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REPORT OF MEETING

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Organizers: **L Ballarin, V Matozzo, F Sandrelli, G Santovito, P Venier**

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

Characterization and functional role of a novel C1qDC from a colonial ascidian

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The complement system is present in all the metazoans as a complex array of soluble and membrane proteins able to orchestrate innate immune responses such as inflammation and phagocytosis. Although the complement system of invertebrates has been much less studied than that of vertebrates, however, it is equipped with at least the alternative and the lectin activation pathways.

The C1q-domain-containing (C1qDC) proteins are a large family of proteins, present in both vertebrates and invertebrates, characterized by one or more globular C1q (gC1q) domain(s) at the C-terminus. C1qDC proteins are distinguished in C1q-like proteins, with a gC1q domain and a collagen-like region at the N-terminus, and globular head C1q proteins (ghC1q) with one or more gC1q domains and a short N-terminus with no defined domains. The latter can be further divided into proteins without a signal peptide (cellular ghC1qs or cghC1qs) and proteins endowed with a signal peptide (secreted ghC1qs or sghC1qs). The gC1q domain has a typical jelly roll topology of five pairs of anti-parallel β-strands creating two β-sheets, with eight conserved hydrophobic amino acids and can interact with a large variety of ligands, both self and non-self. The same topology is present in the tumor necrosis factor (TNF) domain of protein of the TNF family so that a C1q-TNF superfamily of proteins (C1q/TNF-related proteins or CTRPs) has been defined. The mammalian complement component C1q, a subunit of the C1 complex of the classical complement activation pathway, has been the most thoroughly studied vertebrate C1qDC protein. In addition to activating C1r and C1s (and, as a consequence, in C3), C1q can also act as pattern recognition receptor (PRR) as, through its qC1q domain, it can recognize and bind pathogen-associated molecular patterns (PAMPs) on the surface of microbes and modulate their phagocytosis. Most of the C1qDC proteins have only a gC1q domain but the presence of molecules with multiple tandem C1q domains have been reported in both invertebrates and vertebrates; among the latter, CTRP4 is the only protein with two C1q domains described in mammals, birds, reptiles, amphibians and teleosts.

The compound ascidian *Botryllus schlosseri* is a chordate invertebrate that relies only on innate immunity for its defense. Immunocytes (i.e., cells with defined roles in immunity) represent the great majority of the circulating hemocytes: they include cytotoxic morula cells and phagocytes. In this same species, we identified the key components of the lectin and the alternative pathways. All these complement components (C3, Bf, MBL, ficolin and MASP), are expressed by morula cells, the most abundant circulating hemocyte.

In this study, we mined the available transcriptomes and identified, in *B. schlosseri*, a novel multidomain C1qDC protein (BsC1qDC). It belongs to the sghC1q proteins and contains two gC1q domains, a signal peptide and present high similarity with human CTRP4. We followed the expression of BsC1qDC during the colonial

blastogenetic cycle and in colonies injected with Gram (+) bacteria and identified its mRNA location by in situ hybridization (ISH). The expression trends during the colonial blastogenetic cycle suggest the presence of checkpoints modulating the transcription of bsc1qdc. The protein is synthesized and released by morula cells and a minority of phagocytes. When we knocked down the gene, we observed a decrease in phagocytosis of target particles, probably related to the involvement of BsC1qDC in the opsonization of non-self, as well as a decrease in degranulation and is involved in.

Ongoing studies are trying to better clarify the role of bsc1qdc in *Botryllus* immune modulation and its interplay with the other complement components such as complement control proteins.

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

Immune contribution to tentacle regeneration in adult mollusc and cnidarian models

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Adult regeneration is a fascinating process that consists in regrowth and regain of function of tissues and organs. The role and contribution of the immune system and immune-related pathways in adult vertebrate and invertebrate regeneration have been investigated for a long time, but important gaps remain. The freshwater snail *Pomacea canaliculata* and the sea anemone *Nematostella vectensis* are two phylogenetically distant organisms, with regenerative capabilities in adult life. These models present different innate immune components, and we focused on their involvement during tentacle regeneration. The two cephalic tentacles of *P. canaliculata* are sensory components used for food search, co-specific recognition and orienting. In *N. vectensis*, the numerous oral tentacles (4-18) are extensions of the diploblastic body, forming appendages that feed, defend and expand the surface area of the gastric cavity.

Histological studies focusing on the early cephalic tentacle regeneration in *P. canaliculata*, have demonstrated that wound closure and blastema formation took place within 24 h post amputation (hpa). A Matlab® plugin allowed the semi-automated identification and quantification of a phagocytic hemocyte sub-population in the blastema. Flow cytometry analysis showed that the injection of the phagocyte-specific drug Clophosome® (45 µg/g snail) could transiently remove circulating hemocytes, that recovered the pre-treatment level within 24 h. Consistently, histological experiment demonstrated that rare hemocytes were present in the early regenerating tentacles of Clophosome®-injected snails. Moreover, the hemocyte depletion impacted on regeneration time, and the blastema took twice as long to form, i.e., 24 h. This extended time overlaps with the time of recovery from Clophosome® treatment, further suggesting a role for *P. canaliculata* hemocytes in the onset of tentacle regeneration.

Differently from molluscs, *N. vectensis* presents no specialized immune cells, though cells displaying phagocytic activity were recently identified. Moreover, components of the main pathways of invertebrate humoral immunity are present. A transgenic line labelling a highly motile population of cells (mPC), similar in shape to vertebrate macrophages, enabled us to investigate their behaviours in homeostasis and regenerating conditions. Because immunostaining showed an accumulation of mPC to the wound site at 6 hpa, a high-resolution live imaging method was developed to investigate in real time to validate their direct migration to the wound site. In vivo imaging showed that mPC are positive for SoxB2, a neural precursor marker, which suggests that mPC originates from a neural cell lineage during development and differentiate in a migrating cell type. To define the molecular signature of mPC, we used FACS to sort them in view to perform RNA SMART-sequencing and characterize their gene expression.

In all, these data suggest a pivotal role for hemocytes during early stages of tentacle regeneration in *P. canaliculata*. Similarly, our original data on *N. vectensis* suggest the presence of cells imitating the behavior of molluscan hemocytes during early stages of regeneration. Intriguingly, mPC also seem to share their origin with neural cells, thus providing an example of the tight connection between immune and nervous systems also in diploblastic animals.

PLENARY LECTURE I

Immune-microbiota interplay in oyster health and disease

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The presence of complex host-associated microbial communities is a characteristic shared by most animal species and the type of interactions that they establish with their host can range from mutualistic to pathogenic. The capacity for microbes to colonize a host depends on both host and microbial determinants. In the marine environment, bivalve mollusks constitute habitats for bacteria of the Vibrionaceae family. *Vibrio* belong to the microbiota of healthy bivalves, which have the ability to concentrate bacteria in their tissues and body fluids, including the hemolymph. The oyster immune system tolerates rather high amounts of *Vibrio* in its hemolymph. However, *Vibrio* can also proliferate in oyster tissues leading to mass mortalities of oysters. Other microorganisms such as the OsHV-1 virus have the ability to alter oyster immunity leading to

dysbiosis and oyster death. In this process OsHV-1 and *Vibrio* synergize to kill oysters. However, while some *Vibrio* populations actively cooperate, others behave as cheaters. In this presentation, I will review the current knowledge on the complex immune-microbiota interactions at play in oyster health and disease.

Session 1. Immune competence and immune response (part I). Chairmen: Paola Venier, University of Padua, Padua, Italy and Annalisa Grimaldi, University of Insubria, Varese, Italy

Antiviral sensors and ZNFX1 in the innate immunity of invertebrates

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Double-stranded RNAs (dsRNAs) are commonly detectable in metazoan cells. In particular, dsRNAs of viral origin can trigger the immune response in the host. Sensors of nucleic acids (including DNA, ssRNA, or dsRNA) have emerged across the three domains of life as a first step in activating specific and non-specific host defenses against viruses. Sensors, such as Toll-like receptors and cytosolic proteins from the broad family of DExD / H-box helicases, act alone or in combination with other proteins to promote and amplify the antiviral response in infected cells. The zinc finger-type NFX1 containing 1 (ZNFX1) is one of the early sensors of viral dsRNAs. The human ZNFX1 is an outer mitochondrial membrane-associated helicase, capable of promoting a type I interferon-mediated response. We have traced the presence of ZNFX1 in metazoans and, based on 221 sequences, we obtained a polyphyletic tree generally consistent up to the phylum level. Moreover, we examined the ZNFX1 expression profiles of selected invertebrate species for which transcriptomic data after viral infection are available. As a result, we found that ZNFX1 was overexpressed in mollusks (*Crassostrea gigas, Scapharca broughtonii*, and *Haliotis diversicolor*) and in a lepidopteran arthropod (*Trichoplusia ni*), but not in a hydrozoan of the genus Millepora (*Acropora millepora*), a decapod crustacean (*Litopenaeus vannamei*) and in a nematode (*Caenorhabditis elegans*). Overall, our analyses support the role of ZNFX1 as an early antiviral sensor in different invertebrate species.

First morpho-functional characterization of *Anemonia viridis* amoebocytes: a light microscopy study

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Anthozoan, as other cnidarians have ameboid cells into their mesoglea. These cells, called amoebocytes can move through the tissues and aggregate near wound sites reacting also to grafts. For the first time, we evaluated morpho-functional characteristics of amoebocytes of the sea anemones *Anemonia viridis*. For this purpose, we sampled cells from the mesoglea of sea anemones. Under the light microscope, we recognized two subpopulations of amoebocytes: granulocytes and hyalinocytes. Granulocytes showed a high number of cytoplasmatic granules, while hyalinocytes showed a cytoplasm with no or few granules. Amoebocytes showed both round and spreading shapes and were divided in basophils and acidophils, in addition also neutrophils were observed. Amoebocytes actively phagocytized yeast cells and produced intracellular superoxide anion. In addition, we evaluated the presence of hydrolytic enzymes in amoebocytes. We observed positive cells to acid phosphatase, acid esterase and non-specific esterase, with no differences in term of positivity between granulocytes and hyalinocytes. Other studies are needed to fully investigated amoebocytes features in this anemone species, including ultrastructural investigations.

Analysis of structural variants and associated gene presence-absence variation phenomena in the genome of the pacific oyster *Crassostrea gigas*

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In recent decades the advancement of sequencing technology has given the opportunity to assemble very complex genomes on a chromosomal scale and to re-sequence the genome of several individuals belonging to the same species. Through the re-sequencing of different individuals of the same population it has been observed that some genomic regions may be present in some specimens and not in others. This phenomenon is called gene presence absence variation (PAV) and leads to the identification in a population of two sets of genes: the core genes, i.e., genes shared by all individuals, and the dispensable genes, i.e., genes that may be present in some individuals and absent in others. Core genes are mostly linked to housekeeping functions, while dispensable genes are not essential for survival but may provide accessory functions useful for adaptation and survival under certain environmental conditions. PAV appears to be widespread in nature and is likely fundamental to allow an enormous inter-individual diversity in many branches of the tree of life. In this work, a bioinformatics pipeline was developed for the identification of dispensable genes in the genome of diploid eukaryotic species and for the study of the PAV phenomenon. The pipeline was tested and applied on the Pacific oyster *Crassostrea gigas*, demonstrating that PAV phenomena also occur in the genome of this species. Functional enrichment tests have shown that dispensable genes have specific functions related to survival, such as immune response and apoptosis. Further analyses focused on the

functional characterization of some of the many molecular players involved in such processes will be necessary to clarify their involvement in pathogen recognition and elimination, and to define in particular the evolutionary advantages that may arise from their presence/absence in different individuals.

Understanding bivalve physiology through NMR metabolomics

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The increase of abnormal mortalities in marine aquaculture bivalves occurring in this time of global warming has brought more attention to the intricate relationships between host and pathogens. The study of metabolite profiles (relative concentration of small molecules, such as amino acids, carbohydrates and organic acids) in biological fluids and tissues is an expanding field of investigation, successfully applied to human, animal, and plant physiology and pathology. We obtained 1H 1D-NMR tissue-specific metabolic profiles of digestive gland, gills and hemolymph samples of mussels (*Mytilus galloprovincialis*) recovering from functional anaerobiosis or responding to an injection of live *Vibrio* bacteria at 18 °C and 25 °C. We detected many resonances, from 227 in total hemolymph lysates to 883 in the whole flesh samples and 69 - 82 % of the signals were assigned. The assigned signals mostly arise from free amino acids (FAA), osmolytes, organic acids, and sugars, such as mytilitol. Mussel acclimation after functional anaerobiosis led to a significant decrease of succinic acid in hemolymph spectra, accompanied by an increase of most FAA and osmolytes, more pronounced at 18 °C than 25 °C. After injection of 200 µL of 108 CFU/mL of live *Vibrio splendidus*, several FAA, sugars and unassigned chemical species significantly decreased, with similar variations between 18 °C and 25 °C. These variations could match a simultaneous increase of FAA in the hepatopancreas, as reported for *Perna canaliculus* injected with *Vibrio* bacteria (Nguyen *et al*., 2019). Overall, these results support the integration of NMR metabolomics with other 'omics as a convenient tool to study marine bivalves.

ADAR-editing in mollusc species: in between physiology and antiviral response

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RNA editing processes increase the molecular diversity of primary transcripts and can significantly alter the gene product function. The conversion of adenosine to inosine (A-to-I) is considered as the most common type of RNA editing in metazoans and is catalyzed by members of the enzyme family of Adenosine deaminases acting on double strand RNA (ADARs). The ADAR-mediated RNA-editing is crucial for the organism homeostasis, for instance the editing of proteins such as ion channels and neuroreceptors is critical in the development and functioning of nervous system. ADAR is also involved in host-virus interactions, and it can act as proviral or antiviral depending on virus-host combination. As regards Mollusca, the physiological role of ADAR has been investigated only in Cephalopoda. We analyzed 87 and 30 RNA-seq datasets pertaining to *Crassostrea gigas* and *Scapharca (Anadara) broughtonii*, respectively, both exposed to Ostreid Herpesvirus-1 (OsHV-1). We compared the ADAR-editing levels on host and viral transcripts, and we traced the ADAR hyper-editing impact on the host genes. In contrast to the infected blood clam, oyster RNAs were more hyper-edited than viral RNAs and this could relate to the differential amount of OsHV-1 dsRNAs. A core set of genes were constantly hyper-edited in both bivalve hosts, a finding suggesting a physiological role of ADAR hyper-editing. Conversely, host genes involved in antiviral response, miRNA maturation and epigenetic regulation were hyper-edited in specific infection phases only.

Tissue distribution of a rhamnose binding lectin in the colonial ascidian *Botryllus schlosseri* and effects of microinjections of the specific antibody into the circulatory system

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Lectins are non-enzymatic and non-immunoglobulin proteins, or glycoproteins, that bind carbohydrates with their Carbohydrate Recognition Domains (CRDs). They are involved in various biological processes, including host-pathogen interaction and intercellular communication. They play pivotal roles in the immune system of invertebrates by binding pathogens directly and opsonizing them.

Botryllus schlosseri in a cosmopolitan ascidian, considered a reliable model organism for studies on the evolution of the immune system. *B. schlosseri* Rhamnose-Binding Lectin (BsRBL) acts as a chemokine and opsonin by interacting with different cell types. Although described in previous works, many aspects and roles of this lectin remain unknown. Here we studied the changes in tissue distribution of BsRBL during immune responses using light and electron microscopy. In addition, following the hints from extant data, suggesting a possible role of BsRBL in the process of takeover, we investigated the effects of the removal of this protein, using a specific antibody, during the generation change, opening new queries on the roles of this lectin in *Botryllus* biology.

Phylogenetic and transcriptomic analysis of two putative STAT genes in the colonial ascidian *Botryllus schlosseri* and their involvement in immunity.

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The JAK/STAT pathway is an evolutionary conserved signalling pathway triggered by diverse cytokines, interferons, grow factors and related molecules. This pathway represents a remarkably straightforward mechanism whereby extracellular factors control gene expression. 7 STAT family members have been identified in mammals, while most of non-chordate invertebrates have one or two STAT genes. In all species here investigated belonging to tunicates, which are the chordates considered the sister group of vertebrates, two STAT proteins have been found, but their functions have not been elucidated yet.

In this study we present the identification of two putative different STAT transcripts in the transcriptome of the colonial tunicate *Botryllus schlosseri*. These transcripts, found in silico, have been verified experimentally and sequenced, and their translation produces two proteins named STAT1 and STAT2. Once we obtained the full sequences of these transcripts, we performed a Maximum Likelihood phylogenetic analysis to investigate the homology relationship between the STATs in chordates. The analyses indicate that a duplication from a unique STAT gene happened in the chordate ancestor, resulting in the ortholog to vertebrates STAT5 and in an ortholog to all the other vertebrate STAT gene for all chordate, followed by subsequent duplications from the second one in the vertebrate evolutionary lineage. The molecular expression of *B. schlosseri* STAT genes was measured throughout the colonial asexual cycle. Initially, this analysis has been done collecting cDNA from whole colonies, and then extended collecting cDNA from extracted haemocytes, showing that these genes are differentially expressed in the different colonial phases. As reported in literature, the JAK/STAT pathway is triggered by several interleukins in mammals, such as IL17. Three IL17 genes have been found in the solitary tunicate *Ciona intestinalis* and their expression is upregulated through lipopolysaccharide (LPS) stimulation. The latter activates the immune response, producing the cytokine TNF-α. We hypothesized the potential activation of the JAK/STAT pathway via LPS stimulation in *B. schlosseri* and we verified it throughout RT-qPCR, finding over expression of the STAT genes and Myc, an important gene for stemness, which has been proved to be a target of these transcription factors. In conclusion, the results suggest that the JAK/STAT pathway play a role in the innate immune responses of this colonial chordate.

How can we define "self"? Complement system and missing-self theory.

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The complement system, with its main protein C3, is the main humoral system of the innate immunity, that type of immunity possessed by almost all the animal kingdom. In the absence of regulatory proteins, uncontrolled activation of the system can also result in disruption of host cells, resulting in immune-mediated tissue damage.

We know that vertebrate immune system is able to discriminate between self tissues from non-self as well invertebrates. Furthermore, the former are able to have a rejection reaction against allogeneic transplants thanks to the adaptive part of their immune system. The latter, that totally lack of adaptive immune system, are able to discriminate between self body and body parts derived from conspecific organisms very similarly to Vertebrates. How is it possible if they lack of adaptive immunity?

Now we know allorecognition machineries in invertebrate animals has nothing to do with those of Vertebrates partially responding to the question if allorecognition in the animal kingdom are of monophyletic origin or evolved independently.

Cnidarian, annelids and protochordates, e.g., are able to allograft rejection with molecular machineries that has nothing in common with the MHC- based histocompatibility reactions of vertebrates, but (some of them) involved highly variable complement receptor-like protein. Now I'm exploring the hypothesis that proteins controlling complement system might have an evolutionary ancient role not only in immune defense but also in histocompatibility. Hypothesis that associates well with the so called "missing-self theory".

Isolation and characterization of the polymeric Ig receptor gene from the cold adapted teleost *Trematomus bernacchii*

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The polymeric immunoglobulin receptor (pIgR) plays a pivotal role in vertebrate immunity, as it mediates the transport of mucosal antibodies across epithelial layers into the external secretions.

In recent years, much attention has been devoted to the function of pIgR in teleost fish, however, information on its gene structure remains still limited. Not even data are available on pIgR from teleost species living under extreme conditions, such as Notothenioidei (suborder

Perciformes), the dominant group of fish inhabiting the extremely cold environment of Antarctica. To enhance the current knowledge in this field, we characterized the structure of the *pIgR* gene of the Antarctic teleost *Trematomus bernacchii*, through a comparative analysis built on genomic and transcriptomic databases available for multiple species belonging to five perciform suborders. We identified unexpected modifications in the Antarctic *pIgR* genes, e.g., intron lengthening, transposable element content and additional regulatory elements. Furthermore, the full-length cDNA and deduced amino acid sequence were obtained. Multiple sequence alignment highlighted that several amino acid substitutions were exclusive to Antarctic pIgRs. Among notable changes, some led to a gain of N-glycosylation sites, strongly suggesting a putative role in the adaptive response to cold temperatures. Expression analysis through q-PCR and *in situ* hybridization showed that pIgR transcripts were constitutively expressed in the mucosal tissues and liver. Overall, our work unraveled specific features of the *pIgR* gene from cold adapted teleost species, providing additional information regarding the structure and organization of *pIgR* genes in teleost fish.

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PLENARY LECTURE II

Climate change and pollution effects on bivalves: cell culture models as promising tools?

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An overarching aim of the Laboratory of Environmental Marine Sciences (LEMAR) from Université de Bretagne Occidentale (UBO) in France is to better elucidate the interactions between marine organisms and their environment. A better understanding of organism adaptation to biotic and abiotic factors and the role of the microbiota in health and disease are crucial focal points for ecosystem sustainability on a changing planet. To address this challenge, our group is currently developing two key research strategies using two bivalve species: the Pacific oyster *Crassostrea gigas* and the Manila clam *Ruditapes philippinarum*. Bivalves are well-recognized as excellent models to study environmental health and, most recently, host-microbe-environment interactions.

Our first strategy aims at better understanding the health impacts of pollution and climate change on aquatic invertebrates, by performing measurements at cellular level. Cells integrate a multiplicity of signals to ensure organismal defense, survival and growth, thus disruption of cellular function can be detrimental often leading to decreased health or lifespan. It is therefore essential to improve culture protocols of bivalve cells. Like the oyster cardiomyocyte model, a well-standardized and long-term hemocyte culture model would be really useful, as well as a toolbox of cellular tests for functional monitoring, particularly for ecotoxicology analyses. Our group has recently successfully optimized the conditions to maintain *C. gigas* immune cells (hemocytes) viable and functional in culture for at least fifteen days, which is unprecedented for bivalve hemocytes.

Our second research strategy aimed to address the fundamental and timely issue of the Manila clam's response to climate change, and encompasses a unique, multi-level and transdisciplinary experimental research plan. The recently accepted international CLIMCLAM project, joins scientists from two laboratories, LEMAR and the Department of Comparative Biomedicine and Food Science (BCA) from University of Padua (UNIPD) in Italy.

By combining *in vitro* and *in vivo* approaches, our group aims at unravelling environmentally relevant cellular mechanisms and organismal effects of biotic and abiotic factors in the context of global changes to support human, ecosystem, and animal health (One Health).

Session 2. Development and immunity. Chairmen: Giuseppe Scapigliati, University of Tuscia, Viterbo, Italy and Davide Malagoli, University of Modena and Reggio Emilia, Modena, Italy

The role of the immune modulator HvRNASET2 during development

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The *Hirudo verbana* protein *Hv*RNASET2 is a pleiotropic enzyme, acting as a key molecule in inflammation, immune response and regenerative processes. Indeed, following bacterial infection, *Hv*RNASET2 induces macrophages recruitment in order to trigger and potentiate the inflammatory response. Furthermore, *Hv*RNASET2 is also involved in the correct progression of muscle tissue regeneration during wound healing process, by regulating the recruitment of the myoendothelial vessel-associated precursor cells, fibroplasia and synthesis of new collagen. Taken together, these data strongly suggest that during wound healing and tissue regeneration, *Hv*RNASET2 is involved in the restoration and maintenance of tissue homeostasis through extracellular matrix (ECM) remodeling.

Recently, several data in literature have demonstrated a correlation between collagen synthesis, ECM organization and tissue morphogenesis during embryogenesis and post-embryonic development. In order to shed light on the possible role of *Hv*RNASET2 as modulator of collagen deposition during these processes, here we evaluated the expression of this enzyme during different stage of *H. verbana* development, starting from the embryos until the juvenile and adult leeches.

Defining the Antarctic krill's ontogenesis from a transcriptomic point of view

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The Antarctic krill (*Euphausia superba*) has a key role in the Southern Ocean ecosystem. During the last 30 years, the abundance of krill in the southwest Atlantic sector has constantly decreased and the reasons behind this decline are still unclear. The main bottleneck that affects the population abundance is the surviving to the larval life across the first winter. Daily vertical migrations as well as an oscillatory oxygen consumption pattern have been recently observed in krill larvae and they likely take part in a complex behavioural and physiological strategy to increase the recruitment success. Total RNAs extracted from seven different krill's larval stages, belonging to three out of 4 main developmental phases (metanauplius, calyptopis and furcilia), were used to produce and sequence seven stage-specific cDNA libraries (Illumina technology) in order to define the gene expression signature of each developmental stage. Also, the juvenile stage which represents the adult stage that is not sexually mature was included in the analysis. We obtained a total of about 1.2 billions of 100 nt paired-end reads. An unsupervised hierarchical clustering analysis showed specific gene expression signatures for early developmental larval stages with respect to late larval stages and young adult (juvenile). Interestingly, among differentially expressed genes between early and late larval stages we identified the circadian clock components as well as genes involved in light entrainment that could elucidate the role of the time-keeping system during larval development.

Dynamics of formation of stress granules during the colonial blastogenetic cycle of *Botryllus schlosseri*

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Stress granules (SGs) are cellular ribonucleoprotein foci preserving mRNAs for anti-stress proteins and so regulating stress responses. This is possible thanks to the presence of mRNA-binding proteins such as TIA-1 related nucleolysin (TIAR), considered an important core component of SGs.

Botryllus schlosseri is a colonial ascidian easily found in the Lagoon of Venice, which undergoes weekly generation changes called take-overs (TOs). A blastogenetic cycle is defined as the period between two successive TOs. During the TO, lasting 24-36 h, a diffuse apoptosis occurs in tissues of old zooids, which will be replaced by their primary buds representing the new generation. At TO, an increase in oxygen consumption (respiratory burst) takes place with the consequent production of reactive oxygen species representing a stressful condition for the new zooid generation. We suppose that SGs can play a pivotal role in the protection from oxidative damages. To verify this hypothesis, in this work we used the TIAR protein as marker to study the dynamics of formation of SGs during the colonial blastogenetic cycle of *B. schlosseri*. At first, we analyzed the modulation of mRNA transcription levels for TIAR by quantitative Real Time PCR (qRT-PCR) and the location of its transcript in the hemocytes through *in situ* hybridization (ISH). Then, we used an antibody specific for TIAR on hemolymph monolayers, and on colony paraffin sections, to confirm the involvement of immunocytes in detoxification. Our results agree with the idea that immunocytes represent the major detoxification system in ascidians, active in the control of TIAR protein synthesis and, therefore, in SGs formation.

Brain and immunity in the teleost fish *Dicentrarchus labrax* along development: A preliminary study

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The central nervous system (CNS) was traditionally considered an immune privileged anatomical region, unable to produce a pro-inflammatory immune response, as it would be hazardous for the brain tissue integrity. However, it has recently been found that multiple lymphocyte populations reside in and/or migrate to mammalian brain compartments under steady state conditions, playing fundamental roles in cognitive process,

neurogenesis and immunosurveillance. The European seabass (*Dicentrarchus labrax*), an economically relevant marine teleost fish, is a well-known attractive model for developmental, anatomical and functional comparative studies of the immune system (IS). However, despite the recent advances, the link between the adaptive immunity and the CNS is still unclear in this species, and the current knowledge remains very limited. Thus, we report herein a preliminary study aimed at describing the relationship between IS and CNS along its development. Two panels of monoclonal and polyclonal antibodies, currently available for *D. labrax,* were used for the identification and localization of T and B lymphocytes within the CNS, both in larval stages and juveniles. Moreover, the expression of several immune-related genes was evaluated through q-PCR in the brain tissue of juvenile specimens. Finally, a novel RNA-seq experimental approach is being used for an in-depth expression analysis, which has never been carried out before on early sea bass developmental stages. Using the laser microdissection technology, we dissected the brain of sea bass larvae, and the transcriptomes obtained will soon be analyzed for the first expression of its kind of immune and neuro-related genes.

Session 3. Organism interactions. Chairmen Adriana Vallesi, University of Camerino, Camerino (MC), Italy and Valerio Matozzo, University of Padua, Padua, Italy

Structural and phylogenetic evidence supports a key role played by helix-3 of *Euplotes* pheromone structure in autocrine and heterologous pheromone/receptor interactions

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Like many other organisms, ciliates rely on diffusible pheromones to socialize. In *Euplotes*, these cell signals form species-specific families of structurally homologous disulfide-rich globular proteins which bind their receptors on target cells in competition with one another, eliciting cell growth or mating responses according to whether the binding occurs in autocrine, or heterologous fashion, respectively. In each cell type, the receptors' extracellular ligand binding domain is structurally identical with the soluble pheromone, as a result of a common gene determination via an intron-splicing mechanism. Given this structural context, the pheromone/receptor interactions were inquired by carrying out a comparative crystallographic analysis of pheromones E*r*-1 and E*r*-13 each specific to cells with strong mating compatibility. E*r*-1 and E*r*-13 crystals showed to markedly differ in their symmetry space groups, *C*2 and $P4_3$, respectively. Nonetheless, both crystals equally result from a tight association of molecules into linear chains, in which each molecule (i) rigorously takes an opposite orientation with respect to its neighborhood (as expected to be the case for pheromone and receptor molecules interacting on the cell surface), and (ii) forms contact interfaces relying on amino-acid side-chains lying in the great majority on helix-3 of its three-helical fold. This identification of helix-3 as central functional element of the pheromone molecular structure receives strong support from the tight conservation of the backbone structure that this helix shows at both intra- and inter-specific level and suggests a parsimonious explanation for the molecular mechanisms underlying the competitive, autocrine and heterologous, pheromone/receptor binding reactions.

Session 4. Environmental stress and immunity. Chairmen: Maria Giovanna Parisi, University of Palermo, Palermo, Italy and Luigi Abelli, University of Ferrara, Ferrara, Italy

First insights into the mechanisms involved in immune response in *Mytilus galloprovincialis* towards the emerging pathogen *Arcobacter*

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Bacteria of *Arcobacter* spp. are regarded as emerging foodborne zoonotic pathogens affecting both humans and animals. Their presence has been increasingly reported in seafood, suggesting its increasing occurrence in seawater as a consequence of marine water pollution, this raising some environmental concern. More recently, the presence of *Arcobacter* species has been reported in diseased oysters (*Crassostrea gigas*) during mortality events or in stressed bivalve species. However, no data are available so far on the persistence of *Arcobacter* in bivalves, its potential pathogenicity or interactions with the immune system of the host.

In the present work two strains of the genus *Arcobacter*, isolated from moribund oyster during a mortality event in 2019 in Spain (R1 and R2) were investigated for their interactions with the immune system of *Mytilus galloprovincialis* both *in vitro*, in isolated hemocytes, and *in vivo,* in injected mussels at 24 h p.i..

The results obtained suggest that mussels are able to mount an efficient immune response against these *Arcobacter* strains. The most notable effects of *Arcobacter* on mussel hemocytes both *in vitro* and *in vivo* was observed on lysosomal membrane stability, with the presence of swollen lysosomes and large vacuoles, and on the increase in extracellular ROS production and lysozyme activity. These responses contribute to a significant bactericidal activity, with a large contribution of hemolymph serum alone. Indeed, *Arcobacter* observed within the lysosomal system showed signs of degradation over time. In contrast, preliminary experiments with *C. gigas* indicate that *Arcobacter* did not induce ROS production and lysozyme

activity by hemocytes, and the absence of bactericidal activity in hemolymph serum.

The results provide a first insight on the immune responses of different bivalve species towards *Arcobacter* strains and the underlying mechanisms.

Inflammation events occurring upon bacterial infection in *Mytilus galloprovincialis*

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Bivalves, and in particular the Mediterranean *Mytilus galloprovincialis* are important sources of food in several countries in the world. Because of that, mussels farming has a strong economic impact. Due to their status as sessile and filter-feeding animals, bivalves accumulate in their tissues environmental pollutants and a larger amount of microorganisms and between these, a multitude of infective bacteria for higher vertebrates and humans, such as *Vibrio* species. Several immunological responses of *M. galloprovincialis* were investigated and described after *Vibrio* infection both, *in vitro* and *in vivo* conditions, such as hemocytes count and different cellular subpopulations. Particularly, intracellular signaling pathways are activated to trigger the synthesis of antimicrobial effectors Here, were investigated the modulation of immunological cellular markers of the Mediterranean bivalve *M. galloprovincialis* in response to in vivo exposure with *Vibrio splendidus*. The activation of inflammatory cascade was examined through immunolabeling with antibodies involved in the pathway: Toll-like receptors 4 (TLR4), myeloid differentiation factor 88 (MYD88), Allograft inflammatory factor-1 (AIF1) and ribonucleases RNASET2 (T2 family), that trigger the recruitment and activation of macrophages in vertebrates. Results confirmed the activation of TRL4 during bacterial infection and MYD88 adapter suggesting a role in recognition and intracellular signaling. Moreover, Gram-negative bacteria determine the recruitment by the ribonuclease RNASET2 of haemocytes and a huge migration of AIF-1+ cells. This approach is suitable to understand the molecular defense mechanisms in invertebrates during the exposure to possible pathogens, also in order to develop new technics and tools to evaluate mussel immunity response used in aquaculture to prevent mass mortality of these mollusks, economic loss and potential risks for consumers of seafood.

Investigating the role of viral infections in the population of the *Congeria kusceri*

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Congeria kusceri (Bole, 1962), a highly endangered freshwater bivalve endemic to the Dinaric Karst, belongs to the only extant genus of cave-dwelling bivalves. This cave clam lives in a unique habitat, which has been subjected to minimal environmental changes over the past five million years. Since the natural populations of *C. kusceri* have been quickly declining over the past few decades, a key question to be answered is whether the "living fossil" status of this species, i.e. the apparent lack of morphological and physiological changes compared with its fossil relatives, is linked with a scarce resilience to the impact of human activities on the subterranean environment. In particular, the alteration of the seasonal abundance of pathogens, linked with the modified influx of water in Karst caves, might pose a severe threat to the survival of this species. Here, through a transcriptomic approach, we describe the identification in the tissues of *C. kusceri* of 5 nearly-complete genomes of RNA viruses belonging to the Picornaviridae family, which displayed a strong tissue preference and a significantly changes in abundance over the summer season. RNA-seq data also allowed to investigate whether these viral infections had an impact on gene expression in different host tissues. Since numerous reports have previously implicated Picorna-like viruses in the mass mortality events observed in different bivalves, we suggest that the presence of the 5 identified viruses should be closely monitored, together with other biological and chemical parameters, to gather a better understanding of the causes underlying the quick decline of *C. kusceri* populations.

AMPylation: a new facet of host-virus crosstalk in mollusks

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FIC-domain-containing enzymes (FicD) performed post-translational protein modifications from bacteria to humans, known as AMPylations. As part of the toxin-antitoxin system, FicDs of pathogenic bacteria occasionally induce AMPylation to overcome host defenses, whilst vertebrates FicDs drive the Unfolded Protein Response (UPR) and act during neurogenesis. Mining genomic and transcriptomic data of protostome metazoans, with a focus on marine invertebrates and associated dsDNA viruses, we traced a single-copy FicD gene transversally conserved in metazoans and viral pathogens, with structural and functional traits suggesting a preserved AMPylation capacity. Extra-numeral FicD gene copies are present in rotifers, in some bivalves and in the isopod *Armadillidium vulgare* genomes. Less conserved protein features and no syntenic conservation suggested their recent

genome integration by horizontal gene transfers from endosymbiont or microbiome communities. Analyzing dual RNA-seq data of the only host-virus combinations both encoding a FicD gene, we revealed a time-dependent expression for White Spot Syndrome Virus and Ostreid herpesvirus-1 FicDs, with higher expression levels than crab and bivalve genes. The frequent exchange of FicDs and the shift of this enzymatic ability from bacteria to pathogenic dsDNA viruses underlie complex host-pathogen-predator interactions in the marine environment and a possible role for AMPylation at the edge of host-virus interactions in mollusks.

Protein extractions from *Amphistegina lessonii*: a new approach for the evaluation of the effects of heavy metals on benthic organisms

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Different stressing factors such as heavy metals, nanomaterials and others environmental contaminants can induce physiological and biochemical changes on foraminifera, unicellular organisms typically living in sediments. Research on protein content of foraminifera is currently limited and no comparative analysis on the efficacy and reliability of the different assays has been conducted on foraminifera so far. The first purpose of this study is to compare the results of different methods of lysis, protein assays, and protein staining on *Amphistegina lessonii* – a symbiont-bearing foraminiferal species and presents a new protocol. This protocol could be applicable to other benthic foraminiferal species and represents a new experimental approach for the application of *A. lessonii* as a potential biomarker. The second part of this study is related to the evaluation of short-term effects of Hg exposure (10 ppb, 24 h) on *A. lessonii*. Benthic foraminifera have been mostly utilized as pollution bioindicators in marine and transitional marine environments and mercury contamination is a global issue due to its significant toxic effects on human health, and cytotoxicity being higher than many other heavy metals. In this work, we have evaluated the activity of different enzymes involved in antioxidant defence (SOD, GST, GSR and GPx) and several proteins (HSP70 and p38MAPK) are analysed by western blotting. The results show that the activities of the antioxidant enzymes increased in Hg-exposed foraminifera, indicating that heavy metals can induce oxidative stress and stimulate the engagement of antioxidant enzymes as cellular defense mechanisms; moreover, Hg treatment induces expression of HSP70 and activation of p38MAPK. As previously demonstrated, these data support that Heat shock proteins (HSP) play a crucial role in maintaining protein homeostasis under stress conditions in both prokaryotes and eukaryotes and the mitogen-activated protein kinase (MAPK) pathway is an evolutionarily conserved signalling pathway existing in unicellular organisms to humans. The results can be used to obtain information on the environmental quality of marine sediments and the development of the cellular biomarkers might represent a complementary approach to the traditional biomonitoring.

***Hirudo verbana* as a freshwater invertebrate model to assess the effects of polypropylene nano and microplastic dispersion.**

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Plastics represent the most widely employed synthetic materials that, given their physical and chemical properties, are used to produce robust and above all economic objects. Although from their discovery these materials became indispensable, their worldwide distribution caused an uncontrolled accumulation of waste products followed by an indiscriminate release in the environment. In particular, millions of tons are reversed in waters every year, making the aquatic ecosystems the most affected by plastics pollution. Moreover, their degradation, due to biotic and abiotic events, leads to the formation of small-size particles, known as nano (NPs) and microplastics (MPs), that can persist inside organisms for a long time and can accumulate in the trophic chain.

To evaluate the possible tissue accumulation and harmful effects in freshwater dispersion, we have exposed the leech *Hirudo verbana* to fluorescent polypropylene NPs and MPs at different timings (1 and 6 hours, 1 week, 1 and 2 months). Optical and fluorescent analyses demonstrate that these particles penetrate inside the organism both through the integument and food and induce epidermal mucous cell proliferation, angiogenesis, release of the pro-inflammatory molecules *Hm*AIF-1 and *Hv*RNASET2, macrophage-like cell migration and amyloidogenesis. Moreover, qPCR analyses reveal an increasing expression of the specific oxidative stress enzymes superoxide dismutase and glutathione S-transferase. Taken together, these data suggest the medicinal leech *H. verbana* as an innovative environmental biomarker ideal for evaluating the possible harmful effects deriving from NPs and MPs on freshwater animals.

Study of immunotoxicity responses of *Sabella spallanzanii* exposed to copper sulphate

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In the last decade, the growing use of heavy metals in human activities has inevitably altered the health of the aquatic environment. Marine pollution due to heavy metals is an issue that has now a global dimension and has interested many researchers because of their ability to persist,

bioaccumulate and biomagnify in the trophic chain leading to adverse effect also on human health. Copper sulphate is a very soluble xenobiotic, still used today into antifouling paint and in aquaculture like biocide, with high activity against algae, fungi and marine invertebrates. Its main component is copper that at high concentrations have immunomodulating effect on marine organisms.

In this study we have used the polychaete worm *Sabella spallanzanii* to investigate its immune response after exposure to copper sulphate and the combinate effect with the inoculation of *Escherichia coli*, to provide an overview of effect biomarkers for monitoring chemical and bacterial stressors. We aimed also to validate the species as model organism in marine-coastal biomonitoring and to investigate the modulation of TLR-4 as response useful to evaluate environmental alterations. Polychaetes were subjected to five treatments: 1. Control (naïve), 2. Filtered sea water + TBS injection, 3. Filtered sea water + *E. coli* injection, 4. Copper sulphate + TBS injection, 5. Copper sulphate + *E. coli* injection. The exposure to xenobiotic was chosen in relation to the specificity of the animals' response and the bacterial injections were made after the exposure. The immune markers evaluated in the total body extract of the animals were inflammatory and antioxidant markers. Therefore, were investigated the activity of: esterase (EST), alkaline phosphatase activity (ALP), cytotoxicity and detoxifying/antioxidant enzyme such as glutathione peroxidase (GPx). In addition, toll-like receptor (TLR), allograft inflammatory factor-1 (AIF-1), lysozyme (LYS) and hemagglutinating and inhibition activity have also been considered to highlights possible modulations.

The analysis of results indicated a significant activation of TLR-4 and AIF -1 in specimens inoculated with bacteria and treated with combinate exposure factors. The results regarding lysozyme and peroxidase enzyme as well as the hemagglutination activity have well highlighted the differences between the different environmental stimulations. Overall copper sulphate had an immunomodulating effect of the species under investigation, varying the immune response especially after bacterial inoculum.

Herbicide exposure alters the expression of antimicrobial peptide patterns in the mealworm beetle infected with the natural entomopathogen *Beauveria bassiana*

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Herbicide treatments are an integral part of agricultural practice. However, repeated applications lead to soil, water, and food contamination and adverse effects on non-target organisms. In this study, a pendimethalin-based commercial formulation (PND) was tested on the beetle *Tenebrio molitor* Linnaeus, 1758. The effects of herbicides on interspecific relationships, such as host-pathogen interaction are poorly studied. In the laboratory, adults were exposed to two different doses, the concentration corresponding to the contamination of the treated soil and the maximum residue level allowed by the EU in cereals. The beetles were then exposed to an inoculum with the entomopathogenic fungus *Beauveria bassiana* (Bb), commonly used as a bioinsecticide. Survival, sporulation of the fungus in cadavers, and expression levels of antimicrobial peptides (AMPs) Tenecin 1, 2, and 3 were examined. Although the survival rate did not change significantly between control and herbicide-exposed beetles, an alteration in the expression pattern of inducible AMPs was recorded. PND-treated beetles showed up-regulated Tenecin 1 and Tenecin 2 after inoculation with Bb. In addition, a slight increase in Bb sporulation on cadavers was recorded at the highest dose of the herbicide. Our results showed a potential alteration of the host-pathogen interactions, raising the question of bioinsecticide compatibility with synthetic pesticides and the effects of herbicides on interspecific relationships in wild species.

Organ-specific accumulation of PLGA nanoparticles injected into *Pomacea canaliculata* snails: preliminary *in vivo* observations

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Nanoparticle-delivered drugs are attracting growing interest for their potential to minimize side effects and create "patient personalized" therapeutic applications. Currently, preliminary animal testing of nanodrug systems must be performed following the European directives and national laws, which are mainly centered on vertebrates. However, *Pomacea canaliculata*, a freshwater amphibious snail from South America, is emerging as a possible substitute of vertebrates in preliminary bio-accumulation and bio-safety studies of nanodrug systems. *P. canaliculata*, which is easy to breed, manipulate, and presents a similar weight and longevity to mice, has already been proposed as a potentially model suitable for studying the distribution and organ accumulation of Superparamagnetic Iron Oxide Nanoparticles (SPIONs, size 14-80 nm). Here the accumulation of the Cy5 fluorescently labeled FDA approved Polylactic-co-glycolic acid nanoparticles

(120-180 nm, z= ~-25 – -30 mV) (PLGANPs-Cy5), often used in the literature as NP drug delivery systems, was assessed after injection in the snail's foot (20 mg/kg) and incubation for 4 h, 24 h, or 1 week. The considered organs were the posterior (PK) and anterior kidney (AK), the digestive gland (DG), a functional analogue of vertebrate liver, the lung, and the cerebral-pedal ganglion ring (G). At the end of the incubation times, the organs were dissected and processed for paraffin embedding and laser scanning confocal microscopy (LSCM) observations. LSCM evidenced that PLGA-NPs-Cy5 accumulated in the PK more than the other target organs in all the experimental times, but the highest levels of accumulation were seen after 4 h. The accumulation within the PK was not homogeneous but concentrated in regions labeled as hemocyte islets. PLGANPs-Cy5 also accumulated in the AK at all the tested time intervals but, contrary to the PK, the maximal positivity was observed at 24 h post-injection (hpi). Surprisingly, the DG did not seem to retain PLGA-NPs-Cy5, though the presence of auto-fluorescent pigments made the observation difficult. PLGANPs-Cy5 were seen in the lung only at 4 hpi and in the ganglia at 24 hpi. Tissue distribution of fluorescent NPs suggested that they did not massively enter the ganglia, but they remained in the peripheral tissue.

Further studies are necessary for detailing whether the PLGA-NPs-Cy5 enter the cells, but these preliminary results show that PLGA-NPs-Cy5 are distributed by the snail open circulatory system and are retained in a time and organ-specific fashion with an absence of suffering or mortality. These first results could pave the way for using invertebrate models such as Pc in preliminary in vivo observations concerning NP biotoxicity, distribution, and accumulation as a cheaper and more manageable first analysis before involving mammals or other vertebrate models.

Immunotoxic effects of a new-generation antifouling biocide in a compound ascidian

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Dichlofluanid has long been employed as a fungicide in agriculture and has been massively introduced in antifouling paints for boat hulls over the last two decades. One of the most important toxic effects of antifoulants is represented by immunosuppression in marine invertebrates, which can be analysed in vitro with a number of short-term toxicity assays on haemocytes. Among bioindicators, the colonial ascidian Botryllus schlosseri is a useful candidate; it is a filter-feeding organism living in the water-sediment interface that is found worldwide and is sensitive to antifouling xenobiotics. Dichlofluanid adversely affects both immunocyte lines (phagocyte and cytotoxic lines) after exposure to sublethal concentrations. At 0.05 µM (16.65 µg/L), dichlofluanid induced haemocyte apoptosis and cell shrinkage with a decrease in both motility and phagocytosis. At the lowest concentration (0.01 µM, 3.33 µg/L), inhibition of pivotal enzymatic activities of phagocytes and cytotoxic cells occurred. At the highest concentration (0.1 µM, 33.3 µg/L), dichlofluanid increased glutathione oxidation, leading to stress conditions. The effects of dichlofluanid on immune defence responses are similar to those of organometal-based antifoulants (i.e., organotin compounds and zinc pyrithione), and its use in coastal areas requires attention.

Effects of nano- and microplastics in the development of immune memory in the ascidian *Ciona robusta*

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Microplastic contamination appears as one of the world's main environmental concerns. Small plastic fragments dispersed in the marine habitat can be consumed by different marine organisms and ultimately transferred to humans along the food chain.

Microplastics could compromise the health of marine organisms by interfering with the functionality of the digestive system when ingested, but there is concern that they could also interact with the immune system and affect the susceptibility/resistance of animals to the infections. Recently, we have demonstrated the establishment of immune memory in the ascidian *Ciona robusta* by priming and challenging animals with microbial agents. This immune memory relies on the modulation of different cellular and humoral immune mechanisms, aiming to develop a more protective response.

In the present study, we analyzed the expression level of several immune-related genes in pharynx and gut of animals primed by short- or long-term exposure (2 and 18 h) to nano- and micro-plastics (NMPs) and challenged seven days later with a prototypical inflammatory stimulus (bacterial lipopolysaccharide, LPS). We aimed to determine (i) whether the NMPs can induce an immune response in different tissues, (ii) whether the NMPs could prime the animals and drive the development of innate memory, and finally (iii) to which extent filter-feeding animals could stand this kind of pollutants. Transcription data suggest that animals primed with NMPs develop an immune memory that potentiates the secondary response to LPS and that such memory is tissue-specific and depends on both the length of the exposure period and the particle size. This study demonstrates that NMPs can modulate the immune reactivity of marine invertebrates, which can influence their defensive fitness. How this may affect their health and survival capacity is still unknown.

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Metallothionein gene expression in *Trematomus eulepidotus* as a response to environmental variation of metal ion concentrations

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Antarctic waters are characterized by a naturally high concentration of metal ions such as Cu and Cd. This peculiar condition has probably influenced the evolution of the organisms that populate this environment. Indeed, those ions tend to bioconcentrate into the animals that occupy the higher levels of the trophic chain, like fishes. Antarctic fishes are able to reduce negative effects caused by the excess of metal ions thanks to the presence of specific adaptations as the expression of two distinct metallothioneins (MTs) isoforms, which are regulated by a gene expression control mechanism. MTs are a family of cysteine-rich, low molecular weight proteins. They have the capacity to bind both physiological (such as Zn, Cu, Se) and xenobiotic (such as Cd, Hg, Ag, As) metals through the thiol group of its cysteine residues. In this work, we analyzed the gene expression of MTs in the Antarctic fish *Trematomus eulepidotus*, experimentally exposed to an increase of Cu and Cd concentrations. Analyses were performed in liver, white muscle, heart and kidney, using *Real-Time* PCR, and the expression levels were related to the physiological role of these organs and tissues. The data presented in this preliminary study improve our knowledge about the molecular and functional evolution of Antarctic fishes and may be used as a starting point for using MTs as biomarkers both for the exposition to Cu and Cd ions and oxidative stress, considered the antioxidant function of this proteins, using Notothenoids as bioindicator organisms in biomonitoring campaign of Antarctic marine habitat.

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Transcriptomic response of *Trematomus bernacchii* to short- to medium-term mild heat stress and experimental design bias

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Many stenotherm marine species live in the cold and stable environment of the Antarctic Ocean which could be impacted by climate change in the upcoming years. To investigate how antarctic fish would cope with this issue, gene expression analysis was carried out on *Trematomus bernacchii* specimens caught near Mario Zucchelli Station. Brain, gill and muscle tissues were sampled from naïve (right after catch) animals and those kept in control (-1.7 °C) and experimental (-0.2 °C) tanks for six hours, seven and twenty days post acclimation.

The brain was the most affected in terms of gene expression, showing a time dependent pattern. Immune response was up-regulated at seven days of exposure, as the 114 upregulated genes (over a total of 117 differentially expressed genes, FDR < 0.05 and |logFC| > 2) were significantly enriched (FDR < 0.05) in gene ontology terms such as: endopeptidase inhibitor activity, complement activation, inflammatory response. In the same tissue the response to a 20 days heat stress consisted in 519 up-regulated and 490 downregulated genes. The up-regulated set was enriched in terms related to cell adhesion, synapse and glutamate receptor activity, while the down-regulated genes were enriched in terms related to ribosome, mitochondrion, energy management, protein folding/turnover and cytoskeleton. Interestingly, consistent reduction in expression levels of hspa9, hsp90aa1.2, hsp90ab1, hspa14 and hspa8b was observed. Gill tissue showed a mild response after 20 days, with 17 up-regulated and 7 down-regulated genes: the enrichment test suggested that DNA replication and negative regulation of apoptosis processes were perturbed in this tissue. A notable early-starting response to stabling was also observed across the entire experiment in brain (2879 DEGs) and gills (239 DEGs). Expression pattern clustering analysis was performed, allowing the identification of trends in gene expression and a more thorough enrichment analysis of the identified clusters. In brain many synapse-related genes were down-regulated and energy-related genes were up-regulated early after transfer to the small experimental tanks, while several binding processes increased their expression level at the latest time point. In gills gene modulation response was milder and mostly involved cytoskeleton and glycolysis related genes in the early experimental phases. No significant change was observed in muscle. These results show that the brain is the tissue most affected by heat and confinement, demonstrating how sensitive it is to the smallest environmental changes and the importance of careful experimental design when working with captive wild organisms.

The effect of group size on the stress level in an open-channel swimming flumeon *Telestes muticellus*

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Artificial barriers in rivers cause a substantial decline in endemic and migratory fish due to habitat fragmentation, changed reproduction environments and blocked migratory routes. Fishways can ensure a safe passage over these barriers and sustain biodiversity in rivers. For this reason, it is essential to understand fish swimming performance and kinematics to build appropriate fish passages. High

velocities at passages, in which fish cannot consist or counterbalance, can result in physical stress. In schools, better performance is observed due to the hydrodynamic benefit of swimming near other individuals. A multidisciplinary research approach was tested to study interactions of group size at different hydraulic conditions. Therefore, collective behavior was analyzed concerning physiological stress responses. Wild vairone (*Telestes muticellus*) was tested in a portable flume in 1, 2 and 6 fish groups. The stress response was studied through the analysis of the hypothalamic-pituitary-internal axis. The neuroendocrine stress response was evaluated by the cortisol level in the muscle tissue. Additionally, oxidative stress was assessed by evaluating cell damage and gene expression of the protein components of the antioxidant defence system. Lipid peroxidation was studied through the level of malondialdehyde (MDA) in the muscle. Further, non-enzymatic oxidative changes were studied by advanced oxidative proteins (AOPP). Preliminary results suggest that oxidative stress is lower in grouped fish than in single fish.

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Preliminary data on physiological responses induced by PFAS exposure in freshwater fish of the Veneto region

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In recent decades, the interest towards per- and polyfluoroalkyl substances has grown exponentially around the world, due to the toxic effects induced by these chemical compounds in humans, as well as in other animals and plant organisms. However, the knowledge related to the antistress responses that organisms can express when exposed to these substances is still lacking and therefore requires further investigation. For this purpose, this study was launched on the possible physiological responses that exposure to environmental concentrations of PFAS can induce on *Squalius cephalus* and *Padogobius bonelli*, two freshwater fish species widely spread in the Veneto Region, a geographical area directly involved in what is considered one of the most significant cases of PFAS pollution. Specimens of the two species were sampled in three rivers of the Vicenza area, characterized by three different levels of PFAS pollution. Several biomarkers of stress have been evaluated and the results obtained suggest an increase in the expression of mitochondrial antioxidant enzymes. Conversely, no change in total antioxidant capacity was observed, suggesting that the effect of oxidative stress detected in the mitochondria does not correspond to a more extensive cellular response. For both species, various morphological indices were calculated with the aim of determining both the general well-being of the organisms and identifying possible variations in the size of the liver, spleen and gonads. The obtained results are preliminary and certainly require further analyses and investigations, but they suggest an interesting protective mechanism against damage to the protein component based on lipid vacuolation in the liver.

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The skin of Mediterranean cetaceans as model to analyse accumulation and toxicity of POP.

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Stockholm Convention (SC; 2001) on Persistent Organic Pollutants (POP; http://www.pops.int/) aimed at eliminating production or lowering use of 12 long-lasting toxic chemicals, e.g. polychlorinated biphenyls (PCBs), dioxins and PCDH. Production of DDT was not banned, but restricted to limited production, only to fight malaria in endemic regions of the world. Later on same year, the SC added other 4 POP (comprising lindane), becoming operative since 2004 (with adhesion of 181 Nations). The scheduled SC 2022 Meeting is regarding also inclusion of PFOA and BPA in the Annex A.

Due to the lipophilic nature of these chemicals, environmental concerns have arisen about bioaccumulation and biomagnification through the food chain, in both land and water ecosystems. Due to large occurrence of lipids in the skin of Cetaceans, these aquatic mammals represent a valuable tool to analyse accumulation and potential toxic effects of POP. Even in males, large amounts of hypodermic blubber have adaptive roles, but are also sites of POP capture, storage and long-lasting slow systemic release.

Our previous epigenetic study in the Mediterranean (Ligurian sea) fin whale (*Balaenoptera physalus*) skin biopsies (all males) revealed a relationship between levels of blubber contaminants (organochlorines and phthalate) and DNA changes in gene methylation profiles. Differentially methylated genes fell into six main pathways: *Wnt signaling, Cadherin signaling, Angiogenesis, Endothelin signaling, Axon guidance mediated by Semaphorins,* and *Nicotinic AchR signaling*. These results depicted a dystrophic condition of the skin. Collection of new dart skin biopsies from free-ranging fin whales is now allowing a wider analysis about contaminants load and histopathological correlates.

PLENARY LECTURE III

Linking Stem cells and innate immunity research via data, information and knowledge integration

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Stem cell biology and the immune system research should be integrated in order to provide a more holistic understanding of how organisms maintain and restore homeostasis. The scopes related to the interplay between stem cells and the immune system go beyond basic insights of organism's physiology and include also regeneration research, (eco)toxicology, biotechnology, and medicine as well as regulatory and ethical aspects. Traditionally, stem cells and immune cells are considered as parts of two branches of biological research with few interconnections between them. It is a challenge to integrate the conceptual frameworks of these two disciplines to address basic questions in biology in a new and innovative way. Great opportunities for linking scientific disciplines are given in the data driven era. Computation has been an important part of science for more than half a century, and the data explosion is making it even more central. But cross-disciplinary collaboration and data, information and knowledge sharing according to findability, accessibility, interoperability, and reusability (FAIR) data principle is a relatively new concept. The contribution of FAIR data will be discussed and aquatic invertebrates are taken as an example. How to integrate new and traditional approaches in an inclusive way will be emphasized.

Session 5. Immune competence and immune response (part II). Chairmen: Matteo Cammarata, University of Palermo, Palermo, Italy and Piero G. Giulianini, University of Trieste, Trieste, Italy

Conservation and diversity in the inflammatory reaction of *Ciona robusta*: hemocyte populational dynamics and molecules related to internal defense

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Ascidians are marine invertebrate chordates belonging to the earliest branch (Tunicata) in the chordate phylum, therefore, they are of interest for studying the evolution of immune systems. Due to the known genome, the non- colonial *Ciona robusta*, previously considered to be *C. intestinalis* type A, is a model species for the study of inflammatory response and here we further explore the topic of hemocyte and molecules related to internal defense of ascidians involved in the inflammatory reaction. The internal defense of ascidians mainly relies on hemocytes circulating in the hemolymph and pharynx. Hemocytes can be *in vivo* challenged by LPS injection and various granulocyte and vacuolated cell populations differentiated to produce and release inflammatory factors. Molecular biology and gene expression studies revealed complex defense mechanisms involving different inflammatory hemocytes. Furthermore, cloning procedures allowed sequence analyses and molecular studies disclose immune-related gene families including TOLL-like receptors, galectins, C-type lectins, collectins, interlectins, pentraxine-like, peroxinectins, complement factors-like, TNFα-like, IL-17-like, TGF-like, MIF-like. These genes are promptly upregulated by the inflammatory stimulus and show a time course of transcription similar to each other. Domains sequence similarity and phylogenetic relationships with the vertebrate counterparts are shedding some light on immune-related gene evolution. Selective bioassays as well as bioinformatic approaches have allowed the characterization of antimicrobial peptides and the identification of post transcriptional molecular mechanisms able of influencing dynamics of gene regulation are described.

Identification of potential hemocyte markers in the gastropod model *Pomacea canaliculata*

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The immune system of non-model invertebrates remains poorly characterized, although it may contribute to a better knowledge of vertebrate innate immunity. The main cellular immune component of invertebrates is represented by circulating and tissue-infiltrating hemocytes. In absence of specific cell markers, hemocytes are usually classified on morpho-functional bases. In these respects, the investigation of molecules selectively expressed in hemocytes (*i.e.*, hemocyte markers, HM) of the freshwater and invasive gastropod, *Pomacea canaliculata*, would represent a valuable contribution to track hemocyte development, maturation, and distribution. Taking advantage of the available genome and organ-specific transcriptomes, sequence analyses were performed, selecting Allograft Inflammatory Factor-1 (PcAif1), Hematopoietically Expressed Homebox protein (PcHhex), Hemocyanin (PcHc), Runt domain-containing protein (PcRunt) and Transglutaminase-2 (PcTg-ase-2) as potential HM candidates. Single probe Fluorescent *In Situ* Hybridization (spFISH) evidenced all the potential HM in cytocentrifuged hemocytes, with a prevalence of PcHc-positive cells. After dual probe FISH for each combination of HM, double or single positive, and double negative hemocytes were observed,

confirming that similar morphologies can correspond to a different transcriptional pattern, then possibly to a different functional status or a diverse role. spFISH enabled the specific identification of tissue-resident hemocytes in two different histological contexts: a complex and potentially hematopoietic organ (*i.e.*, the posterior portion of the kidney) and the blastema of early regenerating cephalic tentacle (RCT) at 12 h post amputation (12 hpa). In order to corroborate the morphological observations with molecular data, RT-qPCR experiments investigated the HM expression levels in RCT 12 hpa. The increased number of hemocytes observed by confocal microscopy was in agreement with RT-qPCR data, that confirmed the expression of all HM analyzed and the significant induction of PcHc, PcTG-ase2 and PcRunt in RCT 12hpa samples. In all, the combined application of *in silico*, microscopy and molecular techniques has led to the first identification of potential HM in *P. canaliculata*. While these markers are diversely expressed in circulating hemocytes, they evidenced the hemocyte islets in the kidney and also hemocyte-like cells in the blastema of RCT. These markers could also be used for future experiments of cell sorting and for following hemocyte maturation in circulation or within hematopoietic organs.

Insight on the signal transduction pathways involved in morula cell degranulation in the colonial ascidian *Botryllus schlosseri*

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Morula cells are granular cells constituting the majority of hemocytes of the hemolymph of botryllid ascidians. They are the first cells sensing nonself and, as a consequence of the recognition, they synthesize and release cytokines and trigger and inflammatory process by release their granular content through exocytosis (degranulation). They are directly involved in the formation of the necrotic points of rejection along the contact border between incompatible colonies. During this process, they are selectively recruited and gather in the ampullae (the blind endings of the colonial circulation) close to the contact region before crossing the vascular epithelium and entering the tunic where they degranulate releasing their granular content, *in primis* the cytotoxic enzyme phenoloxidase and its polyphenol substrata. The degranulation reaction can be mimicked *in vitro* by exposing hemocytes to cell-free hemolymph from genetically incompatible colonies of to microbial cells such as *Bacillus clausii* cells.

In the present research we induced *in vitro* degranulation to study the signal transduction pathways involved in the process using specific inhibitors of a series of kinases. Preliminary results indicate the involvement, in morula cell degranulation, of the pathways mediated by PKA, PKC and Jnk.

Translational comparative immunology: from fish to bed, new antibiotics and SARS-Cov-1 immunodiagnostic

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Available knowledge shows that a morphological and functional implant of immune defences is remarkably similar and conserved among vertebrate classes, as confirmed by the zebrafish model, widely employed for translational research applied to human health. The knowledge on fish immune defences may find application in biotechnology and, in this respect, we investigated whether natural antimicrobial peptides produced by the Antarctic fish species *Chionodraco hamatus* and *Trematomus bernacchii* might be candidate molecules to be employed as novel antibiotics. The results have shown that a modified synthetic 22-mer peptide derived from Chionodracine sequence shows important features such as being actively lytic against antibiotic-resistant bacterial strains of *Acinetobacter baumannii* and *Klebsiella pneumoniae* (both MIC 1.55 µg/ml): the peptide does not induce lysis to human erythrocyte and is not cytotoxic for some human cells lines at the MIC concentrations useful to kill bacteria. Another antimicrobial peptide, Trematocine, has been investigated for its antibacterial and antimicrobial activity and some mutants designed to be more active against bacterial cell walls are currently under screening.

In vertebrates, the ELISPOT assay is employed to measure and quantitate the presence of antigen-specific antibody-secreting memory B cells *in vitro*, but due to its complexity the assay can be solely performed in research laboratories. We previously described a simplified version of the ELISPOT and described the *in vitro* presence of circulating memory B cells long after immunization of the fish sea bass against bacterial antigens. Due to the necessity of information regarding the mounting of an antibody memory response against the SARS-Cov-2 spike-S1 protein, we applied our CellELISA platform (simplified ELISPOT) to the screening of human blood samples, in order to achieve data on both serological IgG presence and *in vitro* IgG produced by memory B cells. The results have shown that in a cohort of 150 donors a significant number of serology-negatives patients resulted indeed positive to CellELISA, thus revealing an active immunization against spike-S1 even in the absence of circulating IgG. The CellELISA assay showed the presence of an antibody memory 12 months after first virus encounter and suggested that blood leukocytes from SARS-Cov-2-infected patients display a modified metabolomic profile.