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REPORT OF MEETING

XXIII scientific meeting of the Italian Association of Developmental and Comparative Immunology (IADCI), February 13-15, 2023, DaDoM - Darwin Dohrn Museum, Villa Comunale, Naples, Italy

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Award "Soci non strutturati" (Best presentation and curriculum studiorum for members under 35)

A *Bombyx mori* infection model for testing antimicrobial compounds against *Staphylococcus epidermidis* infection

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There is an urgent need to develop new antimicrobial molecules due to the increasing spread of antibiotic resistant bacteria. Mammalian models used for preclinical tests during drug discovery and development raised serious ethical problems in the last decades. As a consequence, after the European Parliament Directive 2010/63/EU on animal's welfare, many restrictions have been posed on the use of mammals for research purposes. In addition, trials are time consuming and very expensive. To overcome these issues, the traditional approach of drug screening must to be reconsidered. In particular, researchers are focusing attention on alternative invertebrate models. In this context, insects are increasingly considered a promising tool, able to accelerate preclinical tests of new drugs. Furthermore, the use of insect infection models can decrease the costs of the experimentation and the number of animals used, according to the 3R principle.

In last years, we focused our efforts on developing a silkworm infection model to test antibiomicrobial compounds against *Staphylococcus aureus*. To this purpose, we assayed cheap and easy to perform markers for evaluating three glycopeptide antibiotics (GPAs, i.e., vancomycin, teicoplanin and dalbavancin) (Montali *et al*., 2020). With these analyses, we were able to assess the different efficacy of the three tested antibiotics in curing bacterial infections.

To validate this silkworm infection model, in the present work we tested, for the first time, the three GPAs against another important Gram positive, nosocomial pathogen, i.e., *Staphylococcus epidermidis*. After the injection of bacteria in the hemocoel, larvae were reared at 37 °C to reproduce the human physiological conditions, and different immunological markers were used to monitor the infection. In addition to the survival of the larvae, cellular and humoral immune responses were assessed through the analysis of the viability of hemocytes, and the activity of phenoloxidase system and lysozyme.

All the three antibiotics proved to be effective, curing infected larvae, increasing their survival rate. Moreover, the administration of GPAs blocked the activity of the immune response.

In conclusion, this study lays the foundations for using *Bombyx mori* as a trustable infection model to test novel antimicrobial compounds with therapeutic potential against staphylococci.

Award "Giovani laureati" (Best presentation and curriculum studiorum for members under 29)

How can climate change affect Antarctic fish physiology? A study on the effects of increasing seawater temperatures on fish heart rates

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It is well known that the oceans and marine organisms are suffering the consequences of climate change. Increasing seawater temperatures are causing a severe threat to the survival of living organisms. Global climate change is expected to cause an increase in water temperatures, especially in polar regions, but it is unclear how this will affect marine fish species in general. The Southern Ocean, which surrounds Antarctica, is a central component of the global absorption of ocean heat. The surface waters within and north of the Antarctic circumpolar current and the abyssal waters are experiencing a gradual temperature increase. Temperature can influence many intracellular dynamics, such as the movement of molecules, the activity of enzymes and the function of ion channels. Fish may respond to these changes in various ways, including behavioural strategies like avoidance tactics, molecular modifications like changes in protein or lipid biosynthesis, and physiological responses that impact the entire organism, like changes in cardiovascular function. This study aims to understand the physiological changes that might occur in an Antarctic fish species and whether these peculiar stenothermal fish can adapt to these changes. It is known from previous studies that various circumstances, including oxygen availability, swimming, activity, feeding, stress and temperature, influence the heart rate of fish. To analyse the effect of temperature, we exposed adult specimens of *Trematomus bernacchii*, a fish species endemic to Antarctica, to different temperature conditions. The control group of fish was exposed to 0°C, while another group experienced a rising temperature range from +1 °C to +3 °C. The exposure time for each experimental group was 15 days. To measure the heart rate, DST micro-HRT loggers, developed by Star-Oddi (Iceland), were implanted on four *T. bernacchii* specimens for each experimental group before the experiment started. The DST micro-HRT uses innovative technology, simultaneously measuring the target animal's long-term heart rate and body temperature. The logger is ideal for a wide range of investigations, such as studying behaviour and stress response in laboratory animal research, wildlife research, animal welfare, and fish physiology. Understanding the physiological reactions to such changes may help determine whether these creatures can withstand future global changes and point out any restrictions (including energy investment and stress) on how they can respond to further warming in the Southern Ocean.

We analysed the data collected after the experiment to obtain some preliminary findings that offer interesting information regarding how variations in water temperatures may affect heartbeat frequency. Two comparisons have been performed: one between the tank that was maintained at 0 °C and the tank that experienced temperature increases, and the other between the various temperature increases that the fish experienced. The heartbeat rates of the fish in the control group did not change statistically during the trial. Still, our analysis revealed a statistically significant increase in heart rates between +1 °C and +3 °C in the tank where the temperature was manually increased. Furthermore, a subsequent correlation analysis showed a strong positive correlation between temperature and heart rate, confirming that the heart rate also increases when the temperature increases. This presumably represents a response to increased ATP and oxygen demand at the tissue level in relation to increased metabolic activity.

The present study, therefore, demonstrates that the application of tags provides a powerful analysis tool to measure the heart rate in fish. It also provides a unique insight into the physiological responses of *T. bernacchii* exposed to increasing temperatures. For example, when *T. bernacchii* specimens are exposed to an acute temperature increase from 0 °C to +3 °C, they tend to show tachycardia. This, in principle, may result in unforeseen energy investment for these organisms.

KEYNOTE LECTURE

Novel insights on fish immunoglobulins and B cell subsets

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Although teleost fish constitute the first animal group in which all the elements of acquired immunity are found, the many structural and functional differences of these elements with those of mammals strongly condition how B cells and immunoglobulin (Ig) production are regulated in these species. One of the main differences when compared to higher vertebrates is the fact that fish only express three antibody isotypes, namely IgM, IgD, and the teleost-specific IgT. Interestingly, IgT+ B cells only express this Ig and constitute a B cell linage that apparently is regulated independently of other B cell subsets and seems to play a major role in mucosal immunity. Nevertheless, systemic IgT responses have also been broadly reported and there are still many aspects of IgT regulation that remain unknown. On the other hand, B cells co-

expressing IgM and IgD on the cell surface (IgM+IgD+ B cells) constitute the major B cell subset in systemic compartments and seem to correspond to naïve mature B cells. These fish B cells down-regulate IgD after encountering antigen as mammalian B cells do, once they start a differentiation program towards plasmablasts/ plasma cells and become IgM+IgD− B cells. Finally, IgD+IgM− B cells have also been detected in some species such as rainbow trout (*Oncorhynchus mykiss*) or catfish (*Ictalurus punctatus*), being this subpopulation numerous in some specific mucosal tissues such as intestine or gills. The precise role of this B cell subset is still lacking in teleosts as in mammals, yet some important discoveries have been made in the past years. In this talk, we will go through the most recent discoveries regarding the functionality of all these fish B cell subsets.

Session 1. Characterization of immune genes. Chairpersons: Umberto Rosani, University of Padua, Padua, Italy and Adriana Vallesi, University of Camerino, Camerino (MC), Italy

How does gene presence/absence variation shape the repertoire of defense molecules in marine mussels? The case of CRP-I, cysteine rich peptides belonging to the knottin superfamily

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We have recently demonstrated that marine mussels (*Mytilus* spp.) have an unusual pan-genomic architecture, characterized by widespread hemizygosity and gene presence-absence variation (PAV). This phenomenon does not randomly affect all genes, but disproportionately targets multigene families involved in immune response and survival, including those encoding antimicrobial peptides and other small effectors. As a result, each individual mussel is endowed with a unique repertoire of defense molecules.

However, the lack of a reference chromosome-scale genome assembly has so far prevented to gather a complete understanding of the architecture of these genomic loci, preventing an accurate ascertainment of the orthology/paralogy relationships among variants.

The analysis of a fully-phased genome assembly of *Mytilus edulis* allowed to fill this knowledge gap, with the identification of over 50 paralogous genes and pseudogenes belonging to the CRP-I family, located in a small genomic region within chromosome 5, characterized by extreme structural variation. We provide evidence that CRP-I genes are subjected to massive gene PAV within the *Mytilus* species complex, display a complex evolutionary history and a peculiar pattern of expression, and belong to the knottin structural superfamily, according to AlphaFold prediction. Although most knottins either act as toxins, antimicrobial peptides or protease inhibitors, the functional role of CRP-I remains elusive. Functional assays, carried out on the synthetic peptide sCRP-I H1, do not support neither a role as an antimicrobial agent, nor as a protease inhibitor, suggesting on the other hand that it may be toxic towards invading eukaryotic parasites.

Comparative gene expression analysis reveals adaptation mechanisms in the Antarctic scallop *Adamussium colbecki*

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Antarctica is the most extreme continent of Earth, with strong winds, freezing temperatures on land, and ocean temperatures constantly below 0 °C.

Nonetheles the Antarctic Ocean is home to an astounding diversity of living organisms, that adapted to the multiple challenges posed by such an environment thanks to a diverse set of evolutive traits. Thanks to the recent advancements in sequencing technologies, it was possible to discover many of the molecular bases of such adaptations in antarctic fish, while little is known about invertebrates and in particular for bivalves. In this preliminary study, we tried to address this knowledge gap using transcriptomic data to obtain insights into some of the adaptations that made *Adamussium colbecki* the only Antarctic pectinid species existing in our era.

We used a transcriptome assembly and RNAseq data we generated for *A. colbecki* and retrieved the reference genomes and RNAseq data from the pectinids *Pecten maximus*, *Mizuhopecten yessoensis*, *Argopecten irradians* and *Azumapecten farreri*. In particular, samples from gill, mantle, and digestive gland tissues were selected. With the reciprocal best hit method, we built a dataset of 461 ortholog genes that were used as reference for RNAseq read mapping. The gene expression data was then aggregated and used to perform Differential Gene Expression analysis, identifying the genes with an increased expression in *A. colbecki* compared to the other species. To functionally characterize the obtained gene set, we performed Gene Ontology, superfamily and pathway enrichment analysis and STRING interaction analysis.

Our results revealed that, specially in gills, *A. colbecki* displays higher expression of several genes primary involved in transcription, alternative splicing and protein degradation via ubiquitination. Additionally, genes encoding for membrane-bound proteins are also included in this set.

Although with some limitations, our analyses are indicative of adaptive traits that compensate for a reduced efficiency of transcription and mRNA

splicing at low temperature, resulting in an increased need for the degradation of degenerated or misfolded protein products. Such adaptations poorly overlap those of Antarctic fish, and represent a novel insight in the survival strategies of *A. colbecki*, that will be expanded with the ever-increasing availability of high quality data.

FicD genes in invertebrates: a tale of transposons, viruses and proviruses

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Many genes are shared across the tree of distantly related species because of horizontal gene transfers (HGTs). However, the frequency of HGTs varies strongly between gene families and biological realms suggesting differential selection pressures and functional bias. One gene family with a wide distribution are FIC-domain containing enzymes (FicDs). FicDs catalyze AMPylation, a post-translational protein modification consisting in the addition of adenosine monophosphate to accessible residues of target proteins. In humans, AMPylation plays a role in neuro-development and neuro-degeneration and similar FicD activities have been reported for *Drosophila* and *Caenorhabditis elegans* FicDs. Moreover, it has been shown that bacterial-promoted AMPylation can induce cytotoxicity in the infected cells by targeting host proteins of the Rho GTPase family.

Beside the known conservation of FicDs in deuterostomes, we report the presence of a conserved FicD gene ortholog in a large number of protostomes and basal eukaryotes, with structural and functional traits suggesting a preserved AMPylation capacity. We also discovered additional FicD gene copies in the genomes of some rotifers, parasitic worms, bivalves and isopods. A few dsDNA viruses of these invertebrates, including White spot syndrome virus, *Cherax quadricarinatus* iridovirus, Ostreid herpesvirus-1 and the beetle nudivirus, carry copies of possibly functional FicDs.

Phylogenetic analysis suggested a common origin of the FicD copies of these viruses and the duplicated FicDs of their invertebrate hosts, and the strong conservation between WSSV and OsHV-1 FicDs indicated an evolutionary advantage in maintaining this gene. HGTs and gene duplications possibly mediated by endogenous viruses or genetic mobile elements seem to have contributed to the transfer of AMPylation ability from bacteria and eukaryotes to pathogenic viruses, where this pathway could have been hijacked to promote viral infection.

Investigating the role of A-to-I RNA editing in bivalves physiology

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Enzymes of the *adenosine deaminase acting on dsRNA* (ADAR) family can perform post-transcriptional modifications on structured RNAs, by converting Adenosine in Inosine (A-to-I editing), eventually diversifying transcriptomes and proteomes. ADAR-mediated editing is involved in the neural development, autoimmune disorders and it has been reported to exert pro- or antiviral effects depending on virus-host combination. However, except for a few species, the extension and functional roles of RNA editing have been poorly investigated across the tree of life.

Our research aims to reveal the presence of ADAR-mediated editing in bivalves, developing appropriate pipelines to detect genuine editing sites, characterizing the involved enzymes and revealing editing patterns conserved among species possibly underpinning functional significance.

Accordingly, we phylogenetically characterized the gene complements involved in RNA editing and we recombinantly produced the *Crassostrea gigas* ADAR1 to verify its editing potential, efficiency and specificity on known human targets. In parallel, we exploited paired DNA- and RNA-sequencing data to develop an editing index based on bivalve repeats, recalling the Alu index used for humans. We tested this novel bivalve editing index in samples collected during an experimental infection with Ostreid herpesvirus-1 carried out in *Scapharca (Anadara) broughtonii*. Finally, we characterized the RNA editing in *Mytilus galloprovincialis, S. broughtonii* and *C. gigas* and we compared their targets and distribution to highlight the conserved and unique editing sites among these organisms.

As results, we demonstrated that *C. gigas* rADAR is able to edit dsRNA structures even if it shows some difference in the RNA editing targets comparing to the human ADAR proteins.

The Editing index based on bivalves repeats is able to rapidly estimate the RNA editing levels in bivalve samples, indeed in the dataset obtained from a time course experiment performed with *S.broughtonii* injected with OsHV-1 and monitored up to 72 hours both RNA editing frequency in several editing sites and the newly defined editing index significantly increased in parallel along the time course.

In conclusion, we validated the presence of a A-to-I editing also in bivalves confirming again the conservation of this mechanism in Metazoans and the pivotal role of the ADAR mediated RNA editing in organism physiology.

Molecular evolution of *Euplotes* pheromones and pheromone-coding genes

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In the ciliate *Euplotes*, species-specific families of water-borne protein pheromones regulate self/not-self recognition phenomena which are responsible for the cell decision to switch between vegetative (mitotic) growth and sexual mating. The knowledge of the pheromone and pheromone-gene structures has recently been widened to a number of species that localize in different positions of the *Euplotes* phylogenetic tree and thrive in different environments, making it possible to seek into how the structures of these molecules evolve in relation to speciation. The development of one or more (usually Gly-rich) random-coil segments is a major trait of the evolution of the pheromone structure, which is basically determined by a common tightly conserved, disulfide-rich helical fold. By determining sites of local flexibility of the molecular backbone, these segments come to serve the double function of greatly improving the pheromone adaptive plasticity and capability to interact with other proteins. In parallel, the pheromone-gene structural evolution primarily involves the inclusion of multiple intron sequences within the 5'-leader region or, more rarely, within the coding region. By determining a mechanism of alternative splicing, these sequences make each pheromone gene (which is expressed in the somatic genome of the cell macronucleus) capable of synthesizing multiple mRNAs in addition to the pheromone-specific transcript.

Lost *IgT* gene in icefish: another chapter of the evolutionary tale of Antarctic fish

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Notothenioidei is a monophyletic lineage that accounts for the majority of teleost fish fauna living in the freezing sea of the Southern Ocean. They are an amazing example of adaptive radiation and an interesting model for the study of cold adaptation. During their evolutionary history, Antarctic fish have undergone significant genome alterations, as also highlighted in a previous work on the gene encoding the IgT heavy chain, lacking most of the second constant exon (CH2). A reconstruction across notothenioid phylogeny revealed that the partial loss of CH2 was shared by representative species from four Antarctic families along with the nearest non-Antarctic sister species *Eleginops maclovinus*.

The present work is aimed at investigating Channichthyidae, since remaining the only family apparently lacking the entire *IgT* gene. The survey of available genomes and trascriptomes for this family revealed a heterogeneous situation, with some species (most notably *Chionodraco*, *Cryodraco* and *Chaenodraco* spp.) having entirely lost the *IgT* gene and others displaying a remnant pseudogene carrying only TM exons and a part of the upstream intron. Since the *IgT* gene was present and complete in all the other closely related taxa (Bathydraconinae, Cignodraconinae and Gymnodraconinae), the timing of the gene loss can be inferred to be coincident with the loss of red blood cells and hemoglobin occurred in the family.

To evaluate how the two other evolutionary conserved isotypes can functionally compensate for the loss of IgT, tissue-specific expression of *C. hamatus* IgM and IgD was performed by qPCR, including *Trematomus bernacchii* for comparison. IgM was found to be the predominant isotype expressed in icefish mucosal tissues. In particular, abundant IgM expressing cells were found in the lamina propria of the posterior intestine, as indicated by *in situ* hybridization analysis. Conversely, *IgD* transcripts were found to be predominantly expressed in the lymphoid organs. Of note, the expression levels of IgM in the intestine and of IgD in head kidney of *C. hamatus* were respectively 6- and 20-fold higher than in *T. bernacchii* tissues. As expected, IgT was the highest expressed isotype detected in mucosal tissues of *T. bernacchii*.

Overall, the preliminary results presented here pave the way for completing the reconstruction of the evolutionary history of the *IgT* gene in Antarctic fish, culminated with its loss in icefish.

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MAIN LECTURE

Reconstructing the evolutionary history of the antibody molecule

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Antibodies, otherwise called Immunoglobulins (Ig), are among the best-known molecules. A notable issue is the origin and evolution of antibody. Here, an integrate view of the emergence of antibody in evolution, based on a literature survey across a wide range of prokaryotic and eukaryotic organisms, is provided.

The Ig molecular architecture relies on the very ancient Ig domain. Its chemical features permit the formation of multiple domain chains, self-dimerization or association with different partners. The amino acid sequence of the Ig domain determines a compact structural core and allows a great conformational variability of flexible loops carrying out recognition functions. This key structural role accounts for its occurrence in numerous recognition molecules. The Ig domains that are found in different taxa, are distinguished in four types, IgV, IgC 1, IgC2 and IgI, based on different numbers and arrangements of the occurring β-strands.

Ig-like domains, defined as BIg domain, have been detected on prokaryotic cell surface, and a role in host cell adhesion has been hypothesized. Whether the similarity between BIg and eukaryotic Ig is related to genetic evolution or to the physico-chemical properties of amino acid motives driving the domain assembly is still under debate. IgV appears to be the oldest Ig domain since it has been found in an adhesion molecule of a living fossil sponge. IgV domain has been widely used by the recognition molecules of earlier vertebrates. However, only in the jawed vertebrates the V domains have gained an important structural role in forming the antigen-binding site. The sequence variation arose from the encounter between IgV domain encoding genes and the evolutionary lines leading to the enzymes involved in the mechanisms of somatic recombination and hypermutation.

While IgC2 and IgI domains are present in both invertebrates and vertebrates, IgC1 domain is limited to jawed vertebrates and has been found only in the molecules of adaptive immunity. This observation supports the idea that key actors of the adaptive immune response, all using the novel IgC1domain type, emerged at the same time during the so-called immunological "Big Bang". Various antibody classes, each containing a different heavy chain isotype, differentiated during vertebrate evolution and acquired distinct functions in mucosal and systemic immunity. In mammals, in addition to heavy chain isotypes, subisotypes are distinguished on the basis of their functions.

The evolution of immunoglobulins can thus be considered as a paradigmatic example of how diversity and specificity of molecular interactions between proteins can increase.

Session 2. From comparative immunological studies to biotechnological applications. Chairpersons: Nicolò Baranzini, University of Insubria, Varese, Italy and Maria Rosaria Coscia, National Research Council of Italy, Naples, Italy

Investigation of the *IgM heavy chain* gene from Antarctic fish inspired a novel engineered monoclonal antibody

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Immunoglobulin M (IgM) is the major circulating Ig isotype in teleost fish. Unique features in crucial parts of the IgM molecule have been uncovered in Antarctic fish species that have experienced a special evolutionary history. The most striking structural characteristic is an extraordinary long hinge region, located between the second and third heavy chain constant domains. It can be viewed as a result of adaptive evolution to enhance the functionality of the molecule under very extreme environmental conditions.

This finding prompted the idea to modify the heavy chain constant region (IgH) of a murine monoclonal antibody (mAb) by replacing its hinge with that from Antarctic fish IgM by using the CRISPR-Cas9 system. This technology has been recently proposed as an RNA-guided DNA targeting platform and widely used as a powerful tool for precise gene editing. Given its simplicity and flexibility, the CRISPR-Cas9 system has been successfully used also in the field of immunology to edit mouse and human Ig genes. A stepwise approach was chosen for targeted genome editing of a hybridoma cell line secreting IgG mAb. The first step was the creation of a targeted DNA double-stranded break at the hybridoma *IgH* gene locus to be modified. Homology-directed repair was then used to insert the “Antarctic” hinge sequence through recombination of a DNA donor template with the target locus. The correct sequence insertion was assessed by using a fluorescent protein as selection marker.

A preliminary characterization of the antigen binding activity of the engineered mAb was performed by the Localized Surface Plasmon Resonance. The association constant k of the engineered mAb was found to be three-fold higher than that of the murine counterpart, suggesting an enhanced ability of the “antartized” mAb to recognize its target antigen, when immobilized on a rigid substrate.

Overall, these results may open a new frontier in the field of antibody engineering by using an innovative and versatile CRISPR-based method.

Medicinal leeches: a promising source of cell lines with multiple biotechnological applications

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Stem cells represent one of the most dynamic research fields in biology and biomedicine. However, the vast majority of research is carried out in mammalian models, which represent only 0.4% of extant metazoans. Aquatic invertebrates show the greatest biodiversity and the widest phylogenetic radiation on Earth, but cell lineages are currently available form only few of them (in particular molluscs and crustaceans, while there are none from annelids).

In the last decades, we have identified and characterized different cell types in the medicinal leeches *Hirudo verbana* and *Hirudo medicinalis*, such as hemopoietic precursor cells, monocytes/macrophages, granulocytes, natural killers, fibroblasts, telocytes, myofibroblast and myoendothelial cells, all sharing the same morphofunctional and molecular features with vertebrates.

In addition, by means of an innovative system developed in our laboratory, based on the injection into the leech body wall of the Matrigel biopolymer (MG) supplemented with selected cytokines, we were able to isolate, expand and differentiate in vitro

hemopoietic precursor cells into muscle and myofibroblasts cells.

These studies enabled us to clarify some key processes concerning the role of cytokine signaling on progenitor cells differentiation, muscle regeneration and wound healing. Starting from these promising results, we propose the medicinal leech, whose experimental use is not subjected to legislative restrictions, as an emerging experimental model for the production of invertebrate cell lines endowed with innovative biotechnological potential and in support of vertebrate cell lines-based research.

To this aim, using the consolidated MG technique and combining it with the cytokines PDGF (Platelet-Derived Growth Factor) and EGF (Epidermal Growth Factor), we have isolated different leech cell populations and cultured them in vitro in a medium containing Fibroblast Growth Factor 2 (FGF2), Transforming Growth Factor-β (TGF-β) or *Hv*RNASET2 enzyme. The cell responses have been then evaluated by both morphological and immunocytochemical assays.

Concurrently, an expression vector (pEGFP-N1) containing the leech's Actin 1 minimal promoter was created in order to perform, for the first time, transfection experiments aimed at producing immortalized leech cell lines which, in addition to the relative scientific interest due to their sheer diversity, will provide the potential for multiple applications, such as assays for ecotoxicological analyses.

Session 3. Model organisms for basic and translational immunology. Chairpersons: Nicola Franchi, University of Modena and Reggio Emilia, Modena, Italy and Daniela Melillo, National Research Council of Italy, Naples, Italy

The multiple potentialities of anthozoans: analyses and comparisons between animal models

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Anthozoans are the richest class of species of the phylum Cnidaria. They are a candidate group for studying the evolution of mutualisms and immunity and despite their morphological simplicity exhibit a repertoire of immunological components with large genomes and gene families similar to those of the Bilateria.

Like other invertebrates, anthozoans immunity is based on self/non-self recognition mechanisms and allorecognition responses, therefore, maintaining their integrity and responding actively to selection pressures.

Highlight and investigate the link between innate immunity, homeostasis maintenance, inflammation, tissue remodelling and regeneration in Anthozoa could be useful to elucidate the adaptive capability features to different stress factors.

We have carried out studies demonstrating that all these processes are highly conserved among the anthozoans species. We have compared the inflammatory responses and the morpho-functional aspects related to regeneration in different species of Mediterranean anthozoans using histological, cellular and molecular technical approaches on organisms, maintained in aquaria under environmental and pathogenic stressful conditions.

This approach appears to be a useful tool from baseline studies in immunology and anthozoans result valid models able to respond to environmental stress conditions. Important results have been obtained with potential biotechnological transferability in pharmacology.

The protochordate *Ciona robusta* as an experimental system for studies of gut microbial immune interactions

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The recognition that animals exist as metaorganisms suggests that attention should be focused on defining host-microbe interactions in not just pathogenic conditions, but during health as well. For example, the gut microbiota serves vital roles in various aspects of animal life, including development of the immune system and influence of host physiology. The gut immune system, and specifically the innate immune components, is at the forefront of the crosstalk between host and microbes, where colonization by commensal microbes is tolerated while pathogens are resisted. This dialogue is evolving and is shaped by encounters with a continuum of microbial species. Establishing diverse experimental systems is essential for understanding the fundamental rules legislating these ecological interactions.

Ciona robusta, a marine invertebrate belonging to the subphylum of Protochordate, a sister taxon to vertebrates, represents an ideal experimental system for such studies: it is a highly tractable model that engages with a complex environment using only innate immunity and develops into transparent juveniles with a digestive tract that is easy to stage, dissect and study the steps shaping microbial colonization dynamics.

Our group is establishing this model for defining critical host-microbiota interactions, combining approaches of microbiology, molecular biology, biochemistry, and functional assays. We have identified and characterized some components of the *Ciona* gut environment, and these include the presence of a gut epithelium layered with chitin-rich mucus, secreted immune effectors, namely the immunoglobulin-like variable region-containing chitin-binding proteins (VCBPs) and a stable gut microbiome in adults that includes abundant and diverse bacteriophages. A large catalog of cultured bacteria and fungi, from which cultured juveniles

can be colonized for experimental manipulation and study, have been isolated and characterized. We have observed that VCBPs are able to interact with distinct elements of the microbiome, mediating transkingdom interactions and shaping biofilm formation and colonization dynamics among microbes.

Finally, we have developed approaches to rear aseptic juveniles to facilitate future studies interrogating each component of the symbiosis, including the role(s) of microbes in animal development. Thus, *Ciona* is a valuable experimental organism that may help to define conserved and unique innate immune adaptations shaping the gut ecosystem and its homeostasis.

Comparing immune responses of *Mytilus galloprovincialis* to different bacterial isolates from oyster mortality outbreaks

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Marine bacteria of the *Vibrio* genus are widely distributed in estuaries and coastal waters and sediments. They include species pathogen for humans (e.g., *Vibrio cholerae)* and for aquatic animals (e.g., *Vibrio splendidus*, *Vibrio aestuarianus*). Several *Vibrio* species have been repeatedly associated with oyster mortality outbreaks in Europe.

Host-pathogen interactions have been investigated in cultured and wild populations of bivalves susceptible to infection by certain *Vibrio spp*. and strains, showing that different *Vibrios* can elicit distinct immune responses in bivalve hemocytes.

V. aestuarianus (*V. a*.) is a common bivalve pathogen detected in samples from *Crassostrea gigas* mortality episodes since 2001, suggesting a strong link between their presence in oyster tissues and development of disease. Most studies on the interactions with the host immune system were carried out with the *V. a*. 01/032 isolate. This strain can secrete extracellular products that inhibit phagocytosis by oyster hemocytes and enable bacterial proliferation. In contrast, the mussel *Mytilus galloprovincialis* can activate efficient immune responses to challenge with *V. a*. 01/032, by a combination of cellular and soluble defences.

During a mortality event in 2019 in Spain, different strains of *Malaciobacter marinus* were isolated from moribund oyster together with V. a. strains. We have recently shown that *M. galloprovincialis* hemocytes efficiently responds to challenge with *M. marinus* strains. In this work, immune responses of mussel hemocytes to the *V. a.* strain 106 isolated from the same mortality event were investigated.

The results of *in vitro* experiments, carried out in the absence and presence of hemolymph serum HS, show that this strain induced strong lysosomal destabilization without stimulating extracellular defences (ROS production, lysozyme release). However, *Vibrio a.* 106 was apparently internalized in hemocytes: accordingly, significant bactericidal activity was observed, that was stimulated by HS.

The results obtained so far suggest that *V. a.* 106 may be more pathogenic to *M. galloprovincialis* with respect to *V. a.* 01/032. Moreover, in the presence of *M. marinus*, *V. a.* 106 may significantly affect the health status of mussels over longer period. Further *in vivo* experiments are in progress to investigate the responses of *M. galloprovincialis* to co-infection with these two bacteria.

Bacterial oral infections: exploring the role of the circadian clock in the modulation of pathogen sensitivity in *Drosophila melanogaster*

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In almost all organisms, circadian clocks regulate physiological and behavioral rhythms, showing a periodicity of 24 h in the absence of any external cue. Moreover, they are synchronized by environmental stimuli (such as light), enabling organisms to adapt to environmental changes in phase with the 24 h day.

In *Drosophila melanogaster*, sensitivity to systemic infection and some immune response components show daily variations in 12 h light: 12 h dark cycles (12:12 LD) and in constant darkness (DD), suggesting these phenotypes are circadian controlled. These data refer to flies exposed to a pathogen *via* an injection route, while a possible role of the circadian clock during oral infections is unclear.

Here we show our first experiments analyzing the role of the circadian clock in the modulation of flies' pathogen sensitivity during oral infections. Using a CApillary FEeder assay, we determined the 24 h feeding profile in wildtype and clock mutant flies, with and without gut microbiota, in LD and DD conditions. We identified the proper moments of the day to orally infect flies with the same amount of pathogen during daytime and nighttime, in different conditions. The first results about the daily sensitivity to oral infection with *Providencia rettgeri* will be shown.

MAIN LECTURE

Biotechnology meets aquaculture needs: a promising support for fish health management

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Seas and oceans are the largest resource of the planet and are of fundamental importance for developing sustainably a strategic asset for a country

with 8700 km of coasts and possessing ca 15% of the surface of the Mediterranean Sea. However, their potential is still largely unexploited. In order to expand the Marine Biotechnology field, the Stazione Zoologica Anton Dohrn has recently opened a new premise: the Department of Marine Biotechnology.

The philosophy beyond this Department is to have a direct access to the marine organisms and to valorise their potential in many fields of industrial sectors, therefore the location of the Department is directly on the sea, at Molosiglio in Naples.

The marine life offers a plethora of novel opportunities, either in terms of seafood, nutraceutical, and bioactive molecules, which are of extraordinary importance in the field of pharmacology, biomaterials and bioremediation. The development of new technologies and biotechnologies can unlock this underexploited potential with positive impacts in several industrial sectors and with positive impact on national economy. The Department of Ecosustainable Marine Biotechnology represents an international strategic implementation of the biotechnological research and is planned to enhance the research potential in specific industrial sectors like pharmaceuticals, nutraceuticals, and aquaculture.

The mission of the Marine Biotechnology Department is to conduct and promote scientific research regarding the possible applications of marine natural products in the biomedical and environmental sectors. We also focus on the isolation and characterization of marine microorganisms, including bacteria and fungi, which can resist and degrade pollutants such as heavy metals and polycyclic aromatic hydrocarbons. Bioremediation and bioaugmentation studies are aimed at validating new technologies for the recovery of marine polluted coastal areas, such as in the aquaculture sector. New and innovative approaches are being developed for the restoration of such sites using seagrasses and corals.

To foster the Blue Economy in our Country, we need a tight cooperation between research and industry capable of identifying smart solutions. In fact, from one side, we need to develop tools for the sustainable use of marine resources, and, from the other side, we need create new occupational opportunities.

Session 4. New promising sources of bioactive compounds. Chairpersons: Marco Gerdol, University of Trieste, Trieste, Italy and Simona Picchietti, University of Tuscia, Viterbo, Italy

NK-lysin homologue from the Antarctic teleost *Trematomus bernacchii* and its Ala mutant: focus on antibacterial and anticancer activities

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Antimicrobial peptides (AMPs) are small molecules naturally produced by all living organisms, with a great diversity in amino acid composition, structural organization and mechanism of action. Given their broad-spectrum activity against multiple classes of pathogens in addition to their immunomodulatory and anticancer activities, AMPs have been proposed as promising candidates for pharmacological applications. Several families of AMPs have been identified from teleost fish to date. Among them, NK-lysin (NKL), predominantly characterized in mammals, garnered scientists' attention due to antimicrobial, immunomodulatory and anticancer activities. In this study, a NKL sequence belonging to the saposin-like protein superfamily was identified in silico from the head kidney transcriptome of the Antarctic teleost *Trematomus bernacchii*. The sequence is rich in positively charged amino acids and contains six cysteine residues that form three disulphide bridges. Tissue-specificity qPCR analysis indicated predominant NKL expression in *T. bernacchii* gills and spleen, followed by head kidney, gut, liver and brain. A putative biologically active 27-residue long peptide, containing 2 cysteines, was identified by multiple sequence alignment of the T*. bernacchii* NKL amino acid sequence with teleost homologues. A NKL mutant (NKL-MUT) was designed by replacing Cys-10 and Cys-20 of the wild type peptide with Ala residues, with the aim of verifying the functional importance of the disulfide bridge. The NKL wild type (NKL-WT) and NKL-MUT peptides were synthesized and their bioactivity was evaluated *in vitro*: they significantly increased membrane permeability in bacterial models and inhibited the growth of clinical isolates with known resistance profiles. Notably, NKL-WT and NKL-MUT exhibited bactericidal activity against the human pathogenic bacteria *Enterococcus faecalis* and *Acinetobacter baumannii*, respectively. Of note, NKL-MUT possessed improved selective cytotoxicity and pro-apoptotic activity compared to NKL-WT towards the melanoma cell line B16F10, without affecting the viability of FB789, a primary human fibroblast cell line. Neither peptide showed any significant haemolytic activity against mammalian erythrocytes. These results provide interesting perspectives on the possible application of such peptides as antimicrobial and/or anticancer agents.

This research was supported by the National Programme for Antarctic Research (PNRA), Project number PNRA18_00077.

Botryllin, a novel antimicrobial peptide from the colonial ascidian *Botryllus schlosseri*

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By mining the transcriptome of the colonial ascidian *Botryllus schlosseri*, we identified a transcript for a novel styelin-like antimicrobial peptide, we named botryllin. The gene is constitutively transcribed by circulating cytotoxic morula cells (MCs). The synthetic peptide, obtained from *in silico* translation of the transcript, shows robust killing activity to bacterial and unicellular yeast cells causing breakages of both the plasma membrane and the cell wall. Specific monoclonal antibodies were raised against the epitopes of the putative amino acid sequence: they label the MC granular content. Upon MC degranulation induced by the presence of nonself, the antibodies recognise the extracellular nets with entrapped bacteria nearby MC remains. The obtained results suggest that the botryllin gene carries the information for the synthesis of an AMP involved in the protection of *B. schlosseri* from invading foreign cells.

Red cells of the black sea urchin *Arbacia lixula*: a promising source of anti-inflammatory compounds

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Many echinoderms' species are regarded as an important source of promising bioactive compounds. Among them, sea urchins are increasingly investigated for their potential pharmacological benefits, producing compounds with several biological properties, such as antibacterial, anticoagulant, antifungal, anti-inflammatory, anti-tumor, and antiviral activities. The globose body of sea urchins possess a cavity filled with a circulatory medium (coelomic fluid) containing a heterogeneous population of cells (coelomocytes) involved in immune defense. Among coelomocytes, also red spherule cells play a role in immune response being able to accumulate around injuries and sites of infection. Red cells contain echinochrome A, a naphthoquinone which gives the cells their characteristic red color. Echinochrome A is released from red cells in the presence of bacteria or virus and has antimicrobial properties against both Gram-positive and Gram-negative bacteria. An antioxidant property of echinochrome A from sea urchins *A. crassispina* and *S. mirabilis* has been also reported.

In this work, we investigated the anti-inflammatory properties of the red cells methanolic extracts from the black sea urchin *Arbacia lixula*.

Human microvascular endothelial cells (HMEC-1) stimulated with the pro-inflammatory cytokine TNF-α were used to assess the anti-inflammatory effect of the methanolic extracts. The total phenol content of the samples was estimated by Folin-Ciocalteau assay, and Trolox Equivalent Antioxidant Capacity (TEAC) assay has been performed to evaluate the antioxidant activity of the extracts. Treatment of HMEC-1 with 100 µg/mL of red cell methanolic extract significantly reduced the TNF-α-induced expression of inflammatory genes, including adhesion molecules (VCAM-1), chemokines and cytokines (MCP-1, IL-6). Moreover, the activation of the transcription factor nuclear factor κ-B (NF-κB), responsible for the induction of inflammatory genes, was significantly inhibited by red cell extracts. These genomic effects translated into a reduced adhesion of monocytes to inflamed endothelial cells treated with methanolic extracts.

These data contribute to characterize the biological activities of the red cells from the sea urchin *A. lixula* and point to beneficial role of its red cells extracts against inflammatory response in vascular endothelial cells, with potential implications for the modulation of inflammatory diseases.

Antimicrobial and antibiofilm activity of a synthetic AMP derived from a *Bombyx mori* Cecropin B natural variant

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A natural variant of the antimicrobial peptide Cecropin B derived from the silkworm *Bombyx mori* showed an effective antimicrobial activity against the opportunistic pathogen *Pseudomonas aeruginosa*. This isoform, named Q53 CecB, also displayed a high stability to pH and temperature variations, as well as low toxicity against human cells. Importantly, Q53 CecB maintained an anti-Pseudomonas activity at a high saline concentration, a typical condition of the respiratory tract of cystic fibrosis patients, which are often affected by multi-drug resistant *P. aeruginosa* infections. However, Q53 CecB was sensitive to enzymatic degradation.

Protease degradation represents one of the main limitations in the use of AMPs as antimicrobial drugs. To overcome this drawback, we produced a synthetic Q53 CecB-derived variant, carrying D-amino acids in its sequence. We are currently characterizing this peptide, evaluating its activity and mechanism of action against both planktonic and biofilm forms of *P. aeruginosa*, sensitivity to enzymatic degradation, and toxicity against human cells.

***Halla parthenopeia* and its defense systems: first steps towards and annelid model in eco-immunology and blue biotechnologies**

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Halla parthenopeia is a marine annelid which colonize soft bottom of the Mediterranean Sea. Due to its endemicity, ability to regenerate and to colonize habitats richly populated by pathogens it has a great potential as a model of study in different fields, including eco-immunology and blue biotechnologies. The latter aim to the identification of new compounds from marine organisms useful for the human health. During feeding, locomotion and predator defense, *H. parthenopeia* produces different types of mucus which are rich of bioactive compounds, with at present unknown biological roles and applications.

In order to observe its behaviour, to study its biology and to obtain a significant amount of mucus for bioactive compound purification, the worm husbandry was optimized in *ad hoc* designed aquarium systems. The easily collectable defense mucus, secreted by *H. parthenopeia*, contains a toxic anthraquinone known as Hallachrome. This compound showed a minimal inhibitor concentration (MIC) of 0.12 mM and 0.06 mM for Gram positive bacteria and for *Candida albicans*, respectively. Given the effect of Hallachrome on human pathogens, it could be used by animals as a defense against pathogens present in the sediment, or to control the microbial environment into the tunnels.

Antimicrobial peptides (AMPs) represent molecules that could integrate the action of Hallachrome. However, as for many non-model animals, studies on *H. parthenopeia* suffer from the lack of omics data and optimized experimental protocols. The whole-body RNA extraction protocol was optimized to obtain good quality RNA for transcriptomic studies. The *de novo* transcriptome, in association with tissue-specific proteomics, will serve as database for *H. parthenopeia* blue biotechnology-oriented studies.

While developing transcriptomic data, the purified mRNA was used for RT-PCR analysis aimed at identifying immune-related soluble factors constitutively expressed by the worm. First positive reactions included the AMP bactericidal/permeability-increasing protein (BPI)-like transcript and the immune mediator Lipopolysaccharide binding protein (LBP)-like transcript, immune-related factors already observed in other annelids.

In conclusion, this study presents the first evidence on the humoral defense of *H. parthenopeia* with the aim to offer a new research organism to the fields of eco-immunology and blue biotechnologies.

Actinins as novel broad-spectrum AMP isolated from the tentacle of Anthozoan *Actinia equina* (Linnaeus, 1758)

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Capturing activities and defense mechanisms of Cnidarian are strongly associated with toxins and peptide with antimicrobial properties. AMP are an important component of many organisms' innate immune system with a good inhibitory or killing effect against invaders pathogens.

We investigated the AMP activity of acid extracts obtained from tentacle and body of *Actinia equina* (Cnidaria, Anthozoa) against Gram positive (*Micrococcus lysodeikticus*) and Gram negative (*Escherichia coli*, *Vibrio alginolyticus*) bacteria. The peptide fractions showed interesting minimum inhibitory concentrations (MIC) values (concentrations up to 0.125 µg/ml) against tested pathogens. Tentacle acid extracts exhibiting a good antimicrobial activity, were further investigated, characterized and the peptides purified by reverse phase chromatography on solid phase Sep-Pak C8 column followed by several HPLC runs on C18 column. A broad-spectrum antibacterial peptides activity was detected in 40 % acetonitrile fractions.

The Peptide 6.2 has a molecular weight of 2612.91 Da and is composed of 27 amino acids (Actinin A); while peptide 7.3 has a molecular weight of 4323.07 Da and is composed of 35 amino acids (Actinin B).

The two peptides were completely sequenced and their aa sequence revealed similarity with the already described AMPs identified in amphibians and fish, with anti-Gram+ & Gram-, antifungal, candidacidal, anti- Methicillin-resistant *Staphylococcus aureus* (MRSA) activity Actinins A and B were chemically synthesized and tested in vitro against the above-mentioned bacterial pathogens. The analysis identified the peptide Actinin B which showed an interesting antibacterial and can be considered good candidates for new therapeutic applications.

Session 5. Embryogenesis, development and regeneration. Chairpersons: Annalisa Grimaldi University of Insubria, Varese, Italy and Jacopo Vizioli, University of Lille, Lille, France

Hemocytes, motile cells and immune-related mediators in adult sensory organ regeneration: evidence from the evolutionary distant models *Pomacea canaliculata* (Mollusca, Gastropoda) and *Nematostella vectensis* (Cnidaria, Anthozoa)

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The apple snail *Pomacea canaliculata* and the sea anemone *Nematostella vectensis* possess different body organization, but they share outstanding regeneration capability in adult life. *P. canaliculata* and *N. vectensis* tentacles are analogue sensory appendages, with a different anatomical organization. Our aim was to search for common cellular and molecular immune-related events during the early regeneration of these two evolutionary distant models.

Confocal laser-scanning microscopy demonstrated that during *P. canaliculata* tentacle regeneration, the hemocytes accumulated at the wound site and infiltrated the forming blastema. By combining flow cytometry and microscopy, it was shown that the transitory reduction of circulating hemocyte number, secondary to the injection of the phagocyte-targeting drug, Clophosome®, corresponded to a delay in the blastema formation. After the number of circulating hemocytes was autonomously restored by the snails, the regeneration process took place regularly, with numerous hemocytes accumulating in the forming blastema. As these results suggested a pro-regenerative role for the phagocytic hemocytes of *P. canaliculata*, their potential involvement was also investigated by qPCR experiments on immune-related genes (IRGs), e.g., *Pc-hemocyanin*, *Pc-AIF-1*, *Pc-TGAse* and *Pc-Runt*, and on genes associated with regeneration and cell-proliferation, e.g., *Pc-Wnt1*, *Pc-Jagged-1*, *Pc-FGF18* and *Pc-PCNA*. Our data demonstrated a significant increase in the expression of IRGs during hemocyte accumulation in the blastema, and a modified gene expression profile for regeneration markers following Clophosome® treatment, indicating a plausible role of hemocytes in modulating regeneration-associated gene expression.

High-resolution live-imaging method applied to control and regenerating specimen of *N. vectensis*, allowed the identification of a highly motile population of cells (mPC), that was found to accumulate at wound site within 6 h after oral tentacle amputation. After FACS sorting, RNA SMART-sequencing was performed on the mPC, demonstrating that they express IRGs such as *AIF-1*, *C2*, *C5*, *DSCAM*, *Elf-1*, *Jagged-1*, *LtA4h*, *Prxl2b*, and the macrophage marker *Mpeg*. This further supports the hypothesis that the mPC could represent an original immunocompetent cell population in cnidarians, with a cell behaviour similar to that of snail hemocytes, during tentacle regeneration.

In all, by combining advanced microscopy and molecular strategies, it has been demonstrated that phylogenetically distant animals such as snails and sea anemones, present similar cell behaviour profile during early regeneration phases, with the accumulation IRGs-expressing motile cells, supporting the view of an ancient and proactive role of the immune system in the onset of sensory organ regeneration.

Role of the extracellular matrix during the postembryonic development of the medicinal leech *Hirudo verbana*

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The extracellular matrix (ECM) is a 3D non-cellular network, composed of collagens, proteoglycans/glycosaminoglycans and various glycoproteins, which regulate different cellular functions. Interestingly, several evidence in literature have demonstrated that cell-ECM physical interactions, and thus the mechanical forces, are the most important factors involved in the maintenance of stem cell phenotype, differentiation, and cell behavior. The remodeling of the ECM and the physical interactions between cell and ECM are two important aspects that could also be involved in muscle development; however, still today, little information is available on this topic.

To obtain new insights on how a fine tuning of the 3D ECM microenvironment can drive both cellular and tissue fates and their spatial organization during post embryonic development, we used the invertebrate *Hirudo verbana* as a valuable inexpensive and easy manipulable experimental model characterized by a simple anatomical organization reproducing many aspects of the basic biological processes of vertebrates and by post-embryonic stages of development easily to stage, to manipulate and whose morphology is well described.

Overall, our data show that during leech developmental stages, ECM remodelling leads to a stiffness gradient that regulates both the migratory cell pathway and the cell fate of the developing body wall. These cells respond to changes in the 3D ECM network by activating the Hippo pathway epigenetic mechanism, of which one of the main players is YAP (Yes-Associated Protein 1), evolutionarily conserved in the medicinal leech as well.

Stress granules-related genes regulation during embryogenesis of an invertebrate chordate

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Stress granules (SGs) allow eucaryotic cells to regulate stress responses and face adverse environmental conditions. Their formation occurs in the cytoplasm with the over-expression of mRNA binding proteins, the aggregation of which blocks the translation of mRNAs for anti-stress proteins and prolong their stability.

The protection from stress represents a critical issue during embryogenesis and is important for survival of the organisms and the perpetuation of the species. The aim of this research was to investigate the transcriptional regulation of the genes for three important protein components of SGs, during early stages of development of the solitary ascidian *Ciona robusta*, the role of which in stress defense was already demonstrated in adult: TIA1 cytotoxic granule associated RNA binding protein like (TIAR), tristetraprolin (TTP) and GAP SH3 domain-binding protein (G3BP).

Electroporation experiments on embryos were carried out with constructs for reporter gene (LacZ) expression, containing the promoter region for TIAR, TTP or G3BP. The gene reporter assays allowed us to study level, time and cellular specificity of the production of TIAR, TTP and G3BP, which reflects the action of the regulatory sequences, especially occurring in notochord and mesenchyme cells of larval stages under normal physiologic conditions and in response to metal-induced stress.

Morphological and functional characterization of hemocytes in *Hermetia illucens* larvae

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Understanding the physiology of saprophagous larvae that are used for waste management is fundamental to monitor the health status of the insect during the bioconversion process. In particular, information on the immune system could provide new insights on the ability of these insects to survive in rearing substrates that are characterized by high bacterial loads and potential pathogenic microorganisms.

In the present study, we analyzed the cellular immune response of *Hermetia illucens*, known as black soldier fly (BSF), which represents the most promising ecological decomposer of organic waste substrates. In particular, although some information on the humoral and cellular response of these larvae to bacterial infections is present in the literature, little is known about their circulating cells. In fact, a characterization of the hemocyte populations of this Dipteron has never been performed, nor the processes carried out by these cells (i.e., phagocytosis, nodulation and encapsulation) have been investigated so far.

Our results demonstrate that, in addition to prohemocytes, five hemocyte types mount the cellular immune response in these larvae (i.e., plasmatocytes, lamellocyte-like cells, granulocytes, crystal cells, and adipohemocytes). Moreover, we shed light on their behavior, role, and morphofunctional changes in response to bacterial infection and injection of chromatographic beads. Noteworthy, differently from other insects, plasmatocytes represent the only circulating phagocytes in the hemolymph of these larvae. In combination with granulocytes and lamellocyte-like cells, they also take part in nodulation and encapsulation, by establishing a starting core for nodule/capsule formation around non-self agents. These processes are supported by the action of crystal cells, which release melanin precursors, and adipohemocytes, which could trophically support the cellular mechanisms through nutrient reserve mobilization.

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Response of *Mytilus galloprovincialis* ovaries to different environmental pressures

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The Mediterranean mussel (*Mytilus galloprovincialis*) is a marine invasive species cultured all over the world. Mussels are an appreciated resource in local aquaculture enterprises, become increasingly economically and ecologically relevant. Being sessile and filtering animals, they have been used for decades in programs to monitor chemical contamination in the marine environment. Although no cases of massive mortalities have been reported, the effects of different environmental pressures and water quality can alter the morphology and functionality of the organs, including the reproductive capacity of these organism.

The objective of this study was to investigate bioaccumulation abilities and biomarker responses in *M. galloprovincialis* ovaries. Analysis was carried on specimens from two different coastal areas of the Gulf of Naples, one graded as A-area and another as B-area. Such areas were differently classified according to *Escherichia coli* levels and other chemical parameters established from Regulation (EC) No. 854/2004 of the European Parliament; mussels from A-area can be sold without purification, whereas mussels from B-area must be purified before the sale.

We determined the accumulation in the ovaries of the bisphenol A (BPA), a chemical compound used to synthetize polycarbonate plastic and, mimicking the estrogen activity, able to modifies gonadal development and reproduction. Then, we investigated variables as condition and gonad-somatic indices (CI and GSI), the morphology of female gonad, the presence of apoptosis, the expression of *estrogen receptors* (*ERs*) genes, both in the non-reproductive (July) and reproductive (October) periods. Significant differences in BPA content and in the expression of *ERs* genes, particularly for ER2, were observed between the individuals collected in the different areas during the two periods. Statistically significant differences were observed also in CI and GSI, as well as in degeneration events affecting the structural organization of the ovary, leading to a considerable

increase of apoptotic cells in gonads of specimens from the B-area, no matter the period. The collected data demonstrate that mussels are excellent sentinel organisms also for the assessment of endocrine disruptors, and point out the importance of water quality parameters on health status of mussel specimens. Understanding the vulnerability of mussel beds to specific contaminants could inform and improve the management of mussel farms.

Session 6.1. Environmental challenges and the immune response. Chairpersons: Piero G. Giulianini, University of Trieste, Trieste, Italy and Gianfranco Santovito, University of Padua, Padua, Italy

Bio-plastic recognition by mussels hemocytes

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The growing use of bio-polymers derivatives poses an increasingly pressing problem regarding their environmental sustainability. In particular, it should be still ascertained the claimed absence of direct and indirect influence on ecosystems and the health of living organisms, including humans.

Our goal was about assessing the potential effects of poly-lactates and polyhydroxyalkanoates, the most widely used bio polymers classes with promising different applications for replacing conventional plastics on natural aquatic environments.

We chose *M. galloprovincialis* as sentinel species since their extensive filter-feeding activity. When it is exposed to microparticles can bioaccumulate them in soft tissues and organs. In the immunobiological investigation, to highlight if bio-polymers can influence the marine ecosystems, *in vitro* exposure assays on bivalve mussel have been carried out, and their impacts have been explored, by evaluating the cellular response of hemocytes referred to their phagocytic and/or encapsulation activity.

Preliminary evidences have shown that bioplastic particles behave in a very similar way to fossil plastic triggering the immuno-system and activating the elimination of non-self particles via cellular response. As future perspectives, although it is widely recognized that *in vitro* testing is an effective method for defining the effects of emerging pollutants, the *in vitro* test will be further deepened with *in vivo* experiments.

The medicinal leech as a valuable model to evaluate the effect of polypropylene micro and nanoplastics on innate immune response activation

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Among the different types of plastic, polypropylene (PP) is one of the most widespread, widely used in the food and textile industries for disposable packaging and to produce surgical masks. Due to the enormous distribution and the consequent abundant presence of PP waste products, it is necessary to investigate the possible toxicity on living organisms. In particular, successful regeneration requires precise coordination of multiple processes, such as clearance of cellular debris, progenitor cell activation and proliferation, immunomodulation, angiogenesis, and granulation tissue formation. The presence of PP micro (MPs) and nanoplastics (NPs) could hinder regenerations as their ingestion depletes energy reserves, reduces nutrition, survival and immune response.

Here we demonstrate that the medicinal leech *Hirudo verbana*, considered as a substitute method not subject to legislative restrictions (Legislative Decree 26 /2014), is a useful and promising model to elucidate the effects of PP on the inflammatory response. Fluorescent PP, in which a probe was introduced into the carbon chains of the polymer, has been used to better follow the plastic fate in tissues and cells of leeches exposed to water dispersed PP MPs and NPs and to evaluate their potential effect on the innate immune response stimulation as compared to not exposed leeches. Data here presented demonstrate that PP debris entering leech tissues cause morphological changes in body wall organization and increase both inflammatory and fibrotic responses, altering proper extracellular matrix and collagen deposition.

Cytotoxicity of ether perfluoro carboxylic acid PFAS congeners in earthworm granulocytes

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Soil pollution has enormously increased in the last decades. From different type of pollutants Per- and polyfluoroalkyl substances (PFAS) are found in the soil due to their various industrial uses (e.g., anti-fire foams, fluoropolymer resins such as Teflon; separation processes; textiles; cosmetics) and their high persistence. Most emerging PFAS congeners do not have toxicity data that would allow an environmental assessment. Biological approaches to soil monitoring, such as the measurement of biochemical and cellular responses to pollutants (i.e., biomarkers) on organisms living in the soil, have become of major importance for the assessment of the quality of soil. Aim of this work was to investigate the effect of these substances from an eco-toxicological point of view on non-target species, using PFAS as a reference standard. For this purpose, our study focused on assessing the potential cytotoxicity and genotoxicity of four different PFAS congeners (PFOA, HFPO-DA, PF4MOBA, PF3MOPrA) on immune system cells

(coelomocytes) of sexually mature earthworm *Eisenia foetida* in a range concentration of 0.6-229 microM. Toxicity tests were carried out according to OECD Test No 207 (by filter paper test) to assess the cytotoxic and genotoxic effect in earthworms coemolocytes.

Coelomocytes were collected from the coelomatic fluid using an insulin syringe prefilled with 0.25 ml saline solution. Coelomocytes morphometric alterations were determined by Diff quick staining; oxidative stress alterations and micronuclei frequency were determined respectively by H2DCFDA staining for ROS and DAPI coupled with fluorescence microscopy. Results showed significant alteration of the investigated patterns. An increased enlargement of granulocytes was usually observed in exposed earthworms with respect to control group. A hormetic pattern was observed. The enlargement was quantified by measuring the area of 2D digitalized granulocyte images. A decrease of oxidative burst and an increase of micronuclei frequency were also seen. Further investigation of impaired cell-mediated immune function is ongoing. The results of this study will lead to the construction of an ecotoxicological database on numerous alternative PFAS congeners allowing a weight of evidence risk assessment analysis of these substances. These data can contribute to regulation and restriction of PFAS at national and European level.

Antioxidant enzyme gene expression in *Trematomus newnesi* from Ross Sea (Antarctica) experimentally exposed to PFOA

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Thanks to its geographical and climatic isolation, Antarctica is the continent with the lowest local human impact, yet it is still vulnerable to contaminants from external sources.

Emerging pollutants, like PFAS, pose an increasing threat to this environment and therefore require more in-depth investigations to understand their environmental fate and biological impacts. This research is part of the "AntaGPS" project, funded by the PNRA, which aims to make Antarctica a global pollution sensor, using its endemic organisms as bioindicators. The project's purpose is to study the antioxidant defence components of Antarctic fish and how this system can be influenced, at the biomolecular level, by exposure to some chemical stress factors. All aerobic organisms have evolved metabolic strategies to reduce the toxicity of reactive oxygen species (ROS), natural by-products of aerobic metabolism and continuously produced by cells, both at the cytoplasmic and mitochondrial levels. Furthermore, several studies confirm the correlation between toxicity induced by xenobiotics and an increase in ROS formation, with a consequent more significant risk of oxidative stress. For these reasons, variations in the content and the activity of antioxidant enzymes can be used as biomarkers for contaminant-mediated oxidative stress in several marine organisms. Specifically, this research focuses on the expression analysis at the transcriptional level of genes coding for four antioxidant enzymes (sod1, sod2, gpx1, gpx4) in 2 different organs of an Antarctic fish species, *Trematomus newnesi*. The kidney showed a higher expression level than the liver of wildlife specimens for each antioxidant enzyme (but the most expressed is sod1). The mRNA levels were assessed in fish exposed to 1.5 µg/L of PFOA for ten days. In the liver, the treatment induced an increase in gene expression for all the considered enzymes, while in the kidney, it induced a general decrease (especially for sod1). The obtained results can contribute significantly to the prediction of the physiological responses of these organisms to environmental changes, which can improve the general understanding of the molecular and functional evolution of Antarctic fishes. Furthermore, our gene expression analysis may provide the basis for using antioxidant enzymes as indicators of oxidative stress and PFOA exposure.

MAIN LECTURE

Ascidian cytotoxic cells: from zero to hero

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Ascidian cytotoxic cells are multivacuolated cells, variable in morphology, abundantly represented in the circulation, playing important roles in ascidian immunosurveillance. The interest of researchers towards these cells arose three decades ago when it was realized that, in colonial species, they were the effectors of the rejection reaction between contacting, genetically incompatible colonies. Indeed, they are the first cells to sense nonself and, upon the recognition of foreign molecules, are selectively recruited to the infection site where they release the content of their vacuoles. Their cytotoxic activity is closely linked to the activity of the enzyme phenoloxidase (PO), a copper-containing enzyme widely distributed in invertebrates, contained inside their vacuoles together with its polyphenol substrata. Recent data indicate that ascidian cytotoxic cells synthesize and release the majority of the complement factors of both the alternative and lectin pathways. In addition, they are also the main source of antimicrobial peptides codified by the ascidian genome. Therefore, these cells, once neglected, are now drawing the attention of researchers for their multiple roles in immune defense.

Session 6.2. Environmental challenges and the immune response. Chairpersons: Maria Giovanna Parisi University of Palermo, Palermo, Italy and Loriano Ballarin, University of Padua, Padua, Italy

Immune response of the ascidian *Ciona robusta* to abiotic and biotic stressors

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Environmental changes provoke huge stresses on sessile marine organisms making them more or less susceptible to infections, and in turn organisms develop complex response mechanisms to keep homeostasis for surviving. The immune system of the solitary ascidian *Ciona robusta* relies on two organs, the pharynx and the gut, and encompasses a wide array of immune and stress-related genes which are involved in the response to environmental challenges and modulated in adapting to changing conditions. Recently, we have demonstrated that *C. robusta* immune response can be modulated after priming and challenging animals with microbial agents. The establishment of "immune memory" relies on the activation of different cellular and humoral immune mechanisms aimed to develop a more protective response.

In the present study, we assessed the primary response in the gut and pharynx of *C. robusta* in terms of expression of several immune-related genes (*C3-1*, *C3ar*, *Il17-1*, *Il17-2*, *Il17r*, *Tnf, Tgfb*, *Lbp*, *Tlr-2*, *Tlr13*, *Cd36*), pharynx- and gut-specific genes involved in mucosal immunity (*VCBP-B* and *VCPB-C*) and oxidative stress-related genes (*Sod-A*, *GST*, *GR*) upon exposure to hypoxia/starvation (H/S) for 2 or 18 h and in presence of polystyrene nanoplastics (Nps) or not. We also evaluated how a previous exposure to abiotic stresses could influence the ability of *C. robusta* immune organs to react to a subsequent bacterial challenge (LPS).

Our preliminary data suggest that i) the immune response to stress vary greatly between the two organs, ii) the presence of Nps attenuate the gene expression induced by H/S in both organs, iii) animals previously exposed to H/S stress when challenged with LPS shown a "tolerance" to LPS and this H/S stress-induced memory response is only partially modulated by the presence of Nps.

In conclusion, while gut and pharynx differently react to abiotic and biotic stresses, the concurrence of abiotic stress seems to reduce the primary response in both organs, and Nps only partially affect the H/S stress-dependent induction of innate memory.

Tyre wear particles effects on growth and some immune parameters in the terrestrial isopod *Armadillidium pallasii*

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About 1.3 million tons of tyre wear are generated on Europe's roads each year and are probably the most important source of polymer-based plastics entering the environment. Therefore, itis of particular interest to know the impact of this material on soil organisms living near busy roads (Baensch-Baltruschat *et al*., 2020). *Armadillidium pallasii* (Brandt, 1833) was collected in Gradisca d'Isonzo (Gorizia), Italy and grown for several months in containers with soil certified for organic farming, pH 7 (Bioterril, Geotec), in an experimental room with controlled photoperiod (12:12, Light:Dark) at a mean temperature of 25 °C. 75 *A. pallasii* (weight: 0.223±0.036 g, length: 17.6±0.9 mm) were evenly distributed in 15 glass terrariums (12x12 cm): 3 replicates for 4 different concentrations of 10%, 5%, 2.5% and 1.25% (tyre particles/soil, w/w) and 3 control replicates without tyre particles were tested for 30 days. The tyre dust was obtained in laboratory by abrasion of the tyre tread with a grinding stone. Haemolymph from *A. pallasii* was collected from described by *Dolar et al.* (2021). For determination of total haemocyte count (THC), one drop was placed in the Buerker counting chamber and about 10 µl were centrifuged and immediately stored at -20 °C for analysis of phenoloxidase (PO)-like activity. After 30 days of treatment, animals in soil containing 5% and 10% tyre particles are significantly shorter (5%: 17.23±1.00 mm; 10%: 17.4±0.06 mm) than at the beginning of the experiment (5%: 17.60±0.76 mm; 18.20±0.04 mm). Average weights decrease during the experiment, but no significant differences were found between animals raised in soils with different proportions of tyre particles. Mean THC levels are highest in animals raised in soil containing 5% (5.44x106±3.14x106 haemocytes/mL) and 10% (4.36x106±1.87x106 haemocytes/mL), but no significant differences were observed. PO activities of animals showed higher values in animals treated with soil containing 1.25%, 5% and 10% tyre particles, compared to the control animals but no significant differences were found. Since the literature emphasises the stronger effect of tyre dusts together with road abrasion dusts, a second experiment was conducted to evaluate the effects of

these dusts collected in Poland also on key behavioural abilities of isopods such as their locomotor activity, anxiety-like responses and a form of learning.

Evaluation of stress biomarkers in the European nightcrawler *Dendrobaena veneta* exposed to PFAS under controlled conditions

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Poly- and Perfluoroalkyl substances (PFASs) are a broad family of synthetic compounds widely used by industries and subsequently released in the environment. Several studies on the effects of PFAS were carried out on animals. These studies showed the danger of these substances to the world community. To investigate the effects of PFASs, an in vivo laboratory study was set up. The nightcrawler *Dendrobaena veneta* has been used as a model organism for an in vivo exposure experiment under controlled laboratory conditions. *D. veneta* has been chosen as an experimental model since earthworms are recognised among the 5 key bioindicators for the ecotoxicological evaluation of persistent environmental pollutants. Earthworms were exposed to three different concentrations of PFAS mixtures: the concentrations applied resulted in average concentrations of soil samples in the Veneto region. The results underlined that, in the short term (30 days), these compounds induce an increase in ROS levels in the mitochondria with a consequent increase in genomic damage. The antioxidant system of these organisms consists mainly of low molecular mass antioxidant molecules such as GSH, metallothionein (MT) and vitamins. No significant differences in the Total Antioxidant Capacity (TAC) between the different treatments were shown, indicating the activation of the physiological stress response induced by xenobiotics. The oxidation of low molecular mass antioxidant molecules mainly drives the depletion of TAC. However, there was a trend in the TAC according to the increasing concentration of PFASs. Furthermore, it was observed that the genomic damage assessed by the Comet test reported DNA damage in the control group and the PFAS-exposed groups. Of course, laboratory conditions may affect the overall health assessment of organisms compared to *in situ* experiments. Furthermore, it has been reported that exposure to PFASs enhances both transcriptional and translational levels of MTs, implying their possible involvement in response to oxidative stress in mitochondria.