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REPORT OF MEETING

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Organizers: **L Abelli, A Mancia, MG Marchetti, D Lunardi, G Zuccon**

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Session 1. Chairmen: L Ballarin, University of Padua, Padua, Italy; P Luporini, University of Camerino, Camerino, Italy

Evolution of the immune system

Lecture

On the origin of lymphocytes: a review

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Each individual of each species must defend against pathogenic microbes and thus is armed with molecules and cells recognizing and eliminating non-self. In this respect, immune defences are as old as first eukaryotes. Main gene products recognizing non-self code for molecules having Immunoglobulin domains, leucine-rich repeats, peptidoglycan-recognising proteins, cysteine-rich scavenger receptors, and lectins.

The cells responsible for microbial recognition and clearance are the immunocytes, as they can be defined for all animal species, but in Vertebrate species new classes of immunocytes appeared as a result of evolutionary changes: the lymphocytes. Lymphocytes have an extraordinary capability of selecting antigens and maintain a memory and thus are indispensable for the homeostasis of Vertebrate species. A main feature of lymphocytes is the presence of RAG enzymes for somatic recombination of immunoglobulin-type and leucine-rich repeats molecules, However, mechanisms of somatic recombination of non-self recognition molecules are present in invertebrates with paralogous sets of genes, molecules, and effector cells.

On the basis of recent discoveries in comparative immunology, and with data obtained from our group, an hypothetical origin of somatic recombination mechanisms and of lymphocytic cells will be discussed.

The earlier the smaller: evidence from the study of the pheromone and pheromone-gene structures in the protozoan ciliate *Euplotes*

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In protozoan ciliates, the mechanism of self/non-self recognition is evolutionarily associated with the genetic mechanism of mating types. Within a species, these mating types may either be only two like sexes, or multiple, with the number limited to four, seven or eight, or virtually unlimited. The nearly 100 species of *Euplotes*, the most ubiquitous and cosmopolitan ciliate, all appear to include virtually unlimited numbers of mating types that cells express by synthesizing water-borne signaling proteins, known as pheromones. The knowledge of the structures of these pheromones and of their coding genes has for long been based only on studies of *Euplotes* species (i. e. *E. raikovi*, *E. octocarinatus* and *E. nobilii*) that branch into different central positions of the *Euplotes* phylogenetic tree. More recently, we had the opportunity to analyze the pheromone and pheromone-gene structures in two *Euplotes* species, i.e. *E. petzi* and *E. focardii*, that lie at the opposed extremities of the tree: *E. petzi* (together with *E. sinicus*) represents the earliest branch of the tree, while *E. focardii* (together with the *E. crassus*/*E. minuta*/*E. vannus* species complex) represents the latest branch. In *E. petzi*, we found that the pheromone sequences extend for 32 amino acids and that the pheromone genes have extremely reduced dimensions (715 nucleotides, telomeres included), because they practically lack the regulatory 5' region (only 72 nucleotides). In *E. focardii*, at the opposite, pheromone sequences extend for 85 amino acids and pheromone genes include a long regulatory 5' region (1357 nucleotides) for an overall gene extension of 1951 nucleotides. Since the dimensions of the known pheromone and pheromone-gene sequences of *E.*

raikovi, *E. octocarinatus* and *E. nobilii* are intermediary between those of their homologs in *E. petzi* and *E. focardii*, it appears that an earmark of *Euplotes* pheromone and pheromone gene evolution is a progressive increase of structural complexity, which presumes a parallel increase in complexity of the pheromone activities and of the regulatory mechanisms of pheromone gene expression.

Characterization of cellular and molecular responses of *Actinia equina* (Linnaeus, 1758)

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Sea anemones (Actininaria, Cnidaria) are benthic sessile species that depend of their toxic venom for survival, providing to defense and predation.

Pore-forming cytolytic hemolysins, sodium channel toxins and potassium channel toxins have been isolated and characterized from a great number of sea anemones. However little is known about toxins compositions ant their cellular origin.

Aim of this research is to describe the cell types of internal cavity of *Actinia equina* and their biological activity and characterize, after purification, cytolytic and antimicrobial molecules from different tissues.

Extracts from mucus and tissues showed cytolytic activity toward rabbit and sheep erythrocytes and human chronic myelogenous leukemia cells K562.

A new lytic peptide of about 6 kDa from percoled body fluid and tentacles extract has been isolated by SEC-HPLC followed by mass spectrometry and identified as a neurotoxin specific for sodium channels (AeI) existing in the database.

Then, we showed clearly that the presence of cytolysins is not exclusive of nematocysts. A plaque-forming assay carried out with cell populations extracted from the percoled body fluid showed for the first time that anthozoan granulocytes are able to form plaque of lysis. We have separated the total population of free cells into three distinct discrete bands by discontinuous Percoll gradient, and we have identified different cell types. Cell lysate of each cellular band showed cytolytic activity towards different erythrocytes types and was inhibited by sphingomyelin.

Tentacles acid extracts were subjected to purification on SEP PAK C8 Vac column followed by several HPLC runs on C18. Among the several peaks, two showed biological activity towards *E. coli, S. aureus and C. albicans* and were pooled and lyophilized.

These first results on *A. equina* immunobiology lead us to explore the evolution of cnidarian immune response.

The ampulla is a hemocyte reservoir but not an hematopoietic organ in the freshwater snail *Pomacea canaliculata*

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Scanty information is available on hematopoiesis in molluscs. In the land slug *Incilaria fruhstorferi*, hematopoiesis has been described in the connective tissue associated with the vascular system, in absence of a hematopoietic organ. The only hematopoietic organs retrieved in gastropods are those located into the pericardial cavity of the freshwater snails, *Biomphalaria glabrata* and *Planorbarius corneus*. The South American freshwater snail *Pomacea canaliculata* has been recently banned as a pest and invading species by the EU Parliament, and novel information on its biology is welcome. We have morphologically analyzed the effects of repeated hemolymph collections on the components inside the pericardial cavity of *P. canaliculata*: the heart, the aortas, the ctenidial/pulmonary veins, the renal veins and the ampulla. The latter is a vesicle located just beside the heart, and it is connected with the anterior aorta. The principal function of the ampulla is to prevent an exceeding pressure into the heart chambers during mollusc retraction, but a role in hematopoiesis was also proposed.

After 4 hemolymph collections, repeated on the same animal after 24-h intervals, significant changes intervened in the ampullar morphology, whereas the other components remained unchanged. Into control ampullae numerous hemocytes were retrievable, especially inside the follicles formed by the inner epithelium. Sporadic hemocytes were observed free in the ampullar lumen. Light microscopy evidenced the absence of ampullar follicles after 4 hemolymph withdrawals and a very few hemocytes were still present into the ampulla. Conversely, the number of circulating hemocytes remained constant after each hemolymph collection. Immunohistochemical staining were performed with the anti-p-H3 (Ser10) polyclonal antibody, which marks cells committed to mitosis. In control snails numerous positive nuclei were observed along the external side of the veins and into the pericardial lumen. No positivity was observed neither into nor around the ampulla or in circulating hemocytes. The number and the distribution of positive nuclei remained unaltered after repeated hemolymph collections.

Our data suggest that the *P. canaliculata* ampulla acts as a hemocyte reservoir but not as a hematopoietic organ. Hemocyte renewal rate is not influenced by withdrawals and it takes place only within the pericardial lumen in correspondence of the principal veins. This situation closely resembles the hematopoietic regions of *B. glabrata* and *P. corneus.*

Lecture

Evolution of *MHC-G* gene in Primates

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Major histocompatibility complex (MHC)-G molecules are non-classical MHC class I antigens that differ from other classical class I MHC (-A, -B and -C) molecules for: (I) limited protein variability, (II) presence of different membrane bound and soluble isoforms, generated by alternative splicing of the primary transcript, (III) unique molecular structure characterized by three alpha domains (α1-3), presenting a reduced cytoplasmic tail, (IV) modulation of the immune response, and (V) restricted tissue expression to immune privileged sites (e.g. fetal tissues, thymus). MHC-G molecules play an important role in the suppression of the immune response. In particular, they interact with leukocyte inhibitory receptors inhibiting T lymphocyte and Natural Killer cell activation.

Considering the lineage that gave rise to Old World and anthropoid monkeys, the New World monkey lineage separated about 38 million years ago (mya). The cotton-top tamarin (*Saguinus oedipus*, Saoe) that inhabits the Central-South American continent is a typical example of this group. Saoes presumably have functional *MHC-G*-like genes instead of *MHC-A* and *-B* genes. Thus, it has been proposed that *MHC-G* could be the ancestral *MHC class I* gene.

Old World primates (*Cercopithecinae* subfamily) live in Eurasia and Africa and their MHC-G isoforms do not bear the α2 MHC-G domain due to the existence of stop codons in homozygosis mainly at position 164, that appeared in the *Cercopithecinae* common ancestors after the separation from the human and *Pongidae* lineages about 35 mya.

Gorillas and chimpanzees do not seem to have high polymorphism at MHC-G, while orangutans, that separated from the human lineage about 15 mya show more *MHC-G* alleles, exhibiting variability at the T cell receptor, NK and antigen binding sites. Similar to gorilla and chimpanzee, humans also present low coding region polymorphisms, encoding only 14 common proteins. The selective forces acting at the regulatory regions of the *MHC-G* gene seem to be different from those acting at the coding region. The pattern of variation of the promoter region is characterized by two divergent lineages of haplotypes, which has been maintained by balancing selection in worldwide human populations and may modulate the fine balance between high- and low expressing MHC-G haplotypes.

Since each primate species seems to have undergone a particular evolutionary pathway, further research studies are needed to understand the MHC-G 40 million year evolution.

The study of MHC variation and evolution in the Alpine chamois (*Rupicapra rupicapra*): from targeted Sanger sequencing to genomic enrichment?

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Major histocompatibility complex (MHC) proteins are essential components of the immune response, and are coded by the most polymorphic of the vertebrate genes. Being mediators between the organism and the environment, MHC-coding genes are excellent candidates for exploring adaptive processes and for estimating the genetic component of resistance to infections. Accurate genotyping is essential to understand the role and evolution of MHC in natural populations, but it may be extremely challenging due to gene duplication and high sequence variation of these genomic regions.

Recently, MHC typing has been greatly improved by the advent of next-generation sequencing methods (NGS). In particular, the high coverage of NGS (*i.e.*, the number of time each base is sequenced) allows an accurate representation of all variants that may be present in a multilocus system. MHC regions of interest are commonly enriched by PCR, and high-coverage NGS is obtained for tens or hundreds of individuals at the same time (amplicon-based approach). Alternatively, MHC loci can be captured by hybridization with a probe and then sequenced by NGS (hybrid capture-based approach). Compared to amplicons, hybridization is less stringent and potentially able to capture duplicated loci.

In this study we set-up a hybrid-capture enrichment coupled with NGS (Illumina Miseq technology) to type the genetic variation at DRB-like loci in the Alpine chamois (*Rupicapra rupicapra*). The approach is based on a probe obtained by the PCR amplification of the extremely variable exon 2 using known primers, followed by a low-stringency hybridization. In parallel, NGS sequencing of amplicons has been used to check the enrichment-by-hybridization results. These approaches were tested as a follow-up of a previous Sanger sequencing study of a single exon that allowed us to infer an evolutionary process of stabilizing selection in different populations.

We are currently analysing about 60 Alpine chamois (*Rupicapra rupicapra*) sampled in the Dolomiti Bellunesi National Park (Eastern Alps). The hybridization approach has been performed in 4 individuals and 2 pooled-samples libraries, producing about 30 million short reads that map on the sheep genome. Preliminary results show that the efficiency of the capture is limited and needs to be improved. Since an epidemic of sarcoptic mange had his outbreak in 2009 the Eastern Alps, and produced a significant decline in many populations, we will also compare healthy and mange-affected individuals to test if a specific pattern of MHC genetic variation significantly predicts resistance to the parasite.

The production of amyloid requires cross-talk between immunocytes in the compound ascidian *Botryllus schlosseri*

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Two main immunocyte types are present in the hemolymph of the compound ascidian *Botryllus schlosseri*, *i.e.*, phagocytes and cytotoxic morula cells (MCs). Previous studies have demonstrated that MCs work as sentinel cells able to sense foreign molecules and, as a consequence, release cytokines which activate phagocytes leading to the synthesis and release of rhamnose-binding lectin. The latter, in turn, acts as a chemotactic factor for phagocytes and opsonizes foreign particles stimulating their clearance. In addition, upon the contact with foreign molecules, MCs can degranulate and release the content of their vacuoles, mainly phenoloxidase (PO) and its polyphenol substrata.

In a recent investigation on *Botryllus* cytotoxic cells, we found abundance of amyloid inside MC vacuoles which likely, once released, act as a scaffold to prevent the diffusion of PO and cytotoxicity to the whole organism. In addition, the study of the molecular cascade leading to the synthesis of amyloid, is revealing a non-classical pathway in which both the phagocytes and MC are involved.

Session 2. Chairmen: E Ottaviani, University of Modena and Reggio Emilia, Modena, Italy; E Adinolfi, University of Ferrara, Ferrara, Italy

The fight against pathogens

Lecture

The P2X7 receptor for extracellular ATP: from mediator of inflammation to regulator of tumoral immune infiltration

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Adenosine triphosphate (ATP) is well known for its central role in intracellular metabolic processes. However, ATP can also be released in the extracellular space activating two classes of receptors: P2X ion channels and G protein coupled P2Y receptors.

Among those, the P2X7 receptor is well characterized for its ability to mediate the production and secretion of IL1β, PGE2 and other inflammatory mediators. P2X7 expression has been reported in a wide range of species including fish, amphibians, birds and mammals. In all these species, the receptor is mediating immune response against pathogens activating cytokine secretion and phagocytosis.

In recent years, we have demonstrated that P2X7 receptor activity can increase intracellular levels of ATP and mediate its secretion. The use of sensors enabling *in vivo* measurement of extracellular ATP, allowed associating an increase in the peri-cellular levels of the nucleotide with almost all know inflammatory situations, including graft-versus-host disease, allergic and anti-cancer immune reactions. Here we show for the first time that P2X7 receptor also regulates immune infiltrate in cancer. Indeed, when developing experimental melanoma, C57/black 6 mice, lacking P2X7 expression, showed a virtually absent immune infiltrate if compared to wild type controls. The main cells involved seemed to be macrophage-dendritic cells and T lymphocytes, as demonstrated by F/480 and CD3 immunostaining. Taken together all these data suggest a central role for P2X7 receptor in regulating a widespread range of immune responses.

Toxic secondary metabolites in predator-prey interactions among ciliated protists

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Predator-prey interactions involving numerous protist species have been extensively studied in the last years, and great attention has been paid to the repertoire of toxic secondary metabolites adopted in the immobilization and capture of prey, as well as in chemical defense against predators. In this context, particular attention was focused on the important role of specialized ejectable membrane-bound organelles, generally called extrusomes, and widely distributed in protists. These organelles are usually localized in the cell cortex and attached to the cell membrane, and they share a common characteristic in discharging their contents to the outside of the cell in response to mechanical or chemical stimuli. The chemical nature of protists' extrusive compounds characterized to date is extremely variable, including proteins, carbohydrates, lipids, and dozens of additional classes of secondary metabolites. However few data are available to date for a particular group of protists, the ciliated protozoa. It is worthy of note that at least some of the secondary metabolites produced by ciliates have been demonstrated to show antibiotic, anti-cancer and pro-apoptotic properties in addition to their physiological functions. Among these secondary metabolites, euplotin C produced by the ciliate *Euplotes crassus,* and climacostol produced by *Climacostomum virens,* have been shown to activate programmed cell death by impairing mitochondrial membrane potential and inducing ROS generation. Interestingly, it was also demonstrated for climacostol, antimicrobial activity against Gram-positive bacteria and some fungal pathogens.

In conclusion, the toxic secondary metabolites from ciliated protozoa may set the basis for the development of a novel series of antitumor or antimicrobial agents.

Phagocytosis-induced apoptosis in the compound ascidian *Botryllus schlosseri*

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Colonies of the ascidian *Botryllus schlosseri* contain three blastogenetic generations: functional zooids, their buds and budlets on buds. Generation change or take-over (TO) occur cyclically and assure the recurrent renewal of the colony. During these events, lasting 24 - 36 h, diffuse apoptosis occurs in zooid tissues, as indicated by morphological, biochemical and molecular investigations. Tissues are rapidly infiltrated by circulating phagocytes, selectively recruited by dying cells, which recognize and greedily ingest them. Using hemocytes as selected cell population for investigation, we studied the transcription rate of three recently characterized genes involved in apoptosis, *bsbax*, *bsaif1* and *bsparp1*, during the TO as compared to colony developmental phases far from it. In addition, we observed that the massive ingestion leads phagocytes themselves to undergo apoptosis, probably as a consequence of the oxidative stress related to the sustained respiratory burst, as suggested by biochemical analysis. Therefore, a large fraction of circulating phagocytes needs to be replaced by new, young hemocytes, entering the circulation at the end of the generation change.

Activity of salmonid antibacterial proteins and regulation of their expression in response to different bacterial pathogens

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Cathelicidins are a family of antimicrobial peptides characterized by conserved pro-peptide sequences. They represent an important component of innate immune response in vertebrates. Two members of the cathelicidin group, Cath-1 and Cath-2, have been identified in salmonids and other fishes. Both peptides have a variable glycine/serine-rich C-terminal domain corresponding to the antimicrobial peptides.

This study purposes to characterize their antimicrobial activity spectrum, their mode of action and tissue expression.

Different peptide fragments representing Cath-1 and Cath-2 of *Oncorhynchus mykiss*, *Salvelinus fontinalis*, *Salmo trutta fario* and *Thymallus thymallus* were chemically synthesized, and their antimicrobial activity tested against the most relevant fish pathogens such as *Yersinia ruckeri*, *Aereomonas salmonicida*, *Aeromonas hydrophila*, *Vibrio anguillarum* and *Lactococcus garvieae*. The different peptides resulted to have medium-dependent antibacterial activity at micromolar concentrations. Membrane permeabilization and hemolytic assays indicated that Cath peptides rapidly kill target bacteria showing at the same time low toxicity against host erythrocytes. Western blot analysis revealed that Cath-1 is expressed in spleen and head kidney.

cath-1 and *cath-2* gene expression was then comparatively studied by *in vivo* experiments inoculating *O. mykiss* with one of the following formalin-inactivated pathogens: *L. garvieae*, *Y. ruckeri*, *A. salmonicida* or *Flavobacterium psychrophilum*. Real-Time PCR analysis performed on tissues collected at different time points demonstrated that both genes are expressed in most tissues and their expression is highly induced 24 h after the challenge. Moreover, the expression pattern of *cath-1* and *cath-2* results different according to the different pathogen. These results suggest that Cath peptides are endogenous antibiotics and an important element of salmonids innate immune response and open the possibility that their upregulation might be exploited in the protection of salmonids from infection and at the same time as a possible biomarker for diagnosis of bacterial diseases.

IgT from Antarctic teleosts

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IgT, a novel teleost-specific Ig isotype, also named IgZ, has been recently identified in the most studied species belonging to the main orders of teleost fish, such as *Salmo salar*, *Oncorhynchus mykiss*, *Takifugu rubripes*, *Cyprinus carpio*, *Ctenopharyngodon idella*, *Dicentrarchus labrax, Gasterosteus aculeatus*, *Siniperca chuatsi* and *Epinephelus coioides.* This immunoglobulin isotype appears to be specialized in mucosal immunity since produced specifically by IgT^+ cells that represent an independent B lineage specialized in mucosal immunity. In the majority of the species investigated the IgT heavy chain comprises four Ig constant domains (CH), except in *G. aculeatus* and *D. labrax* where it presents only three CH domains. In this study, we isolated and analyzed the IgT transcripts in the gut of the Antarctic teleost *Trematomus bernacchii*. We were also able to identify the IgT nucleotide sequence in the liver transcriptome of another Antarctic teleost, *Notothenia coriiceps*. The phylogenetic analysis was carried out using the amino acid sequence alignment of the IgT CH region of Antarctic teleosts and other species using ClustalX. Based on the alignment, we constructed a NJ tree of individual CH domains, supported by 1000 bootstrap replications, which revealed that both Antarctic species had only three CH domains, CH2 being the missing domain. A limited diversity between the temperate and Antarctic species was found, sharing a percentage of amino acid similarity of 24.7 - 80 %

for CH1, 16.7 - 88.5 % for CH3, 24.5 - 79.3 % for CH4. The cysteine residues, crucial for the Ig domain folding, were found to be conserved and two extra-cysteines were identified in the second constant domain and in the secretory fragment. The analysis of N-glycosylation sites revealed the presence of one site in the second domain and two sites in the third domain. Despite Antarctic IgT contained an N-glycosylation site and a cysteine residue in the secretory tail, this region showed low similarity to the canonical motif required for J-chain association. To clarify the polymeric assembly of IgT, a biochemical analysis will be carried out. In addition, a preliminary analysis of amino acid sequences from a number of *T. bernacchii* specimens showed in some sequences the presence of a short insertion between CH1 and CH2. It will require further analysis to determine whether this insertion may act as a hinge, despite a lack of evidence for the hinge features typical of mammalian IgA.

Lecture

IL-18 associates with microvesicles shed from human macrophages in response to P2X receptors stimulation

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Extracellular ATP, released upon cell stress, cell damage or as a consequence of necrotic cell death due to microbial infection or inflammation, acts as a *danger signal* alerting immune and non-immune cells by activating their purinergic P2 plasma membrane receptors (P2R). P2R are classified into two subfamilies named P2YR and P2XR.

In this report we show that pharmacological stimulation with extracellular ATP induced in human macrophages the fast release of membrane microvesicles in which the inflammatory cytokine IL-18 was highly concentrated, while on the contrary it was almost absent from the culture medium. The pan P2R agonist ATP and the P2X7 preferred agonist benzoyl-benzoic ATP (BzATP), but not the P2YR active nucleotides UTP or UDP, evoked microvesicle shedding. The phenomenon was completely abrogated by pre-treatment of macrophages with the P2X inhibitor oxidized ATP (oATP) and partially prevented by the non-covalent P2X7 inhibitors KN-62 A-740003 or A-438079. Microvesicles release and IL-18 content were greatly decreased in the absence of extracellular Ca^{2+}. Disassembling of cytoskeletal microtubules or actin filaments did not impair formation and release of the microvesicles, while inhibition of $iPLA_2$ or $cPLA_2$ delayed it. Interestingly, differently from IL-1β, accumulation of IL-18 did not require pre-treatment of cells with bacterial endotoxin (LPS) as the pro-cytokine was already present in un-primed macrophages and did not decrease by incubating cells with the LPS-binding antibiotic polymyxin B nor with the TLR-4 intracellular inhibitor CLI-095.

Presented data point to a P2X-mediated release of IL-18 loaded membrane vesicles from human macrophage.

New evidences of conserved pathways in complement system dynamics from the colonial ascidian *Botryllus schlosseri*

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In recent years, it has been widely demonstrated that complement, although often depicted as a 'first line of defense', is more than just a defender against microbial intruders and acts as a tightly integrated surveillance system. It is not only important against microorganisms, but also for the clearance of apoptotic cells and corpses. *Botryllus schlosseri* belongs, as vertebrates, to the phylum Chordata and, for this phylogenetic trait, it is unanimously considered a reliable model organism for the studies of the evolution of the immune system. Moreover it is also characterized by a peculiar life cycle with a cyclical, massive apoptosis. This two key features render *B. schlosseri* a good research tool for the study of the evolution of the complement system. Here we report the first results on the expression of BsC3 and BsFactorB, both components of the alternative pathway (AP) of complement activation, which form the AP C3 convertase.

Since studies on mammalian models have shown that 80 % of the observed complement response is derived from AP convertase-mediated C3 amplification, even if initially induced by the classical pathway (CP), studies of complement activation in an organism that lack the adaptive immunity, as *B. schlosseri*, could lead to a better comprehension of the AP cascade and the behavior of C3 convertase not only in invertebrates, but also in vertebrates, mammals included.

As in mammals, BsC3 is highly transcribed at basal level and over-expressed after incubation with non self (zymosan) while BsFactorB always shows limited expression. In the presence of compstatin, a 13-residue cyclic peptide able to inhibit the activation of C3 by C3 convertases, the percentage of phagocytosing hemocytes collapses. In the presence of both zymosan and compstatin, the transcription of BsC3 by hemocytes increases with respect to cells exposed only to zymosan: this suggests the presence of a conserved molecular machinery able to control and modulate *B. schlosseri* as well as the mammalian complement.

Early transcriptional immune responses in *Yersinia*-injected rainbow trouts

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The enterobacterium *Yersinia ruckeri* is the causative agent of enteric red mouth disease (ERM) leading to significant economic losses in salmonid aquaculture worldwide. Infection may result in a septicemic condition with haemorrhages in the oral cavity, on the body surface and in the internal organs.

Knowledge about the immune response of rainbow trout against *Y. ruckeri* is important in terms of control and prevention. Adaptive immune responses related to *Y. ruckeri* infection have been investigated by a number of authors. A few studies have measured in rainbow trout the expression of selected immune-related genes such as Toll-like receptors (*TLR*), *CXCd*, *IL-1β*, and some others. Since the first defence line against bacterial pathogens mainly depends on the innate immune system, we undertook the global study of tissue-specific transcriptomes to depict the early response of juvenile *Oncorhynchus mykiss* to 10^8 formalin-inactivated *Y. ruckeri* cells.

Following i.p. injection, we collected the main tissues at different time points up to 96 h. After preliminary evaluation of the expression of *cathelicidin* genes in real time PCR, we selected 24h post-infection as informative time-point for RNA-seq analysis. Eight samples from gills, intestine, spleen and kidney were subjected to library preparation and Illumina sequencing (paired 50 base reads). Subsequently, a de-novo assembling was performed to obtain a comprehensive reference transcriptome and evaluate the differentially expressed genes for each pair of treated and control samples. About 1928 out of the 62 k assembled transcripts were differentially expressed in at least one tissue. Kidney, spleen and intestine showed the greatest transcriptome modulation in terms of inflammatory and immune response to the bacterial antigens. Pro-inflammatory cytokines like *IL1β*, *IL6* and *TNFα*, complement proteins like *C3*, *C5* and *factorB* and acute phase proteins like *SAA*, *hepcidin* and also c*athelicidins* showed a significant induction and marked the early activation of the immune system in the treated trouts.

Effects of dietary vitamin D_3 administration on innate immune response of sea bass (*Dicentrarchus labrax*)

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The aquaculture sector is a productive force rapidly growing in the world; therefore, over the past 30 years much has been done to improve the performance of fish growth by manipulating diets. Currently, the optimization of diets and disease control are two main objectives to ensure the continued expansion of aquaculture. A new area of great interest to improve growth efficiency and to prevent and/or control fish diseases is the application of probiotics, prebiotics and stimulants of the immune system as additives in the diet, being a promising alternative to antibiotics. Thus, the aim of the current study was to evaluate the potential *in vivo* effects of the dietary administration of vitamin D_3 (vD_3) on the humoral innate immune response (level of total IgM antibodies, the activities of peroxidase, protease, and antiprotease) cellular (phagocytic activity) and biochemical parameters (cortisol, glucose) of the sea bass (*Dicentrarchus labrax*). Vitamin D_3 was orally administered to sea bass specimens in a commercial pellet food supplemented with 0 (control), 3750, 18750 or 37500 U kg^{-1} and fish were sampled after 2 and 4 weeks of treatment. Serum and peritoneal cavity leucocytes were collected from specimens and innate immune biochemical parameters were evaluated. This study showed for the first time in sea bass a modulation in the activities examined in animals fed with the addition of vitamin D_3 after 4 weeks. These results suggested that dietary vitamin D_3 administration has an effect on the innate immune parameters of *D. labrax*, therefore this vitamin could be considered of great interest as immunostimulant when used as food additive in fish farming.

Session 3. Chairmen: M Cammarata, University of Palermo, Palermo, Italy; MR Coscia, IBP CNR, Naples, Italy

Immunity and biotechnology

Lecture

Molecular and cellular basis of mucosal immunity

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Mucosal surfaces represent a first line of defence facing many pathogens, and also monitoring harm-less antigens such as food, airborne particles or symbiotic microorganisms. The latter represent the majority of the encountered epitopes and require precise regulatory mechanisms from the host's immune system to choose between ignorance and active suppression. On the other hand, a strong immune response is needed for the former. Under these constraints, mucosae have evolved a complex immune system, anatomically and functionally distinct from the systemic counterpart. In mammals, mucosa-associated lymphoid tissues (MALTs) are the sites where sampling of antigens occurs and naïve T and B lymphocytes are stimulated. Protection against pathogens is also due to MALT antimicrobial components, in particular enzymes, lytic agents, lysozyme and various immune molecules, such as complement and immunoglobulins (Ig). In teleost fish, MALTs are further distinguished in anatomically distinct compartments such as gut-associated lymphoid tissue (GALT), skin-associated lymphoid

tissue (SALT) and gill-associated lymphoid tissue (GIALT). All MALT types comprise different leukocytes, such as T and B cells, mast cells and macrophages. Not long ago, only two isotypes of Ig, IgM and IgD, have been described in teleosts, IgM being considered the only player either in the systemic or mucosal immune responses. However, a third immunoglobulin isotype, named IgT/IgZ, restricted to teleosts, has been discovered more recently and it has been reported to play a specialized role in mucosal immune responses. A number of studies has shown that in teleosts, like in mammals, the transport of mucosal Ig occurs by a polymeric Ig Receptor which is expressed on the surface of epithelial cells. A review will be given to summarize the current knowledge of fish mucosal immune response with a brief reference to innovative mucosal vaccines.

Antimicrobial response in *Anemonia sulcata* (Cnidaria)

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Recently we analyzed the inflammatory response in *Anemonia sulcata,* following injection of various substances, and observed specific reactions especially after injection of bacteria. Moreover, we analyzed enzymatic activity of protease, phosphatase and esterase, showing how the injection of different bacterial strains alters the expression of these enzymes suggesting a correlation between the appearance of the inflammatory reaction and the modification of enzymatic activities. Our study show for the first time in cnidarian, cellular and molecular responses following injection of bacteria.

The sea anemones are a source of neurotoxins acting on potassium and sodium ion channels. *A. sulcata* is exposed to attacks from predators but is unable to retract the tentacles and actively capturing a broad spectrum of prey is subject to frequent breakage of the tentacles with subsequent bacterial infection. The chemical arsenal of *A. sulcata* is the optimal strategy for survival, mainly through the production of neurotoxins and by using antimicrobial molecules.

By acid extraction, HPLC purification and antibacterial assays, we isolated, purified and characterized an antimicrobial peptide towards *Micrococcus lysodeikticus*. The mass spectrometry analysis and the sequencing, showed that antimicrobial peptide is present in the database as neurotoxin, named Neurotoxin 2 (ATX II), Na^+ channel blocking toxin.

ATX II can then be considered as a neurotoxin having antimicrobial peptide structural and functional characteristics.

This multifunctionality could be an optimal survival strategy that allows these animals to be active predators through the production of neurotoxins and to resist to bacterial infections caused eventual rupture of the tentacles through the functionality by antimicrobial peptides.

Inflammatory effects of multi-walled carbon nanotubes in leeches

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During last years nanotechnologies has developed considerably, occupying a pivotal role in scientific and technological research. Among nanomaterials, multi-wall carbon nanotubes (MWNTs) show high strength and flexibility and for these reasons they are very useful in different fields such as industry, electronics and medicine. Despite these properties, MWNTs seem to be toxic for organisms.

Our research is focused on the possible effects caused by carbon nanotubes exposure in a freshwater invertebrate model: the leech *Hirudo medicinalis.* Animals have been analysed after both a short time and a prolonged exposure to MWNTs (1, 3, 6 , 12 h and from 1 to 5 weeks). Our data obtained by morphological, immunocytochemical histoenzymatic and Western blot analysis, show that MWNTs treatment induce inflammatory responses: angiogenesis, fibroplasia and a massive macrophage migration take place.

Interactive effects of nanoparticles with other environmental pollutants on immune function and larval development of *Mytilus galloprovincialis*

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The increasing production of manufactured nanosized materials is leading to the release of nanoparticles (NPs) and their products into the aquatic environment, thus posing a threat to aquatic biota. Despite the growing concern over the potential biological impact of NPs on marine organisms, little is known about their interactions with other pollutants. Recent evidence indicates that the biological impact of NPs may be due not only to the toxicity of NPs themselves, but also to their capacity to modify the bioavailability, bioconcentration and toxicity of other common environmental pollutants, such as organic xenobiotics and heavy metals.

The bivalve *Mytilus sp.*, largely utilized as a sentinel for marine contamination, has been shown to represent a significant target for different types of NP, including n-TiO_2, one of the most widespread NP in use.

In this work the effects of *in vitro* and *in vivo* exposures to n-TiO_2, alone and in combination with 2,3,7,8-TCDD and Cd^{2+}, chosen respectively as models of organic and inorganic contaminants, on

the immune function of *Mytilus galloprovincialis* were investigated.

The results demonstrate that combined exposure to n-TiO_2 and TCDD exerted interactive effects on phagocytic activity but not on lysosomal membrane stability-LMS *in vitro*. These effects were confirmed in *in vivo* exposure to the mixture.

Antagonistic effects of n-TiO_2 and Cd^{2+} on NO production and lysozyme activity were observed both *in vitro* and *in vivo*. On the other hand, no interactive effects were detected on expression of Metallothioneins and immune-related genes lysozyme and Toll-like receptor (TLR-i). Cd^{2+} alone, but not in combination with n-TiO_2, also affected larval development.

These data indicate complex and often unexpected interactions between NPs and organic contaminants and heavy metals in marine bivalves.

Insights into the structure/activity relationships of *M. galloprovincialis* Myticin C

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One of the most intriguing AMPs of the marine mussel *Mytilus galloprovincialis* is Myticin C (MytC) because of its remarkable constitutive/induced expression levels, high transcript polymorphism and peculiar expression profiles in individual mussels. The conformation of both Myticin C precursor and mature peptide (40 aa, 4.377 kDa) is still unsolved but mainly depends on an evolutionary conserved array of eight cysteine residues.

Following stepwise and optimized protocols of chemical synthesis, we obtained the mature peptide and smaller analogs, useful to investigate the secondary structure in different experimental conditions by circular dichroism spectroscopy and nuclear magnetic resonance.

Aiming to determine the antimicrobial properties of the chemically synthesized MytC peptides and to identify the minimal active fragment we evaluated their bacterial growth inhibition (MIC) and bactericidal (MBC) potency at two pH conditions on Gram negative and Gram positive bacteria able to cause human or fish/shellfish infections and diseases. Opposite to recent findings (Mar Drugs, 2013, 11, 2328-2346), we could not verify any activity of our MytC peptides against the viral hemorrhagic septicemia (VHS) and infectious hematopoietic necrosis (IHN) viruses. Next steps will focus on oxidative refolding, recombinant synthesis and purification of MytC.

Evidences for antimicrobial peptides in the colonial ascidian *Botryllus schlosseri*

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Invertebrates have acquired many mechanisms of defence in order to overwhelm the risk of pathogen attack. In particular, the presence of various types of antimicrobial peptides (AMP) guarantees an efficient response, making them able to kill both gram positive and negative bacteria, fungi and viruses. In ascidians, AMPs have been isolated and described in different species, such as in *Styela clava* (Clavanins and Styelins) and in *Ciona intestinalis* (the recent Ci-MAM). Previous studies have revealed molecules, including phenoloxidase and rhamnose-binding lectins, with an antimicrobial effect also in *Botryllus schlosseri*. Here, we report that the growth of some bacteria strains is highly inhibited by extracts of haemocytes, showing alterations in their surface. Moreover, molecular analyses allowed us to identify a *Botryllus* sequence similar to Styelins that is abundantly transcribed in phagocytes.

Session 4. Chairmen P Venier, University of Padua, Padua, Italy; A Mancia, University of Ferrara, Ferrara, Italy

Immunity and environmental monitoring

Lecture
The Strange Case of Georgia Dolphins: the effects of environmental changes on the transcriptome.

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Land use and population densities influence contaminant concentrations in aquatic organisms. It is increasingly common to monitor the marine environment and establish geographic trends of environmental contamination by measuring contaminant levels in animals from higher trophic levels. In this work we chose the common bottlenose dolphin (*Tursiops truncatus*) as a top level predator to monitor a site on the Georgia coast

the US, known to be heavily contaminated by Aroclor 1268, an uncommon PCB mixture primarily comprised of octa- through deca-chlorobiphenyl congeners. Prior studies have suggested an association between high polychlorinated biphenyls (PCBs) concentrations and increased risk of infectious disease suggesting a causal link between persistent organic pollutants (POPs) exposure, immune function and susceptibility to disease. We are currently investigating the potential for identifying PCBs exposure in bottlenose dolphins through screening for their immunological and/or endocrine perturbations associated with exposure using microarray technology and gene expression profile analysis. A newly developed dolphin oligo microarray representing 24,418 unigene sequences was used to analyze blood samples from 69 dolphins collected from 5 geographic locations (Beaufort, NC, Sarasota Bay, FL, Saint Joseph Bay, FL, Sapelo Island, GA and Brunswick, GA). The Georgia samples were selected due to the measured high concentrations of PCBs contaminants in their blubber. Genes involved in xenobiotic metabolism, in development/differentiation and oncogenic pathways were found to be differentially expressed in GA dolphins compared to the other locations. Hypothyroidism has been previously described in GA dolphins and, interestingly, a few of the genes that we identified are involved in the proper function of the thyroid. The analysis of GA animals alone, correlated with contaminant load measured, showed the activation of genes involved in stress response, DNA repair and skin damage, UV and/or viral infection-induced. If successful, the gene expression profile analysis could provide a cost-effective means to screen for indicators of chemical toxin exposure as well as disease status in top level predators. The transcriptomic data analysis will be a first step towards identification of markers/patterns indicative of exposure to chemical contaminants and will promote an understanding of toxic mechanisms and/or pathways that are currently not well understood in marine mammals.

Characterization of superoxide dismutases in the Antarctic scallop *Adamussium colbecki*

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Superoxide dismutases (SODs) are considered the most important and ubiquitous antioxidant enzymes, involved in cellular antioxidant defenses, maintaining the redox homeostasis during the aerobic cell metabolism. Moreover, SODs are also closely involved in the innate immune response of animals, as evidenced by the rapid modulation of transcription during challenges with endotoxins, bacteria or viruses. During infection, the host often uses reactive oxygen species (ROS) to react to pathogenic invaders via phagocytosis. However, excess ROS generated during the respiratory burst may also be harmful to the host, that use the antioxidant machinery to maintain low ROS concentration in the cytoplasm. Antarctic species are characterized by a large number of special physiological features that allow the life in the extreme environment. First of all, low temperature and salt concentration are physicochemical conditions that increase oxygen solubility and, consequently, the rate of ROS formation. For this reason, a finely modulated antioxidant system is essential to prevent macromolecules oxidation that could result in DNA damage, loss of membrane integrity and changes of protein activities. Despite numerous previous studies on SODs from aquatic animals, only few data are available for the SODs of Antarctic mollusks. In the present work, we studied the Antarctic scallop *Adamussium colbecki*, a bivalve mollusk widely distributed in the Antarctic Ocean; for the first time we characterized the gene structure and expression of *Adamussium* SOD. Specimens were sampled in the Ross Sea (Terra Nova Bay, 74°42'S, 164°7'E) during the XXI Italian Antarctic Expedition. Partial cDNA sequences of Cu,Zn SOD and Mn SOD were obtained from gonadic tissue by RT-PCR and TA-cloning. The obtained nucleotide and deduced amino acid sequences were compared with those of orthologous genes already available in GenBank and the protein phylogenies were reconstructed. We also studied the gene transcription and enzyme activity in various organs and tissues, to investigate the biological fraction of SODs in molluscs living in extreme environmental conditions.

Characterization of immune-related transcripts within the Atlas gene database of *Procambarus clarkii* (Girard, 1852)

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Being *Procambarus clarkii* an invasive species, showing high adaptability and resistance to diversified environmental challenges, such as high pollution levels, high organic and bacterial contents, it results of great interest analyzing its immunome. The knowledge of its immunity could help in characterizing possible aspects of its vulnerability in order to find new strategies for contrasting the continue spread of this alien invasive species. Starting from an Atlas gene database, obtained from a collection of 12 tissues, we depicted and described immune-related transcripts. In particular, eyestalk, brain, hemocytes, hepatopancreas, testes, ovaries, gills, green gland, ventral ganglia, cardiac muscle, Y-organ-epidermis and tail muscle, have been sequenced with the Illumina 2X100bp technology from oriented-cDNA libraries generating 1.9 Gb sequence data and resulting in a non-redundant, high quality subset of 97,976 different transcripts. We focused our attention on the several classes of immune-related transcripts, such as pro-phenoloxidases, anti-lipopolysaccharide factors, lectins, clottable proteins and proteins having

antimicrobial activity. For each immune-related transcript found, we detected its relative percentage expression in all the tissues sequenced creating a collection of immune-related transcripts characterized in *P. clarkii* and reporting the tissue-specificity for each of them.

Hereafter, we present some transcripts that have attracted our attention. Two prophenoloxidases where detected, one had higher expression in the hemocytes and one in the gills. Anti-lipopolisaccharide factors were most expressed in the hemocytes and in the gills. Crustins were also detected and resulted to be mainly expressed in hemocytes, but also in the ovary. Lysozymes where found highly expressed in the hemocytes, but also in the brain, in the hepatopancreas, in the gills and in the Y-organ-epidermis. Numerous lectins and C-type lectins resulted to be mainly expressed in the hepatopancreas.

Moreover, we detected clottable proteins, such class of transcripts was never reported in the red swamp crayfish since now and surprisingly the majority of them resulted to be electively expressed in the brain. This preliminary study helps in outline the possible tissue-specificity of some isoforms of immune-related transcripts and to characterize classes never reported before, such as clottable proteins. Further functional assays could be led considering these data obtained in this preliminary screening, in order to outline the main defense mechanisms of this species.

Functional characterisation of the antioxidant system in *Drosophila melanogaster*, after metals exposure

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Reactive oxygen species (ROS) are a normal byproduct of aerobic metabolism playing an important role in cell signaling and immunity when their production is regulated or controlled by antioxidant enzymes. Reduced expression and/or activity of these proteins lead to an excess of ROS production and oxidative stress, accelerating aging and neurodegeneration. For example, amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease caused by motoneuron loss and some familial cases (fALS) are linked to mutations of superoxide dismutase type-1 (SOD1). Many animal models, such as *Drosophila melanogaster*, are used as useful tool to study this disease, focusing the attention on the different mutant SOD1s but not considering the complex relationships of functional complementarity between SOD1 and the other components of the enzymatic antioxidant system. In the present work we study, for the first time, the gene expression, by qRT-PCR, of SOD1 together with that of superoxide dismutase type-2, catalase, glutathione peroxidases and peroxiredoxins in wild type *D. melanogaster*, exposed to various concentration of copper or cadmium, used as pro-oxidants. The aim was to determine the adequate experimental condition to employ with *D. melanogaster* SOD1 mutants. On the basis of our results, copper is the major inducer of all considered enzymes, and the dose of 1.0 mM seems to be the more suitable. Catalase is temporally co-expressed with SOD1, at least in males and the gene expression of other enzymes seems to be temporally uncorrelated to SOD1. Other important indication will be obtained by biochemical quantification of activity for all considered enzymes and the evaluation of ROS production, that are now in progress.

Analysis of some hematological parameters of *Apis mellifera ligustica* (Spinola, 1806) populations in a polluted and non-polluted site

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Main immune defences of the honey bees are the cellular responses represented by phagocytosis and melanisation. There are a number of factors that could impact on the honeybees immune system and, therefore, increase their susceptibility to disease and lower their survivorship such as: exposure to pesticides, in air, in pollen, in nectar and in water; fungicides from both field and in-hive treatments; varroacides; the pest *Varroa destructor*; antibiotics used in in-hive treatments; fungal pathogens such *Nosema apis* and the emerging *Nosema ceranae*; bacterial and viral infections. More important, it must be highlighted the interactions among different chemicals and their synergic effect with diseases in the immune suppression of individuals and on the colony. The present study use simple and reliable methods that can clearly describe the state of health of the bees, such as total hemocyte counts (THCs) and the activities of the plasmatic phenoloxidase (PO) and its inactive form (proPO) to assess the immune competence of individuals. Specimens of *Apis mellifera ligustica* were collected from beehives located in S. Giovanni (Trieste, “control site”) and from hives placed in Domio (Trieste, “polluted site”) in summer and early autumn. Both in summer and in autumn the statistical comparison showed a greater number of circulating hemocytes in bees from the site of Domio (July: 1159333.3±123073.60 - mean ± SE, n = 15; October: 813333.3±50583.89 - mean ± SE, n = 15) compared to the numbers recorded in bees from S. Giovanni (July: 834166.7 ± 55493.08 - mean ± SE, n = 12; October: 273333.3±33046.38 - mean ± SE, n = 12). The statistical analysis showed a trend towards significant difference in July (Wilcoxon rank sum test, $p = 0.063$) and an highly significant difference in October (Wilcoxon rank sum test, $p < 0.00001$). The honeybees from Domio presented an highly significant lower PO activity than those from S. Giovanni (ANCOVA: $F_{3,206} = 38.73$, $p < 0.01$, $n_{S.Giovanni} = 17$, $n_{Domio} = 18$). Also with regard to the enzymatic activity of the proPO we recorded a significantly higher one in the

honeybees from S. Giovanni in comparison to those from Domio (ANCOVA: $F_{3,206}$ = 23.66, p < 0.01, $n_{S.Giovanni}$ = 17, n_{Domio} = 18). It should be noted that 52 % of the bees collected from hives of Domio had 1 or 2 individuals of *Varroa destructor* on the tergites of the thorax. The higher activities of PO and proPO in the honeybees from S. Giovanni site is probably to be ascribed to the different quality of the environment in the 2 sites and thus it indicates a depression of non-specific immune competence and an increased susceptibility to *Varroa destructor* parasites in the honeybees from Domio.

Characterization of proxiredoxins' coding genes in *Ciona intestinalis*

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Ascidians represent an interesting model from an evolutionary and ecotoxicology point of view, because of their large distribution in temperate sea and their phylogenetic position of invertebrate chordates. Immune responses imply an increase in oxygen consumption with a consequent risk of oxidative stress. With the aim to study the components of the antioxidant defense system in the solitary ascidian *Ciona intestinalis*, we characterized gene sequences encoding peroxiredoxins (Prxs), non-selenium peroxidases that are able to reduce hydrogen peroxide, organic hydroperoxides and peroxynitrite. Thus they represent a class of important antioxidant enzymes, that protect cells against oxidative stress. In the GeneBank database five Prx sequences are present, Prx2, 3, 4, 6a and 6b. The multi-alignment analysis, conducted with orthologous sequences of vertebrates and invertebrates, showed that in *Ciona*'s Prxs the amino acids essential for their enzymatic activity are highly conserved, namely the catalytic tetrad consisting of proline, threonine, cysteine and arginine. Preliminary phylogenetic reconstruction indicates that Prx3 and 4 emerge as sister group of Prxs of the respective vertebrates groups (or isoforms), while Prx2, 6a and 6b have an uncertain position. A partial confirmation of phylogenetic results was obtained with the analysis of homology modeling, according to which Prx3 and 4 present a structure similar to that of two vertebrate proteins, *Bos taurus* Prx3 and *Larimichthys crocea* Prx4, respectively, while Prx2 and 6 six have a three dimensional structure similar to that of two invertebrate proteins, *Ancylostoma ceylanicum* Prx1 and *Arenicola marina* Prx6, respectively. The transcription of all these genes, measured by qRT-PCR, resulted variable in different organs (intestine, ovary, pharynx, stomach). In particular, the ovary is the organ that expresses the highest level of messenger for all Prxs. Preliminary data were also collected about the possible circadian expression of Prx.

Histological and molecular studies on IgM expressing cells of the B-lineage of two Antarctic teleost species

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Life at sub-zero temperatures, such in Antarctic seas, has selected especial adaptations of cell molecules, among them the immunoglobulins (Ig). Differently from species living in temperate water, many Antarctic teleost bony fish showed peculiar protein structure and molecular dynamics of IgM, acquired during radiation of the Suborder Notothenoidei (~24 Mya). To gather further information about IgM producing cells, we sampled two common Antarctic species, the red-blooded *Trematomus bernacchii* (*Tb*) and the haemoglobinless icefish *Chionodraco hamatus* (*Ch*). Our studies were focused on two main lymphoid organs: the head-kidney (HK), thought a primary lymphoid organ for cells of the B-lineage, and the spleen (SPL), a secondary lymphoid tissue involved in effector B cells differentiation. The occurrence of secreted and membrane *Igμ* transcripts, IgM proteins and numerous cells containing IgM has been demonstrated in both tissues of *Tb* and *Ch*. The combined use of rabbit polyclonal antisera against homologous/heterologous IgM (in some instances specific for heavy or light chains) and B-specific transcription factors (with DNA binding domains highly conserved throughout evolution) proved that, at least in *Ch*, the HK houses both B-cell progenitors, B lymphocytes (especially abundant in the peripheral blood) and plasma cells, mainly accumulated around the arteries. These findings strongly suggest that the HK also behaves as secondary lymphoid tissue, in keeping with previous data from rainbow trout and other few teleost species.

The peculiar characteristics of the IgM molecule of many Antarctic teleosts (*e.g.*, three constant Ig*μ* domains in *Tb*, only two constant Ig*μ* domains in *Ch*, regarding the membrane antigen receptor) prompted us to start investigating in detail the organization of the B Cell Receptor (BCR) complex. Preliminary data will be presented about the two accessory molecules CD79A (Ig-α*)* and CD79B (Ig-β).