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REPORT OF MEETING

XIVth scientific meeting of the Italian Association of Developmental and Comparative Immunobiology (IADCI), 14 - 16 February 2013, Department of Biological Chemical Pharmaceutical Science and Technology, University of Palermo, Palermo, Italy

Organizers: **N Parrinello, V Arizza, M Cammarata, MG Parisi, M Vazzana, A Vizzini**

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Plenary lecture

From signal transduction to gene expression in innate immunity: learning from the mussel model

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Antimicrobial peptides (AMP) are certainly one of the major effectors of the anti-infectious innate immunity. They are present in all the living creatures, including bacteria and plants. They are of multiple amino acid sequences and characteristics. In addition, several AMP families are simultaneously present in the same animal, not only in immune circulating cells, but also in various epithelia. Complete molecular cascades of signal transduction from pathogen recognition to AMP activity are known in several vertebrates, but only in *Drosophila* (Ecdysozoa), although still in debate. The present lecture will present the few we know on the mussel *Mytilus* (Lophotrochozoa).

First, biochemical analysis revealed the existence of particular AMPs, grouped in several families, including several isoforms, with different antibacterial capabilities. Synthetic fragments of peptides have been developed for applied biotechnology against bacteria and virus infections.

Second, were molecular biology approaches, which extended the diversity of AMP mRNAs, including within the same mussel, suggesting a complex system of molecular effectors. At that time, we realized how naïve was the idea of a primitive simple innate immune defense.

Third, expression regulations and specificities have been investigated following challenges with bacteria, fungi, and physical stresses. No strict correlation has been yet established between structure and function of AMPs in mussel.

Last, focus was on recognition-signal transduction steps by analogy with *Drosophila* in which the Toll-NF-κB pathway controlled the synthesis of AMPs. Blast of sequences was less successful than looking for conserved domains of partners within EST databases. Thus, Illumina read assemblies have completed identified sequences and revealed new ones. Regulation of gene expression following challenges gave some light on the existence of the cascade in Lophotrochozoa.

In conclusion, mussels possess some of the NF-κB pathway intermediates. But most of them remain to be identified and the functionality of such complex system in the course of real infection is still pending.

Present lecture resulted from data obtained in collaboration with colleagues and students from the Italian Universities of Padua, Trieste, Palermo, and the CSIC-Vigo from Spain.

Session 1. Chairmen: N Parrinello, University of Palermo, Palermo, Italy; E Ottaviani, University of Modena and Reggio Emilia, Modena, Italy; L Abelli, University of Ferrara, Ferrara, Italy; L Ballarin, University of Padua, Padua, Italy
Immunity evolution. Responses and mechanisms

Evolution of the intracellular transport mechanisms in eukaryotes: ciliates and mammals use the same translocation and nuclear localization signals

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In the ciliate *E. raikovi*, self/non-self recognition phenomena are controlled by cell type-specific, water-borne signal proteins (pheromones) by their binding to target cell-surface receptors. The downstream signal transduction pathway activated by the pheromone-receptor interactions of self type (that promote the vegetative, mitogenic cell growth) involves the phosphorylation of a nuclear protein kinase, designated E*r*-MAPK1, which is structurally similar to the "intestinal-cell kinase" and "male germ cell-associated kinase" described in mammals. To identify the sequence segments responsible for E*r*-MAPK1 nuclear localization, mouse fibroblasts were

transfected with plasmids containing the reporter gene for the "Green-Fluorescent Protein" (GFP) associated to different fragments of the E*r*-MAPK1 coding sequence. By expressing GFP-tagged protein constructs in mammalian cells, in the C-terminal domain of E*r*-MAPK1 it was effectively possible to identify an Arg/Lys-rich motif that is required for the nuclear entry of GFP-fused constructs. These results provide evidence that distant related organisms such as ciliates and mammals use the same molecular language for the nuclear translocation and localization of proteins, thus suggesting that this language arose early in the evolution of the eukaryotic cell.

Cloning and expression of methionine sulfoxide reductase genes in the ciliated protozoan *Tetrahymena thermophila*

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Accumulative post-translational modification to proteins, mediated by the action of reactive oxygen species (ROS), is thought to be one of the major causes of aging and age-related diseases. Thus, mechanisms have been evolved to prevent or reverse these protein modifications. While most protein damage by ROS is irreversible, methionine oxidation to proteins can be reversed by the methionine sulfoxide reductase (Msr) system, which includes MsrA (that repairs methioninesulfoxide S-enantiomer) and MsrB (that repairs the methionine-sulfoxide R-enantiomer). The action of the Msr system may prevent irreversible protein damage (e.g. protein carbonylation), contribute to cellular antioxidant resistance resulting in life span extension of the organism. Moreover, many work demonstrated that methionine oxidations in both inhibitor of kappa B-alpha and Ca^{2+}/calmodulin-regulated phosphatase calcineurin may alter their functions and consequently affect transcription levels mediated by NFAT and NFκB especially in T-lymphocytes of the immune system. With the aim to explore this problem we projected some experiments using the ciliated protozoan *T. thermophila*, as model organism, characterizing the genes codifying for Msr. 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. The primers for the amplification of Msr cDNAs were designed after cross analyses between NCBI and *T. thermophila* genome databases. The obtained data seem to indicate that only one of the four annotated Mrs genes is constitutively expressed. The nucleotide and amino acid sequences of all genes were compared with orthologous of other organisms and used for phylogenetic analyses. Time-course of gene expression was analyzed by RT-PCR, using the same primers employed for cloning and sequencing, in *T. thermophila* cells grown in normal condition and after exposure of Cu^{2+}, used as pro-oxidant.

The injection of LPS induces epigenetic changes in *Pomacea canaliculata* neurons

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Epigenetic changes allow to modify gene expression on the basis of cell necessities and environmental stimuli. Recent experiments suggested that resilience or vulnerability to stressful events is sustained by specific changes in gene expression that can affect the behavioral and molecular consequences of stress.

In the present study, we have observed by means of immunocytochemical and western blot analyses that the injection of *Escherichia coli*-derived LPS (O55:B5) in the foot of the apple snail *Pomacea canaliculata* (Gastropoda: Ampullariidae) promotes the phospho-acetylation of histones, a phenomenon usually associated with changes in transcriptional patterns. More in detail, LPS injection provoked an increase in immunoreactivity of anti-phospho (Ser10)-acetyl (Lys14)-Histone H3 pAb in neurons of the pedal ganglia after 2 and 6 hours. In pedal ganglion neurons, the cytoplasmic immunoreactivity of the stress-related CRH-like and ACTH-like factors is also enhanced after LPS injection. Western blot analysis showed also the increase of the transcriptional factor c-Fos after the immune challenge.

In mammals, the expression of c-Fos is promoted by intravenous administration of CRH or exposure to restraint stress. Moreover, several researches have demonstrated a link between immune challenges and activation of the stress axis in vertebrates and invertebrates.

The morphological and molecular data collected in snail neurons allow to surmize that acetylation of Lysin residues in histone H3 may play a role in regulating the expression of molecules involved in the stress response of molluscs, as it has been suggested for mammals.

LPS challenge regulates gene expression and tissue localization of a *Ciona intestinalis* gene through an alternative polyadenylation mechanism

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A subtractive hybridization strategy for the identification of differentially expressed genes was performed between LPS-challenged and naïve *Ciona intestinalis.* This strategy allowed the

characterization of two transcripts (*Ci*8short and *Ci*8long) generated by the use of two Alternative Polyadenylation (APA) sites. The *Ci*8long transcript contains a protein domain with relevant homology to several components of the Receptor Transporting Protein (RTP) family not present in the *Ci*8short mRNA. By means of Real Time PCR, the *Ci*8short and *Ci*8long transcripts showed a different pattern of gene expression with the *Ci*8short mRNA being strongly activated after LPS injection in the pharynx. *In situ* hybridization analysis demonstrated that the activation of the APA site also influenced the tissue localization of the *Ci*8short transcript. This analysis showed that the *Ci*8long mRNA was expressed in hemocytes meanwhile the *Ci*8short mRNA was highly transcribed also in vessel endothelial cells and in the epithelium of pharynx stigmata.

These findings demonstrated that regulation of gene expression based on different polyadenylation sites is an ancestral powerful strategy influencing both the level of expression and tissue distribution of alternative transcripts.

Exosomes and epigenetic information

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The epigenetic changes play an important role in the differentiation of cells and tissues. Among epigenetic factors, the micro RNAs (miRNAs) have assumed a considerable interest, thanks to the availability of new technologies that make these molecules easier to study. Not long ago, non-coding genomic sequences were considered "junk". Today we know that many transcripts play a regulatory role by itself. Non-coding RNAs (ncRNAs) can interact with DNA, RNA, proteins and engage in different structural and functional activities; they have an important role in nuclear and trascriptional organization, in post-trascriptional and epigenetic processes. miRNAs are endogenous ncRNAs of about 22 nucleotides (Bartel, 2004) that negatively regulate gene expression by acting as inhibitors of the translation process and by determining the degradation of the mRNA target. miRNAs are carried by exosomes in biological fluids so they can be considered as circulating molecular messenger that regulate mRNAs. Exosomes are particles of 30-200nm in diameter, derived from multi-vesicular bodies (MBV), delimited by a lipid bilayer containing a wide range of proteins and nucleic acids (particularly mRNA and miRNA) (Martins *et al.*, 2012). Exosomes contain distinct signatures of miRNAs that are characteristic of the cell from which they are derived and operate as signaling platforms that can influence cells destiny. We studied the diffusion of miRNAs in exosomes derived from serum of patients with hepatocellular carcinoma (HCC) to relate the miRNAs identified in tumor tissues with those ones identified in serum. We tested in particular the presence of miRNAs characterizing hepatocellular carcinoma in exosomes isolated from serum and their expression level. Our data are in agreement with the opinion that exosomes could represent the means of transport of metastasizing information. miRNAs have a great potential as diagnostic and prognostic biomarkers and exosomes could be used as drug delivery system.

Molecular cloning, characterization and expression analysis of peroxinectin from *Ciona intestinalis*

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Ascidians occupy a key phylogenetic position and are retained the sister group of vertebrates. Peroxinectins, included into the peroxidase-cyclooxygenase gene superfamily, function as hemoperoxidase and cell adhesion factor and could be involved in invertebrate immune reaction like PO activating system. In this study, the ascidian (*Ciona intestinalis*) peroxinectin gene (*Ci*Pxt) and its expression during the inflammatory response have been examined. LPS was inoculated into the ascidians, total RNA was extracted from pharynx and cDNA produced. The CiPxt full-length cDNA and the deduced amino acid sequence contain a peroxidase domain and an integrin binding motif (Lys-Gly-Asp). Sequence analysis showed high similarity of *Ci*Pxt to echinoderms peroxinectins and mammalian Myeloperoxidase (MPO); Eosinophil Peroxidase (EPO); Thyroid Peroxidase (TPO). Homology modelling process showed the expected molecular structure. *Ci*Pxt, a new member of the peroxidase-cyclooxygenase gene superfamily, is very close to the chordate group and appears to be the out-group of mammalian MPO, EPO and TPO clades. The *Ci*Pxt molecular structure model resulted superimposable to the human myeloperoxidase. Since the *C. intestinalis* pharynx is involved in the inflammatory response to LPS (Parrinello *et al.*, 2010; Vizzini *et al.*, 2012; Giacomelli *et al.*, 2012) tissue expression of the *Ci*Pxt gene was examined by real-time PCR analysis and *in situ* hybridization. *Ci*Pxt gene expression is upregulated by LPS inoculation suggesting it is involved in *C. intestinalis* inflammatory response. The *Ci*Pxt riboprobe was found in vessel haemocytes, in the zones 7-8-9 of the endostyle (a special pharynx organs with a functional homology to the vertebrate thyroid gland) and in stigmata. The present report supports the role of the ascidians' pharynx in the innate immunity.

***In vitro* effect of leptin on rainbow trout *Oncorhynchus mykiss* leucocytes**

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Leptin is an adipocyte-derived hormone discovered in 1994 (Zhang *et al.*, 1994). The finding that the leptin concentration circulating in the plasma is proportional to the body adiposity led to the theory that leptin acts as an "adipostat", a humoral signal carrying information regarding energy reserves (Maffei *et al.*, 1995). In addition to the regulation of appetite and body weight, leptin has been reported to play a role in a different range of physiological functions (Bjorbaek and Kahn, 2004; La Cava and Matarese, 2004).

Leptin three dimensional structure is similar to that of a cytokine consisting of a four α-helix bundle motif which is common to the IL-6 family of cytokines (Zhang *et al*.,1997). Despite the fact that numerous studies carried out in mammals point out at leptin role in the modulation of immune function evidence of immunomodulatory effect of leptin in fish is still unknown. Trout recombinant leptin (rt-lep) has been produced and tested for its biological activity to trigger cellular pathways usually active in the cells of mammals immune system in *in vitro* incubations with blood leucocytes of the rainbow trout *Oncorhynchus mykiss*. NFκB and MAP kinase activation were assessed by immunoblotting with a phospho (anti pIKBα; anti pJNK; anti pp38; anti pERK) specific antibody. rt-lep caused a decrease in the superoxide anion production in trout blood and head kidney leucocytes showing that this hormone plays in teleosts pleiotropic actions as in mammals, however, its actions not always conforms to the picture emerging for mammals.

Putative rhamnose-binding lectin in the solitary ascidian *Ciona intestinalis*

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Lectins are sugar-binding proteins involved in cell-cell interaction and the recognition of carbohydrate-containing molecules. They act as humoral factors in non-self recognition, are a key component of the innate immune systems of many metazoans and are involved in phagocyte activation through their opsonizing activity. In recent years, a new lectin family, the rhamnose-binding lectin (RBL) family, has been described and its members can modulate the inflammatory response in fish (Watanabe *et al.*, 2009) as well as in various invertebrates, including the colonial tunicate *Botryllus schlosseri*.

Recently, we succeeded in purifying, by affinity chromatography using a rhamnose column, a putative RBL, from the hemolymph of the solitary ascidian *Ciona intestinalis*. The molecule is Ca^{2+}-independent and promptly (within 4h) inducible, after LPS inoculation. The eluted fractions, when examined by 15 % SDS-PAGE under reducing condition, showed four bands with apparent molecular masses of 65, 54, 30 and 19 kDa. The agglutinating activity of the isolated fraction was demonstrated using trypsinized rabbit erythrocytes and it was inhibited by glycosides such as rhamnose, galactose and lactose. Moreover, by immunocytochemical analysis using antibodies produced against *B. schlosseri* RBL and *in situ* hybridization with a riboprobe for the annotated *C. intestinalis* RBL, we have observed a positive signal in hyaline and granular amoebocytes and in the endothelium of the pharynx vessels.

Antarctic teleost antibodies: evolution, structure, function

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Over the past 55 million years, the temperature of the Southern Ocean has undergone a progressive reduction from about 15 °C to the present-day -1.86 °C. In consequence, many temperate fish species became extinct and one group, the Notothenioidei, succeeded in adapting to the environmental conditions, and were diversified *in situ*. Eight notothenioid families, including five that are predominantly Antarctic, encompass a total of 44 genera and 129 species, 101 Antarctic and 28 non-Antarctic. Evolution under constant cold conditions was also accompanied by significant gene duplication suggesting that the genomes of notothenioids are evolutionarily dynamic, thus contributing to the overall success of the group.

Due to the plasticity of its gene locus, Immunoglobulin (Ig) is an ideal candidate to contribute to the characterization of the evolutionary modifications undergone by the notothenioid genome.

Investigations conducted during many years, on 23 different notothenioid species allowed us to obtain an overall view of their Ig genes evolution. We purified and characterized Ig molecules, studied the Ig epato-biliary transport and the secretion into the skin mucus, demonstrated the antibody response to nematode parasites, investigated the evolution of the heavy chain (IgH) gene locus, inspected the diversity of the variable gene segments, identified three different Ig light chain (IgL) isotypes, mapped the IgH locus on the chromosome of several species, examined the alternative splicing of the IgH primary transcript and looked at the molecular structure of the IgH transmembrane segment.

The achieved results convinced us that the Antarctic teleost Igs are very fascinating molecules.

T cells and T cell receptor gamma chain of European sea bass *Dicentrarchus labrax* (L.)

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The sea bass is a marine fish species for which much information on its immune system is available, and results obtained from recent investigations on this species can be reassumed as follows. Mucosal tissues are preferential sites for transcription of the T cell-related genes TRB, TRG, CD3, CD4, CD8a, MHC_{II}, as well as are the sites where the highest number of T cells has been measured by mAbs against T cells (DLT15), CD45 receptor (DLT22), and where cells expressing *in situ* mRNA for CD4, CD8a, MHC_{II} are present. Interestingly, the RAG-1 gene is highly expressed in the intestine, suggesting a possible extrathymic somatic rearrangement of TR and Ig in lymphocytes. Leukocytes from intestine and spleen display a spontaneous cytotoxic activity against allogenic and xenogenic cell targets, which was abolished when T cells were depleted by mAb DLT15, suggesting presence of a CTL activity. In agreement, DLT15-enriched leukocytes from intestine and spleen showed an higher expression of TRB and CD8a compared to negative DLT15-cells. Splenocytes respond better than kidney leukocytes to lectin stimulation, and when induced to proliferate by PHA and ConA, a significant increase in the number of T cells and of CD45-bearing cells was measured. We recently cloned in sea bass cDNAs coding for cellular receptors CD45 and CD83, and observed that the transcription of these gene was modulated *in vitro* and *in vivo* by LPS, ConA, PHA, IL-1, poly I:C, and betanodavirus.

A peculiarity of γδ T cells is that they may recognize unconventional without the need of MHCI or MHCII presentation, and we cloned full-length cDNA sequences of sea bass TRG, and TRG-CDR3 length spectratyping revealed the structure of the junction diversity in the thymus and intestine of juveniles. TRG expression can be modulated *in vivo* in the intestine by betanodavirus infection, and in kidney cells by poly I:C stimulation. High basal TRG mRNA expression levels were found in thymus and intestine, that were up-regulated in head kidney and intestinal leukocytes after in vitro stimulation by poly I:C. An *in vivo* infection of juveniles with betanodavirus caused an up-regulation of TRγ expression levels in the head kidney and a down-regulation in intestine. Our data can be of importance for studies on mucosal immunity of Teleost fish.

The vertebrate Allograft Inflammatory Factor-1 (AIF-1) homologous in *Hirudo medicinalis* is involved in wound healing process

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Analysis of an EST library from medicinal leech CNS reveals the presence of a gene, named HmAif-1, showing a high homology with vertebrate AIF-1. This factor is a 17 kDa cytokine-inducible calcium-binding protein that in vertebrates plays an important role in allografts immune response and vasculopathy. Since its expression is mainly limited to the monocyte/macrophage lineage, it was recently suggested that it could play a key role during inflammatory responses, allograft rejection, as well as in the activation of macrophages. To clarify this point we have focused our research on the possible role of AIF-1 during the inflammatory response after injury in the leech *Hirudo medicinalis* (Annelida, Irudinea). This invertebrate is an excellent animal model since the responses evoked during inflammation and tissue repair are clear and easily detectable and have a striking similarity with vertebrate responses. Our preliminary data show that HmAIF-1 is constitutively expressed in unlesioned leeches, but dramatically increase 24 h after a lesion. Immunohistochemical experiments, using an anti AIF-1 polyclonal antibody, shows that HmAif-1 is present in spread, $CD68^+$ / $CD45^+$ macrophage-like cells. A few days after chirurgical wound of the body wall, the amount of these immunopositive cells increases at the lesion site. In conclusion here we propose that in leech HmAif-1 factor is involved in inflammation events like its vertebrate counterparts.

Cellular and humoral components of innate immunity in *Squilla mantis* (Crustacea, Stomatopoda): a preliminary approach

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The morphology and enzyme content of circulating cells of the mantis shrimp *Squilla mantis* from the North Adriatic Sea, together with their ability to phagocytize foreign cells, were studied for deeper insights into the function of crustacean hemocytes in immune responses. The enzyme content and the agglutinating and hemolytic activities of cell-free hemolymph were also assayed.

Three hemocyte types, *i.e*., hyalinocytes, semigranulocytes and granulocytes, were distinguished, according to cell and nuclear morphology and the presence of cytoplasmic granules, in agreement with previous reports. All of them share the same patterns of enzyme activities and are recognized by the same lectins. Spreading cells (hyalinocytes and semigranulocytes) can ingest foreign cells; granules of semigranular and granular cells have similar cytochemical properties. Injection of *Micrococcus luteus* into the heart sinus results in an increase in the frequency of hyaline cells and a decrease in the frequency of granulocytes. After 24 h from the injection, a decrease in the number of phagocytizing hyalinocytes, and a general decrease in the frequency of acid phosphatase-positive cells was reported.

The above results suggest the existence of a single differentiation pathway for *Squilla* haemocytes with the three hemocyte morphs as different stages of cell differentiation. Results also indicate that *Squilla* hemolymph performs immunosurveillance, through rapid changes in hemocyte distribution,

increase of antimicrobial and antioxidant enzymes and secretion of lectins stimulating agglutination, phagocytosis and encapsulation.

Characterization of hemocytes and plasmatic prophenoloxidase from adults of *Pterostichus melas italicus Dejean,* 1828 (Coleoptera, Carabidae)

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Carabid beetles are among the most important groups of beneficial arthropods in the agroecosystem food chain where they are predators of many pests (including aphids, lepidopterans, slugs and Diptera). Previous studies have been shown that they are good models to investigate the negative effects of agrochemical used in agricultural management practices on natural enemies of insect pests. In ecological immunology, variation on immune capacity of insects is an early warning, highly sensitive biomarker to monitor the sub-lethal effect of toxicants introduced into environment as a result of industrial or agricultural activity. However, morpho-functional data about immune system of carabid are absent in spite of their ecological relevance. In this study, we have investigated the immune function of *Pterostichus melas italicus* (Dejean, 1828) for their use in eco-toxicological monitoring. This species is a generalist predator, eurytopic and thermophilous and it is very common in Calabrian (South Italy) agroecosystems (i.e. olive grove) and acts as a predator against insect pests (*i.e.*, *Bactrocera oleae*). Tests performed on adult involve a general screen of immune function and include: characterization of circulating hemocytes, phagocytosis *in vivo* and phenoloxidase activity. The cellular population has been characterized by light and electron microscopy analysis and four morphotypes of circulating hemocytes were found: prohemocytes (1.1 ± 0.35 %), plasmatocytes (76.13 ± 7.00 %), granulocytes (13.40 ± 5.84 %), oenocytoids (1.93 ± 0.96 %). The phagocytosis assays were performed *in vivo* by injection of 0.9 µm carboxylate-modified polystyrene latex beads in order to identify the hemocyte types involved in phagocytosis. After non-self challenge treatment, specimens showed a decrease of granulocyte and prohemocyte percentages (6.13 ± 3.36 % and 0.80 ± 0.41 % respectively) and a non-specific immune response involving phagocytosis performed by plasmatocytes (42.50 ± 3.39 %). Moreover the plasmatic phenoloxidase (PO) activity has been evaluated spectrophotometrically recording the formation of dopachrome by a non-enzimatic reaction from DL-DOPA substrate and it was expressed as absorbance units at 492 nm/µl of hemolymph. The PO level was low in unstimulated specimens (0.0521 ± 0.0026 A_{492}/µl at 30'; n = 20), while we detected an increase of PO activity (0.0816 ± 0.0153 A_{492}/µl at 30'; n = 26) after the activation of the prophenoloxidase (proPO) into PO with methanol.

Effects of *Steinernema carpocapsae* (Nematoda: Steinernematidae) on biological parameters of larvae responses of *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae

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Rhynchophorus ferrugineus (Coleoptera: Curculionidae) known as the Red Palm Weevil (RPW) is becoming more and more of a problem in Italy, and especially in Sicily, where it is well adapted. The infestations are mainly in the urban areas, and for that reason, chemical control is not advisable. Data from literature show that entomopathogenic nematodes (EPN) particularly *Steirnenema carpocapsae,* have a quite successfully control of RPW. However, results coming from the laboratories are often in contrast with each other and no data are available on precise doses and *S. carpocapsae* seems not be able to reproduce itself in the host. The effect of EPN on the RPW immune system is totally unknown. Different dosages of *S. carpocapsae* and varying durations of exposure were assessed. Larval mortality showed a positive linear correlation with both nematode dosage and the duration of exposure. Median Lethal Dose (DL50) and the Median Lethal Time (TL50) were calculated for older larvae. The number of nematodes that gained access to the haemocoel of larvae was always low, but increased with dosage and exposure time. EPN had also a detrimental effect on larval weight. In this paper we also investigate in vivo and in vitro effects of administered *S. carpocapsae* on the phagocytic responses of *R. ferrugineus* later-instar larval haemocytes. After a few hours, the nematodes were measured in the hemolymph of the insect and it appeared that the immune system was not activated by the presence of these foreign bodies. The nematodes suddenly moulted in the hemolymph totally undisturbed by the hemocytes but they were unable to complete the life cycle and to reproduce. After 24 hours, the number of the hemocytes (THC) recorded in the larvae treated with *S. carpocapsae* was dramatically inferior compared to the THC found in the control larvae. The study of the interaction between EPN and RPW could be crucial understanding the mode of action of EPN in the different instars and the reason for the response to different doses. We also evaluated the defence ability of RPW humoral and cellular immunity system *in vitro* against the bacterium *Xenorhabdus nematophila* associated with EPN, through the Minimum Inhibitory Concentrations (MICs) assay. To our knowledge, this is the first time that such assay has been used to evaluate the ability of insect immune system against entomopathogenic bacterium.

Comparison among the responses of the greater

wax moth, *Galleria mellonella* and red palm weevil *Rhynchophorus ferrugineus* to the entomopathogenic nematodes, *Steinernema carpocapsae*

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The entomopathogenic nematode-bacterium complex of *Steinernema-Xenorhabdus* has high potential as lethal biological control agent against many insect pest species. The Red Palm Weevil (RPW) *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) is an important worldwide palm trees pest. This insect is a quarantined pest, accidentally introduced in Sicily in 2005. The pest is killed by *Steirnenema carpocapsae*, but nematodes are unable to reproduce in the RPW larvae. This research try to understand the reasons of the inability of *S. carpocapsae* to complete its life cycle in the host comparing what happens in one of the most suitable host, the greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae), for this nematode species. *G. mellonella* is a pest of beehives; the larvae feed on wax and do considerable damage to the wax and to honey production in Italy. The lethal doses were comparable if pondered at the weight of the hosts. In both *G. mellonella* and *R. ferrugineus* there were no encapsulation or melanisation responses against *S. carpocapsae*. However *S. carpocapsae* successfully complete its life cycle in *G. mellonella* producing thousands of offspring while just few moults were recorded in RPW larvae and not male and female were found in the RPW larvae cadavers. *Xenorhabdus nematophila* is a gram-negative member of the family Enterobacteriaceae that lives in a symbiotic association with *S. carpocapsae* in a highly effective symbiosis of pathogens. We found that RPW was unable to defence its self against the bacterium. Through viable plate counts we measure the quantity in terms of CFU (Colony forming units)/ml of *X. nematophila* in the *G. mellonella* and in the *R. ferrugineus* hemolymph. The results show that the quantity of *X. nematophila* was higher in *G. mellonella* than in RPW already after 24 h from bioassay. The presented combination of nematode species, the two host species and the bacterium studied in terms of reproduction of the complex nematode-bacterium, is unique to this study.

Time-related changes of hemocyte morphology in the gastropod *Pomacea canaliculata*

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The freshwater gastropod *Pomacea canaliculata* is an established model for eco-toxicological and parasitological researches, and a promising model for the analysis of the hemocyte-mediated immune and stress reactions.

In this study, flow cytometry allowed the categorization of *P. canaliculata* hemocytes into two populations, *i.e.*, small and large hemocytes. Histochemical staining demonstrated that small hemocytes are round cells, with agranular and acidophilic cytoplasm. Large hemocyte population displays a more articulated morphology, with either agranular or granular cells. Agranular cells may have acidophilic or basophilic cytoplasm. Cytoplasmic granules are acidic.

This frame is characteristic of just-withdrawn hemolymph. From 20 up to 40 min after hemolymph collection, changes in large hemocytes have been observed by flow cytometry and light microscopy. Morphological observations showed the presence of large hemocytes with vacuolated cytoplasm, and the increase of the percentage of large granular cells.

In order to test the basic immune-related functions of *P. canaliculata* hemocytes, adhesion and phagocytosis assays were performed. *P. canaliculata* hemocytes rarely adhere on a glass surface as individual cells, and they rather clump into loosely adherent aggregates. A spontaneous phagocytic activity of *P. canaliculata* hemocytes has been demonstrated by transmission electron microscopy. Phagocytosis assay confirmed these observations and indicated that more than 30 % of circulating hemocytes phagocitize heat-inactivated *Escherichia coli* but no phagocytizing small hemocytes were observed.

Concluding, *P. canaliculata* hemocytes may be divided into two populations showing distinct morphological and functional features as well as specific dynamic changes after hemolymph withdrawal.

Differential interactions between *Mytilus* hemolymph components and *Vibrio aestuarianus* and *Vibrio splendidus*: *in vitro* and *in vivo* studies

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Marine bivalves are filter-feeding invertebrates that can accumulate large numbers of bacteria in their tissues, in particular *Vibrio* species particularly abundant in coastal waters. Persistence of different bacteria in bivalve tissues largely depends on their sensitivity to the bactericidal activity of mussel hemolymph, resulting from complex interactions between bacteria and circulating hemocytes. Host–pathogen interactions have been increasingly investigated in different bivalves, with the aim of

understanding the pathogenesis of diseases in species susceptible to infection by certain *Vibrio* spp. and strains. *V. splendidus* strains have been associated with the 'summer mortalities' syndrome of juvenile oysters. On the other hand, the mussel *Mytilus galloprovincialis* is considered to be particularly resistant to *Vibrio* infection.

In this work, in order to explore the susceptibility of mussels to different *Vibrio* species in controlled conditions, the interactions between *Mytilus* hemolymph components and *V. aestuarianus* and *V. splendidus* were investigated *in vitro* and *in vivo*. *In vitro*, the bactericidal activity of whole hemolymph towards different vibrios, the capacity of bacteria to adhere to hemocyte monolayers both in the presence and in the absence of different sugars, as well as their effects on hemocyte lysosomal membrane stability (LMS) were evaluated. The results were compared with those obtained in oyster (*Crassostrea gigas*) hemocytes. The results indicate distinct interactions between mussel and oyster hemocytes and different vibrios.

Mussels were also injected with *V. aestuarianus* and *V. splendidus* and different endpoints (hemolymph bactericidal activity, bacterial concentration in mussel tissues, hemocyte LMS and serum lysozyme activity) were evaluated at 6, 24 and 96 h p.i. Moreover, LMS was evaluated in mussel digestive gland as a biomarker of general stress. The results indicate clear differences in the interactions between mussel hemolymph and different *Vibrio* species, with *V. splendidus* inducing more stressful conditions in the host and showing higher resistance to hemolymph bactericidal activity compared to *V. aestuarianus*. However, the effects of *V. splendidus* were transient, thus confirming the high resistance of *Mytilus* to bacterial pathogens.

Characterization of haemolytic activity of coelomocytes of *Holothuria tubulosa*

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Phylogenetic analysis recognizes echinoderms as a key group of deuterostomes, therefore the species in this group are useful for the study of the evolution of innate immunity responses. In addition, this marine invertebrate lives in coastal and estuarine waters that are directly exposed to potentially pathogenic microorganisms and stressful anthropogenic factors. Coelomocyte populations seem to be essential to immune-defence functions such as phagocytosis, ROI production, cytotoxicity, synthesis and release of antimicrobial substances including lectin, cytokine, C3-like expression, prophenoloxidase activity and capsule formation.

Holothurians' coelomocyte populations contain several coelomocyte types, including phagocytes, and can form brown bodies in response to multicellular parasites. *Holothuria tubulosa* coelomic fluid contains three main coelomocyte categories: amoebocytes, spherulocytes and progenitor cells.

The amoebocytes represent about 30 % of the total population, and in living cell preparations exhibit two distinct forms: petaloid and philopodial; spherule cells represent the numerically dominant cell type (about 60 %). Progenitor cells are present in a lesser amount (about 20 %); they are similar to lymphocytes and show a nucleus that is typically prominent with a thin rim of cytoplasm.

In the present report we show that coelomocytes of *Holothuria tubulosa* are able to exert cytotoxic activity against different cellular targets such as rabbit or sheep erythrocytes and the human erythromyeloid leukaemia-derived cell line K562. Moreover, the unseparated coelomocyte supernatant lysate (CLS) exerts a lytic activity, even in the absence of calcium, against the same targets. Analysis of the coelomocyte lysate by overlay assays with sheep and rabbit erythrocytes on PAGE without SDS showed a protein pattern composed by two main hemolytic bands with different electrophoretic mobility. The one with low mobility (I) showed calcium independent haemolytic activity while high mobility band (II) showed calcium dependent activity. The two bands were eluted from the gel and analyzed by SDS-PAGE and stained with silver stain; band I could be separated in three components of different sizes (52, 42 and 41 kDa), and band II had a size of 43 kDa. Spherule cells seemed to be the effector cells, as shown by a plaque lysis assay. Further studies are in progress to identify the lytic proteins and the lytic mechanism at work on both a molecular and cellular level.

Cloning and expression of glutathione peroxidase genes in the chordate invertebrate *Ciona intestinalis*

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Ascidians represent interesting models from an evolutionary and ecotoxicology point of view, because of their large distribution in temperate sea and their phylogenetic position of invertebrate chordates. Immune responses imply an increase in oxygen consumption with a consequent risk of oxidative stress. With the aim to study the components of the antioxidant defense system in the solitary ascidian *Ciona intestinalis*, we have characterized the genes codifying for two glutathione peroxidases (GPx), metalloenzymes that catalyze the reduction of hydrogen peroxide or organic hydroperoxides to water or corresponding alcohols, using reduced glutathione (GSH) as an electron donor. In the GeneBank database five GPx-like sequences from *C. intestinalis* are present, but only two of those genes demonstrated an effective transcription after cloning and sequencing analyses. The respective proteins, named Ci-GPx7 and Ci-GPxb, show a good level of sequence conservation with metazoan orthologs, especially for residues that are important for the catalytic activity.

In the 3'-UTR region of Ci-GPxb cDNA we have found a typical SECIS-element, confirming that this protein may be a selenium GPx. Phylogenetic reconstruction, performed with Bayesian methods using metazoan GPxs, indicate that Ci-GPxb emerges in the tree with the tetrameric GPxs, confirming as previously hypothesized. Preliminary data, obtained by homology modeling, confirmed the tetrameric structure and show that the GPxb is similar to GPx3 from *Homo sapiens*, a selenium protein. Thus, we propose to name this gene *ci-gpx3*. As expected, Ci-GPx7 clusterized with other GPx7s. The transcription of both these genes, measured by RT-sqPCR, resulted inducible by Cd, Cu and Zn, which have different effects. The peroxidase activity decreases in the cell-free extract from specimens treated with each considered metals, probably in relation to metal-induction of GSH biosynthesis, as indicated by the presence of positive correlation by the time-dependent Cd accumulation an increase of ROS and GSH production. The data presented here improved our knowledge about the evolution of the antioxidant system in metazoans and emphasize the importance of a complex regulation for the antioxidant system, including the transcription of *ci-gpx7* and *ci-gpx3* genes, which can create an efficient detoxification pathway allowing *C. intestinalis* to survive in the presence of metals in the environment.

Session 2. Chairman: G Scapigliati, University of Tuscia, Viterbo, Italy
Environmental stress responses

Amyloidogenesis as stress response: winner or loser process?

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Contacts with foreign molecules from bacteria (LPS), fungi (PMA), parasites, and chemicals can provoke serious stress stimuli able in turn to induce protective responses. The defense answers, due to non-self recognition, belong to acquired immunity, or to innate immunity. In the last case any stress event sets off a mix of responses from immune and neuroimmune systems. Animals generally show fundamental biological principles revealing conserved regulation of the involved processes. This is also true for cellular stress responses, a complex and dynamic process of restoring cellular homeostasis characterized by the same specific stages. We have previously shown that cellular stress conditions promote in different animal models (invertebrates and vertebrates) the same massive morphological and physiological modifications (Fowler *et al.,* 2006; Grimaldi *et al.,* 2012). Any kind of insult mimicking a stress condition (sundry chemical, immune, neuroendocrine and inflammatory) provokes, always and in any type of cell/tissue, detectable series of events that start with ROS over expression, ACTH axis activation, and cytokine such as IL-18 production. These general overexpressions sustain a massive amyloid fibril synthesis that provides a resistant scaffold in turn driving melanin deposition. (Falabella *et al.,* 2011; Grimaldi *et al.,* 2012). Now we show that the amyloid production is not only linked to precursor melanin activation and synthesis but it is fundamentally important to guaranty a state of cellular redox equilibrium due to regulation of ROS presence, *i.e.,* amyloid fibrils production could be considered as a basic cellular compensatory response endeavouring to attenuate oxidative stress in different cell types. Moreover, the relationship between amyloidogenesis and stressors allows to surmize a new background of information on the effects of stress.

Molecular and physiological characterization of *in vivo* Sulfamethoxazole response in *Procambarus clarkii*

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Sulfamethoxazole (SMZ) is one of the most widely sulfonamides employed to treat human urinary infections, in veterinary practice and aquaculture. SMZ, acting as broad spectrum antibiotic microbial drug, blocks the folic acid metabolism. Because of SMZ widespread use considerable amount is indeed expected to be introduced into the environment. The SMZ derived cytotoxicity is mediated by an arylamine bioactivation to the arylhydroxylamine metabolites (S-NOH) of SMZ and it is associated to the generation of reactive oxygen species (ROS). There has been very limited information relating to the toxic potential of SMZ at cellular and molecular level particularly in aquatic or non-target organisms. In the present study, the red swamp crayfish (*Procambarus clarkii*), because of its tolerance to extreme environmental conditions and resistance to diseases, was used as a model organism to profile the molecular and physiological response to SMZ.

Haemato-immunological parameters such as glucose serum levels and total haemocyte count (THC) were alterated as compared to controls; moreover a significative increase in Hsp70 serum level was detected for the first time.

Variation at transcriptional levels of proinflammatory genes (*COX 1* and *COX 2*), antioxidant enzymes (*GST* and *MnSOD*), stress response and Fenton reaction inhibitor genes (*HSP70, MT* and *FT*) were evaluated and alteration in the canonical gene expression pattern emerged. Considering the above exposed results, specific mechanisms involved in maintaining physiological homeostasis and adaptation in response to perturbation

are suggested.

Physiological and agonistic behavioural response of *Procambarus clarkii* to an acoustic stimulus

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The impact of human activity on acquatic habitats can produce adaptive alterations and other significant changes in animals. In recent years, many studies have been carried out with the aim of evaluating the effects of anthropogenic acoustic disturbance on marine and freshwater organisms, thus increasing the awareness of the damage done to animals exposed to human related underwater sounds. This study examined the effects of an acoustic stimulus on the agonistic behaviour and on haemolymphatic parameters of the red swamp crayfish *Procambarus clarkii*. The experiment was conducted in a tank equipped with a video recording system using 6 groups (3 control and 3 test groups) of five adult crayfish (30 specimens in total). After one hour of habituation, the behaviour of the crayfish was monitored for two hours. During the second hour, the animals in the test groups were exposed to a linear sweep (frequency range 0.1 - 25 kHz; peak amplitude 148 dBrms re 1 µPa at 12 kHz) acoustic stimulus for 30 minutes. Exposure to the noise produced significant variations in haemato-immunological parameters as well as a reduction in agonistic behaviour. In particular, the acoustic stimulus induced a decrease in the natural aggressive activity (number of fights and tail flip events) and a significant increase in haemolymph glucose levels and on total serum protein concentration. Also, the number of circulating total haemocytes, of the stressed crayfish decreased by approximately 50 % relative to the initial. A different pattern was observed for the differential haemocytes count. In tested crayfish, a significant increase in hyaline cell number (from 20 % to 59 %, P <0.005) was accompanied by significant decreases in the relative proportions of granular and semigranular cells relative to the values determined for the control group. Furthermore we show for the first time that acoustic stimuli induce Hsp70 overexpression in *P. clarkii* haemocytes as expression of a stress status.

Bacillus thuringiensis* treatment modulate the HSP70 expression in larva and adult brain of *Rhynchophorus ferrugineus

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To study the pathogen-host relationship, we used the model of the entomopathogenic bacterium *Bacillus thuringiensis* (Bt) and *Rhynchophorus ferrugineus*, a quarantine pest that attacks palm trees. In particular, we focused on the Bt stress-induced infections. We studied the effect of Bt on larval and adult growth, and on the expression of the heat shock proteins (HSPs), rapidly synthesized in the cell after exposure to stress including pathogens. Bt has negative effects on larval and adult growth, on total hemocytes counts and on the hemocyte type. HSP70 was evaluated in the supernatant of the brain lysate obtained from larvae and adults fed with sublethal doses of Bt. HSP70 expression was modulated in time (3, 6, 12 and 24 h) in response to Bt ingestion, highlighting that Bt is a stress factor for the *R. ferrugineus*. Further investigation is needed to understand the possible correlation between the reduction of hemocytes and HSP70 modulation. The potential costs of the different bioassay used will be compared.

The toxic pesticide lindane affects the immunological competence of the sea urchin *Paracentrotus lividus*

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Pollution of marine environment has become an international problem. This issue is of particular interest in coastal areas usually subjected to a variety of pressures related to the introduction of high nutrient loads, hazardous chemicals and pathogens affecting marine organisms health The presence of pollutants may affect survival, growth, reproduction, metabolism and immunity of marine invertebrates. The scant available studies demonstrated the effect of environmental perturbations on echinoderm immunological competence. In this framework, in previous works, we evaluated the effect of a high zinc concentration on several immunological parameters of the sea urchin *Paracentrotus lividus* as well as the seastar *Marthasterias glacialis* by comparing zinc treated and untreated specimens. The observed modifications in echinoderm immunological competence led us to conclude that they may give an early indication of disease susceptibility thus suggesting to consider the examined defence mechanisms as potential biological indicators of pollution. To generalize our results other xenobiotics effects have to be examined thus, in the present study, we analyzed the effects of the toxic pesticide lindane on the immunological parameters of the sea urchin *P. lividus*. In particular we evaluated the effect of this xenobiotic on celomocytes haemolytic and lysozyme-like activites as well as antibacterial activity on *Vibrio alginolyticus*. In addition, we examined changes in coelomocytes composition and morphology. The inhibitory action of this pollutant on the immunological defence of the sea urchin was evidenced Thus, taken all together, our

results lead to hypothesize the feasibility of using sea urchin coelomocytes and some humoral parameters as novel biosensors of environmental stress useful for sea urchins disease surveillance and environmental health assessment.

Ambient noise of aquaculture systems: impact on *Sparus aurata* welfare

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The present study evaluated the impact of onshore and offshore aquaculture systems' ambient noise on the welfare of gilthead sea bream juveniles (*Sparus aurata*) through primary, secondary and tertiary stress responses. Some biochemical (cortisol and glucose) and haematological (white blood cell count, red blood cell count, haematocrit value and haemoglobin concentration) indexes of stress and the growth performances of fish were measured. The experiment lasted 120 days during which two different playlists of acoustic stimuli were projected inside six experimental tanks (each condition was replicated in three tanks): offshore aquaculture noise condition that recreated the typical acoustic field in proximity of an offshore sea cage for fish farming using a random sequence of quite sea background and boat noises and onshore aquaculture noise that represented the acoustic field inside an onshore open concrete tank for fish farming. The other three tanks were used as a control condition without acoustic projection. The weights and lengths of fish exposed to offshore aquaculture noise were higher than the specimens in the control and onshore aquaculture groups. Moreover, higher levels of serum cortisol, glucose, red blood cell count, haematocrit value and haemoglobin content and lower levels of white blood cells were recorded in fish groups from the control and onshore treatments. These results allow us to hypothesise that offshore aquaculture noise and the sea soundscape in particular positively influence growth performance and could reduce stress and improve the welfare of the sea bream.

Engineered nanoparticles of titanium dioxide (TiO_2) in a fish species (*Dicentrarchus labrax L.*): uptake and biological effects

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The general aim of this work was to investigate if the engineered nanoparticles of TiO_2 (NP-TiO_2) have access to marine species used as food for humans, and monitor if the nanoparticles may affect their physiology.

NP-TiO_2 is widely used in various fields, such as products for personal use, cosmetics and sunscreens and its use is about two million tons a year. The toxicity to humans is well documented but there is a lack of data concerning the effects on animals and its presence in the marine environment, in particular with regard species widely used for human food and therefore considered potential carriers of NP-TiO_2. In addition, it is known that NP-TiO_2 may bind dangerous contaminants present in traces in marine water such as cadmium (Cd), and hence allow magnification of these poisons in trophic chains.

The present work tried to assess how the engineered NP-TiO_2 may be incorporated in a fish model species and evaluated the biological effects in a sea bass cell line (DLEC). The uptake of NP-TiO_2 has been examined by transmission and scanning electron microscopy, this latter coupled with energy dispersive X-ray spectroscopy (EDX) for particle element detection. The effects of controlled exposure of NP-TiO_2 and NP-TiO_2-Cd to DLEC have been studied evaluating different quantitative parameters related to metabolic functions, such as intracellular ATP concentration and cellular viability. Furthermore, we studied the effects of NP-TiO2 on the expression of target genes associated with innate immune defences (MX, IL-8, COX-2, TGF-beta) by real-time PCR.

Finally, considering that there are some evidences that pre-irradiation of NP-TiO_2 with UV light can promote increased production of free radicals and general toxicity, we used UV irradiation to also investigate the cytotoxic potential of photo-activated NP-TiO_2 on DLEC cells.

Our study supports the notion that nanoparticles can enter fish cells quickly and easily. In addition NP-TiO_2 were non toxic, contrary to what is demonstrated, by cell viability measurements, for the photo-activated NP-TiO_2.

These speculations warrant further studies because of the important implications for environmental and human health risk assessment and preventive actions to limit exposure.

Session 3. Chairmen: U Oreste, IBP CNR, Naples, Italy; V Arizza, University of Palermo, Palermo, Italy
Antimicrobial peptides

***De novo* discovery of antimicrobial peptides from invertebrate transcriptomes**

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The innate defense systems of aquatic invertebrates include antimicrobial peptides (AMPs),

usually small, positively charged molecules effective against a broad range of pathogens. Several AMP families have been widely studies and described in a many different phyla (e.g. defensins) many others represent genus- or even species-specific acquisitions. The methodological advances achieved in the last decade now allow a large scale analysis of entire genomes and transcriptomes of non-model animals. Bioinformatics can be used for the development of *in silico* tools aimed at the mining of large sequence databases and identification of both AMPs belonging to known families and novel candidates satisfying specific user-defined requirements. Here we present a bioinformatic pipeline for the whole-transcriptome scale mining of sequences encoding peptides with a potential antimicrobial activity. Based on the known chemical-physical properties of these bioactive peptides, we developed a Perl script which permits to filter a target sequence file based on user-defined parameters, including sequence length, isoelectric point, amino acid composition or the presence of specific amino acid patterns. All these searches can be performed on windows of variable length to deal with the peptide precursors.

To date, linear AMPs rich in specific amino acid residues have not yet been described in the bivalve *Mytilus galloprovincialis*. About 37,500 putative peptides (ranging from 40 to 120 aa) were translated from the 110K coatings that make up the digestive gland transcriptome of the Mediterranean mussel. Out of these, 932 seem to be secreted. Within this reduced dataset, 14 present at least 30 % content in Lys/Arg or Pro in a sliding window of 30 residues. Four of them also show an extremely basic pI (>10). Moreover, 63 peptides are cysteine-rich, bearing at least 3 disulfide bridges within 30 amino acids. As a positive control, several known Cys-rich mussel AMPs were included among these mined sequences. This result indicate that this approach may be successfully applied to *de novo* transcriptome assemblies of non-model marine and freshwater invertebrates. In this respect we are approaching with this pipeline the transcriptome of the crayfish *Astacus leptodactylus,* and we will also take advantage of the wealth of public data available for non model invertebrate organisms.

Characterization of the salmonid cathelicidins and of their biological activities

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Cathelicidins are a family of cationic antimicrobial peptides (AMPs) and are an important component of the innate immune response.

Two members of this family have recently been identified in salmonids and other fishes. Analysis of the *cath-1* and *cath-2* salmonid cathelicidin gene sequences showed a protein organization with a characteristic conserved cathelin-like N-terminal domain and a varied glycine/serine-rich C-terminal domains corresponding to the active peptides.

In this study we characterized the antimicrobial activity spectrum, the mode of action and tissue expression of salmonid cathelicidins. Different peptide fragments representing specific C-terminal regions of CATH1 and/or CATH2 of rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), grayling (*Thymallus thymallus*) and brown trout (*Salmo trutta fario*) have been chemically synthesized and their antimicrobial activity evaluated against standard bacterial strains and some fish pathogens. Most peptides showed a medium-dependent antimicrobial activity with MIC values ranging from 4 μM to 64 μM. Killing kinetics, membrane permeabilization assays and hemolytic assays indicated that these peptides rapidly kill bacteria by permeabilization of their cell membranes, and at the same time show very low toxicity against erythrocytes. To detect CATH-1 in trout tissues and to study its processing a polyclonal antibody was raised against a complete recombinant CATH-1 protein derived from *O. mykiss* spleen cDNA. Western blot analysis, revealed that CATH-1 is abundantly expressed in spleen and head kidney tissues of trout. Experiments are ongoing to determine eventual CATH-1 post translational modifications.

To evaluate gene expression and tissue distribution of CATH-1 and CATH-2, an *in vivo* experiment was performed infecting *O. mykiss* samples with the salmon pathogen *Yersinia ruckeri* and tissues have been collected at different times and analyzed by Real-Time PCR. Preliminary data evidenced a high induction expression level 24 hours post-infection in spleen (average induction i.e. 10- and 180-fold for CATH1 and CATH2 respectively), head-kidney and in intestine 48 hours after the challenge.

Results will contribute to a comparative understanding of the functions of cathelicidins in the vertebrates.

AMPs and biotechnology application for new generation of medical devices

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While a variety of Gram-positive and negative bacteria as well as fungi have been involved as causative organisms in foreign body-related infections (FBRIs), staphylococci, particularly *Staphylococcus epidermidis* and other coagulase-negative staphylococci (CoNS) account for the majority of infections, both of temporarily inserted and of permanently implanted material. Normally, these bacteria are found as normal inhabitants of human skin and mucous membranes. However, in the appropriate clinical setting, specifically when there is a possible infection of a medical device, CoNS may be associated with considerable hospital

expenditures, morbidity and also an increased mortality rate. (Von Eiff *et al.*, 2002). Antimicrobial peptides (AMPs), principle effectors in the innate immunity, are molecules highlighted as potential candidates to produce new antibiotics, able to fight hard-to-treat infections.

The presence of peptides with antimicrobial and antibiofilm activity was discovered in the sea urchin coelomocytes of *Paracentrotus lividus* (Schillaci *et al.*, 2010). Based on these results, we are studying the possibility to develop a new orthopedic device, able to prevent infections, coated with one of these AMPs.

Individual variability and gene expression specificity of the mussel antifungal mytimycin (MytM)

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Four antimicrobial peptides (AMP) families have been identified and characterized in the Mediterranean mussel, *Mytilus galloprovincialis*: defensin, mytilin, myticin and the antifungal mytimycin (MytM). They are synthesized mainly by hemocytes. Nucleotide sequences were reported as different from one mussel to another.

Here, we reported on MytM gene expression and specificity of induction comparing the responses after challenges. Q-PCR showed that MytM gene expression in circulating hemocytes was dose-dependent. Specificity of the MytM gene regulation has been investigated measuring the expression kinetics of mytilin and myticin genes in fungi injected mussels. *In vivo* challenge with yeasts and bacteria did not increase the expression of MytM gene as measured by q-PCR in hemocytes. On the opposite, injection of spores from the filamentous fungus, *Fusarium oxysporum*, resulted in a rapid and significant up-regulation of MytM gene expression at 9 h post injection. The lack of stimulation suggested the existence of two different signal transduction pathways, one activated by bacteria and yeast, the other delayed and triggered by filamentous fungi. The second response to *F. oxysporum* challenge was significantly lower than the first one, suggesting a less efficient response more than a better protection and arguing against memory.

Then, research focused on the expression of MytM gene in individual *M. galloprovincialis*, before and after *F. oxysporum* challenge by q-PCR quantifying MytM mRNA and *in situ hybridization* (ISH). Only some mussels reacted to the injection by increasing the expression of MytM gene. Differences in mRNA quantification values might result from variable numbers of circulating hemocytes expressing MytM gene.

Thus MytM transcript levels have been quantified from mRNA extracted from the entire posterior adductor muscle 9 h after fungi injection. Variability of MytM gene expression measured in hemocytes was not due to the sampling process, but to the fact that not all the mussels reacted to the challenge with *F. oxysporum*.

Cytology of MytM-expressing hemocytes showed that only granulocytes were labeled, suggesting trafficking of MytM mRNA. Gene expression increased in only some of the injected mussels and no correlation has been found between q-PCR quantification and number of labeled hemocytes as observed in ISH. In conclusion we report here different behavior of individual mussels towards the same challenge.

Inflammatory-like reaction following bacterial injection and antimicrobial peptide isolation from *Anemonia sulcata* (Cnidaria)

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The diversity in the body plan, life and habitat of Anthozoa raises crucial questions related to immunity. Inflammation represents a rapid and efficient elimination mechanism of damaged tissue and microbes, and eventually the restoration of tissue functionality. We demonstrate here the presence of an inflammatory-like response in *Anemonia sulcata* after injection of rabbit erythrocytes or bacteria *Escherichia coli*, *Vibrio proteolyticus*, *Vibrio alginolyticus* and *Shigella putrefaciens,* in the upper part of the basal disk.

The reactions occurring mainly with *E. coli* are observed at 24 h post-injection showed the presence of a swelling in the ascending column of the body and in the basal disk. The reaction developed until to the appearance of a brown capsule. We performed histological analysis of injection sites to characterize the cells and tissues involved in the inflammatory response. In addition we analyzed the crude extract of *A. sulcata* body and tentacles by acid extraction, acid-urea PAGE, HPLC purifications and antibacterial assays. The purification procedure consisted of first step on SEP PAK C8 Vac column followed by several HPLC runs on C18 (INTERCHROM UP5ODB-25QS) using different buffers and running conditions. Among the several peaks, two showing the strongest antimicrobial activity was pooled and lyophilized. These fractions named As-AMP1 and 2 show antibacterial activity against *Micrococcus lysodeikticus* evaluated by the minimum inhibitory concentration (MIC) after 16h incubation at 37°C and Minimum Bactericidal Concentration assays counting the colony-forming-units in dilutions lowers than MIC. Further analyses are in progress for obtaining the primary sequence of As-APMs.

Components of hemocyte extracts from marine invertebrates exert antimicrobial activity

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During the last decade, several biologically active peptides, compounds from 12 to 50 amino acids, have been isolated from a wide range of organisms including mammals, insects, plants and bacteria. These molecules are part of the innate immunity of organisms at all levels phylogenetic, also they have a broad spectrum of action against viruses, bacteria, fungi and protozoa pathogens and are able to destroy microorganisms and/or inhibiting their growth (Boman, 2000; Tincu and Taylor, 2004). Present in blood cells and plasma (Taylor *et al.*, 1997), these molecules show a wide range of mechanisms of action, mainly correlated with the destruction of the microbial cell wall. An *in vitro* study about the inhibitory effect (antimicrobial) of samples isolated from different species of marine invertebrates (*Actinia equina*, *Pelagia noctiluca*, *Procambarus clarkii*, *Cancer pagurus*, *Paracentrotus lividus*), on the growth of pathogenic microorganisms or commensals of man, was carried out. In a representative collection of bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Candida albicans*, *Brucella abortus* and *Brucella suis*) assays chosen to evaluate the antimicrobial activity were: Minimum Concentration Inhibitory (MIC) and Minimum Bactericidal Concentration (MBC). In parallel, in order to establish whether the action of extracts from hemocytes is specifically antimicrobial, was estimated their cytotoxic effect on cells of the type J774-A1. Antibiotics, ampicillin and polymyxin B, were used as a control. The results indicate that cellular estracts from *Actinia equina*, *Pelagia noctiluca*, *Procambarus clarkii*, *Cancer pagurus* and *Paracentrotus lividus* at the concentrations tested, have aspecific inhibitory effect and/or bactericidal action. The extract from *Actinia equina* also showed a dose-dependent cytotoxic activity towards target cells. Based on the obtained results and the current literature, the active fractions will be purified and the mechanism of action will be outlined.