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REPORT OF MEETING

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Organizers: **G Scapigliati, AM Fausto, M Mazzini, N Romano, F Buonocore, S Picchietti, MC Belardinelli**

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Session 1. Fish Immunity
Chairman: Giuseppe Scapigliati, University of Tuscia, Viterbo, Italy

Lecture
Evolution of immunity - from invertebrates to vertebrates

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Interspecific communication between various organisms in the environment is relying on a series of basic biological processes which can be found in both invertebrates and vertebrates. Even the most primitive unicellular organisms, such as amoebae, must find a balance between tolerance to surrounding elements and highly protective responses against potentially pathogenic microorganisms. The balance is well illustrated by the process of phagocytosis: this is a way for a cell to import energy and nutrition to sustain life processes but the process can also engulf and inactivate invading pathogens when combined with a range of effector molecules. These events can be supported under different environmental conditions. We presume that primitive unicellular organisms, solitary or residing in colonies, during the relatively early history of the Earth, more than 1000 MYA, occupied anaerobic sediments. This environment challenged survival of the local inhabitants which possessed advanced cellular machinery to extract energy from the substrate and at the same time were able to recognize and respond to pathogen associated molecular patterns. This early development of pathogen and danger recognition receptors is still the basis for survival in more developed animals appearing after the Cambrian explosion. Although photosynthesis and thereby oxygen placed an additional evolutionary pressure on the early organisms, and thereby gave rise to new life forms, a crucial conservation of an anaerobic environment with its associated microorganisms in the intestine of higher animals, plays a pivotal role for host immunity. Concordantly, the importance of the gut microbiota in advanced mammals, such as man, has been well documented for a number of immunologically determined diseases (e.g. diabetes and IBD). Teleosts represent one of the earliest vertebrate groups with a developed adaptive immune system comprising MHC, T-cell receptors and several classes of immunoglobulins. As a range of basic innate effector molecules are available as well, it is worthwhile to study basic immune mechanisms (both innate and adaptive) in fish. This is illustrated by the rainbow trout, *Oncorhynchus mykiss*, which can be immunized against the enterobacterium *Yersinia ruckeri* by several routes. Vaccination studies using this host species show how timing and administration of antigen (injection, immersion, bath, oral) can

provide new insight into the delicate balance between development of vertebrate immunity and tolerance.

In vitro and *in vivo* immunoreactivity of different betanodavirus species

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Betanodaviruses are the causative agents of the viral nervous necrosis (VNN), a devastating disease for the Mediterranean mariculture. Four different betanodavirus species are recognized, Striped jack-, Redspotted grouper-, Tiger puffer-, and Barfin flounder nervous necrosis virus (SJNNV, RGNNV, TPNNV and BFNNV), but there is little knowledge on their antigenic properties.
Recent studies indicate that the SJNNV and the RGNNV are antigenically distinct, constituting serotypes A and C respectively. The natural reassortant viruses RGNNV/SJNNV and SJNNV/RGNNV group within serotypes A and C respectively, indicating that the coat protein encoded by RNA2 acts as major immunoreactivity determinant.
The aim of this study was to investigate the possible *in vivo* cross-protection in sea bass of these two betanodavirus species using a RGNNV and a SJNNV in-house produced vaccine.

Ten ml of RGNNV (strain 283.2009) and SJNNV (strain 484.2.2009) were inactivated with 1% formalin for one week at room temperature. Two groups of 60 juvenile European sea bass were IP immunized with 0.1 ml/fish of one inactivated virus solutions each. An additional group was mock vaccinated with PBS 0.01 M to act as positive control. After 30 days post vaccination, 10 fish from each tank were bled to death and sera collected for immunological analyses. The remaining fish were IM challenged with 283.2009 RGNNV and kept at 25 °C for 28 days. Considering that sea bass is poorly susceptible to SJNNV, challenge was performed with the RGNNV only.

The mortality rate for each experimental condition was calculated and compared through statistical analysis. Results showed a RPS of 81.2 for inactivated RGNNV and 25.3 for SJNNV, confirming that the cross-protection of the two species is very limited. Immunological analyses showed an appreciable seroconversion of the RGNNV- and SJNNV-vaccinated fish against the homologues antigens, but very low cross-reaction against heterologous virus, confirming the *in vivo* results.

Recently, several betanodavirus outbreaks in sea bream occurred in the Mediterranean basin and they were associated to a reassortant strain RGNNV/SJNNV. The data obtained in this study could be highly informative for the development of cross-protective vaccines against different betanodavirus genotypes.

Computational modeling as a tool to predict a more effective fish vaccination

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The validation of vaccine protocols and the determination of the optimal dose for the populations to be treated can be immunological simulated by innovative approach in bioinformatics sciences. Recently we have administered an anti-vibriosis vaccine by an innovative route treatment for the farmed sea bass juveniles were vaccinated against *Listonella anguillarum* (LA) using a commercial formulation (Sharing-Plug) administered by double immersion or by double immersion and subsequent injection *via* i.p.; in both cases, fish have shown an appreciable protection against a subsequent infection. The *in vivo* findings were compared with the *in silico* experimentation through the use of the immunological model C-ImmSim, an Agent Based Model which describes both the humoral and cellular response of immune system. The grid is three-dimensional with periodic boundary conditions, representing a known volume of a lymphoid organ or peripheral blood. The vaccination steps were reproduced by injecting "un-harmful" antigen at those t-steps matching the *in vivo* experiment protocol. The challenge was simulated by injecting a known amount of "active" antigen in the grid. Calibration tests were carried out to select the appropriate doses of vaccine and infectious bacteria concentrations to be used in the simulation experiments. The outputs of the model agree with the immunological analysis of Ab production (anti-L/serum), BcR and TcR gene transcripts in the spleen, allowing to follow the temporal evolution of the immunological response all over the trials. Consequently to appreciable results *in vivo* and *in silico*, a new vaccination protocol has been tested by using two pathogens: *LA* and *P.damselae sb*. *piscicida.* Data were compared with the *silico* model, suitably modified with the

new parameters of the experiment. Thus, for the first time a C-ImmSim model has been applied for a multiple vaccination. The results have indicated a good performance of the model capacity to reproduce the *in vivo* experimentation. The use of an immunological simulator in fish vaccination trials confirms the suitability of ABM models for the simulation of biological systems, providing an useful tool to be exploited as a virtual laboratory where experiments can be made prior to the design of a real *in vivo* or *in vitro* experimentation.

Catalase in Antarctic fish

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When phagocytes are strongly activated, molecular oxygen can be converted into reactive oxygen species (ROS) to damage the microbes. To scavenge the excess of ROS, aerobic organisms have evolved enzymatic and non-enzymatic antioxidant systems. Catalase (CAT, EC.1.11.1.6) is an important member of the enzymatic antioxidant system and can effectively catalyze the decomposition of hydrogen peroxide (H_2O_2) to keep the balance between de novo H_2O_2 generation and efficient elimination, which is essential for innate immunity. With the aim to increase the knowledge on this proteins in Antarctic teleosts, we characterized the genes codifying for CAT in three species: *Trematomus bernacchii*, *Trematomus hansoni* and *Chionodraco hamatus*. The gene sequences were determined by PCR amplification and sequencing, and by transcriptome analysis. Multi-alignment analysis, performed with fish orthologous sequences, demonstrated high conservation of the amino acids involved in catalytic activity also in CAT of Antarctic species. However, some substitutions with polar amino acids, are characteristics of some residues close to the motifs that are important for the functionality of this protein. The CAT gene transcription from various tissues (gills, heart, liver, and skeletal muscle) of *T. bernacchii* and *C. hamatus* was measured by qPCR. In both species, CAT mRNA is preferentially expressed in liver. CAT activity was also measured in the same organs. Again, the highest level is present in liver. The tissue-specific differences in the mRNA and active protein accumulations are probably related to the physiological characteristic of these organs.

More insights into evolution of IgT genes from Antarctic fish

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Immunoglobulin T (IgT) represent the most ancient Ig isotype found only in teleosts, and specialized in mucosal immunity. In our previous studies, *IgT* heavy chain constant region genes of the Antarctic teleost *Trematomus bernacchii*, belonging to the Nototheniidae family, were characterized. These studies highlighted that *T. bernacchii* IgT lack most of the heavy chain second constant domain, retaining only a few amino acid residues, such as proline, glycine and cysteine (Coscia *et al.*, Marine Genomics, 2015). We suggested that this region may act as a hinge region similarly to that found at the CH2-CH3 boundary of the Antarctic IgM heavy chain. Analysis of cDNA clones encoding IgT heavy chain revealed the presence of three different sized IgT transcript variants, named Long (L), Short (S) and Shortest (Sts). Genomic analysis of *T. bernacchii IgH* locus revealed the presence, within the intron between the exons coding for the entire first and second constant domains, respectively, of a reminiscence of the ancestral second exon. The current work is aimed at expanding our knowledge on evolution of *IgT* genes to other Antarctic Notothenioid families. IgT nucleotide sequences have been determined from *Chionodraco rastrospinosus* (Family Channichthyidae), *Gymnodraco acuticeps* (Family Bathydraconidae), *Pogonophryne scotti* (Family Artedidraconidae), *Trematomus hansoni,* and *Gobionotothen gibberifrons* (Family Nototheniidae). The sequences were aligned with the three variants of *T. bernacchii,* and with the sequence from *Notothenia coriiceps*, previously isolated from an online available *N. coriiceps* transcriptome data set. Based on cDNA sequence alignment, our analysis confirmed that the lack of almost the entire CH2 domain is a feature common to Antarctic fish. In particular, *G. gibberifrons* and *N. coriiceps*, both belonging to the Nototheniidae family, showed a shorter CH2 domain than *T. bernacchii* variant S. Moreover, in *C. rastrospinosus* a duplicated region, 9-nt long, was found between CH2-CH3. This duplication is probably a trace of the event leading to the loss of most CH2 domain through transposable elements. Analysis of deduced amino acid sequence of the duplicated region highlights the conservation of cysteine residues, suggesting that these residues could be required for the assembly of the two heavy

chains in light of the loss of most second constant domain.

Diversity evolution and immune function of fish lectins

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Fish are equipped with a complex lectin repertoire that, like mammals, are involved almost all the immune reactions.

Carbohydrate recognition and interactions mediated by lectins have been recognized involved in vertebrate innate immunity, not only for recognition of potential pathogens, but also acting in the agglutination, immobilization and other functional steps.

In fish, C and F types lectins, galectins, rhamnose-binding lectin (RBL) and pentraxin have been identified in both in both cartilaginous and bony fish. In addition, selectins and other genes have been found in the currently available fish genomes.

On the basis of our results on F-type (FBL) and RBL lectins we showed that:

-lectin repertoires in 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 like the FBL and RBL;

-these lectins have been identified in the eggs and embryos but also are present in the serum;

-tissue-specific expression and localization of the diverse lectin repertoires and their molecular partners is consistent with their distinct biological roles in innate and adaptive immunity;

-although some lectins may bind endogenous ligands, others bind sugars on the surface of potential pathogens; in addition to pathogen recognition and opsonization, some lectins display additional effector roles, such as regulation of immune functions.

Session 2. Invertebrate immunity
Chairmen: Magda De Eguileor, Università dell'insubria, Varese, Italy

Allorecognition in the ascidian *Botryllus schlosseri*: the importance of amyloid

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In the compound ascidian *Botryllus schlosseri*, allorecognition manifests primarily as colony specificity, controlled by a highly polymorphic Fu/HC locus, so that contacting colonies sharing at least one allele at the Fu/HC locus can fuse into a chimeric colony; if no alleles are shared, a typical inflammation reaction occurs, with the recruitment of a specific hemocyte type, the cytotoxic morula cells (MCs), inside the tips of the ampullae (the blind termini of the tunic vasculature) extending towards the alien colony, their extravasation in the tunic and their final degranulation with the consequent release of factors inducing autocrine and paracrine cell and the formation of necrotic, melanic spots (points of rejection; PORs) along the contact border.

MCs are the first cells to sense nonself and are the source of cytokines that induce the recruitment of immunocytes at the inflammatory sites and the activation of phagocytes required for the clearance of foreign material. MCs store quinones, polyphenols and the enzyme phenoloxidase (PO) inside their granules, representing their cytotoxic potential. Although PO, quinones and polyphenols are soluble factors, it is remarkable that the deposition of melanin and the cell death is confined to the immediate outside of the ampullar tips, suggesting that the diffusion of the enzyme and the products of its activity is, in some way, prevented in order to limit cytotoxicity to the immediate neighbourhood of degranulating MCs. With this idea in mind, we looked for factors released by MCs that could limit the spreading of cytotoxicity and melanisation. We found that MCs contain amyloid inside their granules and that amyloid fibrils are released in the surrounding medium upon MC degranulation forming a net-like scaffold entrapping PO and melanin, thus limiting their diffusion. In addition, the search for genes and factor controlling both melanogenesis and amyloidogenesis, revealed an evolutionary conserved machinery involved in the processes and an unexpected cross talk between the two *Botryllus* immunocyte types, *i.e.*, phagocytes and MCs. This work represents the first demonstration of a physiological role of amyloid in protochordata immunity.

Bio-molecular perspectives on anti-tumour properties of the ciliate toxin climacostol

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The incidence of melanoma skin cancer has risen sharply over past few decades, making

melanoma one of the most common tumours in Caucasian worldwide. Considering that metastatic melanoma is almost completely resistant to most current therapies and is linked with a poor patient prognosis, the need for additional drugs is urgent. Several modern drugs, including those for cancer therapy, have been isolated from natural sources. Herein we focused on the biological activities of climacostol, a natural toxin, now available by a new and straightforward synthesis, produced by the freshwater ciliated protozoan *Climacostomum virens*. Climacostol inhibits cell growth and reduces viability/proliferation of B16 mouse melanoma cells. To evaluate the climacostol therapeutic potential the pharmacological properties and the molecular signature associated with its actions have been investigated *in vivo*. We showed that climacostol treatment exerts an effective inhibitory action on the progression of B16 melanoma allografts, thus increasing animal survival and, at the same time, decreasing the expression of the proliferation markers pSTAT3, ki67, and survivin. . At molecular level, climacostol rapidly leads to DNA damage and apoptosis. In particular, the signalling events responsible for the climacostol-induced pro-apoptotic effects rely on the up-regulation of p53 network that, in turn, activates the intrinsic programed cell death pathway (mitochondrial dysfunction, ROS, Bax/cytochrome c, caspase-9/3). Finally, our results indicated that climacostol inhibits the autophagic flux in melanoma cells, likely through the modulation of the Akt/mTOR pathway. An increasing number of evidence reveals that perturbation of the autophagic machinery contributes to tumour development. also revealing multiple connection points between pro-survival autophagy and detrimental apoptosis.

Overall these results indicate that climacostol is a powerful and efficacious anti-cancer agent that it may be considered for the design of cytotoxic new drugs for melanoma therapy.

Host-parasite interactions: what is the role of the antioxidant system in immune memory of the red flour beetle *Tribolium castaneum*?

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The consequence of an aerobic metabolism is the production of oxygen derivatives named Reactive Oxygen Species (ROS). The production of ROS, for their unstable electronic configuration, could lead to oxidative stress with harmful consequences on organisms, unbalancing redox status and promoting cell death. Despite the damage potential of ROS, their production has not been eliminated during evolution, since they can be involved in many physiological, immunological and molecular processes that contribute to fitness. The importance of ROS homeostasis on cell survival is also demonstrated by effects of an abnormal level (lack or overproduction) that results in pathologies such as cancer, cardiovascular and neurodegenerative diseases in vertebrates. The role of ROS in host-parasite interactions is defined by their contribution to innate immunity, promoting parasite death through active participation during infection processes. Moreover, ROS are also assumed to play a role in acquired immune responses, such as immune priming, i.e. a form of memory in invertebrates (Roth *et al.,* 2009). Investigating the involvement of ROS in host-parasite interactions, many studies focused on phenoloxidases and NADPH-dependent oxidases only, potentially neglecting the importance of other related proteins, such as antioxidant enzymes. Superoxide dismutases (SOD) and catalases (CAT) have pivotal roles in the antioxidant system and contribute to ROS homeostasis under many physiological conditions. In the present study, we characterize SOD and CAT genes in red flour beetle *Tribolium castaneum*. We used the most recent transcriptomic and genomic databases, in order to identify genomic regions that potentially code for these enzymes, and we studied the promoter regions of their genes for the identification of putative ROS-related regulatory sequences. Moreover, we performed an immune priming experiment with the Gram-positive entomopathogen *Bacillus thuringiensis,* in order to identify the potential role of SOD and CAT in immune memory of this species.

The C1q domain-containing protein from the ascidian *Botryllus schlosseri* manifests *a* cytokine-like behavio

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Genes encoding complement component 3 (C3) have been extensively investigated in invertebrate genomes and traced back in evolutionary history to the early metazoan radiation. However, other components of the complement system, such as those related to the classical activation pathway, are still not much investigated. Currently, the genes encoding for proteins with a C1q domain, probably the main components of the classical pathway, have been only partially investigated from an evolutionary perspective.

These genes exist in many of the sequenced genomes, from both vertebrates and

invertebrates and functions have been described for some of the corresponding proteins.

A C1q-like gene have been identified in the medicinal leech *Hirudo medicinalis* where a C1q-like peptide elicits a chemotactic behavior that could be blocked using a human antibody against the gC1q receptor.

C1q-like genes have also been found in the urochordate *Ciona intestinalis* and the cephalocordate *Branchiostoma floridae* where it has been demonstrated that the globular domain is able to recognize and bind mammalian antibodies initiating the classical pathway of complement activation.

The globular head C1q domain is a lectin domain present in transcriptomes of amphioxus, lamprey, and several teleost fishes. Few of these putative C1q-like proteins have been characterized; however, they can bind to a variety of carbohydrates.

In *Botryllus schlosseri* we have found, in our EST colletion, a single transcript with C1q characteristics (BsC1q-like). The deduced protein contains two globular head C1q domains, a feature unknown in invertebrates. As regard Vertebrates, we can find a similar architecture only in mammals, in the so called C1q/TNF-related Protein 4 (CTRP4). This protein is very poorly studied and seems to be expressed in the hypothalamus and contribute to the modulation of food intake and body weight.

Our data, from the colonial ascidian, suggest a role for the BsC1q-like protein as mediator of the activation and degranulation of the cytotoxic hemocytes. Both ISH and ICC demonstrate that both cytotoxic morula cells and phagocytes express the BsC1q-like mRNA and protein; functional analyses demonstrate that the human antibody against globular head C1q is able to inhibit morula cell degranulation after bacterial challenge.

It is not yet clear if it is possible to considered this molecule as member of the complement system in *Botryllus* but future analyses will be directed to the study of the functional relationships between BsC3 and BsC1q-like as well as of the binding capabilities of the latter.

The medicinal leech as a useful tool for studying haematopoiesis, angiogenesis and innate immune response

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In the last decade, a large body of evidence has demonstrated that hematopoiesis, angiogenesis, immune response and cytokines regulating the occurrence of these processes have been highly conserved across Vertebrates and various invertebrate phyla. Here we provide a broad overview of medicinal leech hematopoiesis and we highlight the benefits of using the medicinal leech, having a simple anatomy and a repertoire of less varied cell types when compared to Vertebrates, as a powerful model for studying basic steps of hematopoiesis and immune responses. In particular, leeches' hematopoietic stem precursor cells (HSPCs), deriving from the activated botryoidal tissue, seem to share with their Vertebrates counterpart most morpho-functional and molecular mechanisms. HSPCs are massively recruited from the bloodstream towards sites of inflammation in response to injury, bacterial injection, or experimental administration of cytokines and contribute to immune response, neovascularization and muscle regeneration. Further, similar to Vertebrates these cells are driven toward a leukocyte, endothelial or myogenic, differentiation pathway, as a function of the time course of VEGF release to target cells. Since investigations on hematopoiesis can greatly help scientists and clinicians to better understand the pathological processes behind blood disorders, cancers and muscle regeneration, HSPCs of medicinal leech can be used as a powerful model system for understanding tissue stem cells and their role in angiogenesis, tissue regeneration and oncogenesis.

Cellular responses induced by multi-walled carbon nanotubes: *in vitro* study on the medicinal leech macrophages

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The intrinsic characteristics of engineered nanoparticles, such as multi-wall carbon nanotubes (MWCNTs) are impressive and attractive for technology, however their environmental dispersion could be potentially hazardous to animal and human health. Since the production and use of MWCNTs is steadily increasing, intentional or unintentional environmental discharges may occur. For this reason, the development of new methods and the identification of reliable models to completely understand MWCNTs effects are critical.

Here we propose a freshwater invertebrate, the medicinal leech, as alternative model to assess the effects of MWCNTs on immune system. Our previous studies have already demonstrated that *in vivo* MWCNT treatment induces the activation of leech's macrophages. In this study, we focus on the direct effects of MWCNTs on these cells. We used the consolidated experimental approach, based on the injection in the leech body wall of the biomatrice Matrigel (MG) added with the cytokine Allograft inflammatory factor-1 (*Hm*AIF-1), to specifically chemoattract macrophages.

Cells extracted from MG were cultured and characterized with the specific markers CD45 and CD68, confirming their belonging to the monocyte-macrophage lineage. Primary macrophage cultures were then subjected to an *in vitro* treatment with MWCNTs at different concentrations (2.5, 5, 10, 25, 50 and 100 µg/ml).

Our results indicate that leech macrophages, once in close contact with MWCNTs, actively produce amyloid material to encapsulate the foreign bodies. We also demonstrated that MWCNTs in vitro treatment cause the decrease of cell proliferation rate and the increase of the apoptotic rate. Furthermore, since oxidative stress is linked with inflammation and amyloid production, reactive oxygen species has been evaluated, confirming that their production rate increases after MWCNT treatment.

Our combined experimental approaches, not only attest the ability of MWCNTs in inducing a potent inflammatory response, but also confirm the medicinal leech as a good alternative model that can be improved and successfully used to study the possible harmful effects of any nanomaterial. Moreover, since autophagic cell death pathway activation is emerging as a possible consequence of MWCNT treatment, in the future we will attempt to clarify this aspect in order to completely understand MWCNT-induces toxicity.

The human recombinant RNASET2 activates the initial phase of the inflammatory response in the medicinal leech

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Recent studies have demonstrated that RNASET2, the only member of the ribonuclease T2 family present in human genome, is involved in the control of tumorigenicity in ovarian cancer cells. Furthermore its capacity to be a chemoattractant for numerous cells of monocytic-macrophage line and the possibility to act as an inducer of the innate immune response in Vertebrates have been established. In fact, in tumor tissues the detectable cross-talk between cancer cells and the surrounding tumor microenvironment is based on these aspects. Although several studies have been reported on the molecular features of RNASET2, the details on the pathways by which this evolutionarily conserved protein regulates the immune system are still poorly defined. In order to better elucidate these aspects, we report here the effect of the human recombinant RNASET2 injection and its role in regulating the innate immune response after bacterial challenge in an invertebrate model, the medicinal leech. This animal has been chosen for its very simple anatomy and for its rapid inflammatory response. Indeed, after few hours from the injection, a large number of fibroblasts are visible in the connective tissue that appears completely remolded and infiltrated by numerous cells expressing the specifics macrophage markers CD68 and *Hm*AIF1. In order to confirm the ability of this ribonuclease to attract macrophages, we used a consolidated experimental approach based on the injection in the leech body wall of the Matrigel biomatrice (MG), supplemented with the human recombinant RNASET2. After one week, the extracted MG sponges was infiltrated by numerous cells $CD68^+$ and *Hm*$AIF1^+$. Moreover, in the leech body wall challenged with lipopolysaccharides (LPS) or with the environmental bacteria pathogen *Micrococcus nishinomiyaensis,* endogenous RNASET2 is highly expressed by the numerous macrophages migrating to the site of inoculation. Taken together, these results clearly suggest that RNASET2 is likely involved in the initial phase of the inflammatory response in leeches.

Session 3. Invertebrate immunity
Chairmen: Davide Malagoli, University of Modena and Reggio Emilia, Modena, Italy

Genomic immune system of ciliates: DNA elimination as a genome defense mechanism

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Whole genome sequencing analyses are providing compelling evidence that pro- and eukaryote microbes, like multicellular organisms, have their life threatened by parasitic attacks. Bacteria and Archea face invading viral nucleic acids with an 'inheritable DNA-encoded immunity' (known as the CRISPR-Cas system) that recognizes foreign DNA from self DNA. In eukaryotic microbes that are exposed to invasions from both bacteria and viruses, bacteria are promptly made harmless either by digestion into food vacuoles, or by 'domestication' as symbionts. But the defence from viral attacks is much less effective. Foreign viral sequences can randomly insert into the cell genome, and may disrupt or deactivate vital genes.

To fight this threat, ciliates rely on a unique model of inheritable genomic immune mechanism based on the evolution of two genomes, a germ-line one lying in the cell micronucleus and a somatic one lying in the macronucleus. The germ-line genome characterized by an orthodox chromosomic organization exposed to invasive viral DNA sequences is maintained transcriptionally silent. Only the somatic genome characterized by a unique sub-chromosomic organization is expressed. It is generated de-novo in

coincidence with every sexual event from a copy of the micronuclear genome that is previously made free of any invasive DNA sequence by the activity of a small RNA-targeted DNA-deletion mechanism, called 'Internal Eliminated Sequences (IES)-associated gene system' by ciliatologists. The discovery, function and effects of this mechanism that eliminates invasive DNAs from the developing somatic genome will be the object of this contribution.

Assessment of morphological and cellular responses after infection with living bacteria in the marine mussel *Mytilus galloprovincialis*

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Bacterial strains of *Vibrio* genus associated with temperate regions are linked to mussels-borne infections. The sedentary nature of marine mussels, *Mytilus galloprovincialis*, together with their filter feeding combine to ensure that they have the potential for considerable exposure to infective agents.

The primary mechanism of bivalve internal defense involves hemocytes responsible for cell-mediated immunity through a panel of activities such as phagocytosis, the release of cytotoxic molecules, reactive oxygen intermediates, lysosomal enzymes, PO enzyme and lysozyme.

In this work *in vivo* infection of *M. galloprovincialis* with living *V. splendidus* to collect hemolymph from the posterior adductor muscle 1, 3, 6, 9, 12, 24 and 48 h post-injection was carried out.

Previously we have found that when bacteria were injected into the circulation of the mussel, the number of living intra-hemocyte bacteria dramatically increased already after thirty minutes, suggesting intense phagocytosis, then decreasing until 24 h. The quantification by flow cytometry indicated a variation of proportions of the three cell categories.

In our study, injection of living bacteria resulted in total hemocyte count (THC) higher than normal 24 - 48 h post-injection, suggesting proliferation and/or recruitment of hemocytes which are mainly concentrated in the site of infection.

To compliment this, here histological and immunohistochemical assessment was performed using adductor musclein order to evaluate the morphological features and cellular response spost bacterial infection. The morphological analysis showed changes in tissue organization, with an altered cell volume and recruitment of hemocytes among fibers.

The change of osmotic equilibrium across muscle cell membranes was observed by increased the staining of Na-K ATPase during the entire period of stimulation, and reduced immunopositivity of aquaporin (AQP) 1 h post infection but with an increasing trend in the all experimental steps.

The investigation on cellular turnover showed a tendency to recover a regular tissue structure, as highlighted by an intense immunopositivity of proliferating cell nuclear antigen (PCNA) from 24 h to 48 h post injection, as well as cell surface death receptor (FAS) and the cysteine-aspartic acid protease (CASP3) untilto 72 h post bacterial injection.

Thus, a detailed overview of the morphological and cellular responses in the mussel adductor muscle following infection with living bacteria was provided herein.

Involvement of exosomes in immune responses

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Exosomes are small-secreted microvesicles interacting with surrounding cells and implicated in intercellular communication. Besides their signaling function, these microvesicles also serve as a mechanism to dispose obsolete cellular material.

Exosomes transport proteins, lipids and nucleic acids in the form of miRNA and mRNA moreover, bearing cytokine or death receptors, they can bind to specific ligand of cells and induce signal transduction. Every tissue fluid analyzed so far (milk, saliva, tears, urine, blood, etc.) has revealed the presence of exosomes and the range of organisms that release small vesicles, including exosomes, covers almost all known life forms. Depending on the tissue of origin, they can regulate a large number of processes as brain development and function, tumor invasiveness and immune system function.

About this last issue, it is already accepted that exosomes play a crucial role in host-pathogen interactions being produced during viral, parasitic, fungal and bacterial infections and could either promote or inhibit host immunity. Just as exosomes can become agents of dissemination, they may otherwise act as agents of control within the body when unaffected. Vesicles secreted by infected cells contain substantial amounts of pathogen molecules, which are sufficient to induce modifications in

non-infected neighboring cells or act as antigen presenters for the immune system. When antigen-presenting cells secrete exosomes bearing MHC bound to pathogen antigens, an activation of humoral and T helper responses occurs.

Studies on exosomes other than mammalian cells are scant, but still support the role of exosomes as a conserved mechanism of communication throughout evolution. The release of these microvesicles has been shown in some processes of invertebrate species (*Drosophila melanogaster, Caenorhabditis elegans, Hirudo medicinalis and Crassostrea gigas*) but their role in invertebrate immunity is poorly investigated. In this framework we searched for the presence of exosomes in the hemolymph of *Mytilus galloprovincialis*, that could represent a good model in some biomedical research areas, such as inflammatory processes. By differential centrifugation, we isolated small microvesicles (10 - 100 nm, exosomes) and we started to study their release in naïve and LPS-stimulated animals. Their proteomic characterization and the investigation on their potential role in inflammatory response are in progress.

Sequence diversity and antimicrobial activity of myticalins

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Myticalins (*Mytilus* cationic linear AMPs), first identified by an *in silico* transcriptome mining approach, represent the first family of antimicrobial peptides devoid of disulphide bridges ever reported in bivalve molluscs. While the protein precursors present conserved signal peptide and pro-peptide regions, they possess highly divergent mature peptide regions, which can be categorized within four subfamilies (A, B, C and D). In stark contrast with the other AMPs previously described in mussels, myticalins are not produced by circulating hemocytes, but they are mainly expressed in the gill tissue. Following the demonstration of a significant antimicrobial activity by myticalin A4b, we selected and synthetized on solid phase six additional peptides, namely myticalin A8, B1, C6, C9, D2 and D5. Minimum Inhibitory Concentration microbiological assays revealed that myticalins have a broad spectrum of activity, targeting both Gram-positive and Gram-negative bacteria, although the specificity and strength of action appears to be strictly dependent on the primary sequence of the peptide. In addition, we revealed that, in spite of the high number of sequence variants identified at the transcriptome level, just a limited number of myticalin loci are present in the genome of *Mytilus galloprovincialis*. We investigated more in depth the sequence diversity of myticalin genes in a mussel population, highlighting that this AMP family is characterized by presence/absence genetic variation. Furthermore, we confirm that myticalins represent a taxonomically restricted gene family only found in *Mytilus* spp.

Session 4. Invertebrate Immunity
Chairman: Loriano Ballarin, University of Padova, Italy

Telocytes in invertebrates

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Vertebrate telocytes (TCs) are stromal cells described in humans and rodens cavitary and non cavitary organs such as heart, gut, gallblader, uterus, lung, pancreas, mammary gland, skeletal muscles. Telocytes, present in many types of tissues, are strategically located among specific resident cells, close to the capillaries and to the nerve endings. TCs are always characterized by a small cell body, containing the nucleus and very thin, long tubular processes named telopodes (TPs) that are considered the ultrastructural peculiar trait of these cells. Each telopode, long and convoluted is moniliform showing thin tubes (podomers) alternated with dilations (podoms). TCs may contact with each other and with virtually all type of cells forming a close network. The cross talk among TCs and the other cells can be direct via gap junctions, and/or indirect via the release of extracellular vesicles (multivescicular bodies, exosomes). TCs are hypothesized as an extensive intercellular information transmission system able to modulate homeostasis, stem cell activity, and other functions working as a primitive nervous system at the cellular level.
Our study shows the existence of these type of cells in invertebrate body wall and we firstly identified telocytes in medicinal leech body. An ultrastructural portrait and immunophenotypic characteristics as well as the possible role played by invertebrate telocytes has been proposed.

First evidences of a complement system lytic pathway in invertebrates: data from the compound ascidian *Botryllus schlosseri*

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The complement system is well studied in mammals, where more than 30 proteins have been described, involved in the activation and regulation of this important humoral effector. However, the evolutionary history of the complement system is not yet fully elucidated and, in recent years, it has been widely demonstrated that complement system is more than just a defender against intruders. For instance, it is important for the clearance of apoptotic cells and corpses.

Botryllus schlosseri is a cosmopolitan ascidian, belonging to the phylum Chordata, considered a model organism for the studies of the evolution of the immune system.

Studying the complexity of the complement system in *Botryllus,* we identified a transcript, in our EST collection, coding a protein containing the MACPF (membrane attack complex/perforin) domain, shared by both most of the proteins involved in the lytic pathway and perforins. In vertebrates, we know that perforins are produced by T-lymphocytes and natural killer cells, whereas the activity of the proteins, with the MACPF domain is regulated by the complement component C3/C5.

Comparing the domain's topology of vertebrate C9 and our protein, called *Botryllus* C9-like protein (BsC9), a high level of similarity results. To demonstrate that our C9-like protein can be considered a part of the complement system in *B. schlosseri* we evaluated the expression of BsC9 with respect to C3 activity. Our previous data demonstrates that *B. schlosseri* C3 is activated by zymosan. We combined the microinjection of zymosan with and without a validated anti-C3 antibody (against human C3) to block the activity of C3. With this approach we studied in, time course, the expression of both BsC3 and BsC9 demonstrating that the anti-C3 antibody is able to inhibit the expression of BsC9. These results are confirmed in both ISH and ICC using the same antibody, and *in vitro* with the C3 inhibitor compstatin. In addition, a significant ($p < 0.05$) decrement of labeling with BsC9 antisense riboprobe and validated antibody anti hsC9 is observed when hemocytes are incubated with zymosan and compstatin. Collectively, these results argue in favor of the presence of components of the lytic pathway in our model organisms.

Allograft Inflammatory Factor 1 (AIF-1) is expressed in CNS and hemocytes of the molluscan pest *Pomacea canaliculata* and shows immune-related function

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In mammals, Allograft Inflammatory Factor 1 (AIF-1), alias Ionized calcium-binding adapter molecule 1 (Iba1), is a 17 kDa cytokine largely synthesized by macrophages during inflammatory responses. AIF-1 is associated to activated microglia in vertebrates and its orthologues have been retrieved in microglial cells of the annelid *Hirudo medicinalis* and in the hemocytes, digestive glands and gills of several molluscs. The analysis of the organ specific transcriptomes of the gastropod pest *Pomacea canaliculata* revealed the presence of an AIF-1-like protein sharing the 49 - 56 % of identity with its vertebrate counterpart and a higher degree of identity with the AIF-1 invertebrate orthologues. Real-time PCR experiments indicated that *PcAIF-1* expression is detectable in all the tested organs, including hemocytes, digestive glands and gills, and the highest level of expression was retrieved in the pedal ganglia.

Twenty-four hours after the injection of 10 mg/ml of *Escherichia coli*-derived LPS, the *PcAIF-1* expression significantly increased in ganglia, digestive glands and gills. These observations suggest the involvement of *PcAIF-1* in the immune responses of *P. canaliculata* and point out a potential marker for microglial cells in one of the most invasive molluscan pests.

First insights in the autophagic processes of *Mytilus* hemocytes

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Autophagy is a highly conserved and regulated catabolic process by which the eukaryotic cell degrades unnecessary, undesirable, or dysfunctional cellular components. The autophagic machinery is also used to remove invading intracellular pathogens and to direct them toward lysosomal degradation. Therefore, autophagy represents an innate immune mechanism against microbial infection.

In invertebrates, that lack acquired immunity,

autophagy will thus play a key role in the protection against potential pathogens. In aquatic molluscs, induction of autophagy by starvation and different environmental stressors has been demonstrated by different types of lysosomal reactions in the cells of the hepatopancreas. However, no information is available so far on the role of autophagy in the immune cells, the hemocytes.

In this work, the possible role of autophagy in the response to bacterial challenge were investigated in the hemocytes of the marine bivalve *Mytilus*. Hemocytes were exposed to different strains of marine vibrios and functional responses were evaluated: lysosomal membrane stability (LMS), lysozyme release, ROS and NO production, bactericidal activity. Autophagy was investigated in the presence different specific inducers/ and inhibitors.

The results show that in mussel hemocytes exposure to the autophagy inducer Rapamycin induced a significant decrease in LMS. A similar decrease was observed after challenge with strains of *V. tapetis* and *V. corallyticus*. In all experimental conditions, lysosomal destabilization was prevented by cell pre-treatment with Wortmannin, a potent inhibitor of class III PI-3Kinase, that plays a key role in autophagosome formation.

Although neither *V. tapetis* or *V. corallyticus* strains were able to induce activation of functional immune responses, no cytotoxic effects were observed. However, incubation with *V. tapetis* in particular resulted in rapid induction of autophagy, as clearly shown by Transmission Electron Microscopy.

The results show that in hemocytes determination LMS may represent a simple and sensitive indicator of induction of autophagic processes. Moreover, these data underline the possible role of autophagy in the protection of bivalve hemocytes towards the potential pathogenicity of certain vibrios.

Identification of nanoparticle-protein coronas in the hemolymph of marine bivalves: implications in particle recognition and immune response

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The development of nanotechnology will inevitably lead to the release of consistent amounts of nanoparticles (NPs) in aquatic ecosystems. Invertebrates are emerging as suitable models for evaluating the environmental impact of NPs. However, NP intrinsic properties, as well as those of the receiving medium, will affect particle behavior, bioavailability, uptake and toxicity. Moreover, once within the organism, NPs will interact with cells in a physiological environment, i.e. in biological fluids. In mammalian serum, NPs associate with soluble components organized into a ''protein corona'', which affects particle interactions with target cells. The corona protein are particle- and species-specific.

The study on NP-protein coronas in invertebrate systems is still at its infancy. The protein composition of extracellular fluids of invertebrates is largely unknown, given the large diversity of phyla and species, and different proteins may be involved in the formation of NP corona in different groups.

In the hemolymph of the marine bivalve *Mytilus,* the formation of a stable protein corona increases the immunotoxicity of amino-modified polystyrene NPs (PS-NH_2). The Putative C1q domain containing protein (MgC1q6) or Extrapallial protein precursor, an immune-related acidic glycoprotein with high affinity for metal cations, was identified as the sole component of the PS-NH_2 hard protein corona. These data represent the first demonstration of the identity of hard-corona proteins in the hemolymph of a marine invertebrate. Since PS-NH_2 retain their positive surface charge in both ASW and hemolymph serum, studies were carried out with other types of NPs (n-TiO_2 and n-CeO_2) characterized by a net negative surface charge. The results show that both n-TiO_2 and n-CeO_2 associated with extracellular SOD (superoxide dismutase), the second most abundant protein in mussel hemolymph. In contrast, no stable association was found between hemolymph serum proteins and neutral/weakly charged n-CeO_2. The results demonstrate that distinct hemolymph proteins are involved in the formation of a stable corona around different types of NPs. These NP - protein interactions are particle specific, and strongly influenced by the net surface charge of the particle. The role of NP-protein coronas formed in biological fluids of different invertebrate species, and their possible consequences in determining the impact of NPs on innate immunity, is discussed.

Innate immune responses of European sea bass (*Dicentrarchus labrax*) induced by dietary administration of vitamin D_3

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Aquaculture - the global production of fish, molluscs, crustaceans and other marine animals - is an ever-growing industry. The major challenges on which its expansion depends comprise the diversification of farmed species, the improvement and optimization of nutrition and the control and prevention of diseases. One of the most important objective is the production of natural commercial diets with no adverse effects on the growth and health of the fish that guarantee high quality for consumers. With respect to the control and prevention of disease, the rapid development of aquaculture, in recent years, has led to the occurrence of diseases and problems associated with its intensive development, involving significant economic losses. A field of great interest is the application of stimulants of the immune system as additives in the diet to improve the efficiency of growth and to prevent and/or control of fish diseases. The effects of dietary administration of vitamin D_3 on some innate immune parameters and on the expression of immune-related genes in head-kidney (HK) and gut were investigated in *Dicentrarchus labrax* L. Vitamin D_3 (vD_3) was orally administered to fish in a commercial pellet food supplemented with 0 (control), 3,750, 18,750 or 37,500 U $kg^{-1}$of dry diet. Furthermore, gut histology was also considered. This study showed a modulation in the activities examined in fish fed with the addition of vD_3. Just after 2 weeks of administration, diet supplementation with the vitamin resulted in an increase in phagocytic ability, while serum peroxidase content was increased in fish fed with all experimental diet after 4 weeks, no significant differences were observed in protease, anti-protease, natural haemolytic complement activities and total IgM level. At gene level, *fucose and rhamnose binding lectin* transcripts were up-regulated in HK in fish fed with highest concentration of vD_3-supplemented diets after 4 weeks while in the gut was observed an up-regulation of *hepcidin* gene in fish fed with the different doses of vD_3. These results suggest that vD_3 may be considered of great interest for immunostimulatory purposes in fish farms.