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Report of Meeting

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Organizers: **L Ballarin, P Burighel, G Zaniolo**

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Session 1. Immunity and soluble factors

Cloning and biotesting of cytokines in fish

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Fish EST and genome projects have advanced at a tremendous speed in the last few years. To date, there exists nearly 1 million fish ESTs and sequence data is still accumulating daily. The third draft of the whole Fugu genome has been published and the zebrafish genome is nearly finished. All this makes it easier than ever before to search for elusive fish genes, especially those with low sequence homology with known molecules in higher vertebrates such as cytokine genes. In this report, by BLAST searching the Fugu genome database with known cytokine sequences or with the conserved genes known to be adjacent to cytokine genes in the human genome, a number of cytokines including IL-6, IL-12, IL-15, IL-18, IL-19, IL-20, and interferon, have been successfully found and their relationship to the mammalian counterparts investigated. Comparative studies have revealed that synteny of some cytokine genes is conserved between Fugu and humans. To characterise their biological functions, trout homologs of some of the identified molecules have been cloned by EST database analysis or homology cloning and expressed in bacterial or mammalian cells. The recombinant proteins have been purified and their bioactivities tested.

Wound repair in *Hirudo medicinalis*: phases and response modulation

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In leeches, different antigens and immunisation conditions lead to responses that may vary both quantitatively and qualitatively. Different antigens can selectively stimulate both proliferation and migration of immune cells that can be classified as macrophage-like cells, NK-like cells or granulocytes (not only for their morphological aspect but also for the expression of specific CD antigens). Defence responses can vary in relation to the dimension of the non-self. Leeches are able to phagocyte small spheres or yeast, but also can incapsulate and subsequently melanize larger non-self, such as parasites.

All these responses towards the non-self, show that leeches can use a variety of selected defence systems.

In addition *Hirudo medicinalis* can react to large and deep explantation through wound healing process that can be divided, as in vertebrates, in three different stages: inflammation, granulation tissue and scar tissue remodelling. In leeches, the granulation tissue stage is characterized by a massive angiogenesis and fibroplasia: -the formation of new vessels is a complex process based on a finely regulated network of cellular and molecular events, involving a great variety of ECM proteins and cytokines; -the production of connective tissue, with the maturation of collagen fibrils, leads to the formation of new solid extracellular matrix network, which can be used as a scaffold not only for tissue reconstruction, but also for migration of immune cells and for directing new vessels growth.

We have used different approaches (light microscopy, scanning and transmission electron microscopy, atomic force microscopy, immunocytochemical studies and selective enzymatic digestions) to evaluate how in leeches angiogenesis and production of new collagen occur in response to surgical lesions.

The first cytokine from Antarctica: IL-1β from the icefish *Chionodraco hamatus*

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Over 200 species of fish from various groups are known to live in Antarctica, the most abundant species of which belonging to the suborder Notothenioidei, family Perciformes. Fishes are estimated to have adapted to polar conditions 20-25 million years ago when there was a physical water barrier established between the Antarctic continent and the temperate oceans. These fish have diversified and developed several peculiar features, among which a protein that is produced which prevents tissues freezing, the lack of a swim bladder and the absence of haemoglobin and functional blood erythrocytes, which is found only in the so called icefishes, members of the family Channichthyidae. Recently, the immune system of icefish has been investigated to study its morphological and functional organisation, to evidence the presence of specific immune humoral responses and to analyse immunoglobulin genes. In the presented study, primers designed to conserved regions of interleukin-1β (IL-1β) were used for the homology cloning of the icefish *Chionodraco hamatus* IL-1β gene, the first cytokine gene sequenced in an Antarctic teleost. The full length *C. hamatus* IL-1β cDNA consists of 1289 nucleotides that translated in a single reading frame to give a predicted 250-amino acid IL-1β molecule. The *C. hamatus* IL-1β sequence had highest nucleotide identity (75.7%) and amino acid similarity (69.8%) and identity (63.2%) with turbot IL-1β, followed by European seabass and Gilthead seabream. The closer relationship between the turbot, seabass and seabream IL-1β with *C. hamatus* IL-1β was also apparent in the phylogenetic tree obtained using amino acid data. Studies of IL-1β expression in *C. hamatus* indicated that the IL-1β molecule is induced by bacterial lipopolysaccharide (LPS) and these data raised questions about Antarctic fish immune responses to Gram-negative bacteria. Similar to the other Perciformes IL-1β genes, seabass and seabream, the *C. hamatus* IL-1β gene is organised in five exons and four introns.

Sea bass (*Dicentrarchus labrax*) recombinant interleukin-1â: purification and biological activities

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Studies on the immune system of Teleost fish are important for the evolution of defence mechanisms within vertebrates and may have relevance in biotechnology. The sea bass *Dicentrarchus labrax* is the major aquacultured seawater fish species in the Mediterranean sea and its health control is a subject of intense research.

Interleukin-1 (IL-1) is a cytokine playing pivotal roles in modulating immune responses in vertebrates and it has been preliminarily employed as immuno-adjuvant in fish vaccination experiments. Our group recently cloned the sea bass IL-1β and characterized its genomic and molecular structure.

The putative mature peptide deduced from the sequence has been expressed as a recombinant protein in *E. coli* by using the plasmid pQE-30. This plasmid is able to add 6xHis tag at the N-terminus of the protein of interest, allowing the purification of the protein on Ni-NTA matrices. In addition, Anti-His Antibodies can be used for detection of the recombinant protein.

The bioactivity of the purified molecule has been studied in some biological assays. Recombinant sea bass IL-1β (rIL-1β) induced the proliferation of murine Th1 cells D10.G4.1, although with less activity with respect to human IL-1β. rIL-1β was able to increase phagocytic activity and phagocytic index of head kidney leukocytes in a dose-dependent fashion. Sea bass rIL-1β was also able to increase the expression levels of IL-1β gene both “in vitro” and “in vivo”. Stimulatory doses of rIL-1β were typically >10 ng/ml. Interestingly, rIL-1β seems to induce sea bass thymocyte proliferation also in the absence of a co-stimulatory factor. Moreover, the effect of rIL-1β as immuno-adjuvant has been tested in sea bass vaccination experiments using a model sea bass pathogen (*Vibrio anguillarum*) as antigen of immunisation. The preliminary results seem to show an increase of the specific antibody production in the fish treated with the recombinant molecule.

Differential lymphocyte proliferation during *in vitro* allogeneic and xenogeneic reactions in the sea bass *Dicentrarchus labrax*

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In the sea bass *Dicentrarchus labrax*, T lymphocytes and B lymphocytes can be recognised by the specific markers DLT15 and DLIg3, respectively. Apart their identification in several species, the immunobiology of teleost lymphocytes is almost unknown, and to study some basical features of these cells, we setup some *in vitro* experimental models using sea bass peripheral blood leucocytes (PBL). These models were an allogeneic mixed leucocyte reaction (MLR), an allogeneic reaction (AR), and a xenogeneic reaction (XR), and lymphocytes were detected by indirect immunofluorescence (IIF) and counted by flow cytometry employing mAb DLT15 and DLIg3.

MLR was performed by incubating donor PBL with irradiated (600 rads) or mitomycin-treated target PBL for 3 and 6 weeks in L15 medium at 18 °C. The mean content of T-cells in PBL was 3.1±0.8%, in the MLR this percent raised to 8.9±4.3% after 3 weeks (n=9, max value 16.7%), and remained almost unchanged at 6 weeks with a value of 8.0±4% (n=9, max value 20.5%). The mean content of immunoreactive B-cells

in PBL was 21.1±3.8%, in the MLR this percent decreased to 6.71±3.18 after 3 weeks (n=9, max value 8.9%), and to 3.0±1.6 at 6 weeks (n=9, max value 6.32%). These MLR data showed the increased proliferation of T-cells and the decrease of B-cells, and were confirmed by RT-PCR experiments using specific primers for Ig (light chain) and TcR (Vβ). These data also showed the high individual variability between animals.

AR was performed against the mitomycin-treated cells of the sea bass embryonic cell line DLEC, and results are under evaluation.

XR was performed against mitomycin-treated sea bream fibroblast cell line SAF-2, and after several attempts, the incubation time of the reaction was fixed at 4 and 7 days. Each sample (n=10) was tested with respect to the same untreated PBL at same incubation time. The mean content of T-cells in PBL was 3.4±1.8% at 4 days and raised to 6.5±3 in the xenoreaction, after 7 days the mean percent of T-cells was 4.8±2 in controls and 3.9±2.2 in the xenoreaction. The mean content of immunoreactive B-cells was 13.2±5.5 at 4 days and raised to 19.8±6.5 (min value 6.9%, max value 35.9%) in the xenoreaction, after 7 days the mean content of immunoreactive B-cells was 13.4±7.3% in controls and 16.6±5.5 (min value 7.3%, max value 28.3%) in the xenoreaction. These XR data indicated that T-cells may have a fast reaction and, surprisingly, that B-cells are involved in this reaction.

Studies are in progress to assess the presence and to evaluate effects of non-specific cytotoxic cells in the XR, and to assess the effects induced by the T-cell function inhibitor cyclosporin in the MLR.

Humoral immune activities *in vivo* and *in vitro* in sea bass *Dicentrarchus labrax*

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During the last years, vaccination has become an important way to prevent infectious diseases in farmed fish and the control of fish diseases is the sole method to prevent mass mortality; this control can be carried out by means of vaccination with different antigens. In this work, we used three different antigens: DNP-KLH (administered intraperitoneally). *Vibrio anguillarum* (administered intraperitoneally and by immersion to juvenile sea bass) and *Photobacterium damselae* spp. *piscicida* that is mainly a mucosal bacterium. The ELISA assay was used to detect the serum specific antibody production. After 30 days post-immunization, a strong serum antibody response was observed against DNP-KLH, with a serum titer of ca. 1:13000; is interesting that, by using the molecule carrier KLH alone as antigen, we have measured a poor antibody response. Fish vaccinated i. p. with *Vibrio anguillarum* were sampled at day 37 after stimulation and this antigen induced an evident production of serum antibody. By employing *P. damselae,* a single immersion vaccination wasn't sufficient to elicit a detectable serum antibody response after 30 days. Consequently, a boosting in the same conditions was performed, and this treatment resulted in the appearance of serum antibody in fish with a titer of ca. 1:3000.

The ELISPOT modified assay was used to detect *in vitro* the presence of Ig-producing B cells for the antigen. In every experiment a control was performed by adding the drug cycloexhimide to the same number of leucocytes to block *de novo* protein synthesis. After 30 days post-immunization with DNP-KLH the production of Ig by head-kidney leucocytes was detected; cells from immunized animals, when plated in wells coated with DNP-KLH, gave a 173-fold higher response with respect to cells coming from control fish. A similar pattern was obtained when using KLH alone; these data suggest the *in vivo* presence of B-T cells cooperation.

The results of experiments with *Vibrio anguillarum* clearly showed that, with respect to controls, leucocytes from fish of 2 g in weight immersion-vaccinated once with *Vibrio* vaccine one year before had memory B-cells for the antigen, since they were able to produce specific Ig against *Vibrio* when re-exposed to immunization antigen.

By using *P. damselae* bacterin in immersion vaccination of juvenile sea bass, it was observed the presence of memory B cells in HK leucocytes in vaccinated fish from 51 days after boosting onwards.

Proliferations experiments with leucocytes from HK, PBL and spleen of fish vaccinated with *Vibrio anguillarum* suggested that PBL were the more effective leucocyte population in this process.

It can be concluded that the humoral immune system of sea bass can be differentially modulated by the type of antigen and the administration protocol.

Expression of T-cell receptor in sea bass, *Dicentrarchus labrax*

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T cell receptor (TcR), responsible in jawed Vertebrates for the recognition of self and foreign cells and molecules is recently demonstrated in Teleost fish, as composed, like in mammals, by αβ or γδ chains [Nam et al., 2003, J. Immunol.,170, 3081-3089]. In this study, RNA probes of sea bass TcRβ were prepared and used for RT-PCR and *in situ* hybridization. Immunohistochemical studies (ABC-peroxidase with nickel enhancement) were made in parallel using the anti-T cell monoclonal antibody DLT15 [Scapigliati et al.,1995, Fish & Shellfish Immunol., 5, 393-405].

In sea bass thymus, $DLT15^+$ cells (70±15 % of cells) were localised mainly in the cortex, while the reactive $TcR\beta^+$cells were numerous at the cortical-medullary border, less in the cortex and a few in the medulla (60±10 % of cells). In the spleen, $TcR\beta^+$cells (7±3,1 % of cells) appeared less numerous compared with the $DLT15^+$cells (10±4,5 % of cells), grouped in white pulp areas. In the head kidney, $TcR\beta^+$ cells

(10±3,2 % of cells) and DLT15$^+$cells (15±4 % of cells) were scattered or grouped close to blood vessels. Sparse TcRβ$^+$cells were more numerous in the midgut (8,3±1,5 % of cells) localised in the lamina propria, while DLT15$^+$ cells distinctly increase in number toward the hindgut (from 11,2±1,2 % in anterior portion to 19±1,5 of cells in the posterior portion. The localisation of intestinal DLT15$^+$ cells was in the basolateral epithelium and lamina propria. These findings suggest that numerous T cells in lymphomyeloid organs express the TcRβ, while a in the gut T-cells do not express TcRβ, but possibly another TcR (γ/δ?). TcRβ$^+$cells (from head kidney and PBL) are significative involved in activities of specific immune response such as the proliferation processes induced by mitogen (PHA) or xenogenic cells (sea bream leucocytes). In allograft rejection, the TcRβ$^+$cells are clearly involved because their percentage (13±2,5) is relevant respect the counting leucocytes.

Biological activity, tissue distribution and preliminary molecular characterization of a serum fucolectin from the sea bass (*Dicentrarchus labrax*)

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Sugar binding proteins (lectins) and free or cell surface-bound sugars constitute an evolutionary conserved recognition system involved in innate immunity. Lectins are widely distributed in both vertebrates and invertebrates. In those taxa endowed of both innate and adaptive immunity, lectins mediate rapid recognition and effector functions that precede adaptive immunity.

Fucose-binding lectins are present in tissues and fluids from invertebrate and vertebrate species. Well-characterized examples, such as the lectin CPL-III from the tunicate *Clavelina picta* and the fucose-binding mammalian collectins, clearly belong to the C-lectin type. Others, such as DLL34 from the sea bass *Dicentrarchus labrax* (Cammarata et al., Biochim. Biophys. Acta, 2001, 1528: 196-202), the serum "fucolectins" from *Anguilla japonica* (Honda et al J Biol Chem, 2000. 275: 33151-33157) and the FBP32 of *Morone saxatilis* lack a typical sequence motif present in any of the lectin families described so far. Furthermore, because of their specificity for carbohydrate moieties present on potential microbial pathogens, and their inducibility upon infectious or inflammatory challenge, these lectins are considered to function as recognition factors in innate immunity. A fucose-binding lectin from serum of the sea bass *D. labrax* was purified and partially characterized. In SDS-PAGE, the lectin has an apparent molecular size of 34 kDa. In this report, studies on the *D. labrax* lectin structural aspects, iological properties, tissue distribution and ontogenetic aspects are described. These results suggest that the *D. labrax* lectin is involved in innate immunity. In addition, the primary structure (nucleotide and amino acid sequence) indicate the presence of a tandem 2-CRD fucolectin that confirm the *D. labrax* lectin can be a component of the recently identified F-lectin family (Vasta et al., ISDCI meeting 2003, Scotland).

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Hepato-biliary transport of immunoglobulin in the antarctic teleost *Trematomus bernacchii*

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Presence of immunoglobulins (Ig) in the liver of *Trematomus bernacchii* was investigated by biochemical and immunochemical assays with polyclonal antisera raised in rabbits against purified *T. bernacchii* serum Ig heavy (IgH) and light chains (IgL).

Bile Ig were quantified by ELISA and purified. Western blot analysis of SDS-PAGE separated bile proteins, performed using IgH- or IgL-specific antisera, revealed two IgH bands (76 and 66 kDa) and the IgL band (25 kDa). Similar results were obtained when extracted liver proteins were analysed by the same method. SDS-PAGE under non-reducing conditions of bile proteins revealed multiple bands (ranging from 200 to 830 kDa) resulting from different Ig polimerization forms. Finally, IEF analysis of the bile Ig showed a wide range of pI values.

Immunohistochemistry detected IgH- and IgL-reactivity in the plasma of hepatic sinusoids, in cells extravasated in the perisinusoidal space, in bile canaliculi and pre-ductules. These findings strongly indicate that Ig, derived from blood and/or activated B-cells, could be transported across the hepatocytes to be secreted into the bile. In the anterior intestine, the intraluminal mucus retained a significant Ig-immunoreactivity, while the mucosa housed a limited density of Ig-bearing cells. In addition, extravasated plasma cells accumulated within identifiable portal tracts and close to the liver capsule that, in turn, was evenly coated by Ig molecules at the peritoneal surface. These peculiar features could be explained in light of liver defence system against ascending infections and/or colonization by nematode parasites and suggest that the Ig could protect the intestinal epithelium via the hepato-biliary transport route.

Immunoglobulin light chain isotypes from the antarctic teleost *Trematomus bernacchii*

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Immunoglobulin light chains (IgL) have been sequenced in eleven different teleost species belonging to order Siluriformes, Gadiformes,

Salmoniformes, Gasterosteiformes, Perciformes, and Cypriniformes. In each species two or three different isotypes have been described. To identify IgL in the Antarctic teleost *Trematomus bernacchii* a cDNA expression library was constructed in ZAP Express vector from spleen poly $(A)^+$ RNA and immunoscreened with rabbit IgG specific for *T. bernacchii* IgL chain. Several immunopositive clones were isolated and sequenced. In an attempt to yield additional clones encoding *T. bernacchii* IgL, a PCR approach was chosen. First-strand cDNA was synthesized by reverse transcriptase using head kidney total RNA. To accomplish PCR amplification a multiple alignment of IgL sequences from different teleost species was used in the design of two oligonucleotide primers complementary to the most conserved part in FR2 (sense) and in the terminus of CL (antisense), respectively. The resulting PCR products were cloned into pGEM-T Easy vector and recombinant clones were isolated and sequenced. Three different isotypes were identified by calculating percent of identity among the nucleotide sequences, and referred to as TrbeCL1 TrbeCL2 and TrbeCL3 based on comparison with the isotypes definied in other teleosts. By Southern blot analysis different DNA fragments were labeled by isotype specific probes. A multiple alignment of CL sequences from *T. bernacchii* and other vertebrate species was obtained with CLUSTAL X and a phylogenetic tree was constructed.

Session 2. Immunity: applicative aspects

Immunity and vaccination in aquaculture

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First reports on fish vaccination date back to the thirties while only since the seventies, following the appearance of antibiotic resistance in trout industry, studies on vaccination reached a renewed interest. During the last twenty years new acquisitions have been obtained on fish immunology and different commercial vaccines have been registered.

Several pharmaceutical products against nearly 15 fish diseases are available up today on the international market and vaccines against Vibriosis, Furuncolosis and Red Mouth Disease are routinely applied in salmonid industry quite often as polyvalent formulations .

First generation vaccines obtained by formalin inactivation of bacterial whole cells still represent a consistent and very efficient product ; they are usually administered to young fish by immersion. Alternatively, when 10-15 g or bigger fish must be vaccinated, i.p. inoculation with or without adjuvants is suggested. Oral vaccination seems to be a very promising system to protect fish mainly due to reduction of handling stress and manpower costs but in spite of substantial research efforts no efficient commercial product, with the exception of a vaccine for *Edwardsiella ictaluri*, have been registered jet.

Besides these unsophisticated products, a few innovative vaccines have been investigated during the recent years and an IPN recombinant vaccine has recently been registered in Norway and Chile. More recently positive results have been published on experimental DNA vaccines particularly against salmonid rhabdoviruses (IHNV and VHSV). The evaluation of vaccine efficacy is measured by the RPS index which reflects the relative% protection of vaccinated groups in comparison with control fish. Experimental results may be affected by several parameters like age of fish, challenge dose, pathogenicity of challenge strains, administration route, environment temperature and fish susceptibility; moreover field trials may be affected by further variables and strict statistical methods are suggested in order to evaluate final results .

The reduction of antibiotic treatments achievable when vaccination strategies are correctly applied , represents an important result not only from an economical point of view but in addition with regard to environment protection and food safety which, during the recent years, became an important growing concern for many people: in Norway following the adoption of routine vaccination the use of antibiotics has been significantly (98%) reduced in ten years .

In conclusion vaccination should be regarded in the future as a fundamental tool for the control of infectious diseases . The appearance of new and severe viral diseases, hard to control by eradication methods, particularly in the marine environment , like salmon anemia and encephalopathy and retinopathy, suggests that an integrated control approach in which vaccination plays a fundamental role, should represent the ideal strategy to avoid disease spreading in aquaculture.

Rearing density may influence seabass (*Dicentrarchus labrax, L.*) stress related protein gene expression

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The worldwide decline of ocean fisheries stocks has provided a rapid growth in fish, shellfish farming and aquaculture and the problems connected to animal welfare in aquaculture gained importance.

In this context, we have looked for molecular markers among those genes whose expression could reasonably result modified by the different farming conditions. With this purpose, we have evaluate, in liver and brain sea bass, grown for two months at different biomass density (<10 Kg/m^3, 80 Kg/m^3 and 100 Kg/m^3), the expression of those genes coding for proteins related to stress such as Heat Shock Proteins (HSPs), Metallothioneins (MT) and Cytochrome P4501A (CYTP4501A).

MT and CYP4501A mRNA resulted induced in liver of animals reared at 80 and 100 Kg/m^3. HSP70 appeared significantly over expressed only at the biomass of 100 Kg/m^3, while apparently, no induction was detectable for HSP90. In brain tissue instead, MT and HSP90 were inducted already at 80 Kg/m^3, CYTP4501A was influenced only at the higher population density of 100 Kg/m^3, while we have not obtained any results for HSP70.

In the last three decades, there has been an exponential increase in the interest concerning the description, classification and functional significance of stress related proteins, in particular HSPs. These proteins represent precious molecular biomarkers able to detect the welfare conditions when they are still recoverable. Here we wish to underline that to detect their mRNA by PCR is fast, easy and relatively unexpensive, therefore we propose this method as a good alternative to monitor fish welfare.

Sequence diversity of antarctic fish IgTM exons

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The secreted (IgH) and membrane-bound forms (mIgH) of the immunoglobulin heavy chains are encoded by a single gene, and alternate pre-mRNA processing determine which mRNA is expressed. In teleost the splicing mechanism is different from that occurring in mammals; in fact the CH4 exon is totally excluded in the teleost rearranged mIgH mRNA. The TM exon encodes the C-terminal sequence of the mIgH comprehensive of the cytoplasmic, the membrane crossing, and the extracellular spacer regions. The region crossing the cell membrane is known to be highly conserved during the evolution.

The aim of the present study is to sequence mIgHs from different Antarctic teleost species was in an attempt to identify specific features accounting for the evolutionary adaptation. RT-PCR was performed on RNA extracted from head kidney of *Trematomus pennelli, Trematomus newnesi, Notothenia coriiceps, Pagetopsis macropterus,* and *Gymnodraco acuticeps* using *Trematomus bernacchii* oligonucleotides as primers. mIgH cDNA were synthesized, cloned and the sequence determined for each of the above mentioned species. In addition the *T. bernacchii* genomic sequence comprising the CH3 and TM exons was completely determined by a RT-PCR approach using testis DNA. The deduced amino acid sequences of mIgH from the Antarcic species were aligned with those from other teleost species reported in data banks, by CLUSTAL X and a phylogenetic tree was constructed. Several specific features of the Antarctic mIgH sequences were found identified: while the cytoplasmic and membrane crossing regions were highly conserved in Antarctic as well as in all the examined species, the extracellular regions were found to be very different in length and composition. In addition mIgH of several Antarctic species showed the lack of the CH3 exon and the presence of an extracellular spacer region about 40 amino acid residue longer than usual. This TM exon elongation may be arisen from a particular evolutive mechanism involving the multiple repeats of a short sequence which is reverse complementary to a portion of the CH3 exon.

NO in the developing gut of *Dicentrarchus labrax:* an early immune role?

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It is known that nitric oxide (NO) plays an important role in the immune-neuroendocrine communications. In this issue, we have examined the appearance and distribution of nitric oxide sinthase (NOS) histochemically, immunohistochemically and biochemically during development of the sea bass gut.

In 4 and 24-day-old larvae the NOS activity evaluated by NADPH-diaphorase was strong in all epithelial cells and in cell and fibers of intestinal wall, at all gut levels. In the same larval stages and localizations immunoreactive material to antibodies against nNOS and iNOS was present. In the 5-month-old adult gut both enzimatic activity for NADPH-diaphorase and immunoreactivity (IR) to anti-nNOS disappeared from epithelium remaining in the gastro-enteric nervous system. The IR to anti-iNOS completely disappeared.

Western blot analysis showed that neuronal (about 150,000 mol. wt band) and inducible (about 135,000 mol wt band) NOS-immunoreactive proteins are present in 24 day-old larvae gut. In the 5 month-old adult gut nNOS and iNOS IR disappeared in the soluble fraction of crude gut homogenates. A small amount of nNOS IR was present in particulate gut fraction.

Our data show that both the calcium-calmodulin dependent nNOS and calcium-independent iNOS are present in the larval gut of sea bass. In this species the maturation of cell-mediated immune responses and humoral immune system takes place respectively around the first and second month post hatching (Scapigliati *et al*., 2002). The presence of inducible NOS in the same regions of the sea bass gut in which the GALT will differentiate, may suggest for NO a role in early defence mechanisms, before of establishment of immune responses in GALT.

Investigation on the phagocytosis operated by the mussel (*Mytilus galloprovincialis*) hemocytes using the 1,2,3-dihydrorhodamine as fluorescent probe

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Different fluorescence techniques, such as flow cytometry, micromethod fluorimetry and confocal laser scanning microscopy have been applied in the study of cellular defense system of Mytilus galloprovincialis. The phagocytosis stimuli was represented by the yeast cells (zymosan), whereas the fluorescent probe utilised for the measuring the synthesis of the reactive oxygen species (ROS) was the 1,2,3-dihydrorhodamine (DHR). Flow cytometry distinguished three hemocyte sub-populations in mussel hemolymph, showing also the relation between hemocyte size, granularity and phagocytosis ability. Micromethod assay confirmed the respiratory burst produced by the hemocytes whereas the use of superoxide dismutase (SOD) and L-N^G-Monomethyl

arginine citrate (L-NMMA) increased fluorescence intensity. In addition, the confocal microscopy assay permitted to valuate the oxidation of DHR, due to ROS synthesis, inside the cellular organelles involved in phagocytosis. In conclusion, these innovatory techniques could represent useful tools for valuating the immune system of marine invertebrates, both in research approach and in applied studies.

Hemocyte circularity (HC) and heat shock protein 70 kda (HSP 70) expression as stress parameters in *Mytilus galloprovincialis*

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The present study aim of investigating, in mussel (*Mytilus galloprovincialis*) specimens exposed to sublethal thermic stress, the variations of two parameters such as the hemocyte circularity (HC) and the mantle HSP 70 expression. A cell viability analyzer has been applied for valuating the hemocyte concentration, the viability and the morphological characteristics, such as circularity and diameter. The assays have been conducted on mussel groups maintained in aquaria at 18°C, then placed for different times at 40°C and finally put back to the original conditions. The heat stress determined a similar increase in the HC in the different groups whereas the recovery to the homeostasis condition showed some differences: the specimens more exposed to the heat stress, indeed, exhibited a slower ability to recovering their HC value to the control condition. Furthermore, mussel specimens maintained at refrigeration conditions displayed a slow progressive increase in the HC. Concerning the HSP 70 induction, the group exposed to the heat treatment for the shorter time developed a subsequent increase compared with the HC variations whereas. One day after the treatment, the HSP 70 induction was still evident. The cold treatment has demonstrated any HSP 70 induction. In conclusion, hemocytes circularity and mantle HSP 70 expression could represent two important stress parameters, morphological and biochemical, immediate and late, respectively. The markers investigated, therefore, could find application in the valuating the general status of the mussel health and, particularly, in studies concerning both the mollusc immunity and their response to environmental stress.

Effects of estrogens on *Mytilus* hemocytes

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Estrogens affect the functioning of several non-reproductive tissues, the immune system in particular. In mammalian immunocytes, 17β-estradiol (E_2) has both dose- and cell- type specific effects that seem to be mediated by rapid, non genomic mechanisms; these may be initiated at either membrane or cytosolic locations, and can result in both direct local effects, such as modification of ion fluxes, and regulation of gene transcription secondary to activation of different kinase cascades, including Mitogen Activated Protein Kinases (MAPKs).

In this work we investigated the possible rapid effects and mechanisms of action of estrogens in the immunocytes of the bivalve mollusc, the mussel *Mytilus galloprovincialis* Lam. Data are reported show that the natural estrogen E_2 induced both morphological (as evaluated by SEM) and functional changes (such as extracellular release of hydrolytic enzymes, lysosomal membrane destabilisation, stimulation of the bactericidal activity) within 10-30 min from addition. E_2 (25 nM) caused a rapid and significant increase in cytosolic [Ca^{2+}]; lower concentrations (5 nM) showed a smaller, not significant effect. Both E_2 concentrations increased the phosphorylation state of the components of tyrosine kinase-mediated signal transduction MAPK- and STAT- (Signal Transducers and Activators of Transcription) -like proteins within 5-15 min from E_2 addition. Experiments with specific kinase inhibitors confirmed that rapid kinase cascades, in particular those leading to p38 MAPK activation, are involved in mediating the effects of E_2. Western blotting revealed the presence of immunoreactive ERα- and ERβ- like proteins in hemocyte extracts. Overall, our data support the hypothesis that the rapid effects and mechanisms of action of 17β-estradiol are extremely conserved and that they may play a crucial role in endocrine-immune interactions in invertebrates. The results are compared to those obtained with synthetic estrogens and estrogenic chemicals. The results address to the importance of the role of kinase-mediated pathways in the action of endocrine disrupters in invertebrate systems.

Gene identification and expression profiling in *Mytilus galloprovincialis*: a new tool for basic and applied research

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Mussels of the genus *Mytilus* constitute an interesting model of study from different points of view. However, the number of available mussel sequences is small and functional studies refer to a few genes only. Messenger RNA was purified from multiple mussel tissues and a 3'-end-specific primary library was prepared in plasmid vector. Massive sequencing of the cDNA inserts (3' fragments of transcribed genes or Expressed Sequence Tags) allowed us to build a collection of 5664 3'ESTs. Such experimental approach allows rapid and large-scale identification of protein coding genes and can provide information on the relative abundance of the most common mRNAs.

Putative identification of the consensus sequences has been published in part (Gene, 2003) whereas the complete set of 1731 consensus sequences deriving from the clustering of rigorously selected ESTs is under refined annotation. In addition, one representative EST for each cluster have been selected and the related cDNA clones used to print on slide such genome-wide collection. Preliminary analysis of gene expression in selected tissues of normal and stressed mussels will be discussed.

The obtained results indicate the great potential of the mussel gene catalogue to increase in size and to become an essential tool for answering specific biological questions.

Commercial transport stress in *Homarus americanus* (Crustacea: Decapoda) investigated by hematological parameters

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Markets for live crustaceans are global and may require shipments over long distance and time, exposing animals to high stressful conditions. Impact of transport condition, emersion and handling were studied on live, commercial *Homarus americanus* imported from North America at the arrival and during the stocking period (3h and 12h) at warehouse. Lobsters maintained in laboratory tanks (in controlled conditions) were used as control and for simulated emersion and handling stress.

Hemolymph sampling was followed by total and differential hemocyte counts (THC and DHC, as immunological indicator of contamination and impaired defence responses) on fresh as well as fixed material, whereas the physiological state of lobsters was studied *in vivo* by measuring pH, glucose (as general stress indicator) and lactate (as indicator of tissue hypoxia) concentration in the hemolymph. Moreover the physiological parameters were related to the vigour index and physical damage of the animals.

At the arrival animals show alterated parameters with a low number of circulating haemocytes (compared with the control) and that may cause a loss in immunocompetence; a highly disturbed metabolic state is revealed by elevated concentrations of lactate and glucose profile and alteration of the hemolymph pH. Undamaged animals start to recover 12 h after reimmersion and cell counts return to control values within 48 h.

Host-parasite relationship: toward a role for entomoparasitic nematodes body surface

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Recent advances in understanding how insects eliminate pathogens and parasites have led to the realization that innate immunity plays a vital role in protecting from infection. This work examines some feature of humoral and cellular defenses involved in innate immune responses, how they act to control parasites and if their engagement or counteracting can explain many immune features characteristic of parasitic infections.

Aim of this work is to examine the interaction between the humoral and cellular defences system of an insect host model (*Galleria mellonella*) and a pathogenic parasitic nematode (*Steinernema feltiae*).

Particularly we are studying the involvement of the parasite body surface in two main strategies leading to immunoevasion/immunodepression mechanisms: molecular disguise and host proteins sequestering.

Our data suggested that the parasite body surface plays a role in the inhibition of host proPO system, interfering with the activation of the proteases cascade involved in prophenoloxidase activity; moreover parasites were not recognized by host immunocompetent cells.

Finally, as confirmed by our preliminary data, *Steinernema* seems to be able to interfere also with the host antibacterial peptides synthesis.

Immunomodulation and apoptotic events in the clam *Tapes philippinarum* after exposure to 4-nonylphenol

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Nonylphenol (NP) is used in the production of nonylphenol ethoxylates (NPEs), NP phosphites and insecticide sprays. NPEs are a large group of nonionic surfactants employed in plastics, latex paints, lubricating oils, emulsifiers, household and industrial detergents and paper and textile industries, whereas NP phosphites are commonly used as stabilisers and antioxidant agents in both rubber and plastic industries. NP is known to be an endocrine disruptor, being able to alter hormonal functions in various aquatic organisms.

In this study, specimens of the clam *Tapes philippinarum* were exposed for 7 days to various sublethal NP concentrations (0, 0+acetone, 0.025, 0.05, 0.1 and 0.2 mg/l) and their effects on the uptake of the vital dye Neutral Red (as index of cell membrane alteration), superoxide dismutase (SOD) and lysozyme activities, the number and volume of haemocytes were evaluated. The capability of NP to induce apoptotic events in haemocytes was also studied. Exposure of clams to 0.2 mg/l NP increased significantly ($P<0.05$) Neutral Red uptake when compared with controls, suggesting that NP may cause alterations in cell membrane stability. Significant ($P<0.01$) decreases with respect to controls in both SOD and lysozyme activities were observed at concentration higher than 0.05 mg /l NP. A significant ($P<0.001$) increase in apoptotic index (i.e., the percentage of haemocytes showing positivity to the TUNEL reaction) was also recorded in the same

concentration range. Apoptotic haemocytes generally showed a shrinkage of cell volume and a spherical shape. Alterations in both circulating haemocyte number and volume, consequent to NP exposure, were recorded using a Coulter counter. In this case, we observed both significant increases (P<0.05, for clams exposed to 0.2 mg /l NP) in total number of circulating haemocytes and different size frequency distribution. Our results, showing a relationship between NP exposure and alterations in functional responses of haemocytes, demonstrated that NP can influence immune responses in *T. philippinarum*.

Immunotoxicity of new antifouling compounds, alternative to TBT, on tunicate haemocytes

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Xenobiotics, which cause severe alterations of the immune responses, can provoke the death of individuals and the local disappearance of the involved species. Organotin compounds were massively introduced since the second half of 1960s in the formulation of antifouling paints for the preservation of submerged structures from the settlement of various aquatic sessile organisms. They resulted highly immunotoxic to benthic marine invertebrates, in particular filter-feeding ones, and most of them persist for a long time in the environment. After their ban, industries turned their attention to new biocidal combinations. About twenty new substances are at present in commerce in Italy in various paint formulations, in which the biocidal compounds play various roles, i.e. as alternative substances to TBT or as boosters to increase the toxic performance of the main biocides towards a wider spectrum of fouling organisms.

We carried out assays of acute toxicity on short-term cultures of haemocytes of the colonial ascidian *Botryllus schlosseri* to evaluate the alterations of the immune responses, as described by a series of biomarkers, by sublethal concentrations of seven active ingredients employed in the formulation of new antifouling paints. Results indicate severe and irreversible effects on immunocyte morphology and functionality with mechanism of action sometimes similar to that of TBT, i.e. induction of apoptosis, cytoskeletal protein disassembly, inhibition of both phagocytic and cytotoxic ability, negative interactions with mitochondrial oxidative phosphorylation, cytosolic Ca^{2+} homeostasis and GSH content. The comparison of our results suggests the following order of immunotoxicity: TBT ~ Cu(I) ~ ZnP > Sea-Nine 211 ~ Chlorothalonil > TCMS pyridine > Diuron > Irgarol 1051.

Owing to the immunosuppressive effects of these compounds on tunicates, which make organisms more vulnerable to both pathogenic agents and other xenobiotics, we remark that more assays of acute and chronic toxicity should be necessary before leading new potentially pollutants into the market, in order to prevent the repetition of the irreversible errors on coastal biocoenoses already occurred with TBT.
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Session 3. Inflammation

Inflammation in ascidians

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Inflammatory responses in solitary ascidians include cell migration, phagocytosis, encapsulation of larger particles, tissue injury, and wound repair. In encapsulation responses in the tunic of *Ciona intestinalis*, an increased expression of type IV-like collagen and elastin-like molecules have been found, apparently produced by the epidermis. Inflammatory cells have been identified as amoebocytes, univacuolar cells, unigranular refringent cells (URG) and morula cells. We show the involvement of a large amount of URGs following LPS injections. These cells contain polyphenols and, *in vitro*, showed a phenoloxidase-dependent cytotoxic activity. Probably, URGs migrate through the epithelium from tissue lining the lacunae under the tunic. Chemotactic stimuli, that induce migration into the inflamed area, could be due to a C3-like molecule while an immunohistochemical study shows that molecules containing interleukin-1-like epitopes are expressed (2-4 hours) by endothelial tissue lining the pharyngeal wall. An IL-1-like functional activity may be indicated by the increased number in the lacunae as a result of the cell proliferation response. Accordingly, we found IL-1-receptor epitopes in cell nodules of the pharyngeal bars ansae. The recently elucidated genome of *Ciona intestinalis* did not reveal IL-1-like genes whereas an IL-1-receptor was found. However, human IL-1 traits can be observed by examining the *Ciona* genome sequence. Finally, the expression of a phenoloxidase component could be stimulated by inflammatory stimuli. Inflammation in ascidians presents invertebrate and vertebrate characteristics.
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The ascidian *Ciona intestinalis* as experimental model for the study of the complement system inflammatory pathway in deuterostome invertebrates

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One of the most challenging problems in immunology is the identification of the molecules that regulate the defence systems and the cross-talk

between innate and adaptive immune systems. A key approach to these issues is the study of how immunity is exploited by and evolved in invertebrates. Many molecules belonging to the arms of the innate immune system have been found scattered in invertebrates at different levels of the phylogenesis. A major breakthrough has been the identification in the echinoderms, in the urochordates and in the cephalochordates of gene homologs of C3, a multifunctional protein that plays a central role in the complement system. However, neither the functions nor involvement of C3 in the inflammation processes have been proved in deuterostome invertebrates. Recently, we have isolated two C3-like genes (CiC3-1 and CiC3-2) from *Ciona intestinalis* blood cell total RNA and shown that these genes are constitutively expressed in only one type of blood cells.

A major contribution to the otherwise scanty panorama of the mechanisms of inflammation in invertebrates, has been provided by our group with the finding that CiC3-1a, the anaphylatoxin peptide generated by the activation of the complement system, exerts a chemotactic activity on *C. intestinails* blood cells. To investigate the presence of complement-mediated chemotaxis in the ascidian *C. intestinails*, as a means of verifying the presence of the inflammatory pathway of the complement system in the deuterostome invertebrates, we have expressed in *E. coli* the fragment of *C. intestinails* C3-1 (rCiC3-1a) corresponding to mammalian complement C3a and assessed its chemotactic activity on *C. intestinalis* hemocytes. We found that the migration of *C. intestinalis* hemocytes toward rCiC3-1a was dose-dependent, peaking at 500 nM, and was specific for CiC3-1a, being inhibited by an anti-rCiC3-1a-specific antibody. As it is true for mammalian C3a, the chemotactic activity of *C. intestinalis* C3-1a was localized to the C-terminus, since a peptide representing the 18 C-terminal amino acids (CiC3-1a$^{59-76}$) also promoted hemocyte chemotaxis. Furthermore, the CiC3-1a terminal Arg was not crucial for chemotactic activity, since the desArg peptide (CiC3-1a$^{59-75}$) retained most of the directional hemocyte migration activity. The CiC3-1a-mediated chemotaxis was inhibited by pre-treatment of cells with pertussis toxin, suggesting that the receptor molecule mediating the chemotactic effect is G protein-coupled. Immunohistochemical analysis with anti-rCiC3-1a-specific antibody and *in situ* hybridization experiments with a riboprobe corresponding to the 3'-terminal sequence of CiC3-1, performed on tunic sections of LPS-injected animals, showed that a majority of the infiltrating labelled hemocytes were granular amoebocytes and compartment cells. Our findings indicate that CiC3-1a mediates chemotaxis of *C. intestinalis* hemocytes, thus suggesting an important role for this molecule in inflammatory processes.

Eosinophilic granulocytes of sparids: cyto-chemical characterization and possible role in inflammatory response

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In mammals eosinophilic granulocytes take part in defence against parasites and hypersensitivity reactions whereas the neutrophilic granulocytes are specialised into phagocytosis and killing of micro-organisms. Basophils contain vasoactive substances and in tissues are known as mast cells. In Teleosts three types of granulocytes, neutrophilic, acidophils and basophils have been reported (Hine *et al*., 1992). However, there are enormous variations amoung the Teleosts in both relative abundance and staining reaction of granulocytes. Most of the information on the Teleosts eosinophils refers to cells found in association with different tissues, mainly in head-kidney. Eosinophils are the most abundant circulating granulocytes in gilthead seabream, as occurs in *Cyrprinus carpio*, *Tinca tinca*, and *Salmo gairdneri* (Lopez-Ruiz *et al*, 1992). Eosinophilic granular cells (ECGs) are a peculiar kind of granulocytes observed in the blood and in many tissues (gills, heart, liver, skin, spleen and kidney) of a variety of fish; in the gut ECGs are located in *stratum granulosum* and *lamina propria*. Some cytochemical and functional characteristic suggested that they are analogous to mammalian mast cells, but other authors have considered them to be akin to eosinophils (Sire & Vernier, 1995; Reite, 1998).

Sparids affected by "Winter Syndrome" showed a severe infiltration of granulocytes involving gastrointestinal tract, pancreas and adipose tissue surrounding these organs. Also in gilthead seabream (*S. aurata*) affected by nephrocalcinosis is evident a considerable presence of these cells in the kidney. Cytochemical analysis of this infiltrate by means of specific and enzymatic staining, and immunocytochemistry for lysozyme, showed that the granules of these cells are acidophilic. They contain peroxidase and acid phosphatase proteins and reveal lysozyme positivity only in few cells. Literature refers that ECGs of gilthead seabream are peroxidase and acid phosphatase negative (Noya *et al*., 1996), whereas in Atlantic salmon (*Salmo salar* L.), the granules of ECGs show strong immunoreactivity for lysozyme. On the basis of morphological, histochemical and enzymatic properties of these cells, we conclude that they could enter into tissue from circulating blood, *in loco* proliferate and act as mature cells. So probably they represent an evolutive stage of eosinophilic granulocytes. The proliferative mechanisms and their role in inflammatory response is under investigation.

A glucocorticoid receptor identified in cells of sea bass innate immunity

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In fish, cortisol is the major corticosteroid produced by the interrenal glands that acts as a component of the neuroendocrine circuit known as the hypothalamo-pituitary-interrenal (HPI) axis.

In *Dicentrarchus labrax* confinement experiments increase in plasma cortisol levels, has been related to inhibition of cytotoxic activities by eosinophilic granule cells (EGCs), were observed. In *in vitro* experiments, using head kidney and peritoneal cavity cells an increased zymosan-induced respiratory burst activity after 1 and 24 hours of incubations with hydrocortisone was increased at 10-100 ng/ml whereas it was reduced after cell incubation with 1000 ng/ml cortisol.

Immunohistochemical studies revealed that exogen cortisol can be found in the leukocytes.

We cloned and sequenced a glucocorticoid receptor (GR) from leukocytes (DLGR1). That present homologies with fish, *Xenopus*, and human GR. In particular, 80% homology between the sea bass and *Hapochromis burtoni* GR1 receptor was found. *In situ* hybridization study demonstrated that mRNA DLGR1 was expressed in head kidney and peritoneal cavity macrophages and neutrophils. GR expression was never observed in eosinophilic granule cells. Present results support a direct effect of cortisol on phagocytic leukocytes containing DLGR1 whereas suggest that an alternative modulation mechanism could be invoked to explain the suppressed peritoneal cytotoxic activity of eosinophils.

Research is in progress to isolate and to sequence the isoforms of DLGR1.

Identification, characterization and phylo-genomic analysis of Toll-like receptor genes in the gilthead seabream (*Sparus aurata* L.)

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Toll-like Receptor (TLR) family consists of at least ten proteins encoded by distinct genes in man and mouse. Each TLR protein recognizes in a specific manner one or more pathogen-associated molecular patterns typical of different group of pathogens and activates the immune system. The aim of the present work was to study TLR proteins in a marine teleost fish, *S. aurata*. All available TLR sequences from the human, mouse and pufferfish genome were aligned and gene-specific primers were designed on conserved regions and used in a RT-PCR approach, obtaining partial cDNA sequences of seabream TLR 2 and TLR 9. Subsequently, the entire sequence for both proteins was obtained using the 5' and 3' RACE method. Expression analysis performed from different adult tissues and from whole larvae at different stages of development provided evidence of broad spatial and temporal distribution of TLR 2 gene, and of the presence of at least two splice variants of the TLR 9 gene, which appear to be differentially regulated. Extensive analysis of sequence similarity and data mining were carried out to obtain all the available TLR proteins in the teleost fishes. All teleost TLRs, including the two seabream genes, were aligned with all human and murine TLRs, and a gene genealogy was reconstructed. Phylogenetic analyses of TLR evolution suggested that in the common ancestor of all gnathostomes were present at least ten TLR genes. Successively, lineage-specific deletions and/or duplications contributed to determine the present gene configuration, eight orthologues and two mammalian specific TLRs in mouse and man, and eight orthologues and four/five teleost specific TLRs in pufferfish and zebrafish, respectively, as confirmed from analysis of completly sequenced genomes.

Thymus response to algal yessotoxin

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Mice thymus responses to algal yessotoxin (YTX) were examined by histochemical and immunocytochemical procedures. Immunoreactivity for different MW cytokeratins (CK) and for cytokines (IL-1α, IL-6, IL-8) was analyzed. Modifications of parameters such as cell proliferation and cell death were also studied. Thymus from male swiss CD1 mice intraperitoneally injected with lethal (420 μg/kg) and not-lethal (10 μg/kg) doses of YTX were examined after 2 and 24 hours, respectively. Histological studies revealed morphological modifications with both YTX doses. Lethal treatment provoked changes in the cortex region that appeared less compact with light areas containing a reduced number of thymocytes and large pale epithelial cells. An increased number of mitotic as well as apoptotic phenotypes was also observed. More severe damages were observed with the lower YTX dose and after 24 h of treatment. Indeed, an increased number of apoptotic cells was observed mainly in cortico-medullary junction and in medulla. Groups of flattened medullary epithelial cells formed single or clustered round structures that resembled Hassall's corpuscles and contained heterogeneous secretory material and necrotic nuclei. The medullary epithelial cells were the most affected cell population. These cells were arranged in a regular reticulum of stellate cells immunoreactive (ir) to high MW CKs, whereas after YTX treatment some cells decreased their ir and some others withdrew cytoplasmic projections modifying to strongly ir round cells. The core of the newly formed medullary structures and true Hassall's corpuscles was also strongly ir to higher MW CKs. With regard to cytokine response, changes were observed in both experimental treatments in comparison to controls. An higher number of cells ir to IL-6 located at the cortico-medullary junction and medulla was found, and they were mostly dendritic cells, while IL-1 a and IL-8 ir cells, observed in the cortex, decreased. The present findings, in disagreement with others reporting little or no toxic effects, indicate that YTX provokes severe morphofunctional damage to thymic microenvironment.

ProCRH in the catfish *Ameiurus nebulosus*: gene structure and tissue-specific responsiveness to LPS

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The procorticotrophin-releasing hormone (proCRH) gene from the teleost *Ameiurus nebulosus* was cloned by direct and inverse PCR-based technologies and characterized. Sequence similarity with the proCRH coding sequences in *Oreochromis mossambicus* (Tilapia) and *Homo sapiens* is 97.7 % and 78 %, respectively. Constitutive expression of the gene was assessed by RT-PCR approach. Western Blot experiments performed with an anti-human CRH (1-41) polyclonal antibody revealed the presence of an immunoreactive molecule with an approximate MW of 18 kDa, value comparable to that of the putative catfish proCRH peptide. Western Blot and immunocytochemical experiments showed modification in proCRH immunoreactivity in the central nervous system (CNS), in the head kidney and in the pancreatic gland after catfish exposure for 15 and 120 min to LPS. An increase in proCRH immunoreactivity in the CNS after 15 min but not after 120 min exposure to LPS was observed, while the increased immunopositivity is detectable throughout the entire period of exposure in the head kidney and only after 120 min of treatment in pancreatic cells. Our findings indicate that CNS responds to the altered conditions for a shorter time with respect to the peripheral organs suggesting a hierarchical and time-regulated response to a persistent stressor.

Stem cell maintenance and committment: the planarian model

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Stem cells are defined by a unique capacity of self-renewal and broad differentiation plasticity. Although the knowledge of the fundamental properties of these cells is the focus of an expanding field of the scientific research, almost nothing is known about the molecular nature of the regulatory mechanisms that determine whether a daughter of a stem cell remains a stem cell or commits to differentiation. The cellular mechanisms that govern these stem cell fate decisions have been explored in a variety of models, including invertebrates. Planarian flatworms, well known for their exceptional regenerative capability, maintain a stable population of totipotent stem cells (neoblasts) throughout their life. Neoblasts are involved not only in regeneration, but also ensure the physiological turnover of all cell types. Pumilio and piwi have recently emerged as regulatory factors involved in asymmetric stem cell division at the post-transcriptional level in *Drosophila*. In order to define the regulatory pathways involved in the rapid events of neoblast fate decisions, we have isolated the planarian homologs of *pumilio* and *piwi* genes of *Drosophila*. *Pumilio* and *piwi* expression pattern is partially overlapping in planarians. In fact both genes are expressed in the same sub-population of neoblasts, with a different antero-posterior gradient. *Pumilio* mRNA is also present in the central nervous system. Knock-down experiments, performed by the RNAi technique, suggest that both these genes are involved in neoblast maintenance. Indeed, planarians injected with dsRNA *pumilio* and dsRNA *piwi* were unable to regenerate. In addition, analyses performed by FACS, in situ hybridization, confocal microscopy as well as TEM observations, revealed a dramatic reduction of the neoblast number in the treated animals, indicating that these genes are fundamental for their maintenance.

Ultrastructure of the hemocytes of *Astacus leptodactylus* (Eschscholtz, 1823): *in vivo* phagocytosis assays

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In the context of comparative studies of immunity cellular defence mechanisms of different taxa of arthropods, the ultrastructure and the *in vivo* phagocytosis of the circulating hemocytes of adults of the crustacean decapod *Astacus leptodactylus* has been investigated by means of light and transmission electron microscopy (TEM). Four types of hemocytes were found in the hemolymph and they were identified as: large-granule cells, medium-granule cells, small-granule cells, and hyaline cells. In order to identify the phagocyte cell, phagocytosis assays were performed *in vivo* by injection of: 1) sterile phospate buffered saline, pH 7.4; 2) 0.9 µm carboxylate-modified polystyrene latex beads; 3) 1.1 and 3 µm unmodified latex beads; 4) *Pseudomonas fluorescens*. 200 µl hemolymph samples (about $1.08x10^4$ hemocytes) were drawn at 0-0.5-1-2-4 hrs from the dorsal vessel of each animal; the hemocytes were pelleted by 14.000 rpm centrifugation, fixed, post-fixed and embedded in epoxy resin for LM and TEM sectioning. Differential cell counts were made from 2 µm semithin transverse sections of the full pellet thickness stained with toluidine blue. The *in vivo* treatments induce changes in relative percentages of hemocyte types. The decrement of hyaline cells compared to the control was concomitant with the increase of the large-granule cells percentage in all treatments. The small-granule cells of *A. leptodactylus* were the main hemocyte type involved in phagocytic response reaching a maximum of 3.6% of the total hemocytes. The percentages of small-granule cells decreased in all treatments suggesting their lysis after the phagocytic activity. The 3 µm latex beads were the only particle not phagocyted at 30 min, advocating that the size of the ingested particle is important for the phagocyting mechanism.

First evidences of Toll like receptor on *Paracentrotus lividus* coelomocytes

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Microbial pathogens use a variety of complex strategies to subvert host defences and to ensure their multiplication and survival. The innate immunity system detects and eliminates invading pathogenic microorganisms by discrimining between self and non-self. The host organism can determine the presence of non-self by recognizing a limited number of conserved structures only produced by pathogen organisms and not by multicellular hosts, called PAMPs (pathogen-associated molecular patterns).

Some of these molecules are lipopolysaccharides (LPS), peptidoglicans, lipoteichoic acid, lipo-arabinomannan acid, lipopeptides and bacterial DNA. Receptors that recognize this molecular pattern have an important role in connecting pathogen detection with innate immune-response. Toll like receptors seem to be the main candidates to perform this function.

In the echinoid *Paracentrotus lividus*, we already characterized phagocytose and cytotoxic activities. Until now, it is not clear the molecular mechanisms for activating these cell responses, and if an inducible system toll dependent, like to that found in *Drosophila*, may be involved.

To assess the presence of these receptors, polyclonal antibodies against human Tlr4 (HTlr IV), were used. Immunoprecipitation and immuno-blotting experiments revealed epitopes that were identified by anty- HTlr IV on protein preparation from coelomocyte membranes, and immunofluorescence reactions showed that they are distributed on coelomocytes.

Using a known DNA sequence of *Strongilocentrotus purpuratus* of Tlr1.1 receptor, degenerated primers were designed. A 350 bp fragment was amplified by PRC using a coelomocyte cDNA, indicating the presence of a Toll-like mRNA. Searches are in progress to clone the fragment and establish the complete sequence and the homologies with the known Toll-like receptors sequences.

Allorecognition in *Botryllus schlosseri*: ultra-structural study of fusion between genetically compatible colonies

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When colonies of the ascidian Botryllus schlosseri contact each other, they can either fuse and anastomise their circulatory systems if genetically compatible, or reject and produce a series of cytotoxic spots along the contact border in the case of incompatibility.

Although many studies have been devoted to the analysis of rejection, few data on fusion are available. In order to fill this gap, we started a preliminary study on the morphology of the various steps of the fusion reaction at both light and electron microscope.

We were able to distinguish at least five different stages in the fusion process. In stage 1 the tunics of the facing colonies contact each other and epithelial cells of the ampullar tips appear cylindrical in shape with a cytoplasm rich in RER with enlarged cisternae containing homogeneous, finely dispersed material. In stage 2, the tunics are strictly juxtaposed and the cuticular papillae are tightly intermingled, but the two tunics are still distinguishable. In this stage, cells of the ampullar tips contain numerous membrane bound granules, with homogeneous electron-dense material, in the supranuclear ("pad") region. Stage 3 is marked by the dissolution of the two cuticles and the local fusion of the tunics in front of the facing ampullae. Less granules are now present in the ampullar pad and some haemocytes leak out from the circulation through the ampullar tips. In stage 4, the pads of the two facing epithelial adhere and new junctional complexes are formed. Basal lamina still delimitate the ampullar lumen and appear highly folded. In stage 5 the juxtaposed epithelia open, thus permitting the communication between the vessels of the two colonies. Cell pads are progressively resorbed and cells of the ampullar tips, now lining a new vessel, return to a cubic shape.

Future studies will investigate the occurrence of apoptotic events in the process of ampullar fusion in *B. schlosseri*.

Cell cooperation among immunocytes of the compound ascidian *Botryllus schlosseri*

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Two different immunocyte types are present in the blood of the colonial ascidian *Botryllus schlosseri*: phagocytes and morula cells (MC). The latter are cytotoxic cells, involved in the inflammatory reaction which occurs when genetically incompatible colonies contact each other. Upon the recognition of non-self molecules, MC release of the proenzyme prophenoloxidase, readily converted to active phenoloxidase which is directly responsible for the induction of cytotoxicity observed along the contacting colonial borders in the forms of a series of pigmented, necrotic spots. We have recently demonstrated that MC respond to the recognition of non-self molecules from the microbial surface through the synthesis of molecules recognised by antibodies raised against mammalian IL-1-α and TNF-α.Here, we demonstrate that MC synthesise the above-reported molecules as a consequence of the recognition of both bacteria and non-self factors from incompatible blood. The positivity is located in the cytosol. In addition, since lysates of MC, previously exposed to bacteria, have been claimed to increase phagocytosis in the solitary ascidian Ciona intestinalis, we investigated whether anti-IL-1-α- and anti-TNF-α-antibodies and recombinant IL-1-α and TNF-α can influence the activity of phagocytes. Data obtained clearly show a

significant ($p < 0.01$) decrease of yeast phagocytosis in the presence (1 µg/ml) of anti-cytokine antibodies whereas, a significant ($p<0.01$) increase is reported when phagocytosis occurred in the presence (10 ng/ml) of the recombinant cytokines. The results support the hypothesis of a role of IL-1-α- and TNF-α-immunopositive molecules in communication among immunocytes.