

Report of Meeting

VIIth scientific meeting of the Italian Association for Developmental and Comparative Immunology (IADCI), 10 and 11 February 2005, Consorzio Universitario Provincia di Trapani, Trapani, Italy

Organizers: **N Parrinello, V Arizza, M Cammarata, M Vazzana**

Dipartimento di Biologia Animale, Università di Palermo, Palermo, Italy

Sessione 1. Teleostean immunity: gene expression and cellular response

Lectins in Innate Immunity: Structural and Functional aspects

GR Vasta

Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 East Pratt Street, Suite 236, Baltimore, MD 21202, USA

Humoral and phagocyte-associated lectins constitute critical components of innate immunity in invertebrates and vertebrates. Their roles include not only self/non-self recognition but also downstream effector functions, such as agglutination, immobilization, and complement-mediated opsonization and killing of potential pathogens. Further, lectins and other pattern recognition molecules may function in antigen processing, and as modulators of adaptive immune responses. The diversity in non-self recognition capacity of the lectin repertoire in ectothermic vertebrates, that possess adaptive immune mechanisms less sophisticated than those described in mammals, has remained an open question. Experimental evidence suggests that: (a) lectin repertoires in teleost fish are highly diversified, and include not only representatives of the lectin families described in mammals, but also members of lectin families described for the first time in fish species; (b) although some lectins such as galectins, may bind endogenous ligands and mediate critical functions in early development, others like C- and F-type lectins bind sugars on the surface of potential pathogens. Fucose-binding lectins are present in tissues and fluids from invertebrate and vertebrate species. Well-characterized examples, such as the lectin CPL-III from the tunicate *Clavelina picta* and the fucose-binding mammalian collectins, clearly belong to the C-lectin type. Others, such as FBP32 from the striped bass *Morone saxatilis*, the agglutinin from *Anguilla anguilla* (AAA), and serum "fucolectins" from *A. japonica*, lack a typical sequence motif present in any of the lectin families described so far. Furthermore, because of their specificity

for carbohydrate moieties present on potential microbial pathogens, and their inducibility upon infectious or inflammatory challenge, these lectins are considered as recognition factors in innate immunity. We have characterized the biochemical properties and primary structure of the carbohydrate recognition domain (CRD) of FBP32 and by comparison with other related lectins,

identified the sequence motif that defines a lectin family (F-lectins) which includes members present in organisms ranging from insects (*Drosophila melanogaster*) to ectothermic vertebrates (*Xenopus laevis*). F-lectins exhibit considerable diversity in organization, with CRDs present as single units, such as in AAA, organized in multiple homologous tandem repeats, or associated to other recognition domains. The crystal structure of the AAA enabled the characterization of a structural fold (F-lectin fold), which is shared not only with other lectins from this family, but also with a glycosidase, a glycooxidase, and a human clotting factor. The identification of novel recognition/effector factors that mediate innate immunity in fish opens new possibilities for their use as genetic markers for disease resistance/susceptibility, and the design and implementation of novel intervention strategies. (Supported by Grants MCB-00-77928 from the NSF and RO1 GM58593-01 from NIH to GRV)

Specificity of innate immune response: evidence from antimicrobial peptides

P Roch, M Toubiana, V Bonnichon, J-R Bonami

Pathogens and Immunity, UMR CNRS EcoLag, University of Montpellier 2, cc 093, Place E. Bataillon, 34095 Montpellier cedex 05, France

Antimicrobial peptides are potent humoral effectors of innate immunity. In acquired immunity, the counterpart would be the immunoglobulins characterized by their high target specificity. As small molecules, acting by disrupting the cell membrane integrity, coded by simple genes and

acting on many targets, antimicrobial peptides were generally considered as non specific. Meanwhile, more and more data revealed they are not acting on all targets and not with the unique efficacy. The present lecture will focus on mollusk and crustacean antimicrobial peptides. Molecular structures, both sequences and 3D, strongly suggest specificity. Precise distribution among circulating cells and particular localization in sub cellular compartments, also suggest specificity. Biological activities on bacteria, virus and protozoa, revealed differences in sensitivity of targets, kinetics and molecular structures involved. Finally, gene structures, signal transduction pathways and regulation of gene expression under various experimental conditions, disclosed the specificity of the antimicrobial peptides. Definite evidence will come from quantification of antimicrobial responses in situations closer to natural stressing conditions.

Antarctic teleost immunoglobulin heavy chain: evolution through genes and pseudogenes

S Giacomelli, M Maglione, MR Coscia, U Oreste
Istituto di Biochimica delle Proteine, CNR, Napoli, Italy

The diversification of Antarctic teleost species was driven by the climatic history of the Southern Ocean. In fact their ecological success was depending on the capacity to alter the structural features of the molecules to optimize their function at low temperature (-1.86 °C). In this context the Immunoglobulin molecule is an interesting model to investigate the adaptive evolution. Because of the relatively fast climatic changes, the evolution used a peculiar molecular strategy to modify the genes. Insertion/deletion of many bases, elongation using direct or inverted repeats, insertion of reverse complementary nucleotide segments occurred in coding regions very frequently, extensively modifying the aminoacid sequences. In addition gene duplication increased the number of the evolutive experiments.

To approach this subject, we determined the sequence of cDNA and genomic clones from the IgH locus of different Antarctic teleost. The selected species were: *Trematomus bernacchii*, *Trematomus eulepidotus*, *Trematomus pennelli*, *Trematomus hansonii*, *Trematomus newnesi*, *Trematomus loennbergii*, *Dissostichus mawsoni*, *Gobionothoten gibberifrons*, belonging to the family of Nototheniidae; *Pagetopsis macropterus* and *Chionodraco hamatus* belonging to the family of Channichthyidae; *Cygnodraco mawsoni* and *Gymnodraco acuticeps* belonging to the family of Bathyracoonidae; *Histiodraco velifer* and *Pogonophrine scotti* belonging to the family of Artedidraconidae. The experimental procedures utilized were PCR or RT PCR, using as primers oligonucleotides reproducing nucleotide sequences of *T. bernacchii* CH1 and CH4 exons.

The results obtained showed a wide diversity concentrated in specific regions as that connecting the CH2 and CH3 exons; differential usage of donor and acceptor splicing sites; different length of the introns. In addition pseudogenes, altered in the polyadenilation signal and in the length of the 3' UTR, were also found. Nucleotide homology among gene and pseudogenes of different

species was analyzed to determine their evolutive relationships.

Immunity in *Dicentrarchus labrax* culture: in vivo effects of alginate immunostimulation

L Abelli¹, N Romano², G Scapigliati², P-G Tiscar³, M Bagni⁴, G Marino⁴

¹Dip. Biologia, Univ. Ferrara; ²Dip. Scienze Ambientali, Univ. Viterbo; ³Dip. Scienze Biomediche Comparete, Univ. Teramo; ⁴ICRAM, Roma, Italy

Parameters of cellular and humoral immunity were analysed in *Dicentrarchus labrax* to evaluate the modulatory activity of Ergosan®, an algal extract containing 1% alginic acid. Four cycles using normal fish feed formulation supplemented with 0.5% Ergosan were performed at 60-days intervals (15-days treatment + 45-days suspension). Serum complement (C), lysozyme and total proteins and tissue HSP were measured 15, 30 and 45 days after the first 15-days feeding cycle (short-term) and 45 days after each feeding cycle over a 35-weeks period (long-term). The percentages of B and T lymphocytes in PBL and gut were measured over long-term trial using mAbs against homologous Ig and T cells. Short-term alginic acid treatment raised serum C activity ($p<0.05$) at 15 days and serum C and lysozyme, gill and liver HSP ($p<0.05$) at 30 days. No significant differences with control group were found at 45 days. Over the long-term period, innate and specific immune parameters, survival, growth performances and conversion index did not differ in treated and control fish.

An experimental group received an oral *Vibrio anguillarum* vaccine (AVL, UK) according to the following administration protocol: 5-days vaccine and 0.5% Ergosan diet, 5-days normal diet, 5-days vaccine and Ergosan diet (vaccination cycle, total dose: 0.2 ml/fish). Controls received the same dose of vaccine and were fed with normal diet throughout the cycle. Total and specific serum Ig, C activity and percentages of lymphocytes in PBL and gut were measured 30 days after the start of the vaccination cycle.

Oral vaccine raised significantly PBL Ig+ cells ($p<0.05$) and gut T-cells ($p<0.01$) in the Ergosan group. The titre of specific serum anti-*Vibrio* Ig was higher ($p<0.05$) in Ergosan group compared with controls, while total Ig were unmodified. These effects were accompanied by a rise of PBL (T-cells +12%, B-cells +27%), intestine lymphocytes (T-cells +17%) and serum C (+19%) compared with vaccinated controls.

The present studies in the sea bass indicate very interesting immunostimulatory properties of alginate. *This work was granted by MIPAF Projects 5C68 and 5C116.*

Cloning and expression of TCR coreceptor CD8 α in sea bass (*Dicentrarchus labrax*)

E Randelli, F Buonocore, S Bird¹, CJ Secombes¹, GF Wiegertjes², G Scapigliati

Dip. Scienze Ambientali, Università della Tuscia, Viterbo, Italy; ¹S.F.I.R.C, Aberdeen, UK; ²Dept. Cell Biol. Immunol., University of Wageningen, NL

Cytotoxic and helper T cells recognize endogenously and exogenously derived peptides presented by MHC class I and II molecules via their α and β T cell receptors. This recognition process also involves the TCR coreceptor molecules, CD8 and CD4, which bind to class I and II molecules, respectively and trigger helper and/or killer T-cell activities.

In mammals, CD8 is a dimeric membrane-bound glycoprotein, present on cytotoxic T cells and consisting of either CD8 $\alpha\alpha$ homodimers or CD8 $\alpha\beta$ heterodimers. Both chains are composed of a single extracellular Ig superfamily V domain, a membrane proximal hinge region, a transmembrane domain and a cytoplasmic tail. Recently, the CD8 gene has been found also in cartilaginous and teleost fish like shark, halibut and rainbow trout.

In this work, degenerate primers corresponding to conserved region of the extracellular Ig superfamily V domain, were used to identify a CD8 α homologous gene from sea bass thymocyte cDNA.

Using these primers we obtained a product of 330 bp that, once sequenced and analysed by BlastX search, corresponded to a fragment of CD8 alpha gene.

From this fragment we designed sea bass specific primers that were used in 3' and 5' RACE - PCR to complete the cDNA sequence.

An alignment was performed using available CD8 α amino acid sequences and a phylogenetic tree was generated with the putative amino acid sequences lacking the signal peptide.

Further, sea bass specific primers giving a 220 bp product, were used to analyse the basal CD8 α expression in organs as gills, brain, liver, intestine, head kidney, spleen, peripheral blood leukocytes and thymus.

Molecular characterization of an antimicrobial peptide isolated from leucocytes of the teleostean *Dicentrarchus labrax*

G Salerno¹, M Cammarata¹, N Parrinello¹, P Roch²

¹Marine Immunobiology Laboratory, University of Palermo, Via Archirafi 18, 90123 Palermo, Italy, ²Pathogens and Immunity, UMR CNRS EcoLag, University of Montpellier 2, cc 093, Place E. Bataillon, 34095 Montpellier cedex 05, France

Endogenous antimicrobial peptides (AMP) are widely distributed in nature and are considered as ancestral components in the evolution of innate immunity. A large variety of AMPs were described in all living creatures, and particularly among aquatic organisms. In most vertebrates, antimicrobial peptides are associated with peripheral blood leukocytes and mucosal surface such as skin, digestive tract and lung. The literature reports the presence of AMPs in many Teleostean fish. In Perciformes (Euteleostei, Acanthopterygii), two families of AMPs have been isolated: moronecidin from striped sea-bass, *Morone saxatilis*, and from white bass, *M. chrysops*, and several hepcidin-related

AMPs from white bass, *M. chrysops*, and red sea-bream, *Pagrus major*.

Dicentrarchus labrax (Perciformes, Moronidae) is a valuable species subjected to intensive farming. The present study was undertaken to identify AMPs from *D. labrax*, starting from head-kidney leukocytes. Consensus PCR probes were designed from moronecidins and a fragment of 282 bp was cloned in *E. coli*. The sequence contained an ORF of 240 bp coding for 79 amino acids and representing the complete cDNA. This sequence was named dicentrarcine (AY303949) and shows a strong homology with moronecidine and pleurocidine. The precursor is constituted by a signal peptide of 22 amino acids, followed by the mature AMP of 22 amino acids, and a C-terminal extension of 35 amino acids. *In situ* hybridization was used for tissue location in both naïve fish and fish challenged by one injection of heat-killed Gram negative bacteria, *Vibrio alginolyticus*. Results show that the dicentrarcine is expressed constitutively. The mRNA is located in monocytes and granular cells from head-kidney leukocytes, and in macrophages and granular cells from peritoneal cavity leukocytes. Protein fractions were separated in reverse phase-HPLC of acidic extract from head-kidney leukocytes collected at the interface 34/46% of percoll. Fractions collected from a C8 column were assayed for *in vitro* antibacterial activity against *Micrococcus lysodeitkus*. Active fractions were loaded onto a C18 column and eluted with 25-50% linear gradient of acetonitrile. Only one fraction revealed a clear inhibition of bacteria growth, but was not in quantity enough for sequencing.

Recombinant IL-1 α directly affects intracellular Ca²⁺ concentration on leucocytes of the sea bass (*Dicentrarchus labrax*)

S Benedetti, M Tiberi, E Randelli, F Buonocore, M Mazzini, G Scapigliati

Dipartimento di Scienze Ambientali, Università degli Studi della Tuscia, Viterbo, Italy

Ca²⁺ is an important second messenger that mediates a large number of cellular processes, and under physiological conditions transient changes in intracellular Ca²⁺ concentration may be related to the transmission of extracellular signals.

In this work we further studied the biological activities of cytokines by investigating the effects induced on intracellular Ca²⁺ of leucocytes by recombinant sea bass rIL-1 α . Leucocytes from peripheral blood (PBL), head kidney, gills, spleen and gut, obtained by density gradient centrifugation were loaded with the calcium-binding fluorochrome Fura 2-AM (4 μ M) at 16°C for 45 min, and then stimulated with various concentrations of rIL-1 α . Positive controls have been made with 5 μ M of a calcium ionophore (ionomycin), and emitted fluorescence read on a dual excitation fluorescence fluorimeter that allows simultaneous excitation of fluorescence at 355 and 460 nm. Results showed that rIL-1 α induced a rapid rise in intracellular Ca²⁺ concentration, and a subsequent decrease until 5 min after stimulation. The stimulating effect is dose-dependent with a maximal amount of rIL-1 α of 200 nM, and can be abolished by heat-treatment of rIL-1 α . The stimulating effect can be also affected in a dose-dependent fashion by treatment of leucocytes with

trypsin, thus suggesting a rIL-1 α -receptor involved in the binding. Considering the difference with mammals, where rIL-1 α do not directly affects intracellular Ca²⁺ concentration, experiments are in progress to verify if the rise in Ca²⁺ concentration may activate cellular processes through tyrosine-kinase enzymes.

IMAQUANIM: an integrated approach to study immunoregulatory genes and molecules of teleosts and molluscs

G Scapigliati, E Randelli, S Meloni, F Buonocore

Dipartimento di Scienze Ambientali, Università della Tuscia, Viterbo, Italy

IMAQUANIM is an EU-funded integrated project involving the participation of 22 partners to study molecular and cellular responses of farmed fish and shellfish to pathogen invasion. In this project, sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) are the species subject of interest by our group and for which the creation of EST libraries for immunomodulatory molecules is in progress. At present, for sea bass we have cloned the cDNA coding for IL-1 α , TcR α , TcR β , Cyclooxygenase-2, CD8, GpcRK, and these sequences will be employed for homology cloning in sea bream. In progress is the cloning of interferon-induced MX protein and of MHC class I. Other interesting sequences available in databases for sea bass are for Ig light chain, RAG-1, RAG-2, HSP-70. These and other sequences will be employed in microarray screening of EST from libraries obtained from pathogen-resistant strains of sea bass to monitor the expression of immunoregulatory molecules. In addition, we have established the continuous embryonic cell line DLEC, and we are establishing a continuous head-kidney leucocyte cell line. These cells will be transfected with interesting recombinant immunoregulatory genes for their expression in an homologous piscine system.

Immunity in *Dicentrarchus labrax* culture: effects of oxygen, carbonic dioxide and nitrate

N Romano, G Scapigliati, L Abelli¹, S Picchiatti, V De Molfetta, L Mastrolia, M Mazzini

Department of Environmental Sciences, Tuscia University, Viterbo; ¹Department of Biology, University of Ferrara, Italy

Both non-infectious and transmittable diseases commonly result from improper rearing management and stressful environments. Critical changes of physical and chemical parameters have been associated with onset of disease. In particular, improper temperature, low oxygen or high ammonia and carbon dioxide concentrations could strongly affect the health of cultured fish.

We evaluated the effects of variation of individual parameters: oxygen (from 6 to 12 ppm), NO₃⁻ (from <50 to 200 ppm) and carbonic dioxide (from <10 to 50 ppm) on specific immune response of the sea bass, *Dicentrarchus labrax* (L.). In control and *Vibrio anguillarum*-immunised specimens we analysed: a) percentage of T and B lymphocytes in the leucocyte fraction of blood and gut (by flow cytometry using specific mAbs), b) anti-*Vibrio* serum Ig by captured-ELISA method.

Oxygen effects

Gut T lymphocytes (54.5 \pm 1.9%, P<0.001) and B lymphocytes (2.3 \pm 1.5%, P<0.05) were significantly enhanced in hyperoxygenated (N=10) fish compared with normo-oxygenated group (N=12), while PBL T and B lymphocytes were not significantly different; whereas PBL did not show significant differences in T and B lymphocytes percentages in the tissues. ELISA did not display significant changes (N=25 per group).

NO₃- effects

Vaccination increased specific Ig and circulating and intestinal T and B lymphocytes, while did not increase head kidney Ig⁺ cells. High concentrations of NO₃- apparently enhanced per se PBL B-lymphocytes (N=8, for each group), without modifying serum Ig in immunised groups (N=15 for each group).

Carbonic dioxide effects

High carbonic dioxide concentration decreased significantly (P<0.001) T and B lymphocytes (N=8) and at least by 50% of anti-*Vibrio* Ig (N=20), evidencing a strong effect in the circulating lymphocytes.

This study remarks the sensitivity of sea bass to oxygen concentration or hypercapnia, evidenced by modification of parameters of specific immunity. Sea bass culture under high-flow water recycling and high oxygen concentration and subjected to frequent vaccination boosts is therefore suggested.

This project was financed by MIPAF 4C047 and 5C176.

Lymphocyte activities "in vitro" of the sea bass *Dicentrarchus labrax*

S Meloni, G Zarletti, S Benedetti, E Randelli, F Buonocore, G Scapigliati

Dipartimento di Scienze Ambientali, Università della Tuscia, Viterbo, Italy

In this work we studied some "in vitro" activities of sea bass peripheral blood leukocytes (PBL) against allogeneic PBL inactivated by irradiation. Stimulator PBL were cultured with inactivated allogeneic PBL, and direct counting of lymphocytes was done after 2 and 4 weeks by immunofluorescence and flow cytometry using the mAb DLT15 and DLlg3 specific for T-cells and B-cells, respectively. Results showed a marked increase of T lymphocytes after two weeks in a one way mixed leukocyte reaction (MLR), whereas B lymphocytes had values similar to control PBL. The proliferation of T-cells in MLR cultures was also confirmed by RT-PCR by analysing the expression of the T-cell receptor mRNA. On the contrary, a significant decrease of T-cell proliferation was observed by adding to MLR 5 μ g/ml of Cyclosporin A (CsA). Leucocytes from MLR cultures displayed an enhanced cytotoxic activity against xenogeneic target cells with respect to control PBL, suggesting the presence of cytotoxic T lymphocytes. Cellular activation of PBL in a 2 weeks MLR was tested by measuring antibody-induced intracellular Ca²⁺ mobilization with Fura-2 AM, and resulted increased in MLR and inhibited by CsA. This work represents the first direct quantitative determination of a "in vitro" T-cell activity in a teleost species.

Developmental expression of TcR in lymphomyeloid tissues of sea bass, *Dicentrarchus labrax*

E. Caccia, F. Buonocore, R. Piergentili, L. Abelli¹, G. Scapigliati, L. Mastrolia, S. Picchietti, N. Romano

Department of Environmental Sciences, Tuscia University, Viterbo; ¹Dept. Biology, Univ. Ferrara, Italy

In jawed vertebrates, T cell receptor (TcR) is a membrane molecule composed by a couple of chains: α - α or β - β [Nam et al., J.Immunol. 170, 3081-3097,2003]. RNA codifying for TcR chains is normally expressed throughout the entire T-lymphocyte life, even on early T-cell that had not still rearranged the membrane protein. With the aim to study the appearance and distribution of T cells, sea bass TcR β probes were prepared and used for *in situ* hybridization studies. Immunohistochemical studies (ABC-peroxidase with nickel enhancement) using the anti-T cell mAb DLT15 were made in parallel.

Lymphocytes were first localised as DLT15⁺/TcR β ⁺ cells in the thymic *anlage* around day 35 post fertilisation (pf). TcR β ⁺ cells are found from day 38 in thymus and day 41 in mucosae (e.g. gut, gills). Five days later, TcR β ⁺ cells were scattered in head kidney and four days later in spleen, whereas DLT15⁺ cells were in head kidney already from day 38.

It can be speculated that early T-cells (DLT15⁺/TcR β ⁺) found in the sea bass may be TcR $\gamma\delta$ ⁺, as observed in mammalian thymus [Parham, The immune system, current trends/Garland, 2001]. TcR β ⁺ cells are first rearranged in thymus, then sent to other tissues including the midgut. Traffic of TcR β ⁺ cells *via* blood occurs from day 41 to day 80 pf; thereafter, these cells are mainly in the lymphoid organs. A similar traffic was hypothesised in mammals [Lefrançois & Puddington, *Immunol. Today* 16,16-21,1995].

When epithelial architecture is completed (from day 75 onwards), TcR β ⁺ thymocytes are confined mainly in the cortex/cortical-medullary border. This finding suggests occurrence and localization of positive/negative selection.

Expression and distribution of DIGR1 in the ontogeny of *Dicentrarchus labrax*

ML Di Bella, Mi Vazzana, A Vizzini, M Cammarata, N Parrinello

Department of Animal Biology, University of Palermo, Palermo, Italy

In Teleost fish cortisol is the major corticosteroid hormone released by the interrenal gland under the control of Hypothalamus-Pituitary-Interrenal axis (HPI). It is considered the stress hormone, and it plays many functions including immunomodulatory and inflammatory ones. Cortisol enters into the cells by passive diffusion and, once inside the cells, it binds to a glucocorticoid GR receptor. cDNA of a GR isoform of *Dicentrarchus labrax* from leucocytes separated from head kidney and peritoneal cavity (Vizzini et al., submitted, 2003) has been identified and sequenced. In the present study we report the expression and distribution of DIGR₁ in the larval stages of sea bass by *in situ* hybridization assay with a riboprobe. The ontogeny of lymphoid organs presents the same pattern than other marine species, and

signals of the riboprobe were present in brain, gills, head kidney, trunk kidney, liver, spleen and anterior gut cells. Positive cells were identified in brain cells 3 days post hatching, while in other examined organs the signal appeared later within 50 days. In particular positive cells in head kidney, spleen and gut were observed at 20-25 days post hatching. Further research are in progress to examine DIGR₁ modulation under stress conditions.

Stress and immunomodulation indicators in gilthead seabream (*Sparus aurata*): preliminary data concerning the alterations due to the social behaviour

M Cammarata, D Accardi, C Romano, M Vazzana, A Mazzola, N Parrinello

Dipartimento di Biologia Animale, Università di Palermo, Palermo, Italy

Fish are highly sensitive to stressful conditions that have an effect on the immune system increasing susceptibility to diseases. We examined social stress caused by aquaculture conditions. The hierarchic position (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, and did not change during one year.

To search for stressing effect of social relationship on natural immunity, leucocytes from peritoneal cavity were identified. Phagocytosis assays with *Saccharomyces cerevisiae* were performed by using a chemiluminescence test (CL) after zymosan stimulation of leucocytes and phagocytic index (PI) was calculated. Blood samples and peritoneal cavity leucocytes were obtained avoiding the animal sacrifice.

Plasma cortisol level, glucose and lactate level were evaluated and agglutinating activity assayed. Results showed that plasma levels of osmolarity, glucose, lactate and cortisol resulted higher in subordinate individuals than in dominant ones. In particular, cortisol reach 1.5 times values than controls.

Related to cortisol increase CL values and PI appeared to be lower in subordinate individuals 24 hours after a hierarchical relationship was established. CL and PI values increase at levels higher than the dominant animals after one month, reaching after 3-6 months. On the contrary, dominant animals maintain constant levels. Finally, *in vitro* cortisol treatment affected the CL response of zymosan-stimulated cells showing a direct effect of this hormone on leucocytes.

Evaluation of biomolecular pattern in fish cell line exposed to chemical compounds

C Messina¹, G Catalano¹, S Manuguerra¹, R Vento², A Santulli¹

¹*Ist. di Biologia Marina, Consorzio Universitario di Trapani.*

²*Dip. di Biologia Cellulare e dello Sviluppo, Sez. Biochimica, Università di Palermo, Palermo, Italy*

Interest in fish cell line utilization to evaluate potential environmental risks, determined by chemical pollutants, is growing rapidly. Tributyltin (TBT), an organotin (IV) compound used in antifouling formulation, determines cytotoxicity *in vitro* e *in vivo* in a wide variety of marine organisms. We evaluated the effects of TBT on an established fish cell line, rainbow trout ovary cells (RTO), by studying some proteins involved in triggering apoptosis. Preliminary results indicated that TBT exposure produced a time- and dose-dependent cytotoxic effect, already evident at 0,1 μM TBT. Light microscopy showed that, in comparison with control, after TBT exposure a large number of RTO cells showed a reduction in size and condensed chromatin. This finding was confirmed by fluorescent microscopy, performed by dual staining with acridine orange and ethidium bromide. By this technique, a large number of cells appeared apoptotic, as revealed by the presence of condensed chromatin. Presence of markers of apoptosis, such as activation by cleavage of caspase 8 and caspase-3 and PARP in TBT exposed RTO cells, was demonstrated by Western analysis. We also evaluated the levels of p53, a well known tumour suppressor whose levels dramatically increase after various types of stress. Observed increase of p53 levels suggests an involvement of p53, as consequence of cytotoxic effects induced by TBT.

Immune depression triggered in insects by the bacteria *Xenorhabdus nematophila* and *Photorhabdus luminescens*

M Brehelin

Ecologie Microbienne des Insectes et Interactions Hôte-Pathogène Université Montpellier II, France

Enterobacteriaceae of the genus *Xenorhabdus* and *Photorhabdus* are potent pathogens of a large spectrum of insect species, some strains of which are toxic for immunocompromised human. Insect larvae die in few days after infection. Because *Xenorhabdus* and *Photorhabdus* septicemia arises in the insect body, it is obvious that these bacteria are able to escape defense reactions and especially the cellular ones that are early settled after pathogen penetration. The means by which entomopathogenic bacteria escape the defense reactions are totally unknown. In this review we show that different toxins are secreted by these bacteria and have the insect immunocytes (haemocytes) as main targets. There is a high redundancy in the kind of immunodepressive toxins and in their mode of action.

Kinase-mediated cell signalling in the response of *Mytilus* hemocytes to bacterial challenge

L Canesi¹, M Betti¹, C Ciacci¹, LC Lorusso¹, C Pruzzo², G Gallo²

¹*Istituto di Scienze Fisiologiche, Università di Urbino Carlo Bo, Loc. Crocicchia, 61029 Urbino (PU);* ²*Dipartimento di Biologia, Università di Genova, 16132 Genova, Italy*

Studies on host-pathogen interactions in mammalian systems have shown that certain bacteria can evade the bactericidal activity of the host cell through dysregulation of the signalling pathways involved in the immune response; in

particular, MAPKs (Mitogen Activated Protein Kinases) represent an important target for pathogenic microorganisms. In marine bivalves, persistence of different bacteria (mainly *Vibrionaceae* and coliforms) largely depends on their sensitivity to the bactericidal activity of circulating hemocytes: however, the signaling pathways involved in bacteria-hemocyte interactions are still largely unknown. In the mussel *Mytilus*, differences in interactions between hemocytes and different *E. coli* and *V. cholerae* strains (*E. coli* MG155, a wild type strain carrying Type 1 fimbriae, and its unfimbriated derivative, AAEC072 Δfim ; *V. cholerae* O1 El Tor biotype strain N16961, carrying the mannose-sensitive hemagglutinin-MSHA, and its MSHA mutant) lead to differences in bactericidal activity in the presence of serum. Here we show that different bacteria induced distinct patterns of phosphorylation of MAPKs, in particular of the stress-activated p38 and JNK MAPKs, in mussel hemocytes. Differences in PKC phosphorylation were also observed. The results support the hypothesis that, like in mammalian host cells, different bacteria can modulate the signaling pathways of mussel hemocytes. In particular, the lower bactericidal activity towards the mutant *E. coli* strain and wild type *V. cholerae* compared with that of wild type *E. coli* may be due to a reduced capacity of these strains of activating MAPKs. Moreover, the mutant *V. cholerae* strain, that was the most resistant to the hemocyte bactericidal activity and showed the strongest cytotoxic effect, induced downregulation of both MAPK and PKC signaling. These data suggest that certain bacteria could evade the bactericidal activity of mussel hemocytes through disruption of the host signalling pathways.

Effects of TNF α in mussel hemocytes: role of kinase pathways

M Betti¹, C Ciacci¹, LC Lorusso¹, B Canonico², L Canesi¹

¹*Istituto di Scienze Fisiologiche,* ²*Istituto di Scienze Morfologiche, Università di Urbino Carlo Bo, Loc. Crocicchia, 61029, Urbino (PU), Italy*

TNF α (Tumor Necrosis Factor α) is a pleiotropic cytokine that plays a pivotal role in orchestrating innate immune responses as well as in apoptotic processes. TNF α triggers several intracellular pathways, including the MAPK (Mitogen Activated Protein Kinase) cascade, which can control gene expression through modulation of transcription factors. Invertebrate counterparts of TNF α signaling have been identified in *Drosophila*; a cytokine network is also active in molluscs, and molluscan hemocytes are responsive to heterologous cytokines.

In this work, the possible effects of TNF α on hemocyte signaling and function were investigated in the bivalve *Mytilus*. The results demonstrate that TNF α induced lysosomal membrane destabilization and depression of phagocytic activity in mussel hemocytes. These effects were rapidly followed by irreversible cell damage, as evaluated by flow cytometric analysis, and were paralleled by persistent increases in phosphorylation of

the stress-activated MAPKs p38 and JNKs and of the transcription factor STAT1.

However, in the presence of hemolymph serum, the cytotoxic effects of TNF α were significantly reduced. Interestingly, a transient and reversible PS (Phosphatidyl Serine) exposure was observed; such an effect was associated to a rapid, large, but transient stimulation of the stress-activated MAPKs and of the transcription factor STAT1.

The results demonstrate the presence of conserved components of TNF α signaling in molluscan immunocytes; moreover, these data indicate that hemolymph serum components actively participate in modulating the pathways involved in maintaining the balance between survival/death stimuli.

Sessione 2. Invertebrate immunity: cellular response

Anticipating Innate Immunity Without a Toll

EL Cooper

Laboratory of Comparative Neuroimmunology Department of Neurobiology School Of Medicine at UCLA University of California, Los Angeles, USA

When we think of the immune system, we can now consider it from an evolutionary viewpoint. There is resurgence of interest in immune-defense systems of invertebrates, such as fruit flies and earthworms. Their properties can be characterized as innate, natural, non-specific, non-anticipatory, and non-clonal. Innate immunity operates through leukocytes that are not components of the macrophage T and B systems that characterize vertebrate adaptive immunity whose properties can be categorized as adaptive, induced, specific, anticipatory, and clonal. In this review, we will focus on the earthworm system. Earthworms, in particular, like other complex invertebrates, possess several leukocytes and the molecules that they synthesize and secrete. Together they effect phagocytosis, encapsulation, agglutination, opsonization, clotting and lysis of foreign components. At least two major leukocytes: small coelomocytes, (SC) and large coelomocytes (LC) mediate lytic reactions against several targets. Destruction of tumor cells *in vitro* reveals the dissociation of phagocytosis from natural killer cell responses. A third type, the chlorogogen cell (CC), synthesizes and sheds effector lytic molecules. Among the lytic molecules, three have been identified and sequenced (fetidins, CCF-1, lysenin) and another has been discovered (eiseniapore) three others H₁ H₂ H₃ share agglutinating and lysing functions and Lumbricin I is the only small antimicrobial but non-lytic molecule. Cellular and humoral components function to distinguish between self and not self, dispose of internal (cancer?), damaged components and external antigens (microbes). Innate immunity evolved as an essential survival strategy in all living species. The innate immune system is capable of recognizing conserved microbial structures or products of microbial metabolism (pathogen associated molecular patterns [PAMPs]) through a set of germ line encoded receptors called pattern recognition receptors (PRRs). The PRRs of the innate immune system, particularly the family of Toll-like receptors (TLRs) is responsible for initiating inflammatory response against invading pathogens. Toll and

Toll-like receptor signaling is essential for phagocytosis and antimicrobial peptide production in insects and vertebrates. There is a need to examine the situation with respect to Toll and TLRs in earthworms in the face of the above information. Their presence or absence would be of enormous interest.

Effect of immunostimulants on immune gene expression in decapod crustaceans

VJ Smith

Gatty Marine Laboratory, University of St Andrews, St Andrews, Fife, Scotland, UK

The expansion of the crustacean aquaculture industry over the last two decades years has been accompanied, like all intensive farming enterprises, by the growing problem of disease in the stock animals. As the use of conventional vaccination programmes are inappropriate for shellfish, interest has focused on non-specific enhancement of the crustacean immune system, particularly by compounds known to activate the prophenoloxide activating system. Several are now marketed commercially. Whilst claims have been made by some authors for the success of these substances when included in the diet, other arguments have been forward that their use may be less than beneficial. Indeed, recent work on lobsters has revealed that some may actually be cytotoxic for the haemocytes *in vitro*. In addition, molecular analyses with quantitative real-time PCR or other approaches are beginning to show that these compounds do not up-regulate immune gene expression, as might be expected. Thus, the efficacy and value of immunostimulation in aquaculture remains questionable. This talk will discuss the effects of immune stimulants on the crustacean host defence system and consider whether or not immune-potentiating strategies are the best way forward for disease control in these animals.

Evolution of Innate Immunity. Components of inflammatory reaction in *Ciona intestinalis*

N Parrinello, F Giaramita, A Vizzini, M Di Bella, D Parrinello, M Vazzana, C Ciancialo, V Arizza, M Cammarata

Dipartimento di Biologia Animale, Università di Palermo, Palermo, Italy

There are several reasons for analyzing tunicate immune systems and phylogenesis. First, they can be established as primitive models, ancestors of vertebrates, for understanding fundamental immunological mechanisms by analyzing their cells and molecular products; inflammation is the first defence system that includes tissue response to foreign agents locally injected. Second, molecular and morpho functional study coupled with molecular approach are requested to characterize molecules (e.g. cytokines) in a phylogenetic context. Third, tunicates are protochordates, pivotal in phylogenetic reconstruction. The body wall of *Ciona intestinalis* mounts a defence inflammatory reaction to

LPS. The kinetics of the response, cell components and histochemical aspects have already been described. In *Botryllus schlosseri* the non fusion reaction shows inflammatory characters. The comparative study is of interest because these ascidians belong to distinct clade and present different life cycle. Cytokine –like molecules have a basic role in inflammatory responses. We will examine cells and molecules, by using several approaches including immunochemistry and immunohistochemistry, protein purification methods, specific antibodies production, functional assay of purified proteins. Protein and gene sequence data will contribute to prepare primers, cDNA sequences and riboprobes to evaluate hemocyte and tissue expression and modulation (*in situ* hybridization, real time PCR). With the aim of verifying the cytokine-like role, functional approaches will be carried out to modulate immune responses as phagocytosis and cytotoxicity. Here we show an opsonizing activity of *C. intestinalis* hemolymph that can be significantly decreased by absorption with anti-human-IL1.

(Research granted by MIUR PRIN Cofin 2004)

Molecular evolution of innate immunity and mitochondrial genome in Ascidiacea

C Gissi, F Iannelli, G Pesole

Dipartimento di Scienze Biomolecolari e Biotecnologie, Università di Milano, Milano, Italy

Systematic comparative analyses of complex gene "categories" in chordates, such as genes belonging to a given metabolic pathway or involved in a particular cellular process, can shed light on the evolutionary dynamics of functionally related genes and reveal specific evolutionary differences between vertebrates and tunicates. Moreover, by comparing the evolutionary dynamics of many genes, suitable molecular phylogenetic markers can be identified to reliably reconstruct the chordate phylogeny.

In general, a molecular phylogenetic tree depicts the relationships between the analysed sequences and is not necessarily coincident with the organism tree. Conversely, congruent phylogenies obtained from several genes strongly suggest that the inferred gene trees reflect the underlying species phylogeny. Therefore, the comparison of phylogenies derived from many unrelated genes, such as nuclear and mitochondrial genes involved in unrelated processes, will provide an opportunity for robust reconstruction of the tunicate/chordate phylogeny.

We have recently started a project focused on molecular evolutionary and phylogenetic studies of unrelated cellular processes, the innate immunity and the mitochondrial system, in Ascidiacea. The molecular phylogeny and evolutionary dynamics of innate immunity nuclear genes will be compared with those of mitochondrial genomes (mtDNA). Indeed, given its reliability as a phylogenetic marker, the mtDNA will be used as reference frame for molecular phylogenetic studies. We want to use incongruencies between the inferred nuclear and mitochondrial gene phylogenies to identify and characterize genes evolving in vertebrates and ascidians under different evolutionary dynamics, as the effect of different selective constraints or change of functions.

In order to increase the sample of ascidian mtDNAs available for phylogenetic studies, the mtDNA of *Phallusia mammillata* and *Phallusia fumigata* (Phlebobranchia, Ascidiidae) were completely amplified and sequenced. Such genomes show extensive gene rearrangements not only compared to other chordate and ascidian mtDNAs, but also at intra-genus level. The base compositional features, the shortness of rRNA genes, and the absence of a main non-coding region indicate that the evolutionary dynamics of ascidian mtDNA differ markedly from those of vertebrates.

Morula cells and non-fusion reaction in the compound ascidian *Botryllus schlosseri*

L Ballarin, F Parisotto, F Cima

Dipartimento di Biologia, Università di Padova, Padova, Italy

Morula cells (MC) are a common haemocyte-type in the compound ascidian *Botryllus schlosseri*, their frequency ranging between 40 and 60% of the circulating blood cells. They are the effector cells of the non-fusion reaction, characterised by the appearance of necrotic foci along the contact border, which occurs when genetically incompatible colonies contact each other. We previously demonstrated that, in the course of this reaction, MC acquire immunopositivity to anti-cytokine (IL-1- α and TNF- α) antibodies, degranulate and release the enzyme phenoloxidase which is responsible of the cytotoxicity observed both *in vitro* (when haemocytes are incubated with blood plasma (BP) from incompatible colonies) and *in vivo* (non-fusion reaction). Subsequently, MC leave the facing marginal ampullae (sausage-like, blind endings of the colonial marginal vessels to reach the tunic, apparently attracted by soluble, diffusing factors, where they degenerate and contribute to the formation of the cytotoxic spots. In the present work, we focussed on the chemotactic recruitment of MC in the course of the non-fusion reaction. As a first approach, we studied the distribution of MC inside the facing marginal ampullae of both contacting colonies (either non-fusible or fusible) and solitary ones. Results clearly indicates a significantly higher concentration of MC inside facing marginal ampullae of incompatible colonies with respect to compatible colonies; the latter concentration is, however, significantly higher than that inside ampullae from solitary colonies. In addition, we used Transwell chambers to evaluate whether incompatible BP has chemotactic properties. We put haemocyte suspensions in filtered sea water (FSW) in the upper wells and BP from either incompatible or compatible colonies in the lower wells. We observed a significant increase migration of haemocytes, and of MC in particular, in the presence of incompatible BP. This migration was significantly decreased by the addition of anti-cytokine (IL-1- α , TNF- α or IL-8) antibodies, suggesting that molecules recognised by these antibodies can be responsible of the chemotaxis observed.

Responses of *Botryllus schlosseri* immunocytes to exogenous cytokines: results and perspectives

A Menin, L Ballarin

Dipartimento di Biologia, Università di Padova, Padova, Italy

We studied the effects of recombinant TNF- α , IL-1- α and IL-8 and of antibodies raised against mammalian cytokines (polyclonal anti-TNF- α and anti-IL-1- α , monoclonal anti-IL-8 and anti-IL-12) on phagocytes of the colonial ascidian *Botryllus schlosseri*. In particular, we analysed the ability of phagocytes to assume an amoeboid shape (expressed as amoebocytic index) and to phagocyte yeast cells (expressed as phagocytic index). rTNF- α and rIL-1- α have no effects on both the amoebocytic and the phagocytic index, whereas rIL-8 significantly increases both the indexes. The observed increase in the phagocytic index was absent in the presence of calphostin C 0.1 μ M, an inhibitor of PKC, and of H89 1 μ M, an inhibitor of PKA, indicating the involvement of both the cAMP and IP3 pathways in signal transduction required for phagocytosis to occur. The IL-8-induced increase in amoebocytic and phagocytic index was not observed when haemocytes were pre-incubated in the presence of suramin 0.7 mM, a protein G inhibitor. Anti-TNF- α , anti-IL-1- α and anti-IL-8 antibodies significantly reduce the above indexes; no effects were observed in the presence of anti-IL-12. Our results suggest the presence of molecules able to cross-react with mammalian antibodies, involved in the regulation of phagocyte behaviour.

Heliothis virescens/Toxoneuron nigriceps: parasitoid strategies for successful endoparasitic development

M de Equileor, R Ferrarese, M Brivio, M Mastore, A Grimaldi, G Tettamanti, R Valvassori

Dipartimento di Biologia Strutturale e Funzionale, Università degli Studi dell'Insubria, Varese, Italy

In the association host/parasitoid, *Heliothis virescens* (Lepidoptera, Noctuidae) shows a combination of different associated mechanisms to kill its parasitoid *Toxoneuron nigriceps* (Hymenoptera, Braconidae) which, in turn, has to escape encapsulation and melanization by using systems that interfere with host capacity for immunologic recognition.

The possibility of recognizing the non-self is due, in *Heliothis*, as for all insects, to humoral and cellular defence reactions. It is well known that eggs of parasitoid *Toxoneuron nigriceps* co-injected with venom (i.e. ovarian calyx fluid) and polydnaviruses (PDV) directly into the host hemocoel are able to re-program the host behaviour, its reproductive potential and its immunity system (Pennacchio et al., 1998). The data presented focus on the dramatic suppression of immune responses by parasitization occurring in *Heliothis* larva at different times from wasp ovodeposition. We report the description of some temporary alterations of the immune defences of *H. virescens* during the very early parasitization stages of *T. nigriceps*. In addition, due to the fact that successful endoparasitic development requires, especially during the first phases of parasitization, numerous strategies enabling parasite to rapidly growth and to protect itself, we also describe the function of serosal membrane enveloping 1st instar larva of *T. nigriceps*.

Biological activities and cytotoxic effects of aqueous extracts from *Petrosia ficiformis* cells

P Pagliara, L Dini

DiSTeBA-Dipartimento di Scienze e Tecnologie Biologiche e Ambientali, Università degli Studi di Lecce, Lecce, Italy

Many marine organisms represent an important source for bioactive natural products with pharmacological properties. It is well known that, also if potential biomedical activity and chemical defence cannot be equated, nevertheless they can be considered strictly correlated, being bioactive compounds often produced by invertebrates as elements of a chemical defence mechanism. Some of these compounds resulted efficient anti-inflammatory, anti-tumor, immunosuppressive and cytotoxic agents.

In this framework, an important role is played by sponges. They are characterised by a simple structural organization, lacking of tissue and organs, and possess cells with the peculiar ability to change shape and function, showing in this way an unusual totipotency capability.

In this work we report our investigation on biological activity of the marine sponge *Petrosia ficiformis* cell lysate. Haemolytic, haemagglutinating and cytotoxic activities of these extracts were monitored. We found that cell extracts do not possess haemolytic or haemagglutinating activity against human blood erythrocytes. On the contrary, a cytotoxic effect on a fibroblast-like cell line was detected: the vitality of Detroit 550 was drastically reduced after treatment with sponge extracts.

The influence of cell aqueous extracts was also evaluated on brine shrimps vitality and on sea urchin development. Sponge cell lysate strongly affected vitality of *Artemia salina* brine shrimps, as well as the sea urchin development, that resulted in an accelerated process with an increase in abnormal embryos production.

Defensive response of the freshwater crayfish *Astacus leptodactylus* following three different challenges in vivo

S Lorenzon, PG Giulianini, S Battistella, M Bierti, EA Ferrero

Dipartimento di Biologia, Università di Trieste, Trieste, Italy

This study assesses the *in vivo* response of the freshwater crayfish *Astacus leptodactylus* (Eschscholtz, 1823) following three different challenges (from a natural system to a completely artificial one) by controlling at the same time the THC (total hemocytes count) as immunological parameter and glycemia as a blood stress one. We investigated the effect of injection of 1) *Pseudomonas* sp. different amounts (200 μ L of 1×10^8 , 1×10^7 , 1×10^6 live bacteria mL⁻¹), 2) different doses of LPS (lipopolysaccharide) from *Pseudomonas* sp (0.01, 0.001,

0.0001 mg g⁻¹ of living weight), 3) three kinds of polystyrene latex beads (1.1, 3.0 µm in diameter and carboxylated polystyrene latex beads 0.9 µm in diameter) and 4) sterile PBS and bled only animals as controls.

Both injection of PBS and bled only experiments showed an increase in THC in the first 2h. Injection of bacteria as well as of LPS caused a dose related decrease of THC that lasted until 24h; the lowest doses of both treatments on the contrary stimulated an increase of THC. All polystyrene latex beads produced an increase of THC that resulted more evident in the case of carboxylated polystyrene latex beads. When considering glycemia, both *Pseudomonas sp.* and LPS induced again a dose related response. No significant variation of glycemia was induced by the response elicited by 1.1 µm in diameter polystyrene latex beads while carboxylated latex beads resulted the most effective in inducing hyperglycemia.

High doses of bacteria and LPS caused a loss in immunodefence and a stress response. On the contrary low concentrations of bacteria, LPS and the three kinds of latex beads induced an activation of the immunosystem causing an increase of THC and, in particular, the carboxylated latex beads result the artificial system that best simulates the natural challenge.

Ultrastructural and functional characterization of circulating hemocytes from adults and larvae of *Carabus lefebvrei* Dejean 1826 (Coleoptera, Carabidae)

A Giglio¹, S Battistella², FF Talarico¹, M Romeo¹, T Zetto¹, PG Giulianini²

¹Dipartimento di Ecologia, Università della Calabria, Arcavacata di Rende (CS); ²Dipartimento di Biologia, Università di Trieste, Trieste, Italy

In the context of comparative studies on immunity defence mechanisms of adults and larvae of the coleopteran *Carabus lefebvrei* Dejean 1826, the circulating hemocytes of the third instar larval stage and adults has been investigated by means of transmission electron microscopy (TEM). Phagocytosis assays were performed *in vivo* by injection of polystyrene latex beads in order to identify the cell types involved in this cellular response. 50 µL of carboxylate-modified polystyrene latex beads 0.9 µm in diameter diluted 1:10 in 0.15 M phosphate buffered saline pH 7.4 (PBS) were injected with a 26-gauge needle in the abdomen of 5 larvae and 5 adults. Parallel controls were run with untreated animals and with animals injected with 50 µL of sterile PBS alone. After 2 h individual animals were cold anesthetized and the last two abdominal segments laterally torn; a 26-gauge needle was inserted in the neck membrane and PBS slowly injected. The two first drops exiting through the tear in the abdomen were collected directly in 2.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M cacodylate buffer pH 7.4 and, the hemocytes pelleted for 10 min. The pellets obtained from pooled hemolymph of the five animals were then post-fixed in 1% osmium tetroxide and embedded in Embed812/Araldite. Sections were observed with a TEM Philips EM 208.

Four types of hemocytes were found in the hemolymph of larvae and adults of *C. lefebvrei* and they were identified as prohemocytes, granulocytes, plasmotocytes, and oenocytoids. Prohemocytes are the smallest circulating

hemocytes with an oval/irregular profile and a high nucleus/cell surface ratio. Granulocytes are oval, elongated cells with a maximum diameter up to 13 µm, they are characterized by electron dense granules (up to 15 per section) with a round to elliptical profile and a mean diameter of 465±118 nm (n=10). Plasmotocytes are large, irregular cells with a maximum diameter up to 15 µm and they represent, after injections, about 70% of total circulating hemocytes, 10% of which presenting up to 11 phagocytized latex beads. They are characterized by numerous electron dense granules with a mean diameter of 214±74 nm (n=18). They present a large euchromatic nucleus with a prominent large nucleolus and a well-developed rough reticulum. It was demonstrated that the plasmotocytes are the only hemocyte types involved in phagocytic response of foreign particles both in larvae and adults.

Mussel response to anthropic pressures measured by HSP70 gene expression in Q-PCR

C Cellura¹, M Toubiana², N Parrinello¹, P Roch²

¹Marine Immunobiology Laboratory, University of Palermo, Via Archirafi 18, 90123 Palermo, Italy; ²Pathogens and Immunity, UMR EcoLag, University of Montpellier 2, cc 093, Place E. Bataillon, 34095 Montpellier cedex 05, France

Coastal environment constitutes a fragile ecosystem submitted to several anthropic pressures. Large variety of marine animals is living in coastal shallow water. Among them, bivalve molluscs are generally considered as good sentinels to evaluate environmental changes. As a first approach to investigate the response of bivalves through quantification of key gene expressions, the present study focussed on experimental heat-shock effect on expression of HSP70 gene in mussel, *Mytilus galloprovincialis*. Adult mussels were submitted to a single heat-shock consisting in 90 minutes immersion in 30°C sea water. Before heat-shock, and at 0, 3, 6, 9, 12, 15, 18 and 24 h after heat-shock, hemocytes were collected from posterior adductor muscle. Total RNA was extracted and converted into cDNA using reverse-transcriptase. As non available, we designed consensus primers to amplify a HSP70 cDNA fragment of 296 bp. Nucleotide sequence revealed identities of 99.4 % with *M. edulis* (AF172607), 80.1 % with oyster *Ostrea edulis* (AJ318883) and 73.6 % with oyster, *Crassostrea gigas* (AJ271444). Quantification of HSP70 gene expression was done by real-time PCR (or Q-PCR) using the Roche Light Cycler and SYBR Green. According to literature, we chosen 28S ribosome as house keeping gene, designed primers and considered its expression as constant through the experiment. Expression of HSP70 gene improved during the first 6 h, and returned to baseline after 24 h. Significance of both, the phenomenon and the calculations, will be discussed.

Early developmental stages of WSSV in *Marsupenaeus japonicus*

VA Di Leonardo¹, N Parrinello², J-R Bonami¹

¹Marine Immunobiology Laboratory, University of Palermo, Via Archirafi 18, 90123 Palermo, Italy; ²Pathogens and Immunity, UMR EcoLag, University of Montpellier 2, cc 093, Place E. Bataillon, 34095 Montpellier cedex 05, France

The White Spot Syndrome Virus (WSSV) is an highly pathogenic agent for marine and freshwater crustaceans, so it is highly dangerous for crustacean farming. We examined two shrimps, *Palaemon sp.* and *Marsupenaeus japonicus* treated *per os* with a WSSV preparation from sick animals. In addition treated animals, after five days, were furtherly infected through intramuscular injection of suitable virus preparation. Among the diagnostic tools we used, PCR and Dot-Blot were able to identify the virus present in the acute phase of injection, whereas *in situ* hybridization (ISH) gave positive signals just at a un precocious stage of the infection. *Palaemon* treated *per os* were not affected by WSSV, but the syndrome was evident when they were furtherly injected. Already, *M. japonicus* was sensitive to *per os* treatment, as revealed by ISH on cells of intestinal epithelium, gills and Oka lymphoid organ. Finally, in *M. japonicus* were observed by TEM, early developmental stages of WSSV in Oka organs and intestinal epithelium. Virus development model is proposed.

Natural immunity in *Paracentrotus lividus*: coelomocyte cooperation

V Arizza, F Giaramita, M Cervello, M Cammarata, N Parrinello

Dipartimento di Biologia Animale, Università di Palermo, Palermo, Italy

Paracentrotus lividus coelomic fluid contains several coelomocyte types that have been identified as amoebocytes, uncoloured spherulocytes (US), red cells and vibratile cells. Previous studies on coelomocytes, revealed that amoebocytes show phagocytic activity and are able to encapsulate foreign particles, in addition they release haemoagglutinins; USs show *in vitro* cytotoxic activity against rabbit erythrocytes (RE) as revealed by plaque forming assay.

In this study, the coelomic fluid was fractionated through a discontinuous Iodixolol gradient, and four cell bands (B1-B4) were obtained. Each of them appeared to be enriched in a particular coelomocyte type: B1 mainly contained amoebocytes; in B2 are present vibratile cells and little amount of amoebocytes; B3 was enriched with USs, and B4 contained red cells. The reported results support the phagocytic activity of amoebocytes assayed with yeast cells, whereas the separated US population were not able to show their *in vitro* cytotoxic activity (plaque forming assay) against RE. When amoebocytes ($1 \times 10^6 \text{ml}^{-1}$) from the enriched fraction were added to USs (1:1), this activity was restored revealing a coelomocyte interaction. Since supernatant from amoebocyte cultures, added to the reaction medium, was able in inducing US cytotoxicity, the interaction seems to be based on substances released from amoebocytes. Western-blot assay with anti-human IL1 α antibodies (Sigma) showed that, in a short time, cultured amoebocytes release interleukin-like molecules. On the other side, protein preparations from US cell membranes cross-reacted with anti-human IL1-R antibodies. Further studies are requested to characterize the molecules that contain human IL1 and IL1-R epitopes.