

REPORT OF MEETING

Third International Symposium AMMR 2019. Advances in Marine Mussel Research, August, 26–28, 2019, Palazzo Grassi, Chioggia, ItalyOrganizers: **L Ballarin, U Rosani and P Venier (Chair)**International Committee: **N Bierne, Á Pérez Diz, A Figueras, M Gerdol, B Novoa, A Pallavicini, U Rosani and P Venier**

Total 58 delegates, from 14 countries and 30 different cities of the world, gathered in Chioggia (VE) in the occasion of AMMR 2019. The event was organized in partnership by the University of Padova, Department of Biology and School of Biosciences, under the patronage of Veneto Region, the town of Chioggia and the Horizon 2020 project VIVALDI (see other sponsors at <https://www.biologia.unipd.it/ammr-2019>). News and views have been exchanged on i) genetic mussel traits, including a significant molecular evidence of gene presence/absence variation, ii) population markers and evolution of the *Mytilus* species complex, iii) functional mussel responses in relation to potential pathogens and other factors. Innovative experimental strategies applied to marine mussels as well as mussel contributions in goods and services confirm these fascinating marine invertebrates as a model of study and strategic resource for the future. The next AMMR symposium is expected in 2021 in Poland!

Oral presentations**Session 1. News and views on genetic and functional traits of the Mediterranean mussel**
Chairpersons: Beatriz Novoa, Angel E. Perez Diz**Advances in genomics and immunity of *Mytilus galloprovincialis*****A Figueras¹, M Gerdol², R Moreira¹, M Sendra¹, B Novoa¹**¹*Institute of Marine Research (IIM), National Research Council (CSIC), Eduardo Cabello, 6, 36208, Vigo, Spain*²*Department of Life Sciences, University of Trieste, Via Giorgieri 5, 34127, Trieste, Italy*

The Mediterranean mussel (*Mytilus galloprovincialis*) is a marine invasive species cultured all over the world and also an appreciated resource in local aquaculture enterprises because of their robust production and resilience that translates into a reliable economic value. The sequencing of the mussel genome has revealed a very complex organization with high heterozygosity, abundance of repetitive sequences and extreme intraspecific sequence diversity among individuals, mainly in immune related genes. Among those genes, antimicrobial peptides are the most expressed gene families in mussels, highly polymorphic and with antimicrobial effect against molluscs pathogens, but also against pathogens of lower vertebrates and humans. The combination of a complex genome with the

adaptation of mussel immune system to a changing environment could explain this high variability, not only in immune-related genes, but also in the functional response among individuals sampled in the same location and date.

Disentangling the impact of PAV by high throughput sequencing. Implications for the interpretation of gene expression data**M Gerdol¹, P Venier², A Figueras³, A Pallavicini¹**¹*Department of Life Sciences, University of Trieste, Trieste, Italy*²*Department of Biology, Padova, Italy*³*Consejo Superior de Investigaciones Científicas (CSIC), Vigo, Spain*

Recent genomic investigations have revealed that the mussel genome is characterized by an extreme level of intraspecific genetic variation, far superior to other marine invertebrates. While the high frequency of single nucleotide polymorphisms can in part explain the high heterozygosity rates observed in mussels, the most significant contribution to inter-individual genetic variability appears to be due to hemizygoty, i.e. to the presence of large genomic regions found in just one out of the two homologous chromosomes. Unexpectedly, such “haploid” regions are not limited to intergenic sequence, but they include a high number of protein-coding genes, which are consequently present in a single copy in the diploid genome.

This unusual genomic architecture determines the presence of a set of “core genes”, invariably

present in all individuals, and underpins gene presence/absence variation (PAV), as the result of the crossing between individual bearing different haplotypes. Genes subject to PAV are “dispensable” (i.e. they can be completely absent in some individuals) and they cover accessory functions, which appear to be mostly related with immune recognition and survival.

This finding has profound implications for the interpretation of gene expression data derived from large-scale (i.e. RNA-sequencing and microarray), as well as from sequence-targeted (i.e. q-PCR) approaches. Indeed, the extreme mussel genetic heterogeneity now emerges as a key factor that should be taken into account since the very early phases of experimental design of genetic and molecular investigations. Unfortunately, this aspect has been nearly completely ignored over the past two decades, often hampering the correct interpretation of gene expression profiles.

We discuss a few key examples of gene families that have been recurrently, but incorrectly, implicated in the response to a multitude of different biotic and abiotic stimuli, showing that such results are artefacts related to the intrinsic dispensable nature of the investigated sequences. We also propose some guidelines for a correct and balanced interpretation of mussel gene expression data in future investigations, suggesting the use of conservative approaches that should always take into account as a paramount concern the extreme levels of inter-individual genetic diversity.

Innate immune memory in mussel (*Mytilus galloprovincialis*): tolerance of hemocytes after a repeated contact with *Vibrio splendidus*

B Novoa¹, M Rey-Campos¹, R Moreira¹, M Gerdol², A Pallavicini^{2,3}, A Figueras¹

¹Institute of Marine Research (IIM), CSIC. Eduardo Cabello 6, 36208, Vigo, Spain

²Department of Life Sciences, University of Trieste, Via Giorgieri 5, 34127, Trieste, Italy

³Istituto Nazionale di Oceanografia e di Geofisica Sperimentale – OGS, via Auguste Piccard, 54, 34151 Trieste, Italy

Mediterranean mussels (*Mytilus galloprovincialis*) are sessile filter feeders that live in close contact with numerous marine microorganisms. As all invertebrates, mussels lack an adaptive immune system but they respond to pathogens, injuries or environmental stress in a very efficient manner. However, it is not known if they are able to modify their immune response when they reencounter the same pathogen. In this work we studied the transcriptomic response of mussel hemocytes before and after two consecutive sublethal infections with *Vibrio splendidus*. The first infection significantly regulated genes related to inflammation, migration and response to bacteria. However, after the second infection, the differentially expressed genes were related to the control and inhibition of the ROS production and the resolution of the inflammatory response. Our results also show that the second infection with *V. splendidus*

led to changes at transcriptional (control of the expression of pro-inflammatory transcripts), cellular (shift in the hemocyte population distribution) and functional (inhibition of ROS production) levels. These results suggest a modified immune response of mussels after the second challenge oriented to tolerate and not to fight the infection, minimizing tissue damage.

What does macrophage Migration Inhibitory Factor do in *Mytilus galloprovincialis*?

U Rosani^{1,2}, S Domeneghetti¹, M Gerdol³, F Vallese⁴, E Bortoletto¹, G Zanotti⁴, A Pallavicini³, R Tavano⁴, P Venier¹

¹Department of Biology Sciences, University of Padova, Padova, Italy

²Alfred Wegener Institute, Waddenseastation Sylt, List auf Sylt, Germany

³Department of Life Sciences, University of Trieste, Trieste, Italy

⁴Department of Biomedical Sciences, University of Padova, Padova, Italy

The presence of cytokines in invertebrates has been debated for a long time. Nowadays, it is clear that ancestral cytokine genes were present before the radiation of deuterostome, although their functional roles remain mostly elusive. Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine initially described as a pro-inflammatory factor in early 1930s. Depending on the functional context and cell type, human MIF can promote the development of inflammatory and malignant diseases or play a protective role supporting resistance to various infections. The current data on MIF receptors and downstream signaling pathways reveal how intricate and multifaceted is the MIF-mediated modulation of cell processes. Hence, human MIF has been renamed ‘Most Interesting Factor’.

Searching for MIF-like sequences in genomic and transcriptomic data of bivalves, we retrieved 148 sequences, with most of the analyzed bivalve species encoding two MIF paralogues, namely one MIF and one D-DT gene. We traced the genomic expansion of D-DT genes in the *Mytilidae* family, describing 10 MIF-like genes in the mussel *M. galloprovincialis*.

The analysis of RNA-seq and RT-qPCR data indicated enhanced tissue-specific expression of MIF-like genes, possibly underpinning neo-functionalization of certain isoform in highly specialized mussel tissues, like the byssus. Conversely, expression data scarcely supported the involvement of the mussel MIF in innate immune responses.

We produced a recombinant *M. galloprovincialis* MIF protein (MgMIF) in *Pichia pastoris* and we tested its effects in mussel hemocytes as well as in human macrophages for comparison. Finally, we verified if MgMIF conserved the enzymatic functions typical of vertebrate MIF and D-DT proteins, namely oxidoreductase and dopachrome methyl ester decarboxylase into 5,6-dihydroxyindole (DHI).

Proteomics analysis of sperm samples from two different *Mytilus* species including hybrids

A Perez Diz¹, MR Romero¹, WJ Swanson², DOF Skibinski³

¹Department of Biochemistry, Genetics and Immunology, University of Vigo, Vigo, SPAIN

²Department of Genome Sciences, School of Medicine, University of Washington, Seattle, USA

³Institute of Life Science, Swansea University Medical School, Swansea University, Swansea, UK

The study of the mechanisms that lead to the formation of new species is of special interest in marine ecosystems due to the lack of obvious barriers to gene flow. Mussels of the genus *Mytilus* are marine organisms with external fertilization able to hybridize where the distribution of two species overlap, allowing the study of reproductive isolation mechanisms in a natural scenario. Because the formation of hybrids is so frequent between *Mytilus* spp., it is likely that different types of reproductive barriers might be playing a role to preserve the genome integrity of each species, though the relative contribution and underlying molecular mechanisms of each are poorly known at present. Choosing gametes as a direct research target is key in order to investigate about specific adaptations that, for example, enhance gamete performance and fertilization success during intraspecific rather than interspecific crosses, hence also helpful to elucidate the molecular mechanisms underlying reproductive isolation. Comparative results from ongoing shotgun proteomics analysis on sperm samples from pure *M. galloprovincialis* and *M. edulis* mussels, including a few wild viable F1-hybrids, will be presented and discussed from an evolutionary ecology viewpoint.

Immunostimulating *Mytilus galloprovincialis* hemocytes: description of transcriptomic, mirnomic and functional capacities

R Moreira, A Romero, M Rey-Campos, B Novoa, A Figueras

Institute of Marine Research (IIM), National Research Council (CSIC), Eduardo Cabello 6, 36208, Vigo, Spain

Mediterranean mussels (*Mytilus galloprovincialis*), as all invertebrates, do not possess adaptive immune response. However, the high resilience of mussels to biotic and abiotic stress is one of the reasons why this species is so interesting to study processes such as immune response. In this work, we sequenced the hemocytes transcriptome and miRnome to study the molecular basis behind this innate immune response. We stimulated the hemocytes with Poly I:C, β -glucans, and LPS to study the hemocytes response to these PAMPs.

An immune transcriptome comprising 219,765 contigs was obtained after the assembly of all the available samples and around 20% of these contigs were identified. After discarding miRNAs with very low expression, a total of 1,550 miRNAs were identified. The expression analyses showed

interesting and opposite results in the transcriptome and miRnome: LPS was the stimulus which triggered the transcriptomic response with 648 differentially expressed genes (DEGs), meanwhile the miRnomic response was led by poly I:C, which triggered the expression of 240 miRNAs.

To further study the sequencing results we performed functional approaches. Viability assays showed that the treatment with these PAMPs did not cause changes in hemocyte count 24hours after the treatment. In the short term, LPS and poly I:C increased phagocytosis and glucans were able to trigger the ROS production. In the long term, the NO production was increased after poly I:C stimulation. PAMPs also affected the hemocytes morphology and mobile behavior: Cells treated with poly I:C showed rounded morphology with condensed cytoplasm and increased their velocity over 20% when compared to controls.

The dark matter of the mussel's genome: long non-coding RNAs as key players in *Mytilus*

C Gallardo-Escárate¹, A Figueras², B Novoa²

¹Interdisciplinary Center for Aquaculture Research (INCAR), Universidad de Concepción, Concepción, Chile

²Instituto de Investigaciones Marinas (IIM), CSIC, Vigo, Spain.

The complexity of the genomes in marine organisms and their functional information have been defined for only a small proportion of them. Here, close to ~1% of the genome is transcribed into protein-coding (mRNA) and non-protein-coding (ncRNA), where DNA elements controlling the gene expression involves ~0.5%. These facts suggest that the genome in marine species is dominated by a "dark matter," which is mostly nonfunctional. However, this portion of the DNA is pivotal for the evolutionary process and greatly exploited in bivalves such as mussel to face with the continuously changing marine environment. This study aimed to explore the role of ncRNAs in *Mytilus*, reviewing the functional implications of long non-coding (lncRNAs) in the immune response of mussels exposed to pathogens and HABS, and how ncRNAs are modulated in individuals exposed to contrasting environments and also to marine pollution such as microplastics. Interestingly, the analysis of lncRNAs revealed that these transcripts are involved in relevant biological processes such as immune system and local adaptation in *Mytilus*. Herein, a comparative transcriptome analysis was conducted between *M. galloprovincialis* and *M. chilensis*. Our results provide the first identification of lncRNAs in *Mytilus* and evidence that lncRNAs are key players in the biology of *Mytilus*.

Distribution and features of DUI in Mytilidae

A Burzynski, B Smietanka, M Lubosny

Department of Genetics and Marine Biotechnology, Institute of Oceanology PAN, Sopot, Poland

Double Uniparental Inheritance (DUI) of mitochondrial DNA was first discovered in *Mytilus*

mussels. Ongoing debate on the origin and age of this phenomenon is hindered by the lack of firm knowledge about its taxonomic span. Here we will present mitogenomic data covering the whole *Mytilidae* family, revealing a complex pattern of DUI – related features.

Session 2. The *Mytilus* species complex in a changing environment: from the past to the future

Chairpersons: Cynthia Riginos, Nicolas Bierne

Mussel melting-pots: hybridization and admixture in the *Mytilus edulis* complex of species

N Bierne

Institut des Sciences de l'Evolution de Montpellier, University of Montpellier-CNRS-EPHE-IRD, Montpellier, France

The *Mytilus edulis* complex of species has become a flagship system for the study of hybridization and genetic admixture in the sea. We have studied natural zones of contact between species of the complex and have demonstrated extensive introgression through semi-permeable species barriers, reconstructed the history of divergence and secondary gene flow, and started to understand the isolation factors at play to maintain the integrity of species genomes. Extensive sampling and surveys of new previously unstudied areas have recently allowed to evidence new zones of contact between native and non-native lineages introduced via human activities (mainly maritime traffic). I will present and compare the patterns of admixture observed in natural hybrid zones, anthropogenic hybrid zones and lab crosses experiments. This comparison of independent admixture events revealed parallel patterns of admixture that suggest a highly polygenic determinism of reproductive isolation in *Mytilus* mussels. Anthropogenic hybridization reshuffles genetic backgrounds which might open new evolutionary routes for invasive populations.

Twin introductions by divergent *Mytilus galloprovincialis* lineages are associated with high levels of genetic admixture with a native congener in Australia

I Popovic¹, A Matias¹, N Bierne², C Riginos¹

¹*School of Biological Sciences, University of Queensland, St Lucia, Queensland 4072, Australia*

²*Institut des Sciences de l'Evolution UMR 5554, Université de Montpellier, CNRS-IRD-EPHEUM, France*

Introduced species can impose profound impacts on the evolution of receiving communities with which they interact. If native and introduced taxa remain reproductively semi-isolated, human-mediated secondary contact may promote genetic exchange across hybrid zones, potentially impacting native genetic diversity and invasive species spread. Here, we investigate the contributions of past and ongoing (post-introduction) gene flow

between the invasive marine mussel, *Mytilus galloprovincialis* and a morphologically indistinguishable and taxonomically contentious native Australian taxon, *Mytilus planulatus*.

Using transcriptome-wide markers, we demonstrate that two contemporary introductions of *M. galloprovincialis* into southeastern Australia originate from genetically distinct lineages from its native range in the Mediterranean Sea and Atlantic Europe. Contingent on resolving species relationships, we also provide strong evidence that both introductions are associated with high levels of recent admixture with the native genomic background. Increased resolution of genomic species relationships and demographic modelling validated that *M. planulatus* sampled in Tasmania are representative of the endemic Australian taxon but share a strong genetic affinity to northern *M. galloprovincialis*.

Demographic inferences indicated late-Pleistocene divergence times and low levels of historical gene flow between the Tasmanian endemic lineage and northern *M. galloprovincialis*, suggesting historical isolation between native and introduced taxa of at least 100,000 years prior to present day contact. Accurate species identification, however, will require the use of many genetic markers, presenting significant impediments for rapid and efficient identification of introduced and endemic populations.

The results of this study build upon previous genetic studies investigating *M. galloprovincialis* introductions and its interactions with endemic southern hemisphere lineages. Our findings also demonstrate the utility of genomic data for detangling contemporary invasive introgression from signatures left by historical gene flow and recent divergence histories in native and introduced marine taxa when species boundaries are not well-defined.

Variable patterns of hybridization and locus-specific introgression in hybrid zones of *Mytilus edulis* and *M. trossulus* assessed by the traditional set of markers

P Strelkov¹, M Skazina¹, M Katolikova^{1,2}, A Gagarina^{1,3}, A Ivanova¹, R Vainola⁴

¹*St. Petersburg State University, St. Petersburg, Russia*

²*Murmansk Marine Biological Institute, Murmansk, Russia*

³*Zoological Institute Russian Academy of Sciences, St. Petersburg, Russia*

⁴*Finnish Museum of Natural History, University of Helsinki, Helsinki, Finland*

Different hybrid zones between the same lineages can vary in extent of hybridization and locus-specific introgression. In blue mussels it was long ago demonstrated on the example of zone between *Mytilus edulis* and *M. trossulus* in the Baltic Sea, where both hybridization and introgression at some but not all the markers appeared to be extensive.

Here we (1) review literature and original data on the genotypic structure of samples from five different zones between *M. edulis* and *M. trossulus*

at historically most commonly used taxonomic molecular markers (allozymes and PCR-based Me 15-16, EFbis, ITS, MAL), (2) assess variation among zones in the extent of hybridization, (3) calculate allele frequencies in regional sympatric populations of hybridizing species and estimate the rate of introgression into their gene pools.

Our direct comparisons demonstrate that not only the Baltic zone, but all of them have individualistic features and that most popular PCR-based “diagnostic” taxonomic markers are not universally informative, in contrast to orthodox allozymes. This should affect the performance of traditional method of taxonomic identification of mussels when mussels are identified by their genotypes characterized by one or few markers from the same limited set. This approach implies that diagnostic loci are universally diagnostic while backcrossing is always limited, which is not true for most hybrid zones considered.

The research was supported by RSF (project No. 19-74-20024).

A farewell to allozymes? Reanalysis of the classical dataset on blue mussels (McDonald, Seed, Koehn 1991)

M Katolikova^{1,2}, P Strelkov^{1,3}

¹St. Petersburg State University, Universitetskaya Emb. 7/9, St. Petersburg 190034, Russia

²Murmansk Marine Biological Institute, Kola Scientific Center, Russian Academy of Sciences, Vladimirska Str. 17, Murmansk 183010, Russia

³Murmansk Arctic State University, Kapitana Egorova Str. 16, Murmansk 183038, Russia

Development of electrophoretic techniques for separating enzyme allelic variation triggered a dramatic recovery of the empirical population genetics in the 1970s. Mussels *Mytilus* were among the model objects for allozyme-based population genetic studies. These studies, firstly, played a remarkable role in the establishment of a global scientific trend to consider natural selection as a predominant cause of spatial population divergence (e.g. salinity-associated variation at Lap locus). Secondly, they were to a large part responsible for substituting this trend by historical divergence and secondary contact paradigm, postulating the existence of three historical lineages of *Mytilus*: *M. edulis*, *M. trossulus* and *M. galloprovincialis* and describing their world-wide distribution and population structuring.

Since that time, numerous genetic methods exploiting different types of molecular markers have been developed for taxonomic and population genetic purposes. A wide range of effective statistical tools has also evolved, allowing a more thorough analysis of genetic data. This helped to reveal hitherto unknown spatial genetic patterns in marine organisms, mussels in particular. Against the background of this technological and informational leap, there has been a rising skepticism concerning the reliability of allozymes as molecular markers. Direct calls to disregard findings inferred by allozymes have even been heard.

In our talk we stand up for allozymes and demonstrate their validity as neutral markers. We claim that allozyme loci can provide accurate information on taxonomy, population structuring and hybridization between ancient lineages in mussels. Noteworthy, primary indication of genetic differentiation between divergent mussel lineages was recognized using a multi-locus analysis of allozyme genes. We consider the old allozyme data against the contemporary background by reanalyzing the initial data set from one of the basic papers addressing *M. edulis*, *M. trossulus* and *M. galloprovincialis* differentiation and distribution (McDonald J., Seed R., Koehn R., 1991. Allozymes and morphometric characters of three species of *Mytilus* in the Northern and Southern hemispheres, Marine Biology, 111, 323-333, <https://doi.org/10.1007/BF01319403>). We use a model-based statistical technique implemented in STRUCTURE (Pritchard et al., 2000) to give a better resolution of this classical example of allozyme based population study and to compare the results with the findings obtained by other types of genetic markers in the last 25 years.

We are deeply grateful to Dr. John McDonald, who kindly shared with us the precious historical data. The research was supported by projects AAAA-A19-119011690138-0 by MES of Russia and 19-74-20024 by RSF.

Adaptive potential in a changing ocean: unraveling the genomic architecture of a climate stress response in blue mussels (*Mytilus edulis* and *M. trossulus*) in the Gulf of Maine

SE Kingston, P Martino, DB Carlon

Department of Biology and Schiller Coastal Studies Center, Bowdoin College, Brunswick, U.S.A.

As coastal marine environments change rapidly, sessile habitat-building benthic species populations will either adapt or face local extinctions. In an effort to assess the adaptive potential of sentinel marine calcifiers in the Gulf of Maine, we used next-generation sequencing techniques to map the response to multivariate climate stress in wild populations of blue mussels, *Mytilus edulis* and *M. trossulus*. Genotype-by-sequencing methods were employed to assay tens of thousands of loci across the genomes of individual mussels (N=655) also phenotyped for calcification rate under predicted future climate-stress (-0.3 pH units, +3 °C, and limited food availability). RNA-seq was used to estimate changes gene expression levels under the predicted future climate-stress (N=24). We leveraged genome-wide association models to infer the underlying genomic architecture of blue mussel calcification under future multivariate climate stress. The stress calcification trait is heritable ($0.19 < h < 0.41$, 95% CI) and likely associated with 2-10 genomic regions; 208 transcripts demonstrate differential expression in future climates stress conditions. Upregulation of heat shock proteins, constituents of metabolic pathways, and membrane processes (including ion binding) appear to be

important components of the stress response at the expression level. Genetic variation associated with resilience to environmental stressors associated with climate change predicted over the next 50-100 years does exist in extant blue mussel populations. Further exploration of this response at the juvenile life stage and through multiple generations of climate stress exposure will further inform predictions of population persistence in the face of a rapidly changing environment.

Analyses of distribution and long-term dynamics of sympatric mussels *Mytilus edulis* and *M. trossulus* in the White Sea using the semi-diagnostic conchological marker

V Khaitov^{1,2}, M Katolikova³, A Zaichikova⁴, P Safonov¹, T Korotkova¹, Y Marchenko¹, P Strelkov¹

¹Biological faculty, Saint-Petersburg State University, Saint-Petersburg, Russia

²Kandalaksha State Nature Reserve, Kandalaksha, Russia

³Murmansk Marine Biological Institute, Kola Scientific Center, Russian Academy of Sciences, Murmansk, Russia

⁴Biological faculty, Moscow State University, Moscow, Russia

Blue mussels *M. edulis* (Me) and *M. trossulus* (Mt) form mixed populations and hybrid zones in the North Atlantic. Dynamic of these systems are poorly understood. Mussels exhibit two discrete morphs: T-morphotype (Tm, underdevelopment nacreous layer under the ligament nympha) and E-morphotype (Em, nacreous layer is well developed). We investigated the association between morphotype and species genotype in the White Sea and revealed that mussels with Tm are mostly Mt whereas mussels with Em - Me. We further analyzed current (2013-2018) distribution of morphotypes along the Gulf of Kandalaksha and inspected old collections from the area, in particular annual samples from four monitoring sites in Kandalaksha harbor area obtained through 2002-2018.

Populations with high concentration of Tm were from areas associated with active or abandoned sea ports. Mussels with Tm were rare in collections sampled before 2000. Rapid rise in Tm frequencies in populations of Kandalaksha harbor area happened between 2002 and 2014. However, Tm frequencies experienced some decreasing therein last years.

High concentration of Tm in areas associated with sea ports is in a good agreement with the hypothesis of introduction of Mt into the White Sea by oceanic transport. Rapid expansion of Tm around Kandalaksha harbor in the very beginning of the century we relate to the massive freshwater discharge from local hydroelectric power plant in 2000, which was catastrophic for marine intertidal communities. After the extinction of intertidal mussel populations, the areas were colonized by more opportunistic Mt. Recent decline in Tm frequencies we relate to competitive exclusion of Mt by Me in

more stable hydrological situation in the area in last years.

The research was supported by RSF (project No. 19-74-20024).

Hybridisation in a future ocean: a mechanism for adaptation of mussels to multiple environmental challenges

RP Ellis¹, RD Houston², MA Urbina³, AE Todgham⁴

¹Department of Biosciences, University of Exeter, Exeter, UK

²The Roslin Institute and Royal School of Veterinary Studies, University of Edinburgh, UK

³Departamento de Zoología, Universidad de Concepción, Chile

⁴Department of Animal Sciences, University of California, Davis, USA

Climate change is one of the greatest challenges facing society in the 21st century, widely considered as the most pervasive threat to global marine biodiversity, as well as the predominant factor determining the value of goods and services that marine ecosystems will continue to provide. Nonetheless, despite an exponential increase in research addressing this topic over the past decade, our understanding of ecosystem level responses remains limited by a significant predominance of single species, short term, single stressor experiments. Adaptive capacity of longer lived, marine metazoan groups, across relevant timescales, however remains unresolved. The marine mussel complex has a unique global biogeography, offering a natural analogue for determining adaptive capacity to climate change stressors and the role hybridisation plays in genetically determining physiological tolerance to abiotic stress.

During a 3.5-year fellowship high-throughput next-generation sequencing technology is being employed to sequence 24 mussel populations from across their global range (12x EU pops.; 6x Californian pops. and 6x Chilean pops.), with populations selected according to prevailing environmental conditions that encompass three key environmental stressor gradients – namely temperature, salinity and elevated CO₂. This preliminary ascertainment of SNPs is subsequently being used to develop a high-density genotyping tool (SNP array), generating a 50K array that will enable segregation of samples based on mussel speciation, introgression and back-crossing, as well as broad scale global biogeographic population structure, and regional local adaptation. Subsequently, by employing this novel technology alongside measures of whole organism physiology and performance the overarching question ‘does hybridisation confer an advantage to multi-stressor exposures in an ecologically and commercially important bivalve species?’ will be addressed. This project will therefore develop a unique, industry-relevant, resource that will significantly advance understanding of ecological physiology and evolutionary biology.

Session 3. Mussels and mussel-associated microorganisms in the light of evolution
Chairpersons: Paola Venier, K. Mathias Wegner

Macrobial and microbial symbionts of mussels. direct and indirect effects of parasite invasions

KM Wegner¹, ME Feis^{1,2}, F Demann¹, C Buschbaum¹

¹Department of Coastal Ecology, AWI Alfred Wegener Institute for Polar and Marine Research, Waddensea Station Sylt, List, Germany

²Station Biologique Roscoff, Roscoff, France

Biological invasions often have negative impacts on native biota. This is particularly true if the invasive species is a parasite. Blue mussels *Mytilus edulis* in the North Sea were invaded as a new host of the parasitic copepod *Mytilicola intestinalis* in the 1930ies starting a new coevolutionary arms race. Here, we explore the evolution of parasite and host traits along separate fronts of the invasion and how infection with the new parasite affects host physiology directly. However, next to direct effects, this host-parasite interaction can also have profound indirect effects that feed back on mussel fitness. These include changes of gut microbiota, resistance to secondary infections but also the interactions with epibionts as well as predators indicating that indirect effects can outweigh the direct effects.

Microbiome diversity in blue mussel (*Mytilus edulis* L.) during larval development

S McMillan¹, A Desbois¹, M Crumlish¹, AD Hughes², AV Laudicella², J Taggart¹, M Bekaert¹, S Carboni¹

¹Institute of Aquaculture, University of Stirling, Stirling, Scotland, UK

²Scottish Association for Marine Science, Oban, Scotland, UK

Global demand for the common or blue mussel (*Mytilus edulis*) continues to increase. One barrier to increased production is the reliable availability of mussel spat. To this end, the SAICHatch consortium was formed to develop a pilot-scale mussel hatchery, supporting the optimization of various production traits via targeted research. This effort includes investigations on feeding regimes and the monitoring for harmful bacteria. Disease outbreaks due to bacterial pathogens are a risk in all intensified aquaculture systems (Kesarcodi-Watson et al., 2009) but especially within bivalve hatcheries. Despite being an essential primary food, marine algae are also an important source of bacteria within a hatchery system (Dubert et al., 2015). Thus, the aims of this investigation were to evaluate the effects of selected microalgae species on growth and survival of the mussel larvae and describe the evolution of the total *M. edulis* microbial community abundance and diversity during larval ontogeny and between the different microalgal feeds.

Five different algal diets (*Chlorella vulgaris*, *Cylindrotheca fusiformis*, *Monodopsis subterranean*,

Nannochloropsis oceanica, and a mix of *Chaetoceros calcitans* f. *pumilus* and *Isochrysis galbana* mix) were fed to *M. edulis* larvae, that otherwise were reared under common conditions. Samples from each dietary group were aseptically collected at 2 days post fertilization, and then weekly for 4 weeks, until animals reached settlement. Total DNA was extracted before libraries of bacterial 16S ribosomal RNA hypervariable regions V3-V4 were amplified, indexed and pooled. Sequence data was analysed at the family level to generate euclidean similarities calculated from standardised and log-transformed OTU abundances. Correlations between family relative abundance over time and between treatments were calculated using Kendall's tau method (Kendall, 1938).

Sequence data revealed that bacterial populations were not significantly different among diets, except for larvae fed with *M. subterranean* ($R^2 = 0.5328$, $p < 0.001$). However, diversity did increase significantly with time ($R^2 = 0.8965$, $p < 0.001$), suggesting microbiome development is largely endogenous indicating that microbiome composition was affected more by development than diet. To our knowledge this is the first report detailing Mussel larvae microbiome diversity during ontogeny and will further our understanding of microbe/host interactions during early development. Methods developed here can be applied in future studies to understand the role of the microbiome and what affects it.

Experimental infection of *Mytilus edulis* by two *Vibrio splendidus*-related strains: evaluation of virulence, the influence of the origin of mussels and season on their sensitivity

M Charles^{1,2}, S Trancart², E Oden², M Houssin^{1,2}

¹Normandy University, University of Caen Normandy, BOREA, CNRS-7208, IRD-207, MNHN, UPMC, UCN, Esplanade de la Paix, 14032 Caen Cedex 4, France

²LABÉO Frank Duncombe, 1 Route de Rosel, 14053 Caen Cedex 4, France

In 2014, France was affected by high and unusual mass mortality of mussels affecting several important production areas along the French coast of the Atlantic and English Channel, where production losses have reached 90 to 100 %. The phenomenon again affected farms on the west coast of France in the first quarter of 2016. The pathogenic bacteria of the genus *Vibrio* has been considered as one of the causes of these events. *Vibrios* are among the major bacterial pathogens of marine organisms and have been identified as pathogens for several bivalve molluscs that may cause high economic losses. During mussel mortality events, different *Vibrio splendidus* strains were isolated from French moribund mussels and were linked to mortality.

In this study, two strains from a *Vibrio* population assembly isolated during disease events in oysters (*Crassostrea gigas*) and mussels (*Mytilus edulis*) in 2014, and described as highly virulent for bivalvia, were used for infection assays.

Pathogenicity in the mussel, *M. edulis*, of these two *Vibrio splendidus*-related strains – *Vibrio crassostreae* 7T4_12 and *V. splendidus* 3G1_6 – were tested every month, respectively for a period of 13 and 9 months; in order to determine whether physiological and morphological conditions, impacted by the season, played a role in susceptibility to bacterial infections. Two concentrations were tested, one close to the natural concentration of *Vibrio* spp. in seawater (~10⁵ CFU/mL) and another much higher (~10⁸ CFU/mL). In addition, those experiments were conducted on mussels of the same age but from five different areas, in order to verify whether the origin of the mussels' capture could affect their sensitivity to Vibriosis.

The observed mortalities are significantly different depending on the strain injected and the tested concentrations. *Vibrio splendidus* is more virulent than *V. crassostreae* at the highest tested concentration and the tested environmental concentration has no pathogenic effect, whatever the used strain. Besides, mussels from one specific origin are significantly less sensitive to infection than others. However, the parameter that seems to be the most decisive in the sensitivity of animals to bacterial infection is the season; a criterion known to have a particular impact on the physiological and morphological conditions of the animals. The highest mortalities due to infection were observed in autumn and in spring. Reproductive status impacts host susceptibility toward infection; the highest concentration seems to cause a specific stress during the reproductive period leading to forced egg laying, promoting the fragility of mussels and their susceptibility to infections.

Nanoparticle exposure affects the microbiota composition of mussel hemolymph: possible interplay with the host immune system

M Auguste¹, A Lasa,¹ T Balbi¹, A Pallavicini², L Vezzulli¹, L Canesi¹

¹Department of Earth, Environmental and Life Sciences, University of Genoa, Genoa, Italy

²Department of Life Sciences, University of Trieste, Trieste, Italy

Bivalves host high microbial abundance and diversity, and alteration of their microbiota, in both tissues and hemolymph, in response to stressful conditions has been linked to a compromised health status and susceptibility to diseases. The hemolymph associated microbiota has been investigated in oysters in response to pathogens and to temperature stress and infection. In contrast, less information is available in other species of widely farmed bivalves such as *Mytilus* spp., that are generally not affected by mortality events, being less sensitive to changes in environmental conditions and microbial infection. However, mussel microbiota may be affected by exposure to environmental contaminants. The recent expansion of nanotechnology has led to increasing concern on the potential consequences of exposure to nanoparticles-NPs for human and environmental

health, thus attracting interest on their potential effects on mammalian gut microbiota.

In this work, data are presented on the effects of NP exposure on hemolymph microbiome of *M. galloprovincialis* evaluated by targeted high-throughput sequencing of the 16S rRNA gene (V4 hypervariable region) using Ion Torrent sequencing technology. Two model NPs were utilized: nTiO₂, one of the most widespread NP in use, and amino modified nanopolystyrene (PS-NH₂), as a model nanoplastics, that represents an emerging threat for marine ecosystems. Exposure conditions were 100 and 10 µg/L, respectively, for 96 h.

The results indicate that both nTiO₂ and PS-NH₂ induced shifts in microbiota composition of mussel hemolymph, with specific effects on abundance of different genera, depending on the NP type. The effects were associated with distinct changes in functional immune parameters, indicating an interplay between the host innate immune system and its microbiota.

These represent the first data on the effects of NP exposure on bivalve microbiome. These studies may represent the basis for better understanding how exposure to both contaminants and natural stressors may shape the interactions between the microbiota and the immune system, and the consequent impact of human activities on the health of aquatic ecosystems.

Work supported by the EU Commission H2020 project PANDORA (GA 671881).

Session 4. The weird case of transmissible cancers in mussel species

Chairpersons: K. Mathias Wegner, Nicolas Bierne

A single clonal lineage of transmissible cancer identified in two marine mussel species in South America and Europe

MA Yonemitsu¹, RM Giersch¹, M Polo-Prieto², A Simon³, F Cremonte⁴, FT Avilés⁵, N Merino-Véliz⁵, M Hammel³, EAV Burioli⁶, AF Muttray⁷, J Sherry⁸, C Reinisch⁸, SA Baldwin⁹, SP Goff^{2,10}, M Houssin^{6,11}, G Arriagada⁵, N Vázquez⁴, N Bierne³, MJ Metzger¹

¹Pacific Northwest Research Institute, Seattle, WA, 98122, USA

²Howard Hughes Medical Institute, Chevy Chase, MD, 20815, USA

³ISEM, Univ Montpellier, CNRS- EPHE-IRD, Montpellier, 34095, France

⁴Laboratorio de Parasitología (LAPA), Instituto de Biología de Organismos Marinos (IBIOMAR) (CCT CONICET - CENPAT), Puerto Madryn, U9120ACF, Argentina

⁵Instituto de Ciencias Biomedicas, Facultad de Medicina, Universidad Andres Bello, Santiago, 2531015, Chile

⁶Research and Development, LABÉO Frank Duncombe, Saint-Contest, 14280, France

⁷Environmental Resources Management, Vancouver, British Columbia, V6E 2J3, Canada

⁸Environment and Climate Change Canada, Water Science & Technology Directorate, Burlington, Ontario, L7S 1A1, Canada

⁹Chemical and Biological Engineering, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada

¹⁰Department of Microbiology and Immunology, Columbia University Medical Center, New York, NY, 10032, USA

¹¹FRE BOREA, MNHN, UPMC, UCN, CNRS-7208, IRD-207, Université de Caen Normandie, Caen, 14032, France

Transmissible cancers, in which cancer cells themselves act as an infectious agent, have been identified in Tasmanian devils, dogs, and four bivalves. We investigated disseminated neoplasia affecting geographically distant populations of two species of mussels (*M. chilensis* in South America and *Mytilus edulis* in Europe). Sequencing alleles from three loci (two nuclear and one mitochondrial) provided evidence of transmissible cancer in both species. Phylogenetic analysis of cancer-associated alleles showed that cancers in both species likely arose in a third species of mussel (*M. trossulus*), but these cancer cells are independent from the previously identified transmissible cancer in *M. trossulus* from Canada. Unexpectedly, cancers from *M. chilensis* and *M. edulis* are nearly identical, showing that the same cancer lineage affects both. Thus, a single transmissible cancer lineage has crossed into two new host species and has been transferred across the Atlantic and Pacific Oceans and between the Northern and Southern hemispheres.

Genotype typing of transmissible cancers in the *Mytilus edulis* complex of species

M Hammel^{1,2}, A Simon¹, C Arbiol¹, J-F Pépin³, JB Lamy³, A Benabdelmouna³, I Bernard⁴, A Villalba⁵, E Burioli⁶, M Houssin⁶, G Charrière², D Destoumieux-Garzon², J Welch⁷, M Metzger⁸, N Bierre¹

¹ISEM, Univ Montpellier, CNRS, EPHE, IRD, Montpellier, France

²IHPE, Univ Montpellier, CNRS, Ifremer, Univ Perpignan, Via Domitia, France

³Eureka modélisation, Brest, France

⁴Santé, Génétique, Microbiologie des Mollusques, IFREMER, La Tremblade, France

⁵Centro de Investigacións Mariñas, Consellería do Medio Rural e do Mar, Xunta de Galicia, Vilanova de Arousa, Spain

⁶LABÉO, Caen, France

⁷Department of Genetics, University of Cambridge, Downing Street, Cambridge, UK

⁸Pacific Northwest Research Institute, Seattle, USA

Cancer cells are egoist entities that derived from and escaped the immune system controls of a multicellular organism. Cancerous cell lines usually become extinct with the death of their host; however some cell lines -called transmissible cancers- acquired the potential to infect a new host from the same or even another species. We studied the population genetics of one of those newly discovered transmissible cancers which affect mussels of the *Mytilus edulis* complex of species. A transmissible cancer originating from *M. trossulus*

was discovered to affect populations of this same species in British Columbia. Moreover, European populations of two other species of the complex, *M. edulis* and *M. galloprovincialis*, have been suspected to be the host of a transmissible cancer that also originated from *M. trossulus*. Here, we used SNP genotyping to screen a sample of 2500 mussels for genetic chimerism, the hallmark of transmissible cancers. We confirmed the existence of *M. trossulus* cancer in European mussels but at low prevalence. We then analyzed the genotypes of infected individuals from each species. We observed polymorphism among neoplasias sampled in different individuals suggesting either multiple emergences or deviation from clonal propagation. The cancer genotypes proved to be intermediate between reference samples of the three species. These results suggest that cancer emerged in individuals from a hybrid or an ancestral population.

Session 5. Mussels as biological models for in vitro and in vivo toxicological testing

Chairpersons: Loriano Ballarin, Laura Canesi

Internalization, uptake routes pathways and effects of polystyrene nanoplastics in hemocytes of *Mytilus galloprovincialis* through an in vitro approach

M Sendra, A Saco-Beiroa, B Novoa, A Figueras
Institute of Marine Research (IIM), National Research Council (CSIC), Eduardo Cabello 6, 36208, Vigo, Spain

Among marine litter, plastic represent 60-80% of litter on the ocean. Plastics trigger a threat for aquatic ecosystems. Degradation and fragmentation of plastics to small fragments led to the formation of microplastics (MPs<5 mm) and nanoplastics (NPs<1µm). The most representative plastic found in the ocean and widely used is polystyrene (PS). Due to primary characteristics of NPs (such as small size, high surface area and high reactivity), PS NPs can stablish colloidal interactions with cells and therefore unwanted effects. Among marine organisms, bivalves and particularly their immune system is recognized as a target to assess nanomaterials toxicity.

Since the hypothesis that internalization and different effects in hemocytes exposed to PS NPs could be observed and that different nominal size of PS NPs could affect from distinct way to internalization routes and immune responses, several goals were proposed: i) to analyze the internalization of PS NPs with different sizes (50 nm, 100 nm and 1 µm) in granulocytes of *Mytilus galloprovincialis*, ii) to study the location of PS NPs in lysosomes as a target organelle as well as the effects in alkalisation of the lysosomal lumen, iii) to assess how the PS NPs exposure could affect to different cell processes and iv) to study the immune capacity of hemocytes infected with *Vibrio splendidus* previously exposed to different size of PS NPs.

The most internalized particles were 1 µm PS NPs when the same number of particles (1.8·10⁷

particles·mL⁻¹) was used for all NPs, however when the same concentration was used (10 mg·L⁻¹) 50 nm PS NPs were the most internalized followed by 100 nm and 1 µm NPs. When hemocytes were exposed to PS NPs differences in the uptake of the particles were observed. The main route for 50 nm NPs were caveolar and clathrin-mediated endocytosis while phagocytosis inhibition did not show significant differences. On the other hand, when hemocytes were exposed to 100 nm and 1 µm PS NPs phagocytosis pathways played a significant role in particles internalization. After internalization all PS NPs were located in lysosomes. Autophagy induction, modulation of gene expression and decrease in phagocytic capacity were also observed after PS NPs exposure.

Using marine mussels for testing the ecosafety and the efficacy of nanostructured cellulose sponges for remediation of zinc in seawater

G Liberatori¹, G Grassi¹, A Fiorati², C Faleri³, G Protano¹, C Punta², I Corsi¹

¹Department of Physical, Earth and Environmental Sciences (INSTM Local Unit), University of Siena, Siena, Italy

²Department of Chemistry, Materials, and Chemical Engineering "G. Natta" (INSTM Local Unit), Politecnico di Milano, Milano, Italy

³Department of Life Sciences, University of Siena, Siena, Italy

Despite the growing interest in using nanotechnologies for environmental remediation, potential impacts on marine ecosystem has been underestimated so far. In order to select ecofriendly and sustainable materials and avoiding unexpected toxic side effects especially on marine biota, in the present study an eco-safety design concept has been developed using marine mussels as environmental indicators.

A novel nanostructured cellulose-based sponge (CNS) has been used for validating this eco-design concept, based on their effective sorbent ability of heavy metals removal from seawater. Specimens of *Mytilus galloprovincialis* widely used as bioindicator of coastal pollution were used as model in laboratory studies, able to show clear response at both cellular and morphological levels even after acute exposure (48 h in vivo). The effects of Zn (< LC50), CNS alone and CNS-treated Zn contaminated waters (CNS t-Zn), were evaluated in mussels' haemocytes and mantle tissue. Lysosomal membrane stability (LMS) by Neutral Red Retention Time assay and micronucleus (MN) frequencies, were both significantly affected by Zn exposure (10 mg/L) while, a significant recovery was observed in mussel's haemocytes exposed to CNS t-Zn waters, which showed no differences with CNS alone. From macroscopic observations, mussels' mantle exposed to Zn resulted clearly damaged with the edge almost lost and absent/wilted siphons, fused together to build deformed and no more functional structures. Upon exposure to CNS t-Zn, they appeared distended and vigorous resembling the normal morphology as observed in controls and CNS alone exposed mussels. From histological

analysis, Zn-treatment caused a significant rupture of the mantle's columnar epithelium, the loss of epithelial cilia and a production of a copious amount of mucus associated with an increase of acidic mucosubstances respect to the neutral ones. Both neutral and acidic mucopolysaccharides of the mantle edge were still present in CNS-t Zn group as well as in controls and CNS alone. Finally, total Zn levels measured in exposure media at time 0 and after 24 hours, confirmed the absorption capacity of CNS toward Zn in seawater and the reduction of Zn bioavailability to exposed mussels.

Ecotoxicological risk assessment for the herbicide glyphosate and its degradation product AMPA: analysis of host and microbiota response in the mussel *Mytilus galloprovincialis*

M Milan¹, S Iori¹, G Dalla Rovere¹, M Smits¹, S Ferraresso¹, M Babbucci¹, MG Marin², L Masiero², J Fabrello², L Carraro¹, B Cardazzo¹, T Patarnello¹, V Matozzo², L Bargelloni¹

¹Department of Comparative Biomedicine and Food Science. University of Padova, Viale dell'Università 16, 35020 Legnaro (PD), Italy

²Department of Biology, University of Padova, Via Bassi 58/B, 35131 Padova, Italy

Glyphosate has been the most widely used herbicide worldwide over the last three decades, raising increasing concerns for its potential impacts on environmental and human health. Recent studies revealed that glyphosate occurs in soil, surface water, groundwater and drinking water, and residues can be found at all levels of the food chain, such as plants, animals, and even in humans. While research has demonstrated that glyphosate can induce a broad range of biological effects in exposed organisms, the global molecular mechanisms of toxicity remain unclear, in particular for marine species. In a recent study, gene expression analysis was used to evaluate the effects of glyphosate on the mussel *Mytilus galloprovincialis*, revealing the disruption of several key biological processes including energy metabolism and Ca²⁺ homeostasis, cell signalling, and endoplasmic reticulum stress response. Here, the impact of glyphosate and its degradation product aminomethylphosphonic acid (AMPA) is assessed for the first time in *M. galloprovincialis* following exposure for 7 and 21 days to environmentally realistic concentrations of AMPA (100 µg/L), glyphosate (100 µg/L), and a mixture of AMPA and glyphosate. Considering that glyphosate targets the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate pathway found in plants and some microorganisms, gene expression analysis (RNA-seq) was analysed in combination with the characterization of the digestive gland microbiota to assess whether glyphosate may affect bacterial symbionts of animals living in the marine environment. Overall, this study provides a novel overview on host-microbiota interactions induced by exposure to realistic concentrations of glyphosate and AMPA.

Multi-level responses of *Mytilus galloprovincialis* to environmental stressors

MG Parisi, A Giacoletti, C La Corte, G Sarà, M Cammarata

Department Distem, University of Palermo, Palermo, Italy

Maintaining homeostasis requires the proper functioning of all individual physiological processes, including the immune system network, both influenced by environmental conditions. Multiple stressors are widely known to affect marine organisms over time and space, exerting negative effects already as individual factors, further generating hard to predict synergistic and interactive effects on ecosystem functioning. No study actually links functional traits (FT) and immune mechanisms to multiple stressors. Here the effects of food concentration, temperature and hypoxia were studied on FT such as clearance rates (CR), assimilation efficiency of food (AE), respiration rates (RR) and on enzymatic scavengers of reactive oxidative species (Catalase, CAT; Superoxide dismutase, SOD; Glutathione S-Transferase, GST and Peroxidase) in the digestive gland of the bivalve *Mytilus galloprovincialis*. Mussels were exposed to three temperatures (12, 20 and 28°C) in normoxic (8 mg O₂ L⁻¹) and hypoxic (0 - 1.5 mg O₂ L⁻¹) conditions and fed with food concentrations ranging from 0.9 to 3.5 mg of chlorophyll (CHL-a) L⁻¹. All three factors significantly influenced CR, which at the maximum temperature resulted negatively correlated to CHL-a concentration. Maximum ingestion rate decreased from 12°C to 28°C, with a significant negative effect highlighted by hypoxia. Instead AE was influenced only by temperature. RRs were modulated by both temperature and food, while different oxygen concentrations did not exert a significant effect until coupled with temperature. In both anoxic and normoxic samples, CAT increases at higher temperatures and at higher food concentrations. Greater availability of food and an optimal temperature of 20°C positively modulated the process of dismutation carried out by SOD. The analysis showed as temperature, oxygen concentration and food availability had a significant effect on the GST of mussels. Peroxidase was highly significant between temperature and food and even a difference emerged from different oxygen conditions. Also in this case it is evident as food can act as a buffer to adverse environmental conditions and specimens under normoxic conditions are better able to maintain homeostasis at high temperatures. Overall, these results can be used to determine the efficacy thresholds and facilitate the interpretation of functional and immunological biomarker monitoring. This approach, applied during significant temperature increases, can be a step forward in determining an environmental evaluation criterion in the population of coastal marine mussels.

Contribution to the study of molecular effects of copper on *Mytilus galloprovincialis* during reproductive period

M Piscopo¹, G Lettieri¹, V Mollo¹, A Ambrosino¹, F Caccavale^{1,2}, J Troisi³, F Febbraio⁴

¹Department of Biology, University of Naples Federico II, Naples, Italy

²Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Napoli, Italy

³Theoreo srl-spin-off, Company of the University of Salerno, Salerno, Italy

⁴Institute of Protein Biochemistry, National Research Council of Italy, Naples, Italy

Successful reproduction is a determining factor for the survival of species. In this century, major risks affecting reproductive health are for those species that fertilize externally, such as several marine invertebrates, since several marine ecosystems are polluted by different types of xenobiotics. Among the xenobiotics present in seawater, a relevant role is played by heavy metals whose release into the marine environment has increased their levels to large extents within the past few decades. Copper is one of the most interesting heavy metals, because in small quantities, it is essential, being involved in several physiological functions, such as redox reactions, oxygen transport, cellular respiration, free radical defense, neurotransmitter synthesis, and neuronal myelination, but can be toxic above certain threshold concentrations. In this study, we have evaluated the effects induced by 24 hr exposure to a subtoxic copper concentration on the reproductive system (gonads, spermatozoa, and protamine-like proteins) of *Mytilus galloprovincialis*. Protamine-like proteins represent the main basic protein component of sperm chromatin of this organism. After exposure, we found accumulation of this metal in gonads, spermatozoa, and protamine-like proteins of exposed mussels, as indicated by inductively coupled plasma-mass spectrometry analyses. Moreover, altered expression levels of mt10 and protamine-like proteins genes in spermatozoa and gonads, respectively, of exposed mussels were registered by real-time polymerase chain reaction analyses. We analyzed also the DNA binding affinity of protamine-like proteins extracted from spermatozoa of exposed mussels. The results showed a higher DNA binding affinity and a different DNA binding mode in exposed mussels. Moreover, an increased amount of NaCl was required for the release from sperm nuclei of PL-III, the main protamine-like proteins component. Interestingly, protamine-like proteins extracted from spermatozoa of exposed mussels promoted DNA oxidative damage in the presence of H₂O₂. These results demonstrate that also tolerable copper amount could affect the properties of protamine-like proteins and determine negative effects on *Mytilus*

galloprovincialis reproductive system. Finally, these analyses could be useful to develop quick and efficient chromatin-based genotoxicity tests for pollution biomonitoring programs.

***Mytilus galloprovincialis* haemocytes activity as biomarker for evaluation of methylmercury effect**

C La Corte, MG Parisi, D Parrinello, M Cammarata
Department Distem, University of Palermo, Palermo, Italy

Biomonitors offer a direct measure of metal pollution, as it affects the local ecosystem. Bioindicator organisms filter feeders, like bivalves, accumulate trace metals in their tissues and, by virtue of their wide geographic distribution, they are been used in many countries to monitor contaminants in the ecosystem.

In an attempt to identify cellular markers for revealing pollution, this study examined the effects of different concentrations of methylmercury (CH₃HgCl) on *Mytilus galloprovincialis* haemocytes activities and morphology. The CH₃HgCl concentrations we used for examining the effect on immunocytes were not toxic, as indicated by Trypan blue dead cell exclusion test.

Thus, we evaluated the effect of three organometal sub-lethal concentrations on cellular morphology and the efficacy of phagocytosis towards yeasts and bacteria, the maintenance of lysosomal membrane integrity and the ability to release cytotoxic molecules.

The results provided evidence of the CH₃HgCl immunotoxic effects on haemocyte viability and morphological changes induced by cytoskeleton alterations. Thus, a morphometric cellular parameter, such as spreading ability, was used as a complementary method. In particular, the haemocytes exposed to the xenobiotic have significantly reduced the phagocytic percentage and phagocytic index of the haemocytes towards the yeast. CH₃HgCl could also act on cytoskeletal network, as indicated by changes in haemocyte morphology and spreading capacity causing immunosuppression. With regard to this, it is known that cytoskeletal alterations lead to reduced phagocytic activity, due to the decreased ability of haemocytes to adhere to the substrate and interact with targets.

The cytotoxic activity of *M. galloprovincialis* haemocytes towards erythrocytes and the activity revealed from lysis plaque assay has not been altered by adequate concentrations of methylmercury in the medium.

The results confirm that the Mediterranean mussel *M. galloprovincialis*, which lives in an anthropogenically influenced environment, is a suitable model organism for environmental assessment of the quality in natural ecosystems.

Session 6. Advancing tools and strategies
Chairpersons: Stefano Carboni, Paola Venier

Effect of temperature during broodstock conditioning in blue mussel (*Mytilus edulis* L.): a lipidomics perspective

VA Laudicella¹, S Carboni², MK Doherty³, PD Whitfield³, AD Hughes¹

¹Scottish Association for Marine Sciences, Oban, UK

²Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling, UK

³Division of Biomedical Sciences, University of the Highlands and Islands, Inverness, UK

Blue mussels (*Mytilus edulis* L.) account for over a quarter of total bivalve produced by European aquaculture. The reliance of the industry on natural spatfall is one of the principal bottlenecks for mussel production in Europe, as these are highly unpredictable and fluctuate between years. A possibility to overcome such limitation is constituted by the development of industrial mussel hatchery technology, which should provide a continuous input of high-quality juveniles all year round.

Conditioning adults' gonads is a fundamental practice in hatcheries, as it allows synchronous spawning and high-quality gametes production. Temperature is between the main factors influencing the gonad development in mollusks; The rearing of adults at higher temperatures triggers gonad development and spawning in several bivalve species and enhances, at the same time, their metabolism and filtration rates. Temperature has a major effect on bivalve lipid compartment; in fact, as poikilothermic organisms, mussels maintain constant the fluidity of membranes by modifying the sterol content and unsaturation degree of their cellular membranes.

In this study, we tested two different conditioning programs on commercial size *M. edulis* adults: a single stage conditioning (SSC) and a cold and hold (C&H) treatment. The conditioning lasted for 80 days with two sampling points (40 and 80 days). The effects of the two conditioning programs were evaluated on the gonad ultrastructure, via histological examination of gonads, and on gonad lipidome, through untargeted lipidomics analysis of the gonads. Lipidomics analyses were performed with a high-resolution LC-MS platform both in positive and negative ionization modes.

The results suggest that SSC enhanced the nutritional requirements of mussels, due to higher metabolic cost, inducing gonad resorption and atresia. At the same time, the two treatments had a profound effect on the mussels' gonad lipidome. Mussels evidenced shifts in gonad membrane lipids after 40 days of conditioning. Such changes were more severe by the end of the trial and included the alteration of triglycerides (TG) composition in female gonads.

The results of this study suggest the importance of a correct broodstock conditioning process. Keeping mussels at higher temperatures resulted deleterious in term of fecundity. Moreover, prolonged exposure to high temperature modified the TG composition in female gonad. TG contained in female gonads are energy source for the offspring during the early development, resulting in possible downstream issues during larval development.

MytiMap: a tool for making publication quality species distribution maps for *Mytilus* mussels

C Riginos¹, J Thia¹, A Simon², P Borsa³, I Popovic¹

¹*School of Biological Sciences, The University of Queensland, St. Lucia, Australia*

²*Institut des Sciences de l'Évolution, Université de Montpellier, Montpellier, France*

³*Institut de Recherche pour le Développement, Montpellier, France*

Creating maps of mussel species distributions can be tedious and time consuming. Existing public resources of species distributions are often confounded by species misidentifications in the absence of genetic validation. Moreover, judgements based on personal (= expert?) knowledge and artistic license make the generative process opaque and non-reproducible. In an attempt to circumvent these issues, we present MytiMap, an R based scripting tool for automating the creation of mussel species distribution maps. Underlying MytiMap is a growing database of species records based on compilations of results from existing genetic studies. MytiMap allows the user to specify criteria for determining species distributions such as types of molecular markers and number of genotyped individuals per location. Known hybrids zones can be added to maps or excluded. Stylistic options include geographic extent, map projections, and color assignment. MytiMap is an open source project hosted on GitHub with an R shiny interface for map creation. The intention is for continued development to be communal such that new studies can add their findings to the database. Creation of a map will produce a list of references underlying the data such that end-users can cite data contributors (thus incentivizing data contribution).

Estrogens in bivalve revisited: lesson learnt from *Mytilus*

L Canesi¹, A Miglioli¹, C Ciacci², T Balbi¹

¹*Department of Earth, Environment and Life sciences-DISTAV, University of Genoa*

²*Department of Biomolecular Sciences -DISB, University of Urbino "Carlo Bo", Italy*

Natural vertebrate estrogens are continuously discharged into the aquatic environment, where they are ubiquitous at ng/L concentrations. In particular, estrone-E1; 17 β -estradiol-E2; and 17- α -ethinylestradiol-EE, have been included in the EU Watch List of contaminants of emerging concern

(CECs) for monitoring of surface waters (Decision 2015/495).

Bivalve molluscs have been widely utilized to investigate the effects of estrogenic compounds, one the most widespread class of Endocrine Disrupting Chemicals-EDCs. However, knowledge on endocrine regulation in bivalves has considerably increased in the last decade, with the identification of receptors/signaling pathways/effectors involved in steroid signaling, synthesis and metabolism: from these studies, a considerable debate emerged on the role of 'natural' steroids in bivalve physiology, in particular in reproduction. This is a key point to understand both the basic endocrine mechanisms and the possible impact of estrogenic chemicals in this relevant invertebrate group.

In this work, we will present available information on the effects and mechanisms of action of estrogens in the mussel *Mytilus* spp, that, widespread in coastal and estuarine ecosystems, are most likely affected by exposure to estrogenic EDCs. Recent advances in steroid uptake and metabolism, and estrogen receptors-ERs in molluscs, as well as in estrogen signaling in vertebrates, will be considered.

The results so far obtained with 17 β -estradiol and different estrogenic compounds demonstrate specific effects on immune function, development and metabolism in mussels, from cellular to organism level. Transcriptomic data obtained from the digestive gland adult mussels exposed to estrogenic compounds confirm alternative estrogen signaling pathways that are supported by new observations at the cellular level. In vitro and in vivo data show, through independent lines of evidence, that estrogens act through, non-genomic signaling pathways in mussels. In this light, regardless of whether bivalves synthesize estrogens de novo or not, and despite their ERs are not directly activated by ligand binding, estrogens can interact with multiple signaling components, leading to modulation of different physiological functions. Increasing knowledge in endocrine physiology of mussels will provide a framework for a better evaluation and interpretation of data on the impact of estrogenic EDCs in bivalves.

***Mytilus galloprovincialis* protamine-like proteins are new bactericidal molecules active also against antibiotic resistant bacteria**

M Piscopo¹, R Notariale^{1,2}, E Montana¹, G Tenore³, M Guida¹, A Basile¹

¹*Department of Biology, University of Naples Federico II, Naples, Italy*

²*Department of Biochemistry, Biophysics and General Pathology, University of Campania "Luigi Vanvitelli", Naples, Italy*

³*Department of Pharmacy, University of Naples Federico II, Naples, Italy*

The discovery of antibiotics with novel mechanisms of action is a critical issue to overcome the serious problem of growing numbers of bacteria resistant to conventional antibiotics.

Sperm nuclear basic proteins are the chromosomal proteins that are found associated

with DNA in sperm nuclei at the end of spermiogenesis. Protamine-like proteins are one of the three types of Sperm Nuclear Basic Proteins, and represent a structurally and functionally intermediate group of proteins between the histone and protamine type. Protamine-like proteins represent the major acid-soluble protein components of the mussel *Mytilus galloprovincialis* sperm chromatin and consist of the protamine-like proteins PL-II, PL-III and PL-IV. The aim of this study was to investigate the antibacterial activity of these proteins since, to date, there are reports on bactericidal activity of protamines and histones, but not on protamine like proteins. We tested the bactericidal activity of these proteins against Gram-negative bacteria: *Proteus mirabilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhmuri*, *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Escherichia coli* as well as on Gram-positive bacteria: *Enterococcus faecalis* and two different strains of *Staphylococcus aureus*. The results show that *Mytilus galloprovincialis* protamine-like proteins exhibited bactericidal activity against all bacterial strains tested with different minimum bactericidal concentration values, ranging from 15.7 to 250 µg/mL and also on the clinical isolates of the same bacterial species. Interestingly, these proteins were active against some bacterial strains tested that are resistant to conventional antibiotics. For their possible therapeutic use, we investigated the toxicity of these proteins. We found that these proteins showed very low toxicity as judged by red blood cell lysis and viability MTT assays and seem to act both at the membrane level and within the bacterial cell. Antibacterial proteins have a potential as alternative treatments to standard antibiotic therapies but oral administration would most likely result in the proteins being degraded in the digestive system. In order to analyze this aspect we generated an in vitro model of gastrointestinal digestion of PL-proteins and tested the bactericidal activity of the product obtained on a Gram-positive and a Gram-negative strain. We obtained the same results with respect to undigested protamine-like proteins on the Gram-positive bacterium. In conclusion, this work presents the first evidence obtained for *Mytilus galloprovincialis* of bactericidal activity of protamine-like-proteins.

Hemolymph extraction sites and 3d-visualization of the cardiovascular system and related structures of the blue mussel (*Mytilus edulis*)

M Eggermont¹, P Cornillie², N Nevejan¹, P Bossier¹, M Dierick³, D Adriaens⁴, P Sorgeloos¹, T Defoirdt⁵, AM Declercq^{1§}

¹Laboratory of Aquaculture and Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

²Department of Morphology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

³Department of Physics and astronomy, Ghent University, X-Ray Engineering bvba Zwijnaarde, Belgium

⁴Research Group Evolutionary Morphology of Vertebrates, Ghent University, Ghent, Belgium

⁵Center for Microbial Ecology and Technology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Bivalve hemolymph is used in a broad range of research domains such as eco(toxico)logy and immunology. However, the lack of a detailed description of hemolymph withdrawal protocols and locations (adductor muscles and heart) raises questions regarding the exact origin of the aspirated hemolymph and doesn't exclude the possibility of contamination with other body fluids which may have led to biased conclusions. A good description of the species-specific anatomy is lacking for many bivalves but is essential for a correct hemolymph withdrawal.

In this study we visualized and discussed the cardiovascular anatomy of the blue mussel (*Mytilus edulis*) and generated three-dimensional (3D) reconstructions based on micro-CT and histological images. Other structures, such as the gastrointestinal system, the muscular system and body cavities, were included as well because of their close relationship to the cardiovascular system.

Hemolymph withdrawn from the posterior adductor muscle originates from small spaces and fissures between the muscle fibers that are connected to at least one hemolymph supplying artery, more specifically the left posterior gastrointestinal artery. Hemolymph withdrawal from the heart is less straightforward. It is possible to puncture the pericard, anterior aorta and ventricle to collect a limited volume of hemolymph, however caution should be taken for contamination from the pallial cavity. Drainage of the pallial fluid prior to hemolymph extraction is therefore essential.

The different hemolymph extraction sites were clearly visualized in 3D. This study resulted simultaneously in a detailed description and visualization of the anatomy of *Mytilus edulis* useful to many research areas. Furthermore the described protocols and techniques to visualize the anatomy in 3D can easily be reproduced and adapted to other bivalve species.

More with mussels

AC Smaal^{1,2}

¹Aquaculture and Fisheries Group, Wageningen University and Research, Wageningen, The Netherlands

²HZ University of Applied Sciences, Aquaculture Team, Vlissingen, The Netherlands

Marine bivalves like mussels have been cultivated for ages and are recognised as a sustainable low food chain resource that acquires feed from natural resources in their environment. They provide a rich source for human nutrition and an associated economic value for local communities. Total mussel aquaculture production amounted 1.9 million tons in 2015 with a landing value of 3.2 billion US \$.

Besides human nutrition, the mussels provide food for birds and benthos, a habitat for a large number of species, they regulate water quality and sequester carbon and nitrogen. Mussels are used to

mitigate fish farming impacts. As eco-engineers, epibenthic bivalve beds are used for coastal defence and nature conservation. Shellfish restoration is a worldwide issue that not only aims to bring back the bivalves but also restore the facilitation functions of bivalve beds. They produce significant amounts of shell material that has many applications. The mussels also provide cultural services through shellfish gardening as a community issue. These functions can be defined as ecological goods and services. This concept provides a framework for description and analysis of the role of bivalves in the ecosystem, and a basis for addressing a wide range of topics, benefits and controversies related to the use of bivalves for production, habitat restoration, water quality and coastal management.

Understanding goods and services may improve management decisions. A way to improve decision making is to valorise the goods and services. The economic revenues of aquaculture are based on the market value of harvestable products, while the economic values of ecosystem services are often not adequately quantified and not fully captured in commercial markets. Both for market and political decisions, techniques are needed that can be used to make economic values of mollusc ecosystem services explicit, provided full knowledge of the goods and services is available. A comprehensive analysis of their ecosystem functions is needed to better make use of the good and services. In the presentation, the state of the art in multiple use of mussels will be reviewed on the basis of a number of case studies.

Poster presentations

Mussel aquaculture in China

C-M Bai^{1,2}, L-S Xin^{1,2}, C Li^{1,2}, C-M Wang^{1,2}

¹Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, China

²Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

Although mussel aquaculture existed since 1950s in China, large-scale commercial aquaculture initiated in the early 1970s and expanded rapidly in the early 1980s due to the development of breeding, seed collection and longline culture techniques. The mussel production has accounted for near 30% of the total mollusk production in late 1980s. In early 1990s, the development of the mussel aquaculture industry began to slow down, because its' space was occupied by the relatively high value oyster and scallop aquaculture. And the proportion of mussel production in molluscs dropped to about 6% in late 1990s. During the last two decades, the annual production of both mussels and molluscs increased steadily in China, while the proportion of mussel production in molluscs was always around 6%, and mussels were always the fourth most cultivated marine bivalves following oysters, clams and scallops. In 2017, the total mussel production from

aquaculture amounted about 0.93 million tons in China, accounting for 53% of global mussel production. More than 50 species belonging to 18 genus of the family Mytilidae distributed naturally along the coastline of China. In northern and southern China, the primary species of mussels cultured are the blue mussel (*Mytilus edulis*) and the green mussel (*Perna viridis*) respectively. In the middle of China, the thick shell mussel (*Mytilus coruscus*) is the primary species farmed. Depending on the abundance of mussel spat in nature and the market value of specific mussel species, mussel seed are either collected from the wild or produced in hatcheries accordingly. For the thick shell mussel, all spats come from hatchery broodstock now. Mussels are cultured by three basic methods in China: rope culture, lantern net culture, and bottom culture. Rope culture is a more widely accepted method for mussel aquaculture due to its high productivity and relatively low cost. The grow-out period may take 6 -12 months after which mussels can attain the market size (8-10 cm). Mussel is relatively resistant to disease compared to its' counterparts, and the loss which mussels might encounter during the grow-out period is attributed mainly to bad weather (e.g. typhoon), predators and bloom of harmful plankton. It is also worth to mention that mussels are viewed as kind of fouling organisms in many aquaculture areas of China, because the large numbers of juvenile mussels produced by wild populations can foul the culture facilities of other species. How to control the fouling of mussels is still a challenge for many farmers and scientists in China.

Preliminary data on immune priming in the mediterranean mussel *Mytilus galloprovincialis*

L Ballarin, V Matozzo

Department of Biology, University of Padova, Padova, Italy

Molluscs, like all the other invertebrate, rely only on innate immunity for their defence. The latter has been traditionally associated with low specificity and lack of immune memory. However, in the last decade, the presence of short-term immune memory, referred to as "immune priming", was revealed in representatives of various invertebrate phyla.

In the present work, we studied the response of the mussel *Mytilus galloprovincialis* to single or double exposure (second one after 7 days from the first) to the gram-positive bacterium *Bacillus clausii*. One day after the 1st or 2nd exposure, the digestive gland and the gills were collected from exposed and unexposed animals (controls) frozen in liquid nitrogen and stored at -20°C. Haemolymph was also collected from the adductor muscle and haemocytes were obtained by centrifugation and resuspended in filtered seawater (FSW).

The following parameters were measured: superoxide dismutase (SOD) and catalase (CAT) activities on tissues; total haemocyte count (THC), haemocyte volume (HV), haemocyte diameter (HD) and the percentage of phagocytosing cells on haemocytes.

No variations in SOD and CAT activities were observed in exposed animals with respect to their control after a single exposure, whereas a significant decrease in CAT activity was observed after the 2nd exposure. No significant differences in the THC and phagocytosing activity were observed whereas significant increases in HV and HD were observed after the exposures without any significant variations between the two exposures.

Collectively, these data represent a first attempt to study immune priming in *M. galloprovincialis*. Further studies are required using a more appropriate stimulus (*B. clausii* is not a natural pathogen for *M. galloprovincialis*) and changing the interval between the 1st and the 2nd exposure.

Risk factors analysis for cadmium contamination in *Mytilus galloprovincialis* harvesting areas of marche region (Italy) for the purposes of chemical sanitary survey

EM Epifanio¹, F Agnetti¹, F Barchiesi², M Latini¹

¹Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", Terni, Italy

²Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", Ancona, Italy

Cadmium (Cd) is the heavy metal most presents in the shellfish (Jernelöv, 2013). Mussels are one of natural accumulators of cadmium from the environment (EFSA, 2009). This happens because metals are accumulated by these filter feeding organisms through water, ingestion of suspended sediments or food (Butler & Timperley, 1996). Sources of Cd into the environment come from human activity but also from natural reservoir. Commission Regulation (EC) 1881/2006 sets maximum levels of Cd in edible bivalve mollusc (wet weight) at < 1.0 mg/kg.

According to Regulation (EC) 854/2004, bivalve molluscs can be harvested and commercialized only from classified areas. The classification is based on *Escherichia coli* contamination of bivalve molluscs. The classification can be correctly established only through a complete and preliminary sanitary survey. The sanitary survey is a procedure that studies the sources of fecal pollution and the environment characteristics of an area used for the bivalve mollusc production. To ensure public health shellfish harvesting areas have to be periodically sampled and monitored. Frequency of sampling and geographical distribution of sampling point are chosen by Competent Authority but, unlike during fecal bacteria monitoring, the plan of a sanitary survey is not a legally requirement for chemical contaminants. Therefore, a correct monitoring for chemical contaminants could be very difficult and expensive for public resources. This study applies the concept of sanitary survey on cadmium control on *Mytilus galloprovincialis* harvesting areas. It was discovered the most representative areas to control the Cd contamination in mussels and created a model that can help to build a better monitoring program following specific risk that is the base for

any type of survey (Shi et al, 2016). The study was applied in the Marche region (Italy) mussels harvesting areas. Six main risk factors for Cd contamination in the environment were studied: type of industrial activity, presence of ports, agriculture land, rainfall, presence of rivers, sea sediments. These factors were compared with the historical Cd contamination in mussels in a ten years period (2009-2018). Five out of six factors (type of industrial activity, presence of ports, agriculture land, rainfall, presence of rivers) were confirmed as risk factors for Cd contamination in mussels. The greater the presence of risk factors the greater the quantity of Cd found in mussels. The health surveillance also for Cd pollution can therefore be used in order to optimize time and locations of sampling for monitoring.

Characterisation of the intracellular protozoan mpx in mussels, *Mytilus edulis* Linnaeus, 1758

G Fichi, S Carboni, JE Bron, J Ireland, MJ Leaver, G Paladini

Institute of Aquaculture, University of Stirling, Stirling, UK

Ciliates have been reported as pathogens of many species of economically important bivalves. Mussel protozoan X (MPX), is an uncharacterised intracellular ciliate of mussels and has been widely reported in *Mytilus spp.* around the world. In order to characterise this ciliate, *Mytilus edulis* samples were collected from a site on the West coast of Scotland, and four different fixatives for histological examination were tested. Fresh preparations of mussel digestive glands were also examined by laser scanning confocal microscopy. Intracellular ciliates were prepared by laser capture microdissection and partial sequences of small subunit ribosomal RNA gene and of large subunit ribosomal RNA gene were generated, using *Phyllopharyngea* primers. Methacarn solution proved to be the best fixative for both histological and molecular characterization. The morphological and molecular investigations confirmed that this ciliate belongs to the class *Phyllopharyngea*, order *Rhynchodida*. However, this organism does not belong to any known family, genus or species, therefore, a new description is necessary, following further morphological analyses. Most mussel samples containing MPX displayed mild to moderate infections, with no signs of necrosis or haemocytic response, although a single sample displayed a severe infection (~103 ciliates per section). The localization of this ciliate in tissues other than the digestive gland, the presence of necrosis in infected tissue of the most severely infected mussel and the binary fission of this ciliate have been observed here for the first time. We also report the first observation of the live ciliate isolated from tissue. Although MPX remains of unknown significance to the mussel industry, tools and protocols described will be useful in further characterizing these and other ciliates (subclass *Rhynchodida*) known as pathogens for bivalves.

NMR-based metabolite profiles in *Mytilus galloprovincialis*: experimental set-up and preliminary data

R Frizzo¹, T Riello¹, E Schievano¹, S Mammi¹, P Venier²

¹Department of Chemical Sciences, University of Padova, Padova, Italy

²Department of Biology, University of Padova, Padova, Italy

Metabolomics studies founded on mass spectrometry (MS) or nuclear magnetic resonance (NMR) have rapidly expanded in the last decade. This and other current 'omics (genomics, transcriptomics, proteomics) complement each other and contribute to a holistic view of organism responses to stimuli. In fact, the profiles of all small molecules detectable in biological fluids or tissues capture a further phenotype level, that of condition-dependent and time-dependent metabolome changes.

We have evaluated different protocols in order to process the whole hemolymph, its acellular fraction and other soft tissues of the Mediterranean mussel *Mytilus galloprovincialis*, relying on available metabolite databases and suitable software for the identification and representation of mussel metabolite profiles.

Mussels maintained in standard conditions or kept in air at 4 °C were used in the procedural set-up. The NMR profiles resulting from the analyzed tissue matrices allowed the identification of amino acids, fatty acids, organic acids and typical osmolytes. These profiles represent the starting point for hypothesis-driven basic and applied research, such as the quality assessment of marketable stocks.

Spat settlement and growth of *Mytilus galloprovincialis* on the ropes in Gökçeada island

I Keskin, A Ekici

Department of Aquaculture, Faculty of Aquatic Sciences, University of Istanbul, Istanbul, Turkey

Rope collectors were used as a settlement material for black mussels in Gökçeada-Kaleköy, Settlement time, quantity, growth rates of black mussels and the occurrence of other fouling organisms were evaluated monthly from June 2017 to November 2018. Average values of chlorophyll-a $0.32 \pm 0.31 \mu\text{gL}^{-1}$, water temperature $19.9 \pm 4.93 \text{ }^\circ\text{C}$, salinity $35 \pm 2.07\text{‰}$, pH 8.12 ± 0.04 and total particulate matter (TPM) $14.95 \pm 10.48 \text{ mgL}^{-1}$ were recorded in the sea water. Mussel spat started to be observed in the rope collectors after the first six months. The spat length was $3.23 \pm 1.17 \text{ mm}$ in March 2018 (N=100) and $31.79 \pm 6.20 \text{ mm}$ in October 2018. The growth of black mussels on the rope collectors was mostly influenced by the temperature ($p < 0.01$). Significant differences in growth

rates were detected in all months, except April and May when mussel spat were detected in abundance ($p < 0.05$). Polychaeta, barnacles, tunicates and *Musculus costulatus* bivalves were also detected in the rope collectors. These results strongly indicate that black mussel farming could be carried out commercially in the region. The spat settlement time was March in the aquaculture operations while large amounts of spat settled on the rope collectors in the following April and May, reaching 3.1 cm length size in a period of eight months. Also, rope collectors are suitable for monitoring the growth of both mussels and fouling species.

Application of monoclonal antibodies in *Mytilus galloprovincialis* hemocytes subpopulation sorting

M Mendoza^{1,2}, S Magadán^{1,2,3}, C Canchaya^{1,2}

¹Department of Biochemistry, Genetics and Immunology. School of Biology, University of Vigo. Vigo, Spain

²Biomedical Research Centre. University of Vigo. Vigo, Spain

³Center for Evolutionary and Theoretical Immunology. University of New Mexico, Albuquerque, United States

All metazoans have evolved in a constant "arms race" with their pathogens, developing specific cell-types to detect and neutralise them. The mussel *Mytilus galloprovincialis*, as well as other molluscs, presents only innate immune system: humoral and cellular mediated response. Carballal *et al.* described two major types of cellular-subtypes in *M. galloprovincialis* hemolymph: basic and acidophilic granulocytes, and hyalinocytes, both similar to other mussel species. Afterwards, García-García *et al.* and Andreyeva *et al.* characterised these populations' functions and responses. These cell populations, which have specific roles in the immune cellular mediated response, have not yet been characterised at molecular level. To characterise them, we need first to separate each population individually. For this purpose, we are improving the isolation of each cell-type using fluorescence activated cell sorting. Because there is not yet any single specific cell-surface antibody described for *M. galloprovincialis* hemocytes, we tested anti-human or mice monoclonal antibodies (MoAbs). These antibodies have been previously used to characterise bivalves hemocytes by flow cytometry (e.g. anti-CD25 and anti-CD34 and other MoAbs suitable to identify human myeloid or lymphoid cells). However, the staining pattern produced by most of them suggested a non-specific reactivity. This led us to make an *in silico* search in *M. galloprovincialis* transcriptomes of homologous sequences to surface markers or CDs useful for the identification of leukocytes. Finally, potential useful sequence candidates were identified to characterize mussel hemocytes.

Halobacteriovorax isolated from marine water of the Adriatic Sea to challenge *V. parahaemolyticus* in *Mytilus galloprovincialis*

D Ottaviani¹, G Angelico¹, S Pieralisi¹, E Rocchegiani¹, M Latini¹, F Leoni¹, F Mosca², PG Tiscar²

¹*Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", Ancona*

²*Facoltà di Medicina Veterinaria, Università degli Studi di Teramo, Teramo*

The public impact of pathogenic vibrios in bivalves is relevant and current decontamination processes are not always effective. The aim of this work was to test predation efficiency "in vitro" of a *Halobacteriovorax* strain isolated from marine water of the Adriatic Sea, named HBXCO1, towards 17 *Vibrio* and 7 non-*Vibrio* strains. Moreover, to test "in vivo", at the laboratory scale, its applicability for decontamination of *Mytilus galloprovincialis* from *V. parahaemolyticus*. 16S rRNA analysis and double layer agar plating technique were used for identification and predation efficiency of HBXCO1, respectively. Mussels artificially contaminated by *V. parahaemolyticus* and HBXCO1 were subjected to depuration and analysed after 24, 48, 72 h. Mussels contaminated with target pathogen without HBXCO1 were used as control. HBXCO1 preyed all *V. parahaemolyticus*, *Vibrio cholerae* non-O1/O139 and *Vibrio vulnificus* strains, but not *V. alginolyticus* and non-*Vibrio* strains. In the depuration experiment, over the test period, we obtain in the control a progressive increase of *V. parahaemolyticus* counts while the opposite trends in the HBXCO1 treated group, with a 2 log reduction of pathogen counts. To the light of these results, we believe that HBXCO1 represents a potential candidate for development of biocontrol strategies of pathogenic vibrios in bivalves from harvesting to trade.

The study was supported by the Health Ministry financed project RC005/2016.

Genetic variability of *Mytilus galloprovincialis* (Mollusca: Bivalvia): the genetic pattern of an invasive marine species

PA Oyarzún¹, JE Toro², JJ Nuñez², JPA Gardner³

¹*Centro de Investigación Marina Quintay (CIMARQ), Universidad Andrés Bello, Quintay, Chile*

²*Instituto de Ciencias Marinas y Limnológicas (ICML), Facultad de Ciencias, Universidad Austral de Chile, Independencia 631, Valdivia, Chile*

³*Centre for Marine Environmental and Economic Research, Victoria University of Wellington, P O Box 600, Wellington 6140, New Zealand*

The genetic characteristics of introduced species have a significant impact on their ability to establish and spread. The blue mussel (*Mytilus galloprovincialis*), native to the Mediterranean coast, is a leading invasive species from the intertidal coasts of the world (i.e. America or South Africa). Here, we use mitochondrial DNA sequence data to investigate the genetic diversity and phylogeographic structure of invasive versus native

populations. We also evaluated whether genetic diversity in invasive populations could be explained by the genetic characteristics of the native sources from which they derived.

The phylogenetic analysis revealed two main lineages indicating a clear separation between the Afro-European and South American populations. On the contrary, we found no evidence of genetic structure in the invasive range in the Southern Pacific. This was probably the result of a recent arrival of *M. galloprovincialis* to the coasts of America.

We also detected the spatial mixture of both lineages in the sampling locations of the Chilean coast, giving rise to high levels of genetic diversity in some areas compared to the population of native mussels. This could lead (potentially) to a better fitness form of these individuals and thus increase the viability of the population and the invasiveness of this species. These results point to the need to better study the populations in which lineages are mixed and, if necessary, to intensify control efforts in them.

Molecular effects on spermatozoa of *Mytilus galloprovincialis* exposed to severe hyposaline conditions: a case of fertility preservation strategy due to gamete plasticity

M Piscopo, G Lettieri, M Maione, MA Ranauda, E Mele

Department of Biology, University of Naples Federico II, Naples, Italy

Climate changes are affecting species physiology, pushing environmental tolerance limits and shifting biogeographic distribution ranges of marine organisms. In addition to temperature and ocean acidification, global climate changes can also occur through changes in seawater salinity. Salinity levels may change also by anthropogenic factors such as mining activity and agricultural and industrial processes. Salinity is considered one of the most significant environmental stressors for marine bivalves. Mussels, in close proximity to coasts and in estuaries, in fact, periodically experience hypo-saline stress, particularly during intense precipitations. Many bivalve species, when experiencing water with high temperatures and low salinity, have the skill to acclimatize, or migrate to deeper water which is cooler and more saline. *Mytilus galloprovincialis*, is distributed in the north Atlantic Ocean, Mediterranean Sea and Black Sea and in recent years has invaded new places, including South Africa, Japan and California. This mussel is a common member of the intertidal estuarine and coastal areas where it can meet different salinities. *Mytilus galloprovincialis* can survive at elevated heat exposure, but it is susceptible to hyposalinity, while the native Pacific coast *M. trossulus* is more tolerant to hypo-saline conditions but vulnerable to heat stress. Since salinity represents a critical factor in reproduction of marine species, we analyzed the responses of *Mytilus galloprovincialis* spermatozoa to hyposaline stress. We exposed mussels, in laboratory tanks, for 24 hours at 18°C to control (35.9 psu) and three

hyposaline (17.1; 22.6 and 26.2 psu) conditions, and evaluated the expression of sperm *hsp70* and protamine-like proteins genes. Further we analyzed the electrophoretic pattern, the DNA binding and the release from sperm nuclei of protamine-like proteins. For all experimental approaches used, the results obtained at 17.1 psu condition were very similar to those obtained in control condition, while alterations were always recorded at 22.6 and 26.2 psu conditions. Particularly, at 22.6 and 26.2 psu, was observed: 42.5 and 17.1-fold increase in *hsp70* expression, respectively and hypo-expression of PL-II/PLIV protamine-like proteins genes. Further, electrophoretic mobility shift assays and salt-induced release of nuclear proteins from sperm nuclei, revealed alterations in the PL proteins/DNA binding, in these two hyposaline conditions. The similarity between the results obtained in control and in the more severe hyposaline condition (17.1 psu) could indicate a phenomenon of fertility preservation strategy due to gamete plasticity.

Traceability of bivalves (*Mytilus galloprovincialis*) through the application of stable carbon and nitrogen isotopes

F Rampazzo¹, O Giovanardi¹, C Gion¹, M Formalewicz¹, G Romanelli¹, A Scarpato¹, F Tosi², G Arcangeli², G Franceschini¹, D Berto¹

¹*Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA). National Institute for Environmental Protection and Research, Chioggia (VE), Roma (RM)*

²*Istituto Zooprofilattico Sperimentale delle Venezie, National Reference Center for Fish, Molluscs and Crustacean Diseases, Legnaro (PD)*

The traceability of agricultural products is regulated by European Parliament Regulation n.178 / 2002, UNI EN ISO 22005: 2008 and Reg. (EU) no. 1379/2013 Chapter IV Article 35 of the European Parliament and of the Council of 11 December 2013 laying down mandatory information to be included on the label of fishery and aquaculture products. Traceability is therefore of great importance in terms of guaranteeing the origin of the products offered to the consumer, but also the sustainability of the fish resource for the protection of the natural heritage. Molluscs, including mussels (*Mytilus galloprovincialis*), have a chemical-nutritional composition that undergoes changes during the year, related to site, season and reproductive period.

The purpose of this paper was to explore the possibility to distinguish the origin of mussels coming from different geographic areas through the analysis of stable carbon and nitrogen isotopes ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$).

Mussel were sampled in different geographic areas during the period 2015-2017. Subsamples of ten specimen from each area were homogenized and freeze dried after separation of muscle from the shell. Isotopic analyses were performed using a Delta V advantage, coupled with an elemental analyzer CHN Flash 2000 (Thermo Fisher Scientific), using glutamic acid and sucrose as standard reference material. Analytical precision was 0.2%.

$\delta^{13}\text{C}$ values of mussels ranged from -23.584 ‰ to -17.101 ‰ (mean value -20.951 ± 1.470 ‰), while $\delta^{15}\text{N}$ from 5.337 ‰ to 8.345 ‰ (mean value 6.627 ± 0.964 ‰). From the plot $\delta^{13}\text{C}$ vs $\delta^{15}\text{N}$ a good separation from mussel from different geographical areas was observed.

The application of IsoSource mixing model to the mussel isotopic data showed as the phytoplankton was the major bivalves carbon source (mean $56 \pm 12\%$), while the urban waste discharges (mean $18 \pm 15\%$), treated and untreated, showed higher influence in the area of Koper with a contribution of 54%. The contribution of the particulate organic matter of terrestrial origin resulted lower ($9 \pm 6\%$), probably due to the distance from the river mouths. Isotopic approach seems to be a promising tool for the traceability of bivalves. This evidence was confirmed also by the shift of the isotopic values of the mussel transplanted in a different geographical area. The isotope analysis of lightweight elements is still at the experimental stage in determining the origin of most matrices, including fish products, while for others agricultural products (wine, honey, vinegar, fruit juice; Camin et al., 2016 and the cited authors) the experimental process has already been concluded and has come to regulate the use of this technique in controls protocols.

Invasions create competitors: how novel interactions among native and invasive parasites influence coevolution with blue mussel hosts

KM Wegner¹, ME Feis^{1,2}, L Gottschalck¹

¹*Department of Coastal Ecology, AWI Alfred Wegener Institute for Polar and Marine Research, Waddensea Station Sylt, List, Germany*

²*Station Biologique Roscoff, Roscoff, France*

Within parasite communities infecting the same host, ecological theory predicts that two species occupying the same niche should evolve distinct niche use to avoid direct competition. Biological invasions can however create situations, where competition could not select for different niche occupancy and closely related parasites species find themselves competing for the same host resources for the first time since their lineages split. Such novel interactions cannot only alter the evolutionary trajectories of both parasite species, but will also feed back onto established coevolutionary interaction of native parasites with the host. Here, we show how the invasion of the parasitic copepod *Mytilicola orientalis* creates potential competition with the established congeneric parasite *Mytilicola intestinalis*, and how this novel ménage à trois feeds back on the immune response of the blue mussel host *Mytilus edulis*. From a series of controlled infection experiments that manipulate competition among the parasites we can show that, although both species occur in the same section of the mussel gut, competition between the parasites is weak and shows similar impact on host condition in either simultaneous or sequential infections. Triplet transcriptomics of matching host (*M. edulis*) and parasite samples (*M.*

intestinalis and *M. orientalis*) however revealed that the novel interaction of the invader with the host changes the transcriptional activity of many more genes and processes than the interaction with the established and coevolved parasite. Our results therefore not only show the utility of biological invasions of parasites to study coevolutionary processes, but also shows that responses to novel host-parasite interactions can lead to massive reactions on the molecular level that are not reflected in host or parasite phenotypes.

Water quality monitoring station in the bivalve purification centre of Giulianova city (Abruzzo Region, Italy): one-year results

F Di Giacinto¹, G Mascilongo¹, M Scattolini², D Pichinelli², M Bertini¹, N Ferri¹

¹*Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Teramo, Italy*

²*CIMAR s.r.l., Lungomare Spalato 17, Giulianova, Teramo, Italy*

European "hygiene package" lays down hygiene requirements to be respected for the commercialization of live bivalve molluscs. One of the first requests for the purification centres is the availability of good quality water. Apart from microbiological parameters, no specific technical references are listed in the legislation to monitor water quality in the purification system. The collaboration between "CIMAR s.r.l.", the purification and dispatch centre (IT 001 CDM CE) located at harbour of Giulianova city (Abruzzo region, Italy), and the research institute "Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM)" has been instituted to enhance management system of water quality to be adopted by the centre. A control station for the continuous monitoring of water

intended for purifying shellfish has been installed at the inlet of water circuit. Chemical/physical and biological parameters, are registered continuously. For over a year, a multiparametric probe has been recording once per hour: pH, oxidation reduction potential (ORP), temperature (T), dissolved oxygen (DO), conductivity (Cond.) and salinity (Sal.). In July 2019, it has been added biomonitoring through the biological early warning system (BEWS) "Mosselmonitor[®]", based on the behavioural responses of *Mytilus galloprovincialis* to disturbances. Electromagnetic sensors, glued on eight mussels, monitor valve movements every one minute. Abnormal behaviors are registered and sent as early warning signals linked to potential water contaminations or to any other mussel disturbances. Three types of alarms are considered: closure of shell valves for a long period (alarm C); rapid openings (alarm A); decrease in the average distance between valves (alarm D).

One-year results of chemical and physical monitoring showed the following average values from July 2018: T = 13 °C; pH = 7,82; ORP = 106 mV; Cond. = 53 mS/cm; Sal. = 34,85 ppt; DO = 95%. No sudden and unusual changes have been registered in the water, only seasonal fluctuations. During the biomonitoring period, no alarms related to water contamination have been registered. Several signals for alarm C and few alarm D were recorded, due to the temperature increasing of almost three degrees.

In the same yearly period, results of official controls and the company HACCP self-controls, were negative for all parameters foreseen in the current legislation.

Innovation is essential to make more efficient and sustainable the management of purification centre, as well as to guarantee food safety.