenge and maintain homeostasis in brain tissue. The vertebrate brain is metabolically one of the most active of all organs and exquisitely sensitive to perturbation of energy metabolism (Magistretti, P.J. 1988). From microarray database analysis, and also from previous experiment in the sea bream brain (Ribas, L. et al. 2004), an up regulation of mRNA enolase levels are required when fish health is threatened. Interestingly, from LPS IP rainbow trout experiment, enolase expression levels in red muscle (RM) were down regulated both 24 and 72 hours post injection whereas in white muscle (WM) enolase mRNA levels were down regulated in 24 hours and enhanced 72 hours after the challenge. WM is the fast muscle used in high velocity bursts and RM is the slow muscle used during sustained cruising (Cole, N.J. et al. 2001). Transcriptomic analysis from rainbow trout LPS IP experiment, red muscle (an aerobic tissue that obtains energy mainly from oxidative phosphorylation) LPS provokes marked changes in representative mitochondrial genes whereas in white muscle (an anaerobic tissue) suffers major expression changes in glycolytic enzymes (Roher, N. et al. under submission). Globally, microarray analysis gives evidence of enolase gene regulation in a wide range of different experiments in which fish health is threatened. In view of the extensive regulation of the enolase enzyme in different fish species, we could consider that enolase mediates a function when the physiological systems responding to stress are activated, and therefore, enolase can be considered as a good biomolecular marker.

As reported in chapter 2, recent findings have suggested that some glycolytic enzymes are more complex and multifaceted proteins rather than simple components of the glycolytic pathway, suggesting that links between metabolic sensors and transcription, are established directly through enzymes that participate in metabolism

(Kim, J.W. et al. 2005). Interestingly, from all 1380 genes disposed on the microarray platform, the most highly correlated gene to enolase expression level pattern, was glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), another metabolic enzyme in the glycolytic pathway (responsible to convert glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate) (Edwards, Y.H. et al. 1976). Like enolase, unexpected functional roles from GAPDH glycolytic enzyme have been recently identified. These new GAPDH functions include transcriptional coactivation (Zheng, L. et al. 2003) and neuronal apoptosis (Ishitani, R. et al. 1996; Shashidharan, P. et al. 1999). Other studies have suggested that GAPDH is also capable of binding to several untranslated regions of viral RNA, although its biological significance is poorly understood (De, B.P. et al. 1996; Choudhary, S. et al. 2000). In addition, GAPDH has been implicated in other processes such as membrane fusion, phosphorylation, tubulin bundling, nuclear RNA export, DNA repair and interaction with cellular components, including RNA, dinucleoside polyphosphate and nitric oxide (Sirover, M.A. 1996, 1997, 1999). Furthermore, involvement of enolase and GAPDH genes in several human disease have been reported, suggesting to be potential gene targets (Xing, C. et al. 2004; Ejeskar, K. et al. 2005; Santini, D. et al. 2006). An increase in anaerobic metabolism in m74 disease compared to healthy fry was observed including an up regulation for several glycolytic enzymes including enolase and GADPH (Vuori, K.A. et al. 2006). However, little is known about the molecular mechanisms of the multifaceted roles of glycolytic enzymes, specifically regarding the relationship between their glycolytic and non-glycolytic functions. Similar expression patterns for both enolase and GAPDH were found in the microarray analysis which may reflect their roles in glycolytic or non-glycolytic functions in different fish tissues and species. Our findings suggest that simple proteins corresponding to central metabolic processes may represent a new generation of reliable molecular marker for fish welfare. Our data opens new insight of considering ancient proteins, enolase and GADPH, as potential fish biomolecular markers. Although the interpretation of future studies will undoubtedly expand our current understanding of metabolic gene activity and contribution to fish health, the enolase gene is a good potential candidate gene for studying fish welfare.

Conclusions

The purpose of this study was to assess enolase gene as a possible biomolecular marker for fish welfare. In this regard, several enolase genes have been cloned in different fish species for the first time which increases the number of enolase fish sequences available. Phylogenetic analysis revealed that in fish, there is a lack of enolase tissue specificity. Although tissue distribution of enolase mRNA expression levels differed to fish specie, enolase expression showed a ubiquitous distribution, with the highest level in the muscle tissue. Microarray analyses gave evidence that the enolase gene is regulated by different fish stressors in different fish species. It can be considered enolase gene as a good biomolecular fish welfare because enolase mRNA levels are regulated when fish health is threatened. Our data opens new insights of classical metabolic genes as a possible fish welfare biomarkers.

5. References

Aaronson, R.M., Graven, K.K., Tucci, M., Mcdonald, R.J. and Farber, H.W. (1995). Non-neuronal enolase is an endothelial hypoxic stress protein. *J Biol Chem*. 270(46): 27752-7.

Alestrom, P., Holter, J.L. and Nourizadeh-Lillabadi, R. (2006). Zebrafish in functional genomics and aquatic biomedicine. *Trends Biotechnol*. 24(1): 15-21.

Babbitt, P.C., Hasson, M.S., Wedekind, J.E., Palmer, D.R., Barret, W.C., Reed, G.H., Rayment, I., Ringe, D., Kenyon, G.L. and Gerelt, J.A. (1996). The enolase superfamily: a general strategy for enzyme catalyzed abstraction of the alpha-protons of carboxylic acids. *Biochemistry*. 35(enolasa): 16489-16501.

Benzie, J.A.H. (1998). Penaeid genetics and biotechnology. *Aquaculture*. 164: 23–47.

Bishop, J.G. and Corces, V.G. (1990). The nucleotide sequence of a *Drosophila melanogaster* enolase gene. *Nucleic Acids Res.* 18: 191.

Chomczynski, P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques*. 15(3): 532-4, 536-7.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 162(1): 156-9.

Choudhary, S., De, B.P. and Banerjee, A.K. (2000). Specific phosphorylated forms of glyceraldehyde 3-phosphate dehydrogenase associate with human parainfluenza virus type 3 and inhibit viral transcription in vitro. *J Virol*. 74(8): 3634-41.

Cole, N.J. and Johnston, I.A. (2001). Plasticity of myosin heavy chain expression with temperature acclimation is gradually acquired during ontogeny in the common carp (Cyprinus carpio L.). *J Comp Physiol [B]*. 171(4): 321-6.

Day, I.N., Peshavaria, M. and Quinn, G.B. (1993). A differential molecular clock in enolase isoprotein evolution. *J Mol Evol*. 36(6): 599-601.

De, B.P., Gupta, S., Zhao, H., Drazba, J.A. and Banerjee, A.K. (1996). Specific interaction in vitro and in vivo of glyceraldehyde-3-phosphate dehydrogenase and LA protein with cis-acting RNAs of human parainfluenza virus type 3. *J Biol Chem*. 271(40): 24728-35.

Edwards, Y.H., Clark, P. and Harris, H. (1976). Isozymes of glyceraldehyde-3-phosphate dehydrogenase in man and other mammals. *Ann Hum Genet*. 40(1): 67-77.

Ejeskar, K., Krona, C., Caren, H., Zaibak, F., Li, L., Martinsson, T. and Ioannou, P.A. (2005). Introduction of in vitro transcribed ENO1 mRNA into neuroblastoma cells induces massive cell death. *BMC Cancer*. 5(1): 161.

Felsenstein, J. (1989). Phylogeny Inference Package (Version 3.2.). *Cladistics*. 5: 164-166.

Fletcher, L., Rider, C.C. and Taylor, C.B. (1976). Enolase isoenzymes. III. Chromatographic and immunological characteristics of rat brain enolase. *Biochim Biophys Acta*. 452(1): 245-52.

Furutani-Seiki, M. and Wittbrodt, J. (2004). Medaka and zebrafish, an evolutionary twin study. *Mech Dev*. 121(7-8): 629-37.

Gerlt, J.A., Babbitt, P.C. and Rayment, I. (2005). Divergent evolution in the enolase superfamily: the interplay of mechanism and specificity. *Arch Biochem Biophys*. 433(1): 59-70.

Gjedrem, T. (1997). Selective breeding to improve aquaculture production. *World Aquaculture*. 28: 33-45.

Gornati, R., Papis, E., Rimoldi, S., Chini, V., Terova, G., Prati, M., Saroglia, M. and Bernardini, G. (2005). Molecular markers for animal biotechnology: sea bass (Dicentrarchus labrax, L.) HMG-CoA reductase mRNA. *Gene*. 344: 299-305.

Gracey, A.Y., Troll, J.V. and Somero, G.N. (2001). Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *PNAS*. 98 (4): 1993-1998.

Harper, J.T. and Keeling, P.J. (2004). Lateral gene transfer and the complex distribution of insertions in eukaryotic enolase. *Gene*. 340(2): 227-35.

Higgs, P.G. and Attwood, T.K. (2005). Bioinformatics and Molecular Evolution. Oxford, Blackwell Publishing.

Hulata, G. (2001). Genetic manipulations in aquaculture: a review of stock improvement by classical and modern technologies. *Genetica*. 111(1-3): 155-73.

Ishitani, R. and Chuang, D.M. (1996). Glyceraldehyde-3-phosphate dehydrogenase antisense oligodeoxynucleotides protect against cytosine arabinonucleoside-induced apoptosis in cultured cerebellar neurons. *Proc Natl Acad Sci U S A*. 93(18): 9937-41.

Kato, K., Asai, R., Shimizu, A., Suzuki, F. and Ariyoshi, Y. (1983). Immunoassay of three enolase isozymes in human serum and in blood cells. *Clin. Chim. Acta*. 127: 353–363.

Kim, J.W. and Dang, C.V. (2005). Multifaceted roles of glycolytic enzymes. *Trends Biochem Sci*. 30(3): 142-50.

Krasnov, A., Koskinen, H., Pehkonen, P., Rexroad, C.E., 3rd, Afanasyev, S. and Molsa, H. (2005a). Gene expression in the brain and kidney of rainbow trout in response to handling stress. *BMC Genomics*. 6(1): 3.

Krasnov, A., Koskinen, H., Rexroad, C., Afanasyev, S., Molsa, H. and Oikari, A. (2005b). Transcriptome responses to carbon tetrachloride and pyrene in the kidney and liver of juvenile rainbow trout (Oncorhynchus mykiss). *Aquat Toxicol*. 74(1): 70-81.

Magistretti, P.J. (1988). Regulation of glycogenolysis by neurotransmitters in the central nervous system. *Diabete Metab*. 14(3): 237-46.

Marangos, P.J., Zis, A.P., Clark, R.L. and Goodwin, F.K. (1978). Neuronal, non-neuronal and hybrid forms of enolase in brain: structural, immunological and functional comparisons. *Brain Res*. 150: 117–133.

Merkulova, T., Lucas, M., Jabet, C., Lamande, N., Rouzeau, J.D., Gros, F., Lazar, M. and Keller, A. (1997). Biochemical characterization of the mouse muscle-specific enolase: developmental changes in electrophoretic variants and selective binding to other proteins. *Biochem J.* 323 (Pt 3): 791-800.

Moghadam, H.K., Ferguson, M.M. and Danzmann, R.G. (2005). Evidence for Hox gene duplication in rainbow trout (Oncorhynchus mykiss): a tetraploid model species. *J Mol Evol*. 61(6): 804-18.

Oliva, D., Barba, G., Barbieri, G., Giallongo, A. and Feo, S. (1989). Cloning, expression and sequence homologies of cDNA for human gamma enolase. *Gene*. 79(2): 355-60.

Pancholi, V. (2001). Multifunctional alpha-enolase: its role in diseases. *Cell Mol Life Sci*. 58(7): 902-20.

Parrington, J. and Coward, K. (2002). Use of emerging genomic and proteomic technologies in fish physiology. *Aquatic Living Resource*. 15: 193– 196.

Peakall, D.B. (1994). The role of biomarkers in environmental assessment. *Ecotoxicology*. 3: 157-60.

Pertea, G., Huang, X., Liang, F., Antonescu, V., Sultana, R., Karamycheva, S., Lee, Y., White, J., Cheung, F., Parvizi, B., Tsai, J. and Quackenbush, J. (2003). TIGR Gene

Indices clustering tools (TGICL): a software system for fast clustering of large EST datasets. *Bioinformatics*. 19(5): 651-2.

Ribas, L., Planas, J.V., Barton, B., Monetti, C., Bernardini, G., Saroglia, M., L., T. and Mackenzie, S. (2004). A differentially expressed enolase gene isolated from the gilthead sea bream (Sparus aurata) under high-density conditions is up-regulated in brain after in vivo lipopolysaccharide challenge. *Aquaculture*. 241: 195-206.

Rider, C. and Taylor, C.B. (1975). Enolase isoenzymes. II. Hibriditzation studies, development and phylogenetic aspects. *Biochim. Biophysys*. 405: 175-187.

Rider, C.C. and Taylor, C.B. (1975). Enolase isoenzymes. II. Hybridization studies, developmental and phylogenetic aspects. *Biochim Biophys Acta*. 405(1): 175-87.

Roche, H. and Boge, G. (1996). Fish blood parameters as a potential tool for identification of stress caused by environmental factors and chemical intoxication. *Marine Environmetal Research*. 41: 27– 43.

Roher, N., Krasnov, A., Mackenzie, S. and Planas, J.V. (under submission). Metabolic and immune changes in skeletal muscle of rainbow trout (oncorhyncus mykiss) after LPS challenge.

Royds, J.A., Parsons, M.A., Taylor, C.B. and Timperley, W.R. (1982). Enolase isoenzyme distribution in the human brain and its tumours. *J. Pathol.* 137: 37–49.

Ryan, J.A. and Hightower, L.E. (1996). Stress proteins as molecular biomarkers for environmental toxicology. *Exs*. 77: 411-24.

Santini, D., Poli, F. and Lega, S. (2006). Solid-papillary tumors of the pancreas: histopathology. *Jop*. 7(1): 131-6.

Segil, N., Shrutkowski, A., Dworkin, M.B. and Dworkin-Rastl, E. (1988). Enolase isoenzymes in adult and developing Xenopus laevis and characterization of a cloned enolase sequence. *Biochem J*. 251(1): 31-9.

Shashidharan, P., Chalmers-Redman, R.M., Carlile, G.W., Rodic, V., Gurvich, N., Yuen, T., Tatton, W.G. and Sealfon, S.C. (1999). Nuclear translocation of GAPDH-GFP fusion protein during apoptosis. *Neuroreport*. 10(5): 1149-53.

Sirover, M.A. (1996). Minireview. Emerging new functions of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in mammalian cells. *Life Sci*. 58(25): 2271-7.

Sirover, M.A. (1997). Role of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in normal cell function and in cell pathology. *J Cell Biochem*. 66(2): 133-40.

Sirover, M.A. (1999). New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochim Biophys Acta*. 1432(2): 159-84.

Tracy, M.R. and Hedges, B.S. (2000). Evolutionary history of the enolase gene family. *Gene*. 259(enolasa): 129-138.

Vuori, K.A., Koskinen, H., Krasnov, A., Koivumaki, P., Afanasyev, S., Vuorinen, P.J. and Nikinmaa, M. (2006). Developmental disturbances in early life stage mortality (M74) of Baltic salmon fry as studied by changes in gene expression. *BMC Genomics*. 7(1): 56.

Wold, F., Ed. (1971). Enolase. The Enzymes. New York, Academic Press.

Xing, C., Laporte, J.R., Barbay, J.K. and Myers, A.G. (2004). Identification of GAPDH as a protein target of the saframycin antiproliferative agents. *Proc Natl Acad Sci U S A*. 101(16): 5862-6.

Zheng, L., Roeder, R.G. and Luo, Y. (2003). S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component. *Cell*. 114(2): 255-66.

Chapter 4

Molecular and bioinformatic studies of potential immune marker genes

1. Introduction

1.1. Expressed Sequence Tag Analysis (EST): Macrophage Project

An expressed sequence tag or EST is a short sub-sequence of a transcribed protein-coding or non-protein coding DNA sequence. It was originally intended as a method to identify gene transcripts, but it has since been instrumental in gene discovery and sequence determination. ESTs act as physical markers for cloning and full length sequencing of the cDNAs of expressed genes. It's produced by one-shot sequencing of a cloned mRNA, and the resulting sequence length only being limited by current technology. These sequence databases describe the kinds of genes and expression levels in a particular tissue or cells.

Macrophages constitute an essential part of the innate immune system and are also important for the activation of the adaptive immune response. The functions of macrophages can be carried out by direct interaction with pathogens (e.g., phagocytosis) or indirectly through the production of a number of factors such as cytokines, growth mediators, and oxygen radicals (Goetz, F.W. et al. 2004). Given the range of activities exhibited by pathogen-activated macrophages, it would appear that they are capable of producing an array of proteins involved in various cellular processes including respiratory burst, inflammation, phagocytosis, cytokine synthesis, and protein processing. Candidate gene approaches have been used to isolate some mRNAs produced by fish macrophages including interleukin 1β (IL-1β) (Zou, J. et al. 1999a), tissue necrosis factor α (TNFα) (Laing, K.J. et al. 2001), nitrous oxide synthase (NOS) (Laing, K.J. et al. 1999), prostaglandin endoperoxide synthase (PGS-2) (Zou, J. et al. 1999b), and chemokines (Dixon, B. et al. 1998). In addition, subtracted cDNA libraries and expressed sequence tags (ESTs) of head kidney cells stimulated with viruses or immunostimulants, have been reported for several fish species (Kono, T. et al. 2001; Liu, L. et al. 2002; Savan, R. et al. 2002). While immune/macrophage related genes have been obtained from these analyses, the number and diversity of these genes have generally been low. A possible reason may be that the cells used for such analyses were primarily monocytes and, therefore, not highly inducible by pathogen associated molecular patterns (PAMPs) such as the lipopolysaccharide component of Gram negative bacterial cell walls (LPS). Recently, it was reported that cells isolated from the rainbow trout (*Oncorhynchus mykiss*) head kidney and cultured for 5 days, exhibited a progressive increase in the expression of TNFα when stimulated with *Escherichia coli* LPS (Mackenzie, S. et al. 2003). Further, head kidney cells incubated for 5 days in culture exhibited the cytologic characteristics of macrophages and displayed an increased phagocytic capacity (Mackenzie, S. et al. 2003). The stimulatory characteristics obtained using this primary macrophage

culture regime have been further characterized (Iliev, D.B. et al. 2005a). This analysis has shown that LPS concentrations from 10 to 100 μg/ml stimulate the expression of TNFα, IL-1β and PGS-2 transcripts which become detectable after 6 h of stimulation and increase to 24 h. The long-term objective for the development and characterization of this trout primary cell culture system has been to use it for the transcriptomic analysis of gene expression in differentiated trout macrophages and other leukopoietic cells. Thus, large-scale macrophage cultures stimulated with LPS were carried out to produce mRNA for cDNA library construction and gene analysis. A large number of immune and cell signal related genes were observed in these libraries, provided the bases for studies of gene regulation in macrophages in salmonid fish (Goetz, F. et al. unpublished data).

1.2. Testing candidate genes after *in vivo* LPS challenge

From the EST database generated from macrophage cell cultures stimulated 12 hours by LPS (10μg/ml) (mEST), a large number of interesting genes were discovered. A significant number of sequences (Fig.4.1) were homologous to general metabolic genes (18.7%), ribosomal proteins (13.8%), immune related genes (11.2%), structural (7.6%), cell signalling (7.2%), proteases and antiproteases (6.8%), transcription/translation (2.6%), replication/division (2.5%) and 29.4% represented similar but unknown, other or novel gene groups. cDNAs related to immune function included structural proteins, cell death regulators, receptors, lectins and immunolglobulins, cytokines and chemokines, cell surface antigens signal transduction proteins, antimicrobial peptides, and enzymes involve in eicosanoid synthesis.

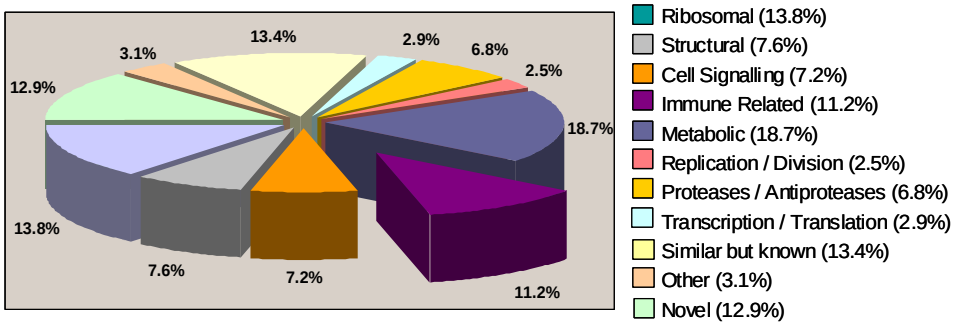


Fig.4.1. Classification of cDNA sequences obtained from LPS-stimulated macrophages into groups based on junction or structural similarity. "Similar but unknown" indicates genes with significant identities to GenBank entries of unknown function; "Other" indicates genes that had significant identities to GenBank entries of known function but could not be categorized into any of the classification listed; "Novel" indicates genes that did not have a significant blastx or blastn comparison.

In vitro systems are well suited to the study of biological processes in an isolated context. *In vitro* culture system has its greatest potential in providing information on basic mechanistic processes in order to refine specific experimental questions to be addressed in the whole animal. It is generally recognized that *in vitro* systems often provide only partial answers to more complex problems; therefore, they can supplement investigations with whole animals. Although there are an increasing number of DNA sequences in fish databases, there is a lack of data concerning the function of those physiological system in lower vertebrates. Consequently, for studying candidate genes and their role as key immune genes, an *in vivo* model consisting of a LPS intraperitoneal injection (LPS IP) to rainbow trout was developed (section 2.3). In recent years, there has been significant progress in understanding the signalling pathways activated by LPS to Toll-like receptor 4 (TLR4), as well as products of TLR4 signalling such as tumor necrosis factor alpha (TNFα), a potent inflammatory cytokine induced by LPS stimulation (Lin, W.J. et al. 2005). However, these TLR4 signalling pathways may not be the same for fish in respect to LPS activation (Iliev, D.B. et al. 2005b). Candidate genes involved in immune function would represent potential molecular markers which allowed a better understanding of the immune system in fish. Furthermore, the application of these molecular markers in aquaculture would improve, not only fish welfare, but also general quality management and prove basic knowledge for the elaboration of novel biotechnological products.

Objectives

1. Selection of candidate immune genes cloned from the EST library constructed using *in vitro* macrophages cultures.
2. In depth, bioinformatic analysis of selected candidate genes.
3. Study the expression pattern of selected candidate genes in different rainbow trout tissues after being subjected to a LPS IP *in vivo* challenge.

2. Materials and Methods

2.1. Expressed Sequence Tag Analysis (EST)

The construction of a cDNA library and gene analysis from the mEST (Goetz, F.W. et al. 2004) reported 1048 sequences. Recently, this large number has been further increased to 5000 sequences (Goetz, F.W. unpublished data). All of them were annotated and classified in the immune-related, cell signalling and proteases/antiproteases categories. The results obtained were rather important because they revealed a large number of immune-related genes, many of them non previously reported for trout. Briefly, cell cultures from 8 head kidney rainbow trout

were isolated and cultured for 5 days when they have typical cytological characteristics and are able to show phagocytic capacity and cytokine expression (Mackenzie, S. et al. 2003). After 12 h incubation with LPS from *Escherichia coli* (10µg/ml), RNA was extracted (TriReagent, MRC), reverse transcribed (Promega) and Zap Express (Stratagene) protocol was followed for cDNA library construction. Plasmid sequences were run on an ABI Prism automated sequencer (Applied Biosystems).

2.2. Sequence data analysis

Sequence chromatogram files were trimmed for quality using phred (http://www.phrap.org/phrap.docs/phred.html), vector screened using cross match (http://www.phrap.org/phrap.docs/phrap.html) and analyzed locally using (1) blastx against the NCBI nonredundant (nr) protein database, (2) blastn against the NCBI nucleotide (nt) database and (3) blastn against the NCBI EST (dbEST) database. All sequences were grouped by category and tentative identification was based initially on a blastx similarity score of $<10^{-3}$ or, in the case of blastx scores of $>10^{-3}$, a blastn score of$<10^{-5}$. All sequences were analyzed for redundancy using CAP3 (Huang, X. et al. 1999), for homology percentage analysis Bioedit v7.0.5. programme and for multiple alignment sequence Clustalw analysis, with a 70% threshold considered (Thompson, J.D. et al. 1994).

2.3. Candidate genes

Based on their homology to known gene databases, candidate genes were consciously chosen depending on their key role in immune functions and gene expression levels. All of these selected genes have relevant biological interest and their functional processes directly contribute to immune system function. Candidate genes chosen from mEST database were PU.1, TNFRs, TNF Decoy receptor, 4-IBB, TNFα, MHCII Ii, CD83, CD209e, CCL4-like, Acute phase serum amyloid A, IL1-Receptor type II and IL1-Receptor like. Interestingly, the enolase gene was represented in the mEST database, so emphasis of the involvement of this gene in immune activation was further studied. The first step was to characterize gene sequences with bioinformatic tools and later, to detect candidate genes directly in tissues by PCR analysis, both conventional PCR and real time PCR, in order to determine if they were differentially expressed after an immune challenge.

2.4. Animals and experimental procedures

Rainbow trout (*Oncorhynchus mykiss*) of 150 g as a mean weight, were obtained from J.Antrés fish farm (St. Privat, Girona). Fish were transported to aquarium facilities at the Universitat Autònoma de Barcelona where they were hold in stock tanks at 19ºC at least two weeks for acclimation period before experiments. Water quality was analysed periodically. Tanks were kept partially covered under a photoperiod of 12h light/12h dark. Trout were fed a maintenance ration of about 0.3–0.5% body weight per day. For this experiment, 48 trout were injected intraperitoneally (IP), half of them with LPS (6mg/kg) and the other half with saline buffer (PBS) and kept in different tanks. After 6, 12, 24 and 72 hours, six fish saline group (controls) and six fish LPS group were sampled by first placing them into a bath with a lethal concentration of 2-phenoxy-ethanol for less than 1 min, and bled. Brain, head kidney, liver, gills, red muscle, white muscle and intestine tissues were carefully dissected and quickly frozen in liquid nitrogen and stored at –80ºC to avoid RNA degradation.

2.5. RNA extraction, reverse transcription and real time PCR

Selected genes were analysed by reverse transcription PCR (RT-PCR) and/or real time PCR to determine differential expression induced by LPS. Total RNA was extracted separately from tissues at each time point and later all animal group were pooled (N=6; C6, LPS6, C12, LPS12, C24, LPS24, C72, LPS72) using TriReagent (Molecular Research Center, Inc.) according to the manufacturer's protocol (Chomczynski, P. et al. 1987; Chomczynski, P. 1993) and 4 μg of that RNA was converted to the first-strand cDNA synthesis by using Super-Script™ II (Invitrogen). PCR primers were carefully designed against the obtained mEST database sequences for all candidate genes (Annex, Tale 1). For conventional PCR the following protocol was used (Amplitaq DNA Polymerase; Biotols): 5 min initial denaturation at 95ºC, followed by cycles of 45 s denaturation at 95ºC, 45 s annealing at certain temperature, and 45 s extension at 72ºC. Samples were taken after 20, 25 and/or 30 cycles and were analyzed on ethidium bromide stained gels. For real time PCR (Brilliant SYBR Green QPCR Master Mix Kit, Stratagene), cDNA from macrophage cultures at different LPS incubation times were run in an Opticon Continuous Fluorescence Detection System (MJ Research) with the following protocol: 10 min initial denaturation at 95ºC, followed by 40 cycles of 30 s denaturation at 95ºC, 60 s annealing at 50ºC, and 90 s extension at 72ºC. Fluorescence was measured at the end of every extension step. C_T (threshold cycle) values were calculated and normalized for each gene against those obtained for β-actin (data not shown). Table 1 in the Annex shows PCR conditions and primers

sequences. Table 2 in the Annex shows selected candidate genes from the mEST database. Table 3 in the Annex, schematise expression results obtained from both RT-PCR and real time. Database homology percentages and multiple alignments for each candidate gene were performed by the BioEdit computer analysis programme.

3. Results and Discussion

Potential molecular marker genes were selected depending on their involvement as a key gene participating in immune function. Specific primers were designed against the obtained mEST sequences (Table 1, Annex). Candidate gene expression was studied at different time points during LPS IP induced inflammation (N=6 individuals each time point) by conventional RT-PCR. Table 3 in the Annex shows expression levels detected at different tissues and times tested by conventional RT-PCR and real time of all candidate genes.

3.1. PU.1 / Spi.1

A full-length cDNA clone from mEST encoding a 1237 bp nucleotide sequence with a 58% homology identities (73% similarities) to human Spi1/Pu.1 transcription factor (Annex, Table 4) was obtained. Characterization of PU.1 nucleotide and putative protein sequences and alignment analysis are discussed in chapter 5 (see Fig.5.2 and Fig.5.3, chapter 5). PU.1 belongs to the E26-Transformation–Specific (Ets) Family and it is required for the development of myeloid and lymphoid cells (Rosenbauer, F. et al. 2004b), as well as microglia in brain (Walton, M.R. et al. 2000; Hwang, C.K. et al. 2004). Its dual name arose originally when it was first identified as an oncogen involved in malignant erythroblastic transformation induced by the acutely leukemogenic Friend and Raushcer spleen focus-forming viruses (Moreau-Gachelin, F. et al. 1988; Moreau-Gachelin, F. et al. 1989; Goebl, M.K. 1990). Later, in mouse, it was described as a DNA-binding protein that binds to a purine-rich sequence (PU box, 5'GAGGAA-3') (Karim, F.D. et al. 1990; Klemsz, M.J. et al. 1990). The PU.1 transcriptor factor is one of the most important regulators of hematopoietic lineage development. However, its role at each hematopoietic stage remains unclear (Iwasaki, H. et al. 2005). During hematopoiesis, PU.1 is required for development along the lymphoid and myeloid lineage, and needs to be downregulated during erythropoiesis (Fisher, R.C. et al. 1998). Different cellular concentrations of PU.1 may direct distinct cell fates (see Fig.5.1, chapter 5), with the highest concentrations of PU.1 required for macrophage development and lower concentrations for granulocytic and B-cell fate adoption (Dahl, R. et al. 2003). Several studies have described this gene in tissues involved in the hematopoietic response and derived-macrophages cell types

(Galson, D.L. et al. 1993; Tondravi, M.M. et al. 1997; Bakri, Y. et al. 2004) where it plays a critical role in the regulation of a large number of genes and multiple nuclear proteins (Yoshida, H. et al. 1990; Witmer-Pack, M.D. et al. 1993; Nagulapalli, S. et al. 1995; Rosenbauer, F. et al. 2004a). Many functions related to growth, development, and functional of the hematopoietic system have been attributed to the PU.1 transcription factor (Henkel, G.W. et al. 2002). The function of this transcriptional factor is so essencial that a dysfunctional expression of PU.1 is lethal for animal survival as shown in PU.1 knock out animals (Cooper, L. et al. 2005). PU.1 is a master gene essential for hematopoietic development and its high expression levels are responsible for the fate of myelopoietic cells (Tondravi, M.M. et al. 1997; Gangenahalli, G.U. et al. 2005). Tissue distribution of PU.1 expression levels in mammals demonstrated that PU.1 expression was high in brain (Walton, M.R. et al. 2000), spleen (Rosenbauer, F. et al. 2004a), bone (Tondravi, M.M. et al. 1997) lung (Hollenhorst, P.C. et al. 2004), and testis (Galson, D.L. et al. 1993). At the moment, PU.1 has been cloned from some non-mammalian species such as chicken (Kherrouche, Z. et al. 1998) but, in fish the PU.1 gene has been scarcely studied. In zebrafish as a research animal model, mainly for developmental studies, some recent reports have been published concerning the role of PU.1 in myelopoietic development (Bennett, C.M. et al. 2001; Lieschke, G.J. et al. 2002; Ward, A.C. et al. 2003; Berman, J.N. et al. 2005; Galloway, J.L. et al. 2005; Rhodes, J. et al. 2005).

In vivo PU.1 PCR expression levels from adult rainbow trout after 24 and 72 hours to LPS IP treatment (6mg/kg) are shown in figure 4.2 resulting in high expression levels in the head kidney, brain and heart tissues, in both control and LPS treated animals. Due to the inflammation caused by LPS IP administration, PU.1 expression levels increased 24 hours after treatment in white muscle and intestine, whereas in red muscle this increase occurred 72 hours post injection. Although PU.1 expression was found in the liver, no changes caused by LPS-IP were detected. From real time PCR results of the *in vitro* macrophage cell cultures treated with LPS (10μg/ml) at different time points (6, 12, 18, 24 and 30 hours), PU.1 mRNA expression levels detected were very high, regardless to LPS treatment. Interestingly PU.1 was the most expressed gene in this cell type, higher than the β-actin gene, independent to the treatment (Annex, Table 3). These results were further completed by using conventional RT-PCR at different cycles from adult rainbow trout head kidney cDNA, where less than 20 cycles were needed to amplify the PU.1 gene cDNA product due to its high level of expression (data not shown). Overall results confirm those described in mammals, where high concentrations of PU.1 is required for macrophage development (Dahl, R. et al. 2003). Overall, PU.1

mRNA expression results show that PU.1 is highly expressed in the head kidney, the primary immune tissue in fish, and also its expression levels are high in the macrophage cells.

In conclusion, from mEST a full length of PU.1 gene has been cloned for the first time in salmonid fish. It is of interest that the transcription factor PU.1 plays an essential role in hematopoietic development and high differential expression levels result in different myelopoiesis cell fates. The study of PU.1 as a key immune gene closely involved in hematopoiesis and its role in fish immune system has never been reported in salmonid fish.

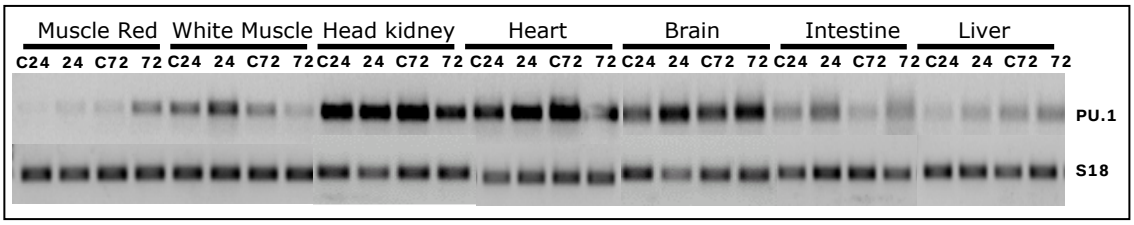


Fig.4.2. PU.1 expression in rainbow trout after LPS (6mg/kg) IP challenge at different hours postinjection. Results of RT-PCR (30 cycles, 312 bp PCR product length) on total RNA (4 µg) extracted from tissues (N=6 per group). Samples were separated on ethidium bromide-agarose gels. S18 was used as a house keeping gene. C24/C72: control animals; 24/72: LPS treated animals.

3.2. Tumor Necrosis Superfamily: factors and receptors

Tumor necrosis factor/Tumor necrosis factor receptor (TNF/TNFR superfamily proteins play a central role in the immune system (Locksley, R.M. et al. 2001). Tumor necrosis factor alpha (TNF-α) is a multifunctional cytokine with pleiotroic actions ranging from a proliferation response (Beutler, B. et al. 1989) to inflammatory effects and the immune response (Locksley, R.M. et al. 2001) being the major mediator of apoptosis (Vassalli, P. 1992; Chen, G. et al. 2002). TNF alpha and beta were isolated in 1984 from human activated macrophages and T cells (Gray, P.W. et al. 1984; Pennica, D. et al. 1984; Aggarwal, B.B. et al. 1985; Kull, F.C., Jr. 1988) while TNFR proteins were identified some years later (Dembic, Z. et al. 1990; Heller, R.A. et al. 1990; Loetscher, H. et al. 1990; Rothe, J.G. et al. 1991). TNFα only has the ability to bind two receptors TNFR1 (also known as TNFRSF1a, p55TNFR, p60, and CD120a) and TNFR2 (also known as TNFRSF1b, p75TNFR, p80 and CD120b) (Gupta, S. 2002; Macewan, D.J. 2002). The interaction of TNFα with TNF receptors triggers a series of intracellular events that ultimately result in the activation of two major transcription factors, nuclear factor κB (NFκB) and c-Jun (Chen, G. et al. 2002; Ghosh, S. et al. 2002). The protein interaction network around TNFα/NFκB pathways have been extensively reported (Bouwmeester, T. et al. 2004). TNFR1 differs to TNFR2, not only for its high affinity

TNF binding (Vandenabeele, P. et al. 1995), but also for having a death domain which gives the ability to induce apoptosis (Tartaglia, L.A. et al. 1993; Thorburn, A. 2004).

In fish, tumor necrosis factors have been recently reported (Goetz, F.W. et al. 2003). TNFα orthologues have been isolated and characterized from several fish species (Laing, K.J. et al. 2001; Garcia-Castillo, J. et al. 2002; Zou, J. et al. 2003b) and a number of studies have provided indirect evidence for the biological role of TNF in fish (Zou, J. et al. 2002; Mackenzie, S. et al. 2003; Zou, J. et al. 2003a; Garcia-Castillo, J. et al. 2004). Recently, the lymphotoxin-beta (LT-beta) sequence has been reported (Kono, T. et al. 2005). In the case of TNFα, two isoforms due to a duplication event of TNFα, TNF1 and TNF2, have been described in rainbow trout (Zou, J. et al. 2002) and carp (Saeij, J.P. et al. 2003a). TNF2 gene is more responsive when cells are stimulated with a range of different activators (Zou, J. et al. 2002; Saeij, J.P. et al. 2003b). Controversially, in view of the abundance of studies on TNF in fish, TNFRs have been poorly documented. TNFR1 and TNFR2 were first cloned in fish with a 35 and 40% homology to extracellular domain to mouse and human respectively from the Japanese founder *Paralichthys olivaceus* (Park, C.I. et al. 2003). There is a lack of TNFRs gene information not only concerning their DNA sequences in fish species databases but also, about their biological function as immune genes responsible for cell signalling transduction pathways which evolved pleiotropic responses. From the mEST database, several TNFRs family putative proteins were identified (Annex, Table 5). Interestingly, by using CAP3 from five different overlapping contigs, a full length sequence TNFRV (TNFRVirtual) was synthetically constructed. TNFRV consists of 1358 base pairs coding for a 319 aminoacid putative protein and possesses cysteine motifs repeated in the extracellular N-terminal domain, therefore certifying it as a member of the TNFR family. TNFRV presented a 91% identity to TNFR rainbow trout (CAD57165) also cloned *in silico*. Another sequence partially cloned, composed with 802 nucleotides was 42% homologous to TNFR2 cloned from Japanese flounder (Park, C.I. et al. 2003).

TNFRSF members are defined as those structural related to the first family members TNFR1 and TNFR2 (Locksley, R.M. et al. 2001). From the mEST, several members of the TNFR superfamily were revealed. For example, two different sequences were similar to TNFRSF6 (Fas) in human (AAP36297) and rat (NP690920), as well as homology to TNFRS14. TNFRSF members and their activating ligands are complex (Bobik, A. et al. 2001). The binding of TNF to TNFRs triggers a series of intracellular processes where a large numbers of proteins are involved (Bouwmeester, T. et al. 2004). Of special interest are TNF receptor-

associated factor (TRAF) which defines a novel group of adaptor proteins involved in signal transduction by most members of the TNF receptor family, IL-1 receptor and IL-17 receptor as well as some members of the Toll-like receptor family (Wajant, H. et al. 2001). Some tumor necrosis receptor associated factors (TRAF) and TTRAF (TRAF and TNFR associated proteins) were also cloned from mEST, for example TRAF3 and TRAF4 (Table 4.1).

Putative Function	EST Accession	Size	Blastx similarity score	Species most similar to	Accession of similar protein
TNFRV	BX316951	606	5 e-173	rainbow trout	CAD57165
TNFR2	BX888381	802	4 e-5	flounder	BAC65226
Decoy Receptor	BX865673	761	4 e-45	brook trout	AAD56428
Fas (TNFRSF6)	BX303060	393	1 e-13	human	AAP36297
Fas (TNFRSF6)	BX321588	607	1 e-6	rat	NP690920
TNFRSF6b	BX869435	675	3,7	human	NP003814
TNFRSF7 precursor	BX865460	375	3,00	human	NP001233
TNFRSF9 precursor	BX318178	522	0,27	human	NP001552
TNFRSF13b	BX862196	771	2 e-26	human	NP006564
TNFRSF14	BX887511	681	2 e-31	mouse	NP849262
TNFR-typeII	BX863609	330	2,50	rat	AAL16021
TRAF3	BX317358	625	3 e-52	rainbow trout	CAD57164
TRAF4	BX869427	716	0,54	mouse	NP033449
TRAF5	BX300254	795	1 e-50	mouse	P70191
TRAF (1F727)	BX302171	661	1,8	nematode	NP491534
TTRAP	BX306203	744	4 e-51	human	NP057698
TTRAP	BX306202	772	1 e-6	mouse	NP062424

Table 4.1. List of all Tumor Necrosis Factor Receptors (TNFRs) family members obtained from the EST macrophage cell culture after 12 h LPS (10μg/ml) stimulation. This table includes most similar blastx against NCBI nr database.

Intensive investigation is ongoing to clarify the annotation of TNF receptor sequences uncovered from public databases compared to the sequences from the mEST database. The TNFR family forms a large list of genes (Baker, S.J. et al. 1998) which in mammals, do not display more than 25% homology overall at the protein level, grouped together by the presence of conserved cysteine residues (repeated two to six times) in their extracellular domain, (Beutler, B. et al. 1989; Nagulapalli, S. et al. 1995; Baker, S.J. et al. 1998). The intracellular domains are largely unrelated with almost no homology between each other (Grell, M. et al. 1994). The lack of homology in the intracellular domains, implies that each receptor utilizes distinct signal transduction pathways and have unique intracellular effects

(Dembic, Z. et al. 1990) which when activated, they recruit and bind cytoplasmatic adaptor proteins (Bridgham, J.T. et al. 2003) to initiate cell signalling. In order to clarify fish database sequences belonging to TNFRs, multiple sequence alignments were assessed within different TNFR proteins from different species (Fig.4.3). Although low homology was found between TNFRs among species, multiple sequence alignments showed that cysteine residues characterizing TNFR family members are localized in the ligand-binding domain.

Expression levels studies using real time PCR (Annex, Table 3), show an up regulation of TNFRV 6 hours after LPS-stimulation and were maintained for at least 30 hours in a LPS-stimulated culture. On the contrary, TNFR2 levels were very low, needing more than 30 cycles to be amplified. Unlike different expression levels of TNFRV in LPS stimulated macrophage cell cultures, no gene amplification was obtained from tissue control brain. TNFR2 tissue distribution results revealed that in brain and head kidney mRNA levels were not affected by LPS IP *in vivo* treatment. Interestingly, in gills TNFR2 shows a slight down regulation after 24 and 72 hours after LPS IP challenge, and in muscle, this detection was only possible after 24 hours LPS treatment (Annex, Table 3).

Another gene belonging to TNF family, cloned by EST technology, was a decoy receptor, similar (32%) to the decoy receptor firstly cloned in brook trout ovary tissue (Bobe, J. et al. 2000). TNF decoy receptor is a soluble protein receptor and lack the death domain (Baker, S.J. et al. 1998). Interestingly, the decoy receptor gene expression was detectable after 24h and 72h LPS treatment in brain tissue but not in control fish (Annex, Table 3). In parallel, decoy receptor expression showed higher levels after LPS treatment in all time points tested by real time PCR in macrophage culture. From mEST another cDNA transcript presenting 46% similarity to rat 1-4BB (CD137) gene was partially cloned. This gene is a T-cell costimulatory receptor, belonging to TNF superFamily (Goodwin, R.G. et al. 1993), and plays a critical role in determining the outcome of T-cell receptor signalling (Mueller, D.L. et al. 1989) which may elicit biological effects such as increase of proliferation and IL-2 production (Alderson, M.R. et al. 1994). Primers designed against sequences obtained from the mEST, did not amplify any gene in control brain tissue although it has been detected in human neurons, astrocytes and microglia cultures (Reali, C. et al. 2003). However, the 1-4BB gene tested by real time PCR from control and LPS macrophages shows moderate level of expression in all time points studied (Annex, Table 3).

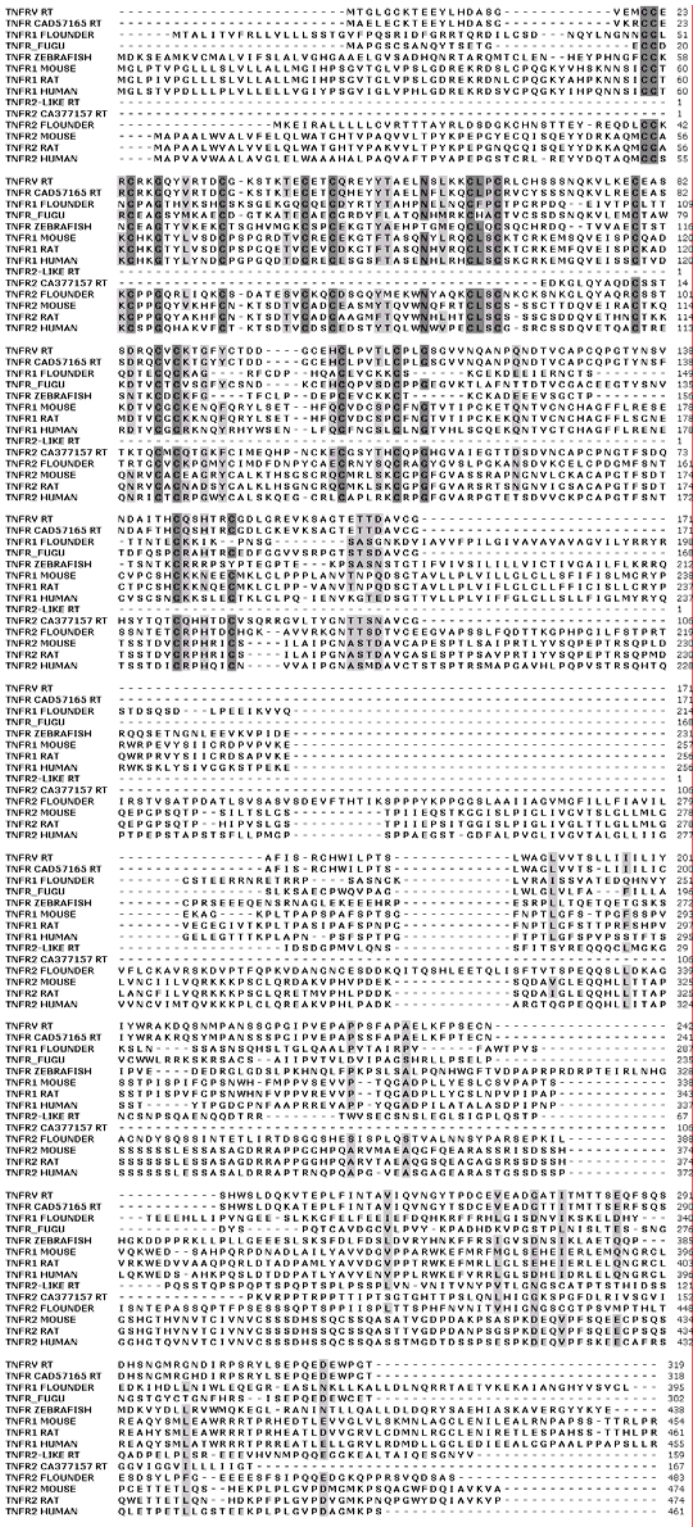


Fig.4.3. TNFRs multiple sequence alignment by ClustalW (Thompson, J.D. et al. 1994), with 70% threshold. Note low homology family where only characteristic conserved cysteines characteristic are shadowed. Accession numbers are indicated in table 5 in the annex section.

3.3. MHCII invariant chain (Ii)

Major histocompatibility complex class II (MHCII) invariant chain (Ii) protein 1 was present in the mEST. The full length cDNA homologue to MHCII invariant chain (Ii) encoding a 278 aminoacid protein and presenting an e-value of e^{-143} to the previously characterized MHCII Ii protein in trout (Dijkstra, J.M. et al. 2003). Sequence homologies of rainbow trout MHCII Ii protein sequence to other species revealed identity percentages of 56%, 37% and 34% to chinese perch, carp and zebrafish respectively (Annex, Table 6). Although these homology percentages are quite low, protein domains are conserved in the MHCII Ii among species where greatest conservation is found within the transmembrane region (Castellino, F. et al. 2001) (Annex, Fig.1).

The major histocompatibility complex (MHC) encodes highly polymorphic polypeptides that serve the immune system as peptide receptors. MHCII invariant chain acts as a chaperone by assisting initial folding and oligomerisation of the MHCII subunits (Roche, P.A. et al. 1991). MHCII invariant chain is a non-polymorphic type II membrane protein playing an important role in the MHCII processing pathway promoting and processing antigen presentation and stabilizes the conformation of the MHCII molecules (Hiltbold, E.M. et al. 2002). MHC class II molecules are responsible to the presentation of antigenic peptides (Pieters, J. 1997) and increase dramatically on the mature dendritic cell (DC) surface (Mellman, I. et al. 2001), in contrast, when not mature, MHCII is accumulated in endosomes and lysosomes (Cella, M. et al. 1997; Pierre, P. et al. 1997). Recently, chromosomes and linkage groups harbouring MHCII have been determined in both rainbow trout (Phillips, R.B. et al. 2003) and lake trout (Noakes, M.A. et al. 2003), and in the last few years, have also made abundantly clear that the teleost genome MHCII is organized in a very different manner from that seen in mammals (Stet, R.J. et al. 2003). Presumed classical class I and class II genes are not linked and are even found on different chromosomes (Ohta, Y. et al. 2000). Several studies in different fish species have been published since the first cDNA MHCII cloned in zebrafish (Bingulac-Popovic, J. et al. 1997), nurse shark (*Ginglymostoma cyrratum*) (Ohta, Y. et al. 2000), salmon (GI:17467197) and trout (Hansen, J.D. et al. 1999). However, studies regarding MHCII expression levels of the invariant chain protein are scarce. *In vitro* results from macrophage cell culture showed that MHCII invariant chain was up regulated by LPS probably associated with the increased membrane expression of antigens derived from pathogens that have been phagocytosed (Goetz, F.W. et al. 2004). *In vivo* results from adult rainbow trout injected with LPS IP (6mg/kg) showed a high expression in brain, head kidney, liver, ovary, intestine and white muscle tissues tested independent to treatment

(Fig.4.4). Only red muscle tissue expression levels were low and they increase was significant 72 hours after LPS IP *in vivo* treatment. High expression levels of MHCII invariant chain in tissues, specifically head kidney and brain, were further studied by RT-PCR expression cycles. These results showed that only 15 PCR cycles are enough for amplifying MHCII in both head kidney and brain and that MHCII expression levels are higher in those animals treated with LPS IP 24 h and 72 h after treatment (data not shown).

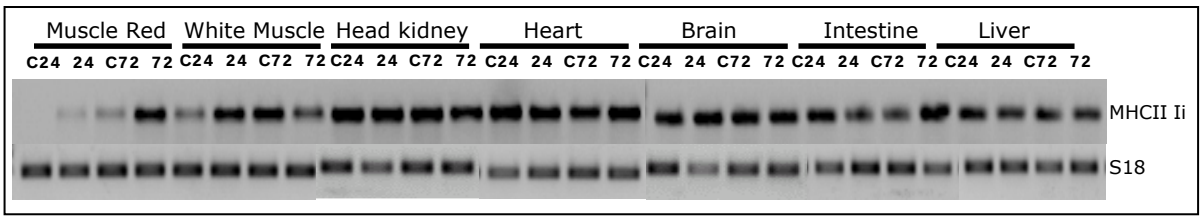
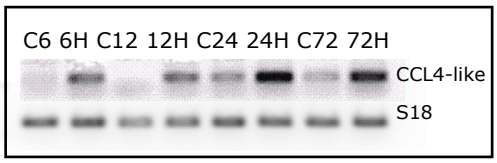


Fig.4.4. MHCII Ii expression in rainbow trout after LPS (6mg/kg) IP challenge at different hours postinjection. Results of RT-PCR (30 cycles, 763 bp PCR product length) on total RNA (4 µg) extracted from tissues (N=6 per group). Samples were separated on ethidium bromide-agarose gels. S18 was used as a house keeping gene. C6/C12/C24/C72: control animals; 6/12/24/72H: LPS treated animals.

3.4. CCL4-like

Chemokines are small inducible proteins that direct leukocyte migration during inflammation and response to infection (Stein, J.V. et al. 2005). From complete sequencing of several of these ESTs, a full-length cDNA (AY561709) was obtained that was 866 nucleotides in length, with an open reading frame of 303 bp, presumably coding for a protein of 101 amino acids. The putative novel trout chemokine CCL4-like was identified with aminoacid homology level of 38% similarity to *Macaca mulatta* CCL4/MIP-1β (macrophage inflammatory protein 1β) (Basu, S. et al. 2002). Table 7 in the annex section shows that the highest homology level corresponds to zebrafish (48%) and pig (40%) chemokine CCL4 sequences. However, these two chemokines are predicted to be the CCL4 protein, and therefore, we considered 38% homology to monkey chemokine as the closest homology protein sequence. Multiple sequence alignment was performed with other CCL4 proteins, where conserved family cysteines are present (Annex, Fig.2). Using conventional RT-PCR (Fig.4.5), a substantial increase in CCL4-like mRNA expression was observed in brain tissue 24 and 72 hours following *in vivo* stimulation of adult trout with LPS, following the same expression pattern as found in intestine, ovary and spleen but not in gills (Mackenzie, S. et al. 2004).



ºFig.4.5. CCL4-like expression rainbow trout brain after LPS (6mg/kg) IP challenge at different hours postinjection. Results of RT-PCR (30 cycles, 415 bp PCR product length) on total RNA (4 µg) extracted from brain tissue (N=6 per group). Samples were separated on ethidium bromide-agarose gels. S18 was used as a house keeping gene. C6/C12/C24/C72: control animals; 6/12/24/72H: LPS treated animals.

3.5. DC-SIGN (CD209)-like

A full length cDNA clone encoding 1602 nucleotides was identified in expressed sequence tags generated from lipopolysaccharide stimulated *in vitro* differentiated macrophages isolated from the head kidney of the rainbow trout (*Oncorhynchus mykiss*). The putative 255 aminoacid protein (AY593994) has 95% homology identity to c-type lectin receptor-A from atlantic salmon (*Salmo salar*) (Soanes, K.H. et al. 2004). *In silico* analysis of the EST database shows that the putative CD209-like EST was homologous to the mouse CD209e antigen (Strausberg, R.L. et al. 2002) with an e-value of $5e^{-12}$ (27% homology identities) (Annex, Table 7). From sequences alignments (Annex, Fig.3), the CD209-like protein possesses CLECT (C-type lectin) domains between 117-248 aminoacids, shared among the lectin family receptors. DC-SIGN (CD209) (dendritic cell-specific ICAM-grabbing non-integrin, where ICAM is intercellular adhesion molecule) is a recently described mannose-specific C-type lectin expressed by dendritic cells (Soilleux, E.J. 2003) which is involved in pathogen capture, processing and antigen presentation to induce immune response (Mcgreal, E.P. et al. 2004). In humans, this family consists of three different receptors (DC-SIGN, L-SIGN and LSECtin) (Koppel, E.A. et al. 2005) and five in mice (mDC-SIGN, mSIGNR1 to mSIGNR4) (Park, C.G. et al. 2001). DC-SIGN was the first SIGN molecule identified and is preferentially expressed on myeloid dendritic cells (Geijtenbeek, T.B. et al. 2000b; Relloso, M. et al. 2002) and it is usually considered as a dendritic cell (DC)-specific phenotypic marker (Dominguez-Soto, A. et al. 2005). Interests for this molecule increased as it was reported to recognize an array of viruses such as human immunodeficiency virus type 1 (Geijtenbeek, T.B. et al. 2000a), Dengue virus (Tassaneetrithep, B. et al. 2003), Cytomegalovirus (Halary, F. et al. 2002), Hepatits-C virus (Lozach, P.Y. et al. 2004) and Ebola (Alvarez, C.P. et al. 2002). Expression studies in the human brain revealed expression of DC-SIGN to isolated primary human brain microvascular endothelial cells (Mukhtar, M. et al. 2002) and perivascular macrophages but not in microglia (Fabriek, B.O. et al. 2005).

Increasing transcriptional levels were detected in the macrophage culture treated with LPS 10μg/ml for 12 h (Goetz, F.W. et al. 2004). From conventional RT-PCR from pooled rainbow trout brain (N=6) expression levels significantly increased 24 h after administration of LPS IP (6mg/kg) (Fig.4.6) (Annex, Table 3). In summary, the CD209e-like cDNA sequence and its putative protein 255 aminoacids in rainbow trout has been reported. The CD209e-like gene is expressed in rainbow trout brain where its expression levels increase as a consequence of LPS IP inflammation.

Fig.4.6. CD209e expression in rainbow trout brain after LPS (6mg/kg) IP challenge at different hours postinjection. Results of RT-PCR (30 cycles, 648 bp PCR product length) on total RNA (4 μg) extracted from brain tissue (N=6 per group). Samples were separated on ethidium bromide-agarose gels. S18 was used as a house keeping gene. C6/C12C24/C72: control animals; 6/12/24/72H: LPS treated animals.

3.6. CD83

CD83 is a cell surface glycoprotein whose surface expression is the standard lineage marker for activated or differentiated dendritic cells (DC) including Langerhans cells (human), circulating DCs, and interdigitating DCs within T cell zones of secondary lymphoid tissues (Zhou, L.J. et al. 1995a) and have been documented in mammalian brain (Kozlow, E.J. et al. 1993; Twist, C.J. et al. 1998). CD83 may play important role in regulation of DC-mediated immune responses (Berchtold, S. et al. 1999) and has been conserved over 450 million years of vertebrate evolution (Ohta, Y. et al. 2004). In fish, DNA sequences of CD83 have been cloned in five different fish species, nurse shark, rainbow trout, zebrafish, flounder and sea bream (Doñate C. et al, unpublished data). Table 9 (Annex) illustrates the homologies of CD83 between species, showing that the closest CD83 rainbow trout sequence is with flounder (45%). Multiple sequence alignment of CD83 from different species is shown in figure 4 in the Annex. Interestingly, the CD83 cloned sequence from the EST, was very similar (98%) to that already reported in rainbow trout (Ohta, Y. et al. 2004). Those two sequences differ in three aminoacids in the extracellular domain and one in the transmembrane domain. In fact, two different CD83 putative aminoacid proteins were reported in rainbow trout in some tissues after virus infection, as a consequence of CD83 duplication event (Ohta, Y. et al. 2004). In mice, CD83 tissue mRNA expression was found in the heart, brain (high), spleen (high), lung, and muscle, and weakly within the kidney, though prolonged exposure or RT-PCR demonstrated expression

within all tissues examined (Twist, C.J. et al. 1998). In humans, CD83 mRNA expression was also detected in the brain, lungs, and mitogen-activated B lymphocytes, and within some T cell populations (Zhou, L.J. et al. 1992; Zhou, L.J. et al. 1995b). In shark, CD83 expression was ubiquitous, though the highest expression level found was in the epigonal (lymphopoietec) tissue. In rainbow trout, expression levels in control fish were hardly or not detected in thymus, pronephros, mesonephros and spleen. After six days of acute infection the expression levels of two CD83 messages are up-regulated in the spleen, pronephos and intestine. However, CD83 expression in rainbow trout brain was not studied (Ohta, Y. et al. 2004). Here, it is reported the RT-PCR results which CD83 amplified in brain and gene expression was significantly higher after 24 hours of LPS administration (Fig.4.7). The overall expression report analysis indicate that CD83 is largely expressed within immunologically important tissues in fish and that expression is up-regulated during acute viral infection (Ohta, Y. et al. 2004) and after LPS IP administration CD83 expression increases in brain tissue. This represents that CD83 could be a putative fish marker for DC lineages, thus opening the door for studies examining DC biology.

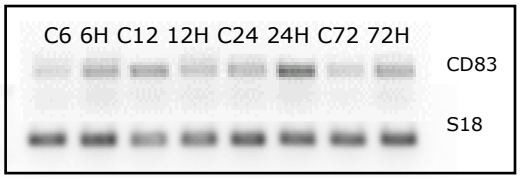


Fig.4.7. CD83 expression in rainbow trout brain after LPS (6mg/kg) IP challenge at different hours postinjection. Results of RT-PCR (30 cycles, 765 bp PCR product length) on total RNA (4 µg) extracted from brain tissue (N=6 per group). Samples were separated on ethidium bromide-agarose gels. S18 was used as a house keeping gene. C6/C12C24/C72: control animals; 6/12/24/72H: LPS treated animals.

3.7. Enolase

Enolase expression levels in rainbow trout tissues where further studied after LPS challenge at different time points. From the mEST results, 6 different cDNA were homologous to enolase gene differing in score value (Table 4.2). The highest e-value of the enolase cloned from mEST, corresponded to both neuron-specific human enolase and enolase 2 (gamma enolase) originated from synthetic construction. The second nearest homologous enolase was zebrafish enolase alpha and the S-enolase (*Sparus aurata*). Interestingly, a high number of clones were sequenced corresponding to zebrafish alpha enolase (9 clones) and S-enolase (3 clones) thus allowing to compare enolase sequences. Multiple sequences alignments are presented in chapter 2 (Fig.2.5) giving evidence of the high protein conservation enolase sequences through evolution. Enolase expression levels were

shown in chapter 3, where highly expressed mRNA levels were found in muscle tissue comparing to other tissues tested. From previous results, the enolase gene appears to be a good molecular marker because it is not only involved in the glycolytic pathway, but also it may participate in coping with disturbances caused by LPS inflammation or by physical stressors in fish. mEST results indicate that enolase is expressed in macrophage cells indicating that enolase may participate in immune functions.

Gene name	EST Accession	Size	Blastx similarity	Isoform described	Species most	Accession of similar
Enolase	BX320000	694	1 e-59	gamma enolase	human	AAA52388
	BX310988	610	2 e-71	enolase alpha-like	sea bream	AAO92646
	BX303009	595	9 e-75	enolase-1, alpha	zebrafish	AAQ97775
	BX302680	653	2 e-93	neurone-specific enolase	human	CAA31512
	BX298597	649	2 e-93	enolase-2, gamma, neuronal	virtual	AAP88878
	BX855755	770	2,4	alpha enolase	turtle	AAD20345

Table 4.2. Enolase sequences identified from EST macrophages cell culture database after 12 hours LPS stimulation (10μg/ml). Six different enolase isoforms were expressed by macrophages.

3.8. Other Genes

Table 2 and 3 in the Annex section shows respectively, a list of all candidate genes tested and their tissue distribution at different time points after LPS challenge. Two interleukin receptors were annotated from ESTs database IL1RII and IL1R-like. However, when brain expression levels were checked, no detectable or low expression levels for both genes were observed. Therefore, we classified them as non candidate immune genes for the present study. Interestingly, real time PCR revealed that macrophages expressed high levels of IL1R-like and IL1RII which were up regulated between 6 and 18 hours of LPS incubation. Considering these results, further experiments are envisaged for the future. Another gene obtained from mEST coding for a putative protein of 120 aminoacids which is 93% similar to an acute phase serum amyloid A (APS) partial cDNA cloned in rainbow trout (Jensen, L.E. et al. 1997). The primary site of production is proposed to be in the liver by hepatocytes, although extrahepatic production has also been observed in animal models and cell culture experiments of several mammalian species and chickens (O'hara, R. et al. 2000; Upragarin, N. et al. 2005). In trout, the production of APS from liver has been reported (Bayne, C.J. et al. 2001) as well as its up regulation by LPS and TNFα (Jorgensen, J.B. et al. 2000). This protein is part of the acute phase response to infection and it is present in blood at substantially different concentrations depending on the species (Petersen, H.H. et al. 2004). Several clones of APS from the mEST were observed and were significantly up regulated after 12 hours in LPS-stimulated macrophages (Goetz, F.W. et al. 2004).

Interestingly, after 30 hours under the same conditions, the number of APS clones was extremely high representing a 20% of the total transcribed genes of cultured macrophages (Goetz F.W., personal communication). These results reflect previous reports in human where the concentration of APS in plasma increases up to 1000-fold within 24 to 48 h after trauma, inflammation or infection (Upragarin, N. et al. 2005). From conventional RT-PCR results (Table 3, Annex), APS was detected and it was highly expressed in tissue control brain tissue although not all LPS-treated tissues were tested. This may be interesting because it shows an extrahepatic production of the APS gene in fish brain with a high expression level in control rainbow trout.

4. Conclusions

The isolation of molecular gene markers has received increasing interest in aquaculture due to their potential capacity to improve fish welfare, and ensure a better quality product. Throughout the past few years, the number of gene sequences has increased dramatically in fish databases for different fish species. More than a thousand of genes discovered from mEST have been reported, some of them for the first time in rainbow trout (Goetz, F.W. et al. 2004). The expression levels of some of these genes in macrophages cell cultures after 12 h LPS stimulation were further reported (Goetz, F.W. et al. 2004). However, *in vivo* expression studies were not conducted. The aim of this study was first, to identify and characterize some of the ESTs genes by using public database annotation and bioinformatic tools. Secondly, to select genes involved in immune function promising to be potentially molecular gene markers. And finally, *in vivo* expression studies were carried out in order to determine the potential molecular markers to be used for a better understanding of immune system in fish.

During the last decade, development of molecular cloning techniques has substantially increased genomic information for several fish species. Emerging genomic science is a powerful tool for discovering an array of genes previously unidentified in fish. Several public databases have been constructed with sequences available in a variety of fish species, although much work remains to be done. The lack of a clear, ordered, reliable and complete annotation work for fish genes in databases currently represents the major bottleneck for researchers. The application of molecular cloning techniques followed to the assistance of bioinformatic tools have resulted in a successful identification of rainbow trout immune genes (Goetz, F.W. et al. 2004). Several genes were completely or partially cloned from the mEST. Interestingly, while 12.9% of all sequences had no significant blastx (against the nr database) or blastn (against the nt database) hits

greater than 50, about one third of these sequences had nearly perfect blastx hits against the NCBI EST database. In most cases these were against the large number of existing rainbow trout ESTs. However, approximately 3% of all the sequences still had no significant hits against protein, nucleotide or EST databases (Goetz, F.W. et al. 2004). From all of these thousand genes, some genes were selected for being potential candidate genes, because they had good gene qualities for our research, such as an importance role in immune function or high homology to known public database genes. In concordance to public database annotations, several sequence analysis were accurately done for all selected genes, such as sequence characterizations and multiple alignment sequence analysis. Furthermore, expression studies in several tissues from rainbow trout challenged with LPS IP (6mg/kg), provide an excellent model to determine *in vivo* expression levels of gene transcripts induced in fish inflammation. After studying and analysing candidate genes, several genes could have been selected, like PU.1, MHCII Ii or CD209e. Unfortunately, working with all of them at the same time was not viable for this thesis. In order to restrict candidate gene selection, several parameters were pointed out in order to choose the best candidate gene to perform further analysis. Table 4.3 shows a schematic resume of gene characteristics and parameters designed for the selection criterion. The parameters used were the following: EST full length cDNA availability, homology to known protein, species conserved protein, immune function relevance, tissue expression, literature reports and research group interests. Depending on the involvement to each parameter, genes were scored from low level to very high level. The roles in immune function as well as tissue expression levels, specifically in the head kidney, were the most valued factors. From the total score, PU.1 resulted as the candidate gene for continuing immune studies in fish. Many articles have demonstrated that PU.1 is required for the development of myeloid and lymphoid cells, playing a critical role in regulation of hematopoiesis (Yoshida, H. et al. 1990; Galson, D.L. et al. 1993; Witmer-Pack, M.D. et al. 1993; Nagulapalli, S. et al. 1995; Tondravi, M.M. et al. 1997; Bakri, Y. et al. 2004; Rosenbauer, F. et al. 2004a) and it is responsible for the regulation of several immune genes like CD209 in dendritic cells (Henkel, G.W. et al. 2002; Dominguez-Soto, A. et al. 2005). Expression levels of rainbow trout treated with LPS IP (6mg/kg) showed an ubiquitous PU.1 mRNA expression pattern from the tissues tested. Higher levels of expression were observed in the head kidney, but also in brain, where a role in microglia development has been well documented (Walton, M.R. et al. 2000; Hwang, C.K. et al. 2004). Other genes, like TNFRs are very interesting and important immune genes, although they lack of homology within TNFR family and have low tissue expression levels. Regarding fish

MHCII Ii in fish, few studies document its real function. Although it may function in a similar way than Ii in higher vertebrates (Dijkstra, J.M. et al. 2003), in zebrafish Ii homologue gene products may not be involved in MHC class II transport but possibly they perform other functions (Sultmann, H. et al. 1993). Therefore, before considering MHCII Ii as a candidate gene, further research is needed to determine the role of this gene in the fish immune system. Both CD209 and CD83 genes play a central role as immune receptors and they might be good candidate genes. In fact CD209 and CD83 have been reported as dendritic cells markers (Zhou, L. et al. 1995; Dominguez-Soto, A. et al. 2005), although CD83 expression in fish macrophages cell cultures may be promiscuous (Doñate, C, personal communication). These genes may represent that they could be a putative fish marker for DC lineages, more than candidate gene markers for the fish welfare.

In conclusion, analysing both laboratory results and literature reports, and after comparing all candidate genes, PU.1 was the most likely candidate gene for determining and better understanding relevant traits fish immune system. PU.1 is considered a master gene required for the hematopoiesis development, owing to its potential ability to regulate the expression of multiple genes specific for different lineages during normal hematopoiesis (Gangenahalli, G.U. et al. 2005). The response of the hematopoietic system and the involvement of PU.1 in the hematopoietic system will give valuable information about immune system development and responses to immune challenge in fish. In this context, the next thesis chapter will aim to study the role of PU.1 in the fish immune system.

CANDIDATE GENES								
Putative Function	**EST Full lenght cDNA**	**Homology to known protein**	**Species Conserved Protein**	**Immune function relevance**	**Tissue Expression**	**Literature reports**	**Group Research interests**	**Total score**
PU.1	yes	high	very high	very high	very high	very high	very high	very high
TNFRV	yes	high	low	very high	low	very high	high	medium
TNF-DR	no	medium	low	high	low	high	medium	low
4-1BB-like	no	low	low	high	low	medium	low	low
CD209e	yes	very high	medium	high	medium	high	high	high
CD83	no	very high	low	high	medium	high	high	high
CCL4-like	yes	medium	medium	high	medium	medium	high	medium
MHCII	no	very high	medium	very high	very high	very high	low	medium
IL1R-II	no	low	medium	high	low	medium	low	low
IL1R-like	no	low	medium	uncertain	low	medium	low	low
APS	no	high	high	medium	high	high	low	low
Enolase	no	very high	very high	medium	high	high	very high	high

Table 4.3. Table showing parameters and the criterion used for candidate gene selection. The score used was determine in very high, high, medium or low.

5. References

Aggarwal, B.B., Eessalu, T.E. and Hass, P.E. (1985). Characterization of receptors for human tumour necrosis factor and their regulation by gamma-interferon. *Nature*. 318(6047): 665-7.

Alderson, M.R., Smith, C.A., Tough, T.W., Davis-Smith, T., Armitage, R.J., Falk, B., Roux, E., Baker, E., Sutherland, G.R. and Din, W.S. (1994). Molecular and biological characterization of human 4-1BB and its ligand. *Eur J Immunol*. 24(9): 2219-27.

Alvarez, C.P., Lasala, F., Carrillo, J., Muniz, O., Corbi, A.L. and Delgado, R. (2002). C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. *J Virol*. 76(13): 6841-4.

Baker, S.J. and Reddy, E.P. (1998). Modulation of life and death by the TNF receptor superfamily. *Oncogene*. 17(25): 3261-70.

Bakri, Y., Sarrazin, S., Mayer, U.P., Tillmanns, S., Nerlov, C., Boned, A. and Sieweke, M.H. (2004). Balance of MafB and PU.1 specifies alternative macrophage or dendritic cell fate. *Blood*.

Basu, S., Schaefer, T.M., Ghosh, M., Fuller, C.L. and Reinhart, T.A. (2002). Molecular cloning and sequencing of 25 different rhesus macaque chemokine cDNAs reveals evolutionary conservation among C, CC, CXC, AND CX3C families of chemokines. *Cytokine*. 18(3): 140-8.

Bayne, C.J., Gerwick, L., Fujiki, K., Nakao, M. and Yano, T. (2001). Immune-relevant (including acute phase) genes identified in the livers of rainbow trout, Oncorhynchus mykiss, by means of suppression subtractive hybridization. *Dev Comp Immunol*. 25(3): 205-17.

Bennett, C.M., Kanki, J.P., Rhodes, J., Liu, T.X., Paw, B.H., Kieran, M.W., Langenau, D.M., Delahaye-Brown, A., Zon, L.I., Fleming, M.D. and Look, A.T. (2001). Myelopoiesis in the zebrafish, Danio rerio. *Blood*. 98(3): 643-51.

Berchtold, S., Jones, T., Muhl-Zurbes, P., Sheer, D., Schuler, G. and Steinkasserer, A. (1999). The human dendritic cell marker CD83 maps to chromosome 6p23. *Ann Hum Genet*. 63 (Pt 2): 181-3.

Berman, J.N., Kanki, J.P. and Look, A.T. (2005). Zebrafish as a model for myelopoiesis during embryogenesis. *Exp Hematol*. 33(9): 997-1006.

Beutler, B. and Cerami, A. (1989). The biology of cachectin/TNF--a primary mediator of the host response. *Annu Rev Immunol*. 7: 625-55.

Bingulac-Popovic, J., Figueroa, F., Sato, A., Talbot, W.S., Johnson, S.L., Gates, M., Postlethwait, J.H. and Klein, J. (1997). Mapping of mhc class I and class II regions to different linkage groups in the zebrafish, Danio rerio. *Immunogenetics*. 46(2): 129-34.

Bobe, J. and Goetz, F.W. (2000). A tumor necrosis factor decoy receptor homologue is up-regulated in the brook trout (Salvelinus fontinalis) ovary at the completion of ovulation. *Biol Reprod*. 62(2): 420-6.

Bobik, A. and Kalinina, N. (2001). Tumor necrosis factor receptor and ligand superfamily family members TNFRSF14 and LIGHT: new players in human atherogenesis. *Arterioscler Thromb Vasc Biol*. 21(12): 1873-5.

Bouwmeester, T., Bauch, A., Ruffner, H., Angrand, P.O., Bergamini, G., Croughton, K., Cruciat, C., Eberhard, D., Gagneur, J., Ghidelli, S., Hopf, C., Huhse, B., Mangano, R., Michon, A.M., Schirle, M., Schlegl, J., Schwab, M., Stein, M.A., Bauer, A., Casari, G., Drewes, G., Gavin, A.C., Jackson, D.B., Joberty, G., Neubauer, G., Rick, J., Kuster, B. and Superti-Furga, G. (2004). A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway. *Nat Cell Biol*. 6(2): 97-105.

Bridgham, J.T., Wilder, J.A., Hollocher, H. and Johnson, A.L. (2003). All in the family: evolutionary and functional relationships among death receptors. *Cell Death Differ*. 10(1): 19-25.

Castellino, F., Han, R. and Germain, R.N. (2001). The transmembrane segment of invariant chain mediates binding to MHC class II molecules in a CLIP-independent manner. *Eur J Immunol*. 31(3): 841-50.

Cella, M., Engering, A., Pinet, V., Pieters, J. and Lanzavecchia, A. (1997). Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature*. 388(6644): 782-7.

Chen, G. and Goeddel, D.V. (2002). TNF-R1 signaling: a beautiful pathway. *Science*. 296(5573): 1634-5.

Chomczynski, P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques*. 15(3): 532-4, 536-7.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 162(1): 156-9.

Cooper, L., Johnson, C., Burslem, F. and Martin, P. (2005). Wound healing and inflammation genes revealed by array analysis of 'macrophageless' PU.1 null mice. *Genome Biol*. 6(1): R5.

Dahl, R. and Simon, M.C. (2003). The importance of PU.1 concentration in hematopoietic lineage commitment and maturation. *Blood Cells Mol Dis*. 31(2): 229-33.

Dembic, Z., Loetscher, H., Gubler, U., Pan, Y.C., Lahm, H.W., Gentz, R., Brockhaus, M. and Lesslauer, W. (1990). Two human TNF receptors have similar extracellular, but distinct intracellular, domain sequences. *Cytokine*. 2(4): 231-7.

Dijkstra, J.M., Kiryu, I., Kollner, B., Yoshiura, Y. and Ototake, M. (2003). MHC class II invariant chain homologues in rainbow trout (Oncorhynchus mykiss). *Fish Shellfish Immunol*. 15(2): 91-105.

Dixon, B., Shum, B., Adams, E.J., Magor, K.E., Hedrick, R.P., Muir, D.G. and Parham, P. (1998). CK-1, a putative chemokine of rainbow trout (Oncorhynchus mykiss). *Immunol Rev*. 166: 341-8.

Dominguez-Soto, A., Puig-Kroger, A., Vega, M.A. and Corbi, A.L. (2005). PU.1 regulates the tissue-specific expression of dendritic cell-specific intercellular

adhesion molecule (ICAM)-3-grabbing nonintegrin. *J Biol Chem*. 280(39): 33123-31.

Fabriek, B.O., Van Haastert, E.S., Galea, I., Polfliet, M.M., Dopp, E.D., Van Den Heuvel, M.M., Van Den Berg, T.K., De Groot, C.J., Van Der Valk, P. and Dijkstra, C.D. (2005). CD163-positive perivascular macrophages in the human CNS express molecules for antigen recognition and presentation. *Glia*. 51(4): 297-305.

Fisher, R.C. and Scott, E.W. (1998). Role of PU.1 in hematopoiesis. *Stem Cells*. 16(1): 25-37.

Galloway, J.L., Wingert, R.A., Thisse, C., Thisse, B. and Zon, L.I. (2005). Loss of gata1 but not gata2 converts erythropoiesis to myelopoiesis in zebrafish embryos. *Dev Cell*. 8(1): 109-16.

Galson, D.L., Hensold, J.O., Bishop, T.R., Schalling, M., D'andrea, A.D., Jones, C., Auron, P.E. and Housman, D.E. (1993). Mouse beta-globin DNA-binding protein B1 is identical to a proto-oncogene, the transcription factor Spi-1/PU.1, and is restricted in expression to hematopoietic cells and the testis. *Mol Cell Biol*. 13(5): 2929-41.

Gangenahalli, G.U., Gupta, P., Saluja, D., Verma, Y.K., Kishore, V., Chandra, R., Sharma, R.K. and Ravindranath, T. (2005). Stem cell fate specification: role of master regulatory switch transcription factor PU.1 in differential hematopoiesis. *Stem Cells Dev*. 14(2): 140-52.

Garcia-Castillo, J., Chaves-Pozo, E., Olivares, P., Pelegrin, P., Meseguer, J. and Mulero, V. (2004). The tumor necrosis factor alpha of the bony fish seabream exhibits the in vivo proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner. *Cell Mol Life Sci*. 61(11): 1331-40.

Garcia-Castillo, J., Pelegrin, P., Mulero, V. and Meseguer, J. (2002). Molecular cloning and expression analysis of tumor necrosis factor alpha from a marine fish reveal its constitutive expression and ubiquitous nature. *Immunogenetics*. 54(3): 200-7.

Geijtenbeek, T.B., Kwon, D.S., Torensma, R., Van Vliet, S.J., Van Duijnhoven, G.C., Middel, J., Cornelissen, I.L., Nottet, H.S., Kewalramani, V.N., Littman, D.R., Figdor, C.G. and Van Kooyk, Y. (2000a). DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell*. 100(5): 587-97.

Geijtenbeek, T.B., Torensma, R., Van Vliet, S.J., Van Duijnhoven, G.C., Adema, G.J., Van Kooyk, Y. and Figdor, C.G. (2000b). Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell*. 100(5): 575-85.

Ghosh, S. and Karin, M. (2002). Missing pieces in the NF-kappaB puzzle. *Cell*. 109 Suppl: S81-96.

Goebl, M.K. (1990). The PU.1 transcription factor is the product of the putative oncogene Spi-1. *Cell*. 61(7): 1165-6.

Goetz, F.W., Iliev, D.B., Mccauley, L.A., Liarte, C.Q., Tort, L.B., Planas, J.V. and Mackenzie, S. (2004). Analysis of genes isolated from lipopolysaccharide-

stimulated rainbow trout (Oncorhynchus mykiss) macrophages. *Mol Immunol*. 41(12): 1199-210.

Goetz, F.W., Mackenzie, S. and J.V., P. (2003). Tumor Necrosis Factor. *In press*.

Goodwin, R.G., Din, W.S., Davis-Smith, T., Anderson, D.M., Gimpel, S.D., Sato, T.A., Maliszewski, C.R., Brannan, C.I., Copeland, N.G., Jenkins, N.A. and Et Al. (1993). Molecular cloning of a ligand for the inducible T cell gene 4-1BB: a member of an emerging family of cytokines with homology to tumor necrosis factor. *Eur J Immunol*. 23(10): 2631-41.

Gray, P.W., Aggarwal, B.B., Benton, C.V., Bringman, T.S., Henzel, W.J., Jarrett, J.A., Leung, D.W., Moffat, B., Ng, P., Svedersky, L.P. and Et Al. (1984). Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. *Nature*. 312(5996): 721-4.

Grell, M., Zimmermann, G., Hulser, D., Pfizenmaier, K. and Scheurich, P. (1994). TNF receptors TR60 and TR80 can mediate apoptosis via induction of distinct signal pathways. *J Immunol*. 153(5): 1963-72.

Gupta, S. (2002). A decision between life and death during TNF-alpha-induced signaling. *J Clin Immunol*. 22(4): 185-94.

Halary, F., Amara, A., Lortat-Jacob, H., Messerle, M., Delaunay, T., Houles, C., Fieschi, F., Arenzana-Seisdedos, F., Moreau, J.F. and Dechanet-Merville, J. (2002). Human cytomegalovirus binding to DC-SIGN is required for dendritic cell infection and target cell trans-infection. *Immunity*. 17(5): 653-64.

Hansen, J.D., Strassburger, P., Thorgaard, G.H., Young, W.P. and Du Pasquier, L. (1999). Expression, linkage, and polymorphism of MHC-related genes in rainbow trout, Oncorhynchus mykiss. *J Immunol*. 163(2): 774-86.

Heller, R.A., Song, K., Onasch, M.A., Fischer, W.H., Chang, D. and Ringold, G.M. (1990). Complementary DNA cloning of a receptor for tumor necrosis factor and demonstration of a shed form of the receptor. *Proc Natl Acad Sci U S A*. 87(16): 6151-5.

Henkel, G.W., Mckercher, S.R. and Maki, R.A. (2002). Identification of three genes up-regulated in PU.1 rescued monocytic precursor cells. *Int Immunol*. 14(7): 723-32.

Hiltbold, E.M. and Roche, P.A. (2002). Trafficking of MHC class II molecules in the late secretory pathway. *Curr Opin Immunol*. 14(1): 30-5.

Hollenhorst, P.C., Jones, D.A. and Graves, B.J. (2004). Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. *Nucleic Acids Res*. 32(18): 5693-702.

Huang, X. and Madan, A. (1999). CAP3: A DNA sequence assembly program. *Genome Res*. 9(9): 868-77.

Hwang, C.K., Kim, C.S., Choi, H.S., Mckercher, S.R. and Loh, H.H. (2004). Transcriptional regulation of mouse mu opioid receptor gene by PU.1. *J Biol Chem*. 279(19): 19764-74.

Iliev, D.B., Liarte, C.Q., Mackenzie, S. and Goetz, F.W. (2005a). Activation of rainbow trout (Oncorhynchus mykiss) mononuclear phagocytes by different

pathogen associated molecular pattern (PAMP) bearing agents. *Mol Immunol*. 42(10): 1215-23.

Iliev, D.B., Roach, J.C., Mackenzie, S., Planas, J.V. and Goetz, F.W. (2005b). Endotoxin recognition: in fish or not in fish? *FEBS Lett*. 579(29): 6519-28.

Iwasaki, H., Somoza, C., Shigematsu, H., Duprez, E.A., Iwasaki-Arai, J., Mizuno, S., Arinobu, Y., Geary, K., Zhang, P., Dayaram, T., Fenyus, M.L., Elf, S., Chan, S., Kastner, P., Huettner, C.S., Murray, R., Tenen, D.G. and Akashi, K. (2005). Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood*. 106(5): 1590-600.

Jensen, L.E., Hiney, M.P., Shields, D.C., Uhlar, C.M., Lindsay, A.J. and Whitehead, A.S. (1997). Acute phase proteins in salmonids: evolutionary analyses and acute phase response. *J Immunol*. 158(1): 384-92.

Jorgensen, J.B., Lunde, H., Jensen, L., Whitehead, A.S. and Robertsen, B. (2000). Serum amyloid A transcription in Atlantic salmon (Salmo salar L.) hepatocytes is enhanced by stimulation with macrophage factors, recombinant human IL-1 beta, IL-6 and TNF alpha or bacterial lipopolysaccharide. *Dev Comp Immunol*. 24(6-7): 553-63.

Karim, F.D., Urness, L.D., Thummel, C.S., Klemsz, M.J., Mckercher, S.R., Celada, A., Van Beveren, C., Maki, R.A., Gunther, C.V., Nye, J.A. and Et Al. (1990). The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. *Genes Dev*. 4(9): 1451-3.

Kherrouche, Z., Beuscart, A., Huguet, C., Flourens, A., Moreau-Gachelin, F., Stehelin, D. and Coll, J. (1998). Isolation and characterization of a chicken homologue of the Spi-1/PU.1 transcription factor. *Oncogene*. 16(10): 1357-67.

Klemsz, M.J., Mckercher, S.R., Celada, A., Van Beveren, C. and Maki, R.A. (1990). The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. *Cell*. 61(1): 113-24.

Kono, T. and Sakai, M. (2001). The analysis of expressed genes in the kidney of Japanese flounder, Paralichthys olivaceus, injected with the immunostimulant peptidoglycan. *Fish Shellfish Immunol*. 11(4): 357-66.

Kono, T., Zou, J., Bird, S., Savan, R., Sakai, M. and Secombes, C.J. (2005). Identification and expression analysis of lymphotoxin-beta like homologues in rainbow trout Oncorhynchus mykiss. *Mol Immunol*.

Koppel, E.A., Van Gisbergen, K.P., Geijtenbeek, T.B. and Van Kooyk, Y. (2005). Distinct functions of DC-SIGN and its homologues L-SIGN (DC-SIGNR) and mSIGNR1 in pathogen recognition and immune regulation. *Cell Microbiol*. 7(2): 157-65.

Kozlow, E.J., Wilson, G.L., Fox, C.H. and Kehrl, J.H. (1993). Subtractive cDNA cloning of a novel member of the Ig gene superfamily expressed at high levels in activated B lymphocytes. *Blood*. 81(2): 454-61.

Kull, F.C., Jr. (1988). Reduction in tumor necrosis factor receptor affinity and cytotoxicity by glucocorticoids. *Biochem Biophys Res Commun*. 153(1): 402-9.

Laing, K.J., Hardie, L.J., Aartsen, W., Grabowski, P.S. and Secombes, C.J. (1999). Expression of an inducible nitric oxide synthase gene in rainbow trout Oncorhynchus mykiss. *Dev Comp Immunol*. 23(1): 71-85.

Laing, K.J., Wang, T., Zou, J., Holland, J., Hong, S., Bols, N., Hirono, I., Aoki, T. and Secombes, C.J. (2001). Cloning and expression analysis of rainbow trout Oncorhynchus mykiss tumour necrosis factor-alpha. *Eur J Biochem*. 268(5): 1315-22.

Lieschke, G.J., Oates, A.C., Paw, B.H., Thompson, M.A., Hall, N.E., Ward, A.C., Ho, R.K., Zon, L.I. and Layton, J.E. (2002). Zebrafish SPI-1 (PU.1) marks a site of myeloid development independent of primitive erythropoiesis: implications for axial patterning. *Dev Biol*. 246(2): 274-95.

Lin, W.J. and Yeh, W.C. (2005). Implication of Toll-like receptor and tumor necrosis factor alpha signaling in septic shock. *Shock*. 24(3): 206-9.

Liu, L., Fujiki, K., Dixon, B. and Sundick, R.S. (2002). Cloning of a novel rainbow trout (Oncorhynchus mykiss) CC chemokine with a fractalkine-like stalk and a TNF decoy receptor using cDNA fragments containing AU-rich elements. *Cytokine*. 17(2): 71-81.

Locksley, R.M., Killeen, N. and Lenardo, M.J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell*. 104(4): 487-501.

Loetscher, H., Schlaeger, E.J., Lahm, H.W., Pan, Y.C., Lesslauer, W. and Brockhaus, M. (1990). Purification and partial amino acid sequence analysis of two distinct tumor necrosis factor receptors from HL60 cells. *J Biol Chem*. 265(33): 20131-8.

Lozach, P.Y., Amara, A., Bartosch, B., Virelizier, J.L., Arenzana-Seisdedos, F., Cosset, F.L. and Altmeyer, R. (2004). C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles. *J Biol Chem*. 279(31): 32035-45.

Macewan, D.J. (2002). TNF receptor subtype signalling: differences and cellular consequences. *Cell Signal*. 14(6): 477-92.

Mackenzie, S., Liarte, C., Iliev, D., Planas, J.V., Tort, L. and Goetz, F.W. (2004). Characterization of a highly inducible novel CC chemokine from differentiated rainbow trout (Oncorhynchus mykiss) macrophages. *Immunogenetics*. 56(8): 611-5.

Mackenzie, S., Planas, J.V. and Goetz, F.W. (2003). LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and in vitro differentiated macrophages. *Dev Comp Immunol*. 27(5): 393-400.

Mcgreal, E.P., Martinez-Pomares, L. and Gordon, S. (2004). Divergent roles for C-type lectins expressed by cells of the innate immune system. *Mol Immunol*. 41(11): 1109-21.

Mellman, I. and Steinman, R.M. (2001). Dendritic cells: specialized and regulated antigen processing machines. *Cell*. 106(3): 255-8.

Moreau-Gachelin, F., Ray, D., Mattei, M.G., Tambourin, P. and Tavitian, A. (1989). The putative oncogene Spi-1: murine chromosomal localization and transcriptional activation in murine acute erythroleukemias. *Oncogene*. 4(12): 1449-56.

Moreau-Gachelin, F., Tavitian, A. and Tambourin, P. (1988). Spi-1 is a putative oncogene in virally induced murine erythroleukaemias. *Nature*. 331(6153): 277-80.

Mueller, D.L., Jenkins, M.K. and Schwartz, R.H. (1989). Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol*. 7: 445-80.

Mukhtar, M., Harley, S., Chen, P., Bouhamdan, M., Patel, C., Acheampong, E. and Pomerantz, R.J. (2002). Primary isolated human brain microvascular endothelial cells express diverse HIV/SIV-associated chemokine coreceptors and DC-SIGN and L-SIGN. *Virology*. 297(1): 78-88.

Nagulapalli, S., Pongubala, J.M. and Atchison, M.L. (1995). Multiple proteins physically interact with PU.1. Transcriptional synergy with NF-IL6 beta (C/EBP delta, CRP3). *J Immunol*. 155(9): 4330-8.

Noakes, M.A., Reimer, T. and Phillips, R.B. (2003). Genotypic characterization of an MHC class II locus in lake trout (Salvelinus namaycush) from Lake Superior by single-stranded conformational polymorphism analysis and reference strand-mediated conformational analysis. *Mar Biotechnol (NY)*. 5(3): 270-8.

O'hara, R., Murphy, E.P., Whitehead, A.S., Fitzgerald, O. and Bresnihan, B. (2000). Acute-phase serum amyloid A production by rheumatoid arthritis synovial tissue. *Arthritis Res*. 2(2): 142-4.

Ohta, Y., Landis, E., Boulay, T., Phillips, R.B., Collet, B., Secombes, C.J., Flajnik, M.F. and Hansen, J.D. (2004). Homologs of CD83 from elasmobranch and teleost fish. *J Immunol*. 173(7): 4553-60.

Ohta, Y., Okamura, K., Mckinney, E.C., Bartl, S., Hashimoto, K. and Flajnik, M.F. (2000). Primitive synteny of vertebrate major histocompatibility complex class I and class II genes. *Proc Natl Acad Sci U S A*. 97(9): 4712-7.

Park, C.G., Takahara, K., Umemoto, E., Yashima, Y., Matsubara, K., Matsuda, Y., Clausen, B.E., Inaba, K. and Steinman, R.M. (2001). Five mouse homologues of the human dendritic cell C-type lectin, DC-SIGN. *Int Immunol*. 13(10): 1283-90.

Park, C.I., Kurobe, T., Hirono, I. and Aoki, T. (2003). Cloning and characterization of cDNAs for two distinct tumor necrosis factor receptor superfamily genes from Japanese flounder Paralichthys olivaceus. *Dev Comp Immunol*. 27(5): 365-75.

Pennica, D., Nedwin, G.E., Hayflick, J.S., Seeburg, P.H., Derynck, R., Palladino, M.A., Kohr, W.J., Aggarwal, B.B. and Goeddel, D.V. (1984). Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature*. 312(5996): 724-9.

Petersen, H.H., Nielsen, J.P. and Heegaard, P.M. (2004). Application of acute phase protein measurements in veterinary clinical chemistry. *Vet Res*. 35(2): 163-87.

Phillips, R.B., Zimmerman, A., Noakes, M.A., Palti, Y., Morasch, M.R., Eiben, L., Ristow, S.S., Thorgaard, G.H. and Hansen, J.D. (2003). Physical and genetic mapping of the rainbow trout major histocompatibility regions: evidence for duplication of the class I region. *Immunogenetics*. 55(8): 561-9.

Pierre, P., Turley, S.J., Gatti, E., Hull, M., Meltzer, J., Mirza, A., Inaba, K., Steinman, R.M. and Mellman, I. (1997). Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature*. 388(6644): 787-92.

Pieters, J. (1997). MHC class II restricted antigen presentation. *Curr Opin Immunol*. 9(1): 89-96.

Reali, C., Curto, M., Sogos, V., Scintu, F., Pauly, S., Schwarz, H. and Gremo, F. (2003). Expression of CD137 and its ligand in human neurons, astrocytes, and microglia: modulation by FGF-2. *J Neurosci Res*. 74(1): 67-73.

Relloso, M., Puig-Kroger, A., Pello, O.M., Rodriguez-Fernandez, J.L., De La Rosa, G., Longo, N., Navarro, J., Munoz-Fernandez, M.A., Sanchez-Mateos, P. and Corbi, A.L. (2002). DC-SIGN (CD209) expression is IL-4 dependent and is negatively regulated by IFN, TGF-beta, and anti-inflammatory agents. *J Immunol*. 168(6): 2634-43.

Rhodes, J., Hagen, A., Hsu, K., Deng, M., Liu, T.X., Look, A.T. and Kanki, J.P. (2005). Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *Dev Cell*. 8(1): 97-108.

Roche, P.A., Marks, M.S. and Cresswell, P. (1991). Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature*. 354(6352): 392-4.

Rosenbauer, F., Wagner, K., Kutok, J.L., Iwasaki, H., Le Beau, M.M., Okuno, Y., Akashi, K., Fiering, S. and Tenen, D.G. (2004a). Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. *Nat Genet*. 36(6): 624-30.

Rosenbauer, F., Wagner, K., Zhang, P., Knobeloch, K.P., Iwama, A. and Tenen, D.G. (2004b). pDP4, a novel glycoprotein secreted by mature granulocytes, is regulated by transcription factor PU.1. *Blood*. 103(11): 4294-301.

Rothe, J.G., Brockhaus, M., Gentz, R. and Lesslauer, W. (1991). Molecular cloning and expression of the mouse Tnf receptor type b. *Immunogenetics*. 34(5): 338-40.

Saeij, J.P., Stet, R.J., De Vries, B.J., Van Muiswinkel, W.B. and Wiegertjes, G.F. (2003a). Molecular and functional characterization of carp TNF: a link between TNF polymorphism and trypanotolerance? *Dev Comp Immunol*. 27(1): 29-41.

Saeij, J.P., Verburg-Van Kemenade, L.B., Van Muiswinkel, W.B. and Wiegertjes, G.F. (2003b). Daily handling stress reduces resistance of carp to Trypanoplasma borreli: in vitro modulatory effects of cortisol on leukocyte function and apoptosis. *Dev Comp Immunol*. 27(3): 233-45.

Savan, R. and Sakai, M. (2002). Analysis of expressed sequence tags (EST) obtained from common carp, Cyprinus carpio L., head kidney cells after stimulation by two mitogens, lipopolysaccharide and concanavalin-A. *Comp Biochem Physiol B Biochem Mol Biol*. 131(1): 71-82.

Soanes, K.H., Figuereido, K., Richards, R.C., Mattatall, N.R. and Ewart, K.V. (2004). Sequence and expression of C-type lectin receptors in Atlantic salmon (Salmo salar). *Immunogenetics*. 56(8): 572-84.

Soilleux, E.J. (2003). DC-SIGN (dendritic cell-specific ICAM-grabbing non-integrin) and DC-SIGN-related (DC-SIGNR): friend or foe? *Clin Sci (Lond)*. 104(4): 437-46.

Stein, J.V. and Nombela-Arrieta, C. (2005). Chemokine control of lymphocyte trafficking: a general overview. *Immunology*. 116(1): 1-12.

Stet, R.J., Kruiswijk, C.P. and Dixon, B. (2003). Major histocompatibility lineages and immune gene function in teleost fishes: the road not taken. *Crit Rev Immunol*. 23(5-6): 441-71.

Strausberg, R.L., Feingold, E.A., Grouse, L.H., Derge, J.G., Klausner, R.D., Collins, F.S., Wagner, L., Shenmen, C.M., Schuler, G.D., Altschul, S.F., Zeeberg, B., Buetow, K.H., Schaefer, C.F., Bhat, N.K., Hopkins, R.F., Jordan, H., Moore, T., Max, S.I., Wang, J., Hsieh, F., Diatchenko, L., Marusina, K., Farmer, A.A., Rubin, G.M., Hong, L., Stapleton, M., Soares, M.B., Bonaldo, M.F., Casavant, T.L., Scheetz, T.E., Brownstein, M.J., Usdin, T.B., Toshiyuki, S., Carninci, P., Prange, C., Raha, S.S., Loquellano, N.A., Peters, G.J., Abramson, R.D., Mullahy, S.J., Bosak, S.A., Mcewan, P.J., Mckernan, K.J., Malek, J.A., Gunaratne, P.H., Richards, S., Worley, K.C., Hale, S., Garcia, A.M., Gay, L.J., Hulyk, S.W., Villalon, D.K., Muzny, D.M., Sodergren, E.J., Lu, X., Gibbs, R.A., Fahey, J., Helton, E., Ketteman, M., Madan, A., Rodrigues, S., Sanchez, A., Whiting, M., Young, A.C., Shevchenko, Y., Bouffard, G.G., Blakesley, R.W., Touchman, J.W., Green, E.D., Dickson, M.C., Rodriguez, A.C., Grimwood, J., Schmutz, J., Myers, R.M., Butterfield, Y.S., Krzywinski, M.I., Skalska, U., Smailus, D.E., Schnerch, A., Schein, J.E., Jones, S.J. and Marra, M.A. (2002). Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc Natl Acad Sci U S A*. 99(26): 16899-903.

Sultmann, H., Mayer, W.E., Figueroa, F., O'huigin, C. and Klein, J. (1993). Zebrafish Mhc class II alpha chain-encoding genes: polymorphism, expression, and function. *Immunogenetics*. 38(6): 408-20.

Tartaglia, L.A., Ayres, T.M., Wong, G.H. and Goeddel, D.V. (1993). A novel domain within the 55 kd TNF receptor signals cell death. *Cell*. 74(5): 845-53.

Tassaneetrithep, B., Burgess, T.H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., Eller, M.A., Pattanapanyasat, K., Sarasombath, S., Birx, D.L., Steinman, R.M., Schlesinger, S. and Marovich, M.A. (2003). DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med*. 197(7): 823-9.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 22(22): 4673-80.

Thorburn, A. (2004). Death receptor-induced cell killing. *Cell Signal*. 16(2): 139-44.

Tondravi, M.M., Mckercher, S.R., Anderson, K., Erdmann, J.M., Quiroz, M., Maki, R. and Teitelbaum, S.L. (1997). Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. *Nature*. 386(6620): 81-4.

Twist, C.J., Beier, D.R., Disteche, C.M., Edelhoff, S. and Tedder, T.F. (1998). The mouse Cd83 gene: structure, domain organization, and chromosome localization. *Immunogenetics*. 48(6): 383-93.

Upragarin, N., Landman, W.J., Gaastra, W. and Gruys, E. (2005). Extrahepatic production of acute phase serum amyloid A. *Histol Histopathol*. 20(4): 1295-307.

Vandenabeele, P., Declercq, W., Beyaert, R. and Fiers, W. (1995). Two tumour necrosis factor receptors: structure and function. *Trends Cell Biol*. 5(10): 392-9.

Vassalli, P. (1992). The pathophysiology of tumor necrosis factors. *Annu Rev Immunol*. 10: 411-52.

Wajant, H. and Scheurich, P. (2001). Tumor necrosis factor receptor-associated factor (TRAF) 2 and its role in TNF signaling. *Int J Biochem Cell Biol*. 33(1): 19-32.

Walton, M.R., Gibbons, H., Macgibbon, G.A., Sirimanne, E., Saura, J., Gluckman, P.D. and Dragunow, M. (2000). PU.1 expression in microglia. *J Neuroimmunol*. 104(2): 109-15.

Ward, A.C., Mcphee, D.O., Condron, M.M., Varma, S., Cody, S.H., Onnebo, S.M., Paw, B.H., Zon, L.I. and Lieschke, G.J. (2003). The zebrafish spi1 promoter drives myeloid-specific expression in stable transgenic fish. *Blood*. 102(9): 3238-40.

Witmer-Pack, M.D., Hughes, D.A., Schuler, G., Lawson, L., Mcwilliam, A., Inaba, K., Steinman, R.M. and Gordon, S. (1993). Identification of macrophages and dendritic cells in the osteopetrotic (op/op) mouse. *J Cell Sci*. 104 (Pt 4): 1021-9.

Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T. and Shultz, L.D. (1990). The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature*. 345(6274): 442-4.

Zhou, L., Szigeti, V. and Miller, R.H. (1995). Characterization of a novel oligodendrocyte cell surface molecule. *J Neurosci Res*. 42(4): 504-15.

Zhou, L.J., Schwarting, R., Smith, H.M. and Tedder, T.F. (1992). A novel cell-surface molecule expressed by human interdigitating reticulum cells, Langerhans cells, and activated lymphocytes is a new member of the Ig superfamily. *J Immunol*. 149(2): 735-42.

Zhou, L.J. and Tedder, T.F. (1995a). A distinct pattern of cytokine gene expression by human CD83+ blood dendritic cells. *Blood*. 86(9): 3295-301.

Zhou, L.J. and Tedder, T.F. (1995b). Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J Immunol*. 154(8): 3821-35.

Zou, J., Grabowski, P.S., Cunningham, C. and Secombes, C.J. (1999a). Molecular cloning of interleukin 1beta from rainbow trout Oncorhynchus mykiss reveals no evidence of an ice cut site. *Cytokine*. 11(8): 552-60.

Zou, J., Neumann, N.F., Holland, J.W., Belosevic, M., Cunningham, C., Secombes, C.J. and Rowley, A.F. (1999b). Fish macrophages express a cyclo-oxygenase-2 homologue after activation. *Biochem J*. 340 (Pt 1): 153-9.

Zou, J., Peddie, S., Scapigliati, G., Zhang, Y., Bols, N.C., Ellis, A.E. and Secombes, C.J. (2003a). Functional characterisation of the recombinant tumor necrosis

factors in rainbow trout, Oncorhynchus mykiss. *Dev Comp Immunol*. 27(9): 813-22.

Zou, J., Secombes, C.J., Long, S., Miller, N., Clem, L.W. and Chinchar, V.G. (2003b). Molecular identification and expression analysis of tumor necrosis factor in channel catfish (Ictalurus punctatus). *Dev Comp Immunol*. 27(10): 845-58.

Zou, J., Wang, T., Hirono, I., Aoki, T., Inagawa, H., Honda, T., Soma, G.I., Ototake, M., Nakanishi, T., Ellis, A.E. and Secombes, C.J. (2002). Differential expression of two tumor necrosis factor genes in rainbow trout, Oncorhynchus mykiss. *Dev Comp Immunol*. 26(2): 161-72.

Chapter 5

PU.1, the immune candidate gene

1. Introduction

1.1. PU.1, an Ets family member

PU.1/Spi1 is an E26-transformation–specific (Ets) family transcription factor that is a key regulator of hematopoietic lineage, required for the development of myeloid and lymphoid cells (Fisher, R.C. et al. 1998b; Rosenbauer, F. et al. 2004b), as well as in the microglia of brain (Hwang, C.K. et al. 2004). The PU.1 locus is a high-frequency integration site for spleen focus-forming virus (SFFV) in Friend murine acute erythroleukemias and was appropriately named SFFV provirus integration site-1 (Spi-1) (Moreau-Gachelin, F. et al. 1988; Moreau-Gachelin, F. et al. 1989; Goebl, M.K. 1990). Later, it was originally further identified in mouse as a DNA-binding protein that binds to a purine-rich sequence (PU box, 5'GAGGAA-3') in the mouse major histocompatibility complex class II gene IAβ present in macrophages and B-cells (Karim, F.D. et al. 1990; Klemsz, M.J. et al. 1990; Galson, D.L. et al. 1993). The PU.1 locus was mapped on human chromosome 11 (Galson, D.L. et al. 1993). The PU.1 protein is composed of three different domains (Klemsz, M.J. et al. 1990; Pongubala, J.M. et al. 1992). The acidic amino-terminal part of the protein corresponds to the activation domain which contains three acidic amino acids region and a glutamic rich region (Gangenahalli, G.U. et al. 2005). The central part corresponds to a proline–glutamic acid–serine–threonine (PEST) domain involved in protein-protein interactions (Rechsteiner, M. et al. 1996). The basic carboxy-terminal sequence contains the DNA-binding or Ets-domain that binds to canonical GAGGAA sequences found in the promoters of many hematopoietic genes (Amemiya, C.T. et al. 1999). Structurally the Ets domain has a helix-loop-helix architecture (Kodandapani, R. et al. 1996; Aittomaki, S. et al. 2004). The Ets family of DNA-binding proteins have become the subject of extensive investigation as key regulators of immune response genes and cell division (Crepieux, P. et al. 1994). In humans, 27 paralogous Ets gene members have been described (Hollenhorst, P.C. et al. 2004), 26 in the mouse genome (Galang, C.K. et al. 2004), 8 in *Drosophila* (Hsu, T. et al. 2000) and 10 in *Caenorhabditis elegans* (Hart, A.H. et al. 2000). The functional diversity of Ets proteins suggest that target selection is critical for biological regulation as well as their activity as DNA binding transcription factors (Hollenhorst, P.C. et al. 2004). Ets family members control important biological processes, including cellular proliferation, differentiation, lymphocyte development and activation, transformation and apoptosis by recognizing the GGA core motif in the promoter (Li, R. et al. 2000).

1.2. PU.1 and its role in hematopoiesis

The PU.1 transcription factor is a key regulator of hematopoietic development, but its role at each hematopoietic stage remains unclear (Gangenahalli, G.U. et al. 2005; Iwasaki, H. et al. 2005). PU.1 expression is initiated as early as the extraembrionic hematopoietic stage at the aorta-gonad-mesonephros region (Delassus, S. et al. 1999). As shown in figure 5.1, PU.1 is required for development along the lymphoid and myeloid lineages but needs to be down regulated during erythropoiesis (Fisher, R.C. et al. 1998b). Different cellular concentrations of PU.1 may direct distinct cell fates, with the highest concentrations of PU.1 required for macrophage development and lower concentrations for granulocytic and B-cell fate adoption (Celada, A. et al. 1996; Dahl, R. et al. 2003). However, high levels of PU.1 induce dendritic cell differentiation at the expense of macrophage fate, which involves repression of the macrophage inducer MafB (Bakri, Y. et al. 2004). PU.1 has lineage-specific transcription factors antagonists, for example GATA-1 (Eichmann, A. et al. 1997) which is essential for the development of erythrocytes, megakaryocytes, mast cells and eosinophils. Another transcription factor antagonist to PU.1, is C/EBPα which is essential for granulocyte development (Koski, G.K. et al. 1999). Some studies have used PU.1 knockout mice as models for understanding haematopoietic development and its involvement in immune function and disease (Mckercher, S.R. et al. 1996; Moreau-Gachelin, F. et al. 1996; Anderson, M.K. et al. 1999; Martin, P. et al. 2003) as well as with zebrafish mutants (Ward, A.C. et al. 2003; Berman, J.N. et al. 2005). PU.1 plays a critical role in mammalian myelopoiesis, evidenced by the quantitative and functional deficiencies in macrophages, granulocytes, and lymphocytes manifested in PU.1-deficient mice immediately after birth (Scott, E.W. et al. 1994; Mckercher, S.R. et al. 1996). Several studies reveal the importance of the PU.1 gene and describe a large number of gene regulations which are involved. The responsibility of this transcriptional factor is so wide that a dysfunctional expression of PU.1 is fatal. For example, PU.1 knockout mice models showed that at 2–3 months, mice had moderately enlarged spleens and the expansion of the progenitor cells and neutrophils was limited to bone marrow and spleen. After 3–8 months age, mice rapidly became moribund or died presenting an accumulation of immature myeloid cells in bone marrow and spleen (Rosenbauer, F. et al. 2004a). Another example of the PU.1 gene as a key gene in immune function directly implicated in disease, is osteopetrosis, a disease where osteoclasts are missing. The osteopetrotic mutation in mice is a recessive lesion in the gene for macrophage colony stimulating factor (M-CSF) (Yoshida, H. et al. 1990). Knock-out PU.1 mice exhibited an absence of both osteoclasts and macrophages suggesting that the transcription factor PU.1

regulates the initial stages of myeloid differentiation (Tondravi, M.M. et al. 1997). A biochemical study of transgenic PU.1 proerythroblasts, were used for investigating the independence of erythroid progenitors to growth factors leading to erythroleukemia (Barnache, S. et al. 2001) where PU.1 was overexpressed in proerythroblasts arrested differentiation (Moreau-Gachelin, F. et al. 1996). Summarizing, PU.1 is a master gene responsible for the hematopoietic linage development, playing a critical role determining immune cell fates, and the PU.1 gene disruption causes a cell-intrinsic defect in hematopoietic progenitor cells.

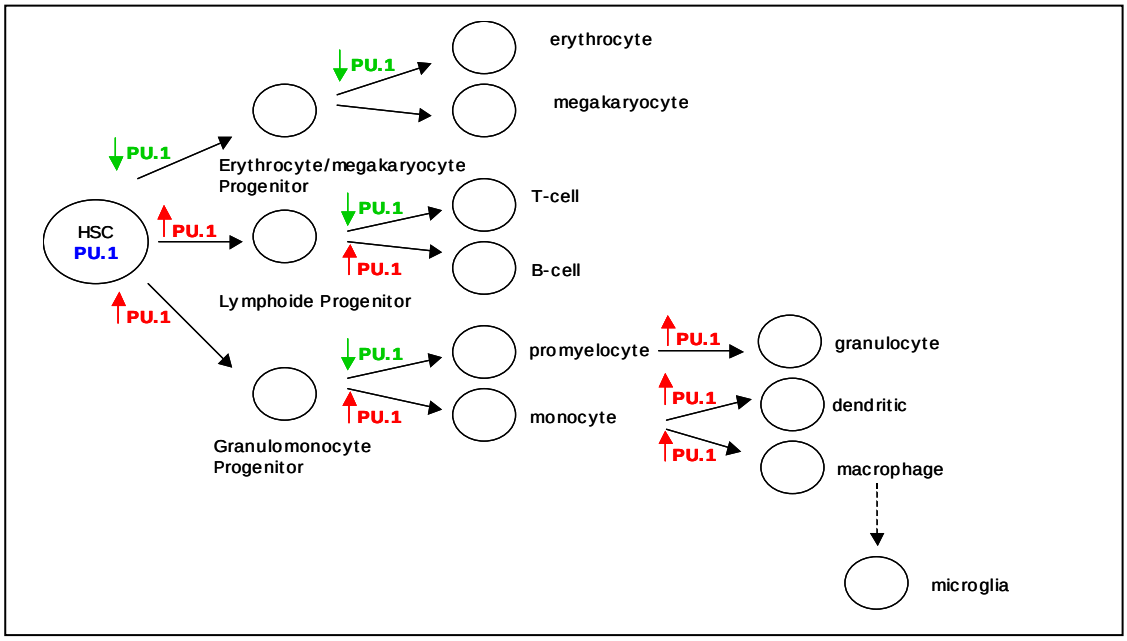


Fig.5.1. Schematic representation of PU.1 expression levels in hematopoiesis development. Picture was based on published figures (Fisher, R.C. et al. 1998b; Dahl, R. et al. 2003; Gangenahalli, G.U. et al. 2005) with some modifications. HSC: hematopoietic stem cell

1.3. Genes regulated by PU.1

The expression pattern of PU.1 has been the primary impetus for *in vitro* based experimental approaches aimed at identifying putative target genes and subsequent functional dissection to locate PU.1 regulatory elements (Fisher, R.C. et al. 1998a). PU.1 is a pleiotropic transcriptional factor that plays a critical role in the regulation of large number of genes and multiple nuclear proteins are capable of physically interacting with PU.1 (Nagulapalli, S. et al. 1995). In the myeloid lineage, a large collection of PU.1-dependent promoters and enhancers have been identified that control an array of genes. Most of them play an important role in growth, development, and function of the hematopoietic system. One mechanism by which transcription factors can control hematopoietic lineage development is through the regulation of the expression of specific promoters of myeloid genes (Henkel, G.W.

et al. 2002); growth factor receptor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF) receptors (Reddy, M.A. et al. 1994; Hohaus, S. et al. 1995; Smith, L.T. et al. 1996). The promoter for CD11b is also regulated by PU.1 and has been considered as a good indicator of PU.1 expression (Henkel, G.W. et al. 2002). It has been described that IL-1β promoter possesses two important transcription factor binding motifs, one for PU.1, in which a mutation in the binding site promoter completely inhibited the autocrine activity and, NF-IL6 transcription factor in which the same mutation caused partial loss of activity (Toda, Y. et al. 2002). The expression of the high affinity receptor for IgG (FcγRI) which mediates phagocytosis, respiratory burst and antibody-mediated cytoxic reactions, is regulated by PU.1 (Perez, C. et al. 1993; Eichbaum, Q.G. et al. 1994) and Stat1 transcription factors (Aittomaki, S. et al. 2004). PU.1 is required for both the basal activity and for the IFN-γ-induced FcγRI promoter activation, while Stat1 alone can not initiate DNA transcription (Aittomaki, S. et al. 2004). For pDP4, an extracellular glycoprotein of the olfactomedin-like family, its expression is strictly dependent on PU.1 binding to its functional promoter site and in mice it is found predominately expressed in mature neutrophils (Rosenbauer, F. et al. 2004b). Three potencial PU.1-regulated genes: MRP-14, Dap12 and CD53 from mice monocyte cultures were identified by a suppressive subtractive hybridisation library (Henkel, G.W. et al. 2002). Finally, other examples of the large number of gene which PU.1 regulates are: the TNFR-associated factor 6, also requiring PU.1 to activate its promoter (Toda, Y. et al. 2002) and the integral membrane protein NADPH oxidase subunits (gp91 and p47), also controlled by PU.1.

1.4. PU.1 and microglial cells

Interestingly, PU.1 has been described as a constitutive transcription factor in microglia and as a marker of this cell type in the brain (Walton, M.R. et al. 2000). Microglial cells contribute to 10% of the total cells in the mammalian central nervous system (CNS) (Vaughan, D.W. et al. 1974) and are located in close vicinity to neurons in the grey matter and between fibre tracts in the white matter (Lawson, L.J. et al. 1990). The origin of microglia has been a long-standing controversial issue (Ling, E.A. et al. 1993). Most authors, however, sustain that microglial cells come from mesodermal cells, probably of hematopoietic lineage (Cuadros, M.A. et al. 1998; Cuadros, M. et al. 2000). Despite the uncertainly of their origin, microglia share most surface molecules with bone-marrow derived macrophages (Flaris, N.A. et al. 1993). Microglia cells are present during early embryonic stages of neural development and are related to blood monocytes

(Streit, W.J. 2001). There are three basic types of microglia cells in brain: resident, ameboid and perivascular microglia. Microglia and macrophages present differences as well as similarities. For example, both microglia and macrophages, express the complement type-3 receptor and lectin binding site, but only microglia express keratan sulfate proteoglucan (Bertolotto, A. et al. 1993). In fact, markers that recognize microglia surface molecules can recognize monocytes and other tissue macrophages (Streit, W.J. 2001). Similar to macrophages (Nathan, C.F. 1987), activated microglia synthesize a variety of potentially harmful soluble factors. These include reactive oxygen and nitrogen intermediates, proteolytic enzymes, arachidonic acid metabolites and proinflammatory molecules, such as the cytotoxic cytokines: TNFα and IL1 (Castellano, B. et al. 1991; Banati, R.B. et al. 1993; Kreutzberg, G.W. 1996). The activities and functions of the microglia in brain arelargely unexplored both during development and throughout adulthood. When the CNS is activated, microglial cells may show morphological changes from a process-bearing (ramified) morphology to a rounded (ameboid) macrophages-like morphology (Streit, W.J. et al. 1999) and become capable of phagocytosis and antigen presentation (Badie, B. et al. 2001). Microglial activation is a key factor in the defence of neural parenchyma against infectious diseases, inflammation, trauma, ischemia, brain tumours and neurodegeneration as microglial cells form a network of immune resident macrophages with a capacity for immune surveillance and control (Kreutzberg, G.W. 1996). Numerous studies aiming to detect microglial cell have been carried out in mammals, using different methods (Hume, D.A. et al. 1983; Schnitzer, J. 1989). However, the descriptions of microglial cells in the central nervous system of non-mammalian vertebrates are scarce (Battisti, W.P. et al. 1995; Velasco, A. et al. 1995; Velasco, A. et al. 1999; Zupanc, G.K. et al. 2003a; Zupanc, G.K. et al. 2003b).

A part from the constitutive PU.1 expression in microglial cells (Walton, M.R. et al. 2000), the PU.1 N-terminal domain interacts and binds to the goosecoid protein, involved in the induction and pattern of the nervous system, repressing neurite outgrowth (Sawada, K. et al. 2000). This PU.1 domain can also bind to retinoblastoma protein promoter (a tumor suppressor gene product involved in erythroid differentiation) and goosecoid protein promoter (Konishi, Y. et al. 1999). PU.1 may be an important regulator of the mouse μ opioid receptor gene, particularly in brain and immune cells, repressing its promoter through the PU.1 binding site (Hwang, C.K. et al. 2004). In conclusion, the role of PU.1 in brain tissue has been related to immune system, where its expression is found in microglial cells and in non-mammalian vertebrates no studies are available.

Objectives

1. Use the transcription factor PU.1 for studying the immune response and its role in hematopoiesis development in fish.

2. Determine and study PU.1 mRNA expression levels in the fish head kidney, in control and LPS IP treated rainbow trout.

3. Determine PU.1 mRNA expression levels in rainbow trout brain, localize PU.1 expression in microglial cells and study differential expression of PU.1 in brain tissue.

4. Provide novel insights into immune cell proliferation and early response in the head kidney cell/tissue to LPS.

2. Materials and Methods

2.1. Animals

2.1.a. First Experiment: PU.1 in head kidney and brain after LPS IP injection

A total of 12 rainbow trout (450 g mean weight) obtained from the Blue Stream Trout Hatchery (Martson Mills, USA) held in 300 gallon tanks with recirculating freshwater maintained at 15ºC, were used for this experiment. The first day of experiment (t=0h), 6 fish were anaesthetised with tricaine methanesulfonate MS-222 (500µl/L) and injected intraperitoneally with 6mg/kg of lipopolysaccharide *Escherichia coli* (LPS Sigma, L8274, 026:B6). The same protocol was followed with six control fish to which phosphate saline buffer (PBS) was injected. After 24 hours post LPS injection, animals were anaesthetised with a lethal dose and rapidly bled and dissected. Brain and head-kidney tissues were removed carefully from all time points, and completely covered by tissue teck (Shandon Cryomatirx, Thermo) and frozen with dry ice. Once frozen, tissues were kept at –80ºC until 20 µm sections were cut in a cryostat for *in situ hybridization* analysis. Frozen sections were mounted on the pre-treated poly-L-lysine slides (Sigma) with a maximum anatomic tissue representation. Slides were run with sense and antisense probes to evaluate the reliability of the technique.

2.1.b. Second experiment: PU.1 in head kidney after an LPS/BrdU IP injection

Adult Rainbow trout (*Oncorhynchus mykiss*) of 150 g as a mean weight were obtained from J.Antrés fish farm (St. Privat, Girona). Fish were transported to aquarium facilities at the Universitat Autònoma de Barcelona where they were held in stock tanks at 19ºC with at least two weeks of acclimation before experiments. Water quality was analysed periodically. Tanks were kept partially covered under a photoperiod of 12h light/12h dark. Trout were fed a maintenance ration of about 0.3–0.5% body weight per day. A total of 28 fish rainbow trout were equally

divided between two tanks. The first day of experiment (t=0h), fish were injected with 6 mg/kg of LPS (Sigma, L8274, 026:B6), and control fish with PBS. In each sampling day, two hours before sampling, all treated fish were injected with 5-bromouracil deoxyriboside (BrdU) (Sigma, B-5002) at a concentration of 50mg/kg. In order to be identified, caudal fins were cut before putting the animals back to the tanks. All solutions were administered via intra-peritoneally (IP) with a 1 ml syringe. Six fish per group, control and LPS treatments, were taken 24 and 72 h post-injections. Animals were anaesthetised with fenoxy-ethanol to a lethal dose and rapidly were perfused transcardially with 20 ml of PBS followed by 100 ml of 4% paraformaldehyde (PFA) solution. Head-kidney tissues were carefully removed at all time points and left overnight in the same fixative. During the following day, tissues were dehydrated and paraffin-embedded. Serial sections were cut at 7 µm using a rotary microtome (Reichert-Jung, 2800 Frigocut E.). Head kidney paraffin sections were mounted on pre-treated poly-L-lysine slides, where two consecutive sections were used for *in situ* hybridization (ISH) detections, with the sense and antisense probes, and the next two in the series for immunohistochemistry (IHC) detections.

2.2. PU.1 probe synthesis and DIG labelling

Primers designed accurately against conservative DNA binding domain PU.1 gene (forward: 5'-gtaacaaggggagaattgggc-3'; reverese: 5'-gtgagacctccccctagcac-3', 55ºC, 30 cycles) amplified a 312 bp sequence from control cDNA brain rainbow trout. Briefly, PCR product was run in 1% agarose gel stained with ethidium bromide. DNA amplified band was cut from agarose gel by MiniElute Gel Extraction kit (Qiagen) and cloned into a bacterial plasmid vector (pGEM-T Easy Vector, Promega). DNA insert was sequenced in order to check insert orientation for the correct use of sense/antisense probes. Transformed bacterial cultures were incubated overnight and plasmid extraction was carried out (Nucleospin, Macherey-Nagel). For generation of both antisense and sense probes, 10 µg of plasmid were digested with restriction enzyme Pst I (Promega) and 10 µg more with SphI and run in 0.7% agarose gel previously stained with ethidium bromide. Digested vector was recuperated from the gel and purified by QIAquick Gel Extraction kit (Qiagen) following manufacturer procedure. Linearized DNA was transcribed into single-stranded RNA probe labelled with digoxigenin-UTP. For this reaction, 3 µg DNA was mixed with T7/SP6 RNA polymerase (Roche), DIG RNA Labeling Mix (Roche), placental ribonuclease inhibitor (Roche) and DTT solution (Promega) and incubated 1 hour. Later, an extra T7/SP6 RNA polymerase volume was added for 1 hour more and left to incubate for 10 min at 65ºC to before stopping the reaction. The reaction

volume was increased and filtered through Sephadex Micro-Spin G-50 columns (Amersham). Transcription quality was checked before using both antisense and sense probes by running an small aliquot on an agarose gel. The RNA probes were kept –80ºC degrees until use.

2.3. *In situ* hybridization (ISH)

Before hybridization, frozen sections were left at room temperature for 45 min, and for paraffin sections, deparaffinized and rehydrated. Thereafter, sections were postfixed in 4% PAF for 10 minutes, rinsed three times in PBS for 3 minutes and treated with a Proteinase-K solution (1 µg/ml in 50 mM Tris-EDTA, pH=7.5). Subsequently, slides were again fixed in 4% PAF for 5 min more. Slides were acetylated for 10 min and permeabilized with 1% Surfact-Amps X-100 (Pierce) in PBS for 30 min and later cleaned with PBS three times for 20 min. All slides were pre-incubated for at least 1 hour with hybridization solutions, containing 50% formamide, 5XSSC ph=4.5, 1% SDS, 5X Denhardt's solution, and 0.25 µg/ml yeast tRNA and 50 µg/ml heparin). The DIG-labelled riboprobes were incubated at 80°C for 5 min and immediately chilled on ice before being diluted to hybridization solution. The slides were then coverslipped and incubated overnight at 55°C in a humidified chamber containing 50% formamide, 5XSSC and DEPC water. The following day coverslips were removed carefully by incubating the slides in a solution containing 5XSSC ph=4.5 at 55ºC. The slides were then rinsed in 0.2XSSC 3 times at 40ºC and another one at RT. Subsequently, slides were next washed in PBS two times for 5 min and the endogenous peroxidase activity was quenched with 3% of H_2O_2 in PBS for 20 min. Continuously, slides were washed two times (10 min), and incubated for 1 hour with the TNB blocking solution containing 0.1 M Tris-HCl pH=7.5, 0.15 M NaCl, 0.5% Blocking reagent (Roche). The same blocking solution was used to dilute the first antibody, Anti-Digoxigenin (Roche) to a final concentration of 0.5µg/ml. The slides were incubated overnight at 4ºC in a humidified chamber without coverslips. The following day, the first antibody was washed four times for 10 min in PBS/0.01% Surfact-Amps X-100 (Pierce). For detection, the ChemMated™ DAKO EnVision™ Detection Kit, Peroxidase/DAB (DakoCytomation) protocol was followed. Slides were incubated for 30 min with a drop of Evision antibody and washed four times for 10 min with PBS/0.01% Surfact-Amps X-100 (Pierce). The probe signal was developed by DAB incubation until signal was revealed. All sections were counterstained with 0.02% toluidene blue, or methyl-green for detailed identification of cell nucleus, dehydrated, incubated with xylol two times 3 min and covered with DPX.

2.4. Immunohistochemistry (IHC): 5-bromouracil deoxyriboside (BrdU) detection

Before IHC detection, paraffin slides were deparaffinized, rehydrated, and postfixed with 4% PFA and washed with TBS (0.9%-Tris buffer saline) 10 min three times. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 minutes at 37ºC and rinsed in PBS for 5 minutes at room temperature (RT). Continuously, DNA was denatured by an incubation of 2N HCl for 30 minutes at 37ºC. After rinsing slides thoroughly in PBS, they were treated with pepsin 30 min at 37ºC and washed in PBS 15 min. Blocking solution was applied for 15 min at 37ºC (0.1 M Tris-HCl pH=7.5, 0.15 M NaCl, 0.5% Blocking reagent (Roche) and following, the monoclonal anti-BrdU (2µg/mL) (Sigma) diluted in 0.01 M phosphate buffered saline (pH=7.4) 15mM sodium acid was incubated for 2 hours at 37ºC. The slides were rinsed in PBS three times for 5 minutes and incubated 2 hours at RT with the second antibody anti-mouse-HP (Sigma) following 2.5 hours at 4°C in a 1:400 solution of peroxidase mouse antiperoxidase (PAP; Nordic) with 4% normal rabbit serum and 0.1% Tween-20. After a further rinse in TBS containing normal rabbit serum, and in 0.05 M Tris buffer (pH=7.6), the sections were stained with a nickelintensified diaminobenzidine (DAB; Sigma, St. Louis, MO) solution (1.14 mM NH4NiSO4, 0.4 mM DAB, 0.01% H_2O_2, pH 8.0) for 5–15 minutes at 4°C. All sections were counterstained with 0.02% toluidene blue for detailed identification of cell nucleus, dehydrated, incubated with xylol two times 3 min and covered with DPX.

3. Results

3.1. PU.1: Cloning and characterization

A full length cDNA transcript of 1237 nucleotides was isolated from an EST generated from a macrophage cell culture stimulated for 12 h with LPS (mEST) (Fig.5.2). The first in frame ATG codon is located at the 297 nucleotide position and the terminator codon at the 1103 nucleotide. Open reading frame is translating a putative protein which consists of 269 aminoacids. Analysis of the 3' end of the clone revealed the motif AATAAA placed 25 nucleotides upstream from the poly(A) track, a characteristic of an appropriate polyadenylation signal. 58% of homology identity was found to PU.1 described in zebrafish (Ward, A.C. et al. 2003) as well as in human (Klemsz, M.J. et al. 1990). The amino-acid sequences of the binding domains of the PU.1 proteins are strictly conserved (Fisher, R.C. et al. 1998a). As shown in figure 5.3, this high homology reflected a high conservation of the PU.1 protein among species, and therefore throughout evolution. Three domains can be defined in the rainbow trout PU.1 protein which correspond to region domains

delineated in mammals (Klemsz, M.J. et al. 1990; Pongubala, J.M. et al. 1992). The acidic sequence found in the amino-terminal part of the protein corresponding to rainbow trout residues 1-111, encodes the activation domain in mammalian orthologues. The basic carboxy-terminal sequence containing the DNA-binding or Ets-domain is located between 152-262 residues. These conserved regions showed a 80% homology identities (87% similarities) to the human PU.1 aminoacid sequence. In between these two domains the PEST domain is found, 112-151 residues, involved in protein-protein interaction (Pongubala, J.M. et al. 1993).

```
   1 gacacactgagatgtgtacacccacaaagatatacagaagtcaaa
     atatcaggttccctttaattttcagtcactgagtagggctaatt
     tattttattttcatagacaaaatttgagagaatagcttggcttac
     aagtggataaagttgtgtaggcagagagagacattttgggtttcg
     tttggtgagaggaaatttggtaaacgttgtgcaatggtgcgggaa
     aagttttgaaagagtgtgagaaacaattgaagctaagtgcctgaa
     gtaggtcagttcaagaagaagccttc
 297 atgttgcatccgtacagaatggaggggtacctcatctcacctggt
     M  L  H  P  Y  R  M  E  G  Y  L  I  S  P  G
 342 cctgcatcagaagacatatacgaacccgacatctatagacaacag
     P  A  S  E  D  I  Y  E  P  D  I  Y  R  Q  Q
 387 atgtcagaatattcatatccctatgtcatcgatgcagagagtcaa
     M  S  E  Y  S  Y  P  Y  V  I  D  A  E  S  Q
 432 ggagaacactgggactatcacgccactcatcatgttcatcctctg
     G  E  H  W  D  Y  H  A  T  H  H  V  H  P  L
 477 gactttgacaacctccaagagggccacttcactgagctccagagt
     D  F  D  N  L  Q  E  G  H  F  T  E  L  Q  S
 522 gtccagcaactacatctacccagtatggctcgttacagcgatgtt
     V  Q  Q  L  H  L  P  S  M  A  R  Y  S  D  V
 567 gacactctctctctggaccctggccttgggggacacaaccatgcc
     D  T  L  S  L  D  P  G  L  G  G  H  N  H  A
 612 ctacccccaccggtgtcatattaccctcgtgccatgggctacttg
     L  P  P  P  V  S  Y  Y  P  R  A  M  G  Y  L
 657 caccctctcctcagacgatgaggagctcagacgatgaggagcca
     H  P  S  P  Q  T  M  R  S  S  D  D  E  E  P
 702 ggaggccgcagccctccactagaagtgtctgatgaagagtgtctg
     G  G  R  S  P  P  L  E  V  S  D  E  E  C  L
 747 agagaccacattgcccaagtaacaaggggagaattgggcaacaag
     R  D  H  I  A  Q  V  T  R  G  E  L  G  N  K
 792 aagaagatccgtctgtaccagttcctgctggatctgctgaggaat
     K  K  I  R  L  Y  Q  F  L  L  D  L  L  R  N
 837 ggggacatgaaggacagtatctggtgggtggacagggacaagggc
     G  D  M  K  D  S  I  W  W  V  D  R  D  K  G
 882 accttccagttctcctcgaaacacaaggaggcactggcacatcgg
     T  F  Q  F  S  S  K  H  K  E  A  L  A  H  R
 927 tggggcgtacagaagggcaaccgcaagaaaatgacctaccagaag
     W  G  V  Q  K  G  N  R  K  K  M  T  Y  Q  K
 972 atggcacgggctcttcgcaactacggcaagacaggcgaggtcaaa
     M  A  R  A  L  R  N  Y  G  K  T  G  E  V  K
1017 aaggtcaagaagaagctgacttatcagttcagtggtgaagtgcta
     K  V  K  K  K  L  T  Y  Q  F  S  G  E  V  L
1062 ggggggaggtctcacttggagaggaggccatattcccattcgtag 1106
     G  G  R  S  H  L  E  R  R  P  Y  S  H  S  *
     agggagaaacatagaggaagtcctgaaaaagggtagtgagagaga
     gagatagttaactgtatatagtatgcaagatggttatcaatgtaa
     taaatattgattgtgttcaaatcaaaaaaaaaaaaaaaaa     1237
```

Fig.5.2. Nucleotide and predicted amino acid sequences of rainbow trout PU.1 cDNA. The completed sequence was cloned from activated macrophages EST. Underlined regions correspond to the Ets domain (binding DNA domain) and red bold and italics aminoacids correspond to the α-helix region. Grey highlight correspond to probe synthesized for experimental studies. In orange bold letters correspond to the PU box identified as a DNA-binding protein. The putative polyadenylation signal is in green bold letters.

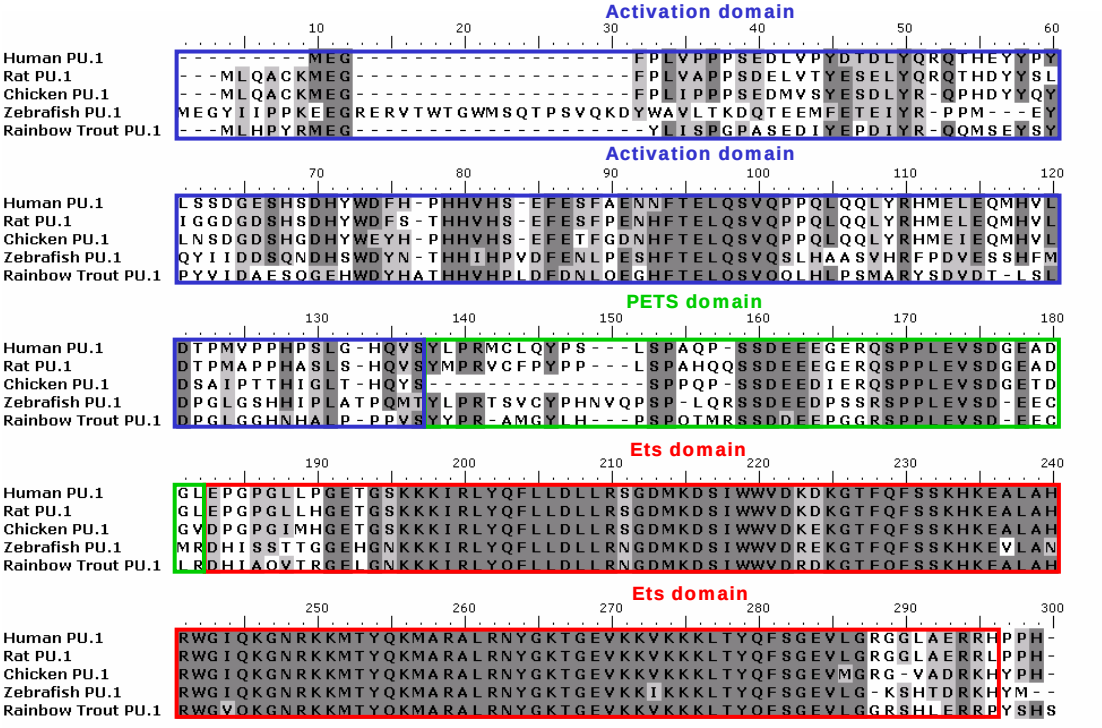


Fig.5.3. Comparison of the translated sequence of the rainbow trout PU.1 putative protein with human (NP003111), rat (BAD35169), chicken (NP990354) and zebrafish (NP932328) protein sequences. Aminoacids are numbered relative to the first putative amino terminus methionine.

3.2. PU.1 expression studies

PU.1 mRNA expression levels in different rainbow trout tissues were studied after 24 and 72 hours LPS-IP (6mg/kg) *in vivo* treatment (see chapter 4). Briefly, PU.1 expression levels analyzed by conventional RT-PCR (Fig.4.2, chapter 4), showed high expression levels in the head kidney, brain and heart tissues, in both control and LPS treated animals. PU.1 expression levels increased 24 hours after treatment in white muscle and intestine, whereas in red muscle this increase occurred 72 hours post injection. Although PU.1 expression was found in the liver, no changes caused by LPS-IP were detected. From real time PCR results of the *in vitro* macrophage cell cultures treated with LPS (10 μg/ml) at different time points (6, 12, 18, 24 and 30 hours), PU.1 mRNA expression levels detected were very high, independently to LPS treatment (Annex, Table 3). These *in vitro* results confirmed what was described in mammals, where high concentrations of PU.1 is required for macrophage development (Dahl, R. et al. 2003). Overall, PU.1 mRNA

expression results show that PU.1 is highly expressed in the head kidney, the primary immune tissue in fish, and also its expression levels are high in macrophage cells, one of the most important cells in the immune system. In brain, PU.1 expression levels were also very high. In mammals, PU.1 has been described as a microglial cell marker (Walton, M.R. et al. 2000), but in fish the presence of PU.1 in brain has never been described.

3.3. *In situ* hybridization

3.3.a. First experiment: LPS injection3.3.a.I. Head kidney results

PU.1 RNA expression levels in head kidney of rainbow trout were detected by ISH. Developed probes showed a clear hybridization signal localized in the cytoplasm of head kidney cells. Positive cells showed a large nucleus and small cytoplasm. Probe signal was positive in head kidney cells of the whole head kidney tissue. These PU.1 positive cells were mostly aggregated to other positive cells, and rarely alone. Although no image analysis software was applied, no clear differences were found between PU.1 mRNA expression levels in control animals compared to LPS treated fish (Fig.5.4). This is in concordance with results found by RT-PCR (chapter 4), were PU.1 expression levels in head kidney were very high, and LPS treatment was not different to the control group. In some control animals PU.1 signals show a reduced expression when comparing to 24 hours LPS treated animals, however this is a qualitative observation and no cell counting was performed. No direct relation was found between positive PU.1 expressing cells to those surrounding melanomacrophagic centre cells. Sense slides, run for each antisense slide, did not give any positive hybridised cell as well as any background detection in the entire tissues, confirming the specificity of the probe designed for the PU.1 mRNA and the reliability of the *in situ* hybridization technique.

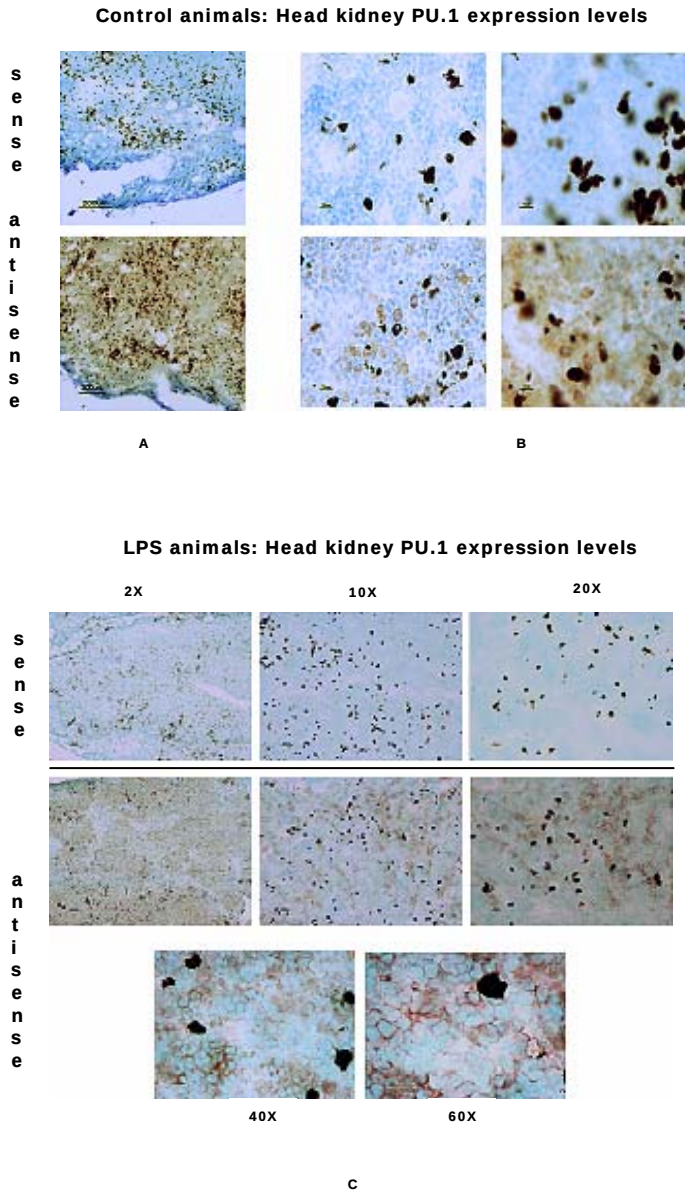


Fig.5.4. *In situ* hybridization of PU.1 expression levels of head kidney rainbow trout. A digoxigenin labeled RNA probe was used to detect PU.1 expression in the head kidney of rainbow trout, counterstained with toluidene blue. A: Control animals with large number of PU.1 positive cells. B: PU.1 expression cells and melanomacrophage centers of control animals. C: PU.1 expression levels in multiple cells of the head kidney after 24 hours LPS IP treatment.

3.3.a.II. Brain results

Sections representing the whole brain tissue of rainbow trout were hybridized with PU.1 probe in order to observe RNA expression levels in microglial cells. Results showed that PU.1 is found in brain tissue and its expression levels are detected by the *in situ* hybridization technique. PU.1 was not distributed equally around all brain sections. PU.1 was not found in the most anterior parts of brain,

such as olfactory bulbs and telencephalon, and neither most external brain regions, such as optic tectum and cerebellum cortex. PU.1 expression levels were found in the most internal areas of the brain, at the mesencephalic area (midbrain), where most abundant signals were situated underneath the beginning of the cerebellum valvula (Fig.5.5). Due to the lack of a reliable steriotaxic atlas for rainbow trout, it is difficult to assign a nucleus area to most PU.1 expression levels. From fish brain neuroanatomy reports, the closest brain nucleus which could be assigned is the nucleus fasciculi longitudunalis medialis (FLM) which is caudally extended to the nucleus fasciculi longitudunalis lateralis (FLL) (Fig.5.5 and Annex, Fig 5) (Billard, R. et al. 1982; Anglade, I. et al. 1999; Muñoz-Cueto, A. et al. 2001; Vacher, C. et al. 2003; Folgueira, M. et al. 2004a, 2004b). *In situ* signal detection of PU.1 shows clear aggregates of positives cell through FLM and FLL. Interestingly, PU.1 has been described as a constitutive transcription factor in microglia and a marker of these cell types in the brain (Walton, M.R. et al. 2000). The histochemical technique of tomato lectin, a specific marker for teleost microglia (Velasco, A. et al. 1995), permits the identification of microglial cell distribution in brain tissue. Among the different microglial markers, the histochemical technique of tomato lectin *Lycopersicum esculentum* has been demonstrated to be a specific marker for teleost microglia (Velasco, A. et al. 1995). The use of lectin histochemical staining in conjunction with cresyl violet counterstaining is a particularly useful and simple method for visualizing microglial cells that engulf fragmented apoptotic cells (Streit, W.J. et al. 1999; Streit, W.J. 2001). The C-type lectins represent a group of pattern recognition receptors (PRRs) which specifically recognise pathogen associated molecular patterns (PAMPS) and deliver appropriate signals to cells of the innate immune system (Janeway, C.A., Jr. 1989). C-type animal lectins are involved in the recognition of carbohydrates at cell surfaces, on circulating proteins as well as those present on pathogens and are consequently involved in diverse processes including cell–cell adhesion, plasma glycoprotein turnover and innate pathogen recognition (Weis, W.I. et al. 1998). These carbohydrate binding molecules are well described but understanding how they influence immune responses is in its infancy (Mcgreal, E.P. et al. 2004). In the Annex, figure 5 shows the histochemistry results of lectin staining on control brain rainbow trout where lectin signals are spread around the whole brain tissue. Interestingly, the brain area with high lectin detection or more microglial cell population, corresponds to the area with most positive PU.1 RNA expression cells by *in situ* hybridization.

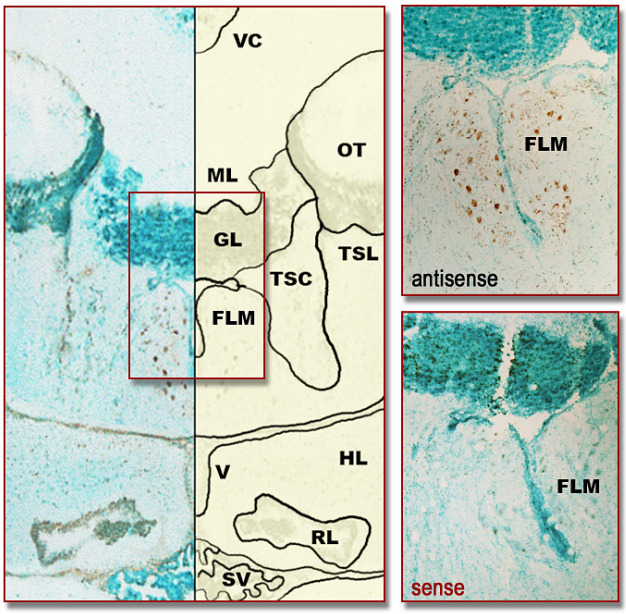


Fig.5.5. Brain transverse section of rainbow trout, showing PU.1 expression levels detected by in situ hybridization, developed by DAB and counterstained with methyl green. Antisense and sense sections are shown. HL:hipotalamic lobe, ML:Molecular layer of the cerebellum; GR:Granular layer of the cerebellum, NFLM:nucleus fasciculi longitudunalis medialis; OT Tectum Optic; RL:Lateral Recess; SV:saccus vasculosus; TSC:Torus semicircularis pars centralis; TSL:Torus semicircularis pars lateralis; V:ventricle; VC:valvula of cerebellum.

3.3.b. Second experiment: LPS/BrdU injection in the head kidney

For this experiment, rainbow trout were first injected by LPS IP and two hours before sampling BrdU was injected IP. Sampling times were 2 and 72 hours (C24, 24LPS, C72 and 72LPS) where head kidney tissues were analyzed both by *in situ* hybridization for PU.1 expression levels and by immunohistochemistry (IHC) for cell proliferation. In just two hours after BrdU, histological examination showed of BrdU positive cells spread through the whole head kidney tissue (Fig.5.6). IHC assay shows a large number of BrdU positive cells giving evidence of their proliferating activity after 72 hours LPS IP post injection. This increase was not so clear after 24 hours LPS stimulation which showed the same proliferating cell profile as control groups. From ISH results, PU.1 was detected after 72 hours LPS injection (Fig.5.7), and contrary to the high levels detected in the first experiments, PU.1 expression levels in control and 24 hours LPS were hardly detected. As observed for PU.1, BrdU positive cells were randomly distributed throughout the head kidney tissue 72 h after LPS IP.

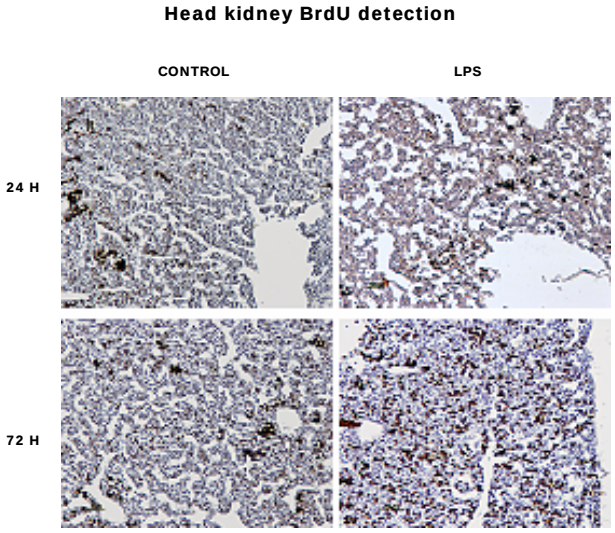


Fig.5.6. Immunohistochemitry of BrdU in the head kidney rainbow trout. Animals were injected with 6mg/kg of LPS and analyzed after 24 and 72 hours. Two hours before fish sampling, BrdU was injected in order to determine cell proliferation. 72 hours after LPS infection BrdU detection was increased.

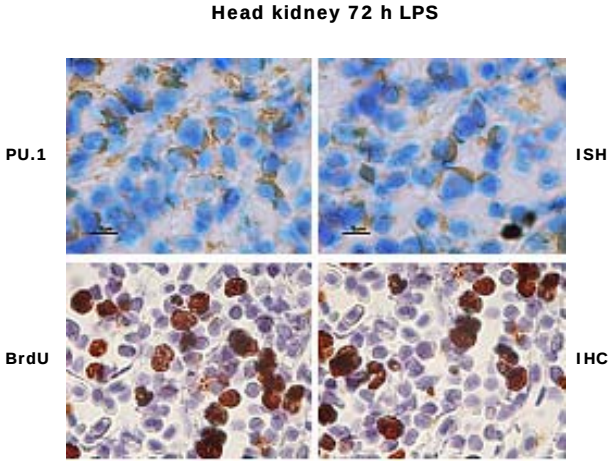


Fig.5.7. PU.1 *in situ* hybridization and BrdU immunohistochemistry in the head kidney rainbow trout 72 hours after LPS infection.

4. Discussion

4.1. Hematopoiesis in the head kidney

Many mammalian genes have been shown to play a role in the development and maturation of hematopoietic and vascular progenitors (Akashi, K. et al. 2000; Thisse, C. et al. 2002). The PU.1 gene plays a critical function for the development of the hematopoietic lineage in the immune system (Iwasaki, H. et al. 2005). From an activated macrophage EST (mEST), the PU.1 full-length cDNA was cloned for the first time in salmonid fish. The putative protein has a 58% homology to the PU.1 gene described in zebrafish (Ward, A.C. et al. 2003) as well as in humans (Klemsz, M.J. et al. 1990) and the DNA-binding or Ets-domain was even more highly

conserved, 80% homology (87% similarities) to human PU.1. This DNA-binding domain classifies PU.1 as a Ets family member, even though it is the most divergent member of the Ets family (Dominguez-Soto, A. et al. 2005). The homology decreased to 38% (data not shown) when the rainbow trout PU.1 protein sequence was compared to the human Spi-B protein, the most closely related Ets family members (Ray, D. et al. 1992) whose expression is critical for normal B cell development and function (Bachmann, M.F. et al. 1996). When comparing the DNA-binding domain between rainbow trout PU.1 to Spi-B human, the homology identity decreases to 64% (data not shown). These data reinforce the evidence that the cDNA sequence cloned from the mEST is PU.1.

PU.1 has been described in several tissues and cells to be involved in the myeloid response. It is present in bone marrow, spleen, interstitial nonhepatocytes of the liver, interstitial nontubular cells of the testis and bone tissues (Galson, D.L. et al. 1993; Tondravi, M.M. et al. 1997; Kherrouche, Z. et al. 1998), as well as in monocytes (Henkel, G.W. et al. 2002), macrophages, B-cells (Klemsz, M.J. et al. 1990; Celada, A. et al. 1996; Fisher, R.C. et al. 1998b; Lloberas, J. et al. 1999), dendritic cells (Bakri, Y. et al. 2004), and microglial cells (Walton, M.R. et al. 2000). As discussed in chapter 4, by using conventional RT-PCR, PU.1 mRNA expression was found in different rainbow trout tissues, where the highest expression was in head kidney, heart and brain. From our research, we report for the first time in salmonid fish the PU.1 hematopoietic transcription factor at a molecular level. From ISH analysis, PU.1 expression levels were detected in the head kidney cells with a large nucleus and little cytoplasm. These positive cells were distributed throughout head kidney, and rarely alone. Results showed a high number of the cell population expressing PU.1 mRNA levels, not only in animals treated with LPS, but control animals also showed high expression levels. The ISH technique works at the individual level, evaluating the expression level responses of specific genes in a single animal. Although RT-PCR results from individuals did not give any different evidence of PU.1 expression levels (Annex, Fig.6), PU.1 *in situ* results showed differences between individual samples. Results show the important of PU.1 in the hematopoietic lineage where the quantity and the expression progenitor-specific expression of PU.1 is critical for its functions in lineage determination (Iwasaki, H. et al. 2005).

Head kidney tissue in fish is a complex tissue containing many cell populations known to occupy the lymphoid tissues in mammals but they do not show the same well defined morphological and functional compartments (Zapata, A. 1979; Imagawa, T. et al. 1995; Takashima, F. et al. 1995; Milano, E.G. et al. 1997). The lymphoid parenchyma in head kidney comprises of a rich population of

leukocytes, including lymphocytes and macrophages (Grove, S. et al. 2006). The lymphoid tissue makes important contributions to the generation of systemic immunity in teleosts and perform the vital function of removal of foreign or antigenic substances from the blood (Ferguson, H.W. et al. 1982). To evaluate whether head kidney cells were proliferating as a consequence of an LPS IP inflammatory challenge, BrdU (a mutagenic substance) was injected IP to rainbow trout. Immunohistochemistry results show that the highest number of BrdU positive cells corresponded to animals treated with LPS and sampled 72 hours post injection. BrdU positive cells were distributed throughout the whole head kidney tissue. From the same experiment, ISH results found the highest PU.1 mRNA expression in the head kidney cells after 72 hours LPS IP treatment. Interestingly, this time point corresponds to the highest BrdU detection levels. Accumulation of BrdU in DNA cells is consequence of their proliferation after the LPS immune challenge. Hematopoiesis is a dynamic development process that ensures a sufficient supply of terminally differentiated blood cells for the survival of the animal. Immature hematopoietic precursors including hematopoietic stem cells (HSC), common myeloid progenitors (CMPs), and common lymphoid progenitors (CLPs) also express PU.1 at a low level (Akashi, K. et al. 2000). These precursor populations promiscuously express other critical transcription factors including megakaryocyte/erythroid (MegE)-related GATA-1, a PU.1 antagonist (Galloway, J.L. et al. 2005; Rhodes, J. et al. 2005) GATA-2, and granulocyte-related CCAAT enhancer-binding protein α (C/EBPα) (Hu, M. et al. 1997; Miyamoto, T. et al. 2002). HSC maturation forms the basis for different blood cell type, including erythrocytes, megakaryocytes, monocytes and lymphocytes (Fisher, R.C. et al. 1998b). In the zebrafish embryo, the erythropoiesis and myelopoiesis are anatomically separated, whereas in the adult these two processes ultimately are colocalized in the head kidney (Lieschke, G.J. et al. 2002). PU.1 is a transcription factor playing an important role in hematopoietic development and it is required for both myeloid and erythroid outcomes, although, at high levels, it represses the latter (Fisher, R.C. et al. 1998b; Ward, A.C. et al. 2003). Although non cell co-localization of PU.1 and BrdU were demonstrated, results give evidence that hematopoietic cells in the head kidney tissue of fish rainbow trout are proliferating as a consequence of the immune LPS challenge, increasing their activity 72 hours after LPS IP challenge. Without specific cell markers, hematopoietic lineage fates can not be assigned therefore it remains unclear whether these proliferating cells pertain to follow erythropoiesis or to myelopoiesis. However, cell lineage differentiation of the head kidney tissue after LPS treatment will be further

discussed in chapter 6, where microarray analysis will give additional information about gene expression profile in this tissue.

Furthermore, although most of the melanomacrophage center functions reported are related to immune system including storage of resistant pathogens, antigen processing in immune response, destruction, detoxification and recycling of endogenous and exogenous materials (Ferguson, H.W. 1976; Lamers, C.H. et al. 1985; Grove, S. et al. 2003; Passantino, L. et al. 2005), from our data, PU.1 expression and BrdU positive cells distribution were not directly related to these macrophage cell types. These results give molecular information about MMCs, and reinforce the intrigue of their real biological function in fish where more efforts are required to characterize MMCs cellular function.

4.2. PU.1 and the rainbow trout brain

It is estimated that more than 28.000 different fish species exist around the world, some still remain to be discovered (Nelson, J.S. 1994). Each fish species has its own particular brain anatomic pattern which interfere in functional brain studies (Bullock, T.H. 1983). Although there is no brain stereotaxic atlas for rainbow trout (*Oncorhynchus mykiss*), some reports studying its anatomical pattern are described (Riddle, D.R. et al. 1991; Pinuela, C. et al. 1992b, 1992a; Teitsma, C.A. et al. 1997; Yanez, J. et al. 1997; Teitsma, C.A. et al. 1999; Mazurais, D. et al. 2000; Folgueira, M. et al. 2002; Folgueira, M. et al. 2003; Menuet, A. et al. 2003; Folgueira, M. et al. 2004a, 2004b, 2005). The lack of reliable information of rainbow trout brain areas difficults interpretation of results. In all vertebrates, a group of structures collectively known as the midbrain, or mesencephalon, link the sensory, motor and integrative components of the hindbrain with those of the forebrain (Butler, A.B. et al. 1996). The midbrain contains topographically organized projections from the auditory, visual, somatosensory systems, intrinsic nucleus with ascending and descending fiber tracts and several nuclei that control the distribution of certain critical neurotransmitters to other brain regions (Butler, A.B. et al. 1996). PU.1 mRNA expression was detected in rainbow trout brain by *in situ* hybridization technique. PU.1 is a microglial cell marker found in both activated and resting microglia, but not in neurons and astrocytes (Walton, M.R. et al. 2000). Results showed PU.1 mRNA expression in the midbrain area of the rainbow trout brain, at the nucleus fasciculus longitudinalis medialis and lateralis (FLM and FLL) (Billard, R. et al. 1982; Anglade, I. et al. 1999; Muñoz-Cueto, A. et al. 2001; Vacher, C. et al. 2003; Folgueira, M. et al. 2004a, 2004b) with a large number of positive cells. The fasciculus longitudinalis (FL) consists of heavily myelinated axons and it is a conspicuous feature of the dorsal brainstem (Muñoz-Cueto, A. et al.

2001). FL is responsible of the eye muscles coordination through reticular formation, a neuronal system that has input from sensory and motor pathways that control pattern generations for rhythmic motor patterns (Butler, A.B. et al. 1996). FL is found next to the oculomotor nucleus and interconnects the oculomotor, trochlear and abducens nucleus with vestibular nucleus (Muñoz-Cueto, A. et al. 2001). Although no cell coexpression of both PU.1 mRNA and extracellular lectin in the microglia cell is reported, histochemical technique results give evidence of a large population of microglia cell localized in the midbrain area (Annex, Fig.5). Microglia cells are a key factor in the defense of the neural parenchyma against infectious disease, inflammation, tissue repair and neural regeneration (Kreutzberg, G.W. 1996). However, the role of PU.1 with high expression levels in the NMF and NFL remains unclear. Some possibilities of the PU.1 role in microglia cells have been suggested, such as production and secretion of various factors, cell proliferation or the differentiation into the macrophage-like phenotype (Walton, M.R. et al. 2000) but it role remains uncertain. Although the interpretation and the study of PU.1 brain results is a complicated issue, we reported the first description of a key immune transcription factor PU.1, related to neuroimmune system in the fish brain. Certaintly, the role of PU.1 in rainbow trout brain as well as its functions in microglial cells requires further investigations.

Conclusions

Here it is reported a full length cDNA transcript of 1237 nucleotides encoding a putative protein of 293 aminoacids isolated from mEST. From ISH results, PU.1 was found in a large number of cells distributed through the head kidney, in both treated and control animals giving evidence of the existence of PU.1 in the major hematopoietic organ in fish. Head kidney cells highly proliferate three days after LPS IP treatment. Our data, provides for the first time, novel insights into overall immune cell proliferation and early response in specific cell/tissue types to LPS in the trout.

5. References

Aittomaki, S., Yang, J., Scott, E.W., Simon, M.C. and Silvennoinen, O. (2004). Molecular basis of Stat1 and PU.1 cooperation in cytokine-induced Fcgamma receptor I promoter activation. *Int Immunol*. 16(2): 265-74.

Akashi, K., Traver, D., Miyamoto, T. and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*. 404(6774): 193-7.

Amemiya, C.T. and Zon, L.I. (1999). Generation of a zebrafish P1 artificial chromosome library. *Genomics*. 58(2): 211-3.

Anderson, M.K., Hernandez-Hoyos, G., Diamond, R.A. and Rothenberg, E.V. (1999). Precise developmental regulation of Ets family transcription factors during specification and commitment to the T cell lineage. *Development*. 126(14): 3131-48.

Anglade, I., Mazurais, D., Douard, V., Le Jossic-Corcos, C., Mananos, E.L., Michel, D. and Kah, O. (1999). Distribution of glutamic acid decarboxylase mRNA in the forebrain of the rainbow trout as studied by in situ hybridization. *J Comp Neurol*. 410(2): 277-89.

Bachmann, M.F., Odermatt, B., Hengartner, H. and Zinkernagel, R.M. (1996). Induction of long-lived germinal centers associated with persisting antigen after viral infection. *J Exp Med*. 183(5): 2259-69.

Badie, B. and Schartner, J. (2001). Role of microglia in glioma biology. *Microsc Res Tech*. 54(2): 106-13.

Bakri, Y., Sarrazin, S., Mayer, U.P., Tillmanns, S., Nerlov, C., Boned, A. and Sieweke, M.H. (2004). Balance of MafB and PU.1 specifies alternative macrophage or dendritic cell fate. *Blood*.

Banati, R.B., Gehrmann, J., Schubert, P. and Kreutzberg, G.W. (1993). Cytotoxicity of microglia. *Glia*. 7(1): 111-8.

Barnache, S., Mayeux, P., Payrastre, B. and Moreau-Gachelin, F. (2001). Alterations of the phosphoinositide 3-kinase and mitogen-activated protein kinase signaling pathways in the erythropoietin-independent Spi-1/PU.1 transgenic proerythroblasts. *Blood*. 98(8): 2372-81.

Battisti, W.P., Wang, J., Bozek, K. and Murray, M. (1995). Macrophages, microglia, and astrocytes are rapidly activated after crush injury of the goldfish optic nerve: a light and electron microscopic analysis. *J Comp Neurol*. 354(2): 306-20.

Berman, J.N., Kanki, J.P. and Look, A.T. (2005). Zebrafish as a model for myelopoiesis during embryogenesis. *Exp Hematol*. 33(9): 997-1006.

Bertolotto, A., Caterson, B., Canavese, G., Migheli, A. and Schiffer, D. (1993). Monoclonal antibodies to keratan sulfate immunolocalize ramified microglia in paraffin and cryostat sections of rat brain. *J Histochem Cytochem*. 41(4): 481-7.

Billard, R. and Peter, R.E. (1982). A stereotaxic atlas and technique for nuclei of the diencephalon of rainbow trout (Salmo gairdneri). *Reprod Nutr Dev*. 22(1A): 1-25.

Bullock, T.H. (1983). Why study fish brains? Fish neurobiology. J. W. S. Canada. Michigan.

Butler, A.B. and Hodos, W. (1996). Comparative Vertebrate Neuroanatomy. New York, A John Wiley & Sons, Inc.

Castellano, B., Gonzalez, B., Dalmau, I. and Vela, J.M. (1991). Identification and distribution of microglial cells in the cerebral cortex of the lizard: a histochemical study. *J Comp Neurol*. 311(3): 434-44.

Celada, A., Borras, F.E., Soler, C., Lloberas, J., Klemsz, M., Van Beveren, C., Mckercher, S. and Maki, R.A. (1996). The transcription factor PU.1 is involved in macrophage proliferation. *J Exp Med*. 184(1): 61-9.

Crepieux, P., Coll, J. and Stehelin, D. (1994). The Ets family of proteins: weak modulators of gene expression in quest for transcriptional partners. *Crit Rev Oncog*. 5(6): 615-38.

Cuadros, M. and Navascués, J. (2000). Early origin and colonization of the developing central nervous system by microglial precursors. *Progress in Brain Research*. 132(microglia).

Cuadros, M.A. and Navascues, J. (1998). The origin and differentiation of microglial cells during development. *Prog Neurobiol*. 56(2): 173-89.

Dahl, R. and Simon, M.C. (2003). The importance of PU.1 concentration in hematopoietic lineage commitment and maturation. *Blood Cells Mol Dis*. 31(2): 229-33.

Delassus, S., Titley, I. and Enver, T. (1999). Functional and molecular analysis of hematopoietic progenitors derived from the aorta-gonad-mesonephros region of the mouse embryo. *Blood*. 94(5): 1495-503.

Dominguez-Soto, A., Puig-Kroger, A., Vega, M.A. and Corbi, A.L. (2005). PU.1 regulates the tissue-specific expression of dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin. *J Biol Chem*. 280(39): 33123-31.

Eichbaum, Q.G., Iyer, R., Raveh, D.P., Mathieu, C. and Ezekowitz, R.A. (1994). Restriction of interferon gamma responsiveness and basal expression of the myeloid human Fc gamma R1b gene is mediated by a functional PU.1 site and a transcription initiator consensus. *J Exp Med*. 179(6): 1985-96.

Eichmann, A., Grapin-Botton, A., Kelly, L., Graf, T., Le Douarin, N.M. and Sieweke, M. (1997). The expression pattern of the mafB/kr gene in birds and mice reveals that the kreisler phenotype does not represent a null mutant. *Mech Dev*. 65(1-2): 111-22.

Ferguson, H.W. (1976). The relationship between ellipsoids and melano-macrophage centres in the spleen of turbot (Scophthalmus maximus). *J Comp Pathol*. 86(3): 377-80.

Ferguson, H.W., Claxton, M.J., Moccia, R.D. and Wilkie, E.J. (1982). The quantitative clearance of bacteria from the bloodstream of rainbow trout (Salmo gairdneri). *Vet Pathol*. 19(6): 687-99.

Fisher, R.C., Olson, M.C., Pongubala, J.M., Perkel, J.M., Atchison, M.L., Scott, E.W. and Simon, M.C. (1998a). Normal myeloid development requires both the glutamine-rich transactivation domain and the PEST region of transcription factor PU.1 but not the potent acidic transactivation domain. *Mol Cell Biol*. 18(7): 4347-57.

Fisher, R.C. and Scott, E.W. (1998b). Role of PU.1 in hematopoiesis. *Stem Cells*. 16(1): 25-37.

Flaris, N.A., Densmore, T.L., Molleston, M.C. and Hickey, W.F. (1993). Characterization of microglia and macrophages in the central nervous system of rats: definition of the differential expression of molecules using standard and novel monoclonal antibodies in normal CNS and in four models of parenchymal reaction. *Glia*. 7(1): 34-40.

Folgueira, M., Anadon, R. and Yanez, J. (2003). Experimental study of the connections of the gustatory system in the rainbow trout, Oncorhynchus mykiss. *J Comp Neurol*. 465(4): 604-19.

Folgueira, M., Anadon, R. and Yanez, J. (2004a). An experimental study of the connections of the telencephalon in the rainbow trout (Oncorhynchus mykiss). I: Olfactory bulb and ventral area. *J Comp Neurol*. 480(2): 180-203.

Folgueira, M., Anadon, R. and Yanez, J. (2004b). Experimental study of the connections of the telencephalon in the rainbow trout (Oncorhynchus mykiss). II: Dorsal area and preoptic region. *J Comp Neurol*. 480(2): 204-33.

Folgueira, M., Anadon, R. and Yanez, J. (2005). Experimental study of the connections of the preglomerular nuclei and corpus mamillare in the rainbow trout, Oncorhynchusmykiss. *Brain Res Bull*. 66(4-6): 361-4.

Folgueira, M., Huesa, G., Anadon, R. and Yanez, J. (2002). The nucleus subglomerulosus of the trout hypothalamus is a link between chemosensory and visual systems: a DiI study. *Brain Res Bull*. 57(3-4): 427-30.

Galang, C.K., Muller, W.J., Foos, G., Oshima, R.G. and Hauser, C.A. (2004). Changes in the expression of many Ets family transcription factors and of potential target genes in normal mammary tissue and tumors. *J Biol Chem*. 279(12): 11281-92.

Galloway, J.L., Wingert, R.A., Thisse, C., Thisse, B. and Zon, L.I. (2005). Loss of gata1 but not gata2 converts erythropoiesis to myelopoiesis in zebrafish embryos. *Dev Cell*. 8(1): 109-16.

Galson, D.L., Hensold, J.O., Bishop, T.R., Schalling, M., D'andrea, A.D., Jones, C., Auron, P.E. and Housman, D.E. (1993). Mouse beta-globin DNA-binding protein B1 is identical to a proto-oncogene, the transcription factor Spi-1/PU.1, and is restricted in expression to hematopoietic cells and the testis. *Mol Cell Biol*. 13(5): 2929-41.

Gangenahalli, G.U., Gupta, P., Saluja, D., Verma, Y.K., Kishore, V., Chandra, R., Sharma, R.K. and Ravindranath, T. (2005). Stem cell fate specification: role of master regulatory switch transcription factor PU.1 in differential hematopoiesis. *Stem Cells Dev*. 14(2): 140-52.

Goebl, M.K. (1990). The PU.1 transcription factor is the product of the putative oncogene Spi-1. *Cell*. 61(7): 1165-6.

Grove, S., Hoie, S. and Evensen, O. (2003). Distribution and retention of antigens of Aeromonas salmonicida in Atlantic salmon (Salmo salar L.) vaccinated with a DeltaaroA mutant or formalin-inactivated bacteria in oil-adjuvant. *Fish Shellfish Immunol*. 15(4): 349-58.

Grove, S., Johansen, R., Reitan, L.J. and Press, C.M. (2006). Immune- and enzyme histochemical characterisation of leukocyte populations within lymphoid and mucosal tissues of Atlantic halibut (Hippoglossus hippoglossus). *Fish Shellfish Immunol*. 20(5): 693-708.

Hart, A.H., Reventar, R. and Bernstein, A. (2000). Genetic analysis of ETS genes in C. elegans. *Oncogene*. 19(55): 6400-8.

Henkel, G.W., Mckercher, S.R. and Maki, R.A. (2002). Identification of three genes up-regulated in PU.1 rescued monocytic precursor cells. *Int Immunol*. 14(7): 723-32.

Hohaus, S., Petrovick, M.S., Voso, M.T., Sun, Z., Zhang, D.E. and Tenen, D.G. (1995). PU.1 (Spi-1) and C/EBP alpha regulate expression of the granulocyte-macrophage colony-stimulating factor receptor alpha gene. *Mol Cell Biol*. 15(10): 5830-45.

Hollenhorst, P.C., Jones, D.A. and Graves, B.J. (2004). Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. *Nucleic Acids Res*. 32(18): 5693-702.

Hsu, T. and Schulz, R.A. (2000). Sequence and functional properties of Ets genes in the model organism Drosophila. *Oncogene*. 19(55): 6409-16.

Hu, M., Krause, D., Greaves, M., Sharkis, S., Dexter, M., Heyworth, C. and Enver, T. (1997). Multilineage gene expression precedes commitment in the hemopoietic system. *Genes Dev*. 11(6): 774-85.

Hume, D.A., Perry, V.H. and Gordon, S. (1983). Immunohistochemical localization of a macrophage-specific antigen in developing mouse retina: phagocytosis of dying neurons and differentiation of microglial cells to form a regular array in the plexiform layers. *J Cell Biol*. 97(1): 253-7.

Hwang, C.K., Kim, C.S., Choi, H.S., Mckercher, S.R. and Loh, H.H. (2004). Transcriptional regulation of mouse mu opioid receptor gene by PU.1. *J Biol Chem*. 279(19): 19764-74.

Imagawa, T., Kitagawa, H. and Uehara, M. (1995). The innervation of the chromaffin cells in the head-kidney of the carp, Cyprinus carpio; regional differences of the connections between nerve endings and chromaffin cells. *Journal of Anatomy*. 188: 149-156.

Iwasaki, H., Somoza, C., Shigematsu, H., Duprez, E.A., Iwasaki-Arai, J., Mizuno, S., Arinobu, Y., Geary, K., Zhang, P., Dayaram, T., Fenyus, M.L., Elf, S., Chan, S., Kastner, P., Huettner, C.S., Murray, R., Tenen, D.G. and Akashi, K. (2005). Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood*. 106(5): 1590-600.

Janeway, C.A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol*. 54 Pt 1: 1-13.

Karim, F.D., Urness, L.D., Thummel, C.S., Klemsz, M.J., Mckercher, S.R., Celada, A., Van Beveren, C., Maki, R.A., Gunther, C.V., Nye, J.A. and Et Al. (1990). The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. *Genes Dev*. 4(9): 1451-3.

Kherrouche, Z., Beuscart, A., Huguet, C., Flourens, A., Moreau-Gachelin, F., Stehelin, D. and Coll, J. (1998). Isolation and characterization of a chicken homologue of the Spi-1/PU.1 transcription factor. *Oncogene*. 16(10): 1357-67.

Klemsz, M.J., Mckercher, S.R., Celada, A., Van Beveren, C. and Maki, R.A. (1990). The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. *Cell*. 61(1): 113-24.

Kodandapani, R., Pio, F., Ni, C.Z., Piccialli, G., Klemsz, M., Mckercher, S., Maki, R.A. and Ely, K.R. (1996). A new pattern for helix-turn-helix recognition revealed by the PU.1 ETS-domain-DNA complex. *Nature*. 380(6573): 456-60.

Konishi, Y., Tominaga, M., Watanabe, Y., Imamura, F., Goldfarb, A., Maki, R., Blum, M., De Robertis, E.M. and Tominaga, A. (1999). GOOSECOID inhibits erythrocyte differentiation by competing with Rb for PU.1 binding in murine cells. *Oncogene*. 18(48): 6795-805.

Koski, G.K., Schwartz, G.N., Weng, D.E., Czerniecki, B.J., Carter, C., Gress, R.E. and Cohen, P.A. (1999). Calcium mobilization in human myeloid cells results in acquisition of individual dendritic cell-like characteristics through discrete signaling pathways. *J Immunol*. 163(1): 82-92.

Kreutzberg, G.W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends Neurosci*. 19(8): 312-8.

Lamers, C.H. and De Haas, M.J. (1985). Antigen localization in the lymphoid organs of carp (Cyprinus carpio). *Cell Tissue Res*. 242(3): 491-8.

Lawson, L.J., Perry, V.H., Dri, P. and Gordon, S. (1990). Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience*. 39(1): 151-70.

Li, R., Pei, H. and Watson, D.K. (2000). Regulation of Ets function by protein - protein interactions. *Oncogene*. 19(55): 6514-23.

Lieschke, G.J., Oates, A.C., Paw, B.H., Thompson, M.A., Hall, N.E., Ward, A.C., Ho, R.K., Zon, L.I. and Layton, J.E. (2002). Zebrafish SPI-1 (PU.1) marks a site of myeloid development independent of primitive erythropoiesis: implications for axial patterning. *Dev Biol*. 246(2): 274-95.

Ling, E.A. and Wong, W.C. (1993). The origin and nature of ramified and amoeboid microglia: a historical review and current concepts. *Glia*. 7(1): 9-18.

Lloberas, J., Soler, C. and Celada, A. (1999). The key role of PU.1/SPI-1 in B cells, myeloid cells and macrophages. *Immunol Today*. 20(4): 184-9.

Martin, P., D'souza, D., Martin, J., Grose, R., Cooper, L., Maki, R. and Mckercher, S.R. (2003). Wound healing in the PU.1 null mouse--tissue repair is not dependent on inflammatory cells. *Curr Biol*. 13(13): 1122-8.

Mazurais, D., Le Drean, G., Brierley, I., Anglade, I., Bromage, N., Williams, L.M. and Kah, O. (2000). Expression of clock gene in the brain of rainbow trout: comparison with the distribution of melatonin receptors. *J Comp Neurol*. 422(4): 612-20.

Mcgreal, E.P., Martinez-Pomares, L. and Gordon, S. (2004). Divergent roles for C-type lectins expressed by cells of the innate immune system. *Mol Immunol*. 41(11): 1109-21.

Mckercher, S.R., Torbett, B.E., Anderson, K.L., Henkel, G.W., Vestal, D.J., Baribault, H., Klemsz, M., Feeney, A.J., Wu, G.E., Paige, C.J. and Maki, R.A. (1996). Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *Embo J*. 15(20): 5647-58.

Menuet, A., Anglade, I., Le Guevel, R., Pellegrini, E., Pakdel, F. and Kah, O. (2003). Distribution of aromatase mRNA and protein in the brain and pituitary of female rainbow trout: Comparison with estrogen receptor alpha. *J Comp Neurol*. 462(2): 180-93.

Milano, E.G., Basari, F. and Chimenti, C. (1997). Adrenocortical and Adrenomedullary Homologs in Eight Species of Adult and Developing Teleosts: Morphology, Histology, and Immunohistochemistry. *General and Comparative Endocrinology*. 108: 483-496.

Miyamoto, T., Iwasaki, H., Reizis, B., Ye, M., Graf, T., Weissman, I.L. and Akashi, K. (2002). Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. *Dev Cell*. 3(1): 137-47.

Moreau-Gachelin, F., Ray, D., Mattei, M.G., Tambourin, P. and Tavitian, A. (1989). The putative oncogene Spi-1: murine chromosomal localization and transcriptional activation in murine acute erythroleukemias. *Oncogene*. 4(12): 1449-56.

Moreau-Gachelin, F., Tavitian, A. and Tambourin, P. (1988). Spi-1 is a putative oncogene in virally induced murine erythroleukaemias. *Nature*. 331(6153): 277-80.

Moreau-Gachelin, F., Wendling, F., Molina, T., Denis, N., Titeux, M., Grimber, G., Briand, P., Vainchenker, W. and Tavitian, A. (1996). Spi-1/PU.1 transgenic mice develop multistep erythroleukemias. *Mol Cell Biol*. 16(5): 2453-63.

Muñoz-Cueto, A., Sarasquete, C., Zhohar, Y. and Kah, O. (2001). An Atlas of the Brain of the Gilthead Seabream (*Sparus aurata*). Maryland, Sea Grant.

Nagulapalli, S., Pongubala, J.M. and Atchison, M.L. (1995). Multiple proteins physically interact with PU.1. Transcriptional synergy with NF-IL6 beta (C/EBP delta, CRP3). *J Immunol*. 155(9): 4330-8.

Nathan, C.F. (1987). Secretory products of macrophages. *J Clin Invest*. 79(2): 319-26.

Nelson, J.S. (1994). Fishes of the world. New York, Wiley & Sons.

Passantino, L., Cianciotta, A., Jirillo, F., Carrassi, M., Jirillo, E. and Passantino, G.F. (2005). Lymphoreticular system in fish: erythrocyte-mediated immunomodulation of macrophages contributes to the formation of melanomacrophage centers. *Immunopharmacol Immunotoxicol*. 27(1): 147-61.

Perez, C., Wietzerbin, J. and Benech, P.D. (1993). Two cis-DNA elements involved in myeloid-cell-specific expression and gamma interferon (IFN-gamma) activation of the human high-affinity Fc gamma receptor gene: a novel IFN regulatory mechanism. *Mol Cell Biol*. 13(4): 2182-92.

Pinuela, C., Baatrup, E. and Geneser, F.A. (1992a). Histochemical distribution of zinc in the brain of the rainbow trout, Oncorhynchos myciss. I. The telencephalon. *Anat Embryol (Berl)*. 185(4): 379-88.

Pinuela, C., Baatrup, E. and Geneser, F.A. (1992b). Histochemical distribution of zinc in the brain of the rainbow trout, Oncorhynchos myciss. II. The diencephalon. *Anat Embryol (Berl)*. 186(3): 275-84.

Pongubala, J.M., Nagulapalli, S., Klemsz, M.J., Mckercher, S.R., Maki, R.A. and Atchison, M.L. (1992). PU.1 recruits a second nuclear factor to a site important for immunoglobulin kappa 3' enhancer activity. *Mol Cell Biol*. 12(1): 368-78.

Pongubala, J.M., Van Beveren, C., Nagulapalli, S., Klemsz, M.J., Mckercher, S.R., Maki, R.A. and Atchison, M.L. (1993). Effect of PU.1 phosphorylation on interaction with NF-EM5 and transcriptional activation. *Science*. 259(5101): 1622-5.

Ray, D., Bosselut, R., Ghysdael, J., Mattei, M.G., Tavitian, A. and Moreau-Gachelin, F. (1992). Characterization of Spi-B, a transcription factor related to the putative oncoprotein Spi-1/PU.1. *Mol Cell Biol*. 12(10): 4297-304.

Rechsteiner, M. and Rogers, S.W. (1996). PEST sequences and regulation by proteolysis. *Trends Biochem Sci*. 21(7): 267-71.

Reddy, M.A., Yang, B.S., Yue, X., Barnett, C.J., Ross, I.L., Sweet, M.J., Hume, D.A. and Ostrowski, M.C. (1994). Opposing actions of c-ets/PU.1 and c-myb protooncogene products in regulating the macrophage-specific promoters of the human and mouse colony-stimulating factor-1 receptor (c-fms) genes. *J Exp Med*. 180(6): 2309-19.

Rhodes, J., Hagen, A., Hsu, K., Deng, M., Liu, T.X., Look, A.T. and Kanki, J.P. (2005). Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *Dev Cell*. 8(1): 97-108.

Riddle, D.R. and Oakley, B. (1991). Evaluation of projection patterns in the primary olfactory system of rainbow trout. *J Neurosci*. 11(12): 3752-62.

Rosenbauer, F., Wagner, K., Kutok, J.L., Iwasaki, H., Le Beau, M.M., Okuno, Y., Akashi, K., Fiering, S. and Tenen, D.G. (2004a). Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. *Nat Genet*. 36(6): 624-30.

Rosenbauer, F., Wagner, K., Zhang, P., Knobeloch, K.P., Iwama, A. and Tenen, D.G. (2004b). pDP4, a novel glycoprotein secreted by mature granulocytes, is regulated by transcription factor PU.1. *Blood*. 103(11): 4294-301.

Sawada, K., Konishi, Y., Tominaga, M., Watanabe, Y., Hirano, J., Inoue, S., Kageyama, R., Blum, M. and Tominaga, A. (2000). Goosecoid suppresses cell growth and enhances neuronal differentiation of PC12 cells. *J Cell Sci*. 113 (Pt 15): 2705-13.

Schnitzer, J. (1989). Enzyme-histochemical demonstration of microglial cells in the adult and postnatal rabbit retina. *J Comp Neurol*. 282(2): 249-63.

Scott, E.W., Simon, M.C., Anastasi, J. and Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science*. 265(5178): 1573-7.

Smith, L.T., Hohaus, S., Gonzalez, D.A., Dziennis, S.E. and Tenen, D.G. (1996). PU.1 (Spi-1) and C/EBP alpha regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. *Blood*. 88(4): 1234-47.

Streit, W.J. (2001). Microglia and macrophages in the developing CNS. *Neurotoxicology*. 22(5): 619-24.

Streit, W.J., Walter, S.A. and Pennell, N.A. (1999). Reactive microgliosis. *Prog Neurobiol*. 57(6): 563-81.

Takashima, F. and Hibiya, T. (1995). Normal and pathological Features. An Atlas of Fish Histology. Tokyo: Kodanska LDT**:** 195.

Teitsma, C.A., Anglade, I., Lethimonier, C., Le Drean, G., Saligaut, D., Ducouret, B. and Kah, O. (1999). Glucocorticoid receptor immunoreactivity in neurons and pituitary cells implicated in reproductive functions in rainbow trout: a double immunohistochemical study. *Biol Reprod*. 60(3): 642-50.

Teitsma, C.A., Bailhache, T., Tujague, M., Balment, R.J., Ducouret, B. and Kah, O. (1997). Distribution and expression of glucocorticoid receptor mRNA in the forebrain of the rainbow trout. *Neuroendocrinology*. 66(4): 294-304.

Thisse, C. and Zon, L.I. (2002). Organogenesis--heart and blood formation from the zebrafish point of view. *Science*. 295(5554): 457-62.

Toda, Y., Tsukada, J., Misago, M., Kominato, Y., Auron, P.E. and Tanaka, Y. (2002). Autocrine induction of the human pro-IL-1beta gene promoter by IL-1beta in monocytes. *J Immunol*. 168(4): 1984-91.

Tondravi, M.M., Mckercher, S.R., Anderson, K., Erdmann, J.M., Quiroz, M., Maki, R. and Teitelbaum, S.L. (1997). Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. *Nature*. 386(6620): 81-4.

Vacher, C., Pellegrini, E., Anglade, I., Ferriere, F., Saligaut, C. and Kah, O. (2003). Distribution of dopamine D2 receptor mRNAs in the brain and the pituitary of female rainbow trout: an in situ hybridization study. *J Comp Neurol*. 458(1): 32-45.

Vaughan, D.W. and Peters, A. (1974). Neuroglial cells in the cerebral cortex of rats from young adulthood to old age: an electron microscope study. *J Neurocytol*. 3(4): 405-29.

Velasco, A., Caminos, E., Vecino, E., Lara, J.M. and Aijon, J. (1995). Microglia in normal and regenerating visual pathways of the tench (Tinca tinca L., 1758; Teleost): a study with tomato lectin. *Brain Res*. 705(1-2): 315-24.

Velasco, A., Jimeno, D., Lillo, C., Caminos, E., Lara, J.M. and Aijon, J. (1999). Enzyme histochemical identification of microglial cells in the retina of a fish (Tinca tinca). *Neurosci Lett*. 263(2-3): 101-4.

Walton, M.R., Gibbons, H., Macgibbon, G.A., Sirimanne, E., Saura, J., Gluckman, P.D. and Dragunow, M. (2000). PU.1 expression in microglia. *J Neuroimmunol*. 104(2): 109-15.

Ward, A.C., Mcphee, D.O., Condron, M.M., Varma, S., Cody, S.H., Onnebo, S.M., Paw, B.H., Zon, L.I. and Lieschke, G.J. (2003). The zebrafish spi1 promoter drives myeloid-specific expression in stable transgenic fish. *Blood*. 102(9): 3238-40.

Weis, W.I., Taylor, M.E. and Drickamer, K. (1998). The C-type lectin superfamily in the immune system. *Immunol Rev*. 163: 19-34.

Yanez, J., Rodriguez, M., Perez, S., Adrio, F., Rodriguez-Moldes, I., Manso, M.J. and Anadon, R. (1997). The neuronal system of the saccus vasculosus of trout

(Salmo trutta fario and Oncorhynchus mykiss): an immunocytochemical and nerve tracing study. *Cell Tissue Res*. 288(3): 497-507.

Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T. and Shultz, L.D. (1990). The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature*. 345(6274): 442-4.

Zapata, A. (1979). Ultrastructural study of the teleost fish kidney. *Dev Comp Immunol*. 3(1): 55-65.

Zupanc, G.K. and Clint, S.C. (2003a). Potential role of radial glia in adult neurogenesis of teleost fish. *Glia*. 43(1): 77-86.

Zupanc, G.K., Clint, S.C., Takimoto, N., Hughes, A.T., Wellbrock, U.M. and Meissner, D. (2003b). Spatio-temporal distribution of microglia/macrophages during regeneration in the cerebellum of adult teleost fish, Apteronotus leptorhynchus: a quantitative analysis. *Brain Behav Evol*. 62(1): 31-42.

Chapter 6

Transcriptome responses to LPS in the head kidney and brain of rainbow trout (*Oncorhynchus mykiss*)

1. Introduction

The use of gene expression profiling to globally address physiological/immunological questions has provided a significant amount of biologically relevant information in recent years (Rho, J. et al. 2002; Chaussabel, D. et al. 2003; Ma, J. et al. 2003; Nicholson, J.K. et al. 2004). Many global genome studies using different mammalian cell and tissue types have been reported, whereas in non-mammalian species, such as fish, relatively few studies have been conducted (Gracery, A.Y. et al. 2001; Rise, M.L. et al. 2004). Currently, little is known concerning global tissue responses in fish at the gene expression level. In recent years, sequence data for salmonid species has greatly increased (Martin, S.A. et al. 2002; Rexroad, C.E. et al. 2003; Goetz, F.W. et al. 2004) providing basic resources for the construction of microarray platforms. Gene chip technology allows for the analysis of large sets of genes, thereby providing a global and integrated vision of the physiological response of an organism to changes in its environment. Merge of high-throughput analytic technologies with bioinformatic and data mining is referred to as a novel scientific discipline; Genomic analysis (Higgs, P.G. et al. 2005). Methods of functional genomics are rapidly expanding towards new species, which are important to gain better understanding of fish physiology and response to immune challenge (Koskinen, H. et al. 2004). The availability of a salmonid-specific microarray platform designed for the first time (Krasnov, A. et al. 2005a) has provided the means to begin the characterisation of the salmonid immune response at a global gene level both *in vitro* and *in vivo* gene chips (Koskinen, H. et al. 2004; Krasnov, A. et al. 2005b; Mackenzie, S. et al. 2005). Basically, microarray analyses provide unique possibility to address many cellular functions, metabolic and regulatory pathways in a single assay. This requires both careful design of the microarray as well as development of data mining approaches.

Microarray technology provides a deeper understanding of overall cellular and tissular processes during immune activation. Therefore, should lead to different physiological/immunological responses *in vivo* upon which the survival of the organism is based. The initial response to pathogens in an organism is fundamental for survival. In order to understand tissue response to LPS inflammation it is first necessary to understand the transcriptional mechanisms which underlie tissue responses to this challenge. In this study we have investigated the response over time of various rainbow trout tissues *in vivo* to Lipopolysaccharide (LPS) (6mg/kg) intraperitoneal (IP) injection, using a salmonid-specific microarray. The endotoxin LPS is a major component of the outer membranes of Gram-negative bacteria and is a strong activator of various immune responses (Wright, S.D. 1999; Brubacher, J.L. et al. 2000). The stimulation of the innate immune system leads to an

inflammatory reaction and the beginning of the proper adaptive immune response (Aderem, A. et al. 2000; Schnare, M. et al. 2001; Janeway, C.A., Jr. et al. 2002). A complex biologic cascade, involving chemokines, cytokines, and the induction of endothelial adhesions, that recruits and activates granulocytes, monocyte/macrophages, and lymphocytes at the damaged or infected tissue sites is a consequence of LPS activation (Idriss, H.T. et al. 2000). Understanding transcriptomic responses in fish to LPS will give reliable information of mechanisms activated to cope with immune challenge.

Objectives

1. Analyse transcriptomic response to LPS IP 24 and 72 hours post injection in rainbow trout.

2. Functional study of activated or repressed genes by Gene Ontology analysis.

3. Understand molecular mechanisms in the rainbow trout head kidney and in the brain after an LPS IP challenge.

2. Materials and methods

2.1. Animals

Rainbow trout (*Onchoryncus mykiss*) of 200 g approximately were obtained from J.Antrés fish farm (St. Privat, Girona) and transported to aquarium facilities at the Universitat Autònoma de Barcelona where they were held in stock tanks at 19ºC at least two weeks acclimation before experiments. Water quality analyses were accurately controlled periodically and tanks were kept partially covered under a photoperiod of 12h light/12h dark. Fish were fed a maintenance ration of about 0.3–0.5% body weight per day, except the day before sampling.

2.2. Experiment design

A total number of twenty four fish, rainbow trout (*Oncorhynchus mykiss*), were distributed equally in two fresh water tanks. Animal conditions during the experiment were the same as said above. Twelve fish were injected intraperitoneally (IP) with 6 mg/kg of lipopolysaccharide *Escherichia coli* (LPS) (LPS IP) and put them back to their corresponding tank. Twelve more fish were IP injected with saline buffer to be used as control fish. After 24 and 72 hours of IP injections, six fish saline group and six fish LPS group, were sampled by first placing them into a lethal concentration of 2-phenoxy-ethanol in less than 1 min, and bled. Briefly, tissues were carefully removed and quickly frozen in liquid nitrogen and stored at –80ºC for avoiding RNA degradation.

2.3. Design of microarray

Methodologically, every gene was presented with six replicate spots. Dye-swap design of experiment provided robust normalization of gene expression data and high power of statistical analyses expression of every gene measured in 12 replicates. This allowed to substantially reduce alterations of expression levels (Krasnov, A. et al. 2005a). Random pooling of six individuals in each sample reduced biological variation, however, representing the population average at an exposure level. Genes in the microarray chip were grouped by functional categories because it is a common way for interpretation of microarray data (Doniger, S.W. et al. 2003; Zeeberg, B.R. et al. 2003). In fact Gene Ontology (Ashburner, M. et al. 2000) is becoming a standard for functional classification of genes because this system appeared most informative and useful.

For preparation of glass cDNA microarray we used subtracted EST libraries and selected clones from a single normalized cDNA library (Rexroad, C.E. et al. 2003). The newly identified cDNA sequences were compared with nucleotide databases using stand-alone blast (Altschul, S.F. et al. 1997) and functional annotation was made by the categories of Gene Ontology (Ashburner, M. et al. 2000). Random clones from common and subtracted cDNA libraries (976) were compared with the known vertebrate proteins using blastx and 686 genes were identified; the functional annotations were transferred from the putative homologs. These clones were supplemented with 297 genes selected by the categories of Gene Ontology. A total of 1380 cDNA inserts of non-redundant clones were amplified with PCR using universal primers and purified with Millipore Montage Plasmid Miniprep-96 Kit. DNA was spotted onto poly-(L)lysine-coated slides and each clone was printed in six replicates. Each microarray was enriched in a number of functional classes, such as stress and defence response (145 and 105 genes, respectively), cell cycle (62 genes), signal transduction (114 genes), chaperone (41), and apoptosis (79).

2.4. Microarray analyses

Total RNA was extracted from six individuals per group (C24, LPS24, C72, LPS72) using TriReagent (MRC) following a commercial protocol. Tissues were randomly pooled in each group. Labelling with Cy3- and Cy5-dCTP (Amersham–Pharmacia) was made using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) primer; cDNA was purified with Microcon YM30 (Millipore). We used a dye swap experimental design (Kerr, M.K. et al. 2001; Yang, Y.H. et al. 2001) and each sample was hybridized to two microarrays. For the first slide, test and control cDNA were libelled with Cy5 and Cy3, respectively, and for the second array dye

assignment was reversed. This methodology resolved the problem of unequal inclusion of label, and each gene was measured in 12 replicates. The slides were pretreated with 1% BSA, fraction V, 5% SSC, and 0.1% SDS (30 min at 50 ºC), washed with 2XSSC (3 min) and 0.2XSSC (3 min), and hybridised overnight in cocktail containing 1.3XDenhardt solution, 3XSSC, 0.3% SDS, 0.67 µg/µl polyadenylate, and 1.4 µg/µl yeast tRNA. All chemicals were from Sigma–Aldrich. Scanning was performed with ScanArray 5000 and images were processed with QuantArray (GSI Luminomics).

2.5. Data analysis

The measurements in spots were filtered by the criteria $I/B \geq 3$ and $(I-B)/(S_I+S_B) \geq 0.6$, where I and B are the mean signal and background intensities and S_I, S_B are the standard deviations. After subtraction of mean background, Lowess normalization (Cleveland, W.S. et al. 1999) was performed. To assess differential expression of genes, the normalized log intensity ratios were analyzed for every pair of dye-swap slides with Student's T-test ($P \leq 0,05$). The genes were ranked by log (P-level) and ranks were calculated separately for the up-regulated and down-regulated genes. Gene Ontology (GO) (Ashburner, M. et al. 2000) were compared by the sums of ranks (Student's T-test, $P \leq 0.05$). All genes that showed significant differential expression in at least one sample were used in these analyses.

3. Results and Discussion

3.1. Microarray in the head kidney

The global gene expression profile in the head kidney of LPS challenged trout was evaluated by high-density microarray analysis 24 and 72 hours after intra-peritoneal injection. In total, 243 genes from a total of 1380 genes, 18% of the genes represented on the microarray, showed significant differential expression under experimental treatment. From this total, 130 genes displayed a differential expression after 24 hours and 113 genes after 72 hours. All differentially expressed genes were classified and grouped depending on their regulation profile. Figure 6.1 shows categories profiles of the differentially expressed genes: Stable up-regulation, Stable down-regulation, Transient induction, Late up-regulation, Transient repression, Late repression, Transient repression/Late induction, Transient induction/Late regression.

3.1.a. Stable up or down regulation

Six genes were up regulated over all experiment; most remarkably, MHC genes show differential responses in LPS treatment. MHC class I gene, well known

to be involved in antigen presentation in dendritic cells (Rossi, M. et al. 2005) was up-regulated with a tendency to increase its expression 72 hours after treatment. This result is in concordance with an up regulation of the beta-2-microglobulin gene, 24 hours post injection and the beta-2-microglobulin precursor 72 hours. Interestingly, the putative HLA class II-associated protein (PHAPI2), a component of the transmembrane signalling pathway that is activated after extracellular binding of ligands during the immune response (Vaesen, M. et al. 1994) was constitutively up-regulated 24 hours after treatment. Another gene up regulated was NADH dehydrogenase which is involved in energy metabolism, playing an important role in cellular respiration and mitochondrial electron transport (Loeffen, J.L. et al. 1998). In contrast, a respectable number of genes were repressed in the head kidney in response to the *in vivo* administration of LPS. Among the genes repressed by LPS it is worth noting that several genes involved in the response of the innate immune system to pathogens have been reported to be activated upon LPS-stimulation in trout macrophages differentiated *in vitro* (Goetz, F.W. et al. 2004): CXC chemokine receptor-4, complement D, and the small inducible cytokine A7. Also repressed by an *in vivo* LPS IP were several proteases, such as MMP-9, MMP-13, collagenase 3 and meprin A [suggested to be a good marker for bacterial infection in carp (Hung, C.H. et al. 1997)]. Other differentially down expressed genes, include several translation-related genes, such as 40S ribosomal proteins and elongation factor 1 alpha; some myeloid transcription factors, such as C/EBP alpha, and several energy and metabolism related genes, such as enolase, transaldolase, glucose-6-phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). It is noteworthy that GADPH is still widely used for normalization of gene expression data despite strong criticism (Bustin, S.A. 2002).

3.1.b. Transient induction/repression

Different genes involved in the immune system were up regulated transiently. Genes related to the immunoglobulin system increased in response to LPS displaying different kinetics. Ig gamma Fc receptor gene, responsible for initiating the allergic response in mammals (Shimizu, A. et al. 1988) increased its expression late in the LPS treatment whereas IgM heavy chain, known to be involved in Ig class switching in B cells (Friedlander, R.M. et al. 1990) increased acutely. Another immune-related gene up regulated 24 hours after LPS IP was the coatomer protein (COP) epsilon, known to play a role in the formation and maturation of phagosomes (Botelho, R.J. et al. 2000). LPS also induced nitric oxide synthase 2 (NOS2) and myeloperoxidase precursor (MPO) genes 24 hours after LPS. NOS2 produce nitric oxide involved bactericidal responses (Lowenstein, C.J. et

al. 1992) as MPO is for microbicidal responses of netrophils (Borregaard, N. et al. 1997; Klebanoff, S.J. 1999). NOS2 requires electron donors as NADH, which was also induced by LPS. Furthermore, LPS enhanced genes involved in transcriptional control such as BACH1 or zinc finger protein 228, myelin transcription factor1 or transcription elongation factor A. Interestingly, genes related to the cell proliferation process were up regulated in response to the LPS challenge. For instance, cell cycle gene 1 protein and prothymosin alpha (Eschenfeldt, W.H. et al. 1986) were enhanced 24 h after treatment whereas granule cell differentiation protein and cyclin B2 genes were enhanced at 72 hours.

Genes involved in the defence and detoxification response presented a transient repression. The MHC class II gene and the annexin A4 were down regulated 24 hours and returned to basal levels after 72 hours post treatment, whereas glutathione S-transferase P was later suppressed at 72 hours.

3.1.c. Differential gene responses

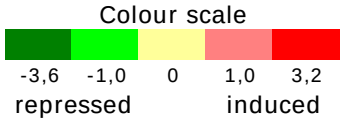
Interestingly, differential gene responses in the HK 24 and 72 hours after treatment were found. LPS down regulated the expression of four hemoglobin genes (hemoglobin $\alpha, \beta, \gamma, \zeta$ chains) and the transcription factor Jun-B, an important regulator of erythroid differentiation (Jacobs-Helber, S.M. et al. 2002) 24 hours after the LPS IP administration. GRAP-2, an hematopoietic cell-associated adaptor protein (Qiu, M. et al. 1998), was up regulated 24 hours to LPS and lately repressed, although granule cell differentiation protein was enhanced 72 hours post treatment. Contrary, 5-aminolevulinate synthase, the key enzyme involved in heme synthesis (Cox, T.C. et al. 1991), was up regulated in a LPS-specific manner at 72 hours in parallel to increase hemoglobin synthesis (hemoglobin α and γ chains) suggesting an overall increase in erythropoiesis in response to LPS 72 hours post injection. These gene expression results suggest that LPS may enhance the progression of hematopoietic cells towards the erythroid lineage 3 days after the immune challenge.

Fig.6.1.(Next page). Genes differentially regulated in the head kidney after lipopolysaccharide (LPS) intraperitoneal administration to rainbow trout (N=6). Experimental samples were hybridised to microarrays on which each gene was printed six times (total of 12 replicates). Differential expression was analyzed with Student's *t*-test ($P<0.05$); the expression ratio is coded with colour scale.

Microarray Head Kindey LPS IP in rainbow trout

3.1.d. Gene Ontology in the head kidney

LPS induced suppression of the inflammatory and defence response, extracellular matrix (collagenases and proteases), several groups of peptidases, protein catabolism, carbohydrate metabolism, and binding of transient metals, especially zinc. These GO groups represent major cellular functions and are composed of large numbers of genes. Table 6.1 shows the differential responses when comparing functional GO categories 24 and 72 hours LPS IP post injection. Signal transduction and cytoskeleton enhancement are observed 24 hours after treatment but suppressed at 72 hours. This suggests processes involved in organization of biogenesis of cytoplasm, organelles and cytoskeleton including myosin and microtubules, as the first response to LPS, whereas this cellular activity is down regulated 3 days after the immune challenge. A series of molecular signals within the cell that are mediated by a member of the Ras family of proteins switching to a GTP-bound active state were differentially expressed 24 to 72 hours. Binding of calcium, phosphate metabolism and oxidative phosphorylation were also augmented in 24 hours but repressed after 72 h. In contrast, transport activity as lipid transport was suppressed in 24 h and up regulated in 72 h of transport activity 24h. Interestingly, the hemoglobin complex necessary for the erythroid cell development was strongly suppressed after 24 h but enhanced 3 days post treatment.



Gene Ontology in the Head Kidney

Function	24H	72H	N°genes
cytoskeleton			36
signal transduction			30
calcium ion binding			19
cytoplasm organization and biogenesis			19
phosphate metabolism			18
organelle organization and biogenesis			17
cytoskeleton organization and biogenesis			16
primary active transporters			13
myosin			9
oxidative phosphorylation			7
small GTPase mediated signal transduction			5
microtubule			5
RAS protein signal transduction			3
ligand-dependent nuclear receptors			2
transporter activity			35
transport			33
oxygen binding			8
hemoglobin complex			6
large ribosomal subunit			5
translation regulator activity			5
iron ion binding			3
translation elongation factor activity			3
ubiquitin-dependent protein catabolism			3
lipid transport			2
protein-synthesizing GTPase activity			2

Table 6.1. Functional categories differentially expressed in head kidney of rainbow trout LPS IP 24 and 72 hours post injection. Differentially expressed genes were grouped by Gene Ontology categories and mean log(expression ratio) were analyzed by Student's *t*-test (P<0,05); the expression ratio is coded with colour scale.

3.2. Microarray in the brain

3.2.a. Genes up or down regulated

In order to examine the transcripcional profile in brain rainbow trout after an immune challenge, a specific salmonid microarray platform was applied. After 24 hours of LPS IP injection, trout brain presented 159 genes (11.5%) differentially expressed to control group, 5.2% of the total platform genes were up regulated and 5.8% of them were repressed (Table 6.2). The gene expression profile revealed that the largest group of genes repressed (20%) corresponded to different ribosomal genes like 60S ribosomal protein L17 or 40S ribosomal protein S11 (Kenmochi, N. et al. 1998), therefore protein biosynthesis in rainbow trout brain after 24 hours LPS IP is decreased. Cytochrome genes represented 10% of total

repressed genes in brain. The most down regulated were Cytochrome oxidase subunits III-1 and 2 (COX-1, COX-2) known to catalyse the rate-limiting step of prostaglandin synthesis and being targets of nonsteroidal antiinflammatory drugs (Funk, C.D. et al. 1991; Vane, J.R. et al. 1994). Myeloperoxidase, which is a protein synthesized during myeloid differentiation that constitutes the major component of neutrophil azurophilic granules for the microbicidal activity, was down regulated in brain 24 h LPS IP (Borregaard, N. et al. 1997; Klebanoff, S.J. 1999). Interestingly, two genes related to the nervous central development were repressed: Nck-associated protein 1, which induce apoptosis of neuronal cells (Suzuki, T. et al. 2000) and Glutathione S-transferase P, an important mediator of inflammation (Jakobsson, P.J. et al. 1997). Among the genes over expressed by LPS, 15% of the total genes up regulated were involved in the immune response: MHC class II invariant chain-like protein 1, which acts as a chaperone by assisting initial folding and oligomeritzation of the MHCII subunits (Roche, P.A. et al. 1991); B-cell-specific transcriptional coactivator OBF-1, which is essential for the response of B-cells to antigens and required for the formation of germinal centers (Gstaiger, M. et al. 1995; Strubin, M. et al. 1995); alpha globin gene, which is involved in the formation of the hemoglobin complex (ζ chain) (Black, J.A. 1976); and adenosine deaminase-3 that catalyses the hydrolysis of adenosine to inosine and its deficiency causes dysfunction of both B and T lymphocytes with impaired cellular immunity decreasing production of immunoglobulins (Adrian, G.S. et al. 1983; Abbott, C.M. et al. 1986). After 72 hours of the LPS treatment, no significant difference in gene expression was found in brain, therefore, from a transcriptional level, brain tissue recovered from the immune challenge three days after the treatment.

Microarray Brain LPS IP in rainbow trout

Gene name	24 H	Gene name
Actin, alpha skeletal 3		ATPase 6
Acyl-Coenzyme A dehydrogenase, long chain,		Basic leucine-zipper protein BZAP45-1
Adenosine deaminase 3		Beta-2-microglobulin-1
Alpha-globin 1-3		C-C chemokine receptor type 2
Apoptosis response zinc finger protein		Collagen alpha 1(VIII) chain precursor
B-cell-specific coactivator OBF-1		Complement factor H-2
Beta actin-2		Creatine kinase, B chain
Bromodomain adjacent to zinc finger domain 2B		Cytochrome b-1
CD2 binding protein 1-2		Cytochrome c oxidase subunit I-1
Chromosome-associated kinesin KIF4A		Cytochrome c oxidase subunit II
Coatomer zeta-1 subunit		Cytochrome c oxidase subunit III-4
Creatine kinase		Cytochrome oxidase subunit III-1
Cytochrome c-2		Cytochrome oxidase subunit III-2
Double-strand break repair protein MRE11A		Cytochrome oxidase subunit III-3
Eukaryotic translation initiation factor 2 subunit 2		Cytochrome P450 2F1
Ferritin heavy chain-1		Cytohesin binding protein HE
Ferritin heavy chain-2		Cytoplasmic dynein light chain
Fructose-bisphosphate aldolase A		Cytosolic nonspecific dipeptidase
G1/S-specific cyclin D2		DnaJ homolog subfamily A member 2
Galectin-3 binding protein		Eukaryotic translation initiation factor 3 subunit 5
GRB2-related adaptor protein 2		Glutathione S-transferase P
Heat shock 70kDa protein 8		Growth hormone inducible transmembrane-1
Heterogeneous nuclear ribonucleoprotein A1-2		Malate dehydrogenase, cytoplasmic
Histone H2Ax		Matrix metalloproteinase-13
Hyaluronan and proteoglycan link protein 2 precursor		Myeloperoxidase
Hyperosmotic protein 21		Myosin heavy chain, skeletal, adult 1-2
Hypothetical protein LOC122618		NAD-dependent deacetylase sirtuin 3
Inhibitor of kappaB kinase gamma		NADH dehydrogenase subunit 2
MHC class II invariant chain-like protein 1		NADH dehydrogenase subunit 4
Multidrug resistance protein 3-1		NADH dehydrogenase subunit 5-1
Myelin basic protein-2		Nck-associated protein 1
Myosin heavy chain, skeletal, adult 1-1		Nucleoside diphosphate kinase 3
Myosin heavy chain, skeletal, fetal		Orphan nuclear receptor NR4A2
NADH-ubiquinone oxidoreductase		Proteasome subunit alpha type 7-1
Nebulin-1		RAC-alpha serine/threonine-protein kinase
p53-regulated protein PA26		Ribosomal protein L35-3
Parvalbumin alpha-2		Ribosomal protein L36a-like-2
PEST-containing nuclear protein		Ribosomal protein L6-1
Polyadenylate-binding protein 4		Ribosomal protein S2
Proteasome subunit alpha type 3		40S ribosomal protein S10
Quinone oxidoreductase		40S ribosomal protein S11
Retinoic acid receptor responder 3-2		40S ribosomal protein S8
SEC13-related protein		40S ribosomal protein S9-3
Serine protease-like protein-3		60S acidic ribosomal protein P1
Stomatin		60S ribosomal protein L17
Transcription regulator protein BACH1		60S ribosomal protein L18
Transposase-6		60S ribosomal protein L23
Transposase-60		60S ribosomal protein L32-3
Tubulin alpha-3 chain		60S ribosomal protein L36
		Selenoprotein T-2
		Serine dependent phospholipase A2
		Small inducible cytokine B14 precursor
		Splicing factor 3b, subunit 3, 130kDa
		Splicing factor, arginine/serine-rich 1-2
		Thymosin beta-4-1
		Triosephosphate isomerase
		Ubiquitin and ribosomal protein S27a-2
		Zinc finger protein 180

Colour scale				
-0,94	-0,4	0	0,4	0,5
repressed			induced	

Table 6.2. Genes differentially regulated in the brain after intraperitoneal lipopolysaccharide (LPS IP) administration to rainbow trout (N=6). Experimental samples were hybridized to microarrays on which each gene was printed six times (total of 12 replicates). Differential expression was analyzed with Student's *t*-test (P<0.05); the expression ratio is coded with colour scale.

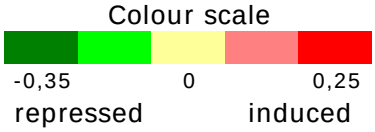
3.2.b. Gene Ontology in brain

To further address molecular mechanisms of LPS IP effects in brain, differently expressed genes were grouped by functional categories of Gene Ontology. Some functional categories show significant differences (Student's t test, $P<0,05$) are shown in Table 6.3. Some of them include genes already discussed above. For instance, genes related to the protein biosynthesis, such as ribosomal genes and translation are down regulated in brain. Most of the categories repressed are related to energy metabolism and mitochondrion. These include proteins of the inner membrane of mitochondrion as well as proteins of electron transport chain, ions and nucleotides. The most enhanced functional category in brain was the response to pathogen which involved a change in state or activity of cells in terms of movement, secretion, enzyme production or gene expression as a result of a stimulus from pathogens. This cell activity is observed by an up regulation of genes involved in cell cytoskeleton.

3.2.c. Brain compared to other tissues

Brain response to LPS challenge was compared to other tissues (head kidney, liver, ovary, red muscle and white muscle) of rainbow trout. As microarray figure 6.4 shows, brain tissue does not respond to LPS challenge as actively as other tissues such as head kidney or liver, but it has higher responses than ovary tissue. In fact, brain tissue is the major protected organ as it is very sensitive to perturbations (Magistretti, P.J. 1999). Table 10 in the Annex shows Gene Ontology categories divided in four main functional levels: metabolism, cellular process, cellular components, molecular function and response to stimulus. Brain is the tissue showing the highest number of metabolic-related genes repressed 24 hours after LPS IP. Although fish brain metabolic depression after food deprivation has been reported (Soengas, J.L. et al. 1998), it has the ability to survive prolonged periods without feeding, which is attributed to the generally low metabolic rate of fish and to the activation of gluconeogenic flux (Sheridan, M.A. et al. 1991; Blasco, J. et al. 1996). Nevertheless, the vertebrate brain is metabolically one of the most active of all organs (Sokoloff, L. 1989) responsible for 2.7-3.4% of the total body energy consumption in ectothermic vertebrates (Van Ginneken, V. et al. 1996). Red and white muscle responses to LPS are involved in organization of biogenesis of cytoplasm, organelles and cytoskeleton including myosin and microtubules more actively than brain tissue. In comparison to brain tissue, liver responses as well as head kidney responses enhance protein biosynthesis within an up regulation of ribosomal proteins and protein polymerisation. Microarray analysis reveals that responses to LPS were almost inexistent in the ovary. In general, brain tissue

compared to other tissues, like head kidney or liver, decreases its transcriptomic activity in response to LPS IP.



Gene Ontology; Brain

Function	24 H	N° genes
protein biosynthesis		41
ribosome		31
mitochondrion		25
electron transport		21
carrier activity		17
primary active transporter activity		16
extracellular space		9
translation		8
lipid binding		7
metallopeptidase activity		7
sodium ion transporter activity		6
ATP synthesis coupled electron transport		5
nucleolus		5
hemoglobin complex		5
antioxidant activity		4
collagen catabolism		3
immune response		48
response to biotic stimulus		31
receptor activity		18
cell-cell signaling		14
cell cycle		7
actin cytoskeleton		5
nuclear transport		4
microtubule-based process		3
response to pest, pathogen or parasite		3

Table 6.3 Examples of functional categories differentially expressed in brain of rainbow trout LPS IP 24 hours post injection. Differentially expressed genes were grouped by Gene Ontology categories and mean log(expression ratio) were analyzed by Student's *t*-test (P<0,05), the expression ratio is coded with colour scale.

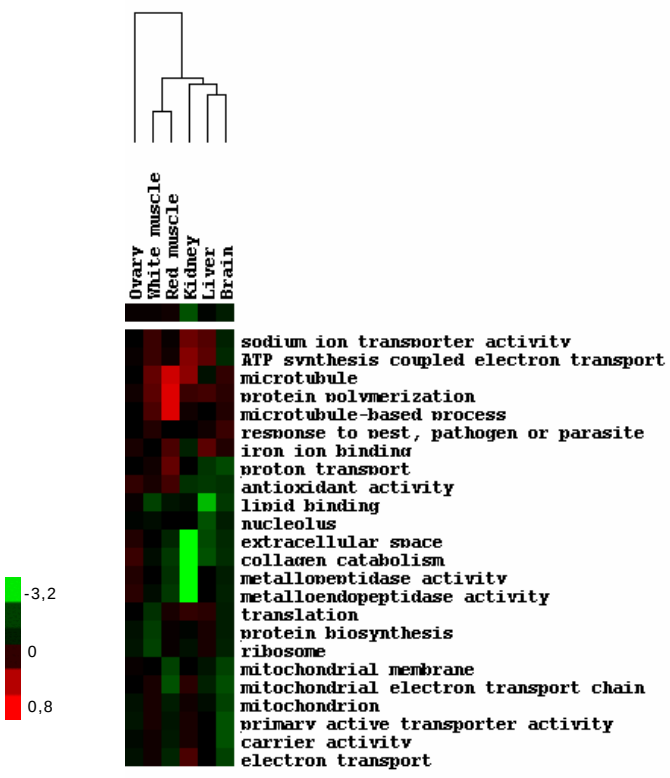


Fig.6.4. Microarray cluster analysis comparing brain tissue responses to other rainbow trout tissue after 24 hours of a LPS IP. Differentially expressed genes were grouped by Gene Ontology categories and mean log(expression ratio) were analyzed by Student's *t*-test (P<0,05); the expression ratio is coded with colour scale.

4. Conclusions

Microarray analyses provide the unique possibility to address many cellular functions, metabolic and regulatory pathways in a single assay and compare the contribution of functional categories in transcriptomic responses. Gene ontology analysis, allows the comparison of transcriptomic responses by functional classes indicating tendencies that could not be seen at a single-gene level. Designed experiments made it possible to compare the contribution of functional categories in the transcriptomic response to LPS IP (6 mg/kg) in the rainbow trout, mainly in the head kidney and brain tissues. Our data reveal and classify several pools of genes, giving insight into their likely functions during an inflammation caused by LPS IP treatment during 24 and 72 hours LPS stimulation. In view of these results gene expression is significantly different in both tissues at an individual gene level and in functional categories at a cellular level. The data obtained provides for the first time novel insights into overall cellular function and early response in specific

cell/tissue types to LPS in the trout. We have identified that time-course analysis *in vivo* will provide important novel insights into the immune response in fish.

Most remarkably, the head kidney presents suppression of genes involved in the immune response 24 and 72 hours after treatment. Among the genes repressed by LPS it is worth noting that several genes involved in the response of the innate immune system to pathogens have been reported to be activated upon LPS-stimulation in trout macrophages differentiated *in vitro* (Goetz, F.W. et al. 2004): CXC chemokine receptor-4, complement D, and small inducible cytokine A7. Repression of several proteases was also observed in the head kidney response, such as MMP-9, MMP-13, collagenase 3 and meprin A. However, microbicidal (MPO) and bactericidal (NSO2) related genes were enhanced 24 h after treatment, as well as MHCI gene indicating activation of the immune system in this tissue. Furthermore, genes related to cell proliferation processes such as cell cycle gene 1 protein, prothymosin alpha were up regulated in the 24 h response whereas granule cell differentiation protein and cyclin B2 genes were later enhanced. In fish, the head kidney tissue is the principal immune organ that assumes hematopoietic functions (Meseguer, J. et al. 1995; Zapata, A.G. et al. 1996). Microarray results show an increase of processes involved in organization of biogenesis of cytoplasm, organelles and cytoskeleton which are enhanced to initiate cell proliferation processes in the head kidney, and suppressed genes involved in the immune immflamatory response such as chemokine receptor, MMP-9, the myeloid transcription factor C/EBP alpha. Such changes in cellular activity could result from changes in the transcriptional profile of the cell, possibly as a consequence of migration into other body compartments in which exogenous factors dictate the cellular response (Mackenzie, S. et al. 2003). These results, suggest that immune cells required for coping LPS challenge in fish may migrate from the head kidney to localize peritoneal inflammation. Head kidney microarray analysis of rainbow trout treated with IHNV (Infectious Hematopoietic Necrosis Virus; kidney and liver are the target organs) IP, induces the opposite response in which LPS IP were repressed: inflammatory and defence response, extracellular matrix (collagenases and proteases), several groups of peptidases, protein catabolism, carbohydrate metabolism, and binding of transient metals, especially zinc (Mackenzie, S. et al. under submission). These clear differences in cellular function may reflect viral invasion to the head kidney, the main target organ (Romero, A. et al. 2005), whereas for LPS treated fish the invasion is in the peritoneal cavity.

Interestingly, hemoglobin complex related genes (hemoglobin α,β, γ,ζ chains) and erythroid transcription factor Jun-B were suppressed after 24 hours. Contrary, after 72 h the 5-aminolevulinate synthase, a key enzyme involved in

heme synthesis (Cox, T.C. et al. 1991) and two hemoglobin α and γ chains were enhanced. These finding give evidence of an initial general stress response 24 hours after LPS IP administration where erythroid lineage development is suppressed. However, 3 days after LPS administration, erythrioid lineage enhances the hematopoietic cell fate by up regulating the expression of genes responsible for heme biosynthesis. The cell proliferation response is not exclusive to the erythroid development lineage as granule cell differentiation protein is up regulated 72 hours post treatment. This cell proliferation processes have already been observed in the head kidney of rainbow trout double injected with LPS IP and BrdU (see chapter 5). Therefore, microarray analysis reinforce the results obtained previously in the head kidney, where cell proliferation process is initiated as a response to LPS IP.

The salmonid microarray platform revealed that after 24 hours of LPS IP injection, trout brain presented 159 genes (11.5%) differentially expressed to control group. The gene expression profile showed that ribosomal proteins such as 40S, S2 or DnaJ, were the gene group most repressed (20%), giving evidence that protein biosynthesis in rainbow trout brain after 24 hours LPS IP is decreased. Large numbers of cytochrome genes were also repressed in brain, such cytochrome c oxidase. Metabolism in brain was down decreasing lipid binding, collagen catabolism, ATP synthesis and protein biosynthesis. 15% of the total genes up regulated were involved in the immune response such as MHC class II invariant chain-like protein 1, B-cell-specific transcriptional coactivator OBF-1, and the adenosin deaminase-3. LPS IP enhanced cytoskeleton and microtubules related genes, as already observed in microarray analysis in rainbow trout brain 1 day after handling stress (Krasnov, A. et al. 2005a). Comparing to other tissues, brain significantly down regulates genes related to mitochondrion. However, the response of brain to LPS is not as high as other tissues like head kidney or liver, but its higher than ovary tissue. Interestingly, after 72 hours of LPS IP brain tissue was practically recovered from the immune challenge, giving evidence of the importance of this protective response for fish survival. Overall results revealed that brain responses caused by LPS to cope with the immune challenge. In conclusion, microarray analysis in rainbow trout brain results in 24 hours after LPS IP administration showed a down regulation of genes involved in energy metabolism, inflammation response and protein biosynthesis whereas response to pathogen and cytoskeleton proteins were enhanced.

5. References

Abbott, C.M., Skidmore, C.J., Searle, A.G. and Peters, J. (1986). Deficiency of adenosine deaminase in the wasted mouse. *Proc Natl Acad Sci U S A*. 83(3): 693-5.

Aderem, A. and Ulevitch, R.J. (2000). Toll-like receptors in the induction of the innate immune response. *Nature*. 406(6797): 782-7.

Adrian, G.S. and Hutton, J.J. (1983). Adenosine deaminase messenger RNAs in lymphoblast cell lines derived from leukemic patients and patients with hereditary adenosine deaminase deficiency. *J Clin Invest*. 71(6): 1649-60.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 25(17): 3389-402.

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M. and Sherlock, G. (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet*. 25(1): 25-9.

Black, J.A. (1976). Human zeta haemoglobin chain. *Nature*. 261(5558): 348.

Blasco, J., Fernandez-Borras, J. and Marimon, I.R., A. (1996). Plasma glucose kinetics and tissue uptake in brown trout in vivo: effect of an intravascular glucose load. *J.Comp. Physiol. B*. 165: 534–541.

Borregaard, N. and Cowland, J.B. (1997). Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood*. 89(10): 3503-21.

Botelho, R.J., Hackam, D.J., Schreiber, A.D. and Grinstein, S. (2000). Role of COPI in phagosome maturation. *J Biol Chem*. 275(21): 15717-27.

Brubacher, J.L., Secombes, C.J., Zou, J. and Bols, N.C. (2000). Constitutive and LPS-induced gene expression in a macrophage-like cell line from the rainbow trout (Oncorhynchus mykiss). *Dev Comp Immunol*. 24(6-7): 565-74.

Bustin, S.A. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol*. 29(1): 23-39.

Chaussabel, D., Semnani, R.T., Mcdowell, M.A., Sacks, D., Sher, A. and Nutman, T.B. (2003). Unique gene expression profiles of human macrophages and dendritic cells to phylogenetically distinct parasites. *Blood*. 102(2): 672-81.

Cleveland, W.S., Grosse, E. and Shyu, W.M. (1999). Local regression models. Statistical Models. J. M. Chambers. Pacific Grove, Wadsworth and Brooks/Cole**:** 309-376.

Cox, T.C., Bawden, M.J., Martin, A. and May, B.K. (1991). Human erythroid 5-aminolevulinate synthase: promoter analysis and identification of an iron-responsive element in the mRNA. *Embo J*. 10(7): 1891-902.

Doniger, S.W., Salomonis, N., Dahlquist, K.D., Vranizan, K., Lawlor, S.C. and Conklin, B.R. (2003). MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. *Genome Biol*. 4(1): R7.

Eschenfeldt, W.H. and Berger, S.L. (1986). The human prothymosin alpha gene is polymorphic and induced upon growth stimulation: evidence using a cloned cDNA. *Proc Natl Acad Sci U S A*. 83(24): 9403-7.

Friedlander, R.M., Nussenzweig, M.C. and Leder, P. (1990). Complete nucleotide sequence of the membrane form of the human IgM heavy chain. *Nucleic Acids Res*. 18(14): 4278.

Funk, C.D., Funk, L.B., Kennedy, M.E., Pong, A.S. and Fitzgerald, G.A. (1991). Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. *Faseb J*. 5(9): 2304-12.

Goetz, F.W., Iliev, D.B., Mccauley, L.A., Liarte, C.Q., Tort, L.B., Planas, J.V. and Mackenzie, S. (2004). Analysis of genes isolated from lipopolysaccharide-stimulated rainbow trout (Oncorhynchus mykiss) macrophages. *Mol Immunol*. 41(12): 1199-210.

Gracery, A.Y., Troll, J.V. and Somero, G.N. (2001). Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *PNAS*. 98 (4): 1993-1998.

Gstaiger, M., Knoepfel, L., Georgiev, O., Schaffner, W. and Hovens, C.M. (1995). A B-cell coactivator of octamer-binding transcription factors. *Nature*. 373(6512): 360-2.

Higgs, P.G. and Attwood, T.K. (2005). Bioinformatics and Molecular Evolution. Oxford, Blackwell Publishing.

Hung, C.H., Huang, H.R., Huang, C.J., Huang, F.L. and Chang, G.D. (1997). Purification and cloning of carp nephrosin, a secreted zinc endopeptidase of the astacin family. *J Biol Chem*. 272(21): 13772-8.

Idriss, H.T. and Naismith, J.H. (2000). TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc Res Tech*. 50(3): 184-95.

Jacobs-Helber, S.M., Abutin, R.M., Tian, C., Bondurant, M., Wickrema, A. and Sawyer, S.T. (2002). Role of JunB in erythroid differentiation. *J Biol Chem*. 277(7): 4859-66.

Jakobsson, P.J., Mancini, J.A., Riendeau, D. and Ford-Hutchinson, A.W. (1997). Identification and characterization of a novel microsomal enzyme with glutathione-dependent transferase and peroxidase activities. *J Biol Chem*. 272(36): 22934-9.

Janeway, C.A., Jr. and Medzhitov, R. (2002). Innate immune recognition. *Annu Rev Immunol*. 20: 197-216.

Kenmochi, N., Kawaguchi, T., Rozen, S., Davis, E., Goodman, N., Hudson, T.J., Tanaka, T. and Page, D.C. (1998). A map of 75 human ribosomal protein genes. *Genome Res*. 8(5): 509-23.

Kerr, M.K. and Churchill, G.A. (2001). Statistical design and the analysis of gene expression microarray data. *Genet Res*. 77(2): 123-8.

Klebanoff, S.J. (1999). Myeloperoxidase. *Proc Assoc Am Physicians*. 111(5): 383-9.

Koskinen, H., Pehkonen, P., Vehniainen, E., Krasnov, A., Rexroad, C., Afanasyev, S., Molsa, H. and Oikari, A. (2004). Response of rainbow trout transcriptome to model chemical contaminants. *Biochem Biophys Res Commun*. 320(3): 745-53.

Krasnov, A., Koskinen, H., Pehkonen, P., Rexroad, C.E., 3rd, Afanasyev, S. and Molsa, H. (2005a). Gene expression in the brain and kidney of rainbow trout in response to handling stress. *BMC Genomics*. 6(1): 3.

Krasnov, A., Koskinen, H., Rexroad, C., Afanasyev, S., Molsa, H. and Oikari, A. (2005b). Transcriptome responses to carbon tetrachloride and pyrene in the kidney and liver of juvenile rainbow trout (Oncorhynchus mykiss). *Aquat Toxicol*. 74(1): 70-81.

Loeffen, J.L., Triepels, R.H., Van Den Heuvel, L.P., Schuelke, M., Buskens, C.A., Smeets, R.J., Trijbels, J.M. and Smeitink, J.A. (1998). cDNA of eight nuclear encoded subunits of NADH:ubiquinone oxidoreductase: human complex I cDNA characterization completed. *Biochem Biophys Res Commun*. 253(2): 415-22.

Lowenstein, C.J., Glatt, C.S., Bredt, D.S. and Snyder, S.H. (1992). Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proc Natl Acad Sci U S A*. 89(15): 6711-5.

Ma, J., Chen, T., Mandelin, J., Ceponis, A., Miller, N.E., Hukkanen, M., Ma, G.F. and Konttinen, Y.T. (2003). Regulation of macrophage activation. *Cell Mol Life Sci*. 60(11): 2334-46.

Mackenzie, S., Iliev, D., Liarte, C., Koskinen, H., Planas, J.V., Goetz, F.W., Molsa, H., Krasnov, A. and Tort, L. (2005). Transcriptional analysis of LPS-stimulated activation of trout (Oncorhynchus mykiss) monocyte/macrophage cells in primary culture treated with cortisol. *Mol Immunol*.

Mackenzie, S., Planas, J.V. and Goetz, F.W. (2003). LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and in vitro differentiated macrophages. *Dev Comp Immunol*. 27(5): 393-400.

Mackenzie, S., Roher, N., Novoa, B., Liarte, C., Ribas, L., Romero, A., Planas, J.V., Tort, L., Goetz, F.W., Krasnov, K. and Figueras, A. (under submission). Transcriptomic analysis of the head kidney response in trout, O.mykiss, to bacterial and viral challenge.

Magistretti, P.J. (1999). Brain energy metabolis. Fundamental Neuroscience. M. J. Zigmond, F. E. Bloom, S. C. Landis, J. L. Roberts and L. R. Squire. San Diego, Academic Press**:** 389–413.

Martin, S.A., Caplice, N.C., Davey, G.C. and Powell, R. (2002). EST-based identification of genes expressed in the liver of adult Atlantic salmon (Salmo salar). *Biochem Biophys Res Commun*. 293(1): 578-85.

Meseguer, J., López-Ruiz, A. and García-Ayala, A. (1995). Reticulo-endothelial stroma of the head-kidney from the seawater teleost gilthead seabream (Sparus aurata L): an ultrastructural and cytochemical study. *Anat Rec*. 241: 303-309.

Nicholson, J.K., Holmes, E., Lindon, J.C. and Wilson, I.D. (2004). The challenges of modeling mammalian biocomplexity. *Nat Biotechnol*. 22(10): 1268-74.

Qiu, M., Hua, S., Agrawal, M., Li, G., Cai, J., Chan, E., Zhou, H., Luo, Y. and Liu, M. (1998). Molecular cloning and expression of human grap-2, a novel leukocyte-

specific SH2- and SH3-containing adaptor-like protein that binds to gab-1. *Biochem Biophys Res Commun*. 253(2): 443-7.

Rexroad, C.E., Lee, Y., Keele, J.W., Karamycheva, S., Brown, G., Koop, B., Gahr, S.A., Palti, Y. and Quackenbush, J. (2003). Sequence analysis of a rainbow trout cDNA library and creation of a gene index. *Cytogenet Genome Res*. 102(1-4): 347-54.

Rho, J., Altmann, C.R., Socci, N.D., Merkov, L., Kim, N., So, H., Lee, O., Takami, M., Brivanlou, A.H. and Choi, Y. (2002). Gene expression profiling of osteoclast differentiation by combined suppression subtractive hybridization (SSH) and cDNA microarray analysis. *DNA Cell Biol*. 21(8): 541-9.

Rise, M.L., Von Schalburg, K.R., Brown, G.D., Mawer, M.A., Devlin, R.H., Kuipers, N., Busby, M., Beetz-Sargent, M., Alberto, R., Gibbs, A.R., Hunt, P., Shukin, R., Zeznik, J.A., Nelson, C., Jones, S.R., Smailus, D.E., Jones, S.J., Schein, J.E., Marra, M.A., Butterfield, Y.S., Stott, J.M., Ng, S.H., Davidson, W.S. and Koop, B.F. (2004). Development and application of a salmonid EST database and cDNA microarray: data mining and interspecific hybridization characteristics. *Genome Res*. 14(3): 478-90.

Roche, P.A., Marks, M.S. and Cresswell, P. (1991). Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature*. 354(6352): 392-4.

Romero, A., Figueras, A., Tafalla, C., Thoulouze, M.I., Bremont, M. and Novoa, B. (2005). Histological, serological and virulence studies on rainbow trout experimentally infected with recombinant infectious hematopoietic necrosis viruses. *Dis Aquat Organ*. 68(1): 17-28.

Rossi, M. and Young, J.W. (2005). Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. *J Immunol*. 175(3): 1373-81.

Schnare, M., Barton, G.M., Holt, A.C., Takeda, K., Akira, S. and Medzhitov, R. (2001). Toll-like receptors control activation of adaptive immune responses. *Nat Immunol*. 2(10): 947-50.

Sheridan, M.A. and Mommsen, T.P. (1991). Effects of nutritional state on in vivo lipid and carbohydrate metabolism of coho salmon, Oncorhynchus kisutch. *Gen Comp Endocrinol*. 81(3): 473-83.

Shimizu, A., Tepler, I., Benfey, P.N., Berenstein, E.H., Siraganian, R.P. and Leder, P. (1988). Human and rat mast cell high-affinity immunoglobulin E receptors: characterization of putative alpha-chain gene products. *Proc Natl Acad Sci U S A*. 85(6): 1907-11.

Soengas, J.L., Strong, E.F. and Andres, M.D. (1998). Glucose, lactate, and beta-hydroxybutyrate utilization by rainbow trout brain: changes during food deprivation. *Physiol Zool*. 71(3): 285-93.

Sokoloff, L. (1989). Circulation and energy metabolism of the brain. Basic Neurochemistry: Molecular, Cellular, and Medical Aspects. G. L. Siegel. New York, Raven Press**:** 565-590.

Strubin, M., Newell, J.W. and Matthias, P. (1995). OBF-1, a novel B cell-specific coactivator that stimulates immunoglobulin promoter activity through association with octamer-binding proteins. *Cell*. 80(3): 497-506.

Suzuki, T., Nishiyama, K., Yamamoto, A., Inazawa, J., Iwaki, T., Yamada, T., Kanazawa, I. and Sakaki, Y. (2000). Molecular cloning of a novel apoptosis-related gene, human Nap1 (NCKAP1), and its possible relation to Alzheimer disease. *Genomics*. 63(2): 246-54.

Vaesen, M., Barnikol-Watanabe, S., Gotz, H., Awni, L.A., Cole, T., Zimmermann, B., Kratzin, H.D. and Hilschmann, N. (1994). Purification and characterization of two putative HLA class II associated proteins: PHAPI and PHAPII. *Biol Chem Hoppe Seyler*. 375(2): 113-26.

Van Ginneken, V., Nieveen, M., Van Eersel, R., Vand Den Thillart, G. and Addink, A. (1996). Neurotransmitter levels and energy status in brain of fish species with and without the survival strategy of metabolic depression. *Comp Biochem Physiol*. 114A: 189-196.

Vane, J.R., Mitchell, J.A., Appleton, I., Tomlinson, A., Bishop-Bailey, D., Croxtall, J. and Willoughby, D.A. (1994). Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Proc Natl Acad Sci U S A*. 91(6): 2046-50.

Wright, S.D. (1999). Toll, a new piece in the puzzle of innate immunity. *J Exp Med*. 189(4): 605-9.

Yang, Y.H., Buckley, M.J. and Speed, T.P. (2001). Analysis of cDNA microarray images. *Brief Bioinform*. 2(4): 341-9.

Zapata, A.G., Chibá, A. and A., V. (1996). Cells and tissues of the immune system of fish. The fish immune system. Organism, pathogen and environment. G. Iwama and T. Nakanishi, Academic Press.

Zeeberg, B.R., Feng, W., Wang, G., Wang, M.D., Fojo, A.T., Sunshine, M., Narasimhan, S., Kane, D.W., Reinhold, W.C., Lababidi, S., Bussey, K.J., Riss, J., Barrett, J.C. and Weinstein, J.N. (2003). GoMiner: a resource for biological interpretation of genomic and proteomic data. *Genome Biol*. 4(4): R28.

Chapter 7

General discussion and conclusions

1. The physiological stress response

All organisms have the capacity to response to stress with physiological mechanisms in order to restore homeostatic mechanisms caused by stress. Connections between nervous-immune-endocrine systems have received particular attention because contribute to understand biological response under stress conditions (Besedovsky, H.O. et al. 1996). In fish, physiological responses including elevations in plasma concentrations of cortisol, glucose, lactate and in marine species, osmolality, are well established as useful indicators of the degree of stress experienced by fish (Barton, B.A. et al. 2002). In chapter 2, physiological parameters of sea bream (*Sparus aurata*) subjected to high density confinement were studied. Remarkably, higher cortisol levels were found in plasma 14 days after chronic confinement. When acute handling stress was applied in fish previously subjected to 14 days of confinement, cortisol plasma levels were significantly lower. Results suggest that there is a limit to the maximum cortisol levels which can be reached, and therefore a limit to the capacity of the interrenal tissue to be stimulated and/or release cortisol (Rotllant, J. et al. 1997).The measurement of these physiological parameters, may not be adequate for a complete biological interpretation (Gornati, R. et al. 2003). The cross talk between neuro-immune-endocrine systems and resultant secretion of regulatory molecules, modulate the physiological response at target tissues at the transcriptional level causing changes in gene expression. The identification of such target genes to be used as molecular markers capable of describing fish welfare is a major aim in the development of diagnostic technologies in modern aquaculture (Thorgaard, G.H. et al. 2002).

2. Enolase

2.1. Enolase in fish species

The Differential Display (DD) technique allows us to study differential gene expression in fish subjected to a particular stress situation. By using DD, differentially expressed genes were identified in the sea bream brain in fish subjected to high density confinement during 14 days. The glycolytic enzyme enolase gene was partially cloned for the first time in the sparid fish *Sparus aurata* (S-enolase AY263379) and by Northern blot analysis a specific 1.9 kb mRNA was obtained. The putative coding protein presents homology (74%) to human alpha enolase gene certifying the high evolutionary conservation of the enolase family (Tracy, M.R. et al. 2000). Enolase is used as a marker for detecting some human diseases, most of them related to carcinomas (Ejeskar, K. et al. 2005; Santini, D. et al. 2006). To date, the possibility of enolase as a candidate gene for molecular marker in fish had never been explored. Animal welfare is one the field of recent

interest in aquaculture, as it encompasses physiology and animal biology with a better quality product and improved ethical procedures. In chapter 3, several enolase sequences were cloned from brain and muscle of several fish species; rainbow trout, leather and mirror carp and stickleback, increasing the number of gene sequences in the fish database. The homology percentage between the enolase sequences obtained from sea bream muscle and brain S-enolase tissue was 81%. In rainbow trout, the enolase sequence was amplified from muscle and brain which presented a homology to S-enolase of 90%. Stickleback and leather carp sequences only differed in three aminoacids to the sequence for mirror carp. From phylogenetic analysis, enolase sequences cloned were clustered with the beta type enolase cluster. Our findings suggest that in fish, enolase isoforms may not present a direct isoform tissue specificity and support the hypothesis that enolase gene is a unique multifunctional protein with unrestricted location diversity (Babbitt, P.C. et al. 1996; Pancholi, V. 2001; Gerlt, J.A. et al. 2005).

2.2. Enolase expression in fish

Differences in gene expression first identified by DD in the sea bream brain, were further checked by Northern analysis where S-enolase was up-regulated during both chronic confinement stress and after 48 hours of an *Escherichia coli* Lipopolysaccaride (LPS) intraperitoneal (IP) administration. The administration of LPS results in the activation of the immune-nervous-endocrine systems (Grinevich, V. et al. 2001) and plays an important role in modulating host susceptibility and resistance to inflammatory disease (Sternberg, E.M. 2001; Haddad, J.J. et al. 2002). This response includes synthesis of various cytokines, especially interleukin IL-1β, IL-6 and TNFα (Grinevich, V. et al. 2001) and elevated plasma cortisol levels (Holland, J.W. et al. 2002). As S-enolase is a glycolytic enzyme, our results are the first evidence in fish of the presence of metabolic regulation in brain as a consequence of an immune system activation or a physical stressor (Ribas, L. et al. 2004). These results give new evidence toward the existence of an interconnection between the neuro-immuno-endocrine network in fish. Interestingly, it has been reported the role of enolase as an immunosuppressive protein (Veiga-Malta, I. et al. 2004; Chandran, V. et al. 2006) and its expression is found on the surface of hematopoietic cells (Miles, L.A. et al. 1991; Redlitz, A. et al. 1995). As reported in chapter 4, from the mEST database (macrophage cell cultures stimulated 12 h with LPS), 6 different cDNA were homologous to the enolase gene. Results revealed that enolase is highly expressed in macrophage cells and it may be involved in immunological functions in fish.

Tissue distribution of enolase mRNA by conventional RT-PCR was assessed for sea bream and rainbow trout. From unstimulated fish, enolase mRNA showed ubiquitous distribution with thevhighest expression levels in muscle, likely due to the co-amplification of the muscle-specific beta isoform of enolase. Similar reports in other species suggest that this may be due to the similarity in nucleotide sequences between enolase isoforms and therefore may result in cross-hybridization (Oliva, D. et al. 1989). Interestingly, differences in enolase tissue expression levels between salmonid and sparid fish were found. The enolase expression levels in sparid tissues were lower than in salmonid tissues. These results revealed that enolase expression may depend to each fish specie influenced throughout evolution.

The availability of microarray analysis for studying enolase mRNA expression from a large number of fish species and tissues gave us the opportunity to study enolase regulation in fish subjected to different stressors. Enolase mRNA levels were significantly up regulated throughout experiments related to toxicity and fish diseases. Chemicals, changes in physiological and pathological conditions, or environmental modifications are capable to elicit responses at the transcriptomic level (Gornati, R. et al. 2005). Remarkably, enolase was highly up regulated in rainbow trout brain one day after handling exposure, maintaining high levels during five days of the exposure. Overall results from Differential Display, Northern blot and Microarray analysis throughout this thesis, give evidence of the transcriptional levels in fish brain of the enolase gene in order to cope with the stressor applied, and the activation of the glycolytic pathway to maintain homeostasis in brain tissue. The vertebrate brain is metabolically one of the most active of all organs and exquisitely sensitive to perturbations in energy metabolism (Magistretti, P.J. 1988). The enolase enzyme has a central role related to metabolic sources in the animal, as it is necessary to convert the glucose reservoir into functional energy for the organism (Voet, D. et al. 1990). The activities of glycolytic enzymes in brain of teleost species support the contention of a high glycolytic potential (Soengas, J.L. et al. 1998a). This is likely, as glucose levels must be maintained in privileged organs to ensure brain function (Pertea, G. et al. 2003). Therefore, from a central nervous system point of view, a threatened fish copes with a stress situation by regulating metabolism in brain tissue. Interestingly, from all 1380 genes on the microarray platform, glyceraldehyde-3-phosphate dehydrogenase (GADPH) presented the highest correlation of expression pattern to the enolase gene. Recent findings support that glycolytic enzymes such enolase or GADPH, are more complicated and multifaceted proteins rather than simple components of the glycolytic pathway (Pancholi, V. 2001). It is reported that enolase acts as a

transcriptional regulator of the c-Myc transcription factor (Feo, S. et al. 2000; Subramanian, A. et al. 2000) and GAPDH acts as a transcriptional coactivator (Zheng, L. et al. 2003) also in neuronal apoptosis (Ishitani, R. et al. 1996; Shashidharan, P. et al. 1999). Enolase has been defined as a 'moonlighting' protein with one function within the cell acting as a glycolytic protein and another role outside acting as a immunosuppressor (Jeffery, C.J. 1999); although the question of how this enzyme is transported to the cell membrane without the presence of a cleavable signal sequence remains unclear (Veiga-Malta, I. et al. 2004). Little is known about the molecular mechanisms of the multifaceted roles of glycolytic enzymes, specifically regarding the relationship between their glycolytic and non-glycolytic functions. From our results it is not possible to distinguish whether changes in enolase mRNA levels may function as a glycolytic or non-glycolytic enzyme, but they show for the first time in fish that enolase mRNA levels are regulated when fish welfare is threatened.

2.3. Enolase conclusions

In view of the previous commentaries; the question remains whether enolase is a good biomolecular marker for fish welfare? From first group of experiments, enolase was up regulated in brain after a physical stress or after an immune challenge to cope with disturbances caused by these stressors. Microarray analysis gave evidence of enolase gene regulation in different experiments with different stressors, different fish species and different fish tissues in which, enolase mRNAs were regulated significantly (up/down). In view of the extensive regulation of the enolase mRNA, it could be considered that enolase mediates some function when fish welfare is threatened. Enolase is a highly expressed key enzyme of glycolysis (Babbitt, P.C. et al. 1996; Tracy, M.R. et al. 2000) which a part from classical involvement in metabolism, acts as a stress protein (Aaronson, R.M. et al. 1995), as a transcriptional regulator (Feo, S. et al. 2000; Subramanian, A. et al. 2000), or as a plasminogen receptor on the surface of a variety of hematopoietic cells (Pancholi, V. 2001). Furthermore, recent studies describe transcriptomic regulation of metabolic genes in fish subjected to different stressors (Krasnov, A. et al. 2005a; Krasnov, A. et al. 2005b; Mackenzie, S. et al. 2005; Vuori, K.A. et al. 2006) and from the mEST database, metabolic genes represented almost 18% of the total transcribed genes being the largest gene group (Goetz, F.W. et al. 2004). Interestingly, microarray data revealed that GAPDH, another glycolytic enzyme, was similarly regulated to enolase. As said above, enolase and GAPDH enzymes perform several functions in addition to their innate glycolytic function, playing an important role in several biological and pathophysiological processes (Sirover, M.A.

1996, 1999). All of our results suggest that in fish, these metabolic genes may be "old" proteins with new faces. It is the first time in fish that classical metabolic proteins with specific glycolytic and other non glycolytic functions are reported to be involved when fish welfare is threatened. In conclusion, the findings of this thesis open new insight into considering classical metabolic proteins, enolase and GAPDH, as possible biomolecular markers for fish welfare.

3. Candidate genes for studying the fish immune system

Several diseases can affect fish at all stages of their life cycle, and knowledge of the immune system is of major importance for their health (Scapigliati, G. et al. 2002). Comparative genomic studies yielding information on how the presence or expression of various genes can affect the innate resistance to certain pathogens or fish welfare conditions, is of increasing interest in fish research (Thorgaard, G.H. et al. 2002). Throughout the past few years, the number of gene sequences has increased considerably in fish databases for different fish species. However, there is a lack of data concerning the physiological function of these cloned genes in fish systems. From the EST database generated from macrophage cell cultures stimulated 12 hours by LPS (10 μg/ml) (mEST), a large number of interesting genes were discovered (Goetz, F.W. et al. 2004). However, the physiological functions in the whole animal were not carried out. From more than a thousand genes represented in the mEST database, candidate genes were selected depending on their key role in immune function and gene expression profiles. Candidate genes chosen from mEST database were: PU.1, TNFRs, TNF Decoy receptor, 4-IBB, TNFα, MHCII Ii, CD83, CD209e, CCL4-like, Acute phase serum amyloid A, IL1-Receptor type II and IL1-Receptor-like. As described in chapter 4, public database annotation and bioinformatic tools, such as multiple alignment sequence analysis or homologies percentage to known proteins, were required to identify and characterize all potential genes likely to be good candidate immune genes. For determining immune expression profiles, an *in vivo* LPS IP experiment were performed for rainbow trout. Expression levels and tissue distribution was conducted by conventional RT-PCR, and furthermore, real time PCR studies in LPS stimulated macrophages, contributed to corroborate our results. After studying and analysing candidate genes more than one gene from the first mEST selection, promised to be a good candidate as an immune gene marker and the selection of interesting genes was assessed considering the following criteria: EST full length cDNA availability, % homology to known protein, species conserved protein, immune function relevance, tissue expression levels, literature reports and group research interests, finally resulting in the PU.1 gene as the most highly

scored gene. Therefore, PU.1 resulted in the best candidate gene for further studies in the immune system in fish. Other genes likely to be potential candidate immune genes to study the immune system were discarded, for instance the Tumor Necrosis Factor Receptors (TNFRs) (Locksley, R.M. et al. 2001). From the mEST database, several TNFRs family putative proteins were identified and a full length sequence for TNF receptor (TNFRV) was synthetically synthesized. There is a lack of TNFRs gene information not only concerning their DNA sequences in fish databases but also, about their biological function as immune genes responsible for cell signalling transduction pathways which develop pleiotropic responses in fish. Furthermore, there is a lack of homology within the TNFRs family members and from our PCR analysis (conventional and real time PCR) they showed low expression levels. Genes coding for proteins related to immune system promise to be good candidate biomarkers for studying the immune response in fish. PU.1 was selected to study and further research relevant aspects of the immune system in fish.

4. PU.1

4.1. Characterization and expression

As reported in the thesis chapter 5, PU.1/Spi1 is an E26-transformation–specific (Ets) family transcription factor that is a key regulator of hematopoietic lineages (Fisher, R.C. et al. 1998). PU.1 is considered a master gene required for hematopoietic development, owing to its potential ability to regulate the expression of multiple genes specific for different lineages during normal hematopoiesis (Gangenahalli, G.U. et al. 2005). A major function of PU.1 is to regulate the expansion of myeloid and lymphoid progenitors by controlling the transcription rates of genes encoding receptors for cytokines (Celada, A. et al. 1996). From the mEST database, a PU.1 full-length cDNA transcript of 1237 nucleotides encoding a putative protein of 269 aminoacids was cloned for the first time in salmonid fish. The putative protein has a 58% homology to the PU.1 gene described in zebrafish (Ward, A.C. et al. 2003) as well as in humans (Klemsz, M.J. et al. 1990) and the DNA-binding domain was even more highly conserved (80%). PU.1 has been found in several tissues and cells involved in the myeloid response. It is present in bone marrow, spleen, interstitial nonhepatocytes of the liver, interstitial nontubular cells of the testis and bone tissues (Galson, D.L. et al. 1993; Tondravi, M.M. et al. 1997; Kherrouche, Z. et al. 1998), as well as in monocytes (Henkel, G.W. et al. 2002), macrophages, B-cells (Klemsz, M.J. et al. 1990; Celada, A. et al. 1996; Fisher, R.C. et al. 1998; Lloberas, J. et al. 1999), dendritic cells (Bakri, Y. et al. 2004), and microglial cells (Walton, M.R. et al. 2000). By using conventional RT-PCR PU.1 mRNA expression was found in different rainbow trout tissues, where the highest

expression was found in head kidney, heart and brain. Further real time PCR results from LPS stimulated macrophage cultures cDNA, confirmed results described in mammals (Celada, A. et al. 1996; Dahl, R. et al. 2003), from which high concentrations of PU.1 are required for macrophage development.

4.2. PU.1 in the head kidney

The structural organization of the immune system within tissues provides information directly relevant to the likely sites of initiation and elaboration of an immune response. Due to the complexity of the immune system, it is important to identify mRNA expression of immune cell types as this will give information on the functional circuits into which the mRNA expression is integrated. This information becomes very important in the light of accumulating evidence that not only the functional, but also the molecular features of immune cells can be modified by activity dependent mechanisms. By using *in situ* hybridization, PU.1 expression levels were detected in the head kidney cells with a large nucleus and small cytoplasm. These positive cells were distributed throughout all of the head kidney, and rarely alone. PU.1 is considered a master gene required for normal hematopoiesic development (Gangenahalli, G.U. et al. 2005) and results showed a high number of the cell population expressing PU.1 mRNA levels, not only in animals treated with LPS, but also in control animals. Although no image analysis software was applied, differences between animal samples were observed. Results show the importance role of PU.1 in the hematopoietic lineage where the quantity and progenitor-specific expression of PU.1 is critical for its functions in lineage determination (Iwasaki, H. et al. 2005). To evaluate whether head kidney cells were proliferating as a consequence of an LPS IP inflammatory challenge, BrdU mutagenic substance was *in vivo* injected to rainbow trout. Immunohistochemistry results show that control animals have positive BrdU cells, but the highest levels corresponded to animals treated with LPS after 72 hours post injection. As PU.1 ISH results, BrdU positive cells were distributed throughout the whole head kidney tissue. From the same experiment, ISH results showed the highest PU.1 mRNA expression in the head kidney cells after 72 hours LPS IP treatment. Interestingly, this time point corresponds to the highest BrdU detection levels. PU.1 is a regulator of hematopoietic cellular fates (Fisher, R.C. et al. 1998) and the immature hematopoietic precursors also express PU.1 at a low level (Akashi, K. et al. 2000). Hematopoietic stem cell maturation originates different blood cells types, including erythrocytes, megakaryocytes, monocytes and lymphocytes (Fisher, R.C. et al. 1998) where PU.1 is required for both myeloid and erythroid outcomes, although at high levels, it represses the latter (Fisher, R.C. et al. 1998; Ward, A.C. et al. 2003).

In fish, the head kidney tissue is the principal immune organ that assumes hematopoietic functions (Meseguer, J. et al. 1995; Zapata, A.G. et al. 1996) and contains many cell populations without defined morphological and functional compartments (Zapata, A. 1979; Imagawa, T. et al. 1995; Takashima, F. et al. 1995; Milano, E.G. et al. 1997). ISH for PU.1 and IHC for BrdU results show that hematopoietic cells in the head kidney tissue of rainbow trout are proliferating as a consequence of immune LPS challenge, with a higher proliferation 72 hours after LPS IP challenge. However, without specific cell markers, hematopoietic lineages can not be assigned, and it remains unclear whether these proliferating cells pertain to an erythropoietic, myelopoietic or lymphopoietic lineages.

4.3. PU.1 in brain

PU.1 was described as a constitutive transcription factor in microglia and as a marker of this cell type in the brain (Walton, M.R. et al. 2000). Microglial cell functions are related to production and secretion of various factors as immune responses (Kreutzberg, G.W. 1996). Although controversial, microglial cells come from mesodermal cells, probably of hematopoietic lineage (Ling, E.A. et al. 1993; Cuadros, M.A. et al. 1998; Cuadros, M. et al. 2000), sharing most surface molecules with bone-marrow derived macrophages (Flaris, N.A. et al. 1993). Markers that recognize microglia surface molecules can recognize monocytes and other tissue macrophages (Streit, W.J. 2001). PU.1 mRNA expression was detected in rainbow trout brain by the *in situ* hybridization technique, at the midbrain area of the rainbow trout brain. Besides the lack of a reliable stereotaxic atlas for rainbow trout brain, from anatomical brain reports (Riddle, D.R. et al. 1991; Pinuela, C. et al. 1992b, 1992a; Teitsma, C.A. et al. 1997; Yanez, J. et al. 1997; Teitsma, C.A. et al. 1999; Mazurais, D. et al. 2000; Folgueira, M. et al. 2002; Folgueira, M. et al. 2003; Menuet, A. et al. 2003; Folgueira, M. et al. 2004a, 2004b, 2005) PU.1 positive cell were localized in the nuclei fasciculus longitudinalis medialis and lateralis and (FLM and FLL). Although, the role of PU.1 in the FLM and FLL remains unclear, some possibilities for the PU.1 role in microglial cells have been suggested such as production and secretion of various factors, cell proliferation or the differentiation into the macrophage-like phenotype (Walton, M.R. et al. 2000). In conclusion, this is the first report of a key immune transcription factor, PU.1, related to neuroimmune system in the fish brain. However, the role of PU.1 in rainbow trout brain as well as its functions in microglial cells needs further investigation.

5. Microarray analysis

Gene chip technology allows for the analysis of large sets of genes, thereby providing a global and integrated vision of the physiological response of an organism to changes in its environment. Microarray analyses provide the unique possibility to address many cellular functions, metabolic and regulatory pathways in a single assay and compare contribution of functional categories in the transcriptomic response. In recent years, sequence data for salmonid species has greatly increased (Martin, S.A. et al. 2002; Rexroad, C.E. et al. 2003; Goetz, F.W. et al. 2004) providing basic resources for the construction of microarray platforms. Microarray technology provides a deeper understanding of overall cellular and tissular processes during immune activation. This thesis chapter addressed the potential of a newly developed rainbow trout cDNA microarray (Krasnov, A. et al. 2005a) to analyse an immune challenge. We investigated the response over time of rainbow trout head kidney and brain tissues *in vivo* to LPS IP injection.

5.1. Microarray in the head kidney

The gene expression profile in the head kidney of LPS challenged trout showed a percentage of 18% of significant differential expression under experimental treatment. From this total, 130 genes displayed a differential expression after 24 hours and 113 genes after 72 hours. A set of genes presented a stable up or down regulation. For instance, MHC class I gene, beta-2-microglobulin gene and HLA class II-associated protein (PHAPI2) were up regulated during 24 and 72 hours after LPS IP. In contrast, a respectable number of genes involved in the response of the innate immune system to pathogens were repressed in the head kidney: CXC chemokine receptor-4, complement D, and small inducible cytokine A7, and also several proteases, such as MMP-9, MMP-13, collagenase 3 and meprin A. Other genes like several translation-related genes, such as 40S ribosomal proteins, myeloid transcription factors, such as C/EBP alpha and several energy and metabolism related genes, such as enolase, transaldolase, glucose-6-phosphate isomerase and GAPDH were also down regulated 24 and 72 hours after LPS IP. Contrary, LPS induced nitric oxide synthase 2, involved in bactericidal responses (Lowenstein, C.J. et al. 1992), and the myeloperoxidase (MPO) precursor involved in microbiocidal responses 24 hours after LPS. Globally, LPS repressed genes were involved in the inflammatory and defence response, extracellular matrix (collagenases and proteases), several groups of peptidases, protein catabolism, carbohydrate metabolism, and binding of transient metals, especially zinc. Such changes in cellular activity could result from changes in the transcriptional profile of the cell possibly as a consequence of migration into other body compartments in

which exogenous factors dictate the cellular response (Mackenzie, S. et al. 2003). Head kidney microarray analysis of rainbow trout treated with IHNV (Infectious Hematopoietic Necrosis Virus) IP, induces the opposite response in which LPS IP were repressed (Mackenzie, S. et al. under submission). Microarray analysis reflect viral invasion to the head kidney, the main target organ (Romero, A. et al. 2005), whereas for LPS treated fish the invasion is in the peritoneal cavity. These results, suggest that immune cells required for coping with the LPS challenge in fish response may migrate from the head kidney to localize at the site of peritoneal inflammation.

Interestingly gene expression in the head kidney responds differently in certain immune related genes. 24 hours after LPS IP genes involved in the immunoglobulin system (IgM heavy chain), in cell proliferation process (cell cycle gene 1 protein and prothymosin alpha) and in the hematopoietic lineage (GRAP-2) were enhanced, whereas erythoid development related genes such the hemoglobin $\alpha, \beta, \gamma, \zeta$ chains and the transcription factor Jun-B, were repressed. On the contrary, at 72 hours post injection genes involved in hemoglobin synthesis, such as hemoglobin α and γ chains and the 5-aminolevulinate synthase as well as the granule cell differentiation protein were increased. Genes involved in cell proliferation process, such as cyclin B2 and genes involved in the immunoglobulin system (Ig gamma Fc receptor) were also up regulated. These gene expression results suggest that LPS enhances cell proliferation in the head kidney72 hours after LPS IP. This transcriptomic gene profile, is in concordance to the results found in animals injected with LPS and BrdU IP (chapter 5), which show a significant increase in the cell proliferation response.

In conclusion, microarray gene expression profiling suggested an increase in mechanisms involved in the organization of cytoplasm biogenesis and the initiation of cell proliferation processes in the head kidney. A large number of genes involved in the response of the innate immune system to pathogens were repressed in the head kidney although microbicidal genes were enhanced. Gene expression results suggest that LPS may enhance the progression of hematopoietic cells towards the erythroid lineage 72 hours post treatment, up regulating genes responsible for heme biosynthesis and hemoglobin genes.

5.2. Microarray in brain

Design of microarray analyses made it possible to compare the contribution of functional categories in the transcriptomic response of the brain tissue to other rainbow trout tissues. The transcriptional profile in rainbow trout brain after an immune challenge was studied. After 24 hours of treatment, trout brain presented

159 genes (11.5%) differentially expressed whereas after 72 hours no significant difference was found. In 24 h after treatment, the gene expression profile revealed that the largest groups of genes repressed corresponded to different ribosomal genes (20%) and cytochrome genes (10%). Genes involved in the inflammatory response, such as MPO, COX-1, COX-2, and chemokine receptor type 2 were also down regulated. However, brain responded to LPS challenge by increasing genes involved in the cytoskeleton, such as tubulin or beta actin, and the immune response such as MHCIi, alpha globin or adenosine desaminase. Gene Ontology analysis reveals that the brain copes with LPS challenge by down regulating metabolism, protein biosynthesis and mitochondrial related genes, whilst enhancing response to stimulus pathogen and cell cytoskeleton activity. Brain is the major protected organ in the organism because it is very sensitive to perturbations (Magistretti, P.J. 1999). Comparison of brain with other tissues, the transcriptional activity was not as high as in other immune related tissues, such head kidney or liver, and brain tissue presented more metabolic genes repressed. It is reported that the fish brain has the ability to survive prolonged periods without feeding, which was attributed to the generally low metabolic rate of fish and to the activation of gluconeogenic flux (Sheridan, M.A. et al. 1991; Blasco, J. et al. 1996). In conclusion, 24 hours after LPS IP, brain tissue down regulated metabolism and protein biosynthesis and enhanced genes involved in immune response to pathogens. After 72 hours of LPS IP brain tissue may be recovered.

6. Thesis overview

The main results of the present thesis can be integrated to a better understanding the stress and the immune responses in fish at a transcriptional level. The application of functional genomic tools, which encloses from using simple PCR analysis to more modern, sophisticate and fashionable microarray technique, allowed us to identified transcriptional regulations of certain set of genes which are enhanced or repressed under stress conditions. Our findings contribute to increase knowledge of molecular mechanism involved in coping the stress and immune responses in fish and provides a better understanding of fish physiology when fish health is threatened. Furthermore, thesis results may be interesting for aquaculture which looks for good biomolecular markers that may improve fish production and fish quality. The isolation, characterization and gene expression study with further microarray analysis of the enolase gene, allowed us to describe enolase as a possible biomolecular marker to determine fish welfare. The *in situ* hybridization study of the hematopoietic transcription factor PU.1, contributed to amplify the knowledge of the development of the fish immune system. Throughout this thesis,

DNA sequences and mRNA expression levels of several genes studied, have contributed to enlarged genomic fish database.

In summary, this thesis described from a transcriptional level, gene expression and molecular mechanisms activated or repressed when fish welfare is threatened and contributes to a better understanding of transcriptiomic mechanisms required to cope with the stress.

7. Thesis Conclusions

1) After 14 days of chronic confinement plasma cortisol levels increased in sea bream (*Sparus aurata*). Fish subjected to acute handling stress after 14 days of chronic confinement exposure show lower plasma cortisol.

2) Enolase gene has been partially cloned for the first time in sea bream by Differential Display. From Northern analysis a specific mRNA of 1.9 kb was obtained. An up regulation of enolase mRNA during both chronic confinement stress and after 48 hours of an LPS IP administration is observed, indicating the existence of an interconnection between neuro-immune-endocrine systems in fish.

3) Enolase microarray analysis from different fish experiments, show a large number of fish species and fish tissues in which enolase mRNAs are regulated. These findings suggest that enolase is directly involved in biological mechanisms when fish welfare is threatened.

4) Correlation in the expression profile of enolase and GADPH throughout different fish experiments in challenged fish, opens for the first time in fish, new insight into classical metabolic target genes with multiple non glycolytic functions, which may be considered as possible biomolecular fish markers.

5) Several enolase fragments have been cloned in different fish species: sea bream (*Sparus aurata*), rainbow trout (*Onchoryncus mykiss*), stickleback (*Culaea inconstans*), leather carp (*Cyprinus carpio*), mirror carp (*Cyprinus carpio carpio*). From phylogenetic analysis, a direct relationship between enolase isoforms and tissue specificity may not exist in fish.

6) Several immune related genes (PU.1, TNFRs, TNF Decoy receptor, 4-IBB, TNFα, MHCII Ii, CD83, CD209e, CCL4-like, Acute phase serum amyloid A, IL1-Receptor type II and IL1-Receptor-like) selected from mEST (macrophage cell cultures stimulated 12 hours by LPS, 10μg/ml) database were characterized and

studied (multiple alignment sequence and species homology percentages). Rainbow trout tissues and macrophage expression profile for each gene was performed using conventional and real time PCR. After following a selected criteria, PU.1 is the highly scored candidate gene for studying and better understanding fish immune system.

7) PU.1, a full-length cDNA transcript of 1237 nucleotides encoding a putative protein of 269 aminoacids was cloned for the first time in salmonid fish. PU.1 mRNA expression in the rainbow trout has an ubiquitous tissue distribution, with high expression in head kidney, heart and brain.

8) In rainbow trout, PU.1 expression levels were detected by *in situ* hybridization in head kidney cells with a large nucleus and little cytoplasm. PU.1 positive cells are distributed throughout the head kidney. PU.1 expression is different between individuals sample.

9) BrdU positive cells are distributed throughout the head kidney with a higher percentage of BrdU positive cells 72 hours after rainbow trout were injected with LPS IP.

10) ISH for PU.1 and IHC for BrdU results show that hematopoietic cells in the head kidney in rainbow trout are proliferating as a consequence of the immune LPS challenge, with a higher proliferation 72 hours after LPS IP challenge.

11) PU.1 mRNA expression is detected in the midbrain area of the rainbow trout brain, specifically in the nuclei fasciculus longitudinalis medialis and lateralis.

12) In the head kidney, LPS repressed Gene Ontology categories involved in the inflammatory and defence response, extracellular matrix (collagenases and proteases), several groups of peptidases, protein catabolism, carbohydrate metabolism, and binding of transient metals, and increased mechanisms involved in the organization of cytoplasm biogenesis and in the initiation of cell proliferation processes.

13) Gene expression results in the rainbow trout head kidney suggest that after 24 hours of LPS IP genes involved in the hemoglobin complex are inhibited, whereas 72 hours post treatment, LPS enhanced the progression of hematopoietic cells towards the erythroid lineage.

14) Rainbow trout brain copes with the LPS challenge by down regulating metabolism, protein biosynthesis and mitochondrial related genes, and enhances response to pathogen stimulus and cell cytoskeleton activity. Brain tissue is recovered 72 hours after treatment.

8. References

Aaronson, R.M., Graven, K.K., Tucci, M., Mcdonald, R.J. and Farber, H.W. (1995). Non-neuronal enolase is an endothelial hypoxic stress protein. *J Biol Chem*. 270(46): 27752-7.

Akashi, K., Traver, D., Miyamoto, T. and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*. 404(6774): 193-7.

Babbitt, P.C., Hasson, M.S., Wedekind, J.E., Palmer, D.R., Barret, W.C., Reed, G.H., Rayment, I., Ringe, D., Kenyon, G.L. and Gerelt, J.A. (1996). The enolase superfamily: a general strategy for enzyme catalyzed abstraction of the alpha-protons of carboxylic acids. *Biochemistry*. 35(enolasa): 16489-16501.

Bakri, Y., Sarrazin, S., Mayer, U.P., Tillmanns, S., Nerlov, C., Boned, A. and Sieweke, M.H. (2004). Balance of MafB and PU.1 specifies alternative macrophage or dendritic cell fate. *Blood*.

Barton, B.A., Morgan, J.D. and Vijayan, M.M. (2002). Physiological and condition-related indicators of environmental stress in fish. Biological Indicators of Aquatic Ecosystem Stress. S. M. Adams. Bethesda. MD. USA, American Fisheries Society**:** 111-148.

Besedovsky, H.O. and Del Rey, A. (1996). Immune-neuro-endocrine interactions: facts and hypotheses. *Endocr Rev*. 17(1): 64-102.

Blasco, J., Fernandez-Borras, J. and Marimon, I.R., A. (1996). Plasma glucose kinetics and tissue uptake in brown trout in vivo: effect of an intravascular glucose load. *J.Comp. Physiol. B*. 165: 534–541.

Celada, A., Borras, F.E., Soler, C., Lloberas, J., Klemsz, M., Van Beveren, C., Mckercher, S. and Maki, R.A. (1996). The transcription factor PU.1 is involved in macrophage proliferation. *J Exp Med*. 184(1): 61-9.

Chandran, V. and Luisi, B.F. (2006). Recognition of Enolase in the Escherichia coli RNA Degradosome. *J Mol Biol*.

Cuadros, M. and Navascués, J. (2000). Early origin and colonization of the developing central nervous system by microglial precursors. *Progress in Brain Research*. 132(microglia).

Cuadros, M.A. and Navascues, J. (1998). The origin and differentiation of microglial cells during development. *Prog Neurobiol*. 56(2): 173-89.

Dahl, R. and Simon, M.C. (2003). The importance of PU.1 concentration in hematopoietic lineage commitment and maturation. *Blood Cells Mol Dis*. 31(2): 229-33.

Ejeskar, K., Krona, C., Caren, H., Zaibak, F., Li, L., Martinsson, T. and Ioannou, P.A. (2005). Introduction of in vitro transcribed ENO1 mRNA into neuroblastoma cells induces massive cell death. *BMC Cancer*. 5(1): 161.

Feo, S., Arcuri, D., Piddini, E., Passantino, R. and Giallongo, A. (2000). ENO1 gene product binds to the c-myc promoter and acts as a transcriptional repressor: relationship with Myc promoter-binding protein 1 (MBP-1). *FEBS Lett*. 473(1): 47-52.

Fisher, R.C. and Scott, E.W. (1998). Role of PU.1 in hematopoiesis. *Stem Cells*. 16(1): 25-37.

Flaris, N.A., Densmore, T.L., Molleston, M.C. and Hickey, W.F. (1993). Characterization of microglia and macrophages in the central nervous system of rats: definition of the differential expression of molecules using standard and novel monoclonal antibodies in normal CNS and in four models of parenchymal reaction. *Glia*. 7(1): 34-40.

Folgueira, M., Anadon, R. and Yanez, J. (2003). Experimental study of the connections of the gustatory system in the rainbow trout, Oncorhynchus mykiss. *J Comp Neurol*. 465(4): 604-19.

Folgueira, M., Anadon, R. and Yanez, J. (2004a). An experimental study of the connections of the telencephalon in the rainbow trout (Oncorhynchus mykiss). I: Olfactory bulb and ventral area. *J Comp Neurol*. 480(2): 180-203.

Folgueira, M., Anadon, R. and Yanez, J. (2004b). Experimental study of the connections of the telencephalon in the rainbow trout (Oncorhynchus mykiss). II: Dorsal area and preoptic region. *J Comp Neurol*. 480(2): 204-33.

Folgueira, M., Anadon, R. and Yanez, J. (2005). Experimental study of the connections of the preglomerular nuclei and corpus mamillare in the rainbow trout, Oncorhynchusmykiss. *Brain Res Bull*. 66(4-6): 361-4.

Folgueira, M., Huesa, G., Anadon, R. and Yanez, J. (2002). The nucleus subglomerulosus of the trout hypothalamus is a link between chemosensory and visual systems: a DiI study. *Brain Res Bull*. 57(3-4): 427-30.

Galson, D.L., Hensold, J.O., Bishop, T.R., Schalling, M., D'andrea, A.D., Jones, C., Auron, P.E. and Housman, D.E. (1993). Mouse beta-globin DNA-binding protein B1 is identical to a proto-oncogene, the transcription factor Spi-1/PU.1, and is restricted in expression to hematopoietic cells and the testis. *Mol Cell Biol*. 13(5): 2929-41.

Gangenahalli, G.U., Gupta, P., Saluja, D., Verma, Y.K., Kishore, V., Chandra, R., Sharma, R.K. and Ravindranath, T. (2005). Stem cell fate specification: role of master regulatory switch transcription factor PU.1 in differential hematopoiesis. *Stem Cells Dev*. 14(2): 140-52.

Gerlt, J.A., Babbitt, P.C. and Rayment, I. (2005). Divergent evolution in the enolase superfamily: the interplay of mechanism and specificity. *Arch Biochem Biophys*. 433(1): 59-70.

Goetz, F.W., Iliev, D.B., Mccauley, L.A., Liarte, C.Q., Tort, L.B., Planas, J.V. and Mackenzie, S. (2004). Analysis of genes isolated from lipopolysaccharide-stimulated rainbow trout (Oncorhynchus mykiss) macrophages. *Mol Immunol*. 41(12): 1199-210.

Gornati, R., Papis, E., Rimoldi, S., Chini, V., Terova, G., Prati, M., Saroglia, M. and Bernardini, G. (2005). Molecular markers for animal biotechnology: sea bass (Dicentrarchus labrax, L.) HMG-CoA reductase mRNA. *Gene*. 344: 299-305.

Gornati, R., Terova, G., Vigetti, D., Mariangela, P., Saroglia, M. and Bernardini, G. (2003). Effects of population density on seabass (*Dientrarchus labrax*, L.) gene expression. *Aquaculture*.

Grinevich, V., Ma, X.M., Herman, J.P., Jezova, D., Akmayev, I. and Aguilera, G. (2001). Effect of repeated lipopolysaccharide administration on tissue cytokine expression and hypothalamic-pituitary-adrenal axis activity in rats. *J Neuroendocrinol*. 13(8): 711-23.

Haddad, J.J., Saade, N.E. and Safieh-Garabedian, B. (2002). Cytokines and neuro-immune-endocrine interactions: a role for the hypothalamic-pituitary-adrenal revolving axis. *J Neuroimmunol*. 133(1-2): 1-19.

Henkel, G.W., Mckercher, S.R. and Maki, R.A. (2002). Identification of three genes up-regulated in PU.1 rescued monocytic precursor cells. *Int Immunol*. 14(7): 723-32.

Holland, J.W., Pottinger, T.G. and Secombes, C.J. (2002). Recombinant interleukin-1β activates the hypothalamic-pituitary-interrenal axis in rainbow trout, *Oncorhynchus mykiss*. *Journal of Endocrinology*. 175: 261-267.

Imagawa, T., Kitagawa, H. and Uehara, M. (1995). The innervation of the chromaffin cells in the head-kidney of the carp, Cyprinus carpio; regional differences of the connections between nerve endings and chromaffin cells. *Journal of Anatomy*. 188: 149-156.

Ishitani, R. and Chuang, D.M. (1996). Glyceraldehyde-3-phosphate dehydrogenase antisense oligodeoxynucleotides protect against cytosine arabinonucleoside-induced apoptosis in cultured cerebellar neurons. *Proc Natl Acad Sci U S A*. 93(18): 9937-41.

Iwasaki, H., Somoza, C., Shigematsu, H., Duprez, E.A., Iwasaki-Arai, J., Mizuno, S., Arinobu, Y., Geary, K., Zhang, P., Dayaram, T., Fenyus, M.L., Elf, S., Chan, S., Kastner, P., Huettner, C.S., Murray, R., Tenen, D.G. and Akashi, K. (2005). Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood*. 106(5): 1590-600.

Jeffery, C.J. (1999). Moonlighting proteins. *Trends Biochem Sci*. 24(1): 8-11.

Kherrouche, Z., Beuscart, A., Huguet, C., Flourens, A., Moreau-Gachelin, F., Stehelin, D. and Coll, J. (1998). Isolation and characterization of a chicken homologue of the Spi-1/PU.1 transcription factor. *Oncogene*. 16(10): 1357-67.

Klemsz, M.J., Mckercher, S.R., Celada, A., Van Beveren, C. and Maki, R.A. (1990). The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. *Cell*. 61(1): 113-24.

Krasnov, A., Koskinen, H., Pehkonen, P., Rexroad, C.E., 3rd, Afanasyev, S. and Molsa, H. (2005a). Gene expression in the brain and kidney of rainbow trout in response to handling stress. *BMC Genomics*. 6(1): 3.

Krasnov, A., Koskinen, H., Rexroad, C., Afanasyev, S., Molsa, H. and Oikari, A. (2005b). Transcriptome responses to carbon tetrachloride and pyrene in the

kidney and liver of juvenile rainbow trout (Oncorhynchus mykiss). *Aquat Toxicol*. 74(1): 70-81.

Kreutzberg, G.W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends Neurosci*. 19(8): 312-8.

Ling, E.A. and Wong, W.C. (1993). The origin and nature of ramified and amoeboid microglia: a historical review and current concepts. *Glia*. 7(1): 9-18.

Lloberas, J., Soler, C. and Celada, A. (1999). The key role of PU.1/SPI-1 in B cells, myeloid cells and macrophages. *Immunol Today*. 20(4): 184-9.

Locksley, R.M., Killeen, N. and Lenardo, M.J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell*. 104(4): 487-501.

Lowenstein, C.J., Glatt, C.S., Bredt, D.S. and Snyder, S.H. (1992). Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proc Natl Acad Sci U S A*. 89(15): 6711-5.

Mackenzie, S., Iliev, D., Liarte, C., Koskinen, H., Planas, J.V., Goetz, F.W., Molsa, H., Krasnov, A. and Tort, L. (2005). Transcriptional analysis of LPS-stimulated activation of trout (Oncorhynchus mykiss) monocyte/macrophage cells in primary culture treated with cortisol. *Mol Immunol*.

Mackenzie, S., Planas, J.V. and Goetz, F.W. (2003). LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and in vitro differentiated macrophages. *Dev Comp Immunol*. 27(5): 393-400.

Mackenzie, S., Roher, N., Novoa, B., Liarte, C., Ribas, L., Romero, A., Planas, J.V., Tort, L., Goetz, F.W., Krasnov, K. and Figueras, A. (under submission). Transcriptomic analysis of the head kidney response in trout, O.mykiss, to bacterial and viral challenge.

Magistretti, P.J. (1988). Regulation of glycogenolysis by neurotransmitters in the central nervous system. *Diabete Metab*. 14(3): 237-46.

Magistretti, P.J. (1999). Brain energy metabolis. Fundamental Neuroscience. M. J. Zigmond, F. E. Bloom, S. C. Landis, J. L. Roberts and L. R. Squire. San Diego, Academic Press**:** 389–413.

Martin, S.A., Caplice, N.C., Davey, G.C. and Powell, R. (2002). EST-based identification of genes expressed in the liver of adult Atlantic salmon (Salmo salar). *Biochem Biophys Res Commun*. 293(1): 578-85.

Mazurais, D., Le Drean, G., Brierley, I., Anglade, I., Bromage, N., Williams, L.M. and Kah, O. (2000). Expression of clock gene in the brain of rainbow trout: comparison with the distribution of melatonin receptors. *J Comp Neurol*. 422(4): 612-20.

Menuet, A., Anglade, I., Le Guevel, R., Pellegrini, E., Pakdel, F. and Kah, O. (2003). Distribution of aromatase mRNA and protein in the brain and pituitary of female rainbow trout: Comparison with estrogen receptor alpha. *J Comp Neurol*. 462(2): 180-93.

Meseguer, J., López-Ruiz, A. and García-Ayala, A. (1995). Reticulo-endothelial stroma of the head-kidney from the seawater teleost gilthead seabream (Sparus aurata L): an ultrastructural and cytochemical study. *Anat Rec*. 241: 303-309.

Milano, E.G., Basari, F. and Chimenti, C. (1997). Adrenocortical and Adrenomedullary Homologs in Eight Species of Adult and Developing Teleosts: Morphology, Histology, and Immunohistochemistry. *General and Comparative Endocrinology*. 108: 483-496.

Miles, L.A., Dahlberg, C.M., Plescia, J., Felez, J., Kato, K. and Plow, E.F. (1991). Role of cell-surface lysines in plasminogen binding to cells: identification of alpha-enolase as a candidate plasminogen receptor. *Biochemistry*. 30(6): 1682-91.

Oliva, D., Barba, G., Barbieri, G., Giallongo, A. and Feo, S. (1989). Cloning, expression and sequence homologies of cDNA for human gamma enolase. *Gene*. 79(2): 355-60.

Pancholi, V. (2001). Multifunctional alpha-enolase: its role in diseases. *Cell Mol Life Sci*. 58(7): 902-20.

Pertea, G., Huang, X., Liang, F., Antonescu, V., Sultana, R., Karamycheva, S., Lee, Y., White, J., Cheung, F., Parvizi, B., Tsai, J. and Quackenbush, J. (2003). TIGR Gene Indices clustering tools (TGICL): a software system for fast clustering of large EST datasets. *Bioinformatics*. 19(5): 651-2.

Pinuela, C., Baatrup, E. and Geneser, F.A. (1992a). Histochemical distribution of zinc in the brain of the rainbow trout, Oncorhynchos myciss. I. The telencephalon. *Anat Embryol (Berl)*. 185(4): 379-88.

Pinuela, C., Baatrup, E. and Geneser, F.A. (1992b). Histochemical distribution of zinc in the brain of the rainbow trout, Oncorhynchos myciss. II. The diencephalon. *Anat Embryol (Berl)*. 186(3): 275-84.

Redlitz, A., Fowler, B.J., Plow, E.F. and Miles, L.A. (1995). The role of an enolase-related molecule in plasminogen binding to cells. *Eur J Biochem*. 227(1-2): 407-15.

Rexroad, C.E., Lee, Y., Keele, J.W., Karamycheva, S., Brown, G., Koop, B., Gahr, S.A., Palti, Y. and Quackenbush, J. (2003). Sequence analysis of a rainbow trout cDNA library and creation of a gene index. *Cytogenet Genome Res*. 102(1-4): 347-54.

Ribas, L., Planas, J.V., Barton, B., Monetti, C., Bernardini, G., Saroglia, M., L., T. and Mackenzie, S. (2004). A differentially expressed enolase gene isolated from the gilthead sea bream (Sparus aurata) under high-density conditions is up-regulated in brain after in vivo lipopolysaccharide challenge. *Aquaculture*. 241: 195-206.

Riddle, D.R. and Oakley, B. (1991). Evaluation of projection patterns in the primary olfactory system of rainbow trout. *J Neurosci*. 11(12): 3752-62.

Romero, A., Figueras, A., Tafalla, C., Thoulouze, M.I., Bremont, M. and Novoa, B. (2005). Histological, serological and virulence studies on rainbow trout experimentally infected with recombinant infectious hematopoietic necrosis viruses. *Dis Aquat Organ*. 68(1): 17-28.

Rotllant, J. and Tort, L. (1997). Cortisol and glucose response after acute stress by net handling in the sparid red porgy previously subjected to crowding stress. *Fish Biology*. 51: 21-28.

Santini, D., Poli, F. and Lega, S. (2006). Solid-papillary tumors of the pancreas: histopathology. *Jop*. 7(1): 131-6.

Scapigliati, G., Romano, N., Buonocore, F., Picchietti, S., Baldassini, M.R., Prugnoli, D., Galice, A., Meloni, S., Secombes, C.J., Mazzini, M. and Abelli, L. (2002). The immune system of sea bass, Dicentrarchus labrax, reared in aquaculture. *Dev Comp Immunol*. 26(2): 151-60.

Shashidharan, P., Chalmers-Redman, R.M., Carlile, G.W., Rodic, V., Gurvich, N., Yuen, T., Tatton, W.G. and Sealfon, S.C. (1999). Nuclear translocation of GAPDH-GFP fusion protein during apoptosis. *Neuroreport*. 10(5): 1149-53.

Sheridan, M.A. and Mommsen, T.P. (1991). Effects of nutritional state on in vivo lipid and carbohydrate metabolism of coho salmon, Oncorhynchus kisutch. *Gen Comp Endocrinol*. 81(3): 473-83.

Sirover, M.A. (1996). Minireview. Emerging new functions of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in mammalian cells. *Life Sci*. 58(25): 2271-7.

Sirover, M.A. (1999). New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochim Biophys Acta*. 1432(2): 159-84.

Soengas, J.L., Strong, E.F. and Andrés, M.D. (1998a). Glucose, lactate, and beta-hydorxybutyrate utilitzation by rainbow trout brain: changes during food deprimvation. *Physiol. Zool.* 71: 285-293.

Sternberg, E.M. (2001). Neuroendocrine regulation of autoimmune/inflammatory disease. *Journal of Endocrinology*. 169: 429-435.

Streit, W.J. (2001). Microglia and macrophages in the developing CNS. *Neurotoxicology*. 22(5): 619-24.

Subramanian, A. and Miller, D.M. (2000). Structural analysis of alpha-enolase. Mapping the functional domains involved in down-regulation of the c-myc protooncogene. *J Biol Chem*. 275(8): 5958-65.

Takashima, F. and Hibiya, T. (1995). Normal and pathological Features. An Atlas of Fish Histology. Tokyo: Kodanska LDT**:** 195.

Teitsma, C.A., Anglade, I., Lethimonier, C., Le Drean, G., Saligaut, D., Ducouret, B. and Kah, O. (1999). Glucocorticoid receptor immunoreactivity in neurons and pituitary cells implicated in reproductive functions in rainbow trout: a double immunohistochemical study. *Biol Reprod*. 60(3): 642-50.

Teitsma, C.A., Bailhache, T., Tujague, M., Balment, R.J., Ducouret, B. and Kah, O. (1997). Distribution and expression of glucocorticoid receptor mRNA in the forebrain of the rainbow trout. *Neuroendocrinology*. 66(4): 294-304.

Thorgaard, G.H., Bailey, G.S., Williams, D., Buhler, D.R., Kaattari, S.L., Ristow, S.S., Hansen, J.D., Winton, J.R., Bartholomew, J.L., Nagler, J.J., Walsh, P.J., Vijayan, M.M., Devlin, R.H., Hardy, R.W., Overturf, K.E., Young, W.P., Robison, B.D., Rexroad, C. and Palti, Y. (2002). Status and opportunities for genomics research with rainbow trout. *Comp Biochem Physiol B Biochem Mol Biol*. 133(4): 609-46.

Tondravi, M.M., Mckercher, S.R., Anderson, K., Erdmann, J.M., Quiroz, M., Maki, R. and Teitelbaum, S.L. (1997). Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. *Nature*. 386(6620): 81-4.

Tracy, M.R. and Hedges, B.S. (2000). Evolutionary history of the enolase gene family. *Gene*. 259(enolasa): 129-138.

Veiga-Malta, I., Duarte, M., Dinis, M., Tavares, D., Videira, A. and Ferreira, P. (2004). Enolase from Streptococcus sobrinus is an immunosuppressive protein. *Cell Microbiol*. 6(1): 79-88.

Voet, D. and Voet, J.G. (1990). Bioquímica. P. Lane. Barcelona, Omega. 6**:** 456-494.

Vuori, K.A., Koskinen, H., Krasnov, A., Koivumaki, P., Afanasyev, S., Vuorinen, P.J. and Nikinmaa, M. (2006). Developmental disturbances in early life stage mortality (M74) of Baltic salmon fry as studied by changes in gene expression. *BMC Genomics*. 7(1): 56.

Walton, M.R., Gibbons, H., Macgibbon, G.A., Sirimanne, E., Saura, J., Gluckman, P.D. and Dragunow, M. (2000). PU.1 expression in microglia. *J Neuroimmunol*. 104(2): 109-15.

Ward, A.C., Mcphee, D.O., Condron, M.M., Varma, S., Cody, S.H., Onnebo, S.M., Paw, B.H., Zon, L.I. and Lieschke, G.J. (2003). The zebrafish spi1 promoter drives myeloid-specific expression in stable transgenic fish. *Blood*. 102(9): 3238-40.

Yanez, J., Rodriguez, M., Perez, S., Adrio, F., Rodriguez-Moldes, I., Manso, M.J. and Anadon, R. (1997). The neuronal system of the saccus vasculosus of trout (Salmo trutta fario and Oncorhynchus mykiss): an immunocytochemical and nerve tracing study. *Cell Tissue Res*. 288(3): 497-507.

Zapata, A. (1979). Ultrastructural study of the teleost fish kidney. *Dev Comp Immunol*. 3(1): 55-65.

Zapata, A.G., Chibá, A. and A., V. (1996). Cells and tissues of the immune system of fish. The fish immune system. Organism, pathogen and environment. G. Iwama and T. Nakanishi, Academic Press.

Zheng, L., Roeder, R.G. and Luo, Y. (2003). S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component. *Cell*. 114(2): 255-66.

Annex

Gene	Primer	Tm (ºC)	Sequence
PU.1	"Forward"	55	5'-GTAACAAGGGGAGAATTGGGC-3'
	"Reverse"		5'-GTGAGACCTCCCCCCTAGCAC-3'
TNFRV	"Forward"	55	5'-GGGGTGGAAATGTGCTGTGAGC-3'
	"Reverse"		5'-GGTCACCACTAGTCCCGCCC-3'
TNFR2	"Forward"	52	5'-CGGGAACAGCAGCAATGTTTG-3'
	"Reverse"		AACATAATTTCCACTCTCCTG
Decoy receptor	"Forward"	59,5	5'-CGACAGGGGATTCCCTTACGTGCG-3'
	"Reverse"		5'-GCCAGGTATAGTGGTGCCACC-3'
4-1BB	"Forward"	56	5'-GTCTGCTGTGAAAGGTGCAATCC-3'
	"Reverse"		5'-GTCATTGAAGGTCCCATCTGGAC-3'
TNFα	"Forward"	55	5'-TCGTTCAGAGTCTCCTGCAG-3'
	"Reverse"		5'-AAGAATTCTTAAAGTGCAAACACACCAAA-3'
CD209	"Forward"	50	5'-ATGGAGGAGGTAGAGAACTATACC-3'
	"Reverse"		5'-CGTCTAGCCGAATCCCATCCAT-3'
CD83	"Forward"	55	5'-ATGTTTTTTCAACTCGTCTGC-3'
	"Reverse"		5'-GCACACAGACATGTTTTATGG-3'
CCL4	"Forward"	50.5	5'-GTGCCATCACTTTTACCG-3'
	"Reverse"		5'-TATCTATTTCCCCAGACCC-3
MHCII	"Forward"	51	5'-TTGAGAGAACTTGGACGC-3'
	"Reverse"		5'-TGGTGCCCTGGATGACTTTG-3'
IL1RII	"Forward"	57	5'-GCTCCTCCCAGCTCGAACTT-3'
	"Reverse"		5'-AGGGTTCGGGGTTAGGGTTG-3'
IL1R-like	"Forward"	59	5'-GGGTCCTGCCCTCAGAGGTC-3'
	"Reverse"		5'-GGGAGTGGGTGAAGGAGGGGG-3'
APS	"Forward"	58	5'-CAGTGGTACCGCTTCCCTGG-3'
	"Reverse"		5'-GTGGTTAGCCTTCTGGTCAGC-3'
ENO	"Forward"	55	5'-AGGCCAAGTACGGAAAGGAT-3'
	"Reverse"		5'-GAGGAGCAGGCAGTTACAGG-3'
β-actin	"Forward"	55	5'-CGATCACACCTTCTACAACGAGCT-3'
	"Reverse"		5'-CTGCTCGAAGTCCAGGGCGACGTA-3'
S18	"Forward"	57	5'-CGAGCAATAACAGGTCTGTG-3'
	"Reverse"		5'-GGCAGGGACTTAATCAA-3'

Table 1. Candidate genes information table with designed primer sequences and annealing temperatures.

Putative Function	EST Accession	Size	Blastx similarity	Species most similar to	Accession of similar
PU.1	BX885779	893	3 e-87	lamprey	AAF78908
TNFRV	BX316950	731	3 e-77	trout	CAD57165
TNFR2-like	BX888381	802	4 e-5	flounder	BAC65226
TNF-α	CF752134	825	1 e-121	trout	CAB92316
TNF-DR	BX865673	761	4 e-45	trout	AAD56428
4-1BB-like	022404L4	779	3 e-24	fugu	CAG03988
CD209e	CF752985	747	5 e-12	mouse	NP570975
CD83	CF752979	854	7 e-17	shark	AAO62993
CCL4-like	BX881152	795	5 e-12	monkey	AAN76071
MHCII Ii	CF752515	850	1 e-143	trout	AAL91668
IL1R-II	CF752524	769	1 e-132	trout	CAC19715
IL1R-like	CA343457	661	1 e-16	fugu	CAG02914
APS	CF752696	624	3 e-50	trout	CAA67766
Enolase	BX320000	694	1 e-59	human	AAA52388
	BX310988	610	2 e-71	sea bream	AAO92646
	BX303009	595	9 e-75	zebrafish	AAQ97775
	BX302680	653	2 e-93	human	CAA31512
	BX298597	649	2 e-93	virtual	AAP88878
	BX855755	770	2,4	turtle	AAD20345

Table 2. Candidate genes table selected from the EST macrophage cell culture database. Similar blastx against NCBI nr database with the score e value is shown.

	Brain			Head Kidney			Gills			Muscle			Macrophages						n° clones	bp	A.Number	Putative Function
	C	24 h	72 h	C	24 h	72 h	C	24 h	72 h	C	24 h	72 h	C	6 h	12 h	18 h	24 h	30 h				
PU.1	++	+++	+++	+++	+++	+++	/	/	/	+	++	+	+++	+++	+++	+++	+++	+++	4	707	AAF78908	Transcriptor factor
TNFRV	-	/	/	/	/	/	/	/	/	/	/	/	+	++	++	++	++	++	5	736	CAD57165	Immune receptor
TNFR2-like	+	+	+	+	+	+	++	+	+	-	+	-	+	-	-	+	+	+	1	802	BX888381	Immune receptor
TNF-DR	-	+	+	/	/	/	/	/	/	/	/	/	++	+++	+++	+++	/	+++	3	788	AAD56428	Immune receptor
4-1BB-like	-	/	/	/	/	/	/	/	/	/	/	/	++	++	++	++	++	++	1	779	CA354408	T-cell immune receptor
TNF-α	+	++	++	+	+	+	+++	++	++	+	++	+	/	/	/	/	/	/	1	825	CAB92316	Cytokine
CD209e	+	++	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	1	747	CF752985	Antigen
CD83	+	++	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	1	854	CF752979	Antigen
CCL4-like	+	++	++	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	4	795	AAN76071	Chemokine
MHCII Ii	+++	+++	+++	+++	+++	+++	/	/	/	+	++	++	/	/	/	/	/	/	1	850	CF752515	Antigen
IL1R-II	+	/	/	/	/	/	/	/	/	/	/	/	+	++	++	++	+	+	1	769	CF752524	Immune receptor
IL1R-like	-	/	/	/	/	/	/	/	/	/	/	/	++	++	++	++	++	++	1	566	CA343457	Immune receptor
APS	+++	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	4	624	CF752696	Serum amyloid protein
Enolase	++	++	++	+++	++	++	/	/	/	+++	+++	+++	/	/	/	/	/	/	2	794	AAO92646	Glycolitic Enzyme
β-Actin/S18	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	/	/	/	House keeping
	RT-PCR												Real Time PCR									

Table 3. PCR results for all candidate genes tested. This table shows conventional RT-PCR results to different tissues from rainbow trout subjected to LPS IP (6mg/kg) administration (N=6), 24 and 72 hours after treatment. Real time PCR results of *in vitro* macrophage cell cultures at different LPS incubation period. Expression levels are represented with symbols: +++: high expression; ++; expression detected, but not high; +: low expression; -: expression not detected; /: expression not studied

MHCII invariant chain (%)	Rainbow Trout (AY081776)		Accession Number
	Similarity	Identity	
Chinese perch	78	56	AAS77256
Carp	60	37	BAC53767
Zebrafish	60	34	NP571665
Caiman	57	33	ABB22797

Table 4. Homology table of the PU.1 protein from rainbow trout compared to other PU.1 protein species.

TNFRV (%)	Rainbow Trout		Accession Number
	Similarity	Identity	
TNFR CAD57165 RT	96	91	CAD57165
TNFR2-like RT	31	12	BX888381
TNFR2 CA377157 RT	32	19	CA377157
TNFR Fugu	61	38	CAAE01014543
TNFR Zebrafish	36	18	AAG24365
TNFR1 Flounder	40	18	BAC65225
TNFR2 Flounder	37	20	BAC65226
TNFR1 Mouse	37	19	AAA39751
TNFR2 Mouse	40	19	M60469
TNFR1 Rat	37	18	AAA42256
TNFR2 Rat	37	19	AF498039.2
TNFR1 Human	36	18	CAA39021
TNFR2 Human	39	20	U52165.1

Table 5. Homology table of the TNFRV putative protein from rainbow trout compared to other TNFR protein species.

MHCII invariant chain (%)	Rainbow Trout (AY081776)		Accession Number
	Similarity	Identity	
Chinese perch	78	56	AAS77256
Carp	60	37	BAC53767
Zebrafish	60	34	NP571665
Caiman	57	33	ABB22797

Table 6. Homology table of the MHCII Ii protein from rainbow trout compared to other MHCII Ii protein species.

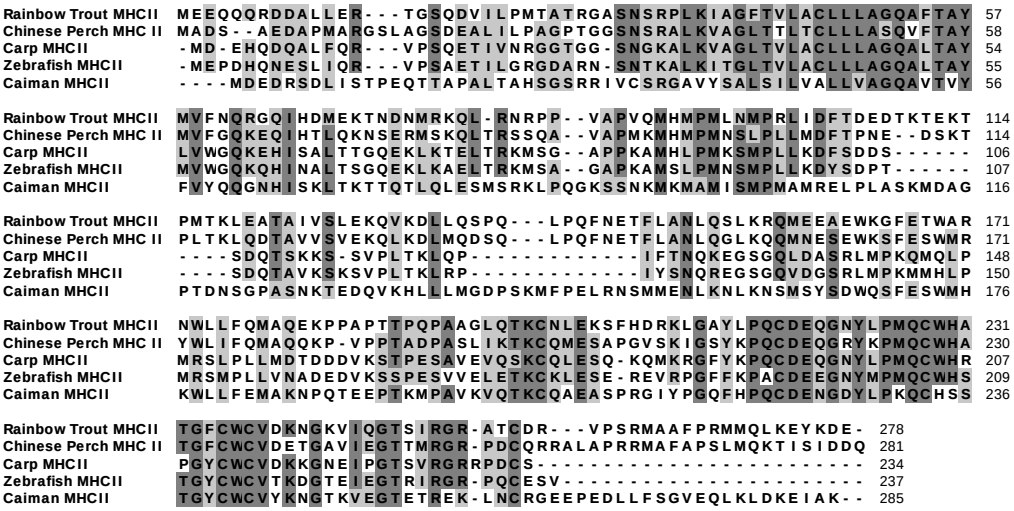


Fig.1. MHCII Ii multiple sequence alignment by ClustalW (Thompson, J.D. et al. 1994) with 70% threshold. Accession numbers are indicated in Table 6.

CCL4-like (%)	Rainbow Trout (AY561709)		Accession Number
	Similarity	Identity	
Zebrafish	77	48	XP696742
Pig	63	40	CAC84398
Monkey	64	38	AAN76071
Human	64	34	P13236

Table 7. Homology table of the CCL4-like protein from rainbow trout compared to other CCL4-like protein species.

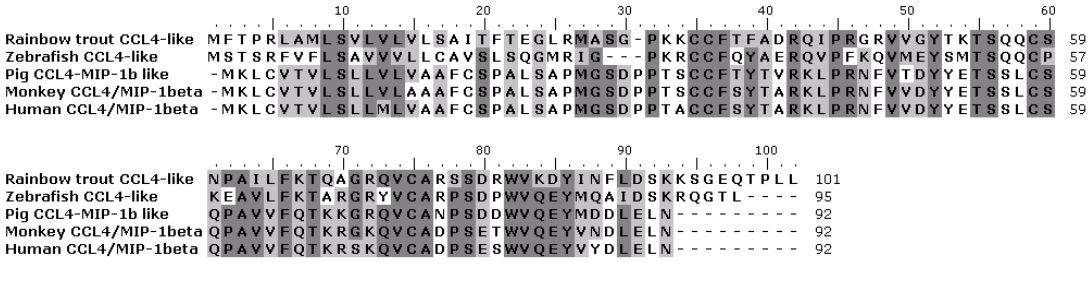


Fig.2. CCL4-like multiple sequence alignment by ClustalW (Thompson, J.D. et al. 1994) with 70% threshold. Accession numbers are indicated in table 7.

CD209e (%)	Rainbow Trout (AY593994)		Accession Number
	Similarity	Identity	
Atlantic salmon c-type lectin receptor A	97	95	AAT77220
Zebrafish similar to CD209	26	14	XP687353
Mouse CD209e	27	49	AAI07190
Mouse CD209a	25	49	NP573501
Human CD209	18	34	NP066978

Table 8. Homology table of the CD209e protein from rainbow trout compared to other CD209 protein species.

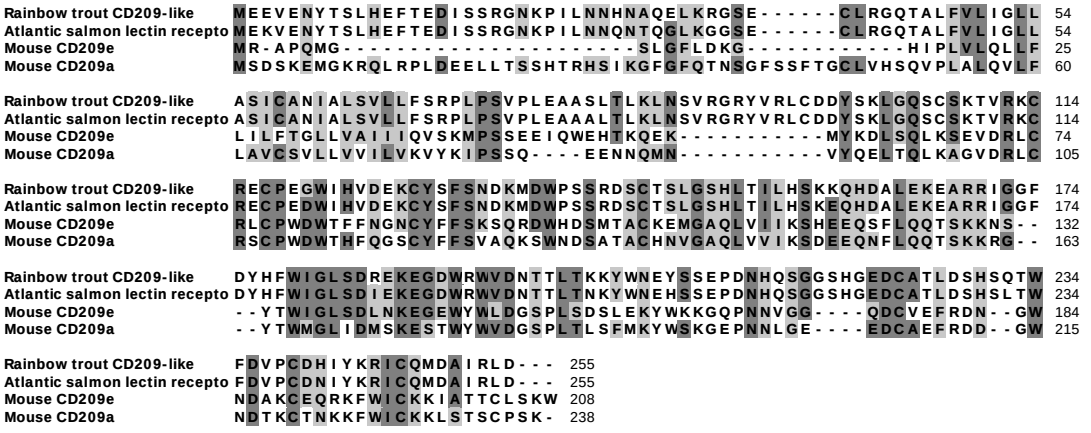


Fig.3. CD209-like multiple sequence alignment by ClustalW (Thompson, J.D. et al. 1994) with 70% threshold. Accession numbers are indicated in Table 8.

CD83 (%)	Rainbow Trout (CF752979)		Accession Number
	Similarity	Identity	
Rainbow trout	99	98	AAP93912
Nurse shark	56	29	AAO62993
Flounder	66	45	AU091120
Zebrafish	63	37	BI671547/BM157226
Mouse	50	25	CAB63843
Rat	42	24	XP225224
Human	50	26	CAB96723

Table 9. Homology table of the CD83 protein from rainbow trout compared to other CD83 protein species.

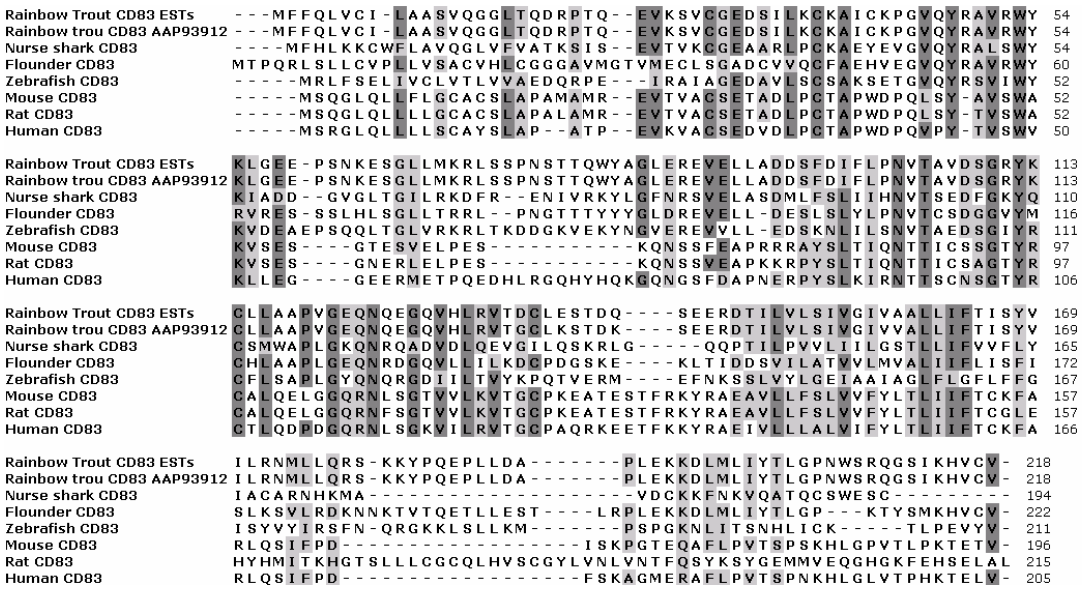


Fig.4. CD83 multiple sequence alignment by ClustalW (Thompson, J.D. et al. 1994) with 70% threshold. Accession numbers are indicated in Table 9.

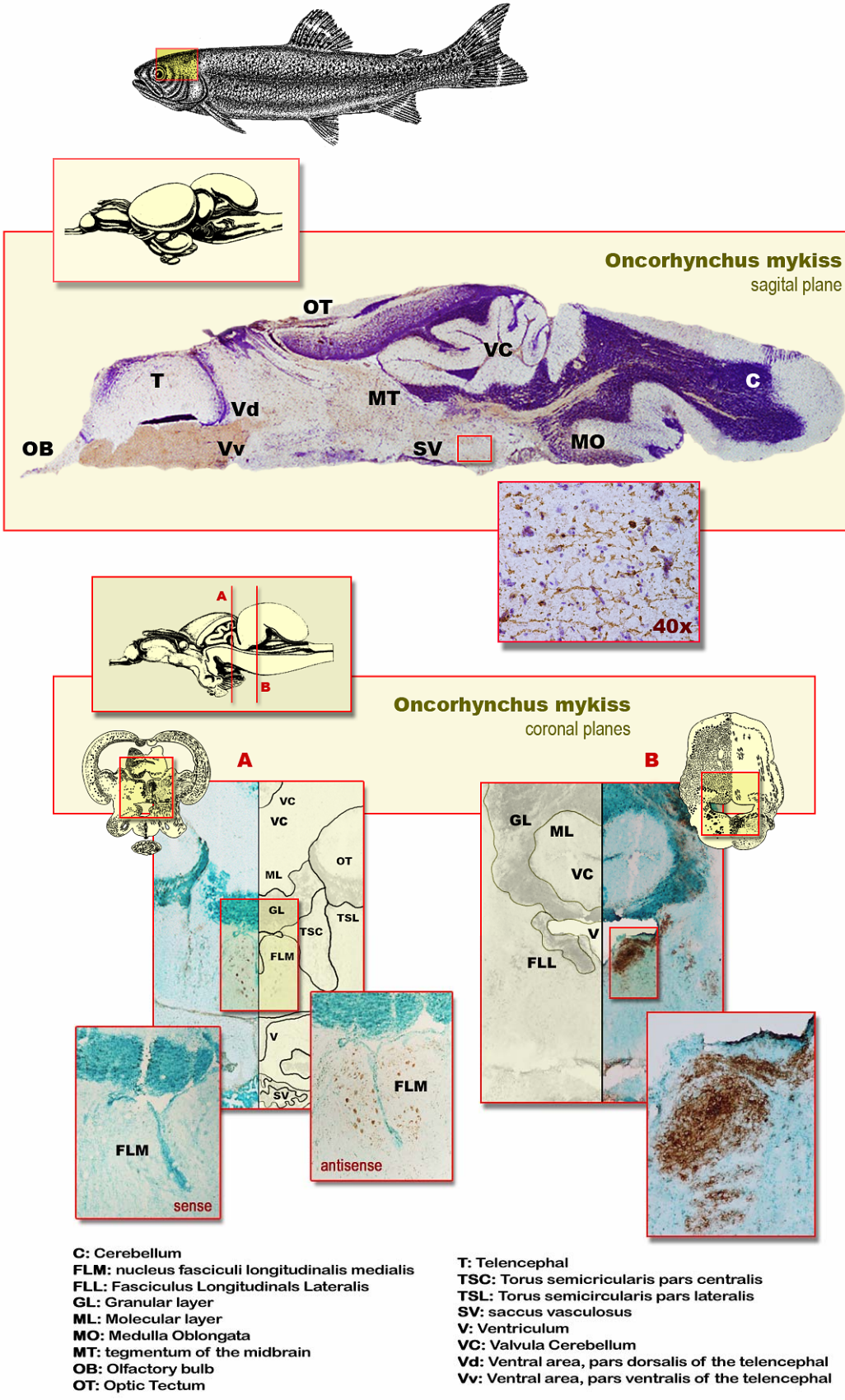


Fig.5. Microglial cell distribution in control brain of rainbow trout, longitudinal sections. Lectin histochemistry was used to for detection of microglial cells.

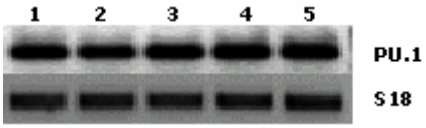
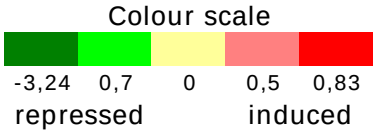


Fig.6. Conventional RT-PCR of PU.1 gene of the head kidney from six individually rainbow trout. 30 cycles, 296 bp PCR product length on total RNA (4 µg) extracted from each individual samples. Samples were separated on ethidium bromide-agarose gels. S18 was used as a house keeping gene.



Brain response to other tissues 24H LPS IP								
Category	Gene Ontology	Brain	Kidney	Liver	Ovary	Red muscle	White muscle	Nº genes
metabolism	collagen catabolism							3
	translation							8
	ATP synthesis coupled electron transport							5
	protein biosynthesis							41
cellular process	microtubule-based process							5
	microtubule							4
	carrier activity							17
	primary active transporter activity							16
	mitochondrial electron transport chain							6
	proton transport							3
	mitochondrial membrane							9
	electron transport							21
cellular components	nucleolus							5
	extracellular space							9
	ribosome							31
	mitochondrion							25
molecular function	antioxidant activity							4
	metalloendopeptidase activity							5
	metallopeptidase activity							7
	iron ion binding							3
	sodium ion transporter activity							6
	lipid binding							7
	sodium ion transporter activity							6
response to stimulus	response to pest, pathogen or parasite							3

Table 10. Gene Ontology analysis comparing brain tissue responses to other rainbow trout tissue after 24 hours of a LPS IP. Differentially expressed genes were grouped by Gene Ontology categories and mean log(expression ratio) were analyzed by Student's *t*-test (P<0,05); the expression ratio is coded with colour scale

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Effects of chronic confinement on physiological responses of juvenile gilthead sea bream, *Sparus aurata* L., to acute handling

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Abstract

Understanding how gilthead sea bream, *Sparus aurata* L., an important Mediterranean Sea species for aquaculture, respond physiologically to stressors commonly encountered in intensive rearing is important for effective production, as managing for stress is a major factor in maintaining healthy fish stocks. Our objective was to determine whether holding juvenile gilthead sea bream at a high density (HD), as a chronic stressor, would affect their physiological responses to a subsequent acute handling stressor. After acclimation at a low density (LD) of 6 kg m^{-3} in 200-L circular tanks containing 33–36 g L^{-1} recirculating seawater at 19 °C under a normal photoperiod, juvenile 37-g gilthead sea bream were confined for 14 days at a HD of 26 kg m^{-3} and then subjected to 30-s aerial emersion in a dipnet. Plasma levels of cortisol, glucose, lactate, osmolality and chloride were determined in fish held in separate lots during LD (control) and HD confinement at 0, 1, 2, 7 and 14 days, and then after handling at 0, 1, 2, 4 and 8 h. Although plasma cortisol levels were similar in LD and HD fish groups after 14 d of confinement (15 and 23 ng mL^{-1}, respectively), the cortisol response in fish from the HD treatment at 1 and 2 h following acute handling (70 and 37 ng mL^{-1}, respectively) was only about half of that measured in the control group (139 and 102 ng mL^{-1}); plasma cortisol was similar in both groups by 4 and 8 h. In contrast, plasma glucose elevations in response to handling were higher at 4 and 8 h in the HD-held fish (94 and 72 mg dL^{-1}, respectively) than in those from the LD treatment (59 and 51 mg dL^{-1}); glucose responses were similar in both groups at 1 and 2 h after handling and throughout confinement. Plasma lactate levels were higher in LD fish than in the HD group at the beginning of the experiment but were similar after 14 d confinement and responses to handling were similar (e.g. 33 and 35 mg dL^{-1} at 1 h). Plasma osmolality showed increases during the first 2 h after acute handling but no differences were evident between the two density treatments at any time during confinement or posthandling. Plasma chloride levels did not change throughout the experiment. The reduced plasma cortisol response to acute handling likely resulted from negative feedback of mildly but chronically elevated circulating cortisol caused by the confinement stressor on the hypothalamic–pituitary–interrenal axis. While other post-handling physiological changes also showed differences between treatment groups, the suppressed cortisol response in the HD-held fish suggests a reduction in the gilthead sea bream's normal capacity to respond to an acute stressor.

Keywords: stress, handling, confinement, cortisol, glucose, lactate, sea bream, *Sparus aurata*

Introduction

Characteristic endocrine and other physiological responses of teleostean fishes to stressors typically encountered in aquaculture have been studied thoroughly during the last two decades (Barton & Iwama 1991; Iwama, Pickering, Sumpter & Schreck 1997; Wendelaar Bonga 1997). Such responses

include elevations in plasma concentrations of cortisol, glucose, lactate and, in marine species, osmolality and major ions, and have become well established as useful indicators of the degree of acute stress experienced by fish (Barton, Morgan & Vijayan 2002). These stress responses, particularly changes in circulating cortisol, however, can be modified extensively by genetic, developmental and internal and external environmental factors (Barton 2002). Prior exposure to other stressors can appreciably alter the response of cortisol to acute stress, for example. The presence of pollutants or contaminants in the water sufficient to chronically elevate plasma levels of cortisol in fish has been shown to attenuate the response to an acute stressor (Hontela, Rasmussen, Audet & Chevalier 1992; Norris, Donahue, Dores, Lee, Laldonado, Ruth & Woodling 1999; Laflamme, Couillard, Campbell & Hontela 2000). This effect possibly occurs as a result of either down-regulation of the hypothalamic–pituitary–interrenal (HPI) axis from continuous negative feedback of cortisol or direct toxic action of the chemical stressor on the axis' functional integrity (Hontela 1997). A reduced corticosteroid response following an acute stressor has been mimicked by prior continuous treatment with cortisol-impregnated feed, demonstrating the negative-feedback effect of elevated circulating cortisol on the HPI axis (Barton, Schreck & Barton 1987; Rotllant, Arends, Mancera, Flik, Wendelaar Bonga & Tort 2000) or on related cellular mechanisms (Basu, Kennedy, Hodson & Iwama 2002).

An altered interrenal response to an acute stressor resulting from chronic stress has been shown in fish exposed to chemical stressors but few studies have demonstrated this phenomenon in fish subjected to a chronic physical stressor such as the continuous crowding or confinement experienced in intensive aquaculture. Pickering and Pottinger (1987) found that continuous crowding suppressed the subsequent cortisol response to an acute stressor in brown trout, *Salmo trutta* L., but suggested that this effect was caused by water quality changes and not the crowding *per se*. Recently, Haukenes and Barton (2004) showed that confining yellow perch, *Perca flavescens* (Mitchill), reduced their cortisol response to an acute lipopolysaccharide (LPS) challenge. In this study, we compared the physiological responses of juvenile gilthead sea bream, *Sparus aurata* L., with an acute handling stressor after holding them at low and high tank densities. Previous studies have documented various responses of this species to handling and confinement stressors (Rotllant, Balm, Pérez-Sanchez, Wendelaar Bonga & Tort 2001; Tort, Montero, Robaina, Fernández-Palacios & Izquierdo 2001; Rotllant, Montero, Cabellero, Robaina, Izquierdo & Tort 2003). Our objective was to determine whether gilthead sea bream subjected to a high-density (HD) confinement stressor would show reduced responses, particularly in cortisol, to a subsequent acute stressor compared with those in fish held at a low density (LD). Further understanding of the nature of this species' responses to aquaculture-related stressors is important practically as gilthead sea bream is one of the most commonly used fishes for commercial marine aquaculture in the Mediterranean Sea (Gasca-Leyva, León & Hernández 2003).

Materials and methods

Acclimation and experimental conditions

Approximately 260 juvenile gilthead sea bream were obtained from Aquadelt Fish Farms, Sant Carles de la Ràpita, Spain. The fish were transported to aquarium facilities at the Universitat Autònoma de Barcelona where they were divided equally into eight rectangular fibreglass tanks. About 33 fish per tank were held in 200 L of 33–36 g L^{-1} seawater at 19 °C and a density of 6 kg m^{-3} for 2 weeks of acclimation before the experiments. Water was recirculated by a small submersible aquarium pump (Enheim, Berlin, Germany) in each tank that passed the water through an individual biofilter unit containing nitrifying bacteria to remove ammonia wastes. During acclimation and experiments, un-ionized ammonia levels [uNH_3-N, determined from dissociation tables developed by Colt (2001) from measured total ammonia-nitrogen (TAN) and compensated for temperature, pH and salinity] ranged from <0.001 to 0.024 mg L^{-1}; pH ranged from 6.35 to 8.13; and nitrite (NO_2-N) levels ranged from 0.08 to 2.85 mg L^{-1} among all tanks (Table 1). Water was vigorously aerated continuously with compressed air supplied by a common diaphragm pump and delivered through two airstones in each tank. Tanks were kept partially covered under a controlled photoperiod of 12 h light:12 h dark using artificial light, with the light period commencing at 07:00 hours. The fish were fed a maintenance ration of about 0.3–0.5% body weight per day with an experimental sea bream growth diet (INVE Technologies NV, Baasrode, Belgium); feeding ceased 24 h before fish were sampled from designated tanks. Any mortality during acclimation and experiments was recorded and those fish were removed from the tanks.

Table 1 Salinity range, and range (low–high) and mean (± SE, $n = 9$) of measured pH, nitrite-nitrogen (NO_2-N) and total ammonia-nitrogen (TAN), and calculated un-ionized ammonia-nitrogen (uNH_3-N) in tanks containing gilthead sea bream at low (LD) and high (HD) densities throughout the experiments

Tank	Salinity (g L^{-1})	pH	NO_2-N (mg L^{-1})	TAN (mg L^{-1})	uNH_3-N (mg L^{-1})
LD 1	33–35	7.50–7.96	0.31–2.16	0.03–0.23	<0.001–<0.008
		7.72 ± 0.06 a,b	0.80 ± 0.22 a,b	0.10 ± 0.02 a	0.002 ± <0.001 a,b
LD 2	34	6.70–7.80	0.11–0.43	0.03–0.12	<0.001–<0.001
		7.48 ± 0.10 a,b	0.19 ± 0.03 a	0.07 ± 0.01 a	<0.001 ± <0.001 a
LD 3	36–37	7.10–7.90	0.12–0.34	0.01–0.14	<0.001–<0.002
		7.50 ± 0.08 a,b	0.23 ± 0.03 a	0.07 ± 0.01 a	<0.001 ± <0.001 a
LD 4	33–34	7.47–7.80	0.08–2.70	0.03–2.61	<0.001–<0.022
		7.69 ± 0.04 a,b	1.54 ± 0.29 b	0.80 ± 0.33 b,c	0.007 ± 0.003 b,c
HD 1	34–36	6.35–7.93	0.51–2.72	0.03–2.44	<0.001–<0.024
		7.39 ± 0.18 a	1.53 ± 0.77 b	1.16 ± 0.32 c	0.011 ± 0.003 c
HD 2	34–36	6.80–7.86	0.14–2.85	0.03–1.79	<0.001–<0.016
		7.60 ± 0.11 a,b	1.11 ± 0.38 a,b	0.38 ± 0.21 a,b	0.004 ± 0.002 a,b
HD 3	34–36	7.60–7.92	0.50–1.39	0.07–0.09	<0.001–<0.004
		7.73 ± 0.04 b	0.83 ± 0.10 a,b	0.20 ± 0.04 a,b	0.002 ± <0.001 a,b
HD 4	35	7.61–8.13	0.24–2.77	0.01–0.84	<0.001–<0.007
		7.78 ± 0.05 b	1.55 ± 0.36 a,b	0.23 ± 0.10 a,b	0.003 ± 0.001 a,b

Mean values followed by letters not in common indicate significant difference ($P < 0.05$) within that parameter column.

Chronic confinement stressor

For the chronic HD confinement treatment, four tanks containing 33 juvenile sea bream per tank (mean weight 37.1 g) were held at a density of 26 kg m^{-3} for 14 days. Water was removed to alter densities initially, and subsequently after fish removal to maintain densities constant, by using a siphon, which allowed for minimal disturbance of the fish in the tanks. The remaining four tanks containing 33 fish per tank at an acclimation density of 6 kg m^{-3} served as LD control groups.

Five fish were removed carefully to avoid unduly disturbing the remaining fish from each of two of the four tanks ($n = 10$) for both LD and HD treatments at the onset of the experiment (day 0). Five fish per tank were then removed from the same two tanks on day 2 only and from the other two tanks on days 1 and 7 only for each treatment. This approach was adopted to keep any possible acute disturbance to remaining fish resulting from fish removal at a minimum; all groups, thus, had at least 2 days to recover physiologically from any such disturbance and both density groups were treated in the same manner. Similar sampling techniques have been used successfully with salmonid fishes with no measurable effect on physiological constituents occurring in remaining unhandled control fish (Barton, Peter & Paulencu 1980; Barton, Weiner & Schreck 1985; Barton, Schreck & Sigismondi 1986). On day 14, two to three fish per tank were taken from all four tanks ($n = 10$) for each treatment just before commencing the acute handling experiment; these samples also served as hour 0 samples for acute handling.

Acute handling stressor

After 14 days of HD or LD confinement, fish in all tanks were subjected to an acute handling stressor by holding them in the air in a net for 30 s. Fish from each treatment group were then divided equally into eight tanks per treatment in order to provide separate replicate sample groups at post-handling times without disturbing remaining fish. Water volumes in tanks were kept similar to those used for the LD and HD chronic treatments accordingly. Five fish from each of two tanks ($n = 10$) were sampled at 1, 2, 4 and 8 h after handling.

Fish sampling

All fish were sampled by first placing them in a lethal concentration of 2-phenoxy-ethanol (Sigma, St Louis, MO, USA) after being removed from the tank with a hand-net; fish were immobilized in < 1 min. Blood was obtained from the caudal vasculature using a 1-mL syringe equipped with a 25-G needle (Houston 1990) and transferred to a 1.5-mL microcentrifuge tube containing 5% sodium heparin. All sampling was completed within 5 min of fish removal from

the tank. Plasma was separated by 5 min centrifugation and stored at − 25 °C for subsequent analysis of cortisol, glucose, lactate, osmolality and chloride.

Sample and data analysis

Plasma cortisol levels were measured by radioimmunoassay following the procedure described for gilthead sea bream in Rotllant and colleagues (2001) using antibody purchased from BioLink SL (Barcelona, Spain). Plasma glucose and lactate were determined by enzymatic colorimetric methods in ELISA plates using commercial kits (BioMérieux 61270 and 61192 for glucose and lactate, respectively; BioMérieux SA, Marcy-l'Etoile, France). Plasma osmolality was assessed by direct reading using an Osmomat 030 cryoscopic osmometer (Gonotec, Berlin, Germany). Plasma chloride was measured using a Corning model 925 chloridometer (CIBA-Corning, Medfield, MA, USA).

Two-way analyses of variance (ANOVA) were conducted with ProStat (Poly Software International, Salt Lake City, UT, USA) using tank means as independent observations to compare treatments and times for the chronic confinement period and the acute handling trial separately. Two-way ANOVA were carried out of water quality parameters to detect differences among tanks and through time. *Post hoc* comparisons to compare means were made using Duncan's Multiple-Range Test with significance level at $P = 0.05$.

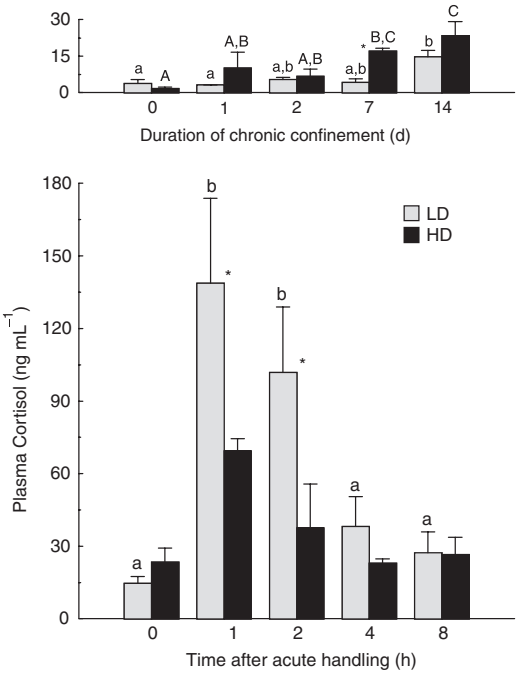

Figure 1 Mean (+SE) levels of plasma cortisol (ng mL^{-1}) in juvenile gilthead sea bream during 14 d of confinement at low (LD) or high (HD) density followed by a 30-s acute handling stressor. Value bars for 14-day confinement are also those for 0 h handling. Bars accompanied by letters not in common indicate significant difference ($P < 0.05$) from other values at different times for that treatment; lower case letters are used for LD treatments and capital letters for HD treatments for ease of interpretation. An asterisk (*) accompanying a pair of bars indicates a significant difference between the two treatments at that time. Bars within a treatment series not accompanied by letters indicate no significant difference among all times.

Results

Chronic confinement

Plasma cortisol increased in both LD- and HD-treatment groups during the 14-day confinement period (Fig. 1); an elevated level of plasma cortisol was apparent by day 7 in the HD fish but not until day 14 in those held at LD. A significant difference in plasma cortisol between LD and HD fish occurred only on day 7 (Fig. 1).

Concentrations of plasma glucose were similar in both sea bream groups throughout the confinement period with no significant differences evident among times within either density treatment or between treatments (Fig. 2). Plasma lactate levels were more variable than glucose; levels were generally lower in the HD group than in the LD fish during confinement and significantly so on day 0 (start of experiment) (Fig. 3). Whereas plasma lactate remained constant in the HD fish, it was significantly lower at day 14 than at day 0 in the LD-treatment group (Fig. 3).

Sea bream in both LD and HD treatments exhibited a significant reduction in plasma osmolality by day 2 compared with that at the start of the experiment but returned to initial (day 0) levels by day 14 (Fig. 4). Plasma chloride concentrations ranged from 154 ± 4.0 (± SE) to 163 ± 0.2 mEq L^{-1} in the LD group and from 158 ± 1.3 to 160 ± 0.3 mEq L^{-1} in the HD group during the 14-day confinement period; no significant differences were evident among times or between treatments (data not shown).

No mortality was observed during the acclimation period but three HD tanks had fish mortalities during

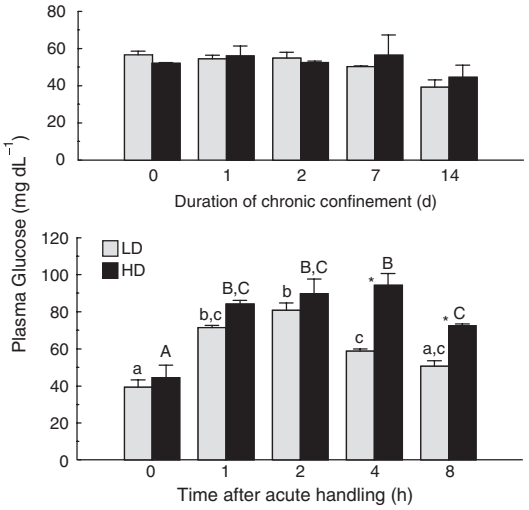

Figure 2 Mean (+SE) levels of plasma glucose (mg dL^{-1}) in juvenile gilthead sea bream during 14 d of confinement at low (LD) or high (HD) density followed by a 30-s acute handling stressor. See Fig. 1 for explanation of letter symbols accompanying the value bars. Bars within a treatment series not accompanied by letters indicate no significant difference among all times.

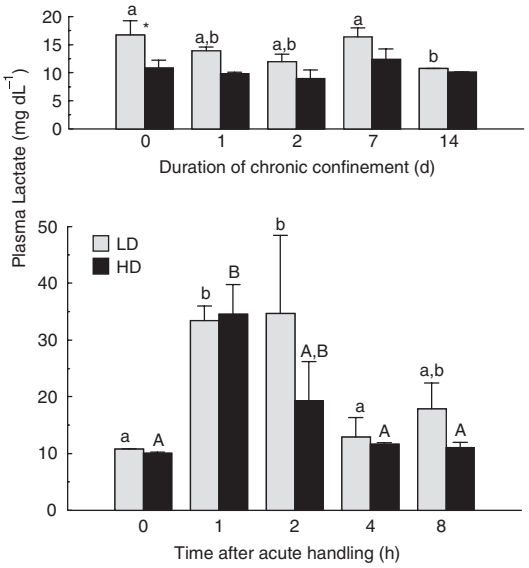

Figure 3 Mean (+SE) levels of plasma lactate (mg dL^{-1}) in juvenile gilthead sea bream during 14 d of confinement at low (LD) or high (HD) density followed by a 30-s acute handling stressor. See Fig. 1 for explanation of letter symbols accompanying the value bars. Bars within a treatment series not accompanied by letters indicate no significant difference among all times.

chronic confinement [HD1 = 2 (6.1%), HD2 = 6 (18%), HD3 = 1 (3.0%)]. No fish mortality occurred in LD tanks.

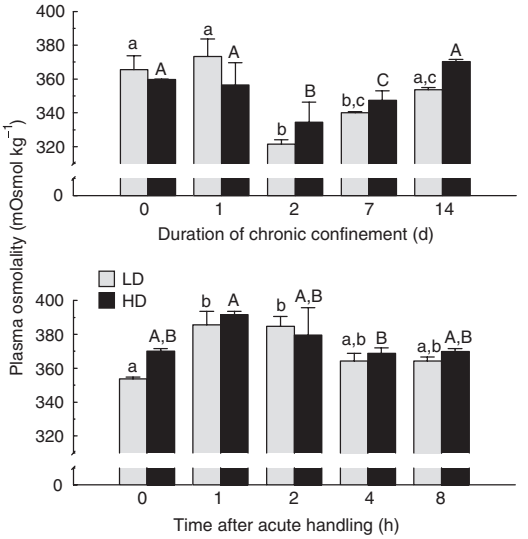

Figure 4 Mean (+SE) levels of plasma osmolality (mOsmol kg^{-1}) in juvenile gilthead sea bream during 14 d of confinement at low (LD) or high (HD) density followed by a 30-s acute handling stressor. See Fig. 1 for explanation of letter symbols accompanying the value bars.

Acute handling

Gilthead sea bream from the LD treatment had significantly elevated levels of plasma cortisol after being subjected to 30 s of handling; these changes were evident at 1 and 2 h after handling (Fig. 1). Plasma cortisol concentrations in fish from the HD treatment, although apparently elevated at 1 h, were not significantly different from each other throughout the post-handling period (Fig. 1).

Plasma glucose in sea bream from both treatments increased in a similar fashion during the first 2 h after handling (Fig. 2). At 4 and 8 h post handling, plasma glucose levels remained significantly higher in the HD fish than in the LD group (Fig. 2). Plasma lactate also increased significantly by 1 h after handling in both groups and concentrations remained elevated in the LD group at 2 h compared with those in the HD fish (Fig. 3). Plasma lactate levels in both groups at 4 and 8 h were similar to those before handling (0 h).

Plasma osmolality increased significantly in the LD fish during the first 2 h after fish were handled, but returned to pre-handling levels by 4 h (Fig. 4). Post-handling changes in plasma osmolality in the HD fish were not significant except at 1 h compared with that at 4 h (Fig. 4). Treatment groups were similar at all post-handling times. Concentrations of plasma chloride ranged from 157 ± 0.6 to

169 ± 1.9 mEq L^{-1} in sea bream held at LD and from 159 ± 0.8 to 168 ± 1.4 mEq L^{-1} in the HD group from the time of initial handling up to 8 h, the end of the trial; no significant differences occurred among times or between treatments (data not shown).

No fish mortality occurred in HD or LD tanks during the acute handling experiment.

Discussion

Pre-stress and post-acute stress responses of plasma cortisol, glucose, lactate and osmolality in the gilthead sea bream used in this study were similar in magnitude to those previously shown for this species (Rotllant *et al.* 2001; Tort *et al.* 2001). Plasma chloride remained unchanged and levels were typical of those in other marine teleosts, as was osmolality (Cech Jr 2000). An explanation for the decline in plasma osmolality after 2–day confinement in both groups of fish is not forthcoming as salinity levels remained relatively constant. We noticed, however, that fish did not start actively taking feed until a few days after their initial transfer and, as suggested by Cech Jr (2000), the short-term dietary deprivation could have altered blood ion levels. Nevertheless, plasma osmolality levels in both groups were similar to their pre-transfer values by the beginning of the handling experiment on day 14.

Elevations in plasma cortisol in the sea bream held under HD confinement for 2 weeks and then handled for 30 s were clearly reduced (e.g. at 1 and 2 h) compared with those in the control group. This phenomenon has been demonstrated very little in fish subjected to a chronic physical stressor, such as confinement, before being acutely handled. In one other study, Haukenes and Barton (2004) showed a trend through time towards reduced corticosteroid responses in yellow perch subjected to LPS treatment that were first either confined at HD or confined and then allowed a day to recover at LD compared with those that were held at the ambient LD. In a similar aquaculture setting, Pickering and Pottinger (1987) reported a reduced elevation of plasma cortisol in handled and acutely confined brown trout after being held in a crowded condition, but indicated that this was likely a result of being exposed chronically to high concentrations of un-ionized ammonia in the tanks. In that case, those authors concluded that continuous interrenal activity caused a down-regulation of the HPI axis from negative feedback by circulating cortisol, which attenuated the response to an additional stressor. In this experiment, and that by Haukenes and Barton (2004), we suspect that a similar phenomenon is occurring as a result of holding the fish at a HD.

Alternatively, the fish may have become sufficiently desensitized to the chronic stressor such that their overall response to the additional stressor was suppressed, similar to what Barton and colleagues (1987) found in rainbow trout, *Oncorhynchus mykiss* (Walbaum), subjected to mild acute stressors daily for a number of weeks. Both plasma cortisol and glucose elevations in response to handling at the end of the 10-week treatment period in that study were lower compared with those in control groups, suggesting not only desensitization of the HPI axis but of the adrenergic mechanisms controlling stress-induced hyperglycaemia as well (Reid, Bernier & Perry 1998). In the present investigation, plasma glucose increased by comparable amounts following 30 s of handling in both LD- and HD-held gilthead sea bream, however, and elevated concentrations were sustained at 4 and 8 h post handling in the HD group, which suggests that stress-response mechanisms were not 'desensitized' in that context. The similarity of increases in plasma lactate and osmolality levels in LD and HD fish at 1 h further support our view that both groups experienced a similar degree of acute stress from being handled. The fish from the HD treatment may have already experienced a higher degree of stress during the 14-day confinement period before handling than those in the LD group as suggested by higher plasma cortisol levels at day 7 and by the fact that three HD tanks had fish mortality whereas the LD tanks had none. Extended periods of confinement have been shown to induce elevations of circulating cortisol in salmonid fishes similar to those we measured in the sea bream (Pickering & Stewart 1984; Pickering, Pottinger, Carragher & Sumpter 1987; Pickering & Pottinger 1989).

The suppression of the acute corticosteroid response in fish following their continued exposure to water-borne contaminants is a well-documented phenomenon (Hontela *et al.* 1992; Brodeur, Daniel, Ricard & Hontela 1998; Wilson, Vijayan, Kennedy, Iwama & Moon 1998; Norris *et al.* 1999; Laflamme *et al.* 2000). Hontela (1997) considered that this interrenal impairment could be due to either down-regulation of the HPI axis by continual negative feedback, as discussed, or as a result of direct toxic action of the compound on the cellular mechanisms or function of the interrenal tissue itself, depending on the type of contaminant. As sea bream were not exposed to a

chemical stressor *per se* in this study, the latter explanation for the attenuated cortisol response in the HD fish is unlikely. It is possible, however, that changes in water chemistry as a result of confinement may have affected interrenal responsiveness by acting as an additional stressor along with HD confinement. Pickering and Pottinger (1987) concluded that confinement-induced alterations in dissolved oxygen (O_2), free carbon dioxide (CO_2) and TAN may have acted in combination to cause a reduction in the response of plasma cortisol to an acute 1-h confinement stressor. In our experiments, the confinement period was for 14 days, thereby allowing for acclimation to occur, and Pickering and Stewart (1984) showed previously that mild but chronic elevation of plasma cortisol from HD confinement can occur independent of water chemistry changes. We concluded that this was likely the case in our study as the water quality parameters of pH, NO_2-N, TAN and uNH_3-N, while showing some variation throughout the experiment, were not appreciably different among the tanks or between the LD and HD groups overall; free CO_2 and dissolved O_2 were not measured, but tanks were vigorously aerated continuously. Moreover, levels of nitrogenous parameters remained well below lethal levels for fish (Tomasso 1994) and those shown to chronically elevate plasma cortisol (Sykes 1999).

Despite considerable research in this area, the implication for a reduced endocrine stress response in fishes used for aquaculture is still not clear. While it may be advantageous to select fish exhibiting low stress responses for intensive commercial aquaculture (Fevolden, Reftsie & Røed 1991; Pottinger, Moran & Morgan 1994), those being reared for stock enhancement could conceivably be at a disadvantage when released into a natural environment because of a lowered capacity to mount the appropriate response to cope with additional stressors. For cage-culture of gilthead sea bream, a possible impairment of the corticosteroid stress response from rearing at overly high densities could result in a reduction in their physiological ability to cope with social stressors from conspecifics or abiotic changes in their confined environment.

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References

Barton B.A. (2002) Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology* **42**, 517–525.

Barton B.A. & Iwama G.K. (1991) Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases* **1**, 3–26.

Barton B.A., Peter R.E. & Paulencu C.R. (1980) Plasma cortisol levels of fingerling rainbow trout (*Salmo gairdneri*) at rest, and subjected to handling, confinement, transport, and stocking. *Canadian Journal of Fisheries and Aquatic Sciences* **37**, 805–811.

Barton B.A., Weiner G.A. & Schreck C.B. (1985) Effect of prior acid exposure on physiological responses of juvenile rainbow trout (*Salmo gairdneri*) to acute handling stress. *Canadian Journal of Fisheries and Aquatic Sciences* **42**, 710–717.

Barton B.A., Schreck C.B. & Sigismondi L.A. (1986) Multiple acute disturbances evoke cumulative physiological stress responses in juvenile chinook salmon. *Transactions of the American Fisheries Society* **115**, 245–251.

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

Barton B.A., Morgan J.D. & Vijayan M.M. (2002) Physiological and condition-related indicators of environmental stress in fish. In: *Biological Indicators of Aquatic Ecosystem Stress* (ed. by S.M. Adams), pp. 111–148. American Fisheries Society, Bethesda, MD, USA.

Basu N., Kennedy C.J., Hodson P.V. & Iwama G.K. (2002) Altered stress responses in rainbow trout following a dietary administration of cortisol and β-napthoflavone. *Fish Physiology and Biochemistry* **25**, 131–140.

Brodeur J.C., Daniel C., Ricard A.C. & Hontela A. (1998) In vitro response to ACTH of the interrenal tissue of rainbow trout (*Oncorhynchus mykiss*) exposed to cadmium. *Aquatic Toxicology* **42**, 103–113.

Cech J.J. Jr (2000) Osmoregulation in bony fishes. In: *Encyclopedia of Aquaculture* (ed. by R.R. Stickney), pp. 614–622. John Wiley and Sons, New York, USA.

Colt J. (2001) Spreadsheet appendices. In: *Online Supplement To: Fish Hatchery Management*, 2nd edn (ed. by G.A.

Wedemeyer), American Fisheries Society, Bethesda, MD, USA (www.fisheries.org/publications/epubs/fhm2e/appendices.shtml).

Fevolden S.E., Reftsie T. & Røed K.H. (1991) Selection for high and low cortisol stress response in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **95**, 53–65.

Gasca-Leyva E., León C.J. & Hernández J.M. (2003) Management strategies for seabream *Sparus aurata* cultivation in floating cages in the Mediterranean Sea and Atlantic Ocean. *Journal of the World Aquaculture Society* **34**, 29–39.

Haukenes A.H. & Barton B.A. (2004) Characterization of the cortisol response following an acute challenge with lipopolysaccharide in yellow perch and the influence of rearing density. *Journal of Fish Biology* **64**, 851–862.

Hontela A. (1997) Endocrine and physiological responses of fish to xenobiotics: role of glucocorticosteroid hormones. *Reviews in Toxicology* **1**, 1–46.

Hontela A., Rasmussen J.B., Audet C. & Chevalier G. (1992) Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. *Archives of Environmental Contamination and Toxicology* **22**, 278–283.

Houston A.H. (1990) Blood and circulation. In: *Methods for Fish Biology* (ed. by C.B. Schreck & P.B. Moyle), pp. 273–334. American Fisheries Society, Bethesda, MD, USA.

Iwama G.K., Pickering A.D., Sumpter J.P. & Schreck C.B. (eds) (1997) *Fish Stress and Health in Aquaculture. Society for Experimental Biology Seminar Series*, Vol. 62. Cambridge University Press, Cambridge, UK.

Laflamme J.-S., Couillard Y., Campbell P.G.C. & Hontela (2000) Interrenal metallothionein and cortisol secretion in relation to Cd, Cu, and Zn exposure in yellow perch, *Perca flavescens*, from Abitibi lakes. *Canadian Journal of Fisheries and Aquatic Sciences* **57**, 1692–1700.

Norris D.O., Donahue S., Dores R.M., Lee J.K., Maldonado T.A., Ruth T. & Woodling J.D. (1999) Impaired adrenocortical response to stress by brown trout, *Salmo trutta*, living in metal-contaminated waters of the Eagle River, Colorado. *General and Comparative Endocrinology* **113**, 1–8.

Pickering A.D. & Pottinger T.G. (1987) Poor water quality suppresses the cortisol response of salmonid fish to handling and confinement. *Journal of Fish Biology* **30**, 363–374.

Pickering A.D. & Pottinger T.G. (1989) Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. *Fish Physiology and Biochemistry* **7**, 253–258.

Pickering A.D. & Stewart A. (1984) Acclimation of the interrenal tissue of the brown trout, *Salmo trutta* L., to chronic crowding stress. *Journal of Fish Biology* **24**, 731–740.

Pickering A.D., Pottinger T.G., Carragher J. & Sumpter J.P. (1987) The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature male brown trout. *General and Comparative Endocrinology* **68**, 249–259.

Pottinger T.G., Moran T.A. & Morgan J.A.W. (1994) Primary and secondary indices of stress in the progeny of rainbow trout (*Oncorhynchus mykiss*) selected for high and low responsiveness to stress. *Journal of Fish Biology* **44**, 149–163.

Reid S.G., Bernier N.J. & Perry S.F. (1998) The adrenergic stress response in fish: control of catecholamine storage and release. *Comparative Biochemistry and Physiology* **120C**, 1–27.

Rotllant J., Arends R.J., Mancera J.M., Flik G., Wendelaar Bonga S.E. & Tort L. (2000) Inhibition of HPI axis response to stress in gilthead sea bream (*Sparus aurata*) with physiological plasma levels of cortisol. *Fish Physiology and Biochemistry* **23**, 13–22.

Rotllant J., Balm P.H.M., Pérez-Sánchez J., Wendelaar-Bonga S.E. & Tort L. (2001) Pituitary and interrenal function in gilthead sea bream (*Sparus aurata* L., Teleostei) after handling and confinement stress. *General and Comparative Endocrinology* **121**, 333–342.

Rotllant J., Montero D., Caballero M.J., Robaina L., Izquierdo M.S. & Tort L. (2003) Differences in interrenal tissue, biosynthetic capacity and ACTH sensitivity in progeny of sea bream from parents selected for high or low cortisol response. *Journal of Fish Biology* **62**, 744–748.

Sykes C.L. (1999) *Evaluation of heat-shock protein 70 and plasma cortisol responses in fathead minnows (Pimephales promelas) exposed to sublethal concentrations of ammonia*. MA thesis, University of South Dakota, Vermillion, SD, USA.

Tomasso J.R. (1994) Toxicity of nitrogenous wastes to aquaculture animals. *Reviews in Fisheries Science* **2**, 291–314.

Tort L., Montero D., Robaina L., Fernández-Palacios H. & Izquierdo M.S. (2001) Consistency of stress response to repeated handling in the gilthead sea bream *Sparus aurata* Linnaeus, 1758. *Aquaculture Research* **32**, 593–598.

Wendelaar Bonga S.E. (1997) The stress response in fish. *Physiological Reviews* **77**, 591–625.

Wilson J.M., Vijayan M.M., Kennedy C.J., Iwama G.K. & Moon T.W. (1998) β-naphthoflavone abolishes the interrenal sensitivity to ACTH stimulation in rainbow trout. *Journal of Endocrinology* **157**, 63–70.

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A differentially expressed enolase gene isolated from the gilthead sea bream (*Sparus aurata*) under high-density conditions is up-regulated in brain after in vivo lipopolysaccharide challenge

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Abstract

To investigate the effect of different population densities on gene transcription in the sea bream brain (*Sparus aurata*), the messenger RNA (mRNA) differential display (DD) technique was used to analyse gene expression. Sea bream were held at different densities, 6 or 26 kg m^{-3}, over a period of 14 days. We identified seven differentially expressed sequences of which one sequence was functionally identified. The *S. aurata* enolase gene homologue (S-enolase), pertaining to the alpha non-neuronal enolase group of the enzyme superfamily, was up-regulated in the brain of fish in the high-density population group. S-enolase mRNA expression was also found in other tissues including heart, liver and head kidney suggesting a ubiquitous nature. Furthermore, brain S-enolase mRNA is highly up-regulated 48 h after intra-peritoneal bacterial lipopolysaccharide (LPS) administration. Therefore, S-enolase gene expression is linked to the incidence of different stressors,

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density and infective agents, in the sea bream and may be a potential molecular biomarker for stress diagnosis in this fish.

Keywords: Enolase; Expression; Density; Challenge

1. Introduction

Stress in fish caused by physical disturbances encountered in aquaculture, such as handling and transport, evokes a variety of responses that may be adaptive or maladaptive and thus, presents a threat to fish health. The persistence of a chronic stressor, such as high population density, may result in decrease in energetic resources, susceptibility to diseases and an overall reduction of performance (Tort et al., 1998). These stress-induced changes are grouped as primary, secondary, and tertiary or "whole animal" responses (Barton and Iwama, 1991). Current measured physiological parameters including cortisol, glucose or haematocrit, may not be adequate for a complete biological interpretation when chronic stresses in fish culture are studied (Gornati et al., 2004).

Multidirectional immuno–neuro-endocrine interactions form the central focus for the stress response (Besedovsky and del Rey, 1996). The cross talk between these systems and resultant secretion of regulatory molecules modulates the physiological response at target tissues acting at a transcriptional level causing changes in gene expression. Therefore, the identification of such 'target' genes for use as molecular markers capable of describing fish welfare is clearly an important aim in the development of diagnostic technologies for increased efficiency in fish farm management.

The enolase enzyme superfamily performs a key role in glycolysis (Babbitt et al., 1996) and forms a group of highly conserved molecules which are found in all organisms. Vertebrates express three tissue-specific isoforms, alpha (α), beta (β) and gamma (γ) encoded by three genes (Tracy and Hedges, 2000). Recent findings have suggested that the enolase enzymes have additional functions aside from those described for glucose catabolism including: acting as a potential plasminogen receptor (Miles et al., 1991), a Myc-binding protein (MBP-1), acting in transcriptional regulation (Subramanian and Miller, 2000) and as a cell associated stress protein (HAP) involved in cellular protection during hypoxia (Aaronson et al., 1995; Roland et al., 2000). Interestingly α-enolase was identified as being up-regulated in liver during hypoxia in the longjaw mudsucker (*Gillicthys mirabilis*) by gene expression profiling (Gracey et al., 2001).

We have isolated the sea bream (*Sparus aurata*) orthologue of α-enolase (S-enolase) in a differential display screening in fish held at different densities. In addition, S-enolase gene expression is up-regulated after intra-peroniteal lipopolysaccharide (LPS) injection suggesting a stress-related function.

2. Materials and methods

2.1. Animals

Sea bream *S. aurata* of 50 g approximately were obtained from Aquadelt Fish Farm, Sant Carles de la Ràpita, Spain. The fish were transported to aquarium facilities at the Universitat Autònoma de Barcelona where they were divided equally into eight rectangular fiberglass tanks. About 33 Fish were held in 200 l of 33–36‰ seawater at 19 °C and a density of 6 kg m^{-3} for 2 weeks acclimation before the experiments. Water was recirculated by a small submersible aquarium pump (Eheim, Berlin, Germany) in each tank that passed the water through an individual biofilter unit containing nitrifying bacteria to remove ammonia wastes. During acclimation and experiments, un-ionized ammonia levels ranged from 0.08 to 2.85 mg l^{-1}, pH ranged from 6.35 to 8.55, and nitrite levels ranged from 0.01 to 2.61 mg l^{-1}. Water was vigorously aerated continuously with compressed air supplied by a common diaphragm pump and delivered in each tank through an airstone. Tanks were kept partially covered under a photoperiod of 12-h light/12-h dark with the light period commencing at 7:00 h. The fish were fed a maintenance ration of about 0.3–0.5% body weight per day with a commercial sea bream growth diet (INVE Technologies, Baasrode, Belgium).

2.2. Chronic confinement

For the chronic high density (HD) confinement treatment, 13 gilthead sea bream (mean weight 37.1±1.2 g) were held at a density of 26 kg m^{-3} for 14 days. Thirteen fish at an acclimation density of 6 kg m^{-3} served as low density (LD) control groups. Ten fish were removed carefully from each of the two conditions (n=10) for both LD (at day 0) and HD treatments (at day 14). Brain tissues were removed from all fish and directly frozen in liquid nitrogen, brains were stored at −80 °C for future RNA extraction.

2.3. LPS IP injection

Twenty fish, *S. aurata*, were used for this experiment. All fish were injected with 8 mg kg^{-1} of the lipopolysaccharide *Escherichia coli* (LPS), except control fish in which saline buffer was used. LPS and saline were administrated via intra-peritoneal (IP) injection. The administration was IP at time 0 h of experiment. Samples from five fish per group were taken 12, 48 and 72 h post-injection. Brain tissues were removed from all fish and directly frozen in liquid nitrogen and stored at −80 °C for future RNA extraction.

2.4. RNA extractions all tissues

Total RNA was extracted from *S. aurata* brain tissues using an established RNA purification method with minor modifications (MacKenzie et al., 2002). In brief, tissues were homogenized with guanidinium thiocyanate homogenisation buffer. Nucleic acids were extracted in phenol/chloroform/isoamyalcohol with 2 M Sodium Acetate, and precipitated overnight at −20 °C with ethanol 100%. The precipitate was washed in ethanol (70%). Samples were dissolved at required concentrations (±1–2 μg μl^{-1}) in MQ water.

2.5. Differential display

Differential display (DD) was performed with RNAimage® kit (GenHunter, US). Brain samples (n=3) from fish subjected to confinement stressor, as described above, at 0 and 14 days were analysed by DD. Three reverse transcription reaction (RT-PCR) for each RNA sample (0.1 μg μl^{-1}) were done. Each reaction contained: 2 μM of one of the three different one-base-anchored H-T_{11}M primers (where M was G, A or C), 5× RT buffer (125 mM Tris–Cl, pH 8.3, 188 mM KCl, 7.5 mM $MgCl_2$ and 25 mM DTT), dNTP Mix 1, Moloney murine leukemia virus (MMLV) reverse transcriptase and DEPC water in a final volume of 20 μl. The thermocycler was programmed to 65 °C for 5 min, 37 °C for 60 min, 75 °C for 5 min and then maintained at 4 °C before amplification.

Amplification of cDNA products was performed in 20 μl reaction for each primer pair combination. Each PCR mixture contained cDNA and 10× PCR buffer, 1 mM dNTPs Mix 2, 2 μM H-T_{11}M primers, 2 μM concentration of one of three 5′-arbitrary primer (49 AAGCTTTAGTCCA, 53 AAGCTTCCTCTAT, 55 AAGCTTACGTTAG), 1 unit of Taq DNA polymerase (Qiagen), 10 μCi $_{35}$S-ATP, DEPC water was added to adjust to final volume. Reaction mixtures were subjected to a PCR using the following parameters: 94 °C: 30 s, 42 °C: 2 min, 72 °C: 30 s 40 cycles followed by 72 °C: 5 min and 4 °C. All PCRs were performed in duplicate and a negative control was checked. The amplified cDNAs produced from duplicate reactions of RNA isolated from HD time 0 days and 14 days of experiment were size-fractionated in parallel by 6% polyacrylamide 8 M urea gels electrophoresis. Following electrophoresis, gels were dried onto a Whatman 3MM paper (Whatman, Maidstone, Kent, UK) on a gel dryer at 80 °C for 2 h and exposed to Kodak X-AR film (Eastman Kodak, Rochester, NY, USA) for 48 h. Differentially expressed cDNAs were visualized by autoradiography.

2.6. Isolation, subcloning and DNA sequence analysis

To isolate differentially expressed cDNA fragments, regions were cut from the gel, and DNA was extracted by incubating the gel slices with 100 μl of MQ water for 15 min at 100 °C. The fragments were then reamplified as above but using dNTP Mix 1 and with a final

Table 1
The percentage of identities and similarities of different species to enolase *Sparus aurata* sequences (S-enolase)

	H. sapiens	*R. norvegicus*	*M. musculus*	*X. laevis*	*D. rerio*	*S. trutta*	*S. aurata*
H. sapiens		0.92	0.93	0.88	0.76	0.81	0.74
R. norvegicus	0.92		0.94	0.86	0.76	0.83	0.74
M. musculus	0.93	0.94		0.88	0.75	0.81	0.73
X. laevis	0.88	0.86	0.98		0.75	0.83	0.75
D. rerio	0.76	0.76	0.75	0.75		0.71	0.65
S. trutta	0.81	0.83	0.81	0.83	0.71		0.9
S. aurata	0.74	0.74	0.73	0.75	0.65	0.9	

Accession numbers to Gene Bank data base are the following: *Homo sapiens* enolase-1; NM_001428, *Rattus norvegicus* neuron-specific enolase; AF019973, *Mus musculus* enolase 3 beta muscle; NM_007933, *Xenopus laevis* enolase-1 similar to enolase-3 beta muscle; BC045082; *Danio rerio* enolase-3; AY130387, *Salmo trutta* enolase alpha-2; AY005162, *Sparus aurata* enolase; AY263379.

volume of 40 μl. PCR products were run in 1% agarose gel stained with ethidium bromide. The reamplified cDNA were extracted from agarose gel by using a Nucleotrap® kit (Macherey-Nagel) and cloned into a bacterial plasmid vector (pGEM-T Easy, Promega). For screening recombinant bacterial colonies for the presence of target plasmid DNA with DNA inserts, CloneChecker™ Kit (GibcoBRL®) were used. DNA inserts were subsequently sequenced using T7 and SP6 primers by a dye terminator cycle sequencing kit (Thermo Sequenase II, Amersham).

2.7. Computer analysis

Analysis of cDNA sequence data including comparisons, alignments and translations to protein, were performed using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). In order to find homology with other gene sequences, cDNA sequence were analysed by Basic Local Alignment Search Tool (BLAST) through the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Significant homology was considered when more than 50% of nucleotide acid composition of bands were found. Multiple Sequence Alignment was carried out via the ClustalW programme at EMBL-EBI (European Informatics Institute) (http://www.ebi.ac.uk/clustalw/index.html).

2.8. Northern blot analysis and PCR analysis

Approximately 10 μg of total RNA from brain tissues was loaded onto a formaldehyde-agarose gel, transferred onto a nylon membrane (Nytran® Super Charge, Schleicher & Shuell) and cross-linked. The membranes were hybridised overnight at 42 °C with a ^{32}P-labelled 1.1-kb cDNA (Random Primers DNA Labeling System, Invitrogen) Enolase fragment (1.1×10^6 cpm μl^{-1}) obtained as a probe by described above. The membrane was washed two times with 2× SSC/0.1% SDS at room temperature, once with 1× SSC/0.1% SDS at 42 °C and once with 0.1× SSC/0.1% SDS at 42 °C. Subsequently, the membrane was exposed to Kodak X-AR film at −80 °C. After stripping, the same blot was rehybridised using 18S ^{32}P-labelled cDNA fragment from *Oncorhynchus mykiss* muscle as a housekeeping gene (18S rRNA with accession number: AJ291668). Bands obtained from developed film of Northern Blot Analysis, were measured by using Bio-Rad Multi-Analyst™/PC Version 1.1 software. The data were analysed statistically as below.

2.8.1. PCR analysis

RNA (4–5 μg) from each tissue (muscle, head-kidney, heart and liver) were treated by RQ1 RNAse-Free DNAse supplied by Promega (Cat. no. M6101). Five microliters of total RNA DNAse-free were reverse transcribed following commercial indications (AMV Reverse Transcriptase, Promega USA). Secondly, cDNA was amplified in a thermal cycler (model MJ Research). Reaction mixtures were subjected to PCR using the following parameters: 94 °C: 4 min, 94 °C: 45 s, 55 °C: 45 s, 72 °C: 1 min. Cycles (35–40) followed by 72 °C: 10 min. Specific PCR primers were designed: enolase (group 1) forward AGGCCAAGTACGGAAAGGAT-/reverse GAGGAGCAGGCAGTTACAGG, enolase (group 2) forward GGCCAAGTACGGAAAGGAT-/reverse ATCAAGGCGTG-TAAACTGGC and β-actin forward 5′-ATCGTGGGGCGCCCCAGGCACC-3′/β-actine

reverse 5′-CTCCTTAATGTCACGCACGATTTC-3′ . PCR products were visualised in a 1.5% agarose gel stained with ethidium bromide (1 μg ml^{-1}). RT-PCR and PCR were performed with negative controls for both primers.

2.9. Data analysis

Significant homology of bands with some gene from BLAST was considered when more than 50% identities of nucleotide composition were found. In order to calculate identities and similarities for sequence alignments, a PAM250 Similarity Matrix was used. Values from RNA quantification of Northern blot were calculated as the mean ± S.E.M. for each experimental group. Results were analysed with the SPSS 10.0 (Statistical Package for Social Sciences) statistical package. Data from chronic confinement was analysed by Student's *t*-test and for LPS experiment a one-way analysis of variance (ANOVA) was applied to detect significant differences with Student–Neuman–Keuls (SNK) as a post hoc test. Significant difference was considered when $p<0.05$.

3. Results

A differential display using total RNAs obtained from the brains of sea bream (*S. aurata*) under control and high-density rearing conditions resulted in the identification of seven cDNA bands. The cDNAs obtained ranged from 200 to 1200 base pairs (data not shown). Five of the cDNA sequences did not correspond to any known gene. However, two of the selected cDNAs displayed homology to available sequences. One of the cDNAs (210 bp) had homology to an EST (accession number: BJ079382) obtained from the tailbud of *Xenopus laevis*.

A second, substantially longer (1106 bp) cDNA displayed significant homology, 777 bp were homologous to the coding sequence region (CDS) of the enolase gene family, with published nucleotide sequences. The CDS content was 55.6% of A+T, and 44.4% of G+C. The *S. aurata* enolase (S-enolase) cDNA nucleotide sequence (accession number: AY263379) displays a very high identity with enolase nucleotide sequences aligned pertaining to major vertebrate groups (90–65%) (Table 1) revealing the highly conserved nature of this gene family. Translation of the *S. aurata* enolase (S-enolase) fragment resulted in a 259-amino acid sequence (Fig. 1) and alignment of proteins (ClustalW) between different species shows a clear conservation of the protein sequence in which protein homology is very high (90% with *S. trutta* alpha-enolase, 74% human enolase-1) (Fig. 2).

3.1. Tissue distribution

Using RT-PCR analysis S-enolase expression was detected in heart, liver, head kidney and muscle albeit at very low levels (35 PCR cycles) (Fig. 3). PCR products were purified and sequenced and showed high homology with the S-enolase nucleotide sequence (data not shown) with the exception of muscle PCR products. The Muscle PCR product was sequenced and found to have a high homology to beta-enolase isoforms (data not shown). To

```
1     TCG AAC TTC CAC GAG GCC ATG AGG ATC GGA GCT GAG GTT TAC CAC   45
1      S   N   F   H   E   A   M   R   I   G   A   E   V   Y   H    15

46    AAC CTG AAG AAC GTG ATC AAG GCC AAG TAC GGA AAG GAT GCC ACC   90
16     N   L   K   N   V   I   K   A   K   Y   G   K   D   A   T    30

91    AAC GTA GGC GAT GAG GGC GGC TTC GCC CCC AAC ATC CTG GAG AAC   135
31     N   V   G   D   E   G   G   F   A   P   N   I   L   E   N    45

136   AAC GAG GCT CTG GAG CTC CTG AAG ACA GCC ATT GAG AAG GCC GGC   180
46     N   E   A   L   E   L   L   K   T   A   I   E   K   A   G    60

181   TAC CCA GAC AAG ATC ATC ATC GGC ATG GAC GTG GCT GCC TCT GAG   225
61     Y   P   D   K   I   I   I   G   M   D   V   A   A   S   E    75

226   TTC TAC AAG GCA GGA AAG TAC GAC TTG GAC TTC AAG TCA CCT GAC   270
76     F   Y   K   A   G   K   Y   D   L   D   F   K   S   P   D    90

271   GAC CCT GCC AGG TAC ATC CCC GGG GAT CAG CTG GGA GAT CTG TAC   315
91     D   P   A   R   Y   I   P   G   D   Q   L   G   D   L   Y   105

316   AAA AGC TTC ATC AAG GGG ATA TCC AGT CCA GTC CAT CGA GGA TCC   360
106    K   S   F   I   K   G   I   S   S   P   V   H   R   G   S   120

361   CTT CGT CAG GAT GAT TGG GCT GCA TGG TCA AAG TTC CCC GCC GCC   405
121    L   R   Q   D   D   W   A   A   W   S   K   F   P   A   A   135

406   GTC GAC ATC CAG GTG GTG GGT GAT GAT CTG ACC GTG ACC AAC CCC   450
136    V   D   I   Q   V   V   G   D   D   L   T   V   T   N   P   150

451   AAG CGT ATC CAG CAG GCT GTG GAG AAA AAG GCC TGT AAC TGC CTG   495
151    K   R   I   Q   Q   A   V   E   K   K   A   C   N   C   L   165

496   CTC CTC AAG GTC AAC CAG ATC GGC TCC GTC ACA GAG TCC ATC AAG   540
166    L   L   K   V   N   Q   I   G   S   V   T   E   S   I   K   180

541   GCG TGT AAA CTG GCC CAG TCT AAC GGA TGG GGT GTG ATG GTG TCT   585
181    A   C   K   L   A   Q   S   N   G   W   G   V   M   V   S   195

586   CAT CGT TCC GGA GAG ACA GAG GAT ACC TTC ATC GCT GAC CTG GTG   630
196    H   R   S   G   E   T   E   D   T   F   I   A   D   L   V   210

631   GTC GGA CTC TGC ACT GGA CAG ATC AAG ACT GGT GCC CCC TGT AGA   675
211    V   G   L   C   T   G   Q   I   K   T   G   A   P   C   R   225

676   TCA GAA CGT CTG GCC AAA TAC AAC CAG CTG ATG AGG ATT GAA GAG   720
226    S   E   R   L   A   K   Y   N   Q   L   M   R   I   E   E   240

721   GAG CTT GGA GCC AAG GCC AAG TTC GCC GGA AAG GAC TAC CGT CGC   765
241    E   L   G   A   K   A   K   F   A   G   K   D   Y   R   R   255

766   CCC AAA ATC AAC   777
256    P   K   I   N
```

Fig. 1. Nucleotide and predicted amino sequence of the partial S-enolase.

confirm the low levels of expression observed, we further designed more primer pairs and repeated RT-PCR analysis. Results obtained concurred with those obtained in the first experimental series. Therefore, S-enolase appears to have a very low basal expression level

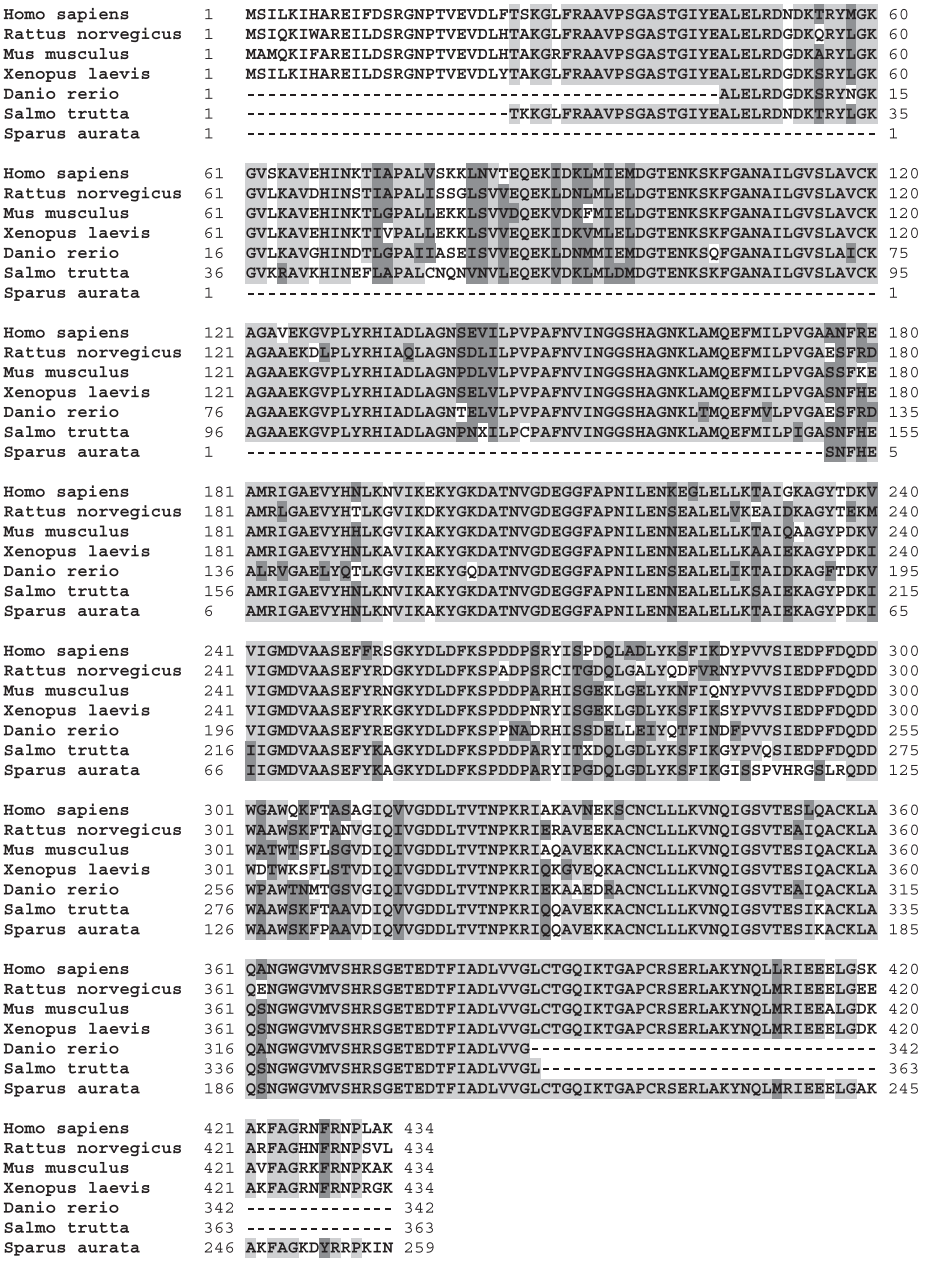

Fig. 2. Protein alignment of different enolase sequences of different species (ClustalW). Accession numbers to Gene Bank data base are the following: *Homo sapiens* enolase-1; NM_001428, *Rattus norvegicus* neuron-specific enolase; AF019973, *Mus musculus* enolase-3 beta muscle; NM_007933 *Xenopus laevis* enolase-1 similar to enolase-3 beta muscle; BC045082; *Danio rerio* enolase-3; AY130387 *Salmo trutta* enolase alpha-2; AY005162, *Sparus aurata* enolase; AY263379.

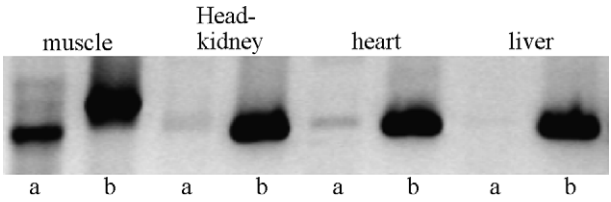

Fig. 3. Results of RT-PCR on total RNA extracted from tissue samples taken from non-stimulated sea bream. For conventional RT-PCR, polymerase chain reactions were initiated and at 35 cycles samples were taken and separated on ethidium bromide-agarose gels and visualized under UV light. a; enolase PCR product: b; β-actin PCR.

in all tissues although we were able to identify expression in the above mentioned tissue samples.

3.2. In vivo regulation

A specific Enolase mRNA of 1.9 kb was identified in total RNA extracted from whole *S. aurata* brain from control fish (Fig. 4). In the parallel experiment where fish were held at a density of 26 kg m^{-3} for 14 days, a slight up-regulation of the S-enolase mRNA was observed (Fig. 4). In order to further study the regulation of the S-enolase mRNA in brain, we injected lipopolysaccharide, LPS (8 mg kg^{-1}) intraperoniteally into groups of sea bream. Significant up-regulation of S-enolase specific mRNA, a 3.95-fold increase, was detected 48 h after of IP-LPS administration (Fig. 5). Furthermore, the observed regulation was transient where S-enolase levels were slightly increased over basal expression 12 h post-injection, peaking at 48 h and returning close to basal levels 72 h post-injection.

4. Discussion

We have applied the Differential Display (DD) technique in an attempt to identify differentially expressed genes in the sea bream (*S. aurata*) brain during acclimation to

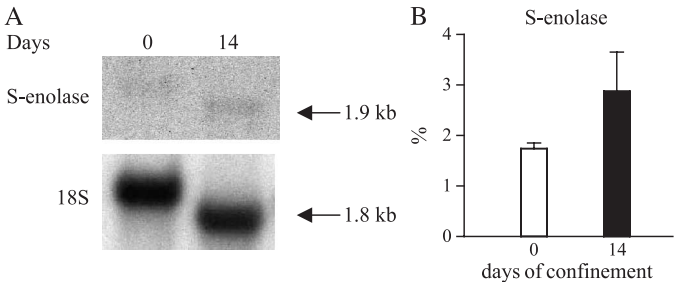

Fig. 4. Enolase gene expression in brain from fish confined for 14 days at 26 kg m^{-3}. (A) Northern blot of S-enolase gene expression, one representative experiment is shown. 18S rRNA expression used as control. (B) Densitometric quantification of bands obtained. Student's *t*-test was used as a statistical test. No significant differences ($p<0.05$) were found. Values are represented as the mean±S.E.M. ($n=3$).

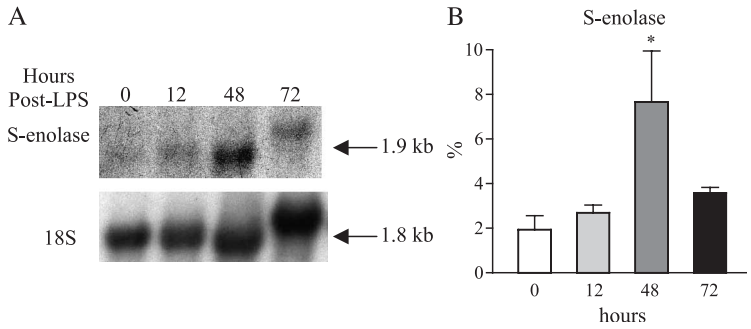

Fig. 5. Enolase gene expression in brain from fish injected with 8 mg kg^{-1} lipopolysaccharide (LPS). (A) Time course of S-enolase gene expression post-LPS administration, one representative experiment is shown. 18S rRNA expression used as control. (B) Densitometric quantification of bands obtained. ANOVA was used as a statistical test. *Indicates significant difference ($p<0.05$).

an increased population density. A partial cDNA sequence (S-enolase) obtained in the DD shows a high sequence homology at both the nucleotide and protein level to all members of the enolase enzyme family. The S-enolase sequence was most similar (90%) to a brown trout (*S. trutta*) enolase alpha2 sequence (Tracy and Hedges, 2000) whereas homology to a published zebrafish (*D. rerio*) enolase 3 (beta isoform) sequence (Takezaki et al., 2003) is relatively low (65%). In addition, homology to alpha isoforms in higher vertebrates ranged between 73% and 74%. Phylogenetic analysis identifies the S-enolase sequence obtained to pertain more closely to the alpha isoform group of enolase sequences (data not shown). However, considering the very high sequence homologies within the enolase gene isoforms particularly the alpha and beta isoforms we suggest that the S-enolase sequence obtained is alpha-like. Recently, we have also cloned a beta-like isoform from sea bream muscle (unpublished data) and a gamma-like isoform from trout macrophages (Goetz et al., 2004) which upon sequence analysis are distinct from the alpha-like isoform presented in this study (81% and 48% amino acid homology, respectively).

S-enolase mRNA expression in different tissues; liver, brain, head kidney and muscle of unstimulated sea bream (*S. aurata*) was detectable by both conventional RT-PCR and Northern blotting. However, expression levels observed were very low and required an elevated number of PCR cycles (35–40). The higher signal obtained in muscle tissue is likely due to the co-amplification of the muscle-specific beta isoform of enolase. We have sequenced the PCR product from sea bream muscle and identified it is beta-like enolase (MacKenzie, unpublished data). Similar reports in other species due to the similarity in nucleotide sequences between enolase isoforms may result in cross-hybridisation (Oliva et al., 1989). Low levels of enolase alpha-1 protein expression have been reported in bovine endothelial cells (Aaronson et al., 1995) and in rat, avian and xenopus tissues (Segil et al., 1988). Enolase alpha is absent in the liver of the longjaw mudsucker under control conditions (*G. mirabilis*) (Gracey et al., 2001). Therefore, it is apparent that enolase alpha expression is low/undetectable under basal expression conditions.

Under increased population density conditions (HD), we did not find any significant differences in S-enolase mRNA expression, although a trend towards up-regulation is

observed (Fig. 4). This may be explained by the population density used which may not represent an intense crowding situation (26 kg m^{-3}). Recently, in a study comparing gene expression in seabass (*Dicentrarchus labrax* L.) at different population densities (80, 100 kg m^{-3}), six bands not identified were found to be differentially expressed (repressed or enhanced) in liver and brain, giving evidence that high densities may be responsible for changes in gene expression in specific fish organs (Gornati et al., 2004).

Northern analysis results obtained after an IP LPS administration, display a significantly up-regulated brain enolase gene expression after 48 h and, at 72 h, a down-regulation is detected (Fig. 5). In a study of gene expression in a euryoxic fish, the longjaw mudsucker (*G. mirabilis*) in which hypoxia was induced, the enolase gene in liver was up-regulated after 72 and 144 h of hypoxia (Gracey et al., 2001). Furthermore, enolase alpha expression was found to be increased in human peripheral blood mononuclear cells (PBMCs) after cadmium exposure (Shin et al., 2003), in bovine endothelial cells under hypoxic conditions (Aaronson et al., 1995) and in immune sera from *C. albicans*-infected mice (Pitarch et al., 2001). These studies show that the enolase alpha gene responds to stressors including heavy metal exposure, environmental variation and infection suggesting that the enolase gene participates in a general stress response similar to that observed with heat shock protein expression.

Administration of LPS results in the activation of the HPA axis (Grinevich et al., 2001). Interaction between the immune and nervous systems plays an important role in modulating host susceptibility and resistance to inflammatory disease (Sternberg, 2001). Although less data is available than in higher vertebrates, cytokines as modulators of the immune response have also been studied in fish and a significant number are functionally active in teleosts (Secombes, 1996). Holland et al. (2002) provided the first direct evidence that directly implicates fish cytokines (IL1-β) as the effectors of the immune system signaling to the neuro-endocrine system and activating the HPI stress axis inducing cortisol secretion. Our results provide new evidence toward the existence of an interconnection between the neuro–immuno-endocrine network in which S-enolase gene expression is up-regulated after LPS administration in the brain. Whether increased S-enolase gene expression represents regulation of metabolism in the brain or a protective cellular response to infection remains an interesting question.

In conclusion, we have identified and characterised an enolase alpha-like gene in the sea bream (*S. aurata*) which is highly conserved with all other available enolase gene sequences. Enolase isoforms not only fundamentally function within the glycolytic pathway but also play a role in the response to hypoxia, infection and heavy metal exposure. S-enolase is up-regulated in the brain after in vivo LPS injection. Therefore, S-enolase represents an interesting potential marker gene for the stress response in fish under diverse conditions including population density and infection.

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References

Aaronson, R.M., Graven, K.K., Tucci, M., McDonald, R.J., Farber, H.W., 1995. Non-neuronal enolase is an endothelial hypoxic stress protein. J. Biol. Chem. 270, 27752–27757.

Babbitt, P.C., Hasson, M.S., Wedekind, J.E., Palmer, D.R., Barret, W.C., Reed, G.H., Rayment, I., Ringe, D., Kenyon, G.L., Gerelt, J.A., 1996. The enolase superfamily: a general strategy for enzyme catalyzed abstraction of the alpha-protons of carboxylic acids. Biochemistry 35, 16489–16501.

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

Besedovsky, H.O., del Rey, A., 1996. Immune–neuro-endocrine interactions: facts and hypotheses. Endocr. Rev. 17, 64–102.

Goetz, F.W., Iliev, D.B., McCauley, L.A.R., Liarte, C.Q., Tort, L., Planas, J.V., MacKenzie, S., 2004. Expressed sequence tag analysis of lipopolysaccharide-stimulated rainbow trout (*Oncorhynchus mykiss*) macrophages. Mol. Immunol. (under review).

Gornati, R., Terova, G., Vigetti, D., Mariangela, P., Saroglia, M., Bernardini, G., 2004. Effects of population density on seabass (*Dientrarchus labrax* L.) gene expression. Aquaculture 230, 229–239.

Gracey, A.Y., Troll, J.V., Somero, G.N., 2001. Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. PNAS 98 (4), 1993–1998.

Grinevich, V., Ma, X.M., Herman, J.P., Jezova, D., Akmayev, I., Aguilera, G., 2001. Effect of repeated lipopolysaccharide administration on tissue cytokine expression and hypothalamic–pituitary–adrenal axis activity in rats. J. Neuroendocrinol. 13, 711–723.

Holland, J.W., Pottinger, T.G., Secombes, C.J., 2002. Recombinant interleukin-1b activates the hypothalamic–pituitary–interrenal axis in rainbow trout, *Oncorhynchus mykiss*. J. Endocrinol. 175, 261–267.

MacKenzie, S., Fernandez-Troy, N., Espel, E., 2002. Post-transcriptional regulation of TNF-alpha during in vitro differentiation of human monocytes/macrophages in primary culture. J. Leukoc. Biol. 71, 1026–1032.

Miles, L.A., Dahlberg, C.M., Plescia, J., Felez, J., Kato, K., Plow, E.F., 1991. Role of cell-surface lysines in plasminogen binding to cells: identification of alpha-enolase as a candidate plasminogen receptor. Biochemistry 30, 1682–1691.

Oliva, D., Barba, G., Barbieri, G., Giallongo, A., Feo, S., 1989. Cloning, expression and sequence homologies of cDNA for human gamma enolase. Gene 79, 355–360.

Pitarch, A., Diez-Orejas, R., Molero, G., Pardo, M., Sanchez, M., Gil, C., Nombela, C., 2001. Analysis of the serologic response to systemic *Candida albicans* infection in a murine model. Proteomics 1, 550–559.

Roland, I., Minet, E., Ernest, I., Pascal, T., Michel, G., Remacle, J., Michiels, C., 2000. Identification of hypoxia-responsive messengers expressed in human microvascular endothelial cells using differential display RT-PCR. Eur. J. Biochem. 267, 3567–3574.

Secombes, C.J., 1996. Cytokines in fish. An update. Fish Shellfish Immunol. 6, 291–304.

Segil, N., Shrutkowski, A., Dworkin, M.B., Dworkin-Rastl, E., 1988. Enolase isoenzymes in adult and developing *Xenopus laevis* and characterization of a cloned enolase sequence. Biochem. J. 251, 31–39.

Shin, H.J., Park, K.K., Lee, B.H., Moon, C.K., Lee, M.O., 2003. Identification of genes that are induced after cadmium exposure by suppression subtractive hybridization. Toxicology 191, 121–131.

Sternberg, E.M., 2001. Neuroendocrine regulation of autoimmune/inflammatory disease. J. Endocrinol. 169, 429–435.

Subramanian, A., Miller, D.M., 2000. Structural analysis of alpha-enolase. Mapping the functional domains involved in down-regulation of the c-myc protooncogene. J. Biol. Chem. 275, 5958–5965.

Takezaki, N., Figueroa, F., Zaleska-Rutczynska, Z., Klein, J., 2003. Molecular phylogeny of early vertebrates: monophyly of the agnathans as revealed by sequences of 35 genes. Mol. Biol. Evol. 20, 287–292.

Tort, L., Gómez, E., Montero, D., Sunyer, O., 1998. Stress and immunosuppression in fish. Comp. Biochem. Physiol. 5, 17–29.

Tracy, M.R., Hedges, B.S., 2000. Evolutionary history of the enolase gene family. Gene 259, 129–138.