ISJ 21: 43-57, 2024 ISSN 1824-307X

REPORT OF MEETING

XXIV scientific meeting of the Italian Association of Developmental and Comparative Immunology (IADCI), February 14-16, 2024, Department of Life Sciences - DSV, University of Modena and Reggio Emilia, National Academy of Sciences, Letters and Arts, Modena, Italy

Organizers: **D Malagoli[1,2], N Franchi[1], M Mandrioli[1,2], L Rebecchi[1,2], S Sacchi[1]**

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

Evolutionary insights into the large repertoire of antimicrobial peptides encoded by the genome of *Mytilus unguiculatus*

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Mussels stand out among marine organisms due to their extensive and diverse array of antimicrobial peptides (AMPs), constituting one of the largest repertoires documented in literature. Initial identification of these molecules dates back to the early '90s, employing traditional purification methods, primarily driven by their strong expression in hemocytes. Over time, the use of sequence homology and Hidden Markov Model-based approaches has considerably expanded the compendium of mussel AMPs. Nevertheless, the precise immunological roles played by these small molecular weight peptides, typically cationic and cysteine-rich, as well as their evolutionary relationships, remain largely unclear.

A data mining approach was applied leveraging the availability of a high-quality genome for the species *Mytilus unguiculatus* and the assembled transcriptome. Coding sequences were predicted and translated to aminoacid sequences. These sequences were then filtered based on criteria shared by the Cys-αβ peptides such as: presence of a signal peptide, even number of cysteine residues in the mature peptide, mature peptide shorter than 100 aminoacids. The candidate sequences were then used for a multiple sequence alignment and clustered to form putative families through neighbor-joining methods. The respective assembled transcripts were also mapped to the reference genome to identify the position and the gene architecture, which were then annotated. Finally, the 3D structures of the candidate sequences were also predicted with alphafold 2 with energy minimization enabled. The structure of the predicted disulfide bridges was also annotated.

In this study, we conducted a comprehensive analysis of AMP-encoding genes within the *M. unguiculatus* genome, highlighting seven novel cysteine-rich AMP families within the Cys-αβ peptide superfamily. This diversification includes peptides exhibiting hairpin-like folds, a structural deviation from the typical Cys-αβ architecture, possibly through the loss of the N-terminal alpha-helix component. Variations within these families also include additional N-terminal tails, predicted to fold into beta-sheets that align on the same plane to the classical two-stranded Cys-αβ structure. These structural adaptations, alongside the displacement, gain, and loss of cysteine residues, impact disulfide bridge formation without departing from the core cys-αβ framework.

Despite significant primary sequence diversity, the shared gene architecture—i.e., the presence of intron 2 in phase 1 and intron 3 in phase 2—suggests a common evolutionary origin with defensins, mytilins, and myticins. The primary

sequences exhibit no detectable homology between the families, implying a rapid evolutionary rate and expansion within these gene families, a theory further supported by the abundance of pseudogenes within the same gene clusters in the genome. Furthermore, not all peptides in these families are cationic, suggesting a potential shift from direct antimicrobial action towards roles that may include cytokine-like functions, observed in other Cys-αβ proteins. This suggests an evolutionary trend towards continuous innovation and assortment of Cys-αβ peptides in *Mytilus*, also favored by the remarkable gene Presence Absence Variation observed in this genus. The observation of hairpin-like structures may represent convergent evolution with similar beta-hairpin structures in antimicrobial peptides across various life forms, emphasizing the evolutionary adaptability of these molecules.

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

Comparative insights into ADAR-mediated RNA editing: developmental and immunological perspectives

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The modification of adenosine to inosine, catalyzed by Adenosine Deaminase Acting on dsRNA (ADAR), occurs as A-to-I in transient double-stranded RNAs. This post-transcriptional modification significantly impacts organism development, neural regulation, autoimmunity, and antiviral responses. In vertebrates, ADAR editing prevents endogenous dsRNA recognition by cytosolic receptors, avoiding inappropriate interferon-mediated immune activation. The deregulation of ADAR editing has been associated with cancer and other human diseases, and the embryonic lethality in mice lacking ADAR1 further supports the essential role of A-to-I editing in animal physiology. Two distinct types of dsRNA editing are detectable: single-site editing, where a limited number of adenosines are targeted, and hyper-editing, characterized by the modification of numerous, more than 20, adenosines within a single RNA molecule. Our phylogenetic analysis of the distribution of ADAR proteins across various Metazoan phyla revealed a consistent presence of both ADAR2 and ADAR1 proteins, with the notable exception of the ADAR1 absence in Crustacea and Insecta. Interestingly, ADAR1 proteins exhibit differences in domain architecture, with the Z-DNA binding domain not universally present. This variability of the structural organization suggests a complex evolution of ADAR1 proteins. Beyond the presence of the protein, our systematic searches of hyper-editing and single-site editing activity in 22 distinct species representative of different taxonomic groups have underscored a broad conservation of such enzyme-mediated RNA editing within Metazoa. Notably, the hyper-editing level varied significantly among the considered species and genome regions. In fact, hyper-editing was primarily traced in repetitive sequences within the genome, suggesting that the primary role of hyper-editing is avoiding the recognition of endogenous dsRNA by the dsRNA cytosolic receptors. As regards the role of hyper-editing and single-site editing in early development, we used the scallop *Chlamys farreri* as a model organism, and we noticed a maximum of hyper-editing activity at 2.5 hours post-fertilization. As already hypothesized for zebrafish, this peak in the hyper-editing activity could support the Maternal-to-zygotic transition, favoring the degradation of the maternal RNA and the initiation of the zygotic genome transcription. Furthermore, ADAR editing of specific editing sites can introduce non-synonymous changes in nascent proteins (recoding effect), enhancing proteome diversity. Substantial single-site editing was reported to occur in the nervous system of cephalopods, where A-to-I RNA changes alter the coding sequence of neurotransmitter receptors, impacting neuronal excitability and synaptic transmission. Also, the significant increase in ADAR editing activity during spawning and in male and female gametes of *Acropora millepora*, would support proteome diversity and the adaptative potential of the species. Since Mollusca and corals share external gamete release and fertilization, increasing proteome plasticity could provide some advantages to the settling larvae. The recoding levels computed for the developmental stages of *C. farreri* are consistent with this hypothesis, as the recoding levels constantly increased in the early scallop development up to the pediveliger stage, which represents the last planktonic larva before the settlement. ADAR editing can also influence gene expression levels by modulating RNA stability, localization, and translation efficiency, thereby regulating protein levels. Depending on ADAR expression patterns, sparse or clustered editing and recoding effects, the overall transcript changes mediated by the ADAR protein family define a new layer of complexity to the biological responses to internal and environmental stimuli. Overall, this study focuses on the evolutionary relationships and functional roles of ADAR proteins, with a particular focus on Mollusca in a comparative perspective.

KEYNOTE LECTURE

Ecoimmunology in a changing world

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The field of ecoimmunology is at the forefront of understanding the intricate relationship between the environment and the immune system. With the ongoing challenges posed by climate change, this relationship becomes increasingly crucial to

decipher the impact of environmental alterations on the health of organisms and their susceptibility to disease. Consequently, it is becoming increasingly important to explore the dynamic interaction between ecological factors and immunological responses of organisms in the context of climate change. In such a scenario, ecoimmunology delves into the mechanisms through which environmental changes, such as fluctuations in temperature, pH, salinity, habitat modifications and alterations in biodiversity, influence immune responses and disease dynamics, in various species. Additionally, ecoimmunology examines the adaptive strategies employed by organisms to cope with these environmental stressors and the potential implications for ecosystem health and resilience. By elucidating these complex interactions, we can pave the way for new insights into ecoimmunological dynamics amid climate change, thus fostering a better understanding of the challenges and opportunities for conservation and public health strategies in a rapidly changing world.

Session I.1: Immunotoxicological and eco-immunological impact of xenobiotics and climate change on animal biodiversity. Chair: Laura Canesi, University of Genoa, Italy and Simona Picchietti, University of Tuscia, Italy

Warmer seawater affects the immune activities of thermophilic coral *Astroides calycularis* under LPS challenge

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A worldwide increase in coral diseases and mortality has been linked to anthropogenic ocean warming due to changes in pathogen virulence and coral immune functions. The anomalous temperature pattern has particularly worried the Mediterranean region over the last 30 years, where intense warming has caused recurring mass-mortality events. To evaluate how warmer seawater conditions influence the immune responses of an endemic coral species, colonies of *Astroides calicularis* were exposed to environmental (23 °C) or elevated (28 °C) temperatures, and subsequently challenged with *Escherichia coli* lipopolysaccharide (LPS). Several enzyme activities, which included phenoloxidase-like, glutathione peroxidase, lysozyme-like, alkaline phosphatase, and esterase, were measured after 6 hours of LPS balneation and over time (0-, 12-, 48-, and 120-h). The five enzyme trends showed upregulation immediately after the LPS balneation under environmental conditions, demonstrating an immune response, while warmer seawater impaired the enzyme activities, delaying it over time. Furthermore, through immunolabeling with specific antibodies, was also detected the regulation of Toll-like receptor 4 (TLR4), nuclear factor kappa B (NF-kB), and heat shock protein 70 (HSP70) activity. The activity of this markers after the LPS stimulation revealed a modulation at environmental temperature. Elevated temperature and LPS-challenge almost suppressed TLR4-NF-kB activity, while HSP70 up-regulation appeared in both treatments under warmer conditions. Such an approach is useful for understanding the pathogen-defence mechanisms in corals in order to disentangle the complex interactive effects related to global climate change.

Acknowledgements: This work was supported by the National Biodiversity Future Center (CUP B73C22000790001)

Could rising seawater temperatures become a challenge for Antarctic fish? Exploring defence responses of *Trematomus bernacchii* to climate change

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The oceans are all facing the repercussions of rising seawater trends, and the Southern Ocean encircling Antarctica stands out as particularly vulnerable to these global changes. Among global climate change projections, the impact on marine fish species, especially in polar regions, remains uncertain. Predicting the adaptive capacity of fish to temperature changes and the potential physiological shifts they may undergo proves to be a complex challenge. Antarctic fish have adapted to constant seawater temperatures below 0 °C for millions of years, developing very peculiar and unique features. In our study, we sampled *Trematomus bernacchii*, an Antarctic endemic fish species, and exposed adult specimens to a gradual temperature increase from 0 °C to +3 °C. Using implanted DST micro-HRT loggers capable of recording both inner body temperature and heart rate, we meticulously monitored each experimental group for 15 days. Analysis of the collected data revealed a robust positive connection between internal body temperature and heart rate among the exposed fish. These findings were subsequently compared with investigating gene expression of antioxidant enzymes within heart tissues of the same organisms. Additionally, biochemical analyses were conducted to detect possible changes in active protein expression, and the main morphological indexes were calculated. Exploring physiological responses becomes imperative for assessing the resilience of Antarctic marine organisms to future global shifts and identifying potential limitations that may reduce their capacity to cope with altered environmental conditions.

Acknowledgements: This work was funded by the Italian National Program for Antarctic Research (PNRA), project No. 2018/B2Z1.01

Epigenetic modifications on the polymeric Ig receptor gene from the cold adapted teleost *Trematomus bernacchii*

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The polymeric immunoglobulin receptor (*pIgR*) arose early in vertebrate evolution with the main role to transport mucosal antibodies across epithelial layers into the external secretions. To date, knowledge of the *pIgR* gene structure remains still limited both in temperate and cold-adapted fish. In our previous work, we identified and characterized the *pIgR* gene of the Antarctic teleost *Trematomus bernacchii*, belonging to the Perciform suborder Notothenioidei, the prevalent component of Antarctic fish fauna. Through a comparative analysis, we discovered that Antarctic *pIgR* gene has a unique structure characterized by longer introns, associated with the presence of transposable elements, and additional regulatory motifs. Interestingly, we have detected a CpG island (CGI), known as indicator of transcription-promoter sequence, located upstream of the 5' region of the Antarctic pIgR gene, but absent in the temperate and non-Antarctic species analyzed. These findings prompted us to investigate the regulation of *pIgR* gene expression in *T. bernacchii* by evaluating the DNA methylation status of CGI in gills and liver. Interestingly, we observed an increased level of unmethylated CpG sites in gills, where we found *pIgR* highly expressed. On contrary, higher methylated CpG sites were found in liver, associated with lower *pIgR* expression. These findings suggest that CpG sites are differentially methylated in a tissue-specific manner in this cold-adapted fish species. Investigation of DNA methylation patterns might help to better understand molecular basis to cold adaptation and in response to environmental challenges.

Acknowledgements: This work was supported in part by ENVIROMED, EU Commission Horizon Europe project GA 101057844.

Evaluation of Per- and Polyfluoroalkyl substances (PFAS) toxic effects on the acute and chronic inflammatory response in the medicinal leech *Hirudo verbana*

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The pollution related to synthetic substances represents a fundamental topic worldwide. In this context, it was recently demonstrated that per- and polyfluoroalkyl (PFAS) compounds, a wide heterogenous group of fluorinated molecules with a high socio-economic impact, possess an elevated toxicity. Given their ability to resist to any kind of degradation, PFAS bioaccumulate inside living organisms, constituting a serious problem also for human health. Although diverse studies have been conducted, more information are necessary. In particular, only few data are available about the new emerging generation of PFAS, more recently introduced on the market. For this reason, in the current study we propose the medicinal leech *Hirudo verbana* as invertebrate model to assess the effects of four diverse PFAS compounds (GenX, PFMoBa, PFOA and PFMOPrA) dispersed in freshwater. In detail, different timings (1 week and 2 months) and concentrations (0.6, 229 µM and 1 nM) were analyzed by means of morphological, immunofluorescent and molecular assays. The results obtained show that PFAS induce an initial physical protection that consists in the mucus secretion, followed by an activation of the inflammatory response, recognizable by an increased angiogenesis, the recruitment of numerous immune cells and the production of pro-inflammatory factors. Moreover, the expression levels of genes related to oxidative stress-induced enzymes have been investigated to evaluate the possible activation of oxidative metabolism. This work allows to deepen the current knowledge on the potential toxic PFAS effects, leading also to new insights about freshwater ecosystems, which often represent the main way between pollutants and human.

Assessing apical toxicity and sublethal responses of earthworms in PFAS-contaminated soils

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Fire training sites and airports are major PFAS contamination sources due to widespread Aqueous Film- Forming Foam (AFFF) use. Recent global policies restrict higher than C8 PFAS congeners, yet PFOS and PFOA legacy persists, impacting ecosystems. Modern AFFF formulations still include short-chain PFAS and their oxidizable fluoroalkylamide precursors. In terrestrial ecosystems, earthworms, play a key role in soil processes. This study assesses PFAS-contaminated AFFF impact on earthworms, highlighting ecological consequences and the need for sustainable firefighting policies. The study investigated apical toxicity and sublethal responses of earthworms across a PFAS contamination gradient from a Trelleborg (Sweden) drill site. PFAS contamination was confirmed, with 22 compounds, mainly PFOS, PFOA, PFNA, and PFHxS, ranging from 960 ppb (B1) to 8.7 ppb (B7). OECD No. 207 (acute toxicity) and No. 222 (reproduction) tests, plus 30-day sublethal assessments, were conducted. Biomarkers included oxidative burst, enzymatic responses, and an escape test. Pristine soil served as an external reference control. Acute toxicity, seen in B1 and B4, indicated a significant

20% and 12.5% mortality increase (n=4), OECD No. 207. Immunological responses showed higher reactive oxygen-producing cells in B1 and B4, with lower phenol oxidase in PFAS- impacted sites. Catalase increased in Trelleborg sites, spiking in B6 (22 PFAS 9.6 ppb). Acetylcholinesterase fluctuated, reducing in B4 and linked to impaired behavior as judged in an escape time, showing PFAS sensitivity. These findings contribute significantly to the broader understanding of PFAS-contaminated site ecology, aiding in the construction of an environmental database crucial for risk assessment analyses.

Acknowledgements: This work was supported by Green Deal H2020 programme (European Grant n° 101037509- SCENARIOS)

Antioxidant responses in the kidney of *Squalius cephalus* chronically exposed to PFAS in Veneto rivers

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Per- and poly-fluoroalkyl substances caused one of the heaviest water contaminations in the Veneto Region. However, their potential impact on the ecosystem and the physiology of freshwater fauna is still largely unknown. For this purpose, this study investigates the physiological responses induced by chronic exposure to environmental PFAS concentrations in a freshwater fish species living in Veneto rivers.

Ten specimens of *Squalius cephalus were* sampled by electrofishing from three rivers in the Vicenza province, characterised by three different contamination levels.

Biochemical and molecular analyses were performed to evaluate some biomarkers of oxidative stress in the kidney. First, the activity of catalase (CAT) and glutathione peroxidase (GPX) was quantified at the level of active proteins. Then, mRNA accumulation was measured to evaluate the gene expression of the same antioxidant enzymes at the transcriptional level. These data were also correlated with two indicators of cell damage: Lipid Peroxidation and Advanced Oxidation Protein Products (AOPP) formation.

The obtained results prove the importance of the specific activation of different components of the antioxidant defence system against the risk of oxidative stress caused by PFAS.

Acknowledgements: Supported by the European Union, PNRR Programme 2021-2027 (Grant Agreement No.PE0000005)

Session II Session in memory of Professor Enzo Ottaviani: Immunocyte, hemocyte and immune-related cell roles in metazoans. Chair: Loriano Ballarin, University of Padua, Italy and Maria Giovanna Parisi, University of Palermo, Italy

Professor Enzo Ottaviani: a pioneer in the study of the "immune-mobile brain" in invertebrates

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Prof. Enzo Ottaviani (Rimini, 1945-Modena, 2017), born in Rimini, spent his career first as a biology student and then as a professor of Cytology and Comparative Immunobiology at the University of Modena and Reggio Emilia (Modena, Italy).

An international leading scientist in the field of comparative immunobiology, founder of the diamond open-access journal "ISJ-Invertebrate Survival Journal", former president of the Italian Association of Comparative and Developmental Immunobiology (IADCI), he was one of the first to demonstrate that circulating immune cells (*i.e.*, hemocytes/immunocytes) from molluscan models, such as freshwater snails or mussels, produce soluble mediators that are associated not only with the immune response but also with the neuroendocrine system. These seminal observations paved the way for increasingly sophisticated and timely research showing that these cells are a true "living laboratory" for studying, in a simplified context, the intersections between immune, nervous and endocrine functions that we also find, at a much higher level of complexity, in vertebrate species such as humans.

Recent advances in comparative immuno-neuroendocrine research have confirmed his far-sighted views and continue to keep his pioneering research at the forefront.

Lecture: History of invertebrate self/non-self recognition: from phagocytes to immunocytes

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It has not always been clear that circulating cells in all metazoans played a significant role in the recognition of 'non-self'. Despite the inception of cell-mediated immunity being credited to Metchnikoff's studies on echinoderms, research thereafter largely focused on mammals, to better understand the mechanisms governing human immunity. Today, things have not changed substantially, but we have uncovered that not only can the immunocytes of gnathostomes engage in extremely complex communication amongst themselves and with the nervous system, but invertebrate immunocytes are capable of this as well. We now recognize that the employment of hypervariable molecules to identify non-self is not exclusive to gnathostomes, nor is the use of immunoglobulin domains or somatic recombination.

Progress in our knowledge of invertebrate immunity is increasingly distancing us from the oversimplified and primitive perspective of a crude innate immunity satisfied with recognizing PAMPs via its PRRs.

The delineation between innate and adaptive immunity is beginning to fade, and perhaps we are beginning to understand that what we consider acquired immunity is merely a form of immunity 'acquired by mammals', which is not substantially different from the 'acquired immunity' observed in arthropods, mollusks, or protochordates

Stem cells and innate immunity: any relationship?

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Stem and immune cells are commonly treated as separated biological entities without any interconnection. However, both the stem and the immune systems operate to ensure the organism survival and contribute to homeostasis by perceiving physiological changes and face them with cell differentiation and immune responses, respectively. Direct evidences from various organisms indicate an interplay between the two systems in which stem cells oh hematopoietic organs influence immune responses by providing it with new immunocytes and, upon the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), immunocytes stimulate stem cell differentiation with the secretion of cytokines. This presentation aims at highlighting the links between stem cells and innate immunity.

Acknowledgements: This work was supported by COST Action 16203

Hemolymph withdrawal transiently alters the ratio of hemocyte populations and promotes an increase in total hemocyte number in the invasive snail *Pomacea canaliculata*

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Hemocytes represent a key cell in invertebrate immunity, but their development and renewal are still poorly characterized in most invertebrate species. The freshwater invasive snail, *Pomacea canaliculata*, offers the unusual possibility of multiple hemolymph sampling and its hemocytes have already been divided into two morphotypes: small and blast-like Group I (GI) and large Group II (GII) hemocytes. The total cell number and the proportion of GI and GII populations (GII/GI ratio) are relatively conserved among individuals, which suggests the existence of mechanisms controlling hemocyte proliferation and differentiation.

In order to establish the basis for studying these mechanisms, the effects of hemolymph collection on hemolymph cell composition were assessed after 1.5, 3, 6, 9, 18, 24 or 48 h. Hemolymph samples were analyzed using the Attune NxT® acoustic flow cytometer, which allows for very rapid analyses and prevents cell clumping. The fluorescent marker CellTracker™ Green CMFDA was used to distinguish live cells from debris. After hemolymph withdrawal GII/GI ratio decreased significantly only after 6, 9 and 18 h, returning to baseline conditions after 24 h. In addition, the total cell number increased significantly after 24 h and returned to normal levels after 48 h. No significant changes in cell number were observed at the other times examined. These data suggest the presence of a control in the proportion of hemocyte populations and the possibility to quickly restore it after a bleeding.

Future experiments focusing on cell markers and cell cycle proteins may provide crucial insights into the mechanisms promoting the increase in total hemocyte number and the recovery of the GII/GI ratio, and may also help to determine whether GI and GII represent two separate cell types or different stages of maturation of the same cell type.

Acknowledgements: This work was supported by National Recovery and Resilience Plan (NRRP), Mission 4, CUP E93C22001090001, Project title “National Biodiversity Future Center-NBFC”

Phylogenetic analysis of the main components of the JAK/STAT pathway and their expression in the haemocytes of the colonial tunicate *Botryllus schlosseri*

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Tunicates, the sister group of vertebrates, represent an interesting cluster of organisms to study stemness and immunity, due to their outstanding regenerative capabilities and articulated immune system responses. The evolutionary conserved JAK/STAT signalling pathway has been demonstrated to be involved in these physiological processes in many metazoans. The structure and role of this pathway have been described in detailed in vertebrates, where each of the main involved genes form a wide gene family including several members, but little is known in chordate invertebrates. In this work, we present the identification of the key molecular components of the JAK/STAT pathway, such as the kinases JAKs, the transcription factors STATs and the negative regulators SOCSs, in the colonial tunicate *Botryllus schlosseri*. Several transcripts belonging to these genes were identified *in silico* analysing the *B. schlosseri* public genomic/transcriptomic resources, and then verified experimentally. A Maximum Likelihood phylogenetic analysis was performed harvesting sequences from all major groups of deuterostomes, including all other ascidians for which genomic/transcriptomic data were available. The evolution of each gene family in ascidians compared to vertebrates was therefore inferred. Moreover, the expression of the main elements of the JAK/STAT pathway was investigated through *in situ* hybridization in haemocytes, as the

haemolymph of *B. schlosseri* comprehends both circulating stem cells and immunocytes. These experiments demonstrated their expression both in the immunocytes and the circulating stem cells. In conclusion, our results suggest that this conserved signalling pathway is involved in immunity and stemness also in this unique colonial chordate.

A potential pathogenic vibrio for *Mytilus galloprovincialis*

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Marine bivalves are exposed to different types of bacteria, in particular of the *Vibrio* genus, that includes species pathogen for aquatic animals. Although mussels (*Mytilus* spp) are usually more resistant to infections compared to other bivalves, the presence of certain bacteria in combination with particular environmental conditions may lead to decreased immunocompetence and disease. In this work, we investigated the effects of a *Vibrio* isolate from *Mytilus galloprovincialis* population suffering a mortality outbreak in Alfacs Bay (Spain) in April 2022. Immune responses of *M. galloprovincialis* were investigated *in vitro* and *in vivo*. For *in vitro* experiments, hemocytes were challenged with several concentrations of live bacteria in two different media: artificial seawater and hemolymph serum. Functional responses were evaluated: lysosomal membrane destabilization, extracellular lysozyme release and oxyradical (ROS) production, nitrite accumulation. Experiments were also carried out with heat-killed bacteria, evaluating by confocal microscopy lysosomal and mitochondrial parameters, intracellular ROS production, and cytoskeletal changes. *In vivo* experiments were also performed testing two different protocols of vibrio exposure: injection and immersion. Overall, in all experimental conditions, no activation of extracellular immune defences by mussel hemocytes was recorded; moreover, the *Vibrio* isolate induced significant lysosomal, mitochondrial and cytoskeletal damages. The results show that mussel hemocytes cannot mount an efficient immune response towards this *Vibrio* isolate, indicating that it may represent a potential threat for mussel populations.

Acknowledgments: This work was supported by National Recovery and Resilience Plan (NRRP) (Project code CN_00000033, CUP: D33C22000960007), Riscos emergents relacionats amb les mortalitats de bivalves al delta de l'Ebre (REMBE). N.Ex: CAT 211CAT00004; TAIS ARP140/20/000011 (SPAIN)

Haemocyte parameters in the clam *Ruditapes philippinarum* feed with BPA analogs-contaminated microalgae

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Bisphenol A (BPA) analogs are plasticizers currently used in many manufacturing activities as substitutes for bisphenol A. The replacement is taking place because BPA is a well-known endocrine disruptor chemical (EDC) that can also cause oxidative stress and genotoxic effects. However, BPA analogs are speculatively considered safer compounds than BPA and their usage is increasing with a consequent higher environmental release. In this study, specimens of the clam *Ruditapes philippinarum* were fed for 7 and 14 days with microalgae previously exposed to an environmentally relevant concentration (300 ng/L) of the three main BPA analogs: BPAF, BPF, BPS, and their mixture (MIX) to allow their bioaccumulation into the microalgae. Effects on biomarkers indicative of cytotoxicity were evaluated. Results showed that the ingestion of microalgae growth in a BPA analogs-contaminated environment caused alterations of hemocyte parameters, with particular reference to hemocyte number and their proliferation. In addition, the activities of both acid phosphatase and alkaline phosphatase were significantly altered in clams fed with contaminated microalgae. These results suggest that BPA analogs can be harmful compounds for clams even through a dietary intake and more effort should be performed to assess the effects of these compounds in marine food chains.

Effects and mechanisms of action of the antidepressant Fluoxetine on *Mytilus* hemocytes

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The serotonin reuptake inhibitor (SSRI) Fluoxetine (FLX), as a result of high consumption, is one of the most frequently detected antidepressant in the aquatic environment, at concentrations from ng/L to µg/L. Although FLX has been shown to exert a number of effects in aquatic species at environmentally relevant levels, little information is available on the possible mode of action (MOA) of FLX in non-target species, marine invertebrates in particular.

In the marine bivalve, the mussel *Mytilus galloprovincialis*, where FLX is bioaccumulated and partially metabolized into norfluoxetine several

biomarker responses from cellular to organism level, were observed, including lysosomal biomarkers.

In this work, the MOA of FLX was investigated in mussel hemocytes in short term *in vitro* experiments (15 min - 4 h) at 0.1, 1 and 10 µM. The FLX affected different functional parameters (e.g., lysosomal membrane stability, phagocytosis, nitrite production, and lysozyme release). Confocal microscopy showed lysosomal acidification and enlargement, increase in neutral lipids, decrease in mitochondrial membrane potential, intracellular ROS production and alteration of actin cytoskeleton. Moreover, the utilization of fluorescent autophagy markers indicated alterations of the autophagic flux. The effects of FLX on lysosomal and autophagic parameters were also confirmed by Flow Cytometry. Interestingly, fluorescent probe colocalization indicated the presence of mitochondria within acidic vacuoles, suggesting that FLX may induce mitophagic processes.

Overall, the present study reveals FLX can act on mussel cells through multiple mechanisms, probably also independent of SSR inhibition. These data shed light on the MOA of this widespread pharmaceutical in marine invertebrates.

Acknowledgements: Project code CN_00000033, CUP: D33C22000960007. National Recovery and Resilience Plan (NRRP)

Session I.2: Immunotoxicological and eco-immunological impact of xenobiotics and climate change on animal biodiversity. Chair: Caterina Ciacci, University of Urbino "Carlo Bo", Italy and Sandro Sacchi, University of Modena and Reggio Emilia, Italy

Monitoring of pharmaceutical impact on marine environment through an eco-immunotoxicological approach: an "ENVIROMED" study

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Pharmaceuticals, even being necessary to ensure longer and healthier human lives, have a critical environmental impact resulting from their manufacturing process, use and disposal. The EU funded project "ENVIROMED" aims to better understand the lifecycle of pharmaceuticals products to reduce their environmental footprint through the study of their occurrence, persistence, and fate in the environment, and toxicity.

The ecotoxicity assessment is focused on the evaluation of immune-related parameters in selected marine invertebrates, the tunicate *Ciona robusta*, the mollusk *Mytilus galloprovincialis* and the sea urchin *Paracentrotus lividus*. In the present study, the mollusk *M. galloprovincialis* was incubated for a short time (2 h) with two different concentrations (10 µg/L, environmental realistic/expected concentration; and 1 mg/L, therapeutic concentration) of selected pure pharmaceuticals (*e.i.*, Diclofenac, Metformin hydrochloride) and stress/immune parameters were measured on freshly collected haemocytes using two immunotoxicity assays, Lysosomal Membrane Stability-LMS and Phagocytic assay.

Very preliminary results showed that both pharmaceuticals induced the loss of neutral red into the cytosol from pathologically enlarged lysosomes of the haemocytes. Conversely, the two pharmaceuticals differently affected the phagocytic capacity of mussel haemocytes. In details, Diclofenac seemed to induce a higher phagocytic rate and index in animals treated with 10 µg/L dose, whilst Metformin hydrochloride seemed to reduce phagocytic rate and index at both the concentrations tested.

These preliminary data show that the two pharmaceuticals have a stressful effect on immune cells. Further analysis will enrich and strengthen these results by using other selected pharmaceuticals and by performing additional *in-vivo*/*ex-vivo* immunotoxicity assays.

Acknowledgements: This work was supported by ENVIROMED, EU Commission Horizon Europe project GA 101057844

Cellular pathway disturbances triggered by the anti-inflammatory dexamethasone in mussel *Mytilus galloprovincialis* under environmental scenarios

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Dexamethasone (DEX), one of the main anti-inflammatory drugs used during the COVID-19 pandemic, belongs to the class of active pharmaceutical compounds (PhACs). It is considered as an emerging contaminant and it is currently detected into the aquatic environment at concentrations ranging from ng/L to µg/L. The global concern on this class of micropollutants is due to the PhACs interaction with non-target organisms, leading to cellular impairments. Therefore, the biological impact of DEX was assessed on marine mussels *Mytilus galloprovincialis* (Lamarck, 1819), challenged with its environmental concentrations (C1: 4 ng/L; C2: 40 ng/L; C3: 400 ng/L; C4: 2 µg/L) for 12 days, with selected sampling time-points (3 days, T3; 6 days, T6; 12 days, T12). The gills, chosen as a key organ for their role in gas exchange, filter-feeding and osmoregulation, exhibited a haemocyte infiltration among the branchial epithelium, as well as a time-dependent pro-oxidant effect combined with lipid peroxidation and altered cholinergic neurotransmission, as revealed by a multi-biomarker approach including histology, molecular and enzymatic assays, and protonic NMR metabolomics. Surprisingly, the impairments on mussel gills occurred from 3 days of exposure even at the low DEX concentration as C2,

with effects more pronounced at T12 particularly in specimens exposed to the highest DEX doses (C3, C4). These findings confirm the ability of PhACs to induce cellular damage on non-target species under realistic scenarios, and therefore underline the urgent need for innovative bio-recovery strategies and effective eco-pharmacovigilance programmes.

Mussels for investigate the effects of bio - based plastic on marine organisms

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Petroleum-based plastics have revolutionized global markets over the last half-century, but today pose a serious question regarding their fate at the end of their usefulness due to the growing amount of plastic waste and their non-biodegradable nature causing serious environmental problems with serious repercussions on the health of ecosystems and organisms, including humans.

To face this challenge, ecological alternatives have been formulated and new bio-based plastics have been widely introduced on the market. Despite their green nature, their growing use poses an increasingly urgent problem regarding their environmental sustainability. In particular, the statements relating to the absence of direct or indirect impacts on ecosystems and the health of living organisms remain to be verified.

Polylactates (PLA), polyhydroxyalkanoates (PHA) and polyhydroxybutyric acid (PHB), as representative polymers of these new materials, were selected for this study given their wide use as a substitute for conventional plastics. This study aims to contribute to the investigation of this topic by evaluating their effects on natural marine aquatic environments based on the investigation of *Mytilus galloprovincialis* exposed to bio-plastic. The results highlight that exposure to bio-based plastic triggers the immune system by activating pathways for the elimination of non-self particles through a cellular response and induces histological alterations and enzymatic stimulation that show a potential harmful effect on organisms. In conclusion, our data should be carefully considered in view of the use of alternative bioplastics and their potential effects on biodiversity and ecosystem components.

Acknowledgements: This work was supported by the National Biodiversity Future Center (CUP B73C22000790001)

Adverse effects of polystyrene nanoplastics on teleost fish: *in vitro* and *ex vivo* evidence

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Nanoplastics pollution in marine and freshwater environments poses a growing global risk to aquatic organisms. In this study, *in vitro* and *ex vivo* approaches were utilized to investigate the effects of 20 and 80 nm commercial polystyrene nanoplastics (PS-NPs) on teleost fish cell lines and primary gill, head kidney and spleen cell culture models. PS-NPs were characterized by transmission electron microscopy and by Dynamic Light Scattering. Subsequently, the severe cytotoxicity of 20 nm PS-NPs was demonstrated. The process of internalization was examined by employing fluorescent PS-NPs, which were localized within the nucleus as early as ½ hour post-incubation. Additionally, the cytoplasmic translocation and the adverse effect on cellular components were observed to be dependent on the size of the internalized nanoparticles. Piscine cells suffered structural damages dependent on both the size, dose and duration of exposure to PS-NPs. The noticeable alterations in cellular morphology, such as cell contraction and plasma membrane bleb formation were distinctive features of the execution phase of apoptosis, as confirmed by the Annexin V/propidium iodide and Tunel assays. The apoptotic responses were unrelated to intracellular ROS signaling. Transcriptional changes at sublethal and lethal PS-NPs doses were confirmed, with a more profound response to the latter consisting in a marked impairment of steroid biosynthesis, TGF-beta signaling pathway, focal adhesion and protein processing in endoplasmic reticulum. Our results clearly indicate that fish face a considerable danger from PS-NPs.

Acknowledgements: This work was supported by the "NANOPISCES" project, Lazio Region (FEAMP 2014-2020, measure 1.40)

Effects of polypropylene micro and nanoplastics on wound healing and tissue regeneration in the medicinal leech *Hirudo verbana*

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Plastics, due to their useful characteristics and versatility, have become indispensable for the

industrial field and everyday use, making them a major contributor to pollution in both terrestrial and aquatic environments. Of all the types of plastics produced, almost 20% is polypropylene (PP), extremely popular in the production of surgical masks and single-use packaging. The accumulation and dispersion of PP plastic waste poses an obvious threat for both animals and humans. Indeed, plastics do not decompose into biodegradable compounds, but instead, by means of biotic and abiotic processes, degrade into smaller pieces known as microplastics (MPs) and nanoplastics (NPs). These particles have harmful effects on both terrestrial and aquatic organisms, as they can accumulate in their tissues and deplete energy reserves, reducing nutrition, survival and immune response. Although numerous studies have already shown the negative impact of MPs and NPs on various vital processes, little is still known about their effect on wound healing and tissue regeneration. To this end, the effect of PP MPs and NPs on tissue regeneration processes following injury was evaluated in the freshwater invertebrate model *Hirudo verbana*, well-suited for evaluating inflammatory and tissue remodelling processes, both involved in proper wound healing.

By means of morphological, immunofluorescence, histoenzymatic and molecular analyses, we have clearly demonstrated that PP MPs and NPs inhibit angiogenesis, activate the innate immune system and induce excessive collagen production leading to the formation of a fibrotic tissue that interferes with the correct muscle tissue regeneration.

***Mytilus galloprovincialis* as sentinel to detect marine hydrocarbon contamination: an integrated approach**

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Oil spills cause significant impacts on the coastal marine ecosystem. Studying and investigating the immune mechanisms that contribute in hydrocarbon (HC) detoxification processes is crucial to better understand the responses of marine organisms under pollutant exposure. The mussel *Mytilus galloprovincialis*, due to its filtration behaviour and the evidence about its ability to response early to environmental stressors, is considered an excellent model species for ecotoxicological studies. In this work, specimens of *M. galloprovincialis* were exposed for 4 days to different concentrations of diesel-engine oil mixture. Increasing concentrations of the mixture significantly decreased the phagocytic activity of the haemocytes. Enzymatic analyses of the main oxidative stress and inflammation markers confirmed the immunomodulation resulting from short-term exposure to the mixture. Histomorphological analyses on digestive gland showed that HCs mixture compromised the microtubules's tissue structures, inducing necrosis especially for the highest concentrations. Furthermore, the modulation of the molecular markers HSP70 and HSC70 was also assessed by western blot, demonstrating their involvement in maintaining the organism's homeostasis. Finally, the effects of HCs mixture on the diversity and structure of microbiome of hepatopancreas and haemolymph were also evaluated by Automated Ribosomal Intergenic Spacer (ARISA) and 16S rRNA gene sequencing analyses. The exposure to increasing HCs concentrations has a positive effect on microbial diversity, with an increase in relative abundance of several known degrading bacterial genera. These results confirmed the role of *M. galloprovincialis* as a sentinel of environmental pollution, thanks to its ability to respond sensitively and quickly to hydrocarbon pollution.

Acknowledgements: This work was supported by the National Biodiversity Future Center (CUP B73C22000790001)

Session III.1: Morpho-functional and molecular advances in comparative and developmental immunobiology. Chair: Annalisa Grimaldi, University of Insubria, Italy and Jacopo Vizioli, Université de Lille, France

***Octopus vulgaris* as a model for studying indeterminate growth and regeneration**

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Regeneration is a widespread phenomenon in which damaged structures regrow after injury. Among invertebrates, coleoid cephalopods are able to regenerate several structures, including appendages and nervous tissue. In *Octopus vulgaris* these processes have been well described; and the involvement of hemocytes have been documented during regeneration in both the arms and pallial nerve. In the latter, hemocytes are involved in debris removal of the degenerating tissues and represent the main source of proliferating cells. Additionally, they appear to be involved in the release of factors that foster regeneration of axons. Knowledge gathered so far allows to hypothesize a possible stem-like behaviour of the immune cells in octopus.

Here, we use the tip of *O. vulgaris* arm as a model for studying regeneration and indeterminate growth, a condition where the body, or part of it, keeps growing throughout life. The arm tip is a zone of the arm putatively representing a stem cells reservoir, due to its low differentiation rate, high number of proliferating cells, and fast regenerative process. Through *in silico* data-mining we identified in the arm tip a characteristic molecular fingerprint with differential expression of genes including transcription- and neurogenic-factors, cell fate and proliferation makers, RAGs and immune-related genes. Through RT-qPCR experiments we evaluated relative gene expression and found it to

be upregulated during different phases of arm regeneration. Together with morphological data, the gene expression profile allowed us at gaining information regarding the biological and molecular fingerprints that characterize the shared mechanisms involved in development, growth and regeneration.

Symphony of resilience: unravelling wound healing, regeneration, and symbiotic dynamics amidst stress

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This research stems from the necessity to delve into the molecular and transcriptomic aspects of the regenerative process, given the knowledge gap concerning this species, which has demonstrated to diverge from other organisms within the same class. The approach employed aims to elucidate events under unstressed conditions and, crucially, to analyse a boundary scenario involving multiple stressors induced by a temperature increase. This allows an assessment of the thermal impact, a plausible condition considering the ongoing rise in seawater temperatures due to climate global changes. To gain a comprehensive understanding, oxidative stress markers (protein carbonylation and total antioxidant capacity), symbiont photosynthetic efficiency assessed through Pulse Amplitude Modulation (PAM) were taken into account. The presence and modulation of proteins related to the inflammation and regenerative processes (HSP70, HSC73 and IL-1ß) were evaluated by blotting. Additionally, to address the query of whether an anthozoan subjected to thermal stress regenerates similarly to an organism undergoing a simple wound, phenotypes were observed over 7 days, measuring tentacular length during regeneration. Results indicate a higher antioxidant capacity in the sea anemone at 20 °C after 6- and 24-hours following tentacle removal compared to values at 27 °C. The redox state imbalance was further affected by reduced photosynthetic efficiency in the symbiont, aligning with previous observations at the histological level. Protein expression and modulation resulted from blotting and RT-qPCR (PCNA, HSP90, CDC37, COL24α1) analyses suggest the activation and production of compensatory molecules to restore internal homeostasis, despite biomarkers of oxidative stress indicating a highly critical situation.

The role of the medicinal leech *Hv*RNASET2 enzyme during tissue remodeling

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The search for new molecules to bring innovative solutions in regenerating therapies represents a fundamental goal in biotechnological research. During wound repair and healing, one of the key aspects consists in fibroblasts activation and proliferation. These mesenchymal cells are the main effectors of tissue remodeling; indeed, not only they are the main producers of the extracellular matrix components, above all collagen, but also physically assist other cells in performing their biological functions. Moreover, fibroblasts are extremely versatile, taking part in the expression of different molecular effectors, among which cytokines and growth factors, and resulting directly involved in the regulation of tissues homeostasis and regeneration.

Of note, the high evolutionary conservation of the mechanisms underlying tissue remodeling and regeneration, which often involve the same cellular and molecular regulators, allows to use and propose low-complexity eukaryotic species as emerging experimental models. In this context, we have previously demonstrated that *Hv*RNASET2, a pleiotropic enzyme found in the leech *Hirudo verbana*, showed a marked ability to induce collagen production in human MRC5 fibroblasts, with a significant increase in COL1α1 gene expression; furthermore, it also induces fibroblasts proliferation and activation when directly injected in the leech body wall, leading to the deposition of spatially organized collagen fibers that form a robust scaffold. Based on this evidence, here we evaluated the molecular relations by which *Hv*RNASET2 regulates this process, in order to identify all its possible interactions with the specific effectors that regulate collagen pathway and tissue remodeling.

Acknowledgements: This work was supported by the FAR - Fondo per Ricercatori a tempo determinato 2022 intestato a Baranzini Nicolò

The medicinal leech *Hirudo verbana* as an innovative model to explore the potential of human Mesenchymal Stem Cells for multiple biomedical applications

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Animal models are essential in biomedical research to validate findings from simpler models to more complex and dynamic organisms. Despite

advanced *in vitro* approaches attempt to replicate multicellular interactions in 3D systems, they still fail in mimicking a whole living creature; conversely, the use of complex animal models faces significant limitations.

For all these reasons, the medicinal leech *Hirudo verbana* is emerging as a promising *in vivo* model for pre-clinical studies. Indeed, despite its simple body organization and the relatively low genetic complexity, it exhibits biological processes, cellular responses, and tissue organization that bear resemblance to those found in vertebrates. Moreover, the easy and cost-effective maintenance of leeches, coupled with the absence of ethical constraints related to *in vivo* experiments, make this organism as an appealing model.

Considering the aforementioned, we propose to use the medicinal leech *H. verbana* as animal model to investigate the potential of soluble factors derived from human Mesenchymal Stem Cells in enhancing tissue regeneration and significantly reducing the healing time by fostering both cellular invasion and capillary growth. These processes play a pivotal role in ensuring an adequate vascularization, crucial for cell survival and in preventing the formation of hypertrophic scars.

The adoption of this cell-free approach might represent an innovative solution for managing various chronic conditions, such as severe burns and tunneling wounds. Furthermore, these investigations also aim to validate the effectiveness of the medicinal leech as a valuable animal model for conducting a rapid and reliable research in the field of regenerative medicine.

KEYNOTE LECTURE

The memory of the enemy: why it took two millennia to discover the immune system

A D'Amico
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In 430 BC, Athens was struck by a plague that decimated its population and contributed to its defeat in the war against Sparta. In his chronicles, Thucydides observed and recorded that those who recovered from the plague did not fall ill again. This historian, often regarded as the "father" of history, described the phenomenon but admitted his inability to explain it. It was the first instance of observations about disease and death, particularly of a horrifying nature, being documented without the reassurance of understanding why. This emergence of a simple yet disconcerting "I do not know" dethroned humans from the apex of nature, undermining their claim to fully comprehend and control it. Yet, it is precisely this admission of ignorance that spurred the search for causes.

The immune system was discovered in 1883, after 2313 years. This discovery was not primarily driven by technological advancements in research tools, such as the microscope. For two thousand years, the cause of the enemy and of "no return" was sought in the heavens, among stars and gods, or established through sophisticated and complex reasoning about "humors," "pneumas," and "miasmas." Never was nature and the human body truly interrogated. Those who did investigate and measure reality were often ignored, ridiculed, slandered, or marginalized. Sometimes, they even paid with their lives.

Session III.2: Morpho-functional and molecular advances in comparative and developmental immunobiology. Chair: Maria Rosaria Coscia, National Research Council of Italy, Naples and N Franchi, University of Modena and Reggio Emilia, Italy

The silkworm as a non-mammalian infection model for screening nanoconjugated-antibiotics

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The excessive and improper use of antibiotics has laid the foundation for the rapid spread of antibiotic-resistant bacteria that cause 1 million deaths per year. As a result, efforts must focus on the development of new molecules with antimicrobial properties, or on the development of alternative and improved formulations. One of the most promising strategies to overcome microbial resistance is the use of nanoparticles as carriers for antibiotics. In this scenario, the development of new antibiotics requires preclinical tests, i.e., *in vivo* assays, that are performed on mammalian models. Following the introduction of European directives on the use of laboratory animals, the research and the introduction of invertebrate models that can be used in these trials are essential. Among them, insects have proved to be a suitable alternative.

This project aims at validating a silkworm infection model for screening nanoconjugated-antibiotics. To this purpose, insects were infected with the nosocomial pathogen *Staphylococcus aureus* and treated with nanoconjugated-teicoplanin. The survival rate, as well as immunological markers of the larvae were evaluated at 37 °C in order to reproduce human physiological condition. The nano-antibiotic proved to be effective, curing infected larvae, increasing their survival rate, and blocking the activity of the immune response.

In conclusion, this study demonstrated the usefulness of this insect alternative infection model for testing the efficacy of nanoconjugated-antibiotics extending the range of its applications.

Acknowledgements: This work was partially supported by Animal Research Tomorrow (3R ART Award)

How bacteria escape from digestion in the phagosome: comparative analysis of the Type 6 Secretion System in pathogenic *Francisella* and non-pathogenic *Parafrancisella* bacteria

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The γ-proteobacterium *Francisella* includes numerous species (*F. tularensis, F. noatunensis*, *F. orientalis, F. halioticida* and others) which are highly pathogenic to humans, fishes and molluscs. These species cause diseases by entering the eukaryotic cell through phagocytosis, and rapidly escaping from the phagosome to multiply into the cytosol. Their ability to avoid phagosome digestion by the target cell is essentially due to a gene cluster known as *Francisella* pathogenicity island (or FPI), which contains genes encoding an atypical type VI secretion system (T6SS). Proteins encoded by the T6SS genes generate a needle-like structure which anchors to the bacterial membrane and is used to inject effector proteins into the target cell. We have analyzed the genomes of three strains of a non-pathogenic species of *Francisella*, recently re-named *Parafrancisella adeliensis*, that have been raised as cytoplasmic endosymbionts of cells of two Antarctic and Arctic species of the ciliate *Euplotes* (*E. petzi* and *E. nobilii*). All three genomes contain a new 23404-bp FPI-like gene cluster, in which the T6SS genes are associated with three other genes encoding effector proteins. Considering that in addition to being not pathogenic, *P. adeliensis* is unable to grow at 37 °C and easy to be cultivated, this identification strongly suggests to use the microbial *Euplotes/Parafrancisella* association as a new model to investigate the role of each T6SS component and the mechanism of bacterial invasion of eukaryotic cells.

Acknowledgements: This work was supported by Programma Nazionale di Ricerche in Antartide (grant PNRA18_00152)

Immune responses to Betanodavirus (RGNNV) in European sea bass central nervous system

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In vertebrates, the intertwined communication between the immune system and the central nervous system (CNS) is increasingly acknowledged. Mammalian CNS harbours both resident and migrating peripheral leukocytes, which play crucial roles in pathogenesis, immune surveillance, and further contribute to the higher functions of the brain and homeostasis. Herein, we investigated, for the first time, the contribution of T cells and CD45+cells in the brain cellular immune responses of *Dicentrarchus labrax* juveniles inoculated intramuscularly with the CNS-infecting virus betanodavirus (strain RGNNV). The time points selected for sampling, 3- and 6-days post-infection (dpi), were those when clinical symptoms appear and fish begun to die. Immunohistochemical and immunofluorescence analyses were performed on brain and retina tissues, as well as brain-derived isolated leukocytes, revealing significant differences between sampling times in the number of T cells and CD45+ cells after RGNNV infection. At 6 dpi, the number of such cells dramatically increase in the brain and eye. The infection triggered the expression of pro-inflammatory and anti-inflammatory cytokines which may be related to the evident damages observed in the brain tissue. Moreover, in infected fish, a notable increase in antiviral, immunomodulatory and T cell-related gene expression in both brain and eye was also recorded. These findings provide compelling evidence of leukocyte involvement in cell-mediated immune responses following RGNNV infection and suggest the potential migration of TCRβ+/CD4+ and CD45-R0+ immune cells into the affected tissues.

The search for invertebrate herpesviruses and their possible association with basal chordates

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Viruses are parasitic mobile genetic elements of almost any living cell, commonly considered to be the most abundant and genetically diverse biological entities on our planet. Besides their obvious pathogenic nature, viruses have the potential to transfer genetic material, even across the species boundaries. However, host-virus co-evolution hampered the transfer of genetic material to distantly-related taxa. Herpesviruses provide excellent examples of virus co-evolution with susceptible hosts, with the families *Orthoherpesviridae* and *Alloherpesviridae* infecting vertebrate hosts, whereas *Malacoherpesviridae* replicating in non-vertebrate mollusk and gastropod species. The connections between these families remain obscure, although the identification of malacoherpes-like viruses associated with the cephalochordates *Branchiostoma floridae* and *B. belcheri* suggested a broader host range and a divergent evolutionary trajectory for *Malacoherpesviridae*. This diversity could be explored through the analysis of integrated viruses (proviruses), endogenous viral elements or circulating viruses. Here, we describe our approaches aiming to extend the knowledge on malacoherpesvirus diversity, such as the screen of

genomic, transcriptomic and metagenomic datasets, including from the Tara Oceans expedition. Moreover, we mined the genomic databanks of lancelets to support the presence of bona-fide herpesviruses associated with these "invertebrate chordates". The lancelet herpesviruses have likely contributed to the exchange of genetic material among distantly related eukaryotes, potentially resulting in genetic innovations.

Session III.3: Morpho-functional and molecular advances in comparative and developmental immunobiology. Chair: Marco Gerdol, University of Trieste, Italy and Adriana Vallesi, University of Camerino, Italy

Underwater noise induces stress on behavioral and physiological performances of the colonial ascidian *Botryllus schlosseri*

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The ability of marine invertebrates to cope with anthropogenic underwater noise is mostly unknown although it is of concern in the European Marine Strategy Framework Directive. Therefore, its effects need to be investigated with the purpose of contributing to a sustainable blue growth. Among marine invertebrates, the ascidians are of interest possessing several mechanoreceptors potentially able to respond to water particle vibrations caused by noise. These include receptors on oral tentacles, the coronal cells, considered homologues of vertebrate hair cells of inner ear. The effect of noise produced by maritime traffic on the colonial ascidian *Botryllus schlosseri* were studied exposing animals to a different continuous noise (for 30 minutes; peak bands 63-125 Hz; at 160 dB; 152,8 dB; 145,5 dB). To evaluate the stress induced by the treatment, we used a behavioral test, the tentacle stimulation test, which specifically monitors coronal cells sensitivity. Their stimulation evokes the atrial siphon closure. Tests were performed in triplicates, before and after noise stimulation. Heartbeats were also counted to assess potential effects on animal physiology. Untreated colonies (genetically identical to treated colonies) were used as control and data were statistically analyzed. Results show that the noise induces stress on animals, decreasing both their sensitivity and heartbeat frequency. Future experiments will verify which is the threshold level that negatively affects animal performances. Moreover, noise effects on immune responses will be evaluated analyzing the ability of phagocytes to ingest target foreign cells and of cytotoxic cells to mount an inflammatory response upon the recognition of nonself.

Stress granule related-genes during the blastogenetic cycle of two colonial ascidians: *Botryllus schlosseri* compared to *Botryllus primigenus*

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Colonial ascidians are the only chordates able to reproduce both sexually and asexually. In the present study we used the Italian species *Botryllus schlosseri* and the Japanese *Botryllus primigenus* to investigate the possible role of *tiar*, *ttp* and *g3bp* in the periodical renewal of the colonies, defined as generation changes or takeovers. In this scenario, the above genes, which codify key components for the formation of stress granules, storing specific mRNAs, can play a pivotal role, allowing the regulation of processes such as stress responses, cell proliferation and stem cell development. We started by characterizing *tiar*, *ttp* and *g3bp* sequences in *B. schlosseri* and *B. primigenus*, then we analyzed gene expressions by *in situ* hybridization in hemolymph cells and colony tissues, and we proceeded with quantification of the gene expressions by quantitative real-time PCR, during the colonial blastogenetic cycle. Our results allowed us to assign to the studied genes a role in defense of the germline of the new colonial generations.

The mytilin gene cluster: shedding light on the enigmatic origin of mussel dispensable genes

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Mussels exhibit a sophisticated innate immune response, marked by a multitude of highly varied molecules responsible for recognizing and killing potential pathogenic microorganisms. The intricacy of this molecular arsenal is heightened by the occurrence of gene presence-absence (PAV), a phenomenon encompassing numerous expanded lineage-specific gene families of recent origin. This phenomenon contributes to substantial inter-individual sequence variability, further enriching the diversity of the molecular repertoire involved in the immune response. However, until now the origin of dispensable genes, which are not shared by all individuals, has remained elusive. In this study, using as a model the mytilins gene family, antimicrobial peptides highly expressed in hemocytes, and analysing the sequences recovered in more than 180 resequenced individuals, we characterize the gene locus architecture, defining the common presence of 4 genes (B, C, D, G1) and 2 pseudogenes (J and P) in 4 distinct species belonging to the *Mytilus* complex. The presence of notable intron length polymorphisms, of allelic variants with small in-frame indels and significant structural variants in intergenic regions highlights the highly dynamic evolution of this locus. In addition, we report the presence of 7 dispensable mytilin genes, often associated with each other in rare haplotypes, in a very small fraction of analysed individuals. Molecular phylogeny supports an ancient origin for dispensable mytilins, which predates the radiation of modern *Mytilus* species,

suggesting that most widespread extant haplotypes derive from a larger ancestral mytilin gene cluster that underwent a gene copy reduction process, which is still presently ongoing.

The defensive anthraquinone Hallachrome: new insight on its antimicrobial potential and histological localization in *Halla parthenopeia* (Polychaeta)

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Halla parthenopeia is a tube dwelling polychaetas that colonizes soft bottoms of the Mediterranean Sea. Recent studies have focused on the indoor maintenance of this worm, which, in addition to its commercial relevance as bait, appears to be potentially significant in the blue biotechnologies field. Indeed, it can produce various types of bioactive mucus, for locomotion, feeding, and defense. Specifically, the defensive mucus contains a toxic purple anthraquinone called Hallachrome, but the structures responsible for its production and release were unknown. Moreover, the knowledge on the antimicrobial potential of this compound was still preliminary. In this study, the antimicrobial activity of Hallachrome was validated against more resistant forms such as bacterial biofilms. We have seen that concentrations lower than 0.5mM of Hallachrome inhibit formation of Gram-positive bacteria biofilms and are active on already formed biofilm as well. Furthermore, histological analyses were performed to investigate the structures responsible for the pigment production. Specific epidermal cells with hematoxylin positive round soma and pigment accumulation at the basal part were identified and seem to be active in the posterior body portion of the worm. This part secretes the Hallachrome when pressure is applied to the epidermis. Given that traumatized tissues are rich in bounded Hallachrome, we propose a defensive role for this pigment as an antibacterial agent which is secreted when the worm, subjected to attacks by potential predators, resulting in wounds or undergoing autotomy.

Constitutive expression of complement C3 and its regulator *Pc*RCA1 in the Gastropod *Pomacea canaliculata*: tissue localization and response to bacterial stimuli

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The complement system is pivotal in the innate immune system, and its characterization in gastropods provides novel insights into invertebrate immunology, development and regeneration. In this study, we investigated the presence and function of complement C3 and its regulator PcRCA1 in the invasive freshwater snail, *Pomacea canaliculata*. Tissue-specific mRNAs were analyzed to map the distribution of C3 and PcRCA1 transcripts, discovering their specific localization in key tissues for immune responses. Additionally, we assessed the variation in transcript levels of C3 and PcRCA1 under control conditions and in response to Gram-positive and Gram-negative bacterial stimuli. Contrary to expectations, our results revealed no significant differences in gene expression between control and stimulated samples. This suggests a high constitutive expression of C3 and PcRCA1 in *P. canaliculata*, indicating a potential constant and ready presence of the complement system for immune defence alongside non-immune roles. These findings are significant as they point to a distinct immune strategy in *P. canaliculata* compared to other systems studied, providing new insights into the regulation of the complement system in gastropods and their overall immune response.

Supported by:

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