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REPORT OF MEETING

IXth scientific meeting of the Italian Association of Developmental and Comparative Immunobiology (IADCI), 27 - 29 February 2008, Biological Departments, University of Insubria, Varese, Italy

Organizers: **M de Eguileor, A Grimaldi, G Tettamanti, R Valvassori**

Department of Structural and Functional Biology, University of Insubria, Varese, Italy

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Session 1. Chairman: M Cammarata, University of Palermo, Italy

The immune system of compound ascidians

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Differently from vertebrates, having an adaptive immunity, ascidians are chordates with a germ-line based innate immunity in which phagocytosis and cytotoxicity are the main effector systems.

In recent years, the comprehension of the immune system of compound ascidian has greatly improved. Most of the research was carried out in species of the genus *Botryllus* and *Botrylloides*. In these organisms, immunocytes constitute a considerable fraction of circulating haemocytes and are represented by phagocytes and phenoloxidase (PO)-containing, cytotoxic cells.

Phagocytes include hyaline amoebocytes (HA) and macrophage-like cells (MLC). The former are wandering cells which quickly recognise and engulf foreign particles or cells: after the ingestion, they withdraw their cytoplasmic projections and acquire a globular shape, turning to MLC. Phagocytosis requires the recognition of molecular patterns on the surface of target particles by receptors on phagocytes and is greatly influenced by the nature of the particle. The recognition triggers signal transduction pathways which lead to the activation of the MAP-kinase cascade and of NF-kB. Soluble lectins can increase the phagocytosis of foreign particles acting as opsonins.

Phagocytes play an important role during the colonial generation change (take-over), when the old adult zooids stop their filtering activity and are progressively resorbed. During this period, tissues of adult zooids undergo diffuse apoptosis and are rapidly infiltrated by blood phagocytes which rapidly and massively engulf senescent cells.

Cytotoxic morula cells are characterised by the presence of vacuoles containing inactive pro-PO and its polyphenol substrata. They are the effectors of the rejection reaction between contacting, genetically incompatible colonies. These cells crowd inside the ampullae contacting the alien colony, cross the epithelium of the ampullar tips and enter the tunic where they degranulate and release the content of their vacuoles. The activated PO acts on substrata and induce necrotic death through the induction of an oxidative stress.

There is an interesting cross-talk between cytotoxic an phagocytic cells: i) phagocytosis can be modulated by soluble molecules (cytokines) released upon the recognition of foreign particles (yeast cells and bacterial spores) by the cytotoxic cells; ii) some HA are exposed to seawater, on the internal side of the siphons, where they act as guard cells. Once recognised foreign materials, they activate morula cells in the tentacular lacunae which, in turn alert the whole immune system towards the potentially dangerous particles that can be phagocytosed or degraded.

Further insights on siphonal guard cells of ascidians

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In the oral siphon of the colonial ascidian *Botryllus schlosseri*, hyaline amoebocytes directly exposed to the sea-water flow entering into the pharynx have been recently observed and described. These cells, named “siphonal guard cells” (SGC), are free of moving on the surface of the tunic that internally covers the siphons. Our previous observations by means of histochemical, histoenzymatic and immunohistochemical techniques showed that they share many morpho-functional characteristics with the phagocytic blood cell line, from which probably they originate, and are able to recognise and phagocytise various foreign particles.

After exposure of colonies to bacterial spores, the observations at both light and electronic microscope revealed that these cells are involved in a complex and unusual series of local and

systemic immune events. Already after 5 min, the SGC showed bacteria inside their heterophagic vacuoles. After 10-15 min, as a transitory plug of floccular and colloidal material formed in the lumen of the siphon by exocytosis of some SGC, other ones with engulfed bacteria crossed the epidermis of the siphon reaching the siphonal sinus; cells of the cytotoxic blood cell line (morula cells) were drawn and crowded into the siphonal sinus, where most of them were positive to anti-TNF-α and anti-CD57 antibodies and degranulated stimulating, after this time and until 12 h, large scavenger phagocytes. The latter showed bacteria engulfed in their large phagosomes, increased in number in the blood circulation and were continuously eliminated through the peribranchial chamber with a mechanism which was never previously described.

As regards the ability to transfer an alert signal, the role of SGC appears important as regards the immunosurveillance of the opening of the alimentary canal, similarly to what occurs in the vertebrate oropharyngeal lymphatic tissues.

Apoptosis signalling pathways in the compoud ascidian *Botryllus schlosseri* during the colonial blastogenetic cycle

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In the colony of the ascidian *Botryllus schlosseri*, three blastogenetic generations are usually present: adult zooids, primary buds on zooids and secondary buds on primary buds. Colonies undergo recurrent generation changes in which adult zooids are gradually resorbed and replaced by new blastogenetic generations. It is possibile, therefore, to define a colonial blastogenetic cycle that begins with the appearance of a new generation, and ends with the generation change, during the take-over phase, in which programmed cell death by apoptosis is largely diffuse.

Using the haemocytes as reference tissue we investigate the extent of cell death during the colonial blastogenetic cycle .

Our results confirm the expression, on cells surfaces, of Fas receptors and theirs ligands Fas-L. Moreover, we showed the presence of members of the caspase family: the initiator caspases 8 and 9 and the executioner caspases 3 and 7. The activated executioner caspases can subsequently cleave distinct cellular proteins such as PARP: using immunoblot assay we observed the cleaveage of proteins recognised by anti-PARP. In Vertebrates, intrinsically mediated initiation begins with mitochondrial membrane disruption resulting in cytochrome c (cyt c) release. We observed an encrease of H_2O_2 in cytoplasmatic contents and a different expression of cyt c during the take-over.

These results confirm *Botryllus* an interesting model organism for the study of apoptosis.

A novel rhamnose-binding lectin from the colonial ascidian *Botryllus schlosseri*

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Lectins are carbohydrate-binding proteins which agglutinate cells and/or precipitate glycoconjugates. The family of Rhamnose binding lectins (RBLs) includes various proteins, previously classified as galectins, with common sugar specificity and one to three homolougous carbohydrate-recognition domains (CRDs), about 100-aminoacids long and characterised by eight highly conserved cysteine residues. From a full-length cDNA library from the compound ascidian *Botryllus schlosseri* we identified five complete transcripts homologous to known RBLs. Comparisons of the predicted amino acid sequences (118 aa) suggest that they represent different isoforms of a novel RBL, called BsRBL-1-5 with only one CRD. Reverse-phase HPLC and mass spectrometry of the affinity-purified material confirmed the presence of four of these isolectins in *Botryllus* homogenate. Analysis of both molecular masses and tryptic digests of BsRBLs indicated that the N-terminal sequence of the purified proteins starts from residue 22 of the putative amino acid sequence, so that residues 1-21 represent a signal peptide. Analysis by mass spectrometry of V8-protease digests confirmed the presence and alignments of the eight cysteines involved in the disulphide bridges characterising RBLs. Functional studies confirmed the enhancing effect on phagocytosis of the affinity-purified material. The phylogenetic relationship of Bs-RBLs with orthologous molecules from protostomes and deuterostomes was also studied.

Enhanced expression of a *CinTNF* gene in the LPS challenged inflammatory responses of the ascidian *Ciona intestinalis*

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In invertebrates immune system, cell proliferation, phagocytosis and chemotaxis are regulated by cytophilic humoral molecules with functional similarities to vertebrate cytokines. These molecules modulate defense responses to exogenous and endogenous insults, tissue repair and recovery of homeostasis by ligand-specific receptor interactions required to initiate and regulate immune responses. tumor necrosis factor (TNF) is a pro-inflammatory cytokine produced as part of the innate response. In invertebrates TNF-like molecules have been identified by using various methods. Since in *Ciona intestinalis* genome (Ensembl) TNF gene has been identified (*CinTNF*),

Real-time PCR analysis was carried out. Results showed a prompt (2-4 hrs) enhanced *CinTNF* gene expression in the inflamed body wall after intratunic LPS injection. *In situ* hybridization assays supported the involvement of pharynx hemocytes in the inflammatory response, and transcript was mainly found in morula cells and in unidentified cells associated with epidermis. Similar results were found by examining hemocytes from the hemolymph. Immunoblotting assay with anti-CinTNF specific antibodies revealed that a 17 kDa CinTNF is released in the hemolymph.

Sphingomyelin as well as carbohydrates are involved in the mechanism of cytotoxic molecules contained and released *in vitro* by *Ciona intestinalis* granulocytes

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The immune-system of invertebrates recognizes and then reacts to foreign particles potentially pathogen by means of cytotoxicity, phagocytosis, encapsulation, and humoral effector mechanisms (agglutination, lysis). Recent studies have shown the presence in invertebrates, including tunicates, of hemocyte/coelomocyte cytolytic molecules (lysins) that can be released into the hemolymph or coelomic fluid, and their lytic activity depends on their integration inside target cell membranes. In some cases membrane lesions appear as circular pores as shown by electron microscopy observations. There are few data on ascidian lysins and their lytic mechanisms. In *Ciona intestinalis* a cytotoxic activity towards mammalian erythrocytes has been reported. The lytic activity, examined using a Tris-buffered saline containing calcium ions and isosmotic to the hemolymph, was inhibited by sphingomyelin (25 µg/ml). To identify the lysin-releasing cells, hemolymph was separated through a discontinuous Percoll gradient. The hemocyte populations were separated in 6 bands, each of them appeared to be enriched with a particular hemocyte population. The hemocytes from band 5 (B5) mainly composed with unilocular refractile granulocytes were responsible for the lysis of rabbit erythrocytes (RE). To characterize the activity, the supernatant of the B5 hemocyte lysate supernatant (B5HLS) or B5 hemocyte culture supernatant (B5HCS) was assayed with different mammal targets. Both B5HLS and B5HCS contain at various extent lytic activity against RE and K562 tumor cell line. A lower level of cytotoxicity was found against sheep erythrocytes. Such an activity was calcium-dependent, thermo-stable (56 °C), inhibited by sphingomyelin (25 µg/ml), phospholypase A2 inhibitors e.g. dibucain and quinacrine, as well as by D-galactose and cell-free hemolymph. Present results suggest that a complex lytic mechanism dependent on sugar-CRD interaction may be involved in innate immune response of *Ciona intestinalis*.

Session 2. Chairman: L Abelli, University of Ferrara, Italy

Leech neuroimmunity: a crossing point between injury and nerve repair

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Lophotrochozoans (Annelids and Molluscs) are increasingly contributing, along with Vertebrates and Ecdysozoans, to a deeper understanding of important biological processes like innate immunity or neurogenesis. Among the Annelids, the medicinal leech *Hirudo medicinalis*, is one of the most extensively used model organism since the XIX century for neurobiology studies. Indeed, the development, the anatomy and the physiology of many nerve cells are now well characterized. In addition, the medicinal leech is a recognized model in central nervous system (CNS) regeneration studies because of its capacity to get in a few weeks a complete morphological and functional repair of the injured nerve cord. Recent results showed that leech CNS is able to activate an innate immune response upon bacterial challenge and that several induced molecules are also known to be involved in regeneration events. Early alert signals, glial cells recruitment, neuronal growth and axonal guidance are the major events occurring during CNS regeneration in *Hirudo*, but the basic molecular mechanisms of these processes are little known. We presently develop a research project aimed to the comprehension of such mechanisms and the characterization of some protagonist of this complex phenomenon. The main points of interest and the guidelines of our research are here briefly exposed to discuss the role of neuroimmunity during leech nerve repair.

Leech hematopoietic cells involved in muscle regeneration

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Recently, myogenic and endothelial (myo-endothelial) cell progenitors were identified in the interstitial spaces of murine skeletal muscle. These are primitive cells, distinct from satellite cells (potent myogenic stem cell population residing in the muscle of vertebrates and responsible for postnatal muscle regeneration and growth), they are located outside from the basal lamina and expressing the hematopoietic precursor marker CD34. These $CD34^+$ precursors cells play a fundamental role during muscle regeneration, differentiating into myogenic cells and contributing to new fibres formation.

Hematopoietic and endothelial precursors cells of leeches show an impressive conservation in

morphofunctional and molecular mechanisms compared to vertebrates. For this reason leeches can be considered a simple model to better understand several mechanism regulating muscle-derived hematopoietic stem cell biology. We have previously demonstrated that in leeches endothelial and circulating precursor cells, express CD34 and the VEGF receptor FLK1 that in vertebrate are specific for both the endothelial and the myo-endothelial cells involved in muscle regeneration.

Initially, leeches show in the area of lesion a plug that is in a short time replaced by a pseudoblastema only made of fibroblasts and macrophages without any type of muscle fibres. After 2 months from injury, new muscle are present in the cicatricial area and the damaged body sac is regenerated.

We focus on the origin of the new muscle fibres since, unlike from vertebrates, no satellite cells are present in the adult muscle body. Our findings suggest that $CD34^+$ circulating precursors cells in leeches, as muscle-derived hematopoietic vertebrate stem cell, can be a source of myogenic precursors, directly recruited from VEGF in the regenerative area.

Self/non-self recognition in the ciliate *Euplotes raikovi*: characterization of E*r*-MAPK1, a downstream component of the autocrine signal transduction pathway

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In the ciliate *Euplotes raikovi*, cell type-specific, water-borne signal proteins (pheromones) control self/non-self recognition phenomena by binding their target cell-surface receptors and activating downstream signal transduction pathways. Immunorecognition analyses of *E. raikovi* cell extracts revealed that at least three distinct protein kinases are activated (phosphorylated) in functional association with the autocrine pheromone-receptor loop that promotes the vegetative (mitogenic) cell growth. One of these kinases, designated as E*r*-MAPK1 (from Mitogen-Activated Protein Kinases), was structurally characterized by molecular cloning of the relevant gene. The E*r*-MAPK1 N-terminal half of 300 amino acids bears unmistakable structural homology with the "intestinal cell kinase" and the "male-germ cell associated kinase", that are involved in the regulation of proliferation and differentiation of specialized animal cells. It contains all the basic structural features that are required for a MAPK catalytic activity, in particular the dual phosphorylation site represented by the Thr-Asp-Tyr motif in the activation loop. In contrast, the E*r*-MAPK1 C-terminal half of 331 amino acids appears to be structurally unique. It is particularly rich in glycine residues and potential sites of regulatory activities, and shows sequence motifs that clearly predicts a nuclear localization of E*r*-MAPK1.

Old and new immunomarkers to assess health status of clams (*Tapes philippinarum*) from the Lagoon of Venice

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The aim of the present study was to examine the health status of clams (*Tapes philippinarum*) from the Lagoon of Venice by means of immunomarkers. Bivalves were collected in June 2007 in 8 sites of the Lagoon characterised by differing contamination levels. Immunomarkers included total haemocyte count (THC), lysozyme-like activity in cell-free haemolymph (as measure of cell membrane stability), and vitellogenin (Vg)-like protein levels in both digestive gland and cell-free haemolymph. The results showed that clams sampled at Marghera and Fusina (highly polluted sites) had significantly reduced THC values with respect to those of animals from the other sites. Conversely, significantly increased THC was observed in clams from Valle di Brenta and Cà Roman, influenced by wastewater from agricultural areas and intense fishing and passage of ships, respectively. Significantly increased lysozyme-like activity was also recorded in cell-free haemolymph of clams collected at the most polluted sites (Campalto, Marghera, Cà Roman), suggesting that destabilisation of cell membranes occurred in haemocytes. Interestingly, altered Vg-like protein levels were observed in digestive gland and haemolymph of both male and female clams from contaminated sites. Vg induction is generally recognised as a biomarker of exposure to estrogenic compounds. However, in the light of recent findings concerning involvement of Vg in immune responses (Zhang *et al.* Fish Shellfish Immunol. 19: 93-95, 2005), application of Vg as potential immunomarker in future laboratory and field studies is suggested. On the basis of the immune responses analysed in the present study, we can conclude that the health status of *T. philippinarum* at contaminated sites is poor. However, influence of both exogenous (i.e., water temperature and salinity, food availability) and endogenous factors (i.e., reproductive cycle) on cell functional responses investigated have to be taken into proper account.

A new hemagglutinin from the coelomic fluid of the sea urchin *Paracentrotus lividus* (Lamarck, 1816) (Echinodermata)

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Agglutinins or lectins are glycoproteins that play a fundamental role in the innate immune responses in invertebrates. They are usually detected in cell-free coelomic fluid or hemolymph by the ability to agglutinate particles such as vertebrate erythrocytes (hemagglutinin), bacteria, protozoa or fungal cells. Hemolymph lectins have been isolated from several

species of invertebrate taxa, while few data are available in Echinodermata. In the present study a hemagglutinin was purified from the coelomic fluid of the sea urchin *Paracentrotus lividus*, by ion-exchange chromatography on DEAE-Sephadex. A fraction, corresponding to a band at 11 kDa in SDS PAGE, was found to have hemagglutinating activity. The amino acid composition of the lectin is in progress by the use of a 2D electrophoresis followed by MALDI TOF analysis.

Session 3. Chairman: R Valvassori, University of Insubria, Varese, Italy

Immunosuppression and host regulation by parasitic Hymenoptera

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The success of parasitism in Hymenoptera is largely influenced by molecules and genes that the ovipositing female injects along with the egg into the host's body or that offspring produces during the course of development. Here we analyze how the basal "toolkit" of gene products in ancestral ectoparasitic idiobionts has changed with the evolution of more intimate developmental strategies, which require very efficient mechanisms of evasion and/or suppression of host immune response. The functional and molecular bases of the major alterations of host immune system, endocrine balance, development and reproduction induced by regulation factors, both of maternal and embryonic/larval origin, are presented and the impact of these changes on host suitability and parasitoid fitness analyzed.

hrIL-8 stimulates *unpaired (upd)-3* expression and other immune-related activities in *Drosophila melanogaster* SL2 cell line

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Invertebrate innate immunity relies on both cellular and humoral components. Among humoral factors, there is less information on soluble molecules able to act as signals during the immune response (i.e. cytokines). To date, only a few cytokines have been observed in different arthropod taxa such as insects (Spätzle and Unpaired [Upd]-3) and crustaceans (astakine). As it has been proposed that a possible involvement of chemotactic factors may increase the expression of *upd-3*, we have studied the effects of human recombinant iInterleukin-8 (hrIL-8) on the immune functions of *Drosophila melanogaster* SL2 macrophage-like cells. We have found that hrIL-8 enhances the expression of *upd-3* and of the putative cytokine, *Drosophila* helical factor (*dhf*). Furthermore, hrIL-8 promotes the transcription of the antimicrobial peptide genes *defensin* and *cecropin A1*. Beside these evidences on humoral factors, hrIL-8 also increases the phagocytic activity of SL2 cells. Our data suggest the existence in *D. melanogaster* of one or more soluble factors that possibly share some structural similarity with IL-8, eliciting an immune response involving simultaneously cellular and humoral components.

IL-1 system in reproduction of invertebrates

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Interleukin-1 (IL-1) is a key regulator of the inflammatory response and an important mediator of materno-fetal immunotolerance in mice. We recently showed that IL1-β and its functional membrane receptor type I (IL-1R tI) are also expressed in the female reproductive tract of vertebrate species with different reproductive strategies, i.e. viviparity and oviparity. In both cases, there are mechanisms of materno-fetal immunotollerance since the sperm enter and cross the female genital tract and then, the fertilized egg/zygote/embryo is transported or retained in the oviduct. To date, research available is limited to vertebrates while no data are reported in invertebrates. Since the cytoplasmic domain of IL-1R tI shares high homology with the cytoplasmic region of Toll protein that recognize many pathogen associated molecular patterns in *Drosophila*, we investigated the expression of Toll-IL1R tI (TIR) domain in reproductive tissues of this invertebrate. By using a specific anti-human antibody against the TIR domain, western blot analysis revealed a band of 117 kDa in membrane lysates corresponding to *Drosophila* Toll protein. Immunohistochemistry showed protein expression in the epithelial cells of oviductal tissues and in the spermathecae.

These findings suggest a potential ubiquitous role of IL-1 system in reproductive tissues of vertebrates and invertebrates.

Differential responses of mussel hemocytes to bacterial challenge

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Marine bivalves are widespread molluscs in coastal waters at different latitudes. Due to their filter-feeding habits, they accumulate large number of both autochthonous and allochtonous bacteria from the harvesting waters.

In this work the functional responses of *Mytilus* hemocytes to in vivo challenge with different

bacteria were evaluated. Two Gram-negative bacteria, *Vibrio splendidus* and *Vibrio anguillarum*, and one Gram-positive bacterium, *Micrococcus lysodeikticus*, were used. Mussels have been injected with heat-killed bacteria and hemocytes sampled at different time post-injection (from 3 to 24 h).

The results demonstrated that different bacteria elicited significant functional responses in mussel hemocytes. All bacteria lead to a significant lysosome membrane destabilisation (*V. splendidus* > *V. anguillarum* > *M. lysodeikticus*) at short time post-injection followed by recovery at longer times. Similar effects have been observed in mussels collected from two different populations (Adriatic Sea and Ligurian Sea). Bacterial challenge also induced a significant increase (about 100 %) in serum lysozyme activity. Moreover, challenge with *V. anguillarum* significantly stimulated the bactericidal activity of hemocytes towards *E. coli*.

The results indicated differential functional responses of mussel hemocytes to different bacteria. The application of this knowledge to the understanding of the actual adaptive responses of bivalve when exposed to micro-organisms in their natural environment can represent significant ecological, economical and public health-related interest.

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Morphological changes of *Mytilus galloprovincialis* hemocytes after bacterial interaction

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Apoptosis represents a physiological process used by multicellular organisms to regulate the cell number (tissue homeostasis), to remove damaged or unwanted cells or to eliminate different pathogens. Protozoan parasites can induce or inhibit apoptosis in host cells, whereas bacteria increased apoptosis in human neutrophils and macrophages, interfering with such essential components of the innate immunity. So, up-regulation of apoptosis due to infection may result in immune suppression. Mainly studied in Vertebrates, only few data are available on the relationships between apoptosis and immunity in Invertebrates.

In the present study, we investigated the response of the marine bivalve *Mytilus galloprovincialis* hemocytes to different bacteria species, as well as the morphological modifications of their nucleus using microscopic observations.. The numbers of hemocytes that detached from the substrate and died by necrosis increased after bacteria contact. The detached hemocytes became round losing their typical cytoplasm extensions. They were also evidences for morphological changes of nucleus with chromatin condensation and bordering at the nucleus periphery, and sometimes its fragmentation. Control of apoptotic induction in mussel has been done on adhering hemocytes treated with H_2O_2 and puromicin.

In conclusion, the changes in nucleus morphology and the rounding of cells suggested apoptosis activation, being more evident with *Vibrio splendidus* than with *Vibrio anguillarum*.

Session 4. Chairman U Oreste, CNR, Naples, Italy

Immune defences and interactions with the environment

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Animal species live in a condition of homeostasis with the microbiome present in their body and in the environment. This microbiome is constituted by a great number of microorganism species, many of which are potential pathogens. The homeostatic condition describes a continuous and dynamic equilibrium present between aggression strategies developed by microorganisms, and defence strategies invented by animal eukaryiotes. During millenia, the continuous development of aggression and defence strategies became an evolutionary machine that produced a great number of orthologous and paralogous genes. Also, the evolution of animal behaviours has been possible by the evolutive capabilities offered by the immune system, that must guarantee full immune defence in every environmental condition. The research performed in our lab considered the morphological and functional organisation of immune defences initially in insects, subsequently in teleost fish and amphibians. Teleost fish represent an experimental model widely employed, since are oldest living vertebrates with a functional platform of immune system conserved in all its components until mammals, they are present in every aquatic environment of the planet, and are of interest for animal and environmental biotechnolgy. In addition, teleosts have a free-swimming larval stage, and it's possible to divide vital stages when only innate defences are present, and when both innate and acquired defences are functional. Employing as main investiagted species the sea bass *Dicentrarchus labrax*, we defined in a teleost species the panel of distribution and the ontogenesis of lymphocytes, the involvement of lymphocytes during antigenic stimulation "in vivo" and "in vitro", the cloning of genes coding for immunoregulatory molecules, and the expression of these genes during immune responses. In the IMAQUANIM consortium, we are developing systems of transcriptomic analysis by using PCR-arrays, to evaluate immune responses through the simultaneous expression of groups of genes coding i) for the whole sea bass immunome (ca 50 genes), ii) for the "innate" immunome (ca 15 genes), and iii) for the "acquired" immunome (ca 35 genes). Of great interest for immune studies is gut-associated

immune system, whose functional organisation could be also related to the intestinal microbiome and food habits. Many T cells are present in the intestinal epithelium of predatory teleosts, and current research is in progress to extend these observations to herbivorous and plancton-eating fish species.

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Thymocyte decisions in sea bass, *Dicentrarchus labrax* (L.): CD4 or CD8? Gene expression profiling and *in situ* hybridisation studies

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In teleosts no precise information is still available on differentiation and selection of T cells, crucial steps to establish a functional immune system, although the role of thymic microenvironment in T-cell development has long been recognized in mammals.

This work first defines the expression levels of *CD4*, *CD8-α* and *TCR-β,* three important genes in T cell function, in the thymus of the marine teleost *Dicentrarchus labrax* (L.). Specific primers were used to analyse by real time PCR gene expression in the thymus of one-year-old specimens. The results reported high *TCR-β* and *CD8-α* transcripts, while *CD4* transcripts were lower ($p<0.05$ vs. *TCR-β*).

The *in situ* hybridization with RNA probes identified *CD4* and *CD8-α* expressing cells in each thymic lobe, $CD8\alpha^+$ and $CD4^+$ thymocytes almost filled the cortex drawing a cortex-medulla demarcation and extended in large cords in the medulla. In sea bass thymus, the expression pattern of *CD4* and *CD8-α* largely overlapped that of *TCR-β*, except in the subcapsular zone where $TCR\beta^+$ double-negative thymocytes were detected. These results provide new information about the thymic compartmentalization in teleosts and leads to new hypotheses about thymocyte differentiation pathways.

Gene expression and functional studies on gut immune system of the sea bass, *Dicentrarchus labrax* (L.)

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Morphological and molecular data have evidenced in a few teleost species a gut-associated lymphoid tissue (GALT), consisting mainly of T cells, whose origin, selection and functions are still unclear.

This work reports about TCR-β, CD8-α and CD4 transcription in the intestine of the marine teleost *Dicentrarchus labrax* (L.) and localization of T cells expressing such genes. Anterior, middle and posterior intestine segments of one-year-old specimens were sampled for RQ-PCR and *in situ* hybridization (ISH) studies.

TCR-β and CD4 transcription did not differ along the intestine, while CD8-α transcripts rose in the posterior segment ($p<0.05$ vs. middle). In the whole intestine TCR-β and CD8-α transcripts were higher than CD4 ones ($p<0.001$). In anterior and middle segments TCR-β expression was higher than CD8-α ($p<0.001$).

The ISH with RNA probes identified numerous TCR-β+ and CD8-α+ cells intraepithelially and in the lamina propria of mucosa, the latter forming aggregates, and rare CD4+ cells. Percoll-purified leucocytes from the whole intestine showed innate cytotoxic activity against a xenogenic tumour cell line.

There results confirm that the sea bass GALT consists mainly of T cells with significant cytotoxic activity.

Early administration of probiotic strains stimulates the gut immune system of the marine teleosts *Dicentrarchus labrax* and *Sparus aurata*

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Early feeding (started during gut metamorphosis) with probiotic-supplemented diets, besides modifying the intestinal microflora, provoked profound effects on physiology of fish larvae.

Using rotifers and Artemia as living vectors, the autochthonous bacterium *Lactobacillus delbrueckii* or a multispecies probiotic formulation (autochthonous *Lactobacillus fructivorans* + *Lactobacillus plantarum* from human faeces) were orally administered to sea bass and gilthead seabream larvae, respectively.

The treatments stimulated the larval rearing (significantly increased body weight, decreased cortisol levels and improved stress response) and the immune system.

In sea bass, the probiotic raised intestinal T-cells (+105 %), in keeping with increased total body TcR-β transcript (+41 %), and acidophilic granulocytes (+118 %) concomitant to lower transcription of pro-inflammatory genes (*IL-1β, TGF-β, IL-10, Cox-2*).

In seabream, the multispecies formulation raised intestinal Ig+ cells (+51 %) and acidophilic granulocytes (+284 %), mainly belonging to the MAb G7+ phagocytic population (+536 % vs. control).

These results point to stimulatory effects of probiotics on the gut immune system that correlates with improvement of fry survival.

A CD4 homologue in sea bass (*Dicentrarchus labrax*): molecular characterisation and structural considerations

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The CD4 is a transmembrane glycoprotein fundamental for cell-mediated immunity. Its action as a T cell co-receptor increases the avidity of association between a T cell and an antigen-presenting cell by interacting with portions of the complex between MHC class II and TR molecules.
The sea bass CD4 cDNA consists of 2071 bp that translates in one reading frame to give the entire molecule containing 480 amino acids. The analysis of the sequence shows the presence of four putative Ig-like domains and that some fundamental structural features are conserved from sea bass to mammals.

By real time PCR analyses very high levels of CD4 mRNA basal transcripts have been evidenced in thymus, followed by gut and gills. *In vitro* stimulation of head kidney leukocites with LPS and PHA-L gave an increase of CD4 mRNA levels after 4 h and a decrease after 24 h.

Homology modelling has been used to create a 3D model of sea bass CD4 and to investigate its interaction with sea bass MHC-II.

Our results will add new insights in sea bass T cell immune response and will help to identify T cell subsets in teleost fishes to better understand the evolution of cell-mediated immunity.

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Effects of exogenous cortisol on the expression of glucocorticoid receptor (*DIGR1*) and *hsp70* in head-kidney and peritoneal cavity cells from *Dicentrarchus labrax*

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Fish are constantly exposed to stressful conditions, and have evolved responses including the prompt release of corticosteroids hormones. The physiological effects of corticosteroids are regulated by cellular glucocorticoid receptors (GR), that mediate gene expression influencing a variety of physiological function related to metabolism, immunity, beahaviour, osmoregulation and cardiovascular transport. The assembly, functionality and transport of GRs are dependent upon the activity of heat shock proteins (HSP) including HSP 90, HSP 70 and HSP 56. In previous papers we showed that high plasma cortisol level due to fish confinement affects sea bass innate immunity, then we cloned and sequenced a GR from sea bass peritoneal cavity leucocytes (*DIGR1*). In this study we examined the expression of *DIGR1* as related with HSP 70 tissue content in the presence of high exogenous plasma cortisol level. Cortisol (20mg/Kg body mass) was injected into fish peritoneal cavity, and, at various times after the injection, *DIGR1* gene expression was evaluated by real time PCR. HSP 70 was identified by specific antibodies an densitometry analysis of western blot pattern was carried out. Results showed that, despite a drop in DIGR1 protein content, there was a significantly enhanced *DIGR1* mRNA expression in head kidney cells due to stress for manipulation procedures, whereas in leucocytes from peritoneal cavity the enhanced expression of *DIGR1* could be related to a cortisol effect. An increased level of HSP 70 protein was also shown. Research are in progress to evaluate *hsp* 70 gene expression.

Session 5. Chairman: N Parrinello, University of Palermo, Italy

Longevity genes across species: conservation versus evolvability

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The search for longevity genes has greatly developed in recent years basing on the idea that a consistent part of longevity is determined by genetics. The ultimate goal of this research is to identify possible genetic determinants of human aging and longevity, but studies on humans are limited by a series of critical restrictions. For this reason, most of the studies in this field have been, and still are, performed on animal models, basing on the assumption that fundamental biological mechanisms are highly conserved throughout evolution and that, accordingly, extrapolation from model systems to humans is quite reasonable.

Indeed, many comparative data obtained on single genes or gene families fit with this assumption. However, it is also clear that, despite such a basic conservative scenario, major changes also occurred in evolution, particularly regarding biological regulatory processes and integration between and among pathways. This consideration raises the fundamental question of the *transferability* of the results obtained from model systems to humans. Indeed, many data obtained on animal models were not confirmed on humans or, on the contrary, some contributions to the genetics of longevity were discovered in humans and not always have been replicated in animals. Recent conceptualizations stressed the importance of *robustness* as a fundamental property of living organisms, a characteristic acquired during evolution that allow them to be error-tolerant and to easily respond to external perturbations. A *"bow-tie" organizational architecture* is likely to be a common feature of highly organized, robust systems. A bow-tie model is present when many inputs converge on, and are integrated by, few elements, and many different outputs come out as the product of the integration. The elements composing the core ("knot") of the bow-tie in a biological system are "hub proteins" and the genes that encode them are likely to be in many cases robustness genes. Much of the core of a bow-tie is often conserved throughout evolution, but this conservation does not prevent, but rather facilitates the variability of the possible outputs. Thus, a bow-tie structure allows both robustness and evolvability. We propose that longevity genes should participate with a core position to a bow-tie metabolic structure and that a new way to identify putative genetic determinants of longevity could be thus the conservation of their products in bow-tie structures all along evolution.

Thymus development is influeced by hypophyseal and thymic allograft after hypophysectomy: expression of PCNA and CD3 markers

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Experiments of hypophysectomy, followed by hypophyseal or thymic allograft were performed in chicken embryos in order to study the influence of thymus or hypophysis on the thymus development.

Experimental and control thymuses, collected at the 18th day of incubation, were tested for anti-PCNA (proliferating cell nuclear antigen) and anti-CD3 immune reaction. The immunoreactive cells were evaluated by computer-assisted statistical analysis.

In the first series of experiments, hypophysectomy was performed on chick embryos at 36-40 hr of incubation. The thymuses collected evidenced in the cortex, a significant reduction of PCNA immunereactive thymocytes (P<0,05). In the second series of experiments hypophysectomized embryos received at the 12th day of incubation or a hypophyseal allograft or a thymic allograft onto the chorio-allantoic membrane from 18 day-old donor embryos. The hypophyseal allograft allowed an increase of the CD3 expression of the cortical and medullary thymocytes but no improvement of PCNA expression in cortical thymocytes. The thymic allograft let a very good recovery (P<0,001) both in PCNA and CD3 expressions in the cortical thymocytes and a good recovery, of CD3 similar to that found in hypophyseal allograft, in the medullary thymocytes.

These findings confirm that the lack of hypophysis decreases the possibility of cortical thymocytes to proliferate, but not to differentiate further on and that a thymic allograft may influence the recovery of the thymus of a hypophysectomized embryo, probably also by an emigration of thymocytes from the thymic graft.

Comparative analysis of fucose binding lectins isolated and characterized from different teleost species, and distribution of a F-Lectin during *Dicentrarchus labrax* ontogenesis

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Fucose-binding lectins (FBL) are contained in tissues and fluids from invertebrates and vertebrates. The lectin repertoire in teleost fish is highly diversified and, recently, the FBL structure with a novel lectin fold of F-type carbohydrate recognition sequence (the eel "F-type") has been described (Bianchet *et al*. Nat. Struct. Biol. 8: 628-634, 2002). Such a lectin shared an unique fucose-binding sequence motif contained in carbohydrate-binding domain of lectins and unrelated proteins.

Recently, we have shown that a serum FBL from sea bass *Dicentrarchus labrax* (DlFBP) and sea bream *Sparus aurata* (SaFBP) are involved in innate immunity. Both lectins were composed of two tandem domains that exhibit the eel "F-type" motif. These lectins were purified, characterized, cloned and sequenced, and biological activity and tissue distribution were examined. A complete coding of teleost F-type lectin CRDs showed that DlFBP and SaFBP are members of the F-lectin family.

The phylogenetic sequence analysis from bacteria to vertebrates, showed that the F-lectin motif is broadly distributed, and significantly diversified in terms of molecular organization and number of domains. Preliminary results on comparative analysis of purified and characterized lectins from different teleost species are also reported. These lectins, that showed calcium independent hemoagglutinating activity against rabbit erythrocytes, have been purified using a fucose-agarose affinity chromatography. Serum fucose binding lectins are present in all the studied species with a molecular size range from 32 to 41

kDa, and share shrinkage property in SDS-PAGE. The relationship with the F-lectin family has been supported with western blot analysis using DIFBP antibody.

Finally, we examined distribution and expression of DIFBP during fish ontogeny and showed lectins are also present in eggs.

Cloning and expression analyses of an interferon (IFN) in sea bass (*Dicentrarchus labrax* L.) after Poly I:C induction

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Interferons (IFNs) are a multigene family of inducible cytokines and posses antiviral activity through a JAK/STAT signalling pathway. IFN action induces the synthesis of IFN-regulated proteins, as Mx, that collectively constitute the antiviral response responsible for the inhibition of virus multiplication.

IFN cDNA cloning was possible after in vitro stimulation of head kidney sea bass (*Dicentrarchus labrax* L.) leukocytes with polyinosinic: polycytidilic acid (Poly I:C). Poly I:C is a synthetic ribonucleotide that acts as a viral RNA to induce the IFN system.

We used the "homology cloning" strategy: with degenerate primers we obtained an initial fragment of 189 bp. From this fragment we designed specific primers for 3' and 5' RACE - PCR to complete the cDNA sequence.

An alignment was performed using available IFN amino acid sequences to study the conservation of characteristic features and a phylogenetic tree was generated using the same sequences.

Finally, specific primers were used to analyse by Real Time-PCR the IFN and Mx expression in head kidney leukocytes stimulated with Poly I:C for 6 and 24 h. The IFN expression after 6 h is higher with respect to 24 h expression, whereas for Mx it is the contrary. This is an agreement with the teory that IFN is produced after viral infection and, successively, it induce Mx synthesis.

The same cloning procedure will be used on genomic DNA, as it is important to determine the number of introns in the IFN sequence to investigate its evolution from fish to mammals.

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Phylogenetic analysis of teleost light chain immunoglobulin

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In the last decade a large number of cDNA IgL sequences have been obtained from teleosts. Because of the extensive IgL diversity within the genome of each species and the very wide phylogenetic distance among teleost orders, the classification of IgL isotypes from teleost species remains questionable. At present, because the number of IgL isotypes identified so far varies in different teleost species, it remains unclear whether all possess the same number of IgL isotypes. The discrepancy in the number of IgL isotypes identified in each species may be explained by incomplete screening of differently expressed isotype specific mRNAs and by a different degree of diversity between sequences identified in each species. To date, IgL genes have been sequenced from 27 different species, two of them being Antarctic species investigated by us: *Trematomus bernacchii* and *Gymnodraco acuticeps.*

In the present work we compared all the available teleost immunoglobulin light chain sequences in order to evaluate the evolutionary relationships among them. We searched the Data Banks for all teleost immunoglobulin light chain, either genomic or cDNA, sequences. For each species we aligned the sequences by ClustalX, distinguished different isotypes and constructed a consensus sequence for each isotype. Then we calculated the distances from the consensus of individual sequences and used the closest one in a data set. The sequences of the data set were aligned and phylogenetic trees were constructed by Neighbor-Joining and Maximum-likelihood methods.

Our results indicated that three different isotypes can be identified in teleosts. In the Acanthopterygean species one isotype can be distinguished into two subisotypes one of them carrying a DNA microsatellite insertion. Specific sequential features of each group of isotypes have also been observed.

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Screening for antimicrobial peptides in zebrafish (*Danio rerio*)

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Antimicrobial peptides (AMPs) are small-sized (from 12 to 45 amino acids in length), cationic and amphipathic molecules able to neutralize pathogenic microrganisms. The AMP are very ancient in evolution life and wide expressed in methazoan species. They were described as being inducible and exert the killing or slow growth of bacteria, thus representing an old immune system that share adaptive and natural characteristics. Probably, the AMPs are early expressed during ontogeny of vertebrate as "evolution memory" before more complicate immune system has to differentiate. With the emergence of antibiotic resistant bacteria the AMPs represent good candidate for therapeutic strategies to counter bacterial infection in animals. The aim of this study is to identify the genes encoding for AMPs in

zebrafish. By analysing zebrafish EST and genome databases we identified those sequences homologous to known antimicrobial peptides genes from higher vertebrates: 1) mucosal (Parasin I, Chatepsin D3 and D5); 2) liver/organs expressed (Defensin beta-DB1, LEAP-2); 3) circulating phagocytes (bactericidal permeability-increasing protein, BPI); 4) the neuroendocrine/immune system–related peptides, Chromogranin A and B (CgA, B). We designed specific primers sets that allowed the amplification of the full or partial length of these genes. The lengths of the seven cDNAs (obtained by retro-trascription of total RNA extracted from adult samples) range from 242 bp (dbi) to 504 bp (cgb). They have been cloned and sequenced and the deduced amino acids sequences have been aligned to the well known antimicrobial peptides to confirm successful amplification of the genes of interest. Here we report the comparison analysis the deduced AMPs amino acid sequences from *Danio rerio* with those of other fishes. Further analysis will be performed in order to investigate the expression of these molecules during ontogeny.

Experimental evidences from EST sequencing and gene expression profiling in *Mytilus galloprovincialis*

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More than three decades since the use of mussels as pollution biosensors, their genes are still almost unknown. In the frame of the European Integrated Project FOOD-CT-2005-007103, EST sequencing and gene expression profiling are providing new interesting evidences. The former refers to the extension of the DNA microarray of *M. galloprovincialis* defined at CRIBI. (University of Padova) and currently used for investigating mussel gene transcription.

Among a number of selected stress factors, mussels of different origin have been challenged with bacterial antigens and the total RNA subsequently purified from haemolymph samples was used to build primary cDNA libraries. The abundant expression and molecular variability of transcripts identified as antimicrobial peptides emphasize the importance of such molecules in the response to bacterial pathogens. *In silico* processing and robust sequence annotation is in progress on the whole bulk of the new mussel ESTs.

Parallel evaluation of gene expression is performed by DNA microarray analysis on indigenous mussels from the Venice lagoon, a coastal transition system (4 sites, summer samplings 2005-2007). A relatively small number of immune-specific probes is present in the available cDNA microarray and, with exception of one defensin, they appear down-regulated in the lagoon compared to offshore mussels. Results will be discussed according to the published literature.

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