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REPORT OF MEETING

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Organizers: **P Pagliara, L Stabili**

Department of Biological and Environmental Sciences and Technologies, University of Salento, Lecce, Italy

Session 1. Chairmen: G Scapigliati, Università della Tuscia, Viterbo, Italy and A Vallesi, University of Camerino, Camerino (MC), Italy
Insights into fish immunity

Lecture
Evolution of the immune response to pathogens

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To survive, organisms must continually adapt to continually evolving invading organisms. Hosts and pathogens are the key players of a continous conflict in which natural selection aids pathogens to increase virulence to escape host surveillance, and hosts to acquire adequate defence strategies. In both cases, these achievements are limited by several factors such as the genetic fitness and the number of genes required, much larger than that available in the genome. Moreover, since the pathogen usually has a very shorter life than the host, it has to fix new mutations favoring virulence much faster than the host can evolve effective defense mechanisms. Another constrain concerns the host that must avoid adverse effects which may derive from the defence system itself. Once resistance and counter resistance are genetically assessed, both the host and the pathogen evolve in response to mutually exerted pressures. This is generally referred to as the “Red Queen Paradigm”, that highlights the significance of biotic versus abiotic factors that lead to constant evolutionary changes. Evolution acts at different levels: biotic factors mainly shape species diversity over short time periods, whereas changes in the physical environment such as climate changes drive evolution at a large scale, during much longer time. Much of our current knowledge of infection biology is based on studies of the immune system in humans and mice. In contrast, much less attention has been paid to immune response in lower vertebrates. Since many features of immune defence mechanisms have been acquired throughout evolution, studying the evolution of successful pathogen virulence mechanisms highlights the potential weaknesses in host immune defenses. On the other hand, investigating the defence mechanisms which species other than tetrapods have evolved to counter infectious agents may allow to identify novel molecules and strategies useful to manage an infection in the host’ s favor. So far, few attempts have been made at considering host and pathogen as interacting partners into a common evolutionary framework. A short overview on how the host-pathogen interaction has been shaped by evolution will be given.

Analysis of Antarctic Teleosts transcriptomes as a tool to explore adaptive immune responses

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During the last decade the use of next-generation sequencing technology has become highly widespread to generate massive amounts of sequence data mainly due to its reduced costs and to the possibility of collecting simultaneously information useful both for transcriptome characterization and quantification. In the present work we analyzed two transcriptomes from the head kidney of the emerald rockcod (*Trematomus bernacchii*) and from gills of the icefish (*Chionodraco hamatus*). The sequences were generated with an Illumina platform and, successively, de novo assembling procedures were performed to obtain the final transcripts. A first aim was to identify new genes related to adaptive immune responses by using as a comparison the

known genome of the Antarctic fish *Notothenia coriiceps*, which contains about 30.900 expected transcripts. We were able to confirm that in the icefish transcriptome about 20400 transcripts were present (66,1 %), whereas in the emerald rockcod we found about 19800 transcripts (64,2 %). Moreover, orthologous proteins showed about 80 % amino acid identity considering *Chionodraco* and *Trematomus transcriptomes*. From these transcriptomes, a relative high number of sequences related to adaptive immune responses genes have been identified and confirmed by cloning from cDNA, *e.g.,* MHC-I, MHC-II, beta2-microglobulin, CD4, CD8alpha, IgT, IgD, etc. The identification of MHC-II molecules provided the opportunity of evaluating the levels of genetic variation at a MHC-II β locus in the icefish population from the Ross Sea. Preliminary data suggest a genetic variability comparable to that reported for other fish species at both inter- and intra-individual levels. This finding allows exploring possible relationships occurring between the levels of genetic variation of the MHC in the icefish with respect to the parasitic load recorded in this fish host.

Preliminary data on the effects of chestnut skin extracted polyphenols on *Oncorhynchus mykiss* blood and GALT

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Agricultural by-products are a rich source of bioactive molecules, including polyphenol compounds or "polyphenols". The Immunostimulant and antioxidant properties of polyphenols may have a potential role for animal welfare and for the production of " healthy " feed. Polyphenol-enriched feed has been finding application in animal farming, thank to polyphenol capability to improve the productive performance, immune response and health of livestock. For this reason, polyphenols may also represent a valid alternative to antibiotics and medicines currently employed in animal farming. The introduction of natural extracts in animal feed represent a booming business after the ban of auxinic antibiotics and it proves that invest resources in the search for plant extracts can deliver significant benefits. The Interest in use natural substances, known and used since ancient times in the care of man, can certainly be considered innovative for the animal diet. The potential immunostimulant activity of polyphenols extracted from chestnut skin has been studied on lymphocytes isolated from blood and lymphocytes extracted from GALT of the rainbow trout *Oncorhynchus mykiss*. The assays used to evaluate the parameters of both innate immunity and acquired immunity were: superoxide anion production; phagocytosis; expression of pro and anti-inflammatory cytokines. Our results indicate that superoxide anion production and phagocytosis decreased in both the blood and GALT leucocytes incubated in vitro with concentrations of chestnut skin polyphenols ranging from 10 to 100 µg/ml. Higher concentrations (500 and 5000 µg/ml) were instead stimulating both anion superoxide production and phagocytosis. Chestnut skin polyphenols used in our experiments were also able to modulate the gene expression of immune-related cytokines, such as TNF-α and IL-10. Specifically, it was observed an upregulation of the pro-inflammatory cytokine TNF-α and a downregulation of the anti-inflammatory cytokine IL-10 in blood and GALT leucocytes. Similar results were obtained with Gallic acid and Ellagic acid, although the effects were less evident, suggesting that the effects of chestnut skin polyphenols are depending on the mixture synergism between the various phenolic compounds.

In the light of these preliminary results, we suggest that the addition of polyphenols to standard diet may improve the immune response of farmed fish. Moreover, this study suggests the possible re-use of agri-food industry wastes as feed additives for farmed animals.

Strategies for detection and vaccination of juveniles european sea bass (*Dicentrarchus labrax*) against betanodavirus

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Encephalopathy and retinopathy virus (VERv) or betanodavirus causes massive mortalities of the most important farmed species in Mediterranean area, the European sea bass. In order to develop strategies for the control of virus infection and virus detection, we have studied the possibilities of vaccinating sea bass at young age (2-10 grams) through mucosal and intraperitoneal immunization using VERv inactivated by differents ways. After inactivation of VERv by differents protocols: formalin, BPL and heat treatment we have performed two control experiments using the differents inactivated VERv by immersion and intraperitoneal administration. Serum antigen-specific IgM titers was determined by Indirect Elisa being specially significant after intraperitoneal immunization with formalin-inactivated VERv. VERv-free juveniles immunised by immersion in formalin-inactivated virus showed the presence and the uptake of VERv in the gills by Immunohistochemistry (IHC). Quantitative PCR expression on gut and head kidney after intraperitoneal administration with formalin-inactivated virus showed modulation in the expression of antiviral gene ISG-12 after 48 h in both organs and Mx gene in gut after 48 h too but

induced detectable modulation of Mx and ISG12 genes was detected in the gills 24 h postimmersion. Finally, challenge experiment using live VERv were performed after immunization with formalin-inactivated VERv, and we observed a 80 % increase in a relative protection value in intraperitoneal immunizated fish with respect to unimmunized fish. In the other hand, bath immunization resulted in no protection in *in vivo* challenge. In addition, by employing a rabbit antiserum against VERv (pAb 283) and a mouse monoclonal against VERv capsid protein (mAb 4C3) we have developed an ELISA system to detect and quantitate the presence of VERv in solutions and biological fluids.

Fish cytokines: IL-4/13 in sea bass (*Dicentrarchus labrax*), molecular characterization and expression analysis after *in vitro* and *in vivo* stimulation

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Interleukin-4 (IL-4) and interleukin-13 (IL-13) are key cytokines in Th2 mediated immune responses. These cytokines have been extensively studied in mammals where they show different roles with some overlapping bioactivities exploited through shared receptors. Usually IL-4 and IL-13 are activated at the same time when the immune system recognizes the presence of an external pathogen. In Teleost fish the situation seems quite different from mammals, in fact only recently, in 2007, a gene with some relation to IL-4 and IL-13 was found in the pufferfish genome and, successively, a second IL-4/13 like gene was identified in zebrafish at a different locus. These two genes were called IL-4/13A and IL-4/13B depending on their position on fish locus and they have been successively identified in other species, like rainbow trout, Atlantic salmon and medaka. In our work we have found in a sea bass gills transcriptome three IL4/13 transcripts that have been mapped on the available sea bass genome and therefore identified as IL4/13A1, IL4/13A2 and IL4/13B. The identified sequences have been confirmed by cloning on sea bass gills cDNA and their expression has been studied by real-time PCR. Basal expression analysis revealed a different expression of the IL-4/13 genes in the various tested organs and tissues: in particular the IL4/13B expression is very high in spleen, while the expression of IL-4/13A1 and IL-4/13A2 is high in head kidney. Moreover, we investigated the expression of the IL4/13 isoforms in sea bass head kidney and spleen leukocytes after in vitro stimulation with the T cell mitogen agents PHA and PMA and after in vivo infection with nodavirus and *Vibrio anguillarum*. The results have showed that IL-4/13B is high responsive to all stimulations, whereas, in contrast, IL-4/13A1 and IL-4/13A2 are less up-regulated. Successively, we have produced the three isoforms as recombinant molecules and we tested their action in vitro on leukocytes from head kidney and spleen. This is the first in-depth analysis of the biological activity of IL-4/13 cytokines in sea bass and it will highly contribute to a broader understanding of the evolution of type-2 immunity in this species.

Session 2. Chairmen: N Parrinello, University of Palermo, Palermo, Italy and P Luporini, University of Camerino, Camerino (MC), Italy
Ascidian immunity

New data on the expression of molecular markers involved in stemness and differentiation in the colonial ascidian *Botryllus schlosseri*

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Cell types are often identified by determining which genes they express specifically. The use of specific antibodies or complementary RNA probes allows the identification of the translational/transcriptional products: These molecules, also called “molecular markers”, show an unique expression pattern, frequently used to identify and isolate stem cell populations. In *B. schlosseri*, a compound ascidian, there are three important processes that suggest the presence of stem cells during the life cycle: i) embryogenesis, in which an embryo develops from a zygote, palleal budding where new buds emerge as thickenings of the peribranchial epithelium and vascular budding, *i.e*., the development of new buds within the vasculature by circulating multipotent or pluripotent cells. During the cyclical generation changes, which characterize the colonial blastogenetic cycle, an increase in the number of hemoblasts, *i.e*., undifferentiated circulating cells, occurs which will replace, once differentiated, the hemocytes 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 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 identification of hematopoietic molecular markers in B. schlosseri, very similar to those of vertebrates, their localization and expression profile during the blastogenetic cycle.

Characterization and genetic variability of Tumor Necrosis Factor alpha (CiTNFα) in *Ciona intestinalis*

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The tumor necrosis factor superfamily (TNFSF) is involved in a lot of cellular signaling pathways like inflammation, apoptosis, lymphocyte homeostasis and tissue development. TNFSF ligands are bound to membrane. About half of different ligands encode proteolytic cleavage sites that can generate soluble forms holding biological activity. The study of evolution of genes and genomes allows to understand the role of different evolutionary forces, including natural selection. The structure and composition of a gene are inherited from the organism's ancestors, so various driving forces could cause the change of structural and functional aspects of a gene, for a best adaptation to environment. In *Ciona intestinalis*, a Tumor Necrosis Factor-alpha (TNFα)-like gene challenged with bacterial lipopolysaccharide (LPS) was cloned and sequenced. It encodes a propeptide of 312 amino acids (35.4 kDa), shows a transmembrane domain from positions 7 to 29, a TACE cleavage site and a mature peptide domain of 185 amino acids (20.9 kDa). The diversity of mRNAs from CiTNFα has been investigated in 30 Ascidians, collected from Termini Imerese (Italy), by denaturing gradient gel electrophoresis (DGGE). DGGE migration revealed different molecular patterns. All patterns observed were sequenced and variants were identified in 30 CiTNF-α. Several comparisons have been made: (i) for each DGGE pattern, all the sequences were aligned and clustered according to nucleotide sequences, (ii) the different cds were translated into pro-peptides and (iii) the resulting aminoacid sequences were compared. The evolutionary relationships were inferred using the Neighbor-Joining algorithm (MEGA-6). Site-by-site frequency spectrum-based statistical assays were used to test the hypothesis of polymorphisms within loci being neutral. All three used tests were in agreement with the hypothesis of negative selection pressure linked to transmembrane domain, propeptide and mature peptide. Fu and Li's D and F tests suggested possible positive selection pressure linked to COOH-terminus region only. All statistical tests indicated possible negative selection pressure when applied to full cds sequences. TNF mRNAs from invertebrate and vertebrate animals were used to construct a phylogenetic tree to study the evolutionary dynamic of TNF family gene. These results show that the TNF family gene has encountered remarkable changes in invertebrates, but conserved in vertebrates during history of evolution.

Growing complexity of the invertebrate complement system: evidences from colonial tunicates

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The evolutionary history of the complement system is not yet fully elucidated. Evolutionary studies suggest that the origin of the complement system can be traced back to the common ancestor of the Eumetazoa as the genes for the C3, factor B and MASP have been identified in sea anemones. Nonetheless, no complement genes are present in the genome of the sponge *Amphimedon queenslandica* or the choanoflagellate *Monosiga brevicollis* and, although their presence in invertebrate protostomes is generally accepted, complement genes are missing also in *Drosophila* and *Caenorhabditis*, probably due to a secondary loss. As regards deuterostome complement system, it is well studied in mammals, where more than 30 different proteins have been described, involved in the activation and regulation of this fundamental humoral system able to modulate immunocytes behaviour, belonging to both innate and adaptive immune response. However, the mere report of the presence of C3, factor B and lectin pathway in a species cannot help in elucidating the evolution of the complement system. Since adaptive immunity evolved in the presence of a functioning complement system, the presence of considerable and important interaction between complement and adaptive responses is not surprising. In particular, referring to the invertebrate-vertebrate transition, the description of the complement-mediated immune modulation requires the identification and characterization of additional factors, such as complement control proteins (*e.g.*, factor H, C4bp, CR1, CD46, CD 55) and receptors in invertebrates chordates.

Here we report on the identification and analysis of transcripts for CR1 (C3b receptor), C3aR and two factors H in the colonial ascidian *Botryllus schlosseri* that, for the first time are described in tunicates, the sister group of vertebrates. The localization of CR1 and C3aR on phagocytes and morula cells, respectively, open the possibility to use such molecules as molecular markers for immunocytes. In addition, the presence of a complement regulator, such as factor H, in tunicates suggests a higher level of complexity than that expected in an invertebrate.

Session 3. Chairmen: L Ballarin, University of Padua, Padua, Italy and P Pagliara, Università del Salento, Lecce, Italy

A spotlight on Echinoderms

Lecture
The Sp185/333 system in the Sea Urchin

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The arms race between a host immune system and its pathogens drives diversity on both sides of the conflict. Pathogens change their approaches for infection and virulence, and the host changes the diversity of the immune detection and effector proteins. The purple sea urchin, *Strongylocentrotus purpuratus*, survives in the marine habitat with an innate immune system of the is both complex and sophisticated. There are several large gene families in the sea urchin genome that encode pathogen detection and immune response proteins. One of these is the Sp185/333 gene family, which is composed of ~50 small genes that are tightly linked

in clusters. The genes share blocks of sequence called elements that are present in mosaic patterns and have a variety of repeats within the second exon, and each gene is surrounded by one or two types of microsatellites. These characteristics suggest that the genomic region may be unstable, which may drive sequence diversification of the genes, a benefit for keeping up in the arms race with pathogens. Gene sequence diversity and expression patterns suggest that the encoded proteins may have effector functions. To test this, we have evaluated one Sp185/333 recombinant protein (rSp0032), which binds specifically to *Vibrio* and yeast, but not to *Bacillus*. It also binds LPS, β-1,3-glucan, and flagellin with specificity and high affinity, but does not bind peptidoglycan. rSp0032 also binds phosphatidic acid (PA) and can deform liposomes composed of 10 % PA. rSp0032 is intrinsically disordered, however, when bound to LPS or PA, it transforms to α helical suggesting "Shapeshifter" activities for binding lipids, sugars and proteins. Given that single sea urchins are capable of expressing up to 260 Sp185/333 protein variants, and if each one has a range of overlapping binding activities that target simultaneously multiple PAMPs, this may facilitate highly effective and flexible host protection against a broad array of potential pathogens encountered in the marine environment.

Antimicrobial and antioxidant activity in some Echinoderm species

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Echinoderms represent one interesting marine renewable resource and produce bioactive compounds related to their innate immune system. These invertebrates indeed are able to differentiate self from non-self through the production of soluble molecules and coelomocytes response playing an important role in the resistance to disease. Therefore, they appear as a promising alternative valuable source of new compounds for drug development. In particular, the application of new marine antioxidants in foods, food supplements, nutraceuticals and medicine is recently considered from the perspective of benefits to human health. In this study, the antimicrobial and antioxidant activity of several echinoderm species was investigated. We focused our attention on the two sea urchins *Sphaerechinus granularis* and *Arbacia lixula* and on the sea star *Echinaster sepositus*. Coelomic fluid and coelomocyte lysate of each species were utilized for antimicrobial activity assay using the Kirby Bauer method (1966). The antioxidant activity of the samples was measured by two in vitro assays: the TEAC (Trolox Equivalent Antioxidant Capacity) assay based on a single electron transfer (SET) reaction, using ABTS [2-2'-azino-bis (3-ethylbenz-thazoline-6-sulfonic acid)] as chromogen and the ORAC (oxygen radical absorbance capacity) based on a hydrogen transfer mechanism (HAT). Both the antioxidant assays showed a higher antioxidant activity in the coelomocyte lysate compared to coelomic fluid for all the Echinoderm species studied. Moreover *A. lixula* cell lysate had the highest antioxidand activity both with TEAC and ORAC assay. These antioxidant values are comparable with those reported in the literature for various high antioxidant fruit and spice extracts. Among the investigated species, the coelomocyte lysate of A. lixula showed a bacteriostatic activity against two emerging pathogenic bacteria *Pseudomonas aerugionsa* and *Staphylococcus aureus* and against the yeast *Candida famata*. These results are noteworthy considering the resistance against antibiotics developed by bacteria and the need to control human infections. The antioxidant activity was also of interest since it is the first record for the investigated species and represents a potential for applicative purposes.

Bacterial challenge induces variation of the Allograft Inflammatory Factor I (AIF-1) gene expression in *Paracentrotus lividus*

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The phylum Echinodermata is a very heterogeneous group of marine invertebrate species. After Chordata, it represents the second largest group of deuterostomes. The interest for echinoderm immune defence system is steadily increasing in the last years, due to their phylogenetic, ecological, biotechnological and economical relevance. Noteworthy, their basal position within the deuterostome lineage makes the analysis of their defense mechanisms highly relevant to understand the evolution of the deuterostome immune system as it now appears in Vertebrata. Suggestions derive from the systematic analysis of the immune genes and of their products, such as cytokines, *i.e*., a specific group of effectors molecules that play a variety of roles in the immune response. The Allograft Inflammatory Factor-1 (AIF-1), a calcium-binding cytokine, has been identified as i) a key regulator of the immune response; ii) a central player of the self-intensifying cycle of inflammation; and iii) an important pathogenic factor in multiple inflammatory diseases in Vertebrata. Recent literature evidences that proteins of the AIF-1 superfamily are present in several phylogenetically distant species, all showing high similarity at the primary protein sequence level. These data suggest a significant conservation of the functional properties of AIF-1 in the immune response. In the present work, we report on the immune response of the sea urchin *Paracentrotus lividus* after challenge with *Micrococcus lysodeikticus*, and we estimate the variation of cellular and humoral responses after bacterial injection. In particular, AIF-1-like protein constitutive expression has been evidenced in *P. lividus* coelomocytes by immunocytochemistry performed

using a human anti-AIF-1 antibody. Subsequent mining of expressed nucleotide sequences (ESTs) databases allowed us to identify an AIF-1-like mRNA fragment sequence and to study its expression. Notably, mRNA levels were found to be up-regulated in coelomocytes at 24 h post-bacterial injection. Overall, our data suggest novel hints on the AIF-1 involvement and responsiveness to immune activation by bacteria in cells and tissues of *P. lividus*.

X-ray photoelectron spectroscopy as a non-conventional analytical technique for bio-organic materials characterization: the sea urchin coelomocytes and the purple photosynthetic bacteria examination

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X-ray photoelectron spectroscopy is a surface technique (depth profiling analysis 5 - 10 nm) that allows to analyze, in terms of chemical speciation, all elements, with the exception of H and He, present in different typologies of solid samples (inorganic and organic), as long as their atomic percent concentrations (At.%) are not below 0.01 - 0.l %, depending on the specific element. This technique potentially can also allow to obtain chemical imaging of surfaces with lateral resolution of 3□m. However, at the state of the art, the employment of this analytical technique as a non-conventional tool for the investigation of bio-organic materials (*i.e.*, microorganisms and their related systems such as biofilms, extracellular polymeric substances (EPS) and bio-adhesions), has been reported only in a limited number of papers. In this study, we report some preliminary results on the XPS characterization of sea urchins coelomocytes and photosynthetic bacteria. The sea urchin *Arbacia lixula* coelomocyte population is characterized by the presence of red cells, which number may increase in response to different stress. As red spherula cells accumulate around injuries and sites of infection, this analysis may help to understand what is the role of cell surface interacting with bacteria in addition to the action of echinochrome A, present inside the cells. In particular, here we compare the red and withe coelomocytes surface. Furthermore, the characterization of purple photosynthetic bacteria (*Rhodobacter sphaeroides*) able to interact with detrimental heavy metal ions, such as nickel and chromium has been performed, successfully highlighting both the immobilization of metals and surface modifications induced by the environmental stress.

Session 4. Chairmen: E Ottaviani, University of Modena and Reggio Emilia, Modena, Italy and L Stabili Università del Salento, Lecce, Italy

Invertebrate models suitable for the study of mammalian diseases

Lecture

Bivalves as a translational model in inflammation and cancer research

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Historically, animal models have been used in the biomedical research to characterize disease progressions, evaluate the action of drugs and also discover new biomarkers. Invertebrate models of human disease first appeared in the scientific literature in the late 19th century. Currently, model species range from terrestrial invertebrates such as nematodes and insects, to freshwater and marine life including planarians, crustaceans and molluscs. Among new invertebrate translational models in biomedical research, mollusc are emerging as a promising one in many areas, such as those concerning immune-neuro-endocrine system, neurodegenerative disorders, inflammatory lesions and cancer. Along with an overview of the main features concerning both inflammation and cancer pathogenesis in bivalve molluscs, we will also stress the rationale concerning the potential use of bivalves as a translational model for early validation of new therapeutic approaches in both human inflammation and cancer. At the same time, we highlight the need for standardization of scientific terminology in this new field of investigation.

Amyloidogenesis in animal models

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The biomedical research depends on the use of animal models that can be utilised to understand, at different levels, the pathogenesis of human diseases. The ultimate goal of this approach is to develop and test new therapies to eradicate the examined diseases. Many researchers interested in embriologically and genetically tractable disease models have opted for zebrafish. This animal can provide a variety of human diseases due to sophisticated mutagenesis and supply economic screening strategies on a large scale. Furthermore, despite the advantages and the pre-eminence of the mouse in modeling human diseases there is a lot of aspects limiting the use of this animal in large-scale genetic and therapeutic screening. Even if invertebrates lack structures and organs that can be involved in human pathologies it is important to bear in mind that there is a surprising degree of functional conservation in basic cellular biological processes between mammals and invertebrates (such as worms and insects). Thus when there is a slow down or a total block in one of complex and basic processes, pathological events take place. Amyloidogenesis represents a primitive, simple response, widespread in invertebrates and

vertebrates where innate immune signalling pathways is linked to stress responses. The critical role played by amyloid synthesis and deposition in several pathologies, could explain the structural resistance of these scaffolds and could provide the basis for developing new diagnostic and therapeutic approaches in all diseases in which the innate branch of the immune system has a pivotal role.

Serum Amyloid A in marine bivalves: an acute phase protein of innate immunity

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Serum amyloid a (SAA) is an evolutionarily conserved acute-phase protein, involved in many vertebrate biological processes, such as lipid metabolism and immunity. The rapid increase of SAA can activate the immune system and promote inflammatory responses after injuries, infections or stress. Although SAA homologs are widespread in vertebrates, to date they have only been identified in limited number of invertebrate species. We traced the presence of SAA genes along metazoan evolution by screening available genomic and transcriptomic data, finally retrieving 51 SAA-like proteins in several protostome taxa. In detail, we identified SAA homologs in 21 marine bivalves and we investigated the gene structure and expression patterns of mussel and oyster SAAs. Although phylogenetic and structural analyses support a certain degree of conservation between vertebrate and invertebrate SAA sequences, vertebrate SAAs are mainly expressed in liver, whereas invertebrate SAAs appear to be expressed in various tissues. Using both qPCR and RNA-seq approaches, we observed that the two mussel SAA genes are mainly expressed in gills (MgSAAa), mantle and posterior adductor muscle (MgSAAb), whereas *C. gigas* SAA is expressed in significant amounts in mantle and gonads. We also confirmed the inducible nature of bivalve SAA transcripts, observing the over-expression of mussel SAAs after challenge with pathogenic bacteria, although timing and extent of the induction were different for the two mussel SAA genes. The overall results provide new insights into the evolution of these ancient immune-related proteins in invertebrates.

Research of inflammatory markers in the medicinal leech, *Hirudo medicinalis*

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The leech *Hirudo medicinalis* is an interesting model to study inflammatory processes both in nervous system and in peripheral tissues. Here we considered two different molecules involved in peripheral tissues as well as neural immune response: the Macrophage Migration Inhibitory Factor (MIF), a chemotactic cytokine which mediate LPS-induced responses, and the Glia Maturation Factor Gamma (GMFG), a member of ADF-gelsolin superfamily, which seems to be involved in actin cytoskeleton remodelling and TLR4 endocytic pathway in response to LPS. We identified in *H. medicinalis* two genes coding for products showing high similarity with MIF and GMFG of Vertebrates, respectively. Immunolocalization experiments show a weak expression of both these proteins in the leech CNS whereas a stronger signal is detected in peripheral tissues macrophages. Further studies are needed to assess the expression levels of these molecules in leech tissues. However, this work shows that these molecules are good selective markers of activated macrophages in *H. medicinalis*, confirming the close correlation between the leech and vertebrates. Moreover, these results suggest the possible presence of more well-conserved molecules across evolution and represent an interesting starting point to analyze the complex crosstalk occurring during the innate immune response as well as the neuroimmunity processes.

Francisella*-like endosymbionts, potentially harmful to human health, are transported by the universally distributed species of the ciliate *Euplotes

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Genome analyses of wild-type strains of two ecologically separated *Euplotes* species, *E. raikovi* living in temperate sea waters and *E. petzi* living in the polar seas, revealed that both host bacteria in their cytoplasm. These bacteria have been identified with facultative intracellular gamma-proteobacteria of the genus *Francisella*, which includes a number of closely related species well known as extremely infectious to a great variety of organisms. *Francisella tularensis*, with its four subspecies, is a specialized intracellular pathogen capable of infecting both invertebrate and vertebrate hosts, humans included; *F. noatunensis* is the etiological agent of the fish disease known as francisellosis, and its two subspecies well adapt to different temperatures of their hosts; the *Francisella*-like endosymbionts *Wolbachia persica*, together with the freely living generalists *F. philomiragia* and *F. novicida* cause diseases in humans with a compromised immune system. The *Francisella*

endosymbionts of *E. raikovi* and *E. petzi* have been successfully isolated and their genomes completely sequenced. They are genetically distant from one another and form two different clades in the *Francisella* phylogenetic tree, which are distinct from the all other well-established *Francisella* clades. The finding that *Francisella* has equally colonized polar and temperate-water species provides evidence that this bacterium is more common and widespread than previously hypothesized, and confirms that free-living *Euplotes* species and ciliates in general, with their worldwide distribution, may represent a natural reservoir of *Francisella* in every aquatic environment.

Lymnaea stagnalis ganglia transcriptional activity after LPS induced immune activation

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The mechanisms by which the neuroendocrine and immune systems communicate and influence each other from invertebrates to vertebrates are well known and are among the most exiting areas of research in biology. Moreover, environmental influences, such as inflammation or stress, play a key role in determining susceptibility to disease and in particular to nervous system linked diseases. Until now, studies regarding the genetic mechanisms underlying neuroendocrine and immune interactions have used rodent models, while invertebrate models have been used to a much lesser extent. Among gastropods, the freshwater snail *Lymnaea stagnalis* is emerging as an important model to study immune-neuroendocrine functions from an ecological, parasitological and immunological point of view. In the present research, *L. stagnalis*, was used as an invertebrate model to study neuronal responses to LPS induced immune activation. More precisely, we tested the hypothesis that transcriptional changes occur in molluscan neural cells in response to LPS. Adult snails were exposed to LPS after which *L. stagnalis* ganglia were dissected, RNA extracted and analyzed for expression levels of genes related to neural and immune plasticity, such as, AIF-1and HSP70. Preliminary data suggest that LPS induced immune and neural activity alters plasticity related gene expression.

Characterization and neurothrophic effects of leech microglia-released Extracellular Vesicles (EVs)

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The leech *Hirudo medicinalis* is a well-studied model in neurobiology because its Central Nervous System (CNS) spontaneously repairs after a mechanical lesion. This process, leading in a few weeks to the synapse regeneration and a complete functional recovery, is linked to the activity of microglial cells migrating to the injured area. In leech, a few hours after the injury the activated microglia release an impressive amount of extracellular vesicles (EVs) that appear to constitute an important element in the cross-talk between microglia and lesioned neurons. By differential centrifugation we separately isolated small (10 - 100 nm, exosomes) and bigger (100 - 1000 nm, ectosomes) EVs. We also investigated the amount of exosomes and ectosomes released by naïve vs. ATP-stimulated microglia. In order to assess the function of these microglia-released EVs we characterized their proteomic and RNA content and we started investigating their potential in neurite outgrowth. Proteomic analyses of leech vesicles revealed the presence of many proteins typically present in mammalian EVs, including several surface molecules, and the presence of specific elements in differentially-stimulated samples. Functional assays were performed to assess the neurothrophic role of microglia-released EVs on a mammalian neuron-like cell line (PC12). Neurite outgrowth was measured upon incubation with extracellular vesicles issued from leech microglial cells. Results show a significant increase in neurites outgrowth indicating that both leech exosomes and ectosomes can exert a neurothrophic effect on mammalian cells. The association of a specific neurothrophic phenotype with its protein and RNA signatures would help to understand the role of these microglial EVs in promoting CNS repair.

Session 5. Chairmen: P Venier, University of Padua, Padua, Italy and L Abelli University of Ferrara, Ferrara, Italy
Immune response in Molluscs and Cnidarians

Structure and distribution of Astakine in the organs of the freshwater snail *Pomacea canaliculata*

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The freshwater snail *Pomacea canaliculata* is an emerging pest in EU, and its immune system is a potential target for developing strategies of pest control. Circulating hemocytes represent the cellular component of the *P. canaliculata* immune system. *P. canaliculata* hemocytes originate in the pericardial fluid, and are maintained in the ampulla, which may act as a hemocyte reservoir. Astakine-1 is a hematopoietic cytokine first described in the crayfish *Pacifastacus leniusculus*, and recently described also in the insect *Lygus lineolaris* and in

the bivalve mollusc *Crassostrea gigas*. Bioinformatic analyses of a comprehensive *P. canaliculata* transcriptome demonstrated the presence of an Astakine 1-like molecule (Pc-Astakine) also in this organism. Pc-Astakine is 121 aa and contains a domain characterized by 8 cysteins with a conserved distribution pattern homologous to the vertebrate domain prokineticin. Further bioinformatic predictions suggest that the structure of Pc-Astakine may be similar to that one of *P. leniusculus* Astakine-1. The distribution of Pc-astakine gene expression was evaluated by qPCR. We have observed that all organs analyzed express Pc-astakine at detectable levels. However, high expression levels were detected in the ampulla, pericardial fluid and circulating hemocytes.

The data suggest that Pc-Astakine may have a wide range of functions, including the regulation of hematopoiesis and the modulation of inflammatory responses, as previously reported for the human Prokineticin-1.

Effects of LPS injection on Pc-astakine expression in the gastropod *Pomacea canaliculata*

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Astakine-1 is a prokineticin-containing factor and the first hematopoietic cytokine described in invertebrates. Astakine-1 was firstly retrieved in the freshwater crayfish *Pacifastacus leniusculus*, and recent experiments have demonstrated the presence of astakine-like molecules also in insects and molluscs, including the freshwater snail, *Pomacea canaliculata*. In control conditions Pc-astakine is expressed in several organs, especially in the ampulla (reservoir of hemocytes and potential district of hemocyte maturation) and in the pericardial fluid (i.e. the hematopoietic tissue). By mean of qPCR experiments, we have analyzed the effects of the injection of 50 µg LPS on the expression of the gene *Pc-astakine*. Our observations indicate that 24 h after the injection, the major modification of the *Pc-astakine* expression was evident in the anterior kidney, a potential hemocyte reservoir, in which the expression of the gene decreased to almost undetectable level. In the pericardial fluid, ampulla and circulating hemocytes, the expression of *Pc-astakine* dropped to less than 50 % with respect to the sham-injected control snails. The drop in the amount of mRNA detected by qPCR could reflect an increased rate of translation and consequent degradation of the available mRNA, rather than a decrease of the transcription rate. Similarly, in the bivalve *Crassostrea gigas*, it has been suggested that accumulated Cg-astakine transcripts are largely translated under some environment stress, including immune stimuli. On the whole, our results indicate that the expression of *Pc-astakine* and the translation rate of its mRNA may be influenced by immune stimuli, and support the hypothesis that Pc-Astakine may be involved in *Pomacea* hematopoiesis and/or may have immune-related functions, as well.

Myticalins: a novel family of linear cationic AMPs from *Mytilus galloprovincialis* identified by de novo bioinformatics analysis

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The *de novo* discovery of bioactive peptides by bioinformatics screening methods is undoubtedly experiencing a renewed impulse due to the increasing availability of genomic and transcriptomic data from non-model organisms. Marine invertebrates in particular appear to be a vast and still largely unexplored resource due to their adaptation to diverse challenging environments. All the antimicrobial peptides (AMPs) described so far in mussels (*Mytilus* spp.) display a structure stabilized by intra-molecular disulphide bridges. No linear AMPs, relatively widespread in other invertebrates, have been identified so far, arguably due to the fact that they are encoded by taxonomically-restricted genes with no sequence homology to other known AMPs. Here we describe myticalins (*Mytilus* cationic linear AMPs), a novel family of AMPs produced as pre-propeptides and characterized by the absence of cysteine residues and by a positive net charge. Myticalins have been fully identified through a bioinformatics approach with no prior knowledge concerning their biological activity, by scanning the *M. galloprovicialis* transcriptome for potential AMP sequences, based on isoelectric point and amino acid composition. Although these AMPs appear to be largely diversified, they can be categorized into 4 main subclasses (A, B, C and D). We selected myticalin A-5, whose mature peptide sequence presents several Pro-Arg-X repeats, and chemically synthetized the predited mature peptide sequence. The synthetic peptide displayed a significant antimicrobial activity both against Gram+ and Gram- bacteria, validating the effectiveness of our de novo approach. Differently from other mussel AMPs, myticalins are mainly expressed in the gills tissue. Although many aspects concerning the biological activity of myticalins remain to be clarified, they represent a promising class of peptides with potential biotechnological applications.

Antiviral immunity in oysters infected by Ostreid herpesvirus-1

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Marine bivalves do not possess an acquired immune system that allows selective responses based on immunological memory. Conversely, bivalves are evolutionary successful species that live in an environment rich of viruses and bacteria, thus meaning that they have effective defense systems against pathogens. Ostreid herpesvirus type 1 (OsHV-1) is characterized by a 207 kb dsDNA genome and has been associated to sporadic mortalities of several bivalve species since mid '60. In 2008, a new OsHV-1 genotype called µVAR was reported to be the causative agent of massive mortalities of young oysters in France and, in the following years, its presence was reported worldwide. Today, OsHV-1 has become a problematic infective agent for the Pacific oyster *Crassostrea gigas*. Genetic susceptibility and critical chemico-physical conditions could facilitate the overwhelming of the oyster immune system and result in an uncontrolled viral replication (lytic phase). Parallel sequencing of host and pathogen RNAs (DualRNA-seq) has the potential to reveal the host-pathogen interactions during infection. In detail, the availability of a *C. gigas* sample (Goro lagoon, North Adriatic Sea, Italy) highly infected by OsHV-1 µVAR (up to 8.4×104 copies/ng DNA) allowed us to analyze both oyster and viral transcriptomes. Owing to the high sequence coverage we were able to describe the complete genome of an Italian virus genotype with strong pathogenicity. In the analyzed oyster sample, we found genes with similarity to elements of the vertebrate interferon pathway and several other defense genes. Overall, these facts indicate that oyster possesses an antiviral-specific immunity, mainly based on an interferon-like pathway, RNA interference and programmed cell death.

Voltage-gated sodium channels neurotoxin from tentacles of sea anemone *Actinia equina* exhibits cytolityc activity

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Sea anemones are sessile benthic organisms and their evolution has led to a high specific richness in the course of millions of years. The presence, diversification and multi-functionality of the toxins may have played an important role in the ability of colonization and adaptation to ecological niches that change over time. Venoms include peptide and protein toxins as 3 to 5 kDa neurotoxins acting on voltage-gated sodium channels, 3.5 to 6.5 kDa neurotoxins acting on voltage-gated Kv1 potassium channels, 20 kDa pore-forming cytolysins inhibited by sphingomyelin and serine protease inhibitors belonging to the Kunitz-type family. Type 1 and 2 Na^+ channel toxins are composed of 46 to 49 amino acid residues, except for *A. equina* toxin AeI of 54 residues and cross-linked by three disulfide bridges. In this study toxic components from acid tissue tentacles extracts of sea anemone *A. equina* were investigated by size exclusion separation to characterize cytolytic molecules of low molecular weight. Tentacles extracts with low molecular mass were filtered through a membrane (10 kDa Nanosep device). Sample was subjected to size exclusion chromatography using BioSuite 250, 10 µm SEC, 7.5×300 mm column on a HPLC system and collected fractions were concentrated through micro-concentrators to be tested for lytic activity. After electrophoretical analysis of lytic fractions, bands were excised and sent to sequencing by N-gel digestion and MALDI TOF analysis. Database searching and blast analysis allowed to identify a protein of 5,5 kDa molecular weight correspondent to the Ae1 toxin from *A. equina*. Then, the peptide AeI was synthesized from GenScript (Chemical peptide Synthesis service) and it was assayed for its hemolytic activity using the sheep erythrocytes (RBCs) by a spectrophotometric quantization assay of percentage of hemolysis. Lysis was recorded after 1 h of incubation in polyethylene tubes and 58 % and hemolysis was found when the targets were put in contact with the neurotoxin AeI. Current studies concerning the mechanism of action of the molecule and its structure are carrying out. The discovery of cytolytic activity in addition to neuro-inhibitory effect shows the evolutionary trend to combine two functions in one compound: ion channel inhibitor and membranolytic activity.

From cnidarian immunobiology to cultural heritage applications

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The study of cnidarians immunity, as model systems of metazoans, lead additional information on the first steps of the immunity evolution. The functions of the genes and cellular pathways in higher vertebrates are conserved during the evolution of metazoans, as shown by the discovery of homologues in cnidarians. These basal metazoans in fact, are far from "simples" in the range of methods at their disposal to deal with potential prey but also invading microbes and pathogens. They can give information about the invertebrates innate immune repertoire. We investigated the immunobiology starting from the inflammatory response in *Anemonia sulcata* (Cnidaria: Anthozoa) following injection of substances different in type and dimension, to understand the effector mechanisms involved in this

process. We observed clear, strong and specific reactions especially after injection of bacteria and the alteration of the expression of enzymes (protease, phosphatase and esterase), showing a correlation between the appearance of the inflammatory reaction and the modification of enzymatic activities. From cnidarian phylum a large number of toxic compounds have been isolated. Tissues and mucus produced by cnidarians may have a role in immune defense and contain a variety of toxins as neurotoxins, cytolysins and antimicrobial peptides, which can have multifunctional role. The bioactive molecules were purified by acid extraction and HPLC purifications and characterized through biological assays, mass spectroscopy and peptide synthesis. Here, we show the cnidarian bioactive molecules as antimicrobial peptides and enzymes in order to draw applications in fields ranging from pharmacology to cultural heritage. Particularly, in the control of the microbic growth and especially in the tuning of biocleaning protocols, bioactive molecules with proteasic and esterasic activity have been used. These novel enzymes are active at temperature lower than 30°C, they need a reduced time of application and are safety for both operators and environment. Thus they could provide an important contribution to the development of sustainable innovative protocols.

Session 6. Chairmen: D Malagoli, University of Modena and Reggio Emilia, Modena, Italy and M Cammarata, University of Palermo, Palermo, Italy

Hotspots in metazoan immunity

Lecture

Insights into the evolution of dendritic cells

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The development in gnathostome vertebrates of an adaptive immune system, that guarantees fine discrimination of antigenic determinants, also involved main specializations of antigen-presenting cells (APC), which govern presentation to lymphocytes of antigens associated to self and non-self MHC molecules. The dendritic cells (DC), discovered first in mammals and later classified as conventional, plasmacitoid, interdigitating (IDC), follicular, intra-epithelial (*e.g.,* Langerhans cells), dermal or tissutal, fall into the APC category.

However, information about their appearance and evolution in vertebrates is still limited and fragmentary. DC were indirectly (high levels of MHC II molecules) hypothesized in Chondrichthyes, whereas are much better documented in Teleostei (Actinopterygii Osteichthyes), paradoxically more than in heteroterm Tetrapods. Studies in rainbow trout, zebrafish, Atlantic salmon and European sea bass (Esb) convincingly reported about DC, at cytological, molecular (specific surface markers, cytokines and CpG receptors, lectin-induced agglutination) and functional (phagocytosis, migration, MHC-restricted antigen presentation, cytokines release) levels. The best characterized populations recall conventional DC, but studies in Esb additionally detailed IDC occurring in developing and adult thymus, likely engaged in thymocyte selection processes. Furthermore, recent studies in rainbow trout identified MHC II+CD8-α+ DC in the skin. This finding prompted to advance the hypothesis for a common origin for all mammalian DC that may exert antigen cross-presentation.

Germ line dynamics in bivalve molluscs

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Studies about germ line specification in Mollusca have been neglected for years. Available data based on few species indicate that molluscs exhibit the same cleavage mechanism of other spiralians, in which much of the mesoderm comes form the 4d blastomere, including the germinal tissue, whose specification has been identified by localizing the products of vasa orthologs (VASA is highly conserved in the animal kingdom). Despite the relevance of bivalve molluscs in marine ecosystems and aquaculture, their mechanism of sex determination is still unknown, as well as the details of their seasonal gonad reconstitution (*i.e.,* the gonad is reabsorbed after spawning and reconstructed at the beginning of the subsequent reproductive season). In order to determine general features of bivalve germ line development, we employed two antibodies produced against the VASA ortholog of *Ruditapes philippinarum* (Subclass Heterodonta, Family Veneridae) to investigate three additional species. We chose two species of the Subclass Pteriomorphia, *Scapharca inaequivalvis* (Family Arcidae) and *Crassostrea gigas* (Family Ostreidae), and another species of the Subclass Heterodonta, *Mya arenaria* (Family Myidae). The immunoreactivity of anti-VASA was confirmed by Western Blot in which single specific bands were obtained in each species, although of different molecular weight. On the other hand, the presence of two different bands in males and females of the gonochoric *R. philippinarum* has been previously related to sex-biased isoforms, already found in other animal species outside bivalves. The detection of a single band in the two Pteriomorphia may be tentatively related to their state of proterandric hermaphrodites. *M. arenaria* is reported to be gonochoric, as *R. philippinarum*, but the analyzed specimens were all females, so we cannot exclude the existence of different sex-related isoforms, although no sign of a second band was present. The immunohistological data obtained support a conserved mechanism of proliferation of primodial germ cells (showing the VASA labeling at one side of the cell cytoplasm) among the simple columnar epithelium of the gut. Moreover, their seasonal migration to the reconstituting gonad appears to be a common feature of bivalves. The

study of bivalve reproductive biology can clarify important aspects of their development, but can also be useful for conservation management and breeding programs.

Managing of *Procambarus clarkii* by X-ray sterilisation of males: immunological competence after the irradiation

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Procambarus clarkii (Girard, 1852) is an invasive alien species spread worldwide. The Sterile Male Release Technique has been chosen in Friuli Venezia Giulia as part of a strategy to control the red swamp crayfish. This technique consists on the release into the environment of sterile males which are sexually active and able to compete with untreated males for mating partners. Recently the gonad damage induced by ionising irradiation were described (Piazza *et al*., 2015), but no data are available on the immunocompetence of males after irradiation. The present study describes the hemocytes of *P. clarkii* males 20 days after irradiation at a dose of 40 Gy by means of light and transmission electron microscopy. Total (THC) and differential number of hemocytes (DHC) and activity of basal (PO) and total plasmatic phenoloxidase (pPO) are also evaluated. Untreated animals (CTRL), unirradiated animals injected with sterile saline (PBS), and carboxylated polystyrene latex beads (LTX) were used as controls. Three types of circulating hemocytes were characterized: granular hemocytes (GH), semigranular hemocytes (SH) and hyaline hemocytes (HH). Irradiated animals present highly significantly lower THCs (35357 ± 9643 hemocytes/mL, n = 7) in comparison with CTRL (666818 ± 78546 hemocytes/mL, n = 11), PBS (1006071 ± 184413 hemocytes/mL, n = 7) and LTX (368437 ± 98895 hemocytes/mL, n = 8) challenged ones. Irradiated animals show significantly higher GH percentages (48.57 ± 6.2) in comparison to all controls (CTRL: 17.97 ± 2.1; PBS: 29.91 ± 3.2; LTX: 21.89 ± 3.4), but significantly lower HH percentages (17.55 ± 5.2) in comparison to controls (CTRL: 59.73 ± 4.9; PBS: 65.83 ± 2.7; LTX: 67.57 ± 4.0). The comparison of PO and pPO activities among different groups do not evidence significant differences. The literature documents a survival of males irradiated with a dose of 20 Gy at laboratory conditions of at least one year (Aquiloni *et al*., 2009). The present study reports a decrease of about 95 % of circulating hemocytes and an increase in percentage of GH in males irradiated with a dose of 40 Gy after 20 days. Interestingly, the activity of PO and pPO are not affected by ionising radiation. The decrease in circulating hemocytes is consistent with the cytological damages to the gonads that are described as progressive from the day of irradiation up to 30 days (Piazza *et al*., 2015). It remains to be seen how long and how animals are able to offset this hemocyte decline and if this would preclude the survival in the wild.