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Report of Meeting

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Organizers: **A Vallesi, C Alimenti, P Luporini**

Department of Environmental and Natural Sciences, University of Camerino, Camerino, Italy

Plenary lecture

Genomics, immune studies and diseases in Bivalve Aquaculture

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Bivalve diseases are one of the critical bottle necks causing important and recurrent losses in bivalve culture. In some cases, the presence of infectious pathogens has caused the complete destruction of bivalve culture as it happened to the flat oyster (*Ostreaedulis*) in Europe.

Research on bivalve diseases has relied mainly on histological techniques and has been mostly focused on pathogen morphology and ultrastructure, effect of external factors on pathogens or infectivity, and on the development of immune and molecular diagnostic techniques.

Although critical advances have been reported in the last years on bivalve pathology, increased efforts should be placed on the application of "omics and physiology" to explain key physiological/immunological processes. Transcriptomic analysis in parallel to detailed functional studies on gene expression and cell biology in *in vitro* and *in vivo* experimental models are needed. Another important aspect is the identification of "resistance traits" and a deeper understanding of the processes which contribute to bivalve welfare in culture. Also the definition of "abnormal mortalities" is critical for managing and legislating bivalve aquaculture. This clearly "opens the door" toward directed manipulation to increase and improve all relevant processes in modern intensive aquaculture systems.

A future exciting research is in front of us. Now, more than ever, the need to develop multidisciplinary international research, involving research groups and growers organizations to work on shellfish pathology, is more than evident.

Session 1. Chairman: E Ottaviani, University of Modena and Reggio Emilia, Modena, Italy
New insights in immune system components

Multiple faces of ascidian host defense

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Cell migration, phagocytosis, encapsulation, activation of complement and proPO systems, cytotoxicity, tissue injury and wound repair, matrix production, endothelial and epidermis activity are components of the *Ciona intestinalis* inflammatory responses. Hemocytes are primarily responsible of the inflammatory activity. In several paper we reported that cytophilic humoral molecules with functional similarity to vertebrate pro-inflammatory factors can be modulated by LPS challenge suggesting their involvement in defense. Cellular responses include enhanced expression of: collagen-like molecules, galectins with opsonic properties, mannose binding (MBL)-like collectin and C3-like factor, *Ci*TNFα, a component of the CAP superfamily, proPO activation. In addition, for the first time we showed that *Ci*TNFα and galectins are constitutively expressed in the adult and larvae, while cytotoxic hemocytes exert a phospholipase-dependent activity. Since hemocyte proliferation has been claimed in the inflamed tissues as indicated by a massive infiltration, recently we examined the proliferative capability of the ascidian circulating hemocytes. The proliferating cell nuclear antigen (PCNA) gene expression in unseparated and separated hemocyte populations was examined. Taking advantage of the *C. intestinalis* whole genome sequencing, an *in silico* search for a PCNA-like sequence (NCBI) was performed. The PCNA putative DNA binding domain was cloned from hemocytes (*Ci*PCNA), the RNA probe was synthesized, *in situ* hybridization (ISH) assay was

carried out, and gene expression in short-time primary hemocyte was evaluated by semiquantitative Real-time PCR. Results revealed that *Ci*PCNA gene expression increased after 24h culture in native conditions, and in the presence of a mitogen such as VEGF. BrdU labeling disclosed incorporation in granular amebocytes with small granules and hyaline amebocytes, while the highest expression characterized lymphocytes-like cells. In conclusion the ascidian inflammatory response appears to be a well preserved process in chordate evolution.

Morphological and functional observations on the circulating cells of the freshwater snail *Pomacea canaliculata*

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The gastropod mollusc *Pomacea canaliculata* is a freshwater snail used as a model for different ecological studies. Recently, the function of this snail as intermediate host for nematode- and trematode-driven human pathologies, made *P. canaliculata* the subject of intense parasitological investigations. At present, the morphology of *P. canaliculata* circulating cells has been described only by means of TEM observations on clumping activity, which evidenced the presence of granular and agranular cells.

In the present study we analyzed the morphology and phagocytic activity of the circulating cells in normal conditions. Morphological and cytochemical stainings showed different morphological figures: a) small cells with high nuclear/cytoplasmic ratio, b) cells with circular nucleus and uniform, smooth cytoplasm, c) cells with irregular nucleus and vacuolated cytoplasm and d) cells with eccentric and polymorphic nucleus and granular cytoplasm.

Histochemical and histoenzymatic stainings revealed specific positivity for each cell morphology. The vacuolated cells were PAS-positive in the perinuclear cytoplasm and neutral red-negative and the acid phosphatase activity assay showed the presence of positive vesicles. In the cells with granular cytoplasm PAS-positivity was limited to the cytosol, while the granular structures were neutral red-positive.

Immunocytochemical experiments indicated the presence of ACTH-like material in all the circulating cells with the exception of those with circular nucleus and uniform cytoplasm.

With regard to the functional assay, we have observed phagocytic activity expecially in the vacuolated cells towards both heat-inactivated *Escherichia coli* and *Staphylococcus aureus*.

Summarizing, the circulating cells of *P. canaliculata* present some features observed in molluscan immunocytes, such as phagocytic activity and presence of ACTH-like material. Further studies will help in elucidating whether the different cell morphologies and stainability correspond to specific activities or different maturation stages.

Age related properties of the Adriatic clam *Chamelea gallina* (L. 1758) hemocytes

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In marine bivalve molluscs, the phagocytosis represents the main immune mechanism and the circulating hemocytes are able to recognize, engulf and destroy the foreign particles.

The hemocytes possess chemotactic ability to migrate towards foreign particles and include them inside phagosomes. This ability is strictly depending on morphological activation through the projections of membrane ruffles or pseudopodia. On the other hand, the respiratory burst represents aspecific mechanism involved in the destruction of foreign particles. The present research was aimed to analyse the phagocytic activity in the clam *Chamelea gallina* hemocyte, monitoring both their morphological spreading by cell circularity parameter and the production of nitric oxide (NO) by micromethod assay and flow cytometry using a specific fluorescent probe. The parameters were monitored in different groups in order to verify the possible influence of the clam size on phagocytic response. A preliminary analysis was conducted by light microscopy to study the hemocytes morphology and the differential hemocyte count on the basis of their morphotype. This approach revealed the existence of granular and agranular hemocytes with a prevalence of agranular type. However, a significant increase of granular cell type was observed in the larger clams (>30 mm length). In such a group, a better phagocytic response was also detected both in terms of morphological spreading and nitric oxide generation. Then, the phagocytosis assay was also applied on each hemolymph type purified by Percoll method, revealing a better response in granular hemocytes. Thus, the more intense phagocytic response observed in the hemolymph of larger clams seems to be ascribed to the greater concentration of granular hemocytes. A such variation in hemolymph morpho-functional characteristics appeared not dependent on environmental factors or on the presence of pathogens monitored by histology, but rather on clam aging. Finally, in the interpretation of our results we have also to consider the potential role of hematopoietic processes in the life-long hemolymph maturation and, in such a way, further studies have to be directed to investigate the origin, life cycle and life span of the different hemocyte types.

Molecular characterization of macrophage migration inhibitory factor (MIF) from mussel, *Mytilus galloprovincialis*

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Macrophage migration inhibitory factor (MIF) is a highly conserved cytokine which exerts wide-ranged activities. It is a central mediator of innate immunity and has been shown to correlate with regulation of macrophage functions, lymphocyte immunity and a number of inflammatory diseases. Its neuroendocrine role as mediator to increase host responses to microbial endotoxins suggested that MIF is at the crossroads of the endocrine and immune systems. MIF homologues have been detected in a wide range of animal species suggesting such molecule has been conserved.

In this study, three *MIF*-related nucleotide sequences were identified from a *M. galloprovincialis* EST library. Structure analysis of consensus sequence, closely related to gastropod *MIFs*, revealed presence of an ORF coding for 115 aa, with a 5' UTR of 32 nt and the 3' UTR of 349 nt. As for other MIFs, *M. galloprovincialis* ORF does not include signal or C-terminus extension.

Among various dissected tissues, mussel *MIF* was constitutively expressed principally in hemocytes and in mantle.

Polymorphism analysis showed several *MIF* mRNA variants in mussels from Palavas (France) and from Palermo (Italy), some of them being translated in different aa sequences. 11 peptide variants in the Palavas mussel and 14 peptide variants in the Palermo specimens have been found. Only 7 of the 18 different aa sequences were common to both mussel populations. Nevertheless, residues involved in thiol-protein oxidoreductase activity and in a tautomerase isomerase activity were conserved in all the 18 *M. galloproviancialis* MIF (*Mg-MIF*) variants.

Mg-MIF gene possesses the same exon-intron organization as in human and mouse structure, except for one α-helix between aa 68 - 76, which is 7 aa shorten in *Mg*-MIF than in human MIF. Among the mollusks, *Mg*-MIF appeared to be closely related to *P. fucata* and *Haliotis*, but not to *C. farreri* and to gastropod *B. glabrata*.

MIF gene expression was quantified by Real-Time PCR System following *in vivo* challenges of the mussels. Results indicated a different mechanism of elimination of yeast, bacteria and fungi. Expression of *MIF* was significantly up regulated in hemocytes after 1 h of stimulation and returned to background 9 - 48 h following the challenge.

Identification and modulation of an Allograft Inflammatory Factor-1 (AIF-1) homolog in the medicinal leech, *Hirudo medicinalis*

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Allograft inflammatory factor (AIF-1) is a 17 kDa cytokine-inducible calcium-binding protein, produced by activated macrophages during chronic transplant rejection and in inflammatory reactions. In vertebrates, AIF-1 plays a significant role not only in immune defense reactions, but also in responses to inflammatory stimuli. In central nervous system (CNS), AIF-1 is a sensitive marker associated to activated microglia and is upregulated following neuronal death or brain lesions. AIF-1-like factors have been described in several metazoan groups and share a well conserved amino acid primary structure throughout evolution suggesting a common, functional role. The analysis of an EST library from medicinal leech CNS, revealed the presence of a gene, named HmAif-1, showing a high homology with vertebrate AIF-1. Immunohistochemistry and expression pattern studies were performed to determine the localization and the modulation of this gene in leech. The presence of HmAif-1 in naïve and experimentally challenged tissues has been established using anti-human Aif-1 polyclonal antibodies. Results showed that HmAif-1 is constitutively present in spread, CD68+ macrophage-like cells; a few days after experimental wounding of the body wall, the amount of these immunopositive cells increases at the lesion site. In CNS, HmAif-1 is constitutively present in some microglial cells and, during nerve repair process, it augments at the cut end of injured nerve fibers. Quantitative RT-PCR analyses on cultured nerve chains showed a weak modulation of the gene in the days following experimental injury. Interestingly, the HmAif-1 gene is strongly upregulated a few hours after bacterial challenge. Also if the functional role of HmAif-1 has to be elucidated, these data suggest that in leech this factor is involved in immune response and in inflammation events like its vertebrate counterparts.

Session 2. Chairman: R Valvassori, University of Insubria, Varese, Italy
New insights in immune system components

Molecular studies on phenoloxidases of compound ascidians

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Phenoloxidases (POs) constitute a family of copper-containing enzymes with orthodiphenoloxidase (catecholase) activity widely distributed among invertebrates. They exert a pivotal role in immune defences as they can induce cytotoxicity through the conversion of phenols to

quinones and the production of reactive oxygen species. In ascidians, PO activity has been described and studied in both solitary and colonial species and the enzyme is involved in inflammatory and cytotoxic reactions against foreign cells or molecules as well as in the formation of the cytotoxic foci along the contacting edges of genetically incompatible colonies which characterises the nonfusion reaction of botryllids. Expressed genes for two putative POs (CiPO1 and CiPO2) have been identified in *C. intestinalis* (Immesberger and Burmester, 2004).

In the present study, we determined the cDNA sequences of the POs from two colonial ascidians: *Botryllus schlosseri* from Mediterranean (Adriatic) Sea and *Polyandrocarpa misakiensis* from Japan. Multiple sequence alignments clearly evidenced the similarity between ascidian PO and crustacean proPOs whereas the analysis of the three-dimensional structure of compound ascidian POs reveal high similarity with arthropod haemocyanins which share common precursors with proPOs. Ascidian POs and arthropod proPOs grouped in the same cluster well separated from mollusc tyrosinases, and share the full conservation of the six histidines at the two copper-binding sites as well as of other motifs, also found in arthropod haemocyanins, involved in the regulation of enzyme activity. Cytoenzymatic studies and *in situ* hybridisation (ISH) indicated that the genes are transcribed inside morula cells (MCs), a characteristic haemocyte type in ascidians, at the beginning of their differentiation. Sequence analysis allowed a better understanding of previous biochemical data and suggest some hypothesis for the regulation of enzyme activity.

Toll-like receptors: an evolutionary approach

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Toll-like receptors (TLR) are key molecules of the innate immune response, shared by vertebrate and invertebrate phyla. The basic Toll/Toll-like molecular structure consists of two well defined portions with different funtional roles, connected by a segment spanning the membrane. The ectodomain, consisting of a series of leucine-rich repeats (LRR), is able to recognize conserved Pathogen-Associated Molecular Patterns (PAMPs); the cytoplasmic region includes a Toll Interleukin-1 receptor (TIR) domain. It is devoted to transduce the ligand-binding signal to the nucleus where transcription of immune effector molecules genes is triggered.

Several mechanisms such as genome duplication, gene expansion, retrotrascription, and alternative splicing, contributed to shape the TLR repertoire in the different phyla. A comparative analysis of the TLR structure could help identify the critical features of the molecular structure.

Porifera represents the most ancient metazoan phylum showing nucleotide sequences reminiscent of TLR. In Cnidaria only one out of many TIR containing receptors shows several LRRs in its ectodomain, arranged in a specific manner, the so called “protostome-type”. A larger number of LRRs has been found in a *Caenorhabditis elegans* TLR sequence and one anellid TLR has been shown to be unique since does not share the “protostome-type” features, and presents a structure similar to mammalian TLRs. A survey of the genome of the purple sea urchin *Strongylocentrotus purpuratus*, a member of the phylum Echinodermata, has revealed the presence of 222 Toll-like receptor gene models. A great number of TLR sequences (36) has been found also in the cephalochordate *Branchiostoma floridae*, while only two have been identified in *Ciona intestinalis* genome.

So far, at least 23 vertebrate TLRs have been identified, based on amino acid similarity, genomic structure, and ligand properties. They can be grouped into six major families. A unique feature of teleost TLRs is the presence, in addition to TLR5, of a soluble TLR5 molecule (TLR5S), which lacks the transmenbrane and TIR domains in *Oncorhynchus mikiss* and *Takifugu rubripes* genomes.

By reviewing the presence of TLRs in different species it appears clear that evolution used TLR molecules for different functions, ranging from immune response to developmental signaling and cell adhesion. Whether the TLR cooptation in insect and vertebrate immunity represents a convergent evolution is still an unsolved question.

Preliminary studies on the complement system in the compound ascidian *Botryllus schlosseri*

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The complement system represents an important humoral component of the mammalian immune system. Complement components can be subdivided in 5 gene families: C3/C4/C5, Bf/C2, MASP/C1r-s, C6/C7/C8A/C8B/C9 and Factor I.

Until 1884, it was generally believed that the complement system was an unique feature of vertebrates since all attempts to identify complement components in invertebrates failed. In recent years, the genomic approach revealed the presence of complement orthologue genes in invertebrate deuterostomes, mainly in sea urchins and tunicates (ascidians). Conversely, no complement genes were found in the genome of protostomes such as *Drosophila melanogaster* and *Caenorhabditis elegans*, suggesting that the complement system was established in the deuterostome lineage.

Genome analyses carried out in the solitary ascidian *Ciona intestinalis* revealed that most complement gene families are present in urochordates.

We recently carried out the assembling of EST collections from the colonial ascidian *B. schlosseri*, obtained in our and other laboratories: we found multiple transcripts showing high similarity with vertebrate complement components such as C3, MASP, MBL and C6. Preliminary *in silico* studies revealed close relationships between *Botryllus* C3, MASP and MBL and orthologues from other chordates. In particular, C6 seems related with the

C6 proteins of the solitary ascidians *C. intestinalis* and *Halocynthia roretzi* and share with them the absence of the FIM domain which is responsible for the interaction with the other complement molecules in vertebrates.

Future studies will be devoted to the analysis of the expression of genes for complement components of *B. schlosseri*.

A CD83-like molecule in sea bass (*Dicentrarchus labrax*) related to immune responses against viral pathogens

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The CD83 cell surface marker is an important and intriguing component of immune system. It is considered the best marker for mature human dendritic cells, but it also plays an important role as a regulator of peripheral B-cell function, homeostasis and for thymic development of T cells.

A CD83-like molecule was identified in sea bass (*Dicentrarchus labrax*) by EST sequencing of a thymus cDNA library; the comparison of sea bass CD83 sequence with its homologs in other fish species and mammals shows some differences, with two cysteine residues conserved from fish to mammals and a high variability both in the total number of cysteines and in mature CD83 sequence peptide length.

Basal transcripts levels of CD83 mRNA are highest in liver, followed by thymus.

The *in vitro* treatment of head kidney leukocytes with LPS resulted in a down-regulation on CD83 mRNA leves both after 4h and 24h, whereas with poly I:C an up-regulation after 4h followed by a down-regulation at 24h was observed.

An *in vivo* infection of sea bass juveniles with betanodavirus induced an increase of CD83 expression on head kidney leukocytes both after 6h and 24h and a decrease after 72h. On the other hand, an *in vivo* infection with *P. damselae* bacteria induced a decrease of CD83 transcipt levels after 6h and 24h and an increase after 72h.

These findings suggest that sea bass CD83 expression could be modulated by viral and bacterial immune response.

Complement System of Antarctic teleosts: sequence analysis and molecular structure of *Trematomus bernacchii* C3*

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The complement system (CS) is an ancient defense mechanism, which in higher vertebrates involves more than 30 secreted or membrane-bound proteins interacting each other when the system is activated. This system plays important roles in the immune response of vertebrate species, such as pathogen cell surface recognition, promotion of inflammation and coordination of the adaptive immune response. It includes three different activation pathways (classical, alternative, and lectin), converging towards the activation of C3, the third component of the CS. C3 activation results in the production of two proteolytic fragments: a small fragment, C3a, that mediates many immunological activities, and a large fragment, C3b, which undergoes a dramatic conformational change, culminating in the exposure of the buried thioester group, that, in turn, allows C3b interaction with the invading bacterial matrix.

The CS of teleost fish has been investigated in many species. In contrast to mammals, it functions also at low temperature and seric titers of several components are drastically higher. Moreover, teleost C3 is present in several isoforms that have been suggested to bind complement-activating surfaces with different competences.

C3 genes have been sequenced, so far, in 14 teleost species, belonging to 8 different orders. Our aim is to extend the C3 literature to an Antarctic species, *Trematomus bernacchii* (Perciformes), which has been selected as model species to study the cold-adaptation of the immune system.

T. bernacchii liver cDNA has been amplified by reverse transcriptase-polymerase chain reaction using oligonucleotides corresponding to highly conserved nucleotide sequences of teleost C3 available in databases. This approach allowed the isolation of two C3-like clones, TbC3-1 and TbC3-2, whose sequencing completion has been performed by 3' and 5' RACE procedure.

Molecular models of TbC3-1 and -2 have been built using as a template the crystallographic structure 2A73 of human C3. In parallel, a model of the C3 molecule of the temperate species *Paralicthys olivaceus* has been constructed using the same template. Molecular Dynamic simulations of TbC3-1 and *P. olivaceus* C3 models have been performed for 15 nsec and the flexibility of the C-alpha atoms of the backbone have been compared at the equilibrium. Significant differences in the RMSF plots suggest higher flexibility of the Antarctic molecule possibly due to the cold-adaptation evolution process.

Special session 1. Chairman: L Ballarin, University of Padua, Padua, Italy
IADCI Award

Gene expression profiles in mussels from the Venice lagoon

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Global gene expression profiles in organisms selected to represent a given ecosystem currently support ecotoxicological investigations and create a conceptual bridge between the early organism responses and late population effects (Steinberg *et al*., 2008). Bivalve molluscs are constantly exposed

to a variety of environmental changes. As regards *Mytilus galloprovincialis*, tens of thousands Expressed Sequence Tags (ESTs) are currently available and a number of DNA microarrays have been developed.

In this study, we propose the analysis of hybridization data based on the whole Mytibase transcript collection (Venier *et al.*, 2009) and referred to digestive gland and haemolymph of mussels sampled from selected Venice lagoon sites in different years.

Essentially, we designed an Agilent 15K oligo-array representing all the 7112 Mytibase EST clusters (3' UTR 60-mer probes, 63 % the fraction of annotated transcripts at the moment). To assess the performance of the 15K oligo-platform we calculated precision, specificity and sensitivity on the whole set of one-color hybridization data, according to Moreau *et al*. 2003. We used the Principal Component Analysis and t-test to analyze the results and obtain lists of differentially expressed genes. In this way, we identified transcripts typical of digestive gland or haemolymph. Annual- and site-related expression trends were also investigated. Spike-in controls allowed us a precise assessment of the expression values for relevant families of immune-related transcripts.

Effects of fluoxetine on immune parameters of the clam *Ruditapes philippinarum*

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Among emerging environmental contaminants, pharmaceuticals and personal care products (PPCPs) are a large group of substances used either by human for personal health and cosmetic reasons or by agribusiness to enhance growth or health of livestock. PPCPs are produced in large quantities and comprise numerous chemicals, including prescriptible drugs, veterinary drugs, diagnostic agents, fragrances, lotions, and cosmetics. As a consequence, main sources of PPCPs in the environment (aquatic in particular) are human activities, residues from both pharmaceutical manufacturing and hospitals, illicit drug use, veterinary drug use and agribusiness.

Among pharmaceuticals, fluoxetine (a selective serotonin reuptake inhibitor) is an antidepressant commonly used for treating depression and other psychological disorders. Despite its wide use, information is lacking about the effects of fluoxetine on non-target species. To fill this gap, in the present study the effects of fluoxetine on some important immune parameters of the clam *Ruditapes philippinarum* were evaluated for the first time. Clams (25 per concentration) were exposed for 7 days to differing fluoxetine concentrations (0, 1, 5, 25, 125, 625 µg/L), and haemolymph was collected from the anterior adductor muscle. Eight pools of haemolymph (from 3 bivalves each) were prepared for each experimental condition, and total haemocyte count (THC), Neutral Red uptake (NRU), lysozyme activity in cell-free haemolymph (CFH) and haemocyte proliferation were measured. An increasing trend was observed in THC values, the difference being significant at 25 µg/L, with respect to controls. NRU was shown to decrease significantly in haemocytes of clams exposed to 1 and 5 µg/L, compared with controls, whereas NRU increased to control levels in clams exposed to the highest fluoxetine concentrations. Haemocyte proliferation increased significantly in animals exposed to 25, 125 and 625 µg/L, with respect to controls. Conversely, no significant alterations were observed in CFH lysozyme activity. Although preliminary, the results obtained demonstrate that fluoxetine influence markedly immune parameters in clams, even at environmentally realistic concentrations.

Expression of genes involved in glutathione biosynthesis in the solitary tunicate *Ciona intestinalis* exposed to heavy metals

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Exposure to metals is known to generate oxidative stress risk in living organisms, which are able to respond with the induction of antioxidant defenses, both enzymatic and non-enzymatic. Glutathione (GSH) is considered to be important components involved in protecting cells, both as metal chelating agent and oxygen radical scavenger. In this work we used molecular techniques to characterise the nucleotide sequence of genes involved in glutathione biosynthesis (*ci-GCLCgclc, ci-gclm* and *ci-gs*) in the solitary tunicate *Ciona intestinalis*. We also studied the expression of the genes in question after *in vivo* exposure to Cd, Cu and Zn, to expand knowledge on the relation of metal-induced oxidative stress and glutathione production, locating mRNA expression by *in situ* hybridisation (ISH). These genes exhibit a good level of sequence conservation with corresponding metazoan homologs, especially for residues important for the activity of the enzymes. Phylogenetic analyses indicate that the three enzymes evolved in different ways, Ci-GCLC and Ci-GS being mostly correlated with invertebrate proteins, Ci-GCLM resulting as sister group of vertebrate GCLMs. Our *in silico* analyses of the *ci-gs* and *ci-gclc* promoter regions revealed putative consensus sequences similar to mammalian metal-responsive elements (MRE) and antioxidant response elements (ARE), indicating that the expression of these genes may directly depend on metals and/or reactive oxygen species (ROS). Our data highlighted a statistically significant increase in gene expression, demonstrating that metal treatments have inducible effects on this gene. They can modulate gene expression not only through MREs but also through AREs, as a consequence of metal-dependent ROS formation. The ISH location of Ci-GS and Ci-GCLC shows that the cells most involved in glutathione biosynthesis are circulating haemocytes. The data presented here emphasise the importance of complex metal regulation of *ci-gclc*, *ci-gclm* and *ci-gs* transcription, which can create an efficient detoxification pathway allowing *C.*

inestinalis to survive in the continued elevated presence of heavy metals in the environment.

Comparative sequence analysis of antimicrobial peptides in *Mytilus galloprovincialis* and *Ruditapes philippinarum*

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Starting from our previous genome wide analysis in *Mytilus galloprovincialis* we have identified by sequence similarity search in our transcriptomics database of clam *R. philippinarum* a total of 43 new AMPs belonging to 5 different families of peptides with antimicrobial properties. In details 16 defensins, 3 mytilins, 3 myticins, 10 macins and 11 big defensins. The search for the mytimycin homologous did not produce any results. For each class of AMP we have also identified from other molluscs all the homologous sequences stored in public databases. All this data was used to produce a phylogenetic representation of sequence similarity through a bayesian approach. These results allow us to have a better picture, although not definitive, of the distribution and the diversity of AMPs in these clam and in general allows us to enrich our knowledge regarding antimicrobial defenses in the phylum Mollusca. Moreover we have demonstrate that a wide transcriptomics approach aiming to identify families of AMPs, can be very useful for organisms whose genome has not yet been sequenced. This approach proves to be very flexible and adaptable to perform research on a wide range of non-model organisms. Although very important aspects related to the specificity and mode of action, expression inducibility and genomic organization of genes coding for these AMPs require a series of devoted downstream studies, bioinformatics approach can provide fairly quick and relatively easy to identify the primary sequence of many AMPs, laying the groundwork for further research.

Special session 2. Chairman: M Cammarata, University of Palermo, Palermo, Italy
IADCI Award

Skin wound repair in adult *Xenopus laevis*

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Restoration of tissue integrity and homeostasis after an injury is a fundamental property of all organisms and there is diversity in how this process occurs. The healing response can lead to complete regeneration of tissue structure or repair that involves collagen deposition and scar formation. The regenerative capacity of anuran amphibians depends on the developmental stage and declines as metamorphosis proceeds. Few studies have addressed the ability of adult anurans to heal their wounds: skin repair occurred with scar synthesis in *Rana catesbeiana*, while it was without scarring in young adult *Xenopus* froglets.

In this work, we investigated the repair of skin wounds in different aged (8 and 15 month old) *X. laevis* adults to determine the quality of the wound healing response. Molecules (TNF-α, iNOS, MMP-9, α-SMA) and selected genes (*SOCS-3*, *TGF-β2*), known to be involved in inflammatory responses and wound healing, were analysed by immunohistochemical reactions and quantitative RT-PCR. The histological results showed similar repair step sequences in different aged frogs: inflammation, new tissue formation and maturation with wound contraction and remodeling. A large infiltrate of neutrophils and macrophages was early seen in the injured area and then lymphocytes were also detected in the granulation tissue. This wound connective tissue was characterized by extensive angiogenesis and transformation of some fibroblasts into anti-α-SMA immunoreactive myofibroblasts which contributed to scar formation. Quantitative RT-PCR analysis demonstrated that the regulator of cytokine signaling, *SOCS-3*, was rapidly up-regulated after the injury and maintained high levels during the process, while *TGF-β2*, an important tissue fibrosis promoter, increased when the new tissue was formed and high induced expression persisted later in the repair.

The results demonstrated that *Xenopus* skin regenerative capacity is lost with increasing age of the adult. The outcome of skin wound healing is similar to that of mammals with the formation of a scar-like tissue that may be related to an intense immune response, abundant granulation tissue and wound contraction. Moreover, α-SMA, SOCS-3 and TGFβ promote the tissue repair with expression patterns that seem to be different from those observed in scarless healing.

The CD45 receptor in the teleost fish *Dicentrarchus labrax*

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The CD45 tyrosine phosphatase plays an important role in regulating T lymphocyte activation in vertebrate species, and in this work we describe some features of the CD45 receptor molecule from the European sea bass *Dicentrarchus labrax.* By immunising mice with fixed thymocytes we obtained a monoclonal antibody (mAb) able to stain in immunofluorescence both live leucoytes at high percentages in thymus and mucosal tissues, and *in situ* immunoreactive cells by immunohistochemistry in sections from fixed tissues.

The obtained mAb DLT22 (IgG_2 subclass) recognized in western blots of lysates from thymus, spleen, intestine and gills leucocytes mainly polypeptides at 180 kDa and 130 kDa, and

immunoprecipitated a 130 kDa polypeptide from thymocytes lysate. The 130 kDa polypeptide was analysed by nano-RP-HPLC-ESI-MS/MS, and gave peptide sequences homologous to *Fugu* CD45, that were employed for the homology cloning of a partial sea bass CD45 cDNA sequence. The obtained cDNA sequence was employed to measure by quantitative PCR the transcription of the CD45 gene in basal conditions and in *in vitro* stimulated leucocytes, showing a modulation induced by LPS, ConA, PHA, IL-1, and poly I:C. When splenocytes were stimulated *in vitro* with ConA and PHA, a cell proliferation was measured together with a corresponding increase of leucocytes stained by DLT22.

These data indicate that the DLT22 mAb may recognise in sea bass CD45-associated functional stages of lymphocytes, like CD45-like developing lymphocytes in thymus, and peripheral CD45RO-like lymphocytes in tissues, thus dating back to teleost fish the functional activities of these cell populations in vertebrates.

Analysis of Antarctic skate Immunoglobulin heavy chain genes

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Cartilaginous fish are the oldest vertebrate class having an immune system consisting of immunoglobulin (Ig), T cell receptors, and major histocompatibility complex. In contrast to sharks, Ig have been poorly investigated in skates despite their high occurrence in all of the major oceans of the world. We have focused on IgM heavy chain from the Antarctic species *Bathyraja eatonii*, *Bathyraja albomaculata*, *Bathyraja brachyurops*, belonging to the subfamily Arhynchobatinae, and *Amblyraja georgiana,* belonging to the subfamily Rajinae. The RT-PCR analysis of the four species of Antarctic skates yielded several secreted IgM heavy chain sequences. The primers used were designed on IgM sequences available in GenBank from various chondrichthyan species allowing us to isolate several cDNA clones partially encoding immunoglobulin heavy chains. Nucleotide sequence identities calculated for the constant region domains ranged from 88.5% to 97.5% between species, and from 91.1% to 99.7% within species. A distance tree, including also sequences from the temperate species *Raja erinacea*, showed two major branches, one containing Arhynchobatinae sequences, the other one Rajinae sequences. Four distinct *D* gene segments were defined, two being present in all the species analyzed. Southern blotting analysis revealed 5 - 15 genomic fragments of different length, carrying the gene locus encoding IgM heavy chain. As revealed by the Shannon analysis, the position variability for the aligned *B. eatonii* amino acid sequences, was distributed through all four constant domains. However, CH3 was the most variable domain, whereas CH4 exhibited the lowest sum entropy value, being the most conserved domain. *B. eatonii* CDR3 region length varied between 11 and 15 amino acid residues, resulting in overall higher length variability than that of *Leucoraja eglanteria* CDR3 (mean length: 7.7 aa). The presence of more extra-cysteine residues, not involved in the intradomain disulfide bridges, observed exclusively in the Antarctic skates, is the most distinct difference between the Antarctic and non-Antarctic skate species analyzed. Notably, in the CH3 domain, the two extra-cysteines fall within the CXXC motif of thiol/disulfide oxidoreductases, which is essential for their catalysis of redox reactions. Taken together, these results may contribute to fill the knowledge gap relative to Antarctic Rajidae in the field of fish immunology.

Session 3. Chairman: M Balsamo, University of Urbino, Urbino, Italy
Environmental stress responses of the immune system

The new contribution of eco-immunology to the deciphering of immune-neuroendocrine integration

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The original aim of eco-immunology has been the description of the modalities by which biotic and abiotic factors may influence the immune functions in free-living organisms and the source of natural variation in the immune response of a selected model. Further conceptualizations have followed, in order to investigate how the many components of the immune system interact and determine the outcome of an immune challenge.

Fundamental concepts introduced by eco-immunology derive from observations on vertebrates. However, the basic principles introduced are of general validity and may be applied to the largest part of multi-cellular organisms. One of the basic assumptions of eco-immunology is that in absence of an immune stimulus, the energy expenditure for immune responses may be kept at a minimum level. The allocation of different amounts of energy in relation with the functional status may be achieved by mean of trade-offs, *i.e.*, the re-distribution of the available energy among different systems and components. The intrinsic plasticity of the immune functions allows energy re-allocation among different organs and activities, resulting in a variety of trade-offs between different systems.

Several experiments have indicated that in vertebrate and invertebrate models the immune and the neuroendocrine systems present a deep functional interconnection, based on the sharing of a common pool of signal molecules. Cells as different as vertebrate lymphocytes and molluscan immunocytes have both and independently been described as immune-neuroendocrine cells. The cross-talk between immune and neuroendocrine systems must have represented a fundamental advantage being maintained during the diversification of evolutionary distant organisms such as mammals and molluscs.

Accordingly to the view of eco-immunology, the advantage could derive by the intrinsic capability of

optimizing energetic costs by mean of trade-offs and a common pool of mediators. These features made the immune-neuroendocrine system an evolvable unit, allowing its great diversification among metazoans, while maintaining intact the functional cross-talk among its various components.

The antioxidant system of *Tetrahymena thermophila*

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The "free radical theory" correlates reactive oxygen species (ROS) production with oxidative stress and cell damaging. The antioxidative potential of organisms is probably affected by stress conditions, but this is an open question due to the multiplicity of ROS scavengers and their overlapping functions. With the aim to study this problem we projected some experiments using the ciliated protozoan *T. thermophila*, as model organism. The first step of the research was to characterize the genes codifying for some antioxidant enzymes: copper-zinc superoxide dismutase (Cu/Zn-SOD), manganese superoxide dismutase (Mn-SOD) and catalase (CAT). Total RNA has been purified from *T. thermophila* cells (SB210 strain) cultured in PPYG medium and the cells were harvested after three days during exponential growth. cDNA sequences were obtained by RT-PCR, 3'- and 5'-RACE techniques. The primers for the amplification of Cu,Zn-SOD, Mn-SOD and catalase cDNAs were designed after cross analyses between NCBI and *T. thermophila* genome databases. The obtained data demonstrated that two Cu,Zn-SODs, one Mn-SOD, and one CAT are constitutively expressed. Our results highlighted that a third Cu,Zn-SOD gene, annotated in the *Tetrahymena* genome database, seems not be transcribed. The nucleotide sequences of all genes have been compared with orthologous of other organisms and used for phylogenetic analyses. Experiments on the *in vivo* quantification of ROS production, performed with specific fluorescent probes, are now in progress to study the physiological responses during the different phases of *T. thermophila* life cycle and under various oxidative stress conditions (exposure to UV, peroxides and metals). The results will be correlated to the gene expression analyzed by RT-qPCR, using the same primers employed for cloning and sequencing. Other genes of antioxidant enzymes such as glutathione peroxidases will be characterized.

Role of MAPK activation in mediating the response to thermal shock in *Trichoplax* (Placozoa)

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Trichoplax adhaerens (Placozoa) appears as a flat disc consisting of two cellular pseudoepithelia, which sandwich a loose, syncitial network of fiber cells. In culture, *Trichoplax* reproduces by binary fission, whereby one half of the animal moves away from the other half until their connection is broken. Sexual reproduction has never been observed, but in culture formation of putative oocytes in degenerating animals is routinely seen. *Trichoplax* has a compact genome compared with that of vertebrates and of many other animals. All essential components of the BMP/TGF and JAK/STAT signalling pathways are present in the *Trichoplax* genome, but these pathways appear to be incomplete in that they lack molecular components critical to signal transduction.

In this work, the possible role of different MAPKs and STAT1 in mediating the response of *Trichoplax adhaerens* (clone Panama) to thermal shock was investigated. Western blot analyses with specific anti-phospho-MAPK and anti-phospho-STAT1 were performed in animals exposed to cold or heat shock. Our results indicate that in *Trichoplax* signal pathways leading to differential MAPK–like and STAT1-like activation are involved in mediating survival to thermal shock.

Heavy metals induced morphometrical alterations in earthworms granulocytes

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Earthworms are very important organisms for soil formation and organic matter breakdown in most terrestrial environments and they are able to accumulate various organic and inorganic contaminants present in the soil. Earthworm coelomic fluid is particularly interesting from a toxicological perspective for the development of novel cellular biomarkers of pollutant exposure. It can transport pollutants throughout the exposed organism and its cells (coelomocytes) are involved in the internal defence system. Five cell types were observed in *Eisenia foetida* and *Lumbricus terrestris* coelomic fluid: leukocytes type I (basophilic) and II (acidophilic), granulocytes, neutrophils, and eleocytes.

The aim of the present work was to investigate possible heavy metals induced alterations in earthworms coelomocytes in view of future application as sensitive biomarker for soil monitoring and assessment applications. Morphometric alterations were determined by image analysis on Diff-Quick® stained cells. A considerable enlargement of granulocytes was observed in heavy metals (Cu, Cd, Hg) exposed earthworms with respect to control group. On the other hand, the other cell types did not show any changes in the cell size. The enlargement was quantified by measuring the area of 2D digitalised granulocyte images. Heavy metals are known to interfere with a wide

range of metabolic functions and membrane transport mechanisms. This could result in an increase of intracellular osmolyte content, followed by osmotic influx of water and cellular swelling. Moreover, in heavy metals exposed animals the increase in the granulocyte dimension was accompanied by cell rounding with loss of pseudopods. This effect could be ascribed to toxic chemical-induced reduction of the microfilament and microspine number. Moreover, granulocyte enlargement was paralleled by an increased frequency of necrosis in earthworm coelomocytes. Due to the important immunological role of granulocytes, the observed heavy metal induced adverse effects on these cells may increase the susceptibility of animals to diseases and reduce their survival ability. Therefore, early subtle alterations in some of the components of the immune system can be used as early indicators of altered organism health. The authors demonstrated that heavy metals induced morphometric alterations in earthworms granulocytes with possible applications as sensitive, simple, and quick biomarker for monitoring and soil risk assessment.

Immunomodulation by TiO_2 nanoparticles in *Mytilus galloprovincialis*

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The potential toxicity of engineered nanoparticles (NPs) for humans and the environment represents an emerging issue, due to the continuous development and production of manufactured nanomaterials. Since NPs tend to end up in waterways, their possible impact on the immune function of aquatic organisms needs investigation.

In mammals, interactions of NPs with immune cells represent a major issue for both therapeutic use and possible detrimental health effects. Conservation of the general mechanisms of innate immunity from invertebrates to mammals is a key feature that can be used as an useful basis for studying common biological responses to NPs.

We have previously shown that in the marine bivalve *Mytilus* short term exposure to $nTiO_2$, one of the most widespread NP in use, significantly affected immune parameters *in vitro* and digestive gland biomarkers *in vivo*. In this work, the effects of $nTiO_2$ on immune function were investigated in mussels exposed to $nTiO_2$ (1, 10 and 100 µg/L) for 4 days. Detailed physico-chemical characterization of NPs was performed.

The results show that exposure to $nTiO_2$ induced significant changes in different functional and molecular immune parameters in mussel hemocytes. In particular, $nTiO_2$ induced lysosomal membrane destabilization, inhibition of phagocytosis and stimulation of ROS and NO production. Flow Cytometry analysis revealed effects also at mitochondrial level, as well as decreased Total Hemocyte Count (THC) and changes in the percentage of hemocyte sub-populations. Changes in expression of selected genes (antimicrobial peptides, stress proteins) evaluated by RT-Q-PCR were also observed. Both lysosomal and antioxidant biomarkers were affected in the digestive gland, indicating general stress conditions.

These data indicate that exposure to $nTiO_2$, at concentrations in the low µg/L range, can affect the mussel immune function, with possible immunosuppressive/inflammatory effects. Moreover, these data support the hypothesis that in bivalves transfer of NPs from the digestive system to the hemolymph and circulating hemocytes may occur. Biomarkers of immunotoxicity may represent sensitive indicators of how NPs may cause alterations in the organism's physiology, providing an indication of the sublethal impacts of NP exposure, as well as an "early warning" of population level impacts.

Session 4. Chairman: L Abelli, University of Ferrara, Ferrara, Italy
Environmental stress responses of the immune system

Amyloidogenesis and melanogenesis: correlated events induced by "stress condition"

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Amyloidogenesis has historically been associated with pathology of the neurodegenerative diseases while recently it has been demonstrated also in non-pathological protein fold utilized by organisms from bacteria to humans.

We showed that the parasitization promotes in insect host hemocytes several massive morphological and physiological modifications that mimic general stress conditions, in which phenomena such as amyloid fibril formation and melanin polymerization, occur.

Moreover the physiological amyloid fibrillogenesis is an evolutionary conserved biological pathway and the relationship between amyloidogenesis and stressors allows to surmise a new background of information on the effects of stress.

Zinc effect on the immunological competence of the sea urchin *Paracentrotus lividus* gametes and embryos

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In recent years, pollution of marine environment by heavy metals has become an international problem. In the Mediterranean Sea the main heavy metals found are cadmium, mercury, lead, tin, copper and zinc. These metals show levels often easily measurable in the marine samples and can exert toxic effects on living organisms causing an alteration of biological activities. In particular in marine invertebrates heavy metals affect survival, growth, reproduction, metabolism and immunity. Effects of these pollutants on gametes and various developmental stages of echinoids were investigated by several authors focusing on the induction of morphological anomalies. By contrast studies on the effect of heavy metals on the immunological competence are scant. Furthermore, being gametes and early life stages usually more susceptible to toxicants than the adult, in the present work we analysed the effects of a high zinc ion concentration on several immunological parametrs of the sea urchin *Paracentrotus lividus* gametes and embryos. Comparing untreated (control) and zinc treated sea urchins, we evaluated the effects of this metal on jelly coat and seminal plasma haemolitic and lysozyme-like activites as well as antibacterial activity on *Vibrio alginolyticus*. All the examined immunological parameters were significantly reduced by the addition of zinc after 24 hours of treatment. The subsequent sea urchin development was negatively influenced. When lower zinc concentrations were employed deviations from the normal developmental process were observed. Our results confirm sea urchin gametes and early developmental stages as suitable sensors for acute toxicity tests and their immunological competence modifications offer the potential for the development of sensitive assays to investigate and monitor heavy metal marine pollution.

Stress and immunomodulation indicators in gilthead seabream (*Sparus aurata*): long-term dominant-subordinates interplay affects phagocytosis by peritoneal cavity cells

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Fish are highly sensitive to stressful conditions that effect their immune system and increase susceptibility to diseases. The social hierarchy (dominant/subordinate) of gilthead seabream (*Sparus aurata*) specimens were identified by using the "feeding order" and "aggressiveness" parameters in specimen pairs. This hierarchic position appeared at 24 - 36 hours after pairing, and did not change during one year in experiment with two (dominant/subordinate) or three fish (dominant/subordinate β/subordinate γ).

To characterise physiological stress, we measured blood plasma levels of cortisol, glucose, and lactate as well as osmolarity and observed that the levels of these stress markers were higher in subordinate individuals than in dominant ones. Since the increased glucocorticoid levels exhibited during stress might also serve to restrain defence mechanisms we examined the relationship between the social rank and the effects on the innate immunity. In particular the activity in the peritoneal cavity cells (PCC) was estimated using a phagocytosis activity assay employing yeast (PA) and a cell chemiluminescent (CL) reaction subsequent to stimulus with zymosan. After a 24 h pairing period, the PCC activity, with respect to PA and CL, was significantly higher in the dominant and control fish as compared with the subordinate ones. However, after 15 days, these responses returned to control levels in dominant fish, and these levels were maintained for 6 months. In contrast, in β animals, the PCC activity was increased by 3-fold within one month and 2-fold after 6 months.

The discriminant analysis revealed the influence of the social status, with a clear allostatic response of the subordinate fishes and the highly significant separation among groups at 15 days.

Wild dolphin transcriptomes: identification of the immune response to environmental contaminants through gene expression information

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As top level predators, bottlenose dolphins (*Tursiops truncatus*), are particularly sensitive to chemical and biological toxins that accumulate and biomagnify in the marine food chain. A dolphin's exposure to such toxins can be assessed using standard analytical methods, but it is costly and requires the collection of multiple tissue samples. We are currently investigating the potential of screening for multiple contaminant and/or algal toxin exposure through their associated immunological and/or endocrine perturbations in bottlenose dolphins using microarray technology and gene expression profile analysis. If successful, the gene expression profile analysis could provide a cost-

effective means to screen for indicators of chemical and biological toxin exposure as well as disease status in dolphins, and potentially other cetaceans. A newly developed dolphin oligo microarray representing 24,418 unigene sequences was used to analyze blood samples from 74 dolphins collected from 4 geographic locations in the South East USA (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 persistent organochlorine 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 damages, UV and/or viral infection-induced.

The transcriptomic data analysis will be a first step towards identification of markers/patterns indicative of exposure to chemical contaminants as well as marine toxins and will promote an understanding of toxic mechanisms and/or pathways that are currently not well understood in marine mammals.

Session 5. Chairman: V Arizza, University of Palermo, Palermo, Italy
Immuno-active and antimicrobial molecules

Molecular basis of self/nonself recognition in ciliate mating type systems

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Like numerous other organisms, ciliates alternate their life cycles between vegetative (mitotic) growth and sex. However, ciliate sex is unique for at least two major aspects: (i) it becomes manifest as temporary cytoplasmic fusion between cells that carry diploid sets of genes and may equally be identical or different in their genotypes, and (ii) is under the control of a genetic mechanism of mating-types which may either be only two as is the case in various species of *Paramecium* and *Blepharisma*, or multiple as is the case in various species of *Tetrahymena*, *Euplotes*, *Stylonychia* and the hypotrichs in general. While the mating type binary systems recall the duality of the sex of the multi-cellular organisms, the multiple systems find much closer counterparts with the self/non-self recognition mechanisms that permit animals to react immunologically against invaders, and fungi and flowering plants to decide their evolution between self-sterility and self-fertility strategies. A persevering research interest on the multiple mating type systems of different species of *Euplotes* lead us to obtain relevant information on the genes and the cell type-specific diffusible signal proteins (pheromones) that interplay in the control of these systems. Functionally most relevant was the finding that, in full accord with their genetic determination provided by multiple sets of single-locus genes, these *Euplotes* pheromones are represented by species-specific family of structurally homologous proteins which, as such, can compete with one another for binding to their cell receptors in either autocrine (self), or paracrine-like (non-self) fashion. Cells grow in response to the pheromone binding that signals self, and temporarily shift to the sexual stage in response to the pheromone binding that signals non-self.

Functional activity of the ciliate *Euplotes raikovi* pheromone in human cell lines

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Ciliates synthesize cell type-specific chemical signals, designated pheromones, that in association with their mating type systems control the switching between the reproductive (mitotic growth) and mating (non-reproductive, sexual) stages of their life cycles.

In the protozoan ciliate *Euplotes*, pheromones have been isolated and characterized for their genetic determination and molecular structures and interestingly, *Euplotes raikovi* pheromones (designed as Er-1, Er-2, and so forth) have been functionally linked with growth factors of higher eukaryotes as for instance the epidermal growth factor and the cytokine interleukin-2 (IL-2). However, the activity of pheromones in mammalian cells and their underlying cellular basis are currently unknown. In an attempt to elucidate the pharmacological features and the functional effects of *Er-1* in human cells, we have performed an *in vitro* study using Jurkat cells (IL-2 producing human T lymphocyte cell line, commonly used to study T cell signalling), that express functional IL-2 receptors. In particular, the present study was designed to investigate the role of *Er-1* on relevant aspects of cell growth and cytokine gene expression and/or release. Since the cross-talk among IL-2 system, T-cells, and malignant cells plays an important role in brain tumors, we also sought to investigate the possible effects of *Er-1* on human glioma cell line (U-373 cells). Our results provide the first insight into the impact of the *Euplotes raikovi* pheromones on the physiology of human cells, showing that *Er-1* may increase the growth of T-cells, but not glioma cells, cultured under restrictive culture conditions. In addition, *Er-1* was found to significantly increase gene expression levels and production of specific cytokines in Jurkat cells. The work allowed us to parallel the events which occur *in vitro* with those occurring in the microorganism ecosystem and discover *Euplotes raikovi* as a fruitful mine of compounds with bioactive properties in mammalian cells.

Hepatopancreatic transcript expression in the crayfish *Astacus leptodactylus* - functional genomic analysis and the effect of two stereomers of the crustacean hyperglycemic hormone (CHH)

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The crustacean Hyperglycemic Hormone (cHH) is present in many decapods in different isoforms, whose specific biological functions are still poorly understood. This hormone plays a key role in crustacean stress responses (Lorenzon, 2005). Recently we obtained the first chemical synthesis of the chiral isoforms of the cHH of *Astacus leptodactylus* carried out by solid phase peptide synthesis coupled to native chemical ligation (Mosco et al., 2012). The synthetic 72 amino acid long peptide amides, containing L- or D- Phe^{3} and (Glp^{1}, D-Phe^{3}) were tested for their biological activity on hepatopancreatic transcript expression. Sixteen *A. leptodactylus* females were used, divided into 4 female groups. Two groups were injected by D- or L-cHH, respectively (0.5 µg/female in 100 µl phosphate buffered saline). A control group was injected by the hormone carrier and a fourth control group of females neither eyestalk ablated nor cHH injected. RNA extracted from hepatopancreas were sequenced using the Illumina technology in order to: 1) establish the hepatopancreatic transcriptome from pooled RNA and 2) to perform gene expression analysis from individual samples. Preliminary results indicated that the expression profiles of three of the experimental groups, namely the native, the sham injected and the L-cHH injected females are qualitatively quite similar and differ from the D-cHH injected group. Consequently, the statistical analysis was divided into two parts. First, the sham injected profiles were compared to those of the native females to demonstrate differences caused by the extirpation. Second, the profiles of the four sham-injected females were compared to the two most different profiles of the D-cHH and L-cHH injected individuals, respectively, to demonstrate putative effect of each of the two hormone isomers. The overall expression trend and level resulted from statistical testing is presented. Our data clearly shown that the eyestalk-ablation did not make dramatic gene expression alterations in the hepatopancreas and that the effect of D-cHH is much more pronounced that of the L-cHH in both terms of number of altered genes and their abundance (higher RPKM). The effect of the D-cHH in terms of transcript number is mostly attenuation of expression.