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Report of Meeting

# XIIth scientific meeting of the Italian Association of Developmental and Comparative Immunobiology (IADCI), 16 - 18 February 2011, Hotel S. Marco, Monteortone, Padua, Italy

Organizers: **L Ballarin, F Cima, V Matozzo, P Venier**

*Department of Biology, University of Padua, Padua, Italy*

***Plenary lecture***

**Live and let die: hemocytes in the crustacean war against infection**

**VJ Smith**
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Whilst it has long been known that the circulating hemocytes are major effectors in invertebrate immune systems much still remains to be learnt about these cells, as new work is starting to show. This presentation will give an overview of the hemocytes in decapod crustaceans and describe their various roles in defense against pathogens and opportunists. It will give consideration to their production, and likely development into mature functional units and discuss the range of bioactive proteins and factors that they produce. Included here will be antimicrobial peptides, opsonins and recognition factors, all of which may act outside the cell. Fluxes and changes in the size and composition of the hemocyte populations caused by certain extrinsic and intrinsic factors will be described and the question asked: is the hemocyte titer a reliable indicator of physiological or environmental stress? Finally, the talk will highlight the importance of hemocyte death in host defense and, crucially, how the nature of this death may be of greater significance than previously thought.

***Session 1. Chairman: N. Parrinello, University of Palermo, Palermo, Italy***
***Non-self recognition and immunocyte activation***

**Expressed sequences denoting receptors and other proteins progressively reveal the molecular basis of the mussel immunity**

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The current years testify the expansion of genomic/post-genomics studies, and the setting up of new software solutions for project/data management. In particular, massive DNA sequencing accomplished with Sanger dideoxy terminators or third-generation machines is rapidly increasing the sequence data publicly available for bivalve mollusks. As regards *Mytilus galloprovincialis*, we further investigated Mytibase (knowledge-base of 7,112 transcript sequences, assembled from 18,788 high-quality ESTs and partially devoid of functional annotation). Mytibase includes almost all the nine domains denoting innate pathogen recognition receptors (PPR). In addition to the AMP precursors, the abundance and molecular variety of mussel sequences with Complement C1q or C-type lectin or fibrinogen-like motifs indicate a large repertoire of recognition molecules.

Evolutionary related to the TNF-like domain, the C1q domain characterizes proteins active in complement activation, modulatory immune functions, apoptotic cell clearance, coagulation, embryonic development and tissue homeostasis. The 168 predicted C1q proteins of *M. galloprovincialis* show remarkable sequence diversity, lack of central collagen-like repeats and possess the globular C1q motif known to provide great flexibility in the ligand binding; hence, they are expected to act as secreted PRR. Recent data referred to the most abundant C1q EST cluster (MGC00284) indicated its wide expression and significant modulation in mussels infected with Gram positive or Gram negative bacteria (DCI 34(9):926-34, 2010). Among the heterogeneous group of mussel transcripts with carbohydrate binding domains, the most abundant and highly diverse in sequence are those denoting ($Ca^{2+}$-dependent) C-type lectins and fibrinogen-related proteins (FREP). They are regarded as candidate PRR since many C-type lectin proteins support pathogen-specific responses in *Caenorhabditis elegans* and species-specific FREP expansion relevant to immunity occurred in snails and mosquitoes.

Using a multiple search strategy, 1820 expressed Mytibase sequences have finally been selected to design probes and build a DNA-microarray of immuno-related sequences. The performance of the new Immunochip has been positively ascertained with hemocytes of *Vibro*-injected mussels.

**Antarctic teleost Toll-like receptors**

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The pattern recognition receptors (PRR) of the innate immune system sense the invariant molecular patterns present in microorganisms. Secreted PRRs comprise ficolins, collectins, and pentraxins, whereas transmembrane PRRs include the Toll-like receptor (TLR) family and the C-type lectins. TLRs are present in both the invertebrate and vertebrate lineages; in teleosts, orthologs of mammalian TLR6 and TLR10 are absent, however the TLR11 family includes additional members. All TLRs share the same molecular architecture consisting of an N-terminal ectodomain including various numbers of leucine-rich repeat motifs, followed by a membrane-proximal cysteine-rich region, a transmembrane domain and a cytoplasmic TIR domain.

The innate immune response of Antarctic teleosts seems to be more relevant than the adaptive response, and, in turn, TLR molecules could assume a more important role in triggering the immune system. In addition cold-adaptive modifications could have shaped the molecular structure of the TLRs in unpredictable manner.

A fragment of cDNA coding for TLR9 and TLR2 was PCR-amplified from *Trematomus bernacchii* and *Chionodraco hamatus* using primers designed on conserved regions across five publicly available teleost TLR9 and six TLR2 sequences. Full-length sequences were subsequently obtained in a Rapid Amplification of cDNA Ends (RACE). *T. bernacchii* and *C. hamatus* TLR9 sequences are respectively 1056 aa and 1054 aa long and share similarities in structure with previously identified teleost TLRs. Structural homology is conserved also for *T. bernacchii* (804 aa) and *C. hamatus* (802 aa) TLR2, both displaying 6 LRRs and an intracellular TIR domain.

All putative orthologs of TLR9 and TLR2 in the teleost fish were identified through Blastp searches against either NCBI or Ensembl databases. Multiple alignments of teleost TLR9s and TLR2s were obtained using different approaches (MUFFIT, MUSCLE, CODONALIGN) and sequence analyses at the amino acid as well as at the codon level were carried out to investigate the role of natural selection in the evolution of Antarctic fish TLRs.

Molecular models of the ectodomains of both the sequenced TLR2s were constructed using the mammalian structure of mouse TLR2 (3A7C) as template. The obtained models, validated using the PROCHECK programme were minimized with the GROMAX3.2 package. Molecular dynamic simulations were performed in water for 10 ns. The resulting models were compared to the mammalian structure to identify the differing regions.

**Phagocyte behavior during the colonial blastogenetic cycle in the compound ascidian *Botryllus schlosseri***

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Colonies of the ascidian *Botryllus schlosseri* experience a cyclical generation change (takeover) during which adult zooids stop their filtering activity, their tissue undergo cell death by apoptosis and are progressively resorbed. In the meantime, a new blastogenic generation reaches its functional maturity, opens siphons and starts filtering. During the blastogenetic cycle, the phagocytic differentiation pathway of the circulating immunocytes plays a key role: the presence of both hydrolytic enzymes and mediators of local inflammation supports the hypothesis that the phagocytic cell line maintains characteristics of a primordial system with high functional versatility. Phagocytes cyclically change their morphology and frequency. During the mid-cycle, hyaline amoebocytes are numerous in blood circulation, inside the tunic and free of moving on the surface of the tunic that internally covers the oral siphon, where they play an immunosurveillance role of the pharynx by recognizing and phagocytizing foreign particles, and, after exposure of colonies to bacterial spores, forming a transitory plug in the siphonal lumen by exocytosis of floccular and colloidal material rich of heparin, histamine and proteases. In the takeover, the frequency of hyaline amoebocytes falls abruptly since, by engulfing apoptotic cells, they change their shape becoming large and spherical macrophage-like cells. These scavenger phagocytes are massively recruited from the circulation to the dying tissues of old zooids, where they assure the clearance of senescent cells. This is fundamental for the progression of the takeover as during this phase, lasting 24-36 h, colonies do not feed and rely uniquely on the recycling of nutrients deriving from the digestion of senescent cells. The massive ingestion of effete cells causes, in turn, an increase in reactive oxygen metabolite production and nitric ion release leading to the death of phagocytes and subsequent clearance by other phagocytes, so that a “Russian doll effect” can be observed. Finally, large macrophage-like cells accumulate in the pharyngeal lacunae and are continuously eliminated through the peribranchial chamber and then the cloacal siphon with a discharging mechanism never previously described which continues in the first phases of the mid-cycle. The consistent disappearance of large phagocytes from the circulation is counterbalanced by a population of new, undifferentiated cells (hemoblasts) already beginning from the late takeover.

**Immune roles of a rhamnose-binding lectin in the compound ascidian *Botryllus schlosseri***

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The present communication describes the immune role played by a recently identified member of the rhamnose-binding lectin (RBL) family from the colonial ascidian *Botryllus schlosseri*. *B. schlosseri* RBL (BsRBL) can activate phagocytes through: i) induction of their directional movement towards the source of the molecule; ii) modification of cytoskeleton, required for shape changes; iii) stimulation of the respiratory burst, and consequent production of reactive oxygen species (ROS) with microbicidal activity, including superoxide anions and peroxides; and iv) increase in the ability to phagocytose foreign particles. RBL also induces the synthesis and release, by cytotoxic morula cells (MCs), of cytokines recognized by anti-IL1$\alpha$ and anti-TNF$\alpha$ antibodies. At high concentrations, BsRBL induces degranulation of MCs and the consequent release of the cytotoxic enzyme phenoloxidase into the medium. Results are consistent with the existence of cross-talk between *B. schlosseri* immunocytes (phagocytes and MCs). In addition, a three dimensional model for BsRBL is presented.

**Evolution of the genetic mechanism of self/non-self recognition in the protozoan ciliate *Euplotes***

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In the phylogenetic trees (mostly based on comparisons of SSU-rRNA gene sequences) of the nearly 100 morpho-species of the most cosmopolitan and ubiquitous ciliate, *Euplotes*, the marine complex of species *E. crassus-E. minuta-E. vannus* systematically appears as the latest, deeply divergent branch. In relation to the mating type systems that represent the genetic mechanism by which *Euplotes*, and ciliates in general, control the switching from the growth (asexual) stage to the mating (sexual) stage of the life cycle, this species complex has historically been credited by the evolution of multiple mating type systems characterized by: (i) a genetic control of the mating types provided by multiple alleles inherited at the single genetic locus *mat* and mutually regulated by relationships of hierarchical dominance (e. g. *mat-1>mat-2>mat-3* ad so forth), and (ii) the synthesis of mating type-factors (pheromones) represented by water-insoluble, membrane-bound proteins.

Working on *E. crassus*, we have first isolated and structurally characterized soluble, water-borne pheromones, thus demonstrating that (contrary to the historically held concept) this species and, most likely, the whole *E. crassus-E. minuta-E. vannus* species complex constitutively secrete their pheromones into the extracellular environment as it occurs in other, earlier branching (more ancient) *Euplotes* species. Then, based on the knowledge of pheromone amino acid sequences, it was possible to clone and structurally characterize a set of different pheromone coding genes. It was found that each cell type synthesizes one pheromone that is shared in common with other mutually mating compatible cell types, plus one (if *mat*-homozygous) or two (if *mat*-heterozygous) other pheromones which are cell type-specific. The implication that arises from these observations is that, in *Euplotes* species, the *mat*-locus underwent evolutionary duplication. While early branching (more ancient) *Euplotes* species (such as *E. raikovi* and *E. nobilii*) show this locus as single copy, *E. crassus* and its late branching related species would carry it replicated into two distinct copies. One copy would retain the ancestral state as multi-allelic locus deputed to control the synthesis of pheromones which are specific for each cell type, while the new copy would be manifested as mono-allelic locus deputed to control the synthesis of a pheromone which is shared in common by a group of different, but mutually interbreeding cell types. As such, this novel pheromone might function as a self-recognition signal not for a single cell type, but for a population of different and interbreeding cell types, and ensure that mating pairs are formed only between different cell types of the same population.

***In vitro* characterization of the cytokine *Drosophila* Helical factor**

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*Drosophila* Helical factor (Hf) is a protein discovered through the QT method, an algorithm specifically designed for finding helical cytokines. Since *in vivo* experiments suggested the involvement of Hf in *Drosophila melanogaster* immunity, we have proceeded with the characterization of Hf functions in the macrophage-like *Drosophila* embryonic hemocytes, SL2 cell line. qPCR results demonstrated that *Hf* gene is induced in the SL2 cell line, after either 6 or 24 h incubation with *Escherichia coli*-purified peptidoglycan. The silencing of *Hf* expression through RNAi resulted in the reduced capability of synthesizing antimicrobial peptides (AMP) after exposure to heat-inactivated *E. coli*. The effects of the recombinant peptide rHf

have also been tested in the SL2 cell line. rHf promotes the expression and triggers the release of Hf from the hemocytes, and stimulates the synthesis of the antimicrobial peptides (AMP) Defensin and Drosomycin, without any further immune stimulation. Consistent with the output of the QT method, which predicts Hf as a secreted protein, chromatin immune-precipitation experiments confirmed that Hf does not bind DNA, excluding that it acts as an immune-regulated transcription factor. Finally, rHf neither exerts chemotactic action nor triggers bacterial phagocytosis in SL2 cells.

Altogether, our data supports the prediction that Hf is an helical cytokine produced and secreted by the hemocytes and it is mainly involved in the regulation of the humoral component of the immune response of *D. melanogaster*.

***Session 2. Chairman: P. Luporini, University of Camerino, Camerino, Italy***
***Effector mechanisms of immune responses***

**The mast cell: comparative aspects**

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Mast cells (MCs) are paracrine cells, ubiquitous in connective tissue of the majority of vertebrates. They are best known for their involvement in hypersensitivity, allergic and anaphylactic reactions, but they are also implicated in angiogenesis, in inflammation as well as in natural and acquired immunity. The mast cells, together with the basophils, are the only cells producing histamine and expressing membrane receptors for IgE (FceRI). Although FceRI receptor represents a relatively recent acquisition, since IgE appeared with the mammals, either histamine either FceRI-like receptor are present in MCs of teleost fishes. Further analogies among vertebrate MCs are: mediator contents (heparin, proteases, serotonin, phosphatases), expression of membrane receptors (c-kit, TLR, CR3) and response to substances inducing MC degranulation (compound 48/80, chlorpromazine, capsaicin, substance P, bacterial/parasitic toxins). The neuroendocrine activity (i.e. secretion of GnRH) in birds and the production of antimicrobial compound in fish (piscidins, pleurocidins) are some unique roles the MCs are known to play.

Among invertebrates MC-like cells has been described in ascidians. They contain histamine and heparin, tryptase and are induced to degranulate by compound 48/80. In Arthropoda, some leucocytes show ultrastructural features of rodent MCs. A fascinating hypothesis suggests that the progenitor of MCs could have origin in an ancestral leukocyte showing phagocytic activity, in the context of a primitive local immunity. The MCs could have acquired new molecular strategies without losing many of the functions accumulated during million years of evolution. They should have preserved their defensive function against pathogens as predominant in non-mammalian vertebrates.

**The thymus in the tail regeneration of *Xenopus laevis* tadpoles**

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The tadpoles of the frog *Xenopus laevis* show significant regeneration capacity and are useful models to examine cellular and molecular mechanisms underlying the appendage regeneration. After amputation, most of the tail can be rebuilt during the larval life and this ability is gradually reduced as development proceeds towards metamorphosis.

Previous studies demonstrated different morpho-functional responses to tail amputation of different aged *X. laevis* tadpoles. Unlike stage (st) 50, an high percentage of st 55/56 larvae showed limited regenerative efficiency and malformed and shorter new tails were observed. The immune cells were found to take part in the response to tissue injury, in determining the inflammation degree and success of repair process.

In this work, the thymus from tail amputated *Xenopus* larvae (st 50 and st 55/56) was investigated by histochemical and immunohistochemical reactions. The examination of st 50 revealed changes of thymic architecture, compared to unoperated controls, characterized by an increased number of multicellular epithelial cysts, mucous and myoid cells, and cells, mainly located in the medulla, immunoreactive to anti-TNF-$\alpha$ antibody. A significant higher number in cortical apoptotic figures was only detected 1 day after tail cut. The thymic structural modifications were more marked, and observed throughout tail regeneration process, in most of the older tadpoles. The cellular responses included significant increase of apoptotic pictures and reduction of the medullary area where large epithelial cysts containing secretory material and cell debris were seen. At the end of regeneration process the organ size was found to be reduced of about 40 %. Compared to unoperated controls, a higher number of TNF-$\alpha$ immunoreactive cells was also observed.

These findings show that tail cut provokes a stimulation of the thymic function and induction of molecules critical for organ constitutive processes indicating a possible role of the lymphocyte component in control of *Xenopus* tail regenerative quality outcomes. The stage-dependent events occurring in regenerating tail microenvironment, i.e. degree of inflammatory response, may be related to thymic structural modifications.

**Immune response in *Carabus lefebvrei* Dejean 1826 (Coleoptera, Carabidae): circulating hemocytes, phagocytosis and plasma phenoloxidase activities at different developmental stages**

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*Carabus lefebvrei* Dejean 1826 (Coleoptera, Carabidae) is a helicophagous Italian endemic ground beetle that lives in central and south Apennines mountain forests. The pathogens and parasites can be the main causes of mortality for all life-cycle stages of carabid beetles. However, few morpho-functional data about immune system are available. In this study we have compared the cellular population in the hemolymph of *C. lefebvrei* adult, larvae and pupae by light and electron microscopy analysis and identified the hemocytes involved in phagocytosis after *in vivo* artificial nonself-challenges. Moreover, the plasma phenoloxidase (PO) activity were detected using a L-DOPA substrate and enzyme activity was expressed as absorbance units at 492 nm/µL of hemolymph. Total hemocyte counts revealed in pupae a higher number of circulating hemocytes compared to larvae and adults. Four morphotypes of circulating hemocytes were found: prohemocytes, granulocytes, oenocytoids and plasmatocytes. The plasmatocytes were always the main circulating hemocyte type and the pupae percentage was lower than adults and larvae ones whilst the granulocytes were higher in pupae than in adults and larvae. After in vivo artificial non-self challenge treatments, all *C. lefebvrei* stages showed a non-specific immune response involving phagocytosis performed by plasmatocytes. The comparison of hemolymph PO activity (measured as the increment of absorbance at 30 min with respect to 0 min readings) between larvae, pupae and adults revealed a significant difference among stages (Kruskal-Wallis rank sum test, p-value < 0.01). In particular PO in adults showed a significant higher activity compared to larvae (pairwise comparisons using Wilcoxon rank sum test with Bonferroni correction, $p < 0.05$) and an highly significant higher activity compared to pupae ($p<0.01$). No difference was recorded among larvae and pupae PO activities. The exarate pupal stage produced a mixture of terpenes, ketones, aldehydes, alcohols, esters and carboxylic acids as defensive secretion from abdominal glands. This glandular secretion has both a deterrent function against predators and a prophylaxis function against pathogens. From an ecological perspective, the chemical protection is an efficient barrier against pathogen, hence, the pupal stage invest more energetic resources in specific cellular defense responses rather than in PO activity.

**Gene expression profiles of the mussel Myticin C, an antimicrobial peptide displaying high sequence variability**

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The Mediterranean mussel *Mytilus galloprovincialis* commonly appear more resilient than other bivalves to the environmental variation and, in particular, to infective agents and disease onset: for this reason mussels should have a potent immune system able to fight against microscopic aggressors. Their hemolymph cells play a fundamental role in defining rapid and effective defense reactions. In response to bacterial antigens and viral-like polynucleotides, the haemocytes of *M. galloprovincialis* synthesize and release antimicrobial peptides (AMP), effector molecules able to execute the killing of microbial cells. The AMP gene expression may represent up to 25-40 % of whole transcriptome in the hemocytes of immuno-stimulated mussels and one of them, myticin C, showed remarkable transcript polymorphism and unique transcriptional profiles in individual mussels. Myticin C is expressed at constitutive levels in the mussel haemocytes and substantially induced after immuno-stimulation. The peptide has neither been isolated nor has its antimicrobial activity been specifically studied. Aiming to the functional characterization of such 'natural antibiotic' we have set up a real time PCR method allowing the accurate quantification of its expression levels, and an ISH system allowing the localization of myticin C transcripts in situ with riboprobes. Using these experimental approaches we investigated the expression of this effector molecule in the hemocytes of mussels injected with heat-killed bacteria (*Vibrio splendidus* and *Micrococcus lysodeikticus*) and Poly I:C at different time points (from 1 to 48 h post-treatment) in individual mussels.

Overall, the antigenic cocktail resulted to be a more potent inducer of the myticin C expression, with the maximum levels observed at 24 h post-treatment. The injection of individual *V. splendidus* or *M. lysodeikticus* produced different myticin C expression patterns, with a certain degree of inter-individual response variation. Preliminary ISH results referred to naïve mussels suggest that myticin C is expressed by specific haemocyte subpopulations.

**Antibacterial activity and immunomodulatory effects on a sea bream cell line of *Myrtus communis* and *Aloe arborescens* extracts**

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The emergence of antibiotic-resistant bacterial strains is a challenging problem that has led to an urgent global call for new antimicrobial drugs. Such compounds, potentially devoid of undesirable side-effects, have been sought in particular from natural resources.

We have been studying the pharmacological potential of leaf components of *M. communis* and *A.*

*arborescens*, deserving special interest to their antimicrobial activity for possible future applications in fish aquaculture.

Ethanolic extracts from the two plant species were essayed *in vitro* for their effects on *Listonella* (*Vibrio) anguillarum* and *Photobacterium damselae ssp. piscicida* strains. These Gram$^-$ bacteria are two major pathogens for the cultured gilthead seabream (*Sparus aurata*), being the causative agents of vibriosis and pasteurellosis, respectively.

Furthermore, the immunomodulatory effects of the plant-derived extracts on a LPS-stimulated *S. aurata* fibroblast cell line (SAF-1) were evaluated.

Obtained results have provided a promising new perspective for the use of medicinal plants to prevent or oppose bacterial diseases in fish.

***Chairman: E. Ottaviani, University of Modena and Reggio Emilia, Modena, Italy***

**Melanin production and innate immunity**

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In vertebrates and invertebrates, melanin biosynthesis is of prime importance in immune responses. Independently from their phylogenetic position, invertebrates can show either a humoral system, responsible for massive production of melanin coupled with a cellular response lesser important in pigment production, or only a cellular system, as in vertebrates, in which the entire melanin synthesis is concentrated in specific organelles, the melanosomes.

We have employed a variety of techniques to confirm and to discuss the new evidences that in animal, from Cnidaria up to men, the same nexus among melanin production/templation, redox status, cytoplasmic pH, acid phosphatase activity, presence of pro-inflammatory cytokines, adrenocorticotropin hormone (ACTH), and neutral endopeptidase-24.11 (NEP)-like activity overexpression occurs.

**Diversity and evolution of piscidin antimicrobial peptides**

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Endogenous antimicrobial peptides (AMPs) are widely distributed in nature and are considered ancestral elements in the evolution of innate immunity. A variety of AMPs has been described in all organisms, particularly in aquatic organisms such as teleost fish.

The piscidins are a family of linear amphipathic peptides initially identified in the hybrid of American bass (*Morone saxatilis* x *Morone chrysops*).

In order to increase knowledge about the role of leukocytes in the innate immune responses of *Dicentrarchus labrax*, we cloned, sequenced and characterized an antimicrobial peptide belonging to the family of piscidins from the head kidney leukocytes. The complete DNA sequence of piscidin 1 was obtained using degenerate primers designed from other piscidins; In situ hybridization experiments revealed that this antimicrobial peptide is expressed in the leukocytes from peripheral blood, peritoneal cavity and head kidney. Finally, the study of the family diversity, biological role in host protection and immunomodulation of all the known piscidins already sequenced in fishes, assumes a crucial importance for the health of fish in aquaculture and for potential biotechnological uses of these molecules.

**Isolation and cytotoxic activity of neurotoxin from the mucus of *Actinia equina* (Anthozoa, Cnidaria)**

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Cnidarians evolved chemical and biological defenses against predators, parasites and pathogens by the production of bioactive substances. Both anthozoans and medusozoans cnidocytes are known to produce cytolytic and neurotoxic molecules with antihistaminic activity or blocking cationic channels. Some cnidarian toxic peptides, listed in the pharmacopoeias, are now used as therapeutic agents.

A number of toxins with a molecular weight of near 20 kDa (equinotoxin I, II, III) have been characterized from *Actinia equine* (Anthozoa), the common beadlet red anemone living in the intertidal waters of the Mediterranean and European temperate Atlantic coasts.

This study aimed to isolate and characterize cytolytic molecules of low molecular weight for potential biotechnological applications. In extracts from mucus and isolated nematocysts, cytolytic activity was detected toward rabbit erythrocytes and human chronic myelogenous leukemia cells K562. Using high performance liquid chromatography followed by mass spectrometry we isolated a new lytic peptide of about 6 kDa from both the mucus and cnidocytes. The complete amino acid sequence of this peptide revealed that it is a 54 aa polypeptide with high sequence homology with sea anemone neurotoxin 1. These cytolytic molecules have also been studied in the animal body, fluid extracts and free cells of tentacles.

**Identification of antimicrobial peptides of beta-defensin type in lizard tissues**

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Defensins are a widely studied family of cationic antimicrobial peptides characterized by the presence of a conserved cysteine-rich motif and a large number of basic amino acid residues. These peptides have been broadly isolated from insects, animals, plants and mammals and they are critical to innate immunity and protection against infection. The high resistance to infections in lizards has prompted us to search for the presence of antimicrobial peptides in these vertebrates. The availability of the *Anolis carolinensis* genome and expressed sequence tag (EST) sequences (Broad Institute, Boston, USA) enabled us to identify 32 different defensin-like genes, using bioinformatic methods and 5'- and 3'-RACE analyses. These *Anolis* defensin genes, designated as AcBD 1 to 32, are clustered within 5 different genomic scaffolds suggesting that multiple defensin loci may be present in reptiles. The deduced peptides vary from 60 to 111 amino acid residues and almost all share the common features for vertebrate defensins, including small size, net cationic charge and six conserved cysteines in the mature peptide. Moreover, based on their cysteine organization, the identified *Anolis* defensin like peptides resemble beta-defensin family members of birds and mammals. Like in avian beta-defensins, one beta-defensin gene of *Anolis* contains two highly divergent, tandem copies of the six-cysteine motif at the C-terminus. The *Anolis* beta-defensin genes present, like in birds, a variable gene organization, ranging from two to four exons. Moreover, the expression of two of these genes in *Anolis* appears to be regulated by alternative splicing processes. The constitutive expression of 10 different beta-defensins in healthy *Anolis* was detected by semiquantitative RT-PCR in brain, testis, stomach, intestine, kidney, muscle, skin, lung, liver and tongue but the levels and patterns varied for individual defensin genes. Further studies on the antimicrobial activity of these peptides are under investigation.

***Session 3. Chairman: L. Abelli, Department of Biology and Evolution, University of Ferrara, Ferrara, Italy***
***Immunity and environment***

**Immune defenses and interactions with the environment**

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Animal species live in a condition of homeostasis with the microbiome present in their body and in the environment, constituted by a great number of microorganisms, many of which can be potential pathogens. A dynamic equilibrium exists between aggression strategies developed by microorganisms and defense strategies invented by animal eukaryotes, with the immune system that must guarantee full immune defense in every environmental condition. Employing as main investigated species the sea bass *Dicentrarchus labrax*, we studied the basic asset of the immune system of a Teleost species by defining the tissue distribution and the ontogenesis of lymphocytes, the involvement of lymphocytes during antigenic stimulation "in vivo" and "in vitro", the cloning of genes coding for immunoregulatory molecules, and the expression of these genes during various immune stimulations. Of great interest is the intestinal immune system of sea bass, very rich in T lymphocytes, and displaying a regional distribution of T cell subclasses. Current research focus on functional organization of gut-associated lymphoid tissue, and our hypothesis is that the intestinal immune system could be related to food habits. Work is in progress to study predatory and planctophagous fish species, searching for differences that could be present between fish species with different food habits.

**Immune system and environmental induced epigenetic and genetic mutations**

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Prokaryotes are able to distinguish between self and non-self DNA by means of the restriction and modification systems and a recently discovered antivirus immunity. Several features of these systems are functionally analogous to eukaryotic epigenetic systems involved both in the uptake of foreign DNA and in the generation of new genetic information. Recent analysis of *de novo* mutations in several complete genomes, demonstrated that the majority of new mutations are G:C→A:T transitions. In particular CpG sites mutate at a rate several times higher than other sites. This very biased spectrum of these mutations may be the result of two main environmental induced processes: deamination of methylated cytosines and ultraviolet light mutagenesis. These data suggest an important role of deaminases in the transformation of acquired epigenetic changes in stable DNA mutations. Mutagenic enzymes as AIDs, APOBECs, ADARs, X family polymerases and reverse transcriptases are involved not only in the generation of new point mutations but also in environmental induced DNA rearrangements, as mobilization of retroelements, virus immunity and micro-recombination. DNA and RNA methyltransferases and deaminases are able to editing the DNA and RNA in the epigenetic processes and have additional roles in the uptake of foreign DNA sequences, in the regulation of the RNAi, in the stress induced mobilization of the formatting

retroelements, in the cell differentiation mechanisms, and in the acquired immunity. With the discovery of molecular machinery that promotes directional genomic changes in response to environmental stresses, a rethinking of evolutionary processes is needed. As proposed in literature, we can recognize that genetic information processing is the fundamental driving force that provides hereditary changes in biological systems.

**Infection, immunity and the environment: connecting the dots using marine mammal transcriptomic signatures**

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Functional genomics analyses of an organism's transcriptome can be informative of the interaction of genetic, disease and environmental factors. Here we used a combination of microarrays and machine-learning analytical approaches to understand the impact of environmental infection and stress on marine organisms. In particular, we studied marine mammals because they are considered an ideal model for the assessment of immunological responses to pathogens and contaminants. In fact, as mammals that live their entire life (or most of it) in the sea, they act as integrators of the stressors present in the marine environment. Marine mammals may have the potential to predict contaminant effects on health, and to be an indicator of infectious disease that may impact humans who have contact with the marine ecosystem through residence, work, or recreation near the coast.

We tested the hypotheses that 1) individual wild dolphins could be assigned to their home regions and 2) individual sea lions could be assigned to a specific disease status category, using only their blood transcriptomic signatures as classifiers. The tools used were a dolphin peripheral blood leukocyte (PBL) cDNA microarray specifically designed for studies of immune function and stress reactions and a custom oligonucleotide microarray generated from cross-hybridization probing of a canine microarray. Microarray data of 151 wild dolphin PBL samples and 73 sea lion blood samples were analyzed using a machine-learning approach. Artificial neural networks (ANNs) were able to correctly classify dolphins according to their site of sampling and sea lions according to their diseased status. These results suggest that a combination of microarrays and machine-learning analytical approaches would significantly improve the knowledge about the marine environment/organism interactions.

**Insights into B cell heterogeneity in two Antarctic teleost species**

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Interest in fish immunity has risen recently due to the discovery of some peculiar features. In particular, many studies have focused on teleost immunoglobulins (Ig) and their adaptation to the environmental conditions. Ongoing research is studying the B lineage of two species living at sub-zero temperatures in Antarctic seas, the red-blooded *Trematomus bernacchii* (TB) and the hemoglobinless icefish *Chionodraco hamatus* (CH).

Transcripts for the secreted and membrane form (1089 and 864 bp, respectively) of TB *Igμ* were amplified by RT-PCR using specific primers. B cells were identified by immunohistochemistry (IHC) using mutually no cross-reactive polyclonal antisera against TB purified serum IgM heavy (μ, 76 kDa) and light (L, 25 kDa) chains, and a polyclonal antiserum against CH purified serum IgM. Proteins extracted from TB tissues and separated by reducing SDS-PAGE were immunoblotted using the antiserum to homologous Igμ.

Both secreted and membrane *Igμ* transcripts as well as 76- and 66-kDa proteins were detected in TB head-kidney (HK), thymus, spleen, blood cells, gills and intestine, at apparently varied expression levels. Quantitative IHC confirmed that $Ig^+$ cells were more numerous in lymphoid than mucosal organs, in all instances with a prevailing perivascular distribution, but also outlined some differences in size and relative density among cells expressing μ and L chains in the different tissues.

Quantitative IHC on available CH tissues also reported more $Ig^+$ cells in the HK and spleen than in the intestine, predominantly perivascular. Furthermore, cross-reacting sera anti-TB μ and L chains suggested some degree of B cell heterogeneity.

The occurrence along the intestine of TB and CH of reverse gradients (decreasing or increasing towards the anus, respectively) in the number of $Ig^+$ cells needs to be carefully interpreted in light of immune regulation and control of a huge parasitic load, qualitatively and quantitatively different at the gut levels. Indeed, evidence is accumulating in both species that liver and exocrine pancreas would provide to Ig transport toward the intestinal lumen as well as to local defence against parasite invasion (peritoneal side) and/or ascending infections.

Reported findings raise important questions about existence and function of other Ig isotypes (and subtypes) in Antarctic fish, that need to be addressed on a wider set of tissues using proper analytical tools.

**Immune challenge is reflected in the expression of a male sexual trait in the peacock blenny (*Salaria pavo*)**

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The information conveyed by male secondary sexual traits and the potential benefits to choosy females are at the core of sexual selection theory. Indicator models of sexual selection assume that sexual displays reflect the phenotypic/genetic quality of males and that females gain benefits from choosing high-quality males. In particular, when male traits signal the individual ability to resist parasite, females can gather both direct benefits in terms of avoidance of diseased mates and indirect benefits in terms of genes for the progeny. Accordingly, if sexual traits represent honest signals of male health status, immune-compromised males are expected to exhibit a reduced signals' expression. We addressed this prediction in the peacock blenny, *Salaria pavo*, by injecting males with lipopolysaccarides (LPS; dosage: 2 mg/kg), eliciting the immune system activation and leading to a remarkable oxidative stress in animals by an increased production of reactive oxygen species. We recorded changes in the expression of male secondary sexual traits (a pronounced head crest and a pair of anal glands), courtship behavior and ejaculate quality (sperm number, velocity and viability) between groups of males respectively exposed to the immune challenge or sham-injected. The immune treatment had a significant negative effect only on the yellow coloration of the head crest, both in terms of color extension and intensity. These results indicate that in the peacock blenny the head crest, a sexual signal whose coloring expression depends on antioxidants (i.e., carotenoids), may represent an honest advertisement of male quality assessable by females during mate choice. Indeed, the immune system activation trough LPS may result in depletion of bodily antioxidants, mobilized to contrast the oxidative stress at the expense of a carotenoid mediated sexual signals. Moreover we found that the proportional change in head crest color area of LPS treated males was positively related to the total area of head crest, i.e. males with less developed head crest are suffering the strongest LPS-induced reduction in head crest color area. In agreement with the predictions of the condition-dependent indicator model of sexual selection, more ornamented males are better able to cope with the simulated infection, suffering lower detrimental effects of the activation of the immune system.

***Chairman: G. Scapigliati, University of Tuscia, Viterbo, Italy***

**Immunoglobulins at the blood-brain barrier in *Trematomus bernacchii***

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Homeostatic regulation of central nervous system microenvironment is crucial for normal neuronal function in vertebrates. A key component in this regulatory process is the blood-brain barrier (BBB), a selective barrier formed by the endothelial cells that line cerebral microvessels regulating the transport of compounds from the blood to the brain's extracellular milieu. Tight junctions between endothelial cells are the most important structural elements of the BBB. Furthermore the choroid plexus epithelium and the arachnoid epithelium are barriers between the blood and the cerebrospinal fluid. Recent data revealed that the brain is neither isolated nor passive in its interactions with the immune system and the so-called neonatal Fc receptor (FcRn) seems to transport Ig molecules in particular conditions.

Due to the unique features of their vascular system, the Antarctic teleosts are very suitable model species to study the BBB and the Ig occurrence in the brain compartments. The morphology of the brain of Antarctic species resembles that of other teleosts but several important differences should be highlighted: regulatory areas of the brain, including the *saccus vasculosus* (probably equivalent to the mammalian choroid plexus) and the subependyma of the third ventricle are more developed. In some species the expanded ependymal lining forms ventricular sacs, not previously described in any other vertebrate.

The data we collected about an unexpected abundance of transcripts of Immunoglobulin heavy chain (*IgH*) in the brain of *Trematomus bernacchii,* prompted us to investigate the presence of the protein. An antiserum produced against Ig light chains was used to perform immunohistochemical analyses on serial sections of a paraffin embedded *T. bernacchii* brain. A clear staining was observed in the epithelial cells surrounding the microvessels, indicating the presence of a specific Fc receptor on their membranes. In addition a staining not assignable to the vascular system was observed at three different localizations: i) between the telencephalon and the tectum of the mesencephalon; ii) lining the *nucleus diffusus* of the inferior lobe, iii) and between the *saccus vasculosus* and the inferior lobe of the diencephalon.

The data presented here, obtained by preliminary experiments, convinced us that the Antarctic species are highly appealing as animal model for encephalic barriers studies.

**Effect of seawater pH and temperature variation on the hemocytes of the crab *Carcinus aestuarii***

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The impact of $CO_2$ increase on marine systems as well as the consequent ecosystem feedback is still largely unknown. The response of organisms to pH reduction represents one of the main steps in evaluating the impact of direct ocean storage of CO2 to overall functioning of ecosystem. Modified pH has additional impact on the immune system of invertebrates and hypercapnia impairs the ability of the crab *Callinectes sapidus* to remove culturable bacteria from its hemolymph.

In this study the effects of exposure were tested on the hemocytes of the crab *C. aestuarii* at two pH (7.0 and 6.5) and temperatures (10 °C and 18 °C) for three weeks and subsequent recovery at normal seawater pH (8.1) for one week. Firstly the reduction of pH induced high mortality in the exposed animals in particular at the higher temperature when no animal survived until the end of the recovery period. Looking at the total hemocytes counts (THC) a reduction of seawater pH induced a significant variation in the number of cells at both temperatures also during the recovery period. Looking at cell size we recorded a significant increment in the hemocytes dimensions (area, mean diameter, length and width) already after 24h of exposure to reduced pH at both temperatures; this increase in dimension is also present during the recovery period.

**Combined effects of temperature, salinity and pH on immune parameters of bivalve molluscs**

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Atmospheric levels of $CO_2$ are increasing owing to human activities and are responsible for progressive acidification of oceans, which may affect calcareous structures of organisms (bivalves in particular), and modify their physiological performance, survival and growth. In bivalve mollusks, hemocytes play an important role in immune responses against foreign materials. Effects of pH, temperature, and salinity - as foreseen in climate change scenarios - on immune parameters of the clam *Chamelea gallina* and the mussel *Mytilus galloprovincialis* were investigated for the first time. An experimental flow-through system was setup to test simultaneously different temperature (22, 28 °C) and pH (8.1, 7.7, 7.4) values in three experiments performed at 28, 34, 40 psu salinity. Total hemocyte count (THC), pinocytotic activity, and hemolymph lysozyme activity were measured in bivalves after 7 days exposure.

At 28 psu, THC was significantly affected by temperature and pH in both species, whereas lysozyme activity was significantly influenced by pH only. At 34 psu, a significant effect of pH on pinocytotic activity of both clams and mussels was observed. At 40 psu, THC was significantly affected by temperature in both species, and by pH in clams only. In addition, a significant effect of pH was detected on pinocytotic activity of clams and mussels.

Results obtained demonstrated that immunomarkers allowed to highlight stress conditions in bivalves, and indicated differing immunomodulation patterns, on the basis of the experimental conditions tested. Of the immune parameters measured, THC showed to be the most influenced by variations in environmental conditions. Interestingly, the same pattern of statistical significance of temperature and pH effects was observed in both species at the three salinities, with lower impact at 34 psu, that represents optimal salinity value for mussels and clams.

**Metal-induced antioxidant defense in the solitary ascidian *Ciona intestinalis***

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Antioxidant enzymes play important roles in antioxidant responses caused by metabolic process or pathogen invasion. In many organisms, it has been reported that antioxidant enzymes participate in their innate immune defense against immuno-stimulant challenges such as β-glucan and sulfated polysaccharide, LPS or viruses. Cu,Zn superoxide dismutase (SOD, EC 1.15.1.1) is one of these key enzymes, highly conserved from invertebrates to vertebrates, involved in scavenging superoxide radicals into molecular oxygen and hydrogen peroxide. However, little is known about the responses of antioxidant enzymes like Cu,Zn SOD in tunicates after exposure to environmental changes in heavy metal concentrations. The present research focuses on structural and functional studies of the Cu,Zn SOD gene in the solitary ascidian *Ciona intestinalis*.

The gene sequence has been identified in GenBank and the amino acid sequence of the codified protein has been compared with those deduced from orthologous genes in other metazoans, both vertebrates and invertebrates, to determine if the amino acids important for catalytic activity were conserved and whether the enzyme of *C. intestinalis* acquired special features, relatively to the primary sequence, during its evolution. The *in silico* analysis was extended to the promoter regions for the presence of regulatory sequences such as antioxidant response elements (ARE), metal response elements (MRE) and xenobiotic response elements (XRE). The available sequences were used for cladistic studies, providing some interesting inference on the molecular evolution of the *C. intestinalis* protein.

Using semi-quantitative RT-PCR technique, Cu,Zn SOD gene expression have been evaluated as a function of exposure to three different metals (Cd, Zn and Cu). For this purpose, specimens of *C. intestinalis* were divided into three experimental groups, which were exposed for 6, 24, 48, 72, 96 and 120 h at equimolar concentrations (10 μM) of single metal. Untreated specimens were used as controls. Cu,Zn SOD is mainly induced by Cu and Zn, metals that are components of the active site. Besides, it was provided an initial estimation on the

localization of expression by *in situ* hybridization. The results indicate that the cells most involved in the expression of the considered genes are the hemocytes.

**Characterization of selenium glutathione peroxidase in the Antarctic teleost *Gymnodraco acuticeps***

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GPX activity consists in the detoxification of hydrogen peroxide, and is one of the most important ubiquitous antioxidant enzymes involved in antioxidant homeostatic control. Moreover, GPXs are closely involved in the innate immune responses of animals, as evidenced by the rapid modulation of transcription during challenges with endotoxins, bacteria or viruses. During infection, the host often uses ROS to destroy pathogenic invaders via phagocytosis; however, excess ROS formed during the respiratory burst may also be harmful to the host. For this reason, the elimination of excess ROS via antioxidant enzymes, is crucial for maintaining cellular homeostasis in the host.

Despite numerous previous studies on GPX from aquatic animals, the gene structure and expression of teleost GPXs have not been comprehensively studied. In particular, very little is known about the GPX of Antarctic teleost. The Antarctic species have an interesting evolutionary history because they have developed some adaptations that allow them to survive and to breed in waters where the temperature, oxygen and salt concentration deviate significantly from the average recorded in the temperate waters. They represent paradigmatic cases for adaptation to different temperatures and salinities in their environment.

Specimens of *Gymnodraco acuticeps*, a teleost fish widely distributed in Antarctic Ocean, were sampled in the Ross Sea (Terra Nova Bay, 74°42'S, 164°7'E) during the XVII Italian Antarctic Expedition. cDNA sequence of Se-GPX has been obtained from hepatic tissue by a combination of RT-PCR, 3' and 5'RACE techniques. The obtained nucleotide sequence and the deduced amino acid sequence have been compared with those of homologous genes already available in Genbank and have been used for phylogenetic analyses. Preliminary results were also obtained regarding the regulation of gene expression.

***Chairman: P. Venier, University of Padua, Padua, Italy***

**Effect of zinc on immunological competence of the sea urchin *Paracentrotus lividus***

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The levels of trace metals into the marine environment can be affected by human industrial and mining activity, as well as agricultural and domestic wastes, the combustion of fossil fuels and also by natural factors such as erosion, volcanic and tectonic activities. Significantly higher concentrations have been detected in some coastal zones compared to those in oceanic regions. In the Mediterranean Sea the main trace metals found are cadmium, mercury, lead, tin, copper and zinc. Since zinc has been known as a heavy metal less toxic to human health unless the concentration is abnormally high, its release is loosely regulated allowing considerable emissions of zinc from anthropogenic sources. A number of studies on the impact of heavy metals on the invertebrate immune system have been carried out in mollusks, crustaceans and oligochaetes whilst, little information is known about echinoderms. In this framework we analyzed the effects of a high zinc concentration on several immunological parameters of the sea urchin *P. lividus* taking into account that a variety of studies demonstrated the effect of environmental perturbations on invertebrate immunological competence. In particular, by comparing control (untreated) and zinc treated sea urchins, we evaluated the effects of this metal on coelomocytes hemolytic, protease and lysozyme-like activities as well as antibacterial activity on *Vibrio alginolyticus.* In addition, we analyzed changes in coelomocytes composition and morphology. All the immunological parameters considered were significantly reduced by the addition of zinc after 24 h of treatment, except for the protease activity. In addition the number of red spherule cells, usually activated by stress conditions, increased significantly. The modifications in sea urchins immunological competence may give an early indication of disease susceptibility thus suggesting to consider the examined defense mechanisms as potential biological indicators of metal pollution.

**Immunomodulatory effects of low doses of hexavalent chromium [Cr(VI)] in *Mytilus galloprovincialis***

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Chromium (Cr) is a transition metal that exists in many different oxidation states in the environment, with Cr(VI) and Cr(III) being the most stable forms. Hexavalent Chromium Cr(VI) is an important contaminant considered a model oxidative toxicant that is released from both domestic and industrial effluents, and represents the predominant chemical form of the metal in aquatic ecosystems. On the other hand, Cr(III) is considered the biologically active form, representing an essential

microelement in mammals involved in regulation of metabolism. Chromium has been also shown to affect the immune function, with immunostimulatory or immunosuppressive processes on lymphocytes, macrophages, cytokine production, and to modulate serotoninergic signaling.

In this work, the immunomodulatory effects of Cr(VI) were investigated in *M. galloprovincialis in vitro* and *in vivo*. Short term *in vitro* exposure of mussel hemocytes to different concentrations of Cr(VI) (0.1-100 μM) induced significant changes in hemocyte lysosomal membrane stability-LMS, extracellular ROS and NO production, phagocytic activity and lysozyme release. Dose-dependent decrease in LMS and lysozyme release were observed, whereas increased ROS and NO production, and inhibition of phagocytosis were observed at lower concentrations.

In *in vivo* experiments, mussels were exposed to sublethal Cr(VI) concentrations (0.2-20 μM) for 4 days and both functional and molecular responses were evaluated. Metal exposure induced dose-dependent decreases in hemocyte LMS and soluble lysozyme activity, and increased NO production at lower concentrations. Significant increases in transcription of the serotonin receptor 5-HTR and of immune genes (Myticin B, MgC1q) were observed at 2 μM, whereas downregulation of lysozyme and Mytilin B were observed at the lowest concentration tested. On the other hand, expression of the antiapoptotic gene p53 was unaffected by metal exposure. Overall, the results demonstrate that low concentrations of Cr(VI) are not toxic to *Mytilus* hemocytes but can modulate the immune response both at the functional and molecular level.

**Stress effects of *Bacillus thuringiensis* on *Rhynchophorus ferrugineus* hemocytes**

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The heat shock proteins (HSPs) are rapidly synthesized in the cells after an environmental stress exposition. Various stress as such as cold, heat, heavy metal, water osmolarity, drying, organic and inorganic pollution, UV exposure, induce in the animal, the HSPs expression. These are grouped in distinct molecular family, according to molecular size. Most organisms have multiple genes that encode for members of this family. In particularly, the genes for the Hsp70s, are traditionally divided into two groups: the majority of organisms have different genes coding for members of this family. The first group of genes can be induced rapidly under stress, but return to a normal level of expression in non-stressful conditions. Previously we found that the entomopathogenic bacterium *B. thuringiensis* (Bt), registered against another family of Coleoptera, could be a potential pathogen of *R. ferrugineus* beetles. This Curculionidae is a quarantine pest that attacks the palm trees. To study the pathogen-host relationship, we used the model of the Bt-*R*. *ferrugineus* interaction. In particular, we focused on the Bt stress-induced infections. We also studied for the first time the interaction between Bt and *R*. *ferrugineus* hematocytes evaluating the expression of HSP70 in the supernatant of the hemocyte lysate (HLS) obtained from larvae fed with Bt. Moreover the modulation of Hsp70 in larvae of *R. ferrugineus* fed with artificial diet containing a sub-lethal dose of commercial Bt was examined.

This is the first time that the presence of Hsp70 has been recorded in *R. ferrugineus*. The western blot analysis, using a mouse monoclonal antibody anti-HSP70, showed that the HSP70 expression was modulated in the time (3 h, 6 h, 12 h, 19 h, 24 h) in the response to Bt treatment, highlighting that Bt is a stress factor for the *R. ferrugineus* larvae. The protein expression was increased approximately seven fold after 3 h from treatment and after 6 h it returning to control value. Previous data showed that Bt interacts negatively with hemocytes of *R. ferrugineus* whose numbers decreased drastically in the hemolymph and that Bt treatment causes growth inhibition. Further investigation is needed to understand the possible correlation between the reduction hemocytes and modulation dell'Hsp70. The mast cell: comparative aspects

**Hemocyte proliferation influences metallothionein and phytochelatin levels in *Ciona intestinalis***

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We studied, through semiquantitative PCR and *in-situ* hybridization, the activities of the newly identified metallothionein and phytochelatin synthase genes from the solitary ascidian *Ciona intestinalis* (Ci-MT and Ci-PCS, respectively) in response to 10 μM CdCl2. Metallothioneins (MTs) and phytochelatins (PCs) are involved in detoxification systems of many organisms. An appreciable number of data are available for metazoan, especially for vertebrate, but very few data are available for urochordates. As the latter occupy the peculiar phylogenetic position of invertebrate chordates, the research on MTs and PCs in tunicates assumes a particular significance. Cd strongly induced Ci-MT, with a maximum at 4 days. Ci-PCS showed maximum expression at the same time. This result is probably related to a cell proliferation event, rather than an effective Ci-PCS gene activation. The hypothesis is supported by the strong induction of Proliferating Cell Nuclear Antigen (PCNA) transcript after 4 days of treatment and the colocalization in the hemocytes of the different riboprobes used. In addition, in literature is reported an expression profile similar to that of CiMT for *C. intestinalis* mannose binding lectins (CiMBL). Collectively, our data and data from the literature support the conclusion that hemocyte proliferation occurs in tunicate immune responses.

***Session 4. Chairman: U. Oreste, Institute of Protein Biochemistry, CNR, Napoli, Italy***
***Immunity: biotechnological and application-oriented aspects***

**Bivalve mollusk notifiable diseases: legislation, monitoring and diagnostic procedures**

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In Italy, the farming of bivalves has become an important sector of the aquaculture industry with nearly 200.000 tons produced per year compared to the 70.000 tons/year from fish farming.

Over the last ten years, the European Community has published several regulations aiming at protecting bivalve production in each member state. These regulations foresee a variety of different actions including prevention and eradication of particularly aggressive diseases, constant surveillance to avoid the spread of new diseases, control of anomalous mortality, registration of farms and their categorization according their health status.

Listed diseases of mollusks, which are all caused by protozoans, are classified as either exotic (i.e. *Bonamia exitiosa* e *Perkinsus marinus* in pacific oysters) or non exotic (i.e *Marteilia refringens* in mussels and flat oyster, *Bonamia ostreae* exclusively in flat oysters)

The O.I.E. (Office International des Epizooties, now referred to a the World Organization for Animal Health), provides updated diagnostic procedures published in the *Manual of Diagnostic Tests for Aquatic Animals* (revised in 2010) containing many different diagnostic procedures including cytology, histology, and molecular assays. Due to the lack of circulating antibodies in bivalves, serological test are not available as diagnostic tools. Likewise, no suitable cell lines are available to perform cytotoxicity studies.

Recently, in addition to the protozoan diseases mentioned above, an important virosis has been affecting pacific oysters, mainly in France and Ireland, with heavy losses in production of up to 80-90 % being reported in some cases. An Oyster herpes virus-1 variant has been associated with the mortality observed although it is believed that it is not the only cause of the syndrome which is thought to be multifactorial (e.g. involvement of *Vibrio* species, classic herpes virus, environmental conditions, farming typologies, etc.).

The anomalous mortalities events, i.e. *Chamelea gallina* and *Callista chione* in the Veneto, are often of uncertain origin. Finally, the interaction between host and parasite is still the object of active study and has become an important challenge for research groups.

**Construction of new antigen delivery systems for innovative vaccine formulations**

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Two non-pathogenic scaffolds represented by the filamentous bacteriophage *fd* and the dihydrolipoyl acetyltransferase E2 protein of the *Bacillus stearothermophilus* pyruvate dehydrogenase (PDH) complex able to deliver antigenic determinants, were designed. Both of these systems offer the potential for a safe, inexpensive and efficacious delivery system to be used as vaccine vectors.

Based on a modification of the phage display technology a delivery vehicle in which antigenic determinants are inserted into the N-terminal region of the major pVIII coat protein of filamentous bacteriophage *fd*, was developed. This system has proven to elicit full-spectrum of antigen-specific immune responses. In order to improve its efficacy, it was engineered a new bacteriophage vector to display, at the N-terminus of the pIII protein, single chain antibody fragments (scFv) able to bind the dendritic cells-restricted surface molecule DEC-205. It was demonstrated by in vivo tumor protection assay a potent inhibition of tumor growth by targeting fd particles displaying tumor antigens to dendritic cells.

Another antigen delivery system based on the E2 component from the PDH complex and capable of displaying large intact proteins on the surface of an icosahedral lattice which assembles into 24nm virus like particles (VLPs), was also developed. In particular E2 self assembles into trimers which, in turn, aggregate to generate a 60-mer scaffold displaying up to 60 copies of a foreign antigen on the surface of the particle. Thus E2 may be structurally advantageous for displaying gp160-HIV-1 trimeric antigenic proteins. In this context it was observed the induction of high titers of anti-HIV-1 neutralizing antibodies by immunizations with HIV antigens displayed on E2. In addition E2 immunization did not induce IFN-$\gamma$ production by CD4+ T cells and, for this low pro-inflammatory profile, epitopes from $\beta$-Amyloid were displayed on E2 and the immune responses were studied.

**3D Modeling of pro-inflammatory molecules in selected Teleost fish species**

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The inflammatory response is the reaction of all Metazoan organisms to pathogen invasion that initiates when pathogen-derived molecules are recognized by specific pattern recognition receptors, expressed mainly on cells of the innate immune system. The successive expression of pro-inflammatory cytokines and chemokines limits pathogen spread, and attracts and activates immune cells to help in the elimination of the invaders. In this study we focused on the analyses of the 3D structures of three pro-inflammatory molecules (interleukin-1$\beta$, tumor necrosis factor-$\alpha$, interleukin-8) from selected Teleost fish species (*Oncorhynchus mykiss*, *Dicentrarchus labrax*, *Chionodraco hamatus*) generated using as template

models those of experimental mammalian homologous proteins. These structures have been discussed with the aim to investigate the differences between them and mammalian counterparts and, moreover, to verify the presence of the actually known structural requirements for their biological activities. Our data demonstrated that 3D modeling of pro-inflammatory cytokines is a potent strategy that helps to show evolutionary differences in the structure of these proteins. Moreover, the differences should be taken into account when comparing the pleiotropic activities of these molecules during evolution especially for the consequences in the binding with their specific cell receptors and should advise against the use of recombinant human or murine proteins and human or murine specific antibodies when studying biological activities in Teleost fish. Finally, the 3D structures give good indication in the choice of the epitopes of these molecules useful for the production of fish monoclonal antibodies and for the designing of synthetic peptides for the binding to the specific cytokine cell receptors.

**Computational prediction and experimental analysis of the secondary structure of the primary transcript encoding the immunoglobulin heavy chain of the Antarctic teleost *Chionodraco hamatus***

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The primary mRNA splicing for the membrane form was found to be atypical in the majority of the Antarctic species, because it excluded two entire constant exons, but included additional 39-nucleotide exons. Genomic DNA analysis, revealed that each 39-nucleotide exon fell within a long sequence that was the reverse complement of an upstream region.

In the present work we analyze the structure of a synthetic RNA corresponding to the pre-mRNA encoding the membrane-bound immunoglobulin heavy chain of the Antarctic Notothenioid teleost species *Chionodraco hamatus*.

RNA was synthesized from a recombinant 4567nt-DNA template of the Antarctic teleost *Chionodraco hamatus* using the RiboMAX Large Scale RNA Production System (Promega). The DNA template was linearized by digestion with *Hind* III prior to *in vitro* transcription. The transcription reaction was carried out under the control of the T7 RNA polymerase promoter.

Computational analyses of the synthesized RNA from the *C. hamatus* species were performed at different temperatures by using mfold web server, and resulted in different pairing of the two antiparallel regions. The conformation obtained at 0 °C involved 3835 Kcal/mol. To experimentally verify the reliability of the predicted structures we performed atomic force microscopy experiments. Atomic force microscopy of the synthesized RNA, unfolded and relapsed at 0 °C was carried out on dried samples with a Nanoscope IIIa instrument. Topographic images showed double stranded regions characterized by a rod-like shape with a height of about 2 nm with globular features at the two ends of the rod corresponding to the more complex base pairing of the 5' and 3' RNA termini and the RNA loop. Agreement between the predicted and the observed RNA structure confirms the validity of the computational model.