SOCIETÀ ITALIANA DI IMMUNOBIOLOGIA COMPARATA E DELLO SVILUPPO (SIICS)

ITALIAN ASSOCIATION OF DEVELOPMENTAL AND COMPARATIVE IMMUNOBIOLOGY

XVI MEETING

TRIESTE – FEBRUARY 18TH-20TH 2015

Veduta del Faro della Vittoria e del Castello di Miramare durante la Barcolana (http://wwwtreveneziei)

ORGANIZERS

SILVIA BATTISTELLA[1], ENRICO FERRERO[1], MARCO GERDOL[1], PIERO GIULIANINI[1], CHIARA MANFRIN[1], SIMONETTA LORENZON[2], ALBERTO PALLAVICINI[1], MARCO SCOCCHI[1]

[1] Department of Life Sciences, University of Trieste
[2] Istituto Nazionale di Oceanografia e di Geofisica Sperimentale

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PATRONAGE

UNIVERSITÀ DI TRIESTE E DIPARTIMENTO DI SCIENZE DELLA VITA

DIPARTIMENTO DI SCIENZE DELLA VITA

MEETING PROGRAM

Wednesday, February 18th

14.30-15.15 Registration

15.15-15.30 Welcome from the organizing committee

SESSION 1: MOLECULAR IMMUNOLOGY

Chairman: Prof Alberto Pallavicini (Università di Trieste)

15.30-16.00 Lecture: M Gerdol[1], P Venier[2], A Pallavicini[1]
[1]Department of Life Sciences, University of Trieste, Italy, [2]Università di Padova
Solving the jigsaw puzzle of bivalve immune response

16.00-16.15 V Torboli[1], M Gerdol[1], F Florian[1], P Venier[2], A Pallavicini[1]
[1]Department of Life Sciences, University of Trieste, Italy, [2]Università di Padova
Identification of host-pathogen interacting molecules of *Mytilus galloprovincialis* using phage-display technology

16.15-16.30 A Accorsi[1], E Ottaviani[1], E Ross[2], K Gotting[2], A Sánchez Alvarado[2,3]
[1]Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy, [2]Stowers Institute for Medical Research, Kansas City, MO, USA, [3]Howard Hughes Medical Institute, Kansas City, MO, USA
Regeneration of adult sensory tentacles and eyes in *Pomacea canaliculata*

16.30-17.00 Coffee break

17.00-17.15 U Rosani[1], L Varotto[1], M Gerdol[2], A Pallavicini[2], P Venier[1]
[1]Università di Padova, [2]Department of Life Sciences, University of Trieste, Italy
IL-17 signaling components in bivalves: comparative sequence analysis and involvement in the immune responses

17.15-17.30 C Manfrin, L Peruzza, LC Bonzi, A Pallavicini, PG Giulianini
Department of Life Sciences, University of Trieste, Italy
Are Crustacean Hyperglycemic Hormone (CHH)-like transcripts involved in immune response in decapods?

17.30-17.45 T Schorn[1], F Drago[2] , R Girardello[1], M de Eguileor[1], R Valvassori[1], J Vizioli[2], A Grimaldi[1]
[1]Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy, [2]Université Lille 1, Laboratoire de Spectrométrie de Masse Biologique Fondamentale et Appliquée, (IFR 147) Villeneuve d'Ascq, France
The homolog of Allograft inflammatory factor-1 induce macrophages migration during innate immune response in medicinal leech

17.45-18.00 N Franchi, L Ballarin
Università di Padova
New data on *B schlosseri* AMP: a transcripts analysis

18.00-18.15 AM Tolomeo[1], R Bakiu[2], SP Place[3], G Santovito[1]
[1]Department of Biology, University of Padua, [2]Department of Aquaculture and Fisheries, Agricultural University of Tirana, Albania, [3]Department of Biology, Sonoma State University, Rohnert Park, CA, USA
Characterization of peroxiredoxin in the Antarctic teleost *Trematomus bernacchii*

18.15-18.30 C Bernini[1], S Mattiucci [2], M Santoro[2], M Gerdol[3], A Pallavicini[3], D De Pascale[4], MR Coscia[4], F Buonocore[1], E Randelli[1], G Scapigliati[1]
[1]Department for Innovation in Biological, Agro-food and Forest Systems, University of Tuscia, Viterbo, Italy, [2]Department of Public Health and Infectious Diseases, University of Rome La Sapienza, Italy, [3]Department of Life Sciences, University of Trieste, Italy, [4]Institute of Protein Biochemistry, CNR, Naples, Italy
Humoral immunity in antarctic teleosts

Thursday, February 19th

SESSION 2: EVOLUTION OF THE IMMUNE SYSTEM
Chairman: Enzo Ottaviani (Università di Modena e Reggio Emilia), Loriano Ballarin (Università di Padova)

9.30-10.00 Lecture: P Macor
Department of Life Sciences, University of Trieste, Italy
Evolution of complement system

10.00-10.15 C Alimenti, A Vallesi, P Luporini
Laboratorio di Microbiologia Eucariotica, Scuola di Bioscienze e Medicina Veterinaria, Università di Camerino
The protein pheromone family of the ciliate *Euplotes petzi*, the earliest branching species in the Euplotes phylogentic tree

10.15-10.30 A Accorsi, A Bellelli, E Ottaviani, D Malagoli
Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy
Autophagic cell death in the insect cell line IPLB-LdFB: evidence in absence of known mediators

10.30-10.45 M Gerdol[1], U Rosani[2], P Venier[2], A Pallavicini[1]
[1]Department of Life Sciences, University of Trieste, Italy, [2]Università di Padova
Tracking the evolution of bivalve antimicrobial peptides

10.45-11.15 Coffee break

11.15-11.30 N Franchi, F Schiavon, L Ballarin
Department of Biology, University of Padua, Padua, Italy
New data on phagocytosis-induced apoptosis in the colonial ascidian *Botryllus schlosseri*

11.30-11.45 F Ballin, N Franchi, L Ballarin
Department of Biology, University of Padua, Padua, Italy
Expression study of molecular markers involved in staminality and differentiation in the colonial ascidians *Botryllus schlosseri*

11.45-12.00 A Vizzini, F Di Falco, D Parrinello, MA Sanfratello, C Mazzarella, N Parrinello, M Cammarata
Marine Immunobiology Laboratory, Department of Biological Chemical Pharmaceutical Science and Technology, University of Palermo, Italy
Interleukin 17 genes as mediators of inflammatory responses in *Ciona intestinalis*

12.00-12.15 A Vizzini[1], A Bonura[2], V Longo[2], M Sanfratello[1], D Parrinello[1] N Parrinello[1] and P Colombo[2]
[1]Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche, Palermo, Italy, [2]Istituto di Biomedicina ed Immunologia Molecolare "Alberto Monroy" del Consiglio Nazionale delle Ricerche, Palermo, Italy
Isolation and characterization of a LPS induced MD2-like protein in *Ciona intestinalis*

12.15-12.30 A Bonura[1], A Vizzini[2], A Longo[1], S Vlah[1], M R Melis[1], F Gervasi[3], N Parrinello[2], P Colombo[1]
[1]Istituto di Biomedicina ed Immunologia Molecolare "Alberto Monroy" del Consiglio Nazionale delle Ricerche, Palermo, Italy, [2]Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche, Palermo, Italy, [3]Unità Operativa di Ematologia, Ospedale Civico, Palermo, Italy
Characterization of a novel LPS-induced peptide from *Ciona intestinalis* with immune modulatory activities

12.30-12.45 S Giacomelli, F Albanese, R Esposito De Lucia, U Oreste, MR Coscia
Institute of Protein Biochemistry, CNR, Naples
Fish IgT: the weird case of Antarctic teleosts

12.45-14.45 Lunch

Chairman: Matteo Cammarata (Università di Palermo)

14.45-15.15 Lecture: A Tossi
Department of Life Sciences, University of Trieste, Italy

Evolution of β defensin and cathelicidin host defense peptides in primates

15.15-15.30 V Stocchi[1], N Nunez Ortiz[1], M Gerdol[2], A Pallavicini[2], E Randelli[1], F Buonocore[1]
[1]Department for Innovation in Biological, Agro-food and Forest Systems, University of Tuscia, Viterbo, Italy, [2]Department of Life Sciences, University of Trieste, Italy
Identification of teleost fish D (IgD) and T (IgT) immonuglobulins in sea bass (*Dicentrarchus labrax*) from a gills trascriptome

15.30-15.45 N Nuñez Ortiz, V Stocchi, E Randelli, F Buonocore, G Scapigliati
Department for Innovation in Biological, Agro-food and Forest Systems, University of Tuscia, Viterbo, Italy
A polyclonal antiserum for sea bass (*Dicentrarchus labrax*) IgT

15.45-16.00 S Picchietti, L Guerra, N Nunez Ortiz, F Buonocore, D Cervia and G Scapigliati
Department for Innovation in Biological, Agro-food and Forest Systems, University of Tuscia, Viterbo, Italy
Localization of IgT expressing cells in sea bass *Dicentrarchus labrax* (L.)

16.00-16.15 F Buonocore[1], N Nuñez Ortiz[1], E Randelli[1], V Stocchi[1], A Toffan[2], F Pascoli[2], S Picchietti[1], Targetfish Consortium[3], G Scapigliati[1]
[1]Dept. for Innovation in Biological, Agro-food and Forest systems, Tuscia University, Viterbo, Italy, [2]Zooprophylactic Institute of Venezie, Legnaro (PD), Italy, [3]www.targetfish.eu
Immunization of sea bass *Dicentrarchus labrax* against Nodavirus

16.15-17.15 Members Assembly

17.15-17.45 Coffee break

17.45-18.45 PRIN Assembly

20.30 Conference dinner

Friday, February 20th

SESSION 3: ENVIRONMENT AND IMMUNITY
Chairman: Piero Giulianini (Università di Trieste)

9.30-10.00 Lecture: A Manfrin, T Pretto
Istituto Zooprofilattico Sperimentale delle Venezie, Adria (Ro), Italy
Alien species and immunity

10.00-10.15 P Pagliara, M Zotti, L Carrozzo, G Mancinelli
Department of Biological and Environmental Sciences and Technologies, University of Salento, Lecce, Italy
Invasive species and their parasites: the case of the Atlantic blue crab *Callinectes sapidus* Rathbun, 1896 and its endoparasitic dynoflagellate *Hematodinium* spp. in the Mediterranean Sea.

10.15-10.30 PG Giulianini[1], P Brandmayr[2], F Cavaliere[2], A Giglio[2]
[1]Department of Life Sciences, University of Trieste, Italy, [2]Department of Biology,Ecology and Earth Science, University of Calabria, Cosenza, Italy
Is the immunocompente gender-related in *Carabus lefebvrei* (Coleoptera, Carabidae)?

10.30-10.45 S Battistella[1], A Giglio[2], A Ammendola[1], A Naccarato[3], E Simeon[1], A Tagarelli[3], PG Giulianini[1]
Department of Life Sciences, University of Trieste, Italy
The size inequality of *Apis mellifera ligustica* hypopharyngeal glands along a gradient of heavy metal pollution.

10.45-11.00 Coffee break

11.00-11.15 R Girardello[1], M de Eguileor[1], R Valvassori[1], J Vizioli[2], F Drago[2], A Grimaldi[1]
[1]Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy, [2]Université Lille 1, Laboratoire de Spectrométrie de Masse Biologique Fondamentale et Appliquée, (IFR 147), Villeneuve d'Ascq, France
AIF-1 impregnated Matrigel: an important tool to study *in vivo* and *in vitro* behaviour of the leech *Hirudo medicinalis* macrophages in response to MWCNTs treatment

11.15-11.30 L Canesi[1], E Pezzati[1], G Damonte[2], A Salis[2], F Marsano[3], C Pruzzo[1]
[1]DISTAV, University of Genoa, Italy, [2]DIMES, University of Genoa, Italy, [3]DISIT, University of Eastern Piedmont, Alessandria, Italy
Susceptibility of *Vibrio aestuarianus* 01/032 to the antibacterial activity of *Mytilus* hemolymph: identification of a serum opsonin involved in mannose-sensitive interactions

11.30-11.45 C Ciacci[2], B Canonico[2], E Bergami[1], L Canesi[3], KA Dawson[4], I Corsi[1]
[1]Dip. di Scienze Fisiche, della Terra e dell'Ambiente, Univ. di Siena, Italy, [2]Dip. di Scienze della Terra, della Vita e dell'Ambiente, Univ. degli Studi di Urbino "Carlo Bo", Italy, [3]Dip. per lo studio del Territorio e delle sue Risorse, Univ. di Genova, Italy, [4]Centre for BioNano Interactions, School of Chemistry and Chemical Biology, Univ. College Dublin, Ireland
Immunomodulation of cationic polystyrene nanoparticles in *Mytilus* hemocytes

11.45-12.00 MG Parisi[1], M Mauro[1], G Sarà[2], M Cammarata[1]
[1]Department of Biological, Chemical and Pharmaceutical Science and Technology, University of Palermo, Palermo, Italy, [2]Department of Earth and Marine Science, University of Palermo, Palermo, Italy
Immunomodulation and physiological responses of *Mytilus galloprovincialis* as bioindicators of environmental change

12.00-12.15 M Mauro[1], M G Parisi[1], G Sarà[2], M Cammarata[1]

[1]Department of Biological, Chemical and Pharmaceutical Science and Technology, University of Palermo, Palermo, Italy, [2]Department of Earth and Marine Science, University of Palermo, Palermo, Italy

Variation of environmental condition and diet act on immune parameters of *Mytilus galloprovincialis*

12.15-12.30 I. Marisa[1], V. Matozzo[1], D. Sheehan[2], M. G. Marin[1]

[1]Department of Biology, University of Padova, Italy, [2]Department of Biochemistry and Environmental Research Institute, University College Cork, Ireland

Investigation on the effects of three nanoparticles (zinc oxide, titanium dioxide, C_{60} fullerene) on haemocyte parameters of *Ruditapes philippinarum*

12.30 OSMIZZA

ABSTRACTS

Solving the jigsaw puzzle of bivalve immune response

M Gerdol[1], P Venier[2], A Pallavicini[1]

[1]Department of Life Sciences, University of Trieste

[2]Department of Biology, University of Padova

The recent developments of –omic technologies has greatly simplified the identification of the molecules involved in the invertebrate immune response, permitting to outline complex pathways linked to pathogen recognition and clearance. In particular, thanks to the long-standing availability of a complete genome and due to its ideal use in laboratory experiments, *Drosophila* can be now considered as a relatively well-known model organism for immunological studies in arthropods, the largest group of invertebrates. Comparatively, the knowledge of the imunity of Lophotrochozoa, and in particular of Mollusca (the second largest group of invertebrates) is still very limited and fragmentary.

The molecular study of filter-feeding marine bivalves has recently revealed only some key aspects of immune response in these organisms, which are constantly exposed to high loads of microorganisms, especially in coastal shallow waters and yet display a remarkable tolerance to these conditions. The strategies used by bivalves to cope with infection include, among the others, the massive expansion of pattern recognition receptors and the diversification of antimicrobial peptides. However, many open questions remain (e.g. Which are the main players in viral recognition? Which signalling pathways are activated in response to PAMP recognition? How is the expression of antimicrobial effectors regulated?), and our knowledge of the interconnection between bivalve immune pathways is still largely deficient.

Using *Mytilus galloprovincialis* as a model organism, we explored the available sequence resources and scientific literature to update our knowledge on the main gene-encoded elements of mussel immune response, from the recognition of pathogens to their elimination. We highlight the high complexity of this system, identifying and describing for the first time several molecular players whose existence had so far just been hypothesized in Lophotrochonzoa.

Identification of host-pathogen interacting molecules of *Mytilus galloprovincialis* using phage-display technology

V Torboli[1], M Gerdol[1], F Florian[1], P Venier[2], A Pallavicini[1]
[1]*Department of Life Sciences, University of Trieste*
[2]*Department of Biology, University of Padova*

Immunocompetent mollusc cells, in particular the circulating hemocytes, provide a rapid line of defence against potential pathogens. Defensive reactions are triggered by the interaction between PAMPs (pathogen associated molecular patterns) and PRRs (pattern recognition receptors). Even though an increasing number of host-pathogen interacting molecules has been characterized in *Mytilus galloprovincialis*, to date a strategy for a large-scale identification of these PRRs has never been implemented. We used phage display technology, based on the ability to express exogenous peptides as fusions to capsid proteins on the surface of bacteriophages, to identify the PRRs of mussel immunocompetent cells possibly involved in the recognition of *Vibrio splendidus* and *V. aestuarianus*. These Gram-negative bacteria are among the most commonly found pathogens in coastal waters, and mussels, compared to other aquacultered bivalves, display a remarkable tolerance to their infection. Our approach permitted us to express peptides representative of a mussel hemocyte cDNA library on the surface of phage virions, which were subsequently selected by their interaction with PAMPs present on the surface of target bacteria. The complete complement of peptides that recognize PAMPs has be thoroughly analyzed by the use of next generation sequencing methods and bioinformatic tools. The comparison between selected and control samples revealed that a *V. splendidus* is likely recognized by a broader range of PRRs compared to V. aestuarianus. Overall, we identified 42 putative *V. splendidus*-interacting proteins, comprising both putative membrane-bound and extracellular PRRs. While some of these results are consistent with literature available for mussels or other invertebrates (C-type lectins, FREPs, C1qDC proteins and apextrin-related proteins), others are completely novel. In conclusion this innovative approach identified a number of previously unknown mussel PRRs whose involvement in the innate immune response will be further characterized.

Regeneration of adult sensory tentacles and eyes in *Pomacea canaliculata*

A Accorsi[1], E Ottaviani[1], E Ross[2], K Gotting[2], A Sánchez Alvarado[2,3]

[1]*Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy*

[2]*Stowers Institute for Medical Research, Kansas City, MO, USA*

[3]*Howard Hughes Medical Institute, Kansas City, MO, USA*

Regeneration is a wide-spread phenomenon in the animal kingdom, but the level of regenerative potential is significantly different among Metazoan *phyla*. For instance, planarians are able to regenerate the entire body from a small fragment of tissue, while the amniote vertebrates display poor regenerative ability. Regeneration is a process activated when body parts are severed, damaged or amputated. Regeneration induces the re-activation of development pathways for the replacement of the missing structures and it promotes the over-expression of genes typically involved in the functioning of the immune system.

The adult freshwater gastropod *Pomacea canaliculata* (Lophotrocozoa, Mollusca) is able to regenerate *de novo* three sensory organs: the long cephalic tentacles, the short oral tentacles positioned near the mouth, and the complex camera eyes composed of cornea, lens and retina. After amputation of one of the three sensory organs, the transcriptome of the regenerating tissue was obtained after 2, 3, 6, 12 and 24 h and daily up to the end of the regeneration process, that lasted 14, 21 and 28 days, respectively. The obtained sequences were assembled using Trinity software and the transcriptome of each time point was compared with that of the original tissue.

This analysis uncovered 8.432 sequences involved in the regeneration process of the cephalic tentacle, 2.725 involved in the oral tentacle regeneration and 5.492 in eye regeneration. The temporal expression profiles of all the identified sequences during the whole regeneration process was analyzed in detail and the time points presenting comparable gene profiles were clustered together allowing the identification of 4 main phases in the regeneration process of the tentacles and the eye. For all the three sensory organs, the early phase is characterized by extensive gene down-regulation, followed by two intermediate phases with the majority of genes markedly up- or down-regulated. The late and final phase displays only a minority of genes with the expression levels significantly different from the original tissue. These studies open the door to a detailed molecular and mechanistic dissection of regeneration in a member of the understudied Lophotrochozoan super-clade.

IL-17 signaling components in bivalves: comparative sequence analysis and involvement in the immune responses

U Rosani[1], L Varotto[1], M Gerdol[2], A Pallavicini[2], P Venier[1]
[1]Department of Biology, University of Padova, Padova, Italy
[2]Department of Life Science, University of Trieste, Trieste, Italy

According to the cellular origin as well as their pleiotropic and synergic actions, the vertebrate cytokines are grouped in interleukins, chemokines and interferons. Interleukin-17 (IL17) was firstly recognized as cytolitic T-cell factor with pro-inflammatory activity and is the unique member of an interleukin class with no homology to any other known cytokine family. IL-17s are produced by activated T lymphocytes and other cell types relevant to the host immunity such as the mucosal epithelial cells. Basically, the IL-17 family members are potent pro-inflammatory cytokines involved in host defense, autoimmunity and cancer development.

The IL-17 signaling pathway starts with the binding of IL-17 homo- or hetero-dimers to specific membrane-bound IL-17R complexes. Then, intracellular signal transduction proceeds through the tumor-necrosis factor receptor-associated factor 6 (TRAF6), which is a key adaptor also in the TLR- and IL-1R-signaling cascades, up to the transcriptional activation of several cytokines, chemokines and also antimicrobial peptides. The crucial element which makes possible the interaction between IL-17Rs and TRAF6 is the SEFIR domain (SEF/IL-17R domain), located on the cytoplasmic tail of IL-17Rs and display similarity with TIR domain. In the context of cross-talking signal transduction cascades, the adaptor protein CIKS, also known as ACT1 and containing TRAF-binding motifs, is expected to activate the canonical transcription factor NF-κB and induce the expression of selected gene sets.

Until some years ago, IL17 was reported as a vertebrate-exclusive molecule, likewise its downstream pathway. In 2006, three IL-17 gene models were detected in the genome of the sea urchin *Strongylocentrotus purpuratus*. Since then, the presence of IL-17, IL17R and CIKS molecules has been reported in several non-vertebrate organisms. In mollusks, IL17 was firstly identified in *C. gigas* in 2008 and subsequently in *P. fucata*. More recently, five IL17 genes have been identified in the *C. gigas* genome.

Following deep scanning of many bivalve sequence datasets, we have identified and phylogenetically compared 52 IL17-like proteins and more than hundred IL17 receptor and adaptor molecules. Aiming to validate the *M. galloprovincialis* sequence findings, we also report expression data on five selected IL17s, two IL-17Rs and the proximate CIKSL adaptor, as measured in the haemocytes of mussels injected with a mixture of heat-killed bacteria.

Are Crustacean Hyperglycemic Hormone (CHH)-like transcripts involved in immune response in decapods?

C Manfrin, L Peruzza, LC Bonzi, A Pallavicini, PG Giulianini
Department of Life Sciences, University of Trieste, Trieste, Italy

Crustacean hyperglycemic hormone (CHH) is a pleiotropic peptide originally identified in the eyestalk X-organ/sinus gland (XO-SG) complex, the major endocrine system of decapods. It belongs to the CHH family, a superfamily of neuropeptides that controls many fundamental physiological functions such as molting, osmoregulation, modulation of glycemia, reproduction and behavioural responses, such as aggression and anxiety. With the aim of creating new autocidal methods based on neuro-endocrine disruptors for invasive populations of *Procambarus clarkii*, we silenced the Crustacean Hyperglycemic Hormone (CHH) by injecting the corresponding dsRNA. The effects of CHH silencing at the glycemic and transcriptomic level were investigated in the eyestalk, as well as the effects on mortality and moulting rates. After 20 days from the dsRNA injection, an unexpected strong hyperglycemic response, achieved after serotonin injection, was recorded in surviving individuals. Since a couple of new CHH-like transcripts (named here CHHip and CHHop) previously found in *P. clarkii*'s eyestalk transcriptome were up-regulated in eyestalks of experimental surviving crayfish, we hypothesized their possible involvement in the immune and stress responses. To preliminarily test our hypothesis, we set up an additional experiment where we analysed the expression levels of CHH and CHH-like mRNAs in eyestalk and hemocytes of *P. clarkii* injected with lipopolysaccharide (LPS). The overexpression of CHHip both in the eyestalk and hemocytes of treated individuals was recorded, along with the up-regulation of well-known immune markers in hemocytes, suggesting a role of CHHip, and thus an involvement of CHH superfamily, in the immune response.

The homolog of Allograft inflammatory factor-1 induce macrophages migration during innate immune response in medicinal leech

T Schorn[1], F Drago[2] , R Girardello[1], M de Eguileor[1], R Valvassori[1], J Vizioli[2], A Grimaldi[1]

[1]Department of Biotechnology and Life Sciences, University of Insubria, Via J. H. Dunant 3, 21100 Varese, Italy

[2] Université Lille 1, Laboratoire de Spectrométrie de Masse Biologique Fondamentale et Appliquée, (IFR 147). Bat. SN3 Cité Scientifique, 59655 Villeneuve d'Ascq, France

(AIF-1) is a 17 kDa cytokine-inducible calcium-binding protein that in vertebrates plays an important role in allografts immune response and its expression is mostly limited to the monocyte/macrophage lineage. Recently it was assumed that Allograft inflammatory factor-1 (AIF-1) was a novel molecule involved in inflammatory responses. To better clarify this aspect in the present study we investigated the expression of AIF-1 after bacterial challenge and its potential role in regulating the innate immune response in an invertebrate model, the medicinal leech (*Hirudo medicinalis*). The analysis of an EST library from *Hirudo* CNS, revealed the presence of a gene, named *Hmaif-1*/alias *Hmiba1*, showing a high homology with vertebrate *aif-1*. Immunohistochemistry using an anti-*Hm*AIF-1 polyclonal antibody showed that this protein is constitutively present in spread $CD68^+$ macrophage-like cells. A few hours after pathogen bacterial injection in the body wall, the amount of these immunopositive cells increases at the injected site, co-expressing *Hm*AIF-1 and the common leukocyte marker CD45. Moreover here we demonstrated that the recombinant protein *Hm*AIF-1 induces a massive angiogenesis and it is also a potent chemoattractant for macrophages. After r*Hm*AIF-1 stimulation, macrophage-like cells co-express the macrophage marker CD68 and the surface glycoprotein CD45, which in Vertebrates is implicated in the integrin-mediated adhesion of macrophages and plays a key role in regulating the functional responsiveness of cells to chemoattractants. We therefore hypothesized that CD45 could play a role for leech macrophage-like cells activation and migration towards the inflammation site and we examined its potential effect on *Hm*AIF-1-induced signaling.

New data on *B. schlosseri* AMP: a transcripts analysis.

N Franchi, L Ballarin
University of Padova, Dept. of Biology

The innate immune system provides an immediate response against infections in animals and plants. Endogenous antimicrobial peptides (AMPs) are essential effector molecules of this first line of defence allowing the direct killing of invading micro-organisms. Moreover AMPs have attracted increasing interest because of their potential as new antibiotics.
To date various natural peptides from all kinds of organisms and synthetic derivatives have been characterized for their potential use as novel therapeutics. However, the number of candidate peptides undergoing preclinical or clinical evaluation is still very low. Marine invertebrates are source of several AMPs and same of these have been isolated and characterized from different urochordate such as *Styela plicata* (clavanins and styelins) and *Ciona intestinalis* (Ci-MAM).
In the colonial ascidian *B. schlosseri,* exploiting the transcriptome and the genome, we have been able to identify a styelin-like AMP. We also carried out some preliminary experiments to investigate the chemical properties and the expression pattern of such gene in the presence of different PAMPs.

Characterization of peroxiredoxin in the Antarctic teleost *Trematomus bernacchii*.

AM Tolomeo[1], R Bakiu[2], SP Place[3], G Santovito[1]

[1]Department of Biology, University of Padua, Padua, Italy

[2]Department of Aquaculture and Fisheries, Agricultural University of Tirana, Tirana, Albania

[3]Department of Biology, Sonoma State University, Rohnert Park, CA, USA

Immune responses imply an increase in oxygen consumption and formation of reactive oxygen species, with a consequent risk of oxidative stress. With the aim to study the components of the antioxidant defense system in the Antarctic teleosts, we have characterized the genes codifying for peroxiredoxins (Prdxs) in the emerald rockcod *Trematomus bernacchii*. Prdxs are a family of small (22 – 27 kDa) non-selenium peroxidases that are able to reduce hydrogen peroxide, organic hydroperoxides and peroxynitrite, thus representing a class of important antioxidant enzymes that protect cells against oxidative stress. In the genome of *T. bernacchii* we have verified the presence of five of the six Prdx's isoforms so far known in vertebrates: Prdx2, Prdx3, Prdx4, Prdx5, Prdx6. For isoforms 3 and 6, we also characterized two variants (A e B). Multi-alignment analysis, performed with fish orthologous sequences, demonstrated high conservation of the amino acids involved in catalytic activity of the various Prdx's isoforms of *T. bernacchii*. However, some substitutions with polar amino acids, are characteristics of some residues close to the motifs important for the functionality of these proteins. The gene transcription of all *T. bernacchii*'s Prdxs has been measured by RT-sqPCR, in various tissues (gills, heart, liver, spleen, and skeletal muscle). The gills are the organ in which the highest levels of mRNA accumulation is present, with the exception of isoforms 3B and 6A which have a low tissue-specificity of expression. Prdx 2-Cys activity have been measured in the same organs, spleen and liver showing the highest levels. The tissue-specific differences in the mRNA and active protein accumulations are probably in relation to the physiological function characteristic of these organs. (Supported by P.N.R.A. and M.I.U.R. grants).

Humoral immunity in antartic teleosts

C Bernini, S Mattiucci [a], M Santoro[a], M Gerdol[b], A Pallavicini[b], D De Pascale[c], MR Coscia[c], F Buonocore, E Randelli, G Scapigliati

Department for Innovation in Biological, Agro-food and Forest Systems, University of Tuscia, Largo dell'Università snc, Viterbo, VT, Italy

a Department of Public Health and Infectious Diseases, University of Rome La Sapienza, Italy

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

c Institute of Protein Biochemistry, CNR, Via P. Castellino 111, Naples, Italy

The humoral immunity of antarctic teleosts is an aspect of particular interest due to their physiological adaptation to the extreme environment in which they live. The cold-blooded vertebrates evolved particular features to perform physiological functions at temperatures as low as -2°C so the immune system must defend fish from infections even in such conditions and humoral defences play a pivotal role.

First of all we analysed the head kidney transcriptome of the Antarctic fish *Trematomus bernacchii*, an important model species for environmental and immunological studies. RNA-seq data was generated using Illumina paired-end sequencing, obtaining ~7 Gbp of sequence data, which were assembled into 96,641 contigs. The sequence collection contains a relevant number of immunity-related transcripts among which, interestingly, the presence of genes relative to the Toll-like receptor 4 signalling pathway not so common in fish species.

We next examined the basal expression of different genes involved in immunity processes such as TCRγ, TCRβ, IgM, IgT, and then we focused on the kinetics of expression of the same genes in spleen and head kidney tissues after TAD-1 (*Psychrobacter* sp.) *in vivo* stimulation ($1{,}06x10^{10}$/ml in PBS) for 0 (control), 8, 24 and 72 hours. The results showed that a stimulatory effect was appreciable for immune related genes that are expected to be involved in different aspects of the immune responses.

Moreover we performed indirect ELISA assays in order to evaluate the immunoglobulin secretion after *in vivo* stimulation of *T. bernacchii* with three different antigens TAD-1 ($1.58x10^{8}$/ml), Conalbumin (1.25 mg/ml) and DNP-KLH (1 mg/ml)). After 60 days the analysis of the serum immunoglobulins suggested the presence of an immune humoral response against all these antigens (TAD-1 = 0,123 ± 0,035 ; Conalbumin = 0.212 ± 0.19 ; DNP-KLH = 0.309 ± 0.105 values of absorbance at 492 nm)

Finally, considering that there are evidences of massive infestation by parasites in icefish *Chionodraco hamatus* (differently from what observed for *T. bernacchii*); we focused on investigating by indirect ELISA assays the capability of these fishes to recognize nematodes as self or non-self.

The results taken together suggested relevant immunity-related results providing a new perspective for future immunological studies in these species.

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Evolution of complement system

P Macor
Department of Life Sciences, University of Trieste, Italy

The complement system is part of the immune system and in particular of the innate immunity. It is not adaptable and does not change over the course of an individual's lifetime. However, it can be recruited and brought into action by the adaptive immune system. The complement system is most famous for its role in immunity, orchestrating an exquisitely refined system for immune surveillance. At its core lies a cascade of proteolytic events that ultimately serve to recognize microbes, infected cells or debris and target them for elimination.

The human complement system is composed of more than 30 serum and cell surface components, and most of these components show a characteristic domain structure, enabling to trace the evolution of the genes based on their structures. Ongoing projects in both vertebrates and invertebrates revealed that most domains used by mammalian complement components are found in invertebrates. Unexpectedly, the complement system of an invertebrate shows a similar level of complexity and efficacy as that of mammals. Moreover complement components from different species demonstrated the capacity to cross-react.

Mounting evidence has shown that a number of proteins and proteolytic intermediaries in this cascade have, in themselves, other functions in the body, signalling through receptors to drive events that appear to be unrelated to immune surveillance. It seems, then, that the complement system not only functions as an immunological effector, but also has cell–cell signaling properties that are utilized by a number of non-immunological processes.

The scope of the complement system’s function is indeed much greater than we might have imagined only a few years ago.

The protein pheromone family of the ciliate *Euplotes petzi*, the earliest branching species in the *Euplotes* phylogentic tree

C Alimenti, A Vallesi, P Luporini
Laboratorio di Microbiologia Eucariotica, Scuola di Bioscienze e Medicina Veterinaria, Università di Camerino.

Self/non-self recognition in ciliates relies on signaling proteins (pheromones) synthesized in association with a genetic mating-type mechanism, that regulates the cell switching between the growth (mitotic) and sexual (mating) stages of the life cycle. In *Euplotes* species, these pheromones are freely released into the medium from where they can be purified in relative abundance. The knowledge of their molecular structures has so far been limited to four pecies, namely *E. raikovi*, *E. octocarinatus*, *E. nobilii* and *E. crassus*, that occupy varied positions in the *Euplotes* phylogenetic tree. Most research interest has now been focused on the pheromone family of *E. petzi* because of a major distinctive, phylogenetic trait of this species. Together with *E. sinicus*, *E. petzi* forms the earliest branching clade in *Euplotes* evolution.

Four structurally distinct *E. petzi* pheromones have so far been structurally characterized together with their coding genes. With respect to the other known *Euplotes* pheromones, they show smaller dimensions (only 32 amino acids vs. up to 108 in *E. octocarinatus*), a higher density of disulfide bonds (four), and a folding in which molecular districts with no regular structures equal in extension districts with regular structures represented by one extended and two single-turn alpha-helices. Considering that in the other *Euplotes* species pheromones have structures dominated by a bundle of three regular alpha-helices, the minimal dimensions and the relatively simple architecture of *E. petzi* pheromones thus indicates that the structural evolution of *Euplotes* pheromones involves a progressive increase of architectural complexity. And the finding that the *E. petzi* pheromone genes are practically half in dimensions the pheromone genes of the other *Euplotes* species reinforced this indication.

Autophagic cell death in the insect cell line IPLB-LdFB: evidence in absence of known mediators

A Accorsi, A Bellelli, E Ottaviani, D Malagoli
Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy

The larvae of holometabolous insects are privileged models for studying the programmed cell death (PCD). The IPLB-LdFB cell line has been derived from the larval fat body of the gypsy moth *Lymantria dispar*. IPLB-LdFB cells can undergo apoptosis after oxidative stress, but they also present autophagic cell death after a 2 h treatment with the F1-F0 ATP synthase inhibitor, oligomycin A. Autophagic cell death seems activated in IPLB-LdFB with an established program because the oligomycin A-treated cells can condition the culture medium making it lethal for untreated IPLB-LdFB cells.
Our observation demonstrated that conditioning time is critical for the lethality of the conditioned medium (CM), and that 1 h is enough to attribute lethal effects to the CM. In order to characterize which molecules could work as lethal signals into CM, we first tested the effects of the steroidal molting hormone, ecdysone, on the IPLB-LdFB. Unexpectedly, ecdysone did not induce neither autophagic nor other types of PCD in IPLB-LdFB cells. Proteomic analysis of CM revealed that several survival factors are significantly down-regulated in oligomycin A-treated cells, but no ligands able to promote the autophagic PCD were identified. To explore the potential role of CM proteins as pro-death signals, proteases were added to CM. The removal of proteins from CM increased its lethal effects, supporting the hypothesis that ligands other than proteins may intervene in promoting the autophagic PCD. In order to shed light on the targets of the uncharacterized pro-death signals of CM, we assessed the expression and the activity of some factors in cells incubated in CM. On the basis of the proteomic analysis, we investigated the expression of the survival molecule Imaginal Disk Growth Factor (IDGF)-like. qPCR experiments demonstrated that IDGF-like expression remains at control levels in the IPLB-LdFB cells incubated for 6, 12 or 24 h in CM. The activity of the cell-death related proteases, caspase 8, 9 and 3/7 was then analyzed through luminometric assays. The caspase activity in IPLB-LdFB cells maintained for 12 or 24 h in CM is similar to that of control cells, allowing to exclude the involvement of caspase 8, 9 and 3/7 in the autophagic PCD of IPLB-LdFB cells.
In all we conclude that oligomycin A-treated IPLB-LdFB cells quickly release in the medium one or more steroidal or glucidic factors able to induce autophagic PCD, that occur without the involvement of caspases.

Tracking the evolution of bivalve antimicrobial peptides

Marco Gerdol[1], Umberto Rosani[2], Paola Venier[2], Alberto Pallavicini[1]
[1]Department of Life Sciences, University of Trieste
[2]Department of Biology, University of Padova

Antimicrobial peptides (AMPs) are key components of the invertebrate immune system, acting as a first line of defense against invading pathogens. These highly diversified peptides are usually classified according to their amino acid sequence, structure and mode of action, but due to their rapid evolution driven by the host/pathogen interplay, the relationship between different families in distantly related phylogenetic classes are often still obscure.
Almost all the AMPs so far characterized in bivalve mollusks are cysteine-rich, and their disulfide pattern is generally used as the main criteria for their classification within one of the currently known families. Nevertheless, to date, a clear and unambiguous scheme of classification for these AMPs is still missing, leading to potential problems in the correct identification of novel peptides, especially in the next generation sequencing era. Furthermore, due to the limited genetic and genomic knowledge of these organisms, a comprehensive view of the evolution and the spread of different AMP families across highly diversified bivalve species is still completely missing.
Here, taking advantage of a genomic and transcriptomic data-mining approach, we highlight that mytilins, myticins and mytimycins are AMP families which are present in a very narrow taxonomical range of bivalves, which only include the order Mytiloida. The analysis of novel sequences revealed by NGS approaches as well as conserved genomic features permit to investigate with an unprecedented depth the relationship among these AMP families and their possible evolution from ancestral defensin-like genes.

New data on phagocytosis-induced apoptosis in the colonial ascidian *Botryllus schlosseri*

N Franchi, F Schiavon, L Ballarin
Department of Biology, University of Padua, Padua, Italy

Colonies of the ascidian *Botryllus schlosseri* undergo cyclical generation change or take-over (TO) during which diffuse apoptosis occurs in zooid tissues, as indicated by morphological, biochemical and molecular investigations. Tissues are rapidly infiltrated by circulating phagocytes, selectively recruited by dying cells, which recognize and greedily ingest them. Previous observations led us to suggest that phagocytes, once ingested apoptotic cells or corpses, undergo phagocytosis-induced apoptosis (PIA). We already demonstrated, by Western blot and immunocytochemical analyses, the release of cytochrome c by haemocytes and the expression of apoptosis-related molecules, such as BAX and caspases, in phagocytes during the TO (Cima et al., 2010). In order to corroborate the above assumption, we carried out new morphological analyses at the transmission electron microscope (TEM) and started a molecular analysis of apoptosis in *Botryllus* phagocytes looking for transcripts differentially expressed in phagocytes at TO. We identified and characterized transcript sequences for BAX, AIF and PARP (poly ADP ribose polymerase), and studied their expression and the location of the corresponding mRNA in haemocytes. The collected data clearly indicate the diffuse occurrence of PIA among phagocytes which guarantee the disposal of apoptotic cells or corpses. In addition, they extend the classical view of PIA, intended as a mechanism for the prevention of microbial diffusion within the organism, and reveal an undescribed role of the process in the control of asexual development and colonial homeostasis.

Expression study of molecular markers involved in staminality and differentiation in the colonial ascidians *Botryllus schlosseri*.

F Ballin, N Franchi, L Ballarin
University of Padova, Department of Biology

All multicellular organisms originate from a small set of totipotent embryonic cells that differentiate into a structured body plan during embryogenesis. The ability to generate an embryo from a single cell and the regenerative capabilities of metazoans suggest the presence of cell types with stem cell attributes. Compound ascidians, like *Botryllus schlosseri*, offer the opportunities to investigate the biology of both embryonic and adult stem cells thanks to the presence of sexual and asexual reproduction. In *B. schlosseri* new buds emerge as thickening of the peribranchial epithelium in a process call palleal budding. Sometimes, a vascular budding occurs, with the development of new buds formed by circulating multipotent or pluripotent cells. These two kinds of budding processes render *B. schlosseri* a good research tool for the study of staminality.
In *B. schlosseri*, during the cyclical generation change, an increase in the number of hemoblasts occurs which will replace, after their differentiation, the circulating cells undergoing apoptotic cell death. Ascidian hematopoiesis occurs in close proximity to the pharyngeal vessels, in the so-called “hematopoietic nodules” and in the endostyle, the cells of which proliferate and migrate to regenerating organs in developing buds. Despite the morphologic suggestions that hemoblasts are the precursors of all the circulating cell types, immunocytes included, there is a general lack of biochemical and molecular data supporting this assumption.
Here we report the first results on the characterisation of staminality and differentiation molecular markers such as ABCG2, CD133 and GATA2/3 considered hematopoiesis molecular marker in other deuterostomes.

Interleukin 17 genes as mediators of inflammatory responses in *Ciona intestinalis*

A Vizzini, F Di Falco, D Parrinello, MA Sanfratello, C Mazzarella, N Parrinello, M Cammarata
Marine Immunobiology Laboratory, Department of Biological Chemical Pharmaceutical Science and Technology, University of Palermo, Via Archirafi 18,Palermo, Italy

Inflammation is a complex reaction of host defence mechanisms aiming at neutralization of an insult and restoring normal tissue structure and function. In human IL-17 is T-cell derived cytokine plays a key role in the clearance of extracellular bacteria promoting cell infiltration and production of several cytokines and chemokines. Here, we report on three *Ciona intestinalis* IL-17 homologues (*Ci*IL17-1, *Ci*IL17-2, *Ci*IL17-3). The gene organization, phylogenetic tree and modeling supported the close relationship with the mammalian IL-17A and IL-17F suggesting that the *C. intestinalis* IL-17 genes share a common ancestor in the chordate lineages. Real time PCR analysis showed a prompt expression induced by LPS inoculation showing that they are involved in the first steps of inflammatory response. *In situ* hybridization assays disclosed that the genes transcription was upregulated in the pharynx, the main organ of the ascidian immune system, and expressed by hemocytes (granulocytes and univacuolar refractile granulocyte) inside the pharynx vessels. As in human, we can assume that *Ci*IL-17-like stimulates the release of *Ci*TNF*alpha*, which synergizes with *Ci*IL-17 in its effects on cells and molecules of *Ciona intestinalis* immunity system. In addition, a comparative evaluation with others molecules upregulated by LPS challenge as *Ci*TNF alpha, phenoloxisases, peroxinectin, galectins and mannan binding lectin, have been evaluated in terms of temporal and quantitative gene expression.

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Isolation and characterization of a LPS induced MD2-like protein in *Ciona intestinalis*

A Vizzini[(1)], A Bonura[(2)], V Longo[(2)], M. Sanfratello[(1)], D Parrinello[(1)] N Parrinello[(1)] and P Colombo[(2)]
[(1)]Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche, Via Archirafi 18, Palermo, Italy;
[(2)]Istituto di Biomedicina ed Immunologia Molecolare "Alberto Monroy" del Consiglio Nazionale delle Ricerche, Via Ugo La Malfa 153, Palermo, Italy

The MD2 (Myeloid Differentiation factor-2) protein belongs to the ML superfamily. This group of proteins contain a specific lipid binding domain (ML domain) that plays an important role in lipid recognition and metabolism. In vertebrates, MD-2 is involved in innate immune response as co-receptor in the LPS/TLR4 signaling pathway; MD2 recognizes and binds the bacterial lipid A and drives the TLR4 activation. Two TLR isoforms, *CiTLR-1* and *CiTLR-2*, were identified in *Ciona intestinalis* with a TIR domain most similar to human TLR4 and TLR 6 respectively. Using a PCR-based subtractive hybridization strategy for isolation of differentially expressed genes between LPS-challenged and naïve *C. intestinalis*, we identified a full-length cDNA (855 bp) encoding for a 150 a.a. protein (*Ci*MD-2-like). *In silico* analysis showed that the deduced protein contains a signal peptide (1-19 a.a.) and an E1/Der p2/Der f2/ML domain-MD2 related lipid recognition domain (21-148 a.a) with similarities to ML(MD-2 related Lipid-recognition) domain identified in MD-2 and NPC2 (Niemann-Pick disease type C2). Phylogenetic and structural analysis supported the close relationship with MD-2 and NPC2 suggesting that *Ci*MD-2-like originated from a common ancestor gene. Furthermore, gene expression studies by Real-time PCR demonstrated that this cDNA is up-regulated after LPS injection in the body wall. *In situ* hybridization performed in controls and LPS-induced animals has shown that this gene is expressed in granular amoebocytes, large granules hemocytes and URG (univacuolar refractile granulocyte) in pharynx, the main organ of the ascidian immune system.

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Characterization of a novel LPS-induced peptide from Ciona intestinalis with immune modulatory activities

A Bonura[1], A Vizzini[2], A Longo[1], S Vlah[1], M. R Melis[1], F Gervasi[3], N Parrinello[2], P Colombo[1*]
[1]Istituto di Biomedicina ed Immunologia Molecolare "Alberto Monroy" del Consiglio Nazionale delle Ricerche, Palermo, Italy; [2]Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche, Via Archirafi 18, Palermo, Italy; [3]*Unità Operativa di Ematologia, Ospedale Civico, Palermo, ITALY*

We previously published that LPS injection in the body wall of the Tunicate *Ciona intestinalis* induced the activation of an alternative poly-adenylation signal leading to the up regulation and modified tissue localization of a cDNA (C8short) containing the first exon of a Ci-RTP-like mRNA. *In silico analysis revealed that the* synthetic polypeptide derived from the primary sequence of the C8short cDNA displays a peculiar amino acids composition with a high percentage of hydrophobic residues (36%), a negative net charge (-1) and a high content of proline residues (21%) suggesting the possibility that this peptide can act as an Host Defense Peptide. For these reasons, the immunological properties of the C8short peptide were studied by using human peripheral blood mononuclear cells *in vitro*. As a first result, we were able to demonstrate that C8short peptide did not show cytotoxic or/and haemophilic activities *in vitro*. Furthermore, we observed that the C8 peptide displays some immune regulatory activities showing the ability to preferentially induce the proliferation of human $CD4^+$ cells. Following this line of evidence, we decided to perform a time course looking at the appearance of $CD4^+/CD25^+$ cells after C8short stimulation demonstrating that this peptide was able to select peptide-specific effector cells in healthy subjects. Finally, by means of magnetic cell sorting, we were able to show that the C8short induces the secretion of the IFN- and IL-17 inflammatory cytokines from human $CD4^+$ cells. *Taken together,* our data demonstrated that during the inflammatory response induced by LPS injection in the body wall of *Ciona intestinalis*, the organism reacts inducing an alternative poly-adenylation event leading to the over-expression of a truncated form of a longer protein that may have potential to be developed as a novel immune regulatory adjuvant.

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Fish IgT: the weird case of Antarctic teleosts

S Giacomelli, F Albanese, R Esposito De Lucia, U Oreste, MR Coscia
Institute of Protein Biochemistry, CNR, Naples

Abstract
Teleost fish are known to possess three immunoglobulin heavy chain isotypes, Igμ, Igδ and the lastly discovered Igτ. In the present study, Igτ genes of the Notothenioid Antarctic teleost *Trematomus bernacchii* and of a non Antarctic Notothenioid species, *Bovichtus diacanthus*, were cloned and characterized. Surprisingly, compared to Igτ from other teleost species, including the non Antarctic Notothenioid one, *T. bernacchii* Igτ lack the entire second constant domain with only a few amino acid residues left; the latter can be aligned to the C-terminus of *B. diacanthus* CH2 domain. Furthermore, genomic analysis of both species revealed that, in the case of *T. bernacchii*, within the intron between the first and the second costant exons a reminiscence of the ancestral second exon is present. This remnant falls within a 29 bp duplicated region and shows different sizes of 51, 33, and 42 bp according to the genomic sequence analysed. In all cases this exon segments preserved the donor and acceptor splicing sites to be correctly spliced into the mature transcript, giving rise to different cDNA variants. These findings are likely to have resulted from duplication events. Phylogenetic analysis performed on each constant domain and on the entire constant region from all teleost IgT available to date revealed that the loss of almost an entire exon together with the conservation of some amino acids in the remaining domain, such as proline, glycine and cysteine, could be interpreted as another specific and distinctive feature of the evolution of the Antarctic fish genome.

Evolution of β defensin and cathelicidin host defense peptides in primates

A Tossi
Department of Life Sciences, University of Trieste, Italy

Beta-defensins and cathelicidins are the principal families of host defence peptides in mammals and other vertebrate animals. The defensins are encoded by several paraloguous genes in each animal, and some play relevant and differentiated roles in immunity. Four of these (BD1 – BD4) show quite different patterns of evolutionary variations in primates, ranging from conservation to neutral evolution or positive selection for variation. Cathelicidins are structurally quite different to defensins and only one is present in each primate species, including man. These peptides can have both antibiotic and host cell modulating activities, and were found to be under strong positive selection. By chemically synthesizing and comparing the structures and activities of several defensins and cathelicidins from different primates, as well as rationally designed variants, it has been possible to gather useful information on their roles, modes of action, and factors underlying their differing evolutionary patterns.

Identification of IgD and IgT immonuglobulins in sea bass (*Dicentrarchus labrax*) gill transcriptome

V Stocchi, N Nuñez Ortiz, M Gerdol[1], A Pallavicini[1], A Facchiano[2], E Randelli, G Scapigliati, F Buonocore.
Dipartimento DIBAF, Università della Tuscia; [1]Dipartimento di Scienze della Vita, Università di Trieste; [2]Laboratory of Bioinformatics and Computational Biology - CNR, Istituto di Scienze dell'Alimentazione, Via Roma 52 A/C, 83100 Avellino.

The gills of fish are, in terms of exposed surface, the biggest tissue of most Teleost species.They are involved in the maintaining of fish homeostasis by the uptake of nutrients and substances, and by forming an active barrier against the entry of pathogens. The thin gill epithelium is a mucosal tissue at direct contact with the water environment, and contains a gill-associated lymphoid tissue (GIALT) with macrophages/granulocytes, lymphocytes.
To achieve a more comprehensive knowledge of GIALT asset and function in fish we used the European sea bass as a model and we produced a whole gill RNA transcriptome by deep sequencing using an Illumina platform.
Immune-related sequences identified in the transcriptome contained all know components of fish innate and acquired immune system and, interestingly, we identified all T cell gene subsets including Th1/Th2/Th17/Treg cell subpopulations, thus suggesting their possible presence in the branchial epithelium.
Regarding B cells, different sequences of possible IgT and IgD transcripts have been evidenced and these sequences have been confirmed by cloning from gill cDNA.
We obtained a full-length sequence of a heavy chain secretory IgT (accession number KM410929), a partial membrane-bound IgT and a full-lenght heavy chain IgD. Heavy chain secretory IgT has been aligned with secretory form of *O. mykiss* (rainbow trout) that shows 46% of sequence identity with sea bass, and important amino acids involved in fundamental structural features of IgT are conserved, like cysteines that forms inter- and intra-disulphide bridges and tryptophans that are required for the folding of IgSF domains.
Modelling of 3D structure of IgT has been performed based on the mouse Ig gamma-2A chain C region (PDB file: 1IGT) and it showed a beta-sheet sandwich architecture of immunoglobulin-like topology. Moreover, the basal expression of IgT and IgD was studied by real-time PCR in different organs and tissues of unstimulated juvenile sea bass. The highest IgT and IgD expression was found in peripheral blood leukocytes (PBL) followed by gills, liver and head kidney. Finally, after an alignment of the different cloned IgT isoforms, we identified a well conserved region in all sequences and we designed three peptides in this region that have been used to produce a polyclonal antibody against sea bass IgT.

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A polyclonal antiserum for sea bass (*Dicentrarchus labrax*) IgT

N Nuñez Ortiz, V Stocchi, E Randelli, F Buonocore, G Scapigliati
Department for Innovation in Biological, Agro-food and Forest Systems, University of Tuscia, Viterbo, VT, Italy.

Immunoglobulin T (IgT) is one of the key effector molecules of jawed vertebrate's adaptive immune system. IgT is a monomeric immunoglobulin in serum and polymeric in the gut mucus, was first discovered in trout and has an important role in mucosal responses, being analogous to mammalian secretory IgA. We are investigating immune responses in sea bass after mucosal (immersion) vaccination against inactivated nodavirus, and we believe that IgT should play a role in anti-viral immunity.
A necessary step is to have an antibody against sea bass IgT in order to investigate by ELISA the presence of IgT in serum and mucus, and the presence of IgT-bearing cells in tissues. In this respect, we immunized rabbits with synthetic peptides deduced from the full length cDNA sequence and located in the surface-exposed sequence of sea bass IgT peptide. Of the two antisera we obtained, we selected one (RaIgT1) that resulted able to stain in western blot of splenocytes lysates a polypeptide at 74 kDa in reducing conditions and at 150 kDa in non-reducing conditions.
By IIF and flow cytometry of leukocytes, the RaIgT1 stained 40±24% in head kidney, 34±24% of IELs, 28±17% in spleen, 20±5% of PBL and 5±3% in gills. At the fluorescence microscope, live cells from these tissue showed a typical membrane-associated positivity.
Moreover, by using RaIgT1 we performed an indirect ELISA assay platform to evaluate the presence of IgT in sera and intestinal mucus of control and immunized fish. Preliminary results showed a very poor content of IgT in serum and a high content of IgT in mucus, in line data obtained in trout.
Experiments are in progress to obtain IgT-enriched fractions of leukocytes by immunopurification procedures, and in a first attempt in spleen we obtained a 70% of positive cells in the RaIgT-purified fraction. The next step will be to analyze by RT-PCR the expression of B- and T-lymphocytes marker genes in the obtained fractions.

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Localization of IgT expressing cells in sea bass Dicentrarchus labrax (L.)

S Picchietti[1], L Guerra[1], N Nunez Ortiz[1], F Buonocore[1], D Cervia[1], G Scapigliati[1]
[1]*Dep. for Innovation in Biological, Agro-food and Forest systems, Tuscia University, Viterbo, Italy*

Teleost fish have three heavy chain isotypes: μ and δ that correspond to the IgM and IgD classes found in all vertebrates with jaws, and τ that encodes the IgT class, which is specific to fish. The expression of IgM and IgT are mutually exclusive, leading to the existence of two B-cell subsets expressing either both IgM and IgD or only IgT. To define the sea bass IgT positive cells and to investigate their distribution in systemic and mucosal sites, a specific polyclonal antibody (RaIgT1) has been developed. By immunohistochemical analysis, IgT^+ cells were distributed more commonly within the epithelium and LP of the juveniles sea bass posterior intestine. In particular, the gut mucus was strongly positive to the RaIgT1, confirming, as previously reported in other teleost species, that the high concentration of secreted IgT found in the mucus could reflect a specialized role in gut mucosal immunity. In addition, IgT^+ B cells appeared located in the epithelium of the gill lamellae, in the blood vassels and in the spleen and head kidney parenchyma. The presence of unique stained IgT^+ and IgM^+ B cells subsets were also analyzed. I*n situ* hybridization of IgT-mRNA labelled cells further substantiates the presence of IgT expressing cells in sea bass lymphoid tissues, revealing in the intestine a gradient from the anterior segment towards the anus.

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Immunization of sea bass *Dicentrarchus labrax* against Nodavirus

F Buonocore, N Nuñez Ortiz, E Randelli, V Stocchi, A Toffan[1], F Pascoli[1], S Picchietti, Targetfish Consortium[2], G Scapigliati

Dept. for Innovation in Biological, Agro-food and Forest systems, Tuscia University, Viterbo, Italy.
[1]Zooprophylactic Institute of Venezie, Legnaro (PD), Italy.
[2]www.targetfish.eu

The sea bass is particularly sensitive to viral encefalophaty and retinopathy (VER) caused by betanodavirus, a RNA retrovirus that induces high mortalities in wild and farmed fish species. We are currently investigating immune responses of sea bass against nodavirus by immunizing fish with formalin-inactivated VERv, strain 283/I09. To evaluate humoral responses we used an indirect ELISA for sea bass IgM, and developed a capture ELISA assay employing a rabbit antiserum against whole virus and a monoclonal antibody specific for the capsid protein. By using these assy we detected measurable amounts of specific IgM in fish injected intraperitoneally with VERv, whereas after mucosal (immersion) vaccination no or very few specific IgM have been detected. Immunohistochemistry (IHC) analysis of gills in immersion-immunized fish showed uptake of VERv inside the epithelium and expression analysis of antiviral genes showed their upregulation. From these data we conclude that inactivation of virus by formalin damaged antigenicity of the virus. Alternative strategies of immunization have been by oral administration of VERv by adding to food recombinant capsid protein produced in *Pichia pastoris* yeast or in *Tricoplusia ni* insect model. IHC analysis of the intestinal tract showed uptake of the antigen in Pichia-treated fish, and ELISA assays revealed little but measurable IgM titers in sera. Experiments are in progress to evaluate results from additional immunization experiments and to evaluate alternative VERv inactivation methods.

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Alien species and their impact on aquatic animal health

A Manfrin[1], T Pretto[1]
[1] Istituto Zooprofilattico Sper.le delle Venezie – Centro Specialistico Ittico di Adria (RO)

Invasive Alien Species (IAS) are considered to be one of the major threats to native biodiversity, with the World Conservation Union (IUCN) citing their impacts as “immense, insidious, and usually irreversible”. It is estimated that 11% of the *c*. 12,000 alien species in Europe are invasive, causing environmental, economic and social damage and it is reasonable to expect that the rate of biological invasions into Europe will increase in the coming years. Among them, marine and freshwater invasive species are really important not only from an evolutionary point of view, but even for their impact on aquatic animal health. 38 of 82 (46,3 %) fresh water species in Italy are aliens and some of them can be carrier of old or new pathogens, which could be highly pathogenic for autoctonous animals. As many of them are endangered, an exotic disease or a more virulent pathogen could increase the prevalence of mortality and destroy all the population in a limited area but also all along the water catchment. Epizootic Ulcerative Syndrome (*Aphanomyces invadans*) in finfish and Crayfish Plague (*Aphanomyces astaci*) in crustacean are typical examples of pathogens with the potential to cause an adverse effect on aquatic animal health. The first, which is exotic for Europe, can also be transmitted by many ornamental fish species, imported from many extra EU countries every day, while the second one is an “old pathogen” who found “new carriers” like red swamp crayfish (*Procambarus clarkii)* making its spread easier. New pathogens (e.g. Red Sea Bream Iridovirus, shrimp Taura Syndrome Virus, etc.) or new genotype of old ones can spread not only in fish farms but also in the aquatic environment, leading to the destruction of entire populations. To avoid this catastrophic event is important to keep in mind that sanitary and import measures must be taken in order to prevent their introduction and establishment.

Invasive species and their parasites: the case of the Atlantic blue crab *Callinectes sapidus* Rathbun, 1896 and its endoparasitic dynoflagellate *Hematodinium* spp. in the Mediterranean Sea

P Pagliara, M Zotti, L Carrozzo, G Mancinelli
Department of Biological and Environmental Sciences and Technologies, University of Salento, Via Provinciale Lecce-Monteroni, 73100 Lecce, Italy

Coastal marine and transitional water ecosystems are threatened world-wide by invasions of non-indigenous invertebrate species, altering community structure and ecosystem functioning. Noticeably, the Mediterranean Sea is to date experiencing a massive rate of invasion of marine species. A number of alien mollusks, crustacean and fish have established in the Mediterranean basin becoming, in some cases, invasive species, altering native benthic communities. Sometimes the success of these introduced species is due to their ability to escape their natural enemies. Moreover, alien species may transmit disease to native hosts (pathogen spillover), an event that has been given little consideration in the context of biological invasions.
The blue crab *Callinectes sapidus* is an alien species originating in western Atlantic Ocean and that was introduced, accidentally or deliberately, into Asia, Europe, Hawaii and Japan.
Especially common in estuaries, this species eats a large range of foods (eats clams, oysters, and mussels as well as almost any vegetable or animal matte). In its native habitats, the blue crab is subject to infection by the parasitic dinoflagellate *Hematodinium perezi* representing a significant cause of mortality in many crab populations.
In a survey performed along the Apulian coast, we sampled individuals of *C. sapidus* and of native crab species, such as *Eriphia verrucosa.* Their hemolymph was screened for detection of parasite infections. Our results indicated only in very few cases the presence of *Hematodinium* spp. in *C. sapidus* and never in native crabs species. Furthermore, the abundance of parasite cells was very low. A morphological characterization on the blue crab haemocytes was also carried out evidencing the presence of 2 main haemocyte types: the granulocytes and the hyalinocytes.

Is the immunocompente gender-related in *Carabus lefebvrei* (Coleoptera, Carabidae)?

PG Giulianini[1], P Brandmayr[2], F Cavaliere[2], A Giglio[2]
[1]Department of Life Sciences, University of Trieste, via L. Giorgieri, 5, Trieste, Italy
[2]Department of Biology,Ecology and Earth Science, University of Calabria, via P. Bucci, I-87036 Arcavacata di Rende, Cosenza, Italy

Trade-offs exist between immunity and other traits in order to maximize fitness. Moreover, an insect's ability to mount an immune response is related to their gender and age. This study tests this assumption by examining two current immunity markers in insect ecological immunology, phenoloxidase and lysozyme-like enzyme activities in *Carabus lefebvrei* adults. This species is a helicophagous Italian endemic ground beetle that lives in central and south Apennines mountain forests. The plasma phenoloxidase (PO) activity was detected using a L-DOPA substrate and enzyme activity was expressed as absorbance units at 492nm/µL of hemolymph. Moreover, a turbidometric assay was used to measure lysozyme-like enzyme activity in the hemolymph at 450nm. Lysozyme-like activity was quantified using a standard curve and expressed as UE/mL. Total and basal phenoloxidase (PO) and baseline lysozyme-like enzyme activities were compared between females and males according to their age. Results show no significant difference in PO activity levels between females and males at the same age. Higher levels in basal PO activity were found in females (Wilcoxon rank sum test: $p= 2.57x\ 10^{-8}$) and males (p= 0.001) in prereproductive phase than adults displeasing reproductive behaviour. Total PO activity increase with age in males (p= 0.009). The females displeasing reproductive behaviour show lower level of total PO than males ($p= 4.13x10^{-6}$). Baseline lysozyme-like activity was significantly higher in adults displeasing reproductive behaviour than in young adults ($p= 1.564x10^{-6}$) for both females ($p= 1.4x10^{-5}$) and males (p= 0.002). No significant differences were recorded regarding gender in young adults while high levels of enzyme activity were found in females displeasing reproductive behaviour ($p= 4.79x10^{-5}$) than males ones. Results suggest that the basal PO was significantly affected by age because in young adults resources were allocated to strengthen the cuticle by melanization after the metamorphosis forming a barrier against pathogens. The total PO and lysozyme-like enzyme activities were affected by gender but only in adults displeasing reproductive behaviour. Our hypothesis was that resources were invested to increase the activity of two enzymes involved in the humoral response in the reproductive phase preserving the fecundity and longevity of females and males. Moreover, as longevity is of more importance for female than for male fitness, *C. lefebvrei* females invest more in immune defense.

The size inequality of *Apis mellifera ligustica* hypopharyngeal glands along a gradient of heavy metal pollution.

S Battistella[1], A Giglio[2], A Ammendola[1], A Naccarato[3], E Simeon[1], A Tagarelli[3], PG Giulianini[1]
[1]Dipartimento di Scienze della Vita, Università di Trieste [2]Dipartimento di Biologia, Ecologia e Scienze della Terra, [3]Dipartimento di Chimica e Tecnologie Chimiche, Università della Calabria

A number of anthropogenic activities, including farming and urbanization, have a significant impact on the environment and can produce an irreversible damage at the population level. To assess the effects of environmental stressors as metal pollution on *Apis mellifera ligustica* populations, we analyzed the hypopharyngeal gland size and morphology of foraging bees along a urban-suburban gradient. Foraging bees are collected from beehives located in S. Giovanni (Trieste, "urban site") and in Domio (Trieste, "suburban site") over two full activity periods in early summer (July). Foraging bee heads from urban site (n=4) and from suburban site (n=2) were fixed and embedded in Epon 812-Araldite mixture for light microscopy analyses. In order to perform morphometrical analyses of hypopharyngeal glands, the minimum and maximum diameters of the acini was measure used ImageJ program. Diameters were expressed as mean±SE and differences were assessed by nonparametric statistics. Statistical analyses were performed using R version 3.0.1 software (R Development Core Team 2013). The statistical comparison showed a smaller diameter of the acini of hypopharyngeal gland of bees from suburban site (0.08041818 mm ± 0.003134224), compared to those of urban site (0.11400893 mm ± 0.004431358). Statistical analysis showed a trend toward a significant difference in the size of the acini between the site of Domio (Wilcoxon rank sum test, $p = 1.651\ 10^{-7}$) compared to the site of S. Giovanni. Metal contents were recorded for foraging bees (n=20 from each site) and measured by inductively coupled plasma-mass spectrometry (ICP-MS). Recorded elements are As, Bi, Cd, Co, Cr, Cu, Ni, Pb, Sr, V, and Zn. They are accumulated in two different rank order Zn> Cu> Sr=Bi> Ni> Cr> Pb=Co> V>Cd >As in bees from urban site and Zn> Cu> Sr >Cr >Ni>B =Co=V>Pb>As>Cd in bees from suburban site. Significantly differences in concentration of Cd, Cr and Cu were recorded in bees from the two sites (Wilcoxon rank sum test $p<9.229x10^{-5}$ for Cd, $p<0.00021$ for Cr and $p<0.00053$ for Cu). Metal concentrations in the animal's body reflect quantitatively pollution levels of sites. Moreover, morphological data demonstrate that metal pollutions in the environment may have detrimental effects on the individual level and then on *A. mellifera ligustica* population.

AIF-1 impregnated Matrigel: an important tool to study *in vivo* and *in vitro* behaviour of the leech *Hirudo medicinalis* macrophages in response to MWCNTs treatment

R Girardello[1], M de Eguileor[1], R Valvassori[1], J Vizioli[2], F Drago[2], A Grimaldi[1]
[1]Department of Biotechnology and Life Sciences, University of Insubria, Via J. H. Dunant 3, 21100 Varese, Italy
[2] Université Lille 1, Laboratoire de Spectrométrie de Masse Biologique Fondamentale et Appliquée, (IFR 147). Bat. SN3 Cité Scientifique, 59655 Villeneuve d'Ascq, France

The rapid development of nanotechnology and nanoscience during the last decade has led to the discovery of nanomaterials such as multi-wall carbon nanotubes (MWCNTs), widely used in industry. Aquatic ecosystems seem to be particularly susceptible to contamination by MWCNTs with harmful consequences for the aquatic animals as well as for humans, who may come into contact with this nanomaterial through many pathways, such as inhalation, injection, penetration but also ingestion.
We have recently demonstrated that *in vivo* treatment of *H. medicinalis* with water dispersed MWCNTs causes a strong inflammatory process, inducing a massive angiogenesis and migration of $CD45^+$ and $CD68^+$ macrophages throughout the animal body wall. In order to better understand the mechanisms through which this cell population interacts with the nanomaterial, we used a consolidated experimental approach based on injection in the body wall of the leech of the biomatrice Matrigel (MG), added with a specific macrophage chemoattractant, the cytokine Allograft inflammatory factor-1 (AIF-1) and/or with MWCNTs.
We observe that MWCNTs alone are able to induce the migration of a reduced number of macrophages into the MG sponges while the presence of r*Hm*AIF-1 invokes a larger number of cells within the Matrigel and MG pellets are rich in cells which are positive for both CD68 and *Hm*AIF-1, specific monocyte-macrophage markers.
Ultrastructural analysis at TEM suggests that short and curled MWCNTs may be internalized by phagocytosis or during the process of matrix degradation, while straight and rigid MWNCTs seem to be able to pierce cell membranes during cells migration and are then found free in the cytosol.
Starting from these preliminary results, the next goal of our work will be to obtain *in vitro* expansion of macrophages primary leech cells that could be used as a sensitive method to evaluate the presence of the nanomaterial in contaminated water. For this purpose, nanotubes at different concentration will be then added at primary cell cultures to study some aspects of their effects on cell morphology, cell stress response, cell viability and death events.

Susceptibility of *Vibrio aestuarianus* 01/032 to the antibacterial activity of *Mytilus* hemolymph: identification of a serum opsonin involved in mannose-sensitive interactions

L Canesi[1], E Pezzati[1], G Damonte[2], A Salis[2], F Marsano[3], C Pruzzo[1]
[1]DISTAV, University of Genoa; Italy; [2]DIMES, University of Genoa; Italy; [3]DISIT, University of Eastern Piedmont, Alessandria, Italy.

Bacteria persistence in bivalve tissues largely depends on their sensitivity to the hemolymph bactericidal activity. In *Mytilus galloprovincialis* mannose sensitive hemagglutinin (MSHA) expressed by *Vibrio cholerae* El Tor strains mediates adhesion to, and killing by hemocytes; such interactions occur *via* specific serum opsonins. *Vibrio aestuarianus* 01/032, a strain positive for the *mshA* gene, has been associated to oyster mortality outbreaks; however, this strain was only moderately pathogenic to *M. galloprovincialis* compared to *Crassostra gigas.*

In this work, the interactions of *Vibrio aestuarianus* 01/032 with hemolymph of *M. galloprovincialis* and *C. gigas* were investigated to identify the soluble components involved in the higher resistance of mussels to this strain in comparison to oysters. Although 01/032 bacteria adhered to hemocytes of both bivalves, they were sensitive to the bactericidal activity of whole hemolymph from mussel but not from oyster; in addition, adhesion to mussel (but not oyster) hemocytes was affected by D-mannose.

Mussel serum opsonins were purified by ConA sepharose affinity column eluted with D-Man and subjected to SDS-PAGE electrophoresis, yielding a single band of 40 kDa. The purified protein was analyzed by nano-HPLC-ESI-MS/MS: the results matched to the protein precursor EP [M. edulis] and with MgC1q6, a member of the putative C1q domain containing proteins [M. galloprovincialis] (Mascot score:192). Both native serum and the purified protein increased hemocyte association with *V. aestuarianus* 01/032. In the presence of *M. galloprovincialis* EP protein (MgEP), *C. gigas* hemocytes acquired the ability to V. aestuarianus 01/032 almost as efficiently as mussel hemocytes.

The results further support existence of specific interactions between *Mytilus* serum proteins and bacterial strains expressing mannose sensitive ligands, and indicate novel properties of MgC1q6, the homologue of the EP protein, in immune recognition.

These findings suggest that the different sensitivity of 01/032 strain to the antibacterial activity of oyster and mussel hemolymph might partly depend on the fact that C. gigas serum lacks MgEP-like opsonins. These results represent the basis for understanding the different sensitivity to microbial infections of different bivalve species.

Immunomodulation of cationic polystyrene nanoparticles in *Mytilus* hemocytes

C Ciacci[2], B Canonico[2], E Bergami[1], L Canesi[3], KA Dawson[4], I Corsi[1]

[1]Dip. di Scienze Fisiche, della Terra e dell'Ambiente, Univ. di Siena, Italy
[2]Dip. di Scienze della Terra, della Vita e dell'Ambiente, Univ. degli Studi di Urbino "Carlo Bo", Italy
[3]Dip. per lo studio del Territorio e delle sue Risorse, Univ. di Genova, Italy
[4]Centre for BioNano Interactions, School of Chemistry and Chemical Biology, Univ. College Dublin, Ireland

Plastic debris and their degradation products are ubiquitously present in the world's seas and oceans. Engineered plastic nanoparticles derived from post-consumer waste as well as from meso-/microplastics via degradation pose a specific challenge to the ecosystem. The fate and impact of nanoplastics in the marine environment is almost unknown but it is very important due to their increasing abundance in the water column and food webs. The impact of microplastics on marine organisms will depend on a combination of parameters that determine the position of these particles in the water column. Even though the existing data are too limited to determine a realistic natural concentration of microplastics in seawater, the potential for ingestion by commercially important species, however, remains a cause for concern. Bivalves are of particular interest since their extensive filter-feeding activity exposes them directly to microplastics present in the water column.

Polystyrene (PS) is one of the most largely used plastics worldwide, used in food and industrial packaging, disposable cutlery, compact disc cases, building insulation, medical products and toys and can be considered as model for studying both fate and toxicity of nanoplastics in marine organisms. Our recent findings on amine PS NPs (PS-NH_2) toxicity in sea urchin embryos underline that marine invertebrates can be biological targets of nanoplastics. The present study aims to investigate pathways of toxicity of 50 nm cationic PS-NH_2 in hemocytes of Mediterranean mussel *Mytilus galloprovincialis*: a battery of functional immune assays was applied to investigate the short term *in vitro* effects of PS-NH_2. Several functional parameters have been evaluated: lysosomal membrane stability and lysosomal enzyme release, extracellular oxyradical production and Nitric Oxide (NO) production, phagocytic activity, as well as pro-apoptotic processes at both plasma membrane and mitochondrial level.

The results suggest that mussel hemocytes and their immune activity are affected by PS-NH_2 NPs. Therefore, further research is necessary on specific mechanisms of nanoplastic toxicity and its cellular uptake in order to assess the impact on marine biota.

Immunomodulation and physiological responses of *Mytilus galloprovincialis* as bioindicators of environmental change

MG Parisi[1], M Mauro[1], G Sarà[2], M Cammarata[1]
1-Department of Biological, Chemical and Pharmaceutical Science and Technology, University of Palermo, Palermo, Italy
2-Department of Earth and Marine Science, University of Palermo, Palermo, Italy

Oxygenation level temperature increases and changes in food availability are predicted to occur in the future. In such scenario, a global climate change (GCC), there is growing concern for the health status of wild and farmed organisms.
Bivalve molluscs, are important components of coastal marine ecosystems, and as sedentary and filter feeders, are good bioindicators of environmental conditions. The ability of organisms to maintain the immunosurveillance unaltered under adverse environmental conditions may enhance theirs survival capability.
Only a few studies have investigated the effects of changing environmental parameters on the mussels immunity.
In the present study, the effects of different food concentration, temperature and oxygenation treatments were evaluated on immune parameters of *Mytilus galloprovincialis* detected on digestive gland and haemocytes.
Bivalves were exposed to experimental conditions by increasing of six food treatment, to three different temperatures under conditions of normoxia and anoxia.
The multifactorial analysis applied to the responses of the immune variables has showed a direct dependence of various enzymes production by temperature and food concentration. The stability of the lysosomial membrane was altered under conditions of thermal stress and food changing.
The protein concentration of the lysate of haemocytes instead was most affected by the lack of adequate oxygenation.
In addition, a correlation was carried out between mussels immunological effectors and physiological responses as clearance rate, measured by the removal of suspended particles from water flowing through experimental chambers, food absorption efficiency and rates of oxygen consumption by individual mussels.
Overall, information summarized in the present study indicated that climate changes can affect haemocyte and enzymatic functionality and the immune responses of this bivalve could be used as good environmental biomarkers.

Variation of environmental condition and diet act on immune parameters of *Mytilus galloprovincialis*

M Mauro[1], MG Parisi[1], G Sarà[2], M Cammarata[1]
1-Department of Biological, Chemical and Pharmaceutical Science and Technology, University of Palermo, Palermo, Italy
2-Department of Earth and Marine Science, University of Palermo, Palermo, Italy

The knowledge of the immunity mechanisms as environmental indicators and their alterations in the case of physical stress can be of fundamental importance in the environmental management programs.
Recently has been shown that environmental factors affect immune responses in some species of bivalves.
In this study we assessed different enzymatic activities from digestive gland of *M. galloprovincialis* such esterase, phosphatase and phenoloxidase (PO), involved in digestive inflammatory, detoxification and melanization processes.
Particularly, esterases catalyze the hydrolysis reaction of the ester bond. Phosphatases modulates the removal of phosphate groups by producing phosphoric acid from esters and participate in the metabolism of sugars, nucleotides and phospholipids.
The melanization cascade, in which phenoloxidase is the terminal enzyme, plays a key role in recognition of microbial infections in molluscs.
It was also evaluated, as potential biomarker, the lysosomal membrane stability through neutral red assay on haemocytes taken from the posterior adductor muscle.
Specimens were maintained under conditions of normoxia and anoxia and they were subjected to various food amount and different temperatures.
The results showed that the enzymatic activities of esterases, phosphatases and PO are higher during treatments with lowest temperatures and food amount. Moreover, the production of PO is higher in the conditions of anoxia.
The lower values of enzymatic production have been detected under the levels of temperature, oxygen availability and food different than the optimum conditions for the mussels life cycle. During the normoxic treatment, the stability of the lysosomal membrane is highest at the average values of temperature and food concentration. The lowest values, instead, were measured at a temperature of 12 °C and 28 °C in anoxic conditions.

Investigation on the effects of three nanoparticles (zinc oxide, titanium dioxide, C_{60} fullerene) on haemocyte parameters of *Ruditapes philippinarum*

I. Marisa[1], V. Matozzo[1], D. Sheehan[2], M. G. Marin[1]
[1]*Department of Biology, University of Padova, Italy*
[2]*Department of Biochemistry and Environmental Research Institute, University College Cork, Ireland*

Bivalve haemocytes are involved in various biological processes, including wound and shell repair, shell production, transport and digestion of nutrients, excretion and immune defence. It is well known that variations in environmental parameters, such as temperature, salinity, pH, oxygen and nutrients can affect haemocyte functionality in bivalves. However, most of the studies (both *in vitro* and *in vivo*) have been focused on the evaluation of contaminant effects on haemocytes parameters. In this context, attention has recently been addressed to the evaluation of the effects of nanoparticles (NPs) on bivalve haemocytes. NPs are a class of emerging contaminants, and the immune system of aquatic organisms is considered to be a sensitive target for these contaminants.
In order to provide information concerning NP toxicity in clam species, in this study *in vivo* effects of nZnO, $nTiO_2$, C_{60} fullerene on haemocyte parameters of the clam *Ruditapes philippinarum* were investigated for the first time. In addition, protein damage caused by exposure to a mixture containing the three NPs was assessed using 1-D redox proteomics.
Clams were exposed for 7 days to two concentrations of each NPs and the effects on various cellular and biochemical parameters were evaluated in clam haemolymph at time intervals (after 1, 3 and 7 days).
Results showed that $nTiO_2$ is more active in promoting modulation of haemocyte parameters than nZnO and C_{60}. Indeed, in nZnO-exposed clams only a significant increase in haemocyte proliferation and low levels of DNA damage were observed. C_{60} exposure caused alterations in haemocyte diameter, volume and proliferation at the beginning of the exposure only, and increased slightly DNA damage and Neutral Red uptake (NRU) at the end of the exposure. Conversely, $nTiO_2$ exposure significantly increased total haemocyte count, diameter and volume of haemocytes, haemocyte proliferation and DNA damage. Variations in both NRU and cell-free haemolymph lysozyme activity were also observed.
Clams exposed to the mixture showed a high level of protein damage, probably due to NP-induced oxidative stress. Although preliminary, results of the present study can contribute to understand better the mechanisms of action of NPs in *R. philippinarum*, and in bivalve molluscs in general.