

Haematopoietic and proteomic responses to wounding stress in the common sea star *Asterias rubens*

Akademisk avhandling

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Dissertation Abstract

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Many species of echinoderms have a remarkable ability to regenerate lost tissues, including the sea star *A. rubens*. The initial step in regeneration is wound healing, necessary to prevent disruption of body fluid balance, and to limit the invasion of pathogens. Injury initiates an immune response, where the circulating cells are activated. In general, invertebrates have a well developed innate immune system that is mediated by circulating blood cells. In the sea star these kinds of cells, the coelomocytes, respond with a rapid and massive accumulation at the wound site.

The aim of this thesis was to identify and localize the haematopoietic tissues, the source of stem cells for renewal of the coelomocytes, as well as to increase knowledge of response to wounding, with focus on the coelomocytes and their protein expression, in the common sea star *A. rubens*. Synergistic effects of hypoxia were also investigated.

It could be concluded that cells in the coelomic epithelium respond with proliferation when triggered with mitogenic factors and show a protein expression pattern very similar to the pattern of circulating coelomocytes. The shape and behaviour of cells migrating out of the coelomic epithelium show high similarities with the behaviour of coelomocytes, in terms of phagocytosis and network formation. Tiedemann body and axial organ are also proposed as haematopoietic tissues, since a significant increase in proliferation was seen also in these tissues after triggering with mitogens as well as a pattern of protein expression similar to coelomocytes.

Total coelomocyte count (TCC), as well as the expression of the heat shock protein (HSP) 70, is known to increase with severe stress in sea stars. The stresses investigated here, wounding together with hypoxia, induced a significant increase in TCC even after 1 hour while 6 hours after wounding TCC had increased approximately two-fold. Western blot analysis revealed highly elevated coelomocyte cytoplasmic HSP70-expression 3 hours after wounding. Non-wounded sea stars exposed to hypoxia and wounded animals kept in normoxia, showed enhanced HSP70 expression only after 24 hours. This synergistic stress response of wounding together with hypoxia may suggest ecological consequences, since the hypoxic areas in the ocean are growing.

Protein fractions separated by size from the coelomic fluid of wounded *A. rubens*, had slightly different effects on coelomocytes/haemocytes from three groups of invertebrates, the mollusc *Mytilus edulis*, the tunicate *Ciona intestinalis* and the echinoderm, *A. rubens* itself, where also effects on explants of coelomic epithelium were examined. The fraction containing proteins of the size 15-70 kDa showed a significant cytotoxic effect on the sea star coelomocytes and tissue samples, but seemed not to be cytotoxic for mussel or tunicate cells. This fraction, with smaller proteins, may contain cytokine-like molecules such as interleukin (IL)-1 and IL-6, but also the invertebrate form of lysozyme, molecules previously described in the sea star.

The two-dimensional gel electrophoresis method was developed for analyzing the protein content of coelomocytes. The methodology was optimized in terms of sample preparation, pI interval, gel gradient and staining procedure. The analysis of protein spots using MALDI-TOF/TOF mass spectrometry resulted in 9 identified protein homologues out of 18, of which 6 were found significantly up- or down-regulated. Sample preparation and methodological choices can and should be developed depending on the purpose of a study. The protocol developed here will be useful in future proteomic studies, maybe also for other marine organisms. The databases searched included the sea urchin *Strongylocentrotus purpuratus*, but the search result show no closer relationship between *S. purpuratus* and *A. rubens*, than to other invertebrates or even vertebrates within the database. Future proteomic studies of *A. rubens* may give valuable information about the wound healing and regeneration processes in sea stars as well as in other animals including humans.

Keywords: echinoderm, sea star, *Asterias rubens*, coelomocyte, TCC, haematopoietic tissue, HSP70, 2-DE, mass spectrometry

Det mesta vet man inte.

Björn, 1999

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List of papers

This thesis is based on the following papers which are referred to in the text by their Roman numbers:

- I** **Holm K., Dupont S., Sköld H., Stenius A., Thorndyke M. and Hernroth B.** (2008). Induced cell proliferation in putative haematopoietic tissue of the sea star, *Asterias rubens* (L.). *J Exp Biol* 211, 2551-2558.

- II** **Holm K., Hernroth B. and Thorndyke M.** (2008). Coelomocyte numbers and expression of HSP70 in wounded sea stars during hypoxia. *Cell Tissue Res.* Published Online.

- III** **Holm K., Voronkina I., Sharlaimova N., Thorndyke M. and Hernroth B.** Functional properties of sea star coelomic fluid proteins. Manuscript.

- IV** **Holm K., Björhall K., Hernroth B. and Thorndyke M.** Proteomic analysis of sea star coelomocytes. Manuscript.

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Introduction

Asterias rubens, the common sea star

The sea stars are members of the phylum echinodermata (Fig. 1), and belong to the deuterostomes that also include the vertebrates. The echinoderms have no heart, no centralized nervous system, no respiratory organs and no specialized excretory organs (Fig. 2). They are exclusively marine and most species are unisexual (Dahl, 1972).

Asteroids, sea stars, have an open body cavity and three additional circulatory systems, with poorly understood functions. One of them, the water vascular system, is used for locomotion achieved through hydrostatic and muscular contractions of the tube feet. Respiration is carried out directly across the outer epidermis and probably also through the tube feet. Except for the eye spots, light-sensitive cells on the tips of the arms, do sea stars not have any specialized sense organs (Ruppert and Barnes, 1994).

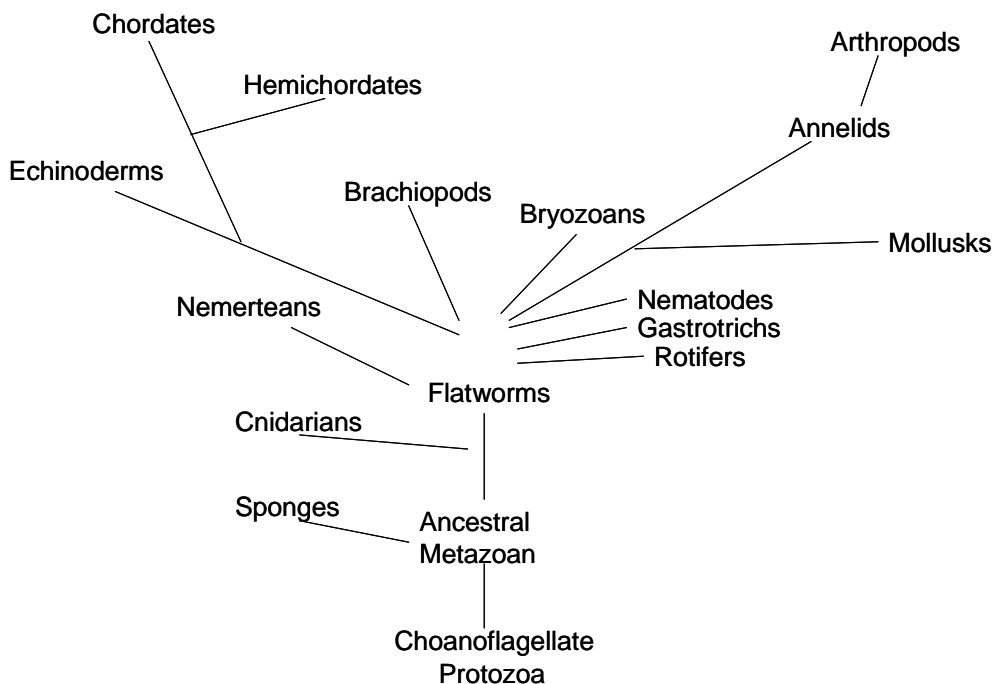


Figure 1. Evolutionary tree (after Ruppert and Barnes, 1994).

The common sea star *A. rubens* (Fig. 3) is a predator and scavenger, with blue mussels as the main prey item. When the sea stars are ready to spawn, the gonads fill the arm cavities and the gametes are released directly through the body wall, with fertilization in the open water (Dahl, 1972). *A. rubens* is found from the surface down to a depth of 200 meters and it reaches an age of 7-10 years. The color is highly variable, from orange and brown to blue and purple. They can reach the size of half a meter in diameter, where the size depends not only on age but also on food availability and probably quality of the food. As usual among

the echinoderms, *A. rubens* has a pentamerous symmetry, with identical organs in all arms. It can move with a speed of 3-5 meters per hour. Predators of *A. rubens* are the sea star *Crossaster papposus*, gulls, fish and crabs (Dahl, 1972).

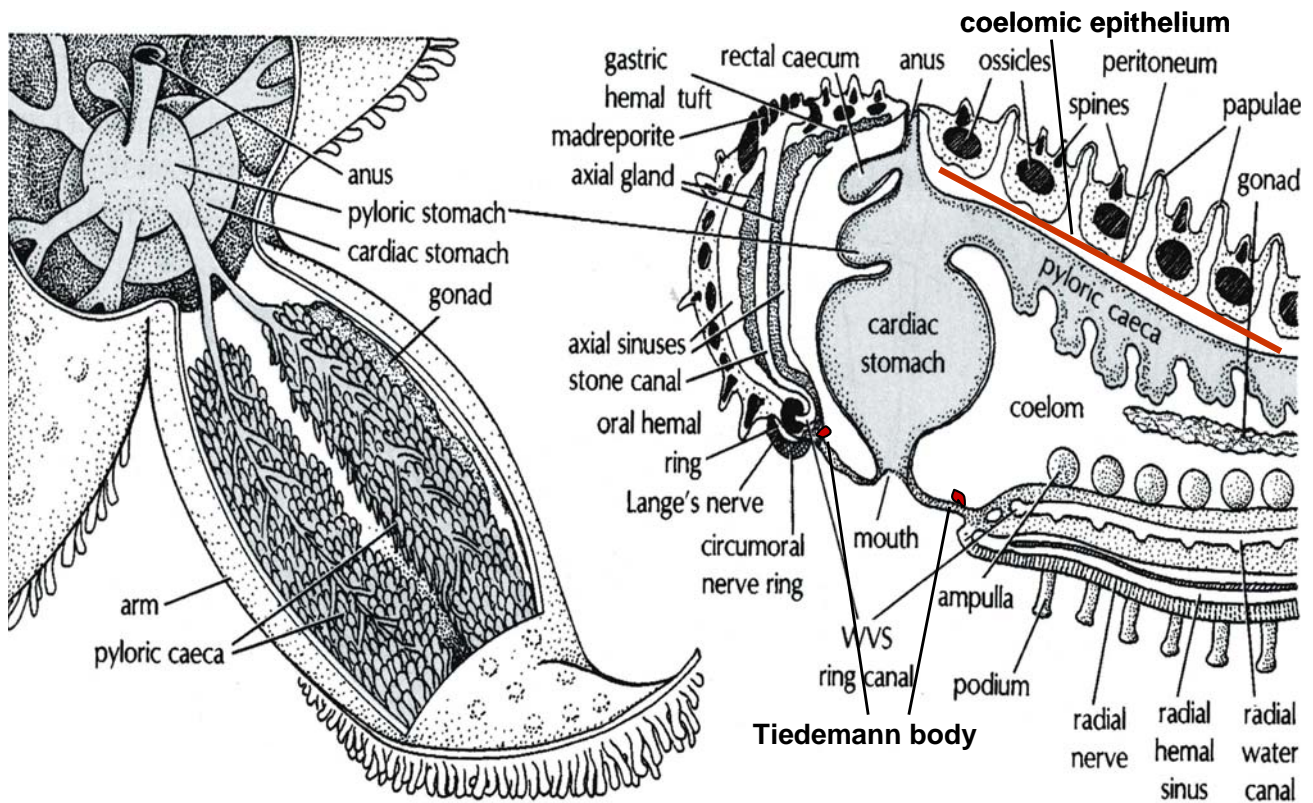


Figure 2. The inside of the sea star, including the tissues examined as HPT, coelomic epithelium, Tiedemann body, axial organ and pyloric caeca (modified after Pechenik, 2000).

Sea stars are easily collected and maintained in the laboratory and have a remarkable capacity for regeneration. These combine to make the common sea star *A. rubens* a very useful research animal for the exploration of key issues in wound healing, immunology and regeneration research.

Regeneration and wound healing

The ability to regenerate, re-grow lost or damaged tissue, exists in many groups throughout the animal kingdom, for example in insects, fish, spiders, amphibians, reptiles, crustaceans and worms (for review see Maginnis, 2006). Regeneration is particularly common in echinoderms, where it is very dramatic and extensive, perhaps more than in any other group (Candia Carnevali and Bonasoro, 2001A). All animals have a response to injury or damage, and are able to heal a wound, but there is a large variability in morphological and physiological recovery, even between closely related species and for different organs of the

same individual (Candia Carnevali and Bonasoro, 2001A). The regenerative abilities amongst different animal groups and species are not completely understood and there are many remaining questions concerning regeneration.

For some echinoderms, as well as in other animal groups, the sacrificing of body parts is a way of escape from predators. The sea star has an autotomy plane, close to the central disc, where it can “release” the attacked arm. *Asterias* has a single autotomy plane for each arm, while other echinoderms have the capacity to autotomize at various points along the whole arm length (Wilkie, 2001).



Figure 3. The common sea star *Asterias rubens* with three regenerating arms. (Borrowed with kind permission from Mike Thorndyke).

Regeneration for some species is a cloning strategy, a way of asexual reproduction with examples as the sea star species *Linckia sp.* and *Coscinasterias sp.* which can re-grow a complete new animal from one arm. An adult sea star of the family *Asterias* does not have this extensive capacity but is capable of survival and regeneration, if up to one fifth of the central disk remains attached to an arm (Ruppert and Barnes, 1994).

Traditionally, there are two alternative ways to describe the mechanism of regeneration; epimorphosis and morphallaxis. In epimorphosis, stem cells or de-differentiated cells form a blastema at the wound site, where the proliferation occurs and from where the new tissue regenerates and differentiates. In morphallaxis in contrast, no blastema is formed. The cells that take part in tissue regeneration are recruited from the surrounding differentiated tissues

or through migration, and are rearranged in the wounded area, and only a limited proliferation occurs at the wound site (reviewed in Candia Carnevali, 2006). Within the echinoderm group, both epimorphosis and morphallaxis occur, and it seems that in species with a rapid and effective regeneration, for example brittle stars and feather stars, epimorphosis occurs, with a fast proliferative blastema. The morphallactic process seems to be more complex and slow, and is seen more obviously in sea star regeneration. However, it has now been shown that these two mechanisms are not discrete, the system is in many species much more flexible and epimorphosis and morphallaxis overlap each other, with both possible in the same individual (Candia Carnevali and Bonasoro, 2001A ; 2001B)

The basic description of how the new tissue forms in *A. rubens* has been described by Moss et al, (1998). By following proliferation over time they were able to follow cell cycle activity in the regenerating tissue. The first week was primarily a wound healing phase, with accumulation of cells at the wound, and just a little proliferation. At the end of the second week, proliferation increased, located to the distal part of the arm, the tip. From the fourth week, the regenerating arm started to regain the normal structure. The coelomic epithelium was the tissue with the highest proliferation. These cells and coelomocytes are a potential source of progenitor/stem cells, which move into the tip and give rise to the new tissue. The coelomocytes did not show any sign of proliferation even within the regenerating area, but it was concluded that the coelomocytes may be incorporated in regenerating tissue before they start to proliferate. Knowledge about potential dedifferentiation or transdifferentiation of cells involved in the wound healing and later regeneration in these animals is scarce.

It has been suggested that the nervous system has great importance for successful regeneration of any kind (Brockes and Kumar, 2005). This is probably due to the production of growth factors of nerve cells, since it has been found that a single protein was able to induce regeneration in absence of the nerve (Kumar et al, 2007). Some of the genes involved in echinoderm regeneration have been also been described, such as the *Hox* genes (Thorndyke et al, 2001)

The initial step in regeneration is wound healing, necessary for all animals to prevent disruption of body fluid balance, and to limit the invasion of pathogens. An injury initiates an immune response, where the circulating cells are activated. In echinoderms, it is evident with a rapid and massive accumulation of coelomocytes at the wound site, which plug and heal the wound and help to maintain homeostasis (Smith, 1981). Invertebrates lack the acquired (adaptive) immune system and do not express the lymphoid cell line (antibody producers), but they have a well developed innate immune system. The circulating coelomocytes mediate this immunity and are the key players in clotting reactions, phagocytosis, encapsulation, nodule formation and release of factors for humoral defense (Smith, 1991).

The molecular differences between wound healing, scar formation, and regeneration capacity among species and different developmental stages is not clear and finding the key molecules

would be a major step in fundamental biological understanding of these processes (Gurtner et al, 2008).

Proteins and protein expression

Protein expression is the amount of all proteins in a cell or tissue at a certain moment. A change in the protein expression, that one or more proteins are up- or down-regulated, demonstrates a reaction, a change of activity in the cell, e.g. a stress response. Protein expression is often complex, humans may express 50000 proteins in total (Brändén, 2001). Knowledge about protein expression in invertebrates is scarce.

The behavior of cells in the body is coordinated through cell communication, with release of signal molecules from the signaling cell, and receptors on the target cell. Proteins are common as signaling molecules, and are in majority of the molecules that influence cell growth, proliferation and survival. The same signal molecule, can also give different effects depending on the target cell (Alberts et al, 2004). Cytokines are low molecular weight proteins that stimulate or inhibit the differentiation, proliferation or function of immune cells, and include molecules such as interleukins (IL), transforming growth factors (TGF) and tumor necrosis factors (TNF) (Roitt, 1997). In echinoderms, signaling molecules such as bone morphogenic protein (BMP), nerve growth factor (NGF) and TGF- β -like molecules have been found (Candia Carnevali et al, 1998A; 1998B; Patruno et al, 2002; 2003; Bannister et al, 2005). In coelomocytes from the sea star *Asterias forbesi*, cytokine-like molecules IL-1 and IL-6, and the receptor for IL-1 have been found (Beck and Habicht, 1986; Beck and Habicht, 1996; Beck et al, 2000).

Heat-shock proteins (HSPs) are a large group of proteins with the sizes ranging from 10 kDa to 170 kDa. HSPs are highly conserved through evolution and HSP70 and HSP90 have been found in all organisms examined (Kiang and Tsokos, 1998). HSPs were first described as stress proteins and were named when found expressed during heat shock, but are now known to take part in a number of intracellular processes by acting as chaperones involved in protein folding (Becker and Craig, 1994; Buchner, 1996), in protection against apoptosis (Parcellier et al, 2003) and in inflammation, both sterile and septic (Quintana and Cohen, 2005; Bruemmer-Smith et al, 2001), as well as mediating extracellular effects on immune active cells (Browne et al, 2007).

In echinoderms, HSP70 increased in sea urchin coelomocytes in response to temperature changes, polluted seawater, UV-B exposure, acid pH and heavy metals (Matranga et al, 2000; 2002; 2006) and *A. rubens* has shown elevation of HSP70-expression in both coelomocytes and tissues due to wounding and during regeneration (Pinsino et al, 2007; Patruno et al, (2001). The 70 kDa HSP has also frequently been used as a biomarker for environmental stress (Hallare et al, 2005; Hamer et al, 2004; Lee et al, 2006).

Coelomocytes in Asteroidea

The coelomic fluid of *A. rubens* contains large populations of circulating cells, the coelomocytes. Three to four different morphotypes have been described for the sea star, with the phagocytes (also called amoebocytes), as the dominating type. The definition and the nomenclature of echinoderm coelomocytes is still not clear (Smith, 1981; Kozlova et al, 2006; Pinsino et al, 2007). The most recent descriptions by Pinsino et al, (2007), include four cell types; phagocytes, which constitute up to 95% of the coelomocyte population, with amoebocytes, vibratile cells and haemocytes as the remaining 5%. Kozlova et al, (2006), defined three main cell types in the coelomic fluid of intact sea star: agranulocytes (phagocytes), (55-80%) varying in size and form, granulocytes (15-45%), and small cells (up to 2 %) with a high nuclear-cytoplasmic ratio. Phagocytes are the main cell type studied, since it is the dominating kind, and these cells act by phagocytosis together with a fast clotting reaction, in responses to trauma such as injury. The other cell types have, so far, no defined roles in asteroids (Smith, 1981; Pinsino et al, 2007).

The total number of circulating coelomocytes, i.e. the total coelomocyte count (TCC), shows an individual variation in *A. rubens* of $3-9 \times 10^6$ cells/ml. The numbers of circulating coelomocytes of *A. rubens* seems to be quite stable despite changes in salinity and temperature, but elevated coelomocyte numbers have been found due to parasitism, wounding, exposure to manganese and as a response to injection of bacteria (Coteur et al, 2004; Pinsino et al, 2007; Oweson et al, 2008; Coteur et al, 2002).

Haematopoietic tissue

Knowledge of the recruitment of coelomocytes is poor and the haematopoietic tissue (HPT) of *A. rubens* has still not been clearly identified.

Haematopoietic tissue is a source of stem cells that give rise to all the blood cell types. For mammals and most other vertebrates, this tissue is the bone marrow. For invertebrates, these stem cells are located in other tissues or nodes, for example in the freshwater crayfish, as a thin tissue covering the dorsal side of the stomach (Söderhäll et al, 2003). Several different tissues have earlier been suggested to be haematopoietic tissues in sea stars, e.g. Tiedemann body, the axial organ and the coelomic epithelium, as well as self replication of the circulating coelomocytes.

It was Friedrich Tiedemann who first described the small appendages attached to the interradius of the ring canal of the sea star (Fig. 2). The Tiedemann bodies are present as four to five pairs, gland-like and their lumen opens into the water ring canal (Ruppert and Barnes, 1994). The tissue has been suggested to have a role in the control of hydrostatic or osmotic pressure in the body, as it has connections with the water vascular system. Tiedemann bodies

have earlier also been described as producing coelomocytes, but this has been questioned since the late 19th century (reviewed by Lawrence, 2001).

The coelomic epithelium, lining the dorsal part of the coelomic cavity throughout the animal (Fig. 2), has been suggested as the most probable source of the coelomocytes in echinoderms (Muñoz-Chápuli et al, 2005).

The axial organ is part of the haemal system, which has not been especially well explored. Experiments have shown transport of nutrients through the haemal system and the connected axial organ after feeding, even though the specific role for the axial organ has not been determined (Ferguson, 1984). Anteunis et al, (1985), proposed cells from the axial organ to be immune active cells.

The fast recruitment of coelomocytes due to stress, indicates the presence of a “storage” tissue or reservoir of mature coelomocytes. This site may be the same as a HPT, but could also be another tissue, with separate storage abilities.

Stress

Homeostasis keeps the physiological balance in cell, tissues and body for optimal function. Stressors may act to disturb and influence the balance and normal conditions. Examples of stressors are changes in temperature, oxygen level or salinity, pollutants, infections or injury.

Wounding

As described in the Regeneration and wound healing section, wounding induces an immune response, with the activation of several signaling pathways. Even for the sea star, with good regeneration capability and even an autotomy plane, wounding is probably a dramatic event and should be regarded as stressful and will trigger the immune system and a wound healing process.

Hypoxia

Hypoxia is defined as dissolved oxygen less than 2.8 mg O₂/l (<30%), (Diaz and Rosenberg 1995).

Hypoxic conditions are a growing problem on soft bottoms around the world. The most important reason is eutrophication as a result of human impacts, such as the use of fertilizers and burning of fossil fuels (reviewed by Diaz and Rosenberg, 2008). Hypoxic events may have consequences for bottom communities also after re-oxidization since the composition of functional groups can be altered. Species with high tolerance to low oxygen levels and species with shorter life cycle may be more competitive and resist hypoxic events (Diaz and Rosenberg, 1995; Wu, 2002).

It has been found that hypoxia has a negative influence on the reproductive and the regenerative capacities of soft-bottom echinoderm species such as *Amphiura filiformis*.

During moderate hypoxia of 19%, the regeneration ability decreased by 25% in length and 36% in area (Nilsson and Sköld, 1996). The arms of the brittle star *A. filiformis* is a large food source for e.g. bottom-living fish, and rapid regeneration is highly convenient for survival and reproduction for the individual. The regenerated tissues also contribute significantly to the biomass and nutrient turnover in benthic communities, and the ecological consequences can be large due to lower regeneration rate (Sköld et al, 1994). Asteroids such as *A. rubens* and *A. forbesis* have also been shown to be sensitive to hypoxia (Theede et al, 1969; Diaz and Rosenberg, 1995).

Aims of the thesis

In recent years interest in the immune system and the role of circulating cells in invertebrates has grown. Comparative studies have been conducted to understand invertebrate immune defence mechanisms, wound healing and regeneration processes, as well as evolutionary relationships to vertebrates. However there is still a great deal to explore in this area. The echinoderms provide a good link to the vertebrates since, as deuterostomes, they are evolutionarily close to vertebrates. Many species amongst the echinoderms have a remarkable ability to regenerate lost tissues and are therefore also interesting from the wound healing aspect. Wounding, by autotomy or injury, activates immunoactive circulating cells, the coelomocytes. The aim of this thesis was to identify the location of haematopoietic tissues, where the stem cells for the coelomocytes are located, as well as to increase knowledge of the response to wounding, with focus on the coelomocytes and their protein expression, in the common sea star *Asterias rubens*.

To meet this aim the following topics have been addressed:

- * The haematopoietic tissues:
 - Location and rate of proliferation

- * Cellular responses to wounding and hypoxia:
 - Responses measured as a change in total coelomocyte count and expression of stress proteins

- * Protein expression in coelomocytes and coelomic fluid in response to injury:
 - Functional properties of coelomic fluid proteins on tissues and coelomocytes/haemocytes
 - Coelomocyte protein expression and identification; Screening and methodological development

Methodological considerations

Cell- and molecular biology is a very large and rapidly developing research area, and some of the methods used here are widely relevant. Applying these sophisticated methods to new species, or even tissues, often requires further development and optimization to suit the specific organism or tissue. Developments from the original protocol, together with details of pilot studies, are therefore further explained here.

Cell count and cell separation

The coelomocytes are, as described earlier, very important cells from aspects such as wound healing, production of signaling molecules and the immune response. In addition to counting the coelomocytes and measuring protein content, attempts were made to examine changes in the cell population due to stress, and to separate the different kinds of coelomocytes for further identification, culturing and antibody studies.

The circulating coelomocytes were counted by using two different methods. A Bürker Chamber with manual counting was used for determination of total coelomocyte count (TCC) in **Papers I** and **III**, and in **Paper II**, the NucleoCounter from ChemoMetec A/S, Denmark, was used for TCC. This cell counter operates by loading 40 µl of lysed cells (lysis buffer provided by the manufacturer), into a disposable NucleoCounter cassette, pre-coated with the nucleus-staining dye propidium iodide. The stain enters only dead (lysed) cells. The fluorescence of propidium iodide is recorded by a camera and the image analysed by NucleoView software. It is possible then to see any clots and get an overview of the cells in the sample. The measured volume is approximately 1.5 µl and the result is presented as cell counts /ml. This cell counter has not earlier been tried with invertebrates, but was easy to use and showed a good agreement with the microscopical determination using the Bürker Chamber. The NucleoCounter was also tried with bacteria-contaminated samples, and the conclusion was that the NucleoCounter only counted the coelomocytes.

To investigate sub-populations of circulating coelomocytes, a particle counter (Hintze), which simultaneously counts the cell and measures cell size was used. The purpose was to investigate possible changes in the cell population before, compared to some hours after wounding. There were some difficulties encountered in keeping a constant flow rate, together with determining the exact flow rate, but otherwise it is a straight-forward method which could be useful especially in comparative studies when the exact cell number is not critical. In the pilot study performed, with determination of the size distribution of cells in a time series after wounding, no significant changes in the cell population due to wounding were found using this method (Fig. 4).

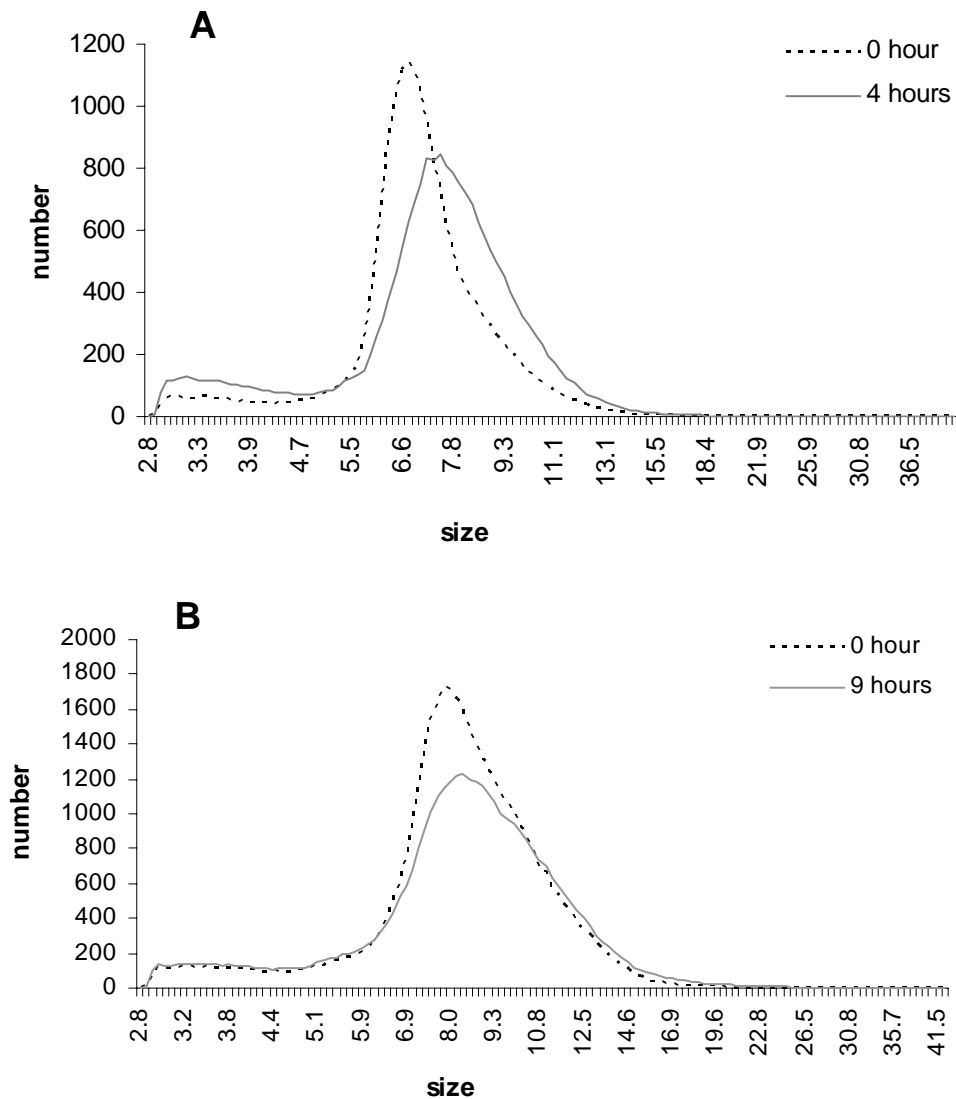


Figure 4. The pilot study regarding cell size of the circulating coelomocytes, before (0 h), and after 4 h (A) and 9 h (B) of wounding. The conclusions are that the sub-populations are not regulated due to wounding. The sample dilutions are not comparable, why focus should be on cell size and not cell count.

Another method used to determine changes in cell profile and possibly also to sort sub-populations of coelomocytes, was flow cytometry. The results from flow cytometry, using size (forward scatter) and granularity (side scatter) as cytometric parameters, showed a long cloud of cells without distinct sub-populations, and it was hard to draw any conclusions about the cell population from this study, but no changes due to wounding could be seen (Fig. 5). The results from these pilot studies are consistent with the result of Pinsino et al,

(2007), who did not find any changes in the cell population due to wounding, but it is in contrast to Kozlova et al, (2006), who found an increase of small cells after loss of large amounts of coelomic fluid in *A. rubens*. During wounding and sampling of coelomic fluid at time zero in the pilot study with flow cytometry, a larger amount of coelomic fluid was sampled and is comparable with bleeding in the study by Kozlova et al, (2006). Flow cytometry has previously been used on cells from *A. rubens*, using monoclonal antibody-labelled cells from the axial organ (Leclerc et al, 1993), and on coelomocytes, where injection of bacteria gave a significantly changed pattern in the sub-populations of coelomocytes, in terms of size and granularity (Coteur et al, 2002). This demonstrates that the method can be applicable to sea star coelomocytes, even though there are limiting factors in the use of flow cytometry for sea star coelomocytes, since the population of phagocytes dominate so much, and there are no known antibodies for marking the different sub-populations.

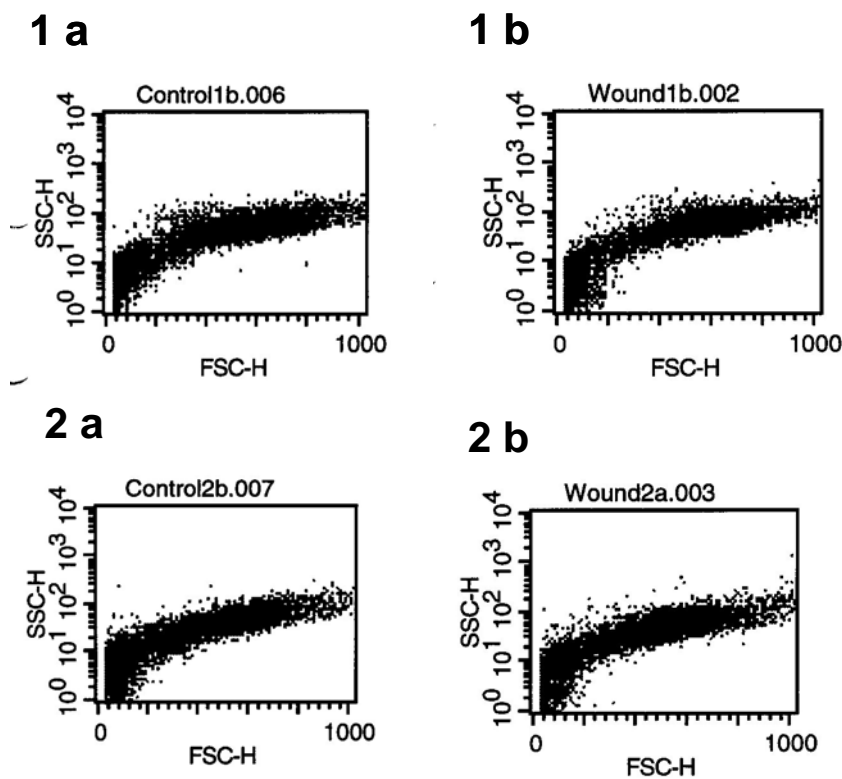


Figure 5. The pilot study on coelomocyte populations using flow cytometry. The cell population of two individual sea stars, 1 and 2, are shown, with a) representing control samples before wounding and b) representing 3 h after wounding. No change in cell population was found due to wounding.

Another commonly used method for cell separation is based on the density of the cells, separated in a fluid, e.g. Percoll (Amersham Bioscience), (Stolen et al, 1995). The separation is achieved by high speed centrifugation of coelomic fluid added on top of the Percoll fluid, diluted in a gradient. Sea star coelomocytes did not separate properly with this method. Since

phagocytes constitute such a large part of the total cell population, the other cell types are probably too few to give clear bands of cells. It was decided to exclude this method after recommendations from our collaborators who found that sea urchin cells lost their activity during the handling with Percoll (Annalisa Pinsino, personal communication).

The purpose of separating the different cell types in sub-populations was to increase knowledge about their functions, and to investigate the possibility of culturing the coelomocytes. However, because of the large proportion of phagocytes present and no clear distinction in size, granularities and density between phagocytes and the other cell types present, it is necessary to find molecular markers for the different cell types to be able to separate them. However, even so it is possible to keep the whole population of coelomocytes in short term cultures, and culturing of sea star tissue is very promising.

Immunocytochemistry

Immunological methods on cells were performed on a monolayer of cells using antibodies against the target molecules. This method is used both in **Paper I**, tracing dividing cells, and in **Paper II**, when localizing the expression of the stress-induced protein HSP70. In this method there are two steps that have to be considered; cell attachment to the surface, and labeling with antibodies.

The coelomocytes of *A. rubens* attach very well to surfaces (Pinsino et al, 2007). For the analysis of proliferation in **Paper I**, SuperFrost®Plus glass slides were used, and for analysis of HSP70-expression in **Paper II**, glass slides coated with poly L-lysine were used. Both types of slides give the cells a preferable surface for attachment, and the settling time was at least 10 min. When investigating the proliferation of cells in the potential haematopoietic tissues it was possible to release the cells by treatment with collagenase Type I and IV (**Paper I**), with further attachment to the glass slide. Of the tissues examined, it was only cells from pyloric caeca that did not attach satisfactory to the glass. This result further strengthens the view of pyloric caeca as a non HPT.

Another method to attach cells to glass slides, is to use a cyto-centrifuge that concentrates the cells to a closed area during centrifugation. With this method it is possible to obtain a more representative settling, both with a more even layer of cells and the possibility of including cells with lower or no phagocytic ability. This method was used in a pilot study for screening a battery of antibodies, for possible labeling of coelomocyte cell types. Preliminary results showed labeling of sea star cells with antibodies against toll like receptor 4 (TLR4) and Piwi, but further experiments need to be done for confirmation and before any conclusions can be drawn.

The two methods presented of applying cells to glass slides, have different advantages. When searching for cell markers with cross reactive antibodies, the cyto-centrifuge is highly

preferable, assuming that all kinds of cells are attached to the surface. In other cases, as when investigating the expression of HSP70, with the main type of coelomocytes attached to the slides and a study based on the comparison of treatments, the methodology of settlement of cells is satisfactory and easy to perform.

Working with immunological methods using antibodies not raised in that particular species, often includes uncertainties, such as specificities of antibodies. When no labeling is found with an antibody, it can be a genuine result, or it may be a non optimized method or a too large molecular difference. On the other hand, if there is a labeling with the antibody, there is a risk that the binding is not specific. All these factors must be taken into account.

Immunohistochemistry

Immunochemical methods were also used on tissue sections. For preparing the sections in **Paper I**, the original protocol could be followed for all tissues except for Tiedemann body, which was hard to identify after the fixation. It might have been better to use a colored fixative such as Bouin to visualize such small tissues. Smears of Tiedemann body were also performed but this was not satisfactory for antibody labeling, and these results were excluded in **Paper I**.

In tissue sections of *A. rubens*, a high background autofluorescence often quenched or reduced the specific fluorescence. It was found that an antibody conjugated with Texas red is preferable compared to an antibody labeled with FITC, since the Texas red fluorescence was much easier to distinguish from the background (**Paper I**).

Western blotting and 2-dimensional gel electrophoresis

Western blotting (WB) is used to identify proteins with the added advantage of the possibility of quantification. In **Paper II**, WB was used to quantify the expression of the stress protein HSP70. HSPs are evolutionary conserved proteins and have earlier been used to measure the stress response in both sea urchins (Matranga et al, 2000; 2002; 2006), and in *A. rubens* (Pinsino et al, 2007).

When sampling coelomocytes for the proteomic methods in **Paper II** and **Paper IV**, approximately 5 ml of coelomic fluid was collected and the cells were concentrated by centrifugation. For satisfactory sample preparation, it was concluded that an amount of 400 µl of buffer was enough to properly solubilize the proteins, but not too large to dilute the sample more than necessary. Sonication on ice for 4 X 10 sec, level 5, of the coelomocytes gave good result.

In our hands, WBs gels from Invitrogen worked the best. An additional coloring step with Ponceau after blotting was found optimal, providing a quick and easy way to ensure that the blot worked properly, and also provided an indication of the amount protein loaded in each well. The color of the Ponceau fades completely during the following antibody treatments. The last step during WB in the protocol used in **Paper II**, was the development of the signal of the horseradish peroxidase-linked antibody. The stronger ECL Plus Western blotting detection reagent (Amersham Bioscience) gives a shorter developing time than other kits tried, and is to be preferred if the signal is fairly weak.

In 2-dimensional gel electrophoresis (2-DE), the expression of a large number of proteins can be compared using size and charge (iso-electric point, pI). The separation in two dimensions gives a protein pattern as a map of the proteins in the sample, with, in the best case, one spot corresponding to one protein. Commonly, the method is used to compare the protein pattern of different treatments, using software analysis, with or without further identification of the proteins.

When starting with 2-DE experiments on an animal where this technique has never been used before, or even a new tissue, the protocol often needs to be modified. In sample preparation, the same procedure as for WB was used, but with a different buffer (described in **Paper IV**). In the case of the sea star coelomocytes, a cleaning step for the sample was required. The first dimensional run, the focusing, where the proteins are separated due to charge, is easily disturbed by e.g. salts, fatty acids or DNA. In this case it was likely to be salts, since sea star tissues have a salt concentration of around 3%, compared to 0.9% for vertebrates, but there can be more than one disturbing factor. Several cleaning procedures were tried, without much success, and since the sample was also somewhat low in protein content, a dilution of the sample could not be afforded. For example, using Float-A-Lyzer (Spectrum) gave too high dilution of the sample. Changing buffer using centrifugation and Ultrafree MC centrifugal filter devices (Millipore) created difficulties to collect the concentrated protein sample from the filter and to re-dissolve the proteins. ReadyPrep Sequential Extraction kit (BIO-RAD), is based on three buffers of different ability to dissolve proteins. After treatment with a first buffer, the remaining cell pellet after centrifugation is treated with buffer two, and the proteins most hard to dissolve, are after centrifugation treated with buffer three. The third fraction, with two previous buffer changes, should be very low in salt, but still there were problems with the focusing, even with fraction three. The DeStreak Reagent (Amersham Bioscience) did not purify the samples enough. Finally, satisfactory results were achieved using 2-D Clean up kit from Amersham Bioscience, which has the positive advantage that it is possible to concentrate the sample during the cleaning process.

After screening the coelomocyte proteins in the pI-range 3-10, different strips of narrower range were tested, and finally the pI-range of 5-8 was used, since a large majority of the proteins were located in this interval. By using a narrower strip a better separation of the proteins is obtained, but at the same time others outside the range could be missed. It is also

possible to loose proteins in the cleaning step, as well as proteins that are hard to solubilize, like membrane proteins. Some proteins may also, even if soluble, stay in the strip and never come out during the run of the second dimensional gel. The length of the gel was 11 cm, and a larger gel would have been preferable, since more than 400 spots could be detected on the gels with the largest amount of protein loaded from sea star coelomocytes (**Paper IV**). The choice of staining method should be based on the purpose of the experiment. Coomassie Brilliant Blue (CBB) staining was chosen to optimized identification of the proteins. Staining with CBB does not give the highest sensitivity, but it is compatible with Mass spectrometry (MS), used for protein analysis, and when a spot is detected by CBB, it is also enough protein to get a good result in MS. It would be interesting to do a screening with a more sensitive staining e.g. silver staining. If possible, the use of a fluorescent stain is a good choice. The possibility to run up to three samples labeled with different fluorescent markers in the same gel, together with a very good detection level makes the comparison between samples much easier (López, 2007).

Wild animals, even if collected at the same time at the same site, have a slightly different protein pattern between individuals. To be able to detect any changes in protein expression, it is therefore necessary to examine the same individual before and after treatment, thus the sampling in **Paper IV** was carried out using the same individuals, before and after wounding. This individual variation found using 2-DE has been described in the study by Diz and Skibinski, (2007), who found significant differences in protein pattern between individual mussels of the same species, sharing the same conditions. During sampling, a large proportion of the coelomic fluid was drained from the animal. The amount of fluid lost during natural wounding may not be as extensive as during sampling, and there is a risk that the effects found at least in part could be an effect of the loss of blood at the first sampling, rather than a response to the subsequent wounding.

Results and Discussion

The haematopoietic tissues

Recent studies on stem cells in sea stars have pointed to the coelomic epithelium as a possible haematopoietic tissue (HPT) (Muñoz- Chápuli et al, 2005), while earlier hypothesis also have included Tiedemann body and the axial organ.

Out of the five tissues examined as HPT, three responded with an increasing proliferation profile when triggered with a mitogenic compound. These were the coelomic epithelium, the axial organ and Tiedemann body (**Paper I**). The proliferation rate of cells from the tissues was measured four hours post injection, and there were significant increases in proliferation after injection with lipopolysaccharides (LPS) and/or concanavalin A (ConA) for these three tissues, with proliferation rates around 10%. In the longer term, after 16 and 24 hours post injection with LPS, proliferation decreased to the basic rate found in the control animals,

approximately 2%. The qualitative measurement of proliferation, by immunohistochemical analysis on tissue sections, showed proliferation after triggering with ConA in coelomic epithelium and axial organ, but not in pyloric caeca. These results are consistent with the quantitative results, using the released cells from the tissues. Tiedemann body was not examined using tissue sections.

The mitogenic factors LPS and ConA induce proliferation of fish cells (Sizemore et al, 1984), with LPS also inducing proliferation in crustacean cells (Van de Braak et al, 2002). The controls were injected with calcium- and magnesium-free saline buffer (CMFSS), and increased proliferation was found at 16 and 24 h post injection for all three tissues. This response could be from the wounding by the needle during injection. In the measurement of stress protein levels in the sea star coelomocytes (**Paper II**), there was an increase of the stress proteins HSP70 24 h after wounding, which indicates that stress response to non-severe stress could be quite slow. The increase in proliferation could also be a response to the volume of CMFSS injected that, even if sterile, may act as a trigger. Such a response has earlier been observed in crustacean HPT (Van de Braak et al, 2002).

By using confocal microscopy, the coelomocytes and cells released from the coelomic epithelium could be compared. Cells from the coelomic epithelium showed very large similarities in both shape and behavior with the circulating coelomocytes (**Paper I**). The most common form of coelomocytes, the phagocytes, are adhesive, phagocytotic, known to change shape to petaloid and filopodial forms, and form networks and clotting formations, when stimulated (Pinsino et al, 2007), where sampling of coelomic fluid without using an anticoagulant is enough as a stimulus. This change in shape and network formation, together with phagocytosis, could be followed from cells released from coelomic epithelium in the study in **Paper I**.

There is very rapid recruitment of coelomocytes after fluid loss in the sea star, probably too rapid to be due only to newly proliferated cells. It is most likely that “storage” (or a reservoir) of coelomocytes exists and the HPTs have been implicated. The coelomic epithelium is one of the most probable “storage sites” for coelomocytes, based on results concerning the shape and behavior of these cells using the confocal microscope. Together with the proliferation results, it seems that the coelomic epithelium is both a HPT and a storage tissue for circulating coelomocytes in *A. rubens*.

The other two putative HP-tissues responding, Tiedemann body and the axial organ, increased the proliferation when triggered (**Paper I**), but it is too early to conclude the role of these cells. The axial organ has been described as releasing cells into the coelomic fluid (Millot, 1969), and there is a hypothesis that Tiedemann bodies act as water cleaning organs for incoming seawater, holding a large number of coelomocytes (Ferguson, 1984). A complementary study of the shape and behavior of these cells would be of great interest. It may be speculated that there may be several tissues producing the same kind of cells, or that different tissues are producers for the different kinds of coelomocytes.

Finally, in the two other tissues examined in **Paper I**, pyloric caeca and circulating coelomocytes, the proliferation rate was around 1%, and did not change due to any treatment. In the case of the coelomocytes it may even be that the proliferation occurred before release. In other animal groups, like crustaceans, proliferation has been found among circulating cells (Van de Braak et al, 2002), and also maturation can occur in the circulation (Söderhäll et al, 2003). Pyloric caeca is not considered as a HPT, and was included as a control. This is a liver-like tissue located in all the arms of the sea star (Fig. 2) The proliferation of approximately 1% seen in this tissue is probably the normal renewal/turnover of cells, as no increase could be seen when triggered. When dissected, a large number of very small cells, of a size of 2-3 μm , are released from the tissue. The function of these cells is unclear.

In **Paper I** examination of the protein pattern has been carried out for the coelomic epithelium, axial organ, Tiedemann body, coelomic fluid and pyloric caeca. The protein pattern of the four first tissues showed large similarities in protein content, while the pyloric caeca gave a different pattern. This further strengthens the connection of coelomic epithelium, axial organ and Tiedemann body with the circulating coelomocytes.

In the search of stem cells and the haematopoietic tissues, features distinguishing human and other vertebrate stem cells have been considered. For example, human stem cells produce specific antibodies such as CD34, expressed on the surface of haematopoietic and pluripotent stem cells. The expression of CD34 is quite specific for early progenitor cells, and expression decreases with differentiation (Coico et al, 2003), but these markers are not reliable in invertebrates. For the Norway lobster, *Nephrops norvegicus*, the stem cell marker CD34 marked HPT cells, but also the circulating granular haemocytes (Bodil Hernroth and Irene Söderhäll, personal communication). Methodological limitations like these make it difficult to study these mechanisms in less explored organisms as sea stars.

The proliferation rate of the HPT in *A. rubens* is quite low, with a basic level of approximately 2% (**Paper I**), compared to e.g. the HPT in the *N. norvegicus*, which has a proliferation rate of 10% without trigger (Hernroth et al, 2004). The coelomic epithelium in *A. rubens* covers a large area, and this may compensate for a lower rate of proliferation of the stem cells.

It is established that the coelomocytes have important roles during wound healing, but haematopoiesis and the role of coelomocytes and stem cells in regeneration in the sea star is still not clear. Here, it was concluded that coelomic epithelium probably is a HPT and a storage tissue (reservoir) for coelomocytes, and that both Tiedemann body and the axial organ respond with proliferation when triggered. Further studies with coelomic epithelium, axial organ and Tiedemann body, together with cultures of these cells are needed. Short-term studies, up to three weeks, with sea star tissue and coelomocyte cultures have been performed (Irina Voronkina, personal communication), as well as functional studies of cell behavior (**Paper III**), with very promising results and good possibilities for future studies.

Cellular response to wounding and hypoxia

TCC

The total coelomocyte count (TCC) was examined in *A. rubens* following treatments with the stresses of wounding and hypoxia (**Paper II**). The combined stress of wounding together with hypoxia significantly increased the cell number already 1 h after injury, and even further after 6 h, with a more than 2-fold increase (Fig. 6). When measuring the TCC 1 and 6 h after wounding or hypoxia treatment alone, increasing trends were found but not as substantial as those seen with the combined stress. Even though Pinsino et al, (2007) found an increasing trend with wounding and repeated blood sampling of *A. rubens*, this large increase during the combined stress of wounding and hypoxia is very noteworthy. The increase of TCC during low oxygen levels found for *A. rubens*, is not in agreement with that reported for the crustacean *Callinectes sapidus*, where the numbers of haemocytes did not alter during a short time exposure (few hours) to hypoxia (Holman et al, 2004). There are certainly differences in tolerance to low oxygen levels and most probably this is also linked to exposure time. A long term exposure to hypoxia (5 days) in the freshwater prawn *Macrobrachium rosenbergii* induced a decrease in haemocyte numbers (Cheng et al, 2002).

Following injection of LPS, the TCC increased more than 2-fold after 4 hours, and 3-fold after 24 hours (**Paper I**). The increase following ConA injection was not as pronounced, but still with an increasing trend (Fig. 6). Similarly, *A. rubens* exposed to Manganese (Mn), gave a significant increase in TCC, after 5, 10 and 25 days of exposure (Oweson et al, 2008). A change in the total number of coelomocytes during stress is common, but in several other species there is a decrease rather than the increase in cell number seen for *A. rubens*. In a study on *N. norvegicus* exposed to Mn, the number of circulating cells gradually decreased in number, affected by time and concentration (Oweson et al, 2006). An explanation may be differences in cell population. Some cell types act through degranulation, and the cells may be sacrificed by the reaction, while sea star coelomic fluid contains mainly phagocytes, that act by engulfing and attaching, and may therefore remain intact after an immune response. It is notable, however, that the phagocytotic ability of *A. rubens* coelomocytes significantly decreased when exposed to Mn (Oweson et al, 2008), so even though the TCC number increases with Mn, the cells may not function as expected. This could be the case also during other elevated stress situations such as hypoxia, and should be very well worth further explorations.

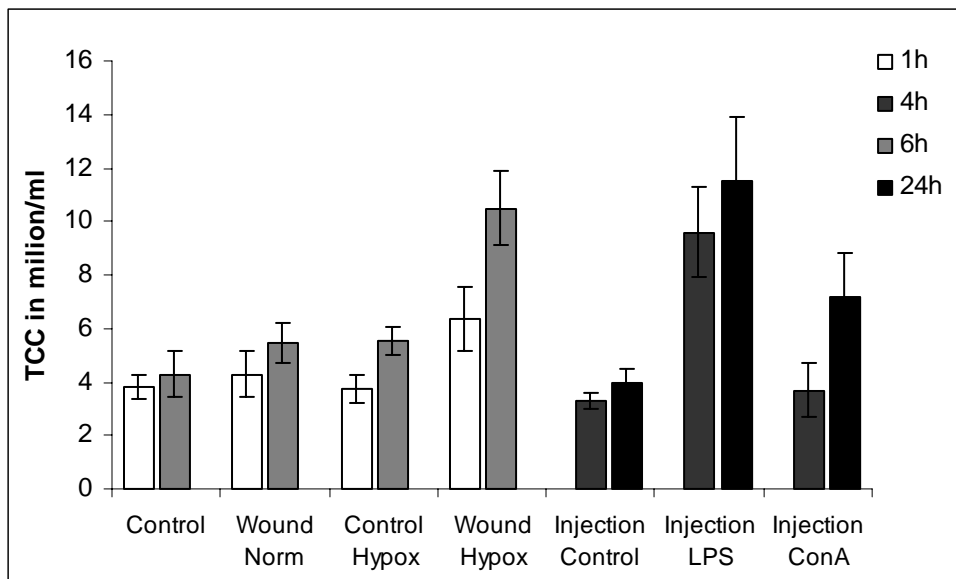


Figure 6. The total coelomocyte count (TCC) concluded from **Paper I** and **Paper II**, with the treatments wounded normoxia, control hypoxia, wounded hypoxia, injection control, injection LPS and injection ConA, at the different times. The error bars are showing St. Err. Significant increases of TCC were after treatment wounded hypoxia and injection with LPS.

It seems that TCC in *A. rubens* is not affected by changes in e.g. salinity or temperature, but tends to increase with more severe stress. The data in **Paper II** show a coelomocyte concentration of $2-8 \times 10^6$ cells/ml for non stressed animals, which agrees with that previously found, $3-9 \times 10^6$ cells/ml for *A. rubens* (Coteur et al, 2004). The un-affected numbers of dead coelomocytes determined for wounded and hypoxia treated animals in **Paper II**, further indicated that neither hypoxia nor wounding have lethal effects on the circulating coelomocytes.

HSP70

The rapid increase of TCC in response to hypoxia and wounding was accompanied by an up-regulation of the stress protein HSP70 (**Paper II**). During hypoxic conditions, the coelomocytes showed an elevation in HSP70 expression already 3 hours after wounding that after 6 hours was highly pronounced. When stressed with injury or hypoxia alone, there was a slower response, with an up-regulation later than 6 h after wounding, but before 24 h. The control group did not show any up-regulation, which also fits with the conclusions that sea stars are not affected by handling stress (Coteur et al, 2004). In comparison with Mn incubation, HSP70 expression in sea stars was not especially pronounced in free coelomocytes, but seen more in tissue sections of the coelomic epithelium after 5 days (Oweson et al, 2008).

HSPs are up-regulated due to changes in the environment that causes stress for the individual, and an expression of HSP70 was found in all sampled sea stars, with an individual variation. After treatment, the expression in each individual was always much

stronger (**Paper II**). It is also a possibility that HSP70 has several roles. In sea urchin embryos, a translocation of HSP70 from the cytoplasm to the nucleus has been observed (Roccheri et al, 1981), however, no translocation of HSP70 from the cytoplasm to the nucleus was found in sea star coelomocytes (**Paper II**). The HSP70-family consists of several forms e.g. the constitutive HSP73 and the inducible HSP72. In the study of Patruno et al, (2001), both forms, HSP72 and 73, were found in tissues of un-handled (control) *A. rubens*. The measurement showed that HSP72 in tissues was up-regulated during wound healing and regeneration up to four weeks after wounding, while HSP73 did not change. Chong et al, (1998) found that the constitutive HSP73 form was over-expressed in heat-treated rat heart derived cells and a translocation from cytoplasm into the nucleus was recognized. The examination of HSP in sea star coelomocytes in **Paper II**, could not distinguish between the different forms of HSP70. There is therefore a possibility that the noted elevation of the HSP70 family was only HSP72, and not HSP73, which is the form found to be translocated, and this may be an explanation of the differing results concerning translocation, compared to sea urchin.

The very diverse functions of HSPs also includes extracellular effects (Browne et al, 2007). In the investigations of the wounded sea star protein pattern in cell free coelomic fluid by 1-D (**Paper III**), there are bands in the region of 72 kDa, indicating the presence of HSP72. Extra cellular HSP70s have been shown to affect the spreading of immune-active coelomocytes in sea urchin (Browne et al, 2007) and an up-regulation of HSPs from an initial stressor has been shown to protect cells against a second stressor (Tedengren et al, 1999; Bond and Bradley, 1995). Many of the roles of HSPs in sea stars are still to be explored, as well as the mechanisms that induce the HSP-expression.

Wounding during exposure to hypoxia clearly stressed the sea star *A. rubens*, indicated by measures of elevated TCC and HSP70-expression. The synergistic stress response of wounding and hypoxia seen agrees with observations of the brittle star *Amphiura filiformis*, which showed a decrease in both regeneration and reproduction during hypoxic conditions (Nilsson and Sköld, 1996). It is not possible at this stage to interpret the consequences for e.g. regeneration and survival for *A. rubens* if exposure to severe and long periods of hypoxia, even though it seems to be a highly stressful condition.

Protein expression in coelomocytes and coelomic fluid, with changes in response to injury

Functional properties of coelomic fluid proteins

Fractionated cell free coelomic fluid from *A. rubens* has been shown to have different functional properties (**Paper III**). The proteins were fractionated by size, with the chosen fractions named Fraction 1, which contained proteins >95 kDa, Fraction 2 containing proteins in the range of 30-70 kDa, and Fraction 3 mainly proteins from 15-70 kDa. When tested on coelomocytes/haemocytes from three invertebrate species: *A. rubens*, the mollusc

Mytilus edulis (blue mussel) and the tunicate *Ciona intestinalis*, a difference was found both between fractions and between species. Viability was measured as the metabolism of cells and adhesive ability assessed as the numbers of cells attached to glass slides. The coelomocytes/haemocytes from the different species showed similar patterns for Fraction 1, and gave an increase in viability and a significant increase in adhesion for sea star and blue mussel, compared to the control. Fraction 2 gave a decreasing trend in viability for sea star cells, while no change compared to the control for mussel and tunicate. The largest species difference was found for Fraction 3. Significant differences between *A. rubens* and the other two species were found, where Fraction 3 showed a significant cytotoxic effect compared to Fraction 1 for *A. rubens*.

The patterns of the protein fractions were analyzed using gel-electrophoresis (**Paper III**). From the gel-electrophoresis it was also possible to control for proteins earlier described for *A. rubens* or other echinoderms. Fraction 1 showed strong adhesive effects on all cells, and possible active molecules could include fibronectin-like molecules. Fibronectin is a protein with many functions, for example during adhesion and motility of cells and in wound healing (Grinnell et al, 1981; Martin, 1997). Fibronectin or similar molecules have earlier been found to be involved in regeneration processes in sea cucumber (Quiñones et al, 2002) and sea stars (Irina Voronkina, personal communication), and also sea urchin cells have been shown to react by initiating migration when treated with fibronectin (Katow and Hayashi, 1985).

Fraction 2 showed a strong band around 39 kDa, that indicates the possible presence of sea star factor (SSF), and that SSF might be involved in the response from Fraction 2. Whole cell lysates from *A. forbesi* coelomocytes, produce an inflammation response in mammalian tissue (Prendergast and Suzuki, 1970), later identified SSF. Very low amounts of purified SSF have shown to decrease the mitogenesis of spleen cells (Prendergast and Liu, 1976) and SSF has also been shown to have immunosuppressive effects on T- cell-dependent antibody responses (Kerlin et al, 1994). The responses of SSF on invertebrate circulating cells or in the context of an immune response in invertebrates, has not been investigated earlier, moreover the species differences in effects found in **Paper III**, are especially interesting.

Fraction 3, which had the cytotoxic effects on sea star, but increased the viability of mussel and tunicate cells, includes smaller proteins. Cytokines, which have the size-range of 8-30 kDa are important molecules during an immune response, and cytokine-like molecules are found in echinoderms as well as in molluscs and tunicates. For example, IL-1-like molecules are found in all three species, and in *M. edulis*, tumor necrosis factor (TNF)-like molecules have also been detected (Raftos et al, 1991; Hughes et al, 1990; Beck and Habicht, 1986; Beck and Habicht, 1996; Beck et al. 2000). TGF- β s are cytokines that are very important in wound healing (Braun et al, 2002), and molecules which are possible members of the TGF- β super family have also been found earlier in the three groups of animals examined in **Paper III** (Patruno et al, 2002; Patruno et al, 2003; Miya et al, 1996; Ottaviani et al, 1993). Study of the protein pattern of Fraction 3, indicate that the sizes of the sea star IL-1, 17 and 22 kDa (Beck and Habicht, 1991), and IL-6, 30 kDa (Beck and Habicht, 1996) correlate with the bands seen for Fraction 3. Another possible molecule is the

invertebrate form of lysozyme, with an approximate size of 15.5 kDa for *A. rubens* (Jollès and Jollès, 1975). Metalloproteinase, an enzyme expressed during regeneration, has not yet been described in *A. rubens*, but four different metalloproteinases have been described in sea cucumber, with sizes between 43 and 59 kDa (Quiñones et al, 2002). There are corresponding bands for both Fraction 2 and 3 in this size range, suggesting the possibility of metalloproteinases also in *A. rubens*.

The sea star tissue coelomic epithelium is considered to be a haematopoietic tissue and examination of functional properties of coelomic fluid proteins on the coelomic epithelium, showed similarities with the response of free coelomocytes from the sea star (**Paper III**). Tissue pieces from both wounded and non-wounded sea stars were compared, and treatment with Fraction 1 and Fraction 2 showed no differences between wounded and non-wounded, and no damage to the tissue samples was seen. Fraction 3 induced a cytotoxic effect with disruption of the tissue, an effect that was more pronounced in the wounded animal. The control with buffer without added serum, gave a disruptive effect on the tissue from wounded animal and whole non fractioned coelomic fluid showed a slight disruptive effects on the tissue, but less than the effect of Fraction 3. Since the controls also had toxic effects on tissue from wounded animals, the conclusion might be that except for cytotoxic compounds in Fraction 3, there might also be protective molecules present in Fraction 1 and 2.

During a response to wounding the protein patterns are changed by e.g. the release of immunoactive compounds, which earlier have been shown in the sea star *A. rubens* (Voronkina et al, 2001). In **Paper III** it is shown that groups of proteins have different effects on coelomocytes/haemocytes from different invertebrate species, as well as on sea star explants. Regulatory factors such as cytokines are known to produce different effects, depending on target cell type (Alberts et al, 2004). The difference in cell types or difference in regulation of receptors on target cells, may be responsible for the differences in response among species and due to wounding.

Coelomocyte protein expression

Screening and searching for homologs of sea star coelomocyte proteins in **Paper IV** is the first work of this type on sea star coelomocytes, using 2-DE, and very few studies with proteomic focus have been performed overall on echinoderms. The main reasons for developing the 2-DE for sea star cells, were to explore the possibilities of further proteomic studies of this kind, screen the protein pattern of sea star coelomocytes, explore the occurrence of proteins regulated due to wounding and the possibilities for identifying protein homologs. The most striking finding from **Paper IV** was that the sea star *A. rubens* did not seem to be more homologous to the sea urchin *Strongylocentrotus purpuratus* than to other invertebrates or even vertebrates in the database. Since the genome of *S. purpuratus* had recently become available (Sea Urchin Genome Sequencing Consortium et al, 2006), efforts were concentrated on finding homologs from this species. Because few protein sequences are available in the public databases for sea stars, protein identification had to entirely rely on

searches of homologous sequences in closely related species. From the protein pattern of the the 2-D gels, 18 spots were chosen for analysis with mass spectrometry (MS), after analysis with the software PDQuest (**Paper IV**). These chosen spots showed either up- or down-regulation due to treatment, where six spots showed significant regulation after the statistical analysis. Out of these 18 chosen spots, possible homologues were found for nine of the spots. Several of the remaining nine proteins gave no hits, even when the protein content and resolution was good. Earlier studies on the sea urchin *Evechinus chloroticus*, which is of the same order, but different family as *S. purpuratus*, Sewell et al, (2008), showed protein homologues with 138 gonad proteins from MudPIT with a MASCOT-score of 50 or above, and using 2-DE, the identification rate was 18 out of 20 spots. A study on sea star oocytes performed before the sea urchin genome was available, could only identify of five out of 37 spots (Santella et al, 2000), but as seen in **Paper IV**, the availability of the sea urchin genome in the database has still not yet been able to increase the score of identification.

The nine protein homologs identified in **Paper IV** were common proteins with roles in normal functions of cells, with one of the proteins, Cdc42, especially interesting to be found in sea star coelomocytes. Cdc42, belonging to the Rho family, was not regulated. It has been found to be involved both in wound healing and epithelial cell polarization processes (Redd et al, 2004; Cowan and Hyman 2007). In sea urchins it has been shown to be involved in forming thin, actin-rich surface projections, the filopodia (Passey et al, 2004), also well known in sea stars (Smith, 1981; Pinsino et al, 2007).

Protein expression of sea stars has been shown to change pattern and increase 6 hours after wounding, as earlier discussed (Voronkina et al, 2001), while this time period was used as a comparison for the first screening of sea star coelomocytes (**Paper IV**). The protein pattern found is one representative of a much longer time scale of the wound healing and regeneration process. This is important to have in mind, when discussing the results of up- and down-regulation. Another important factor is the amount of proteins, and the limitations there are with the methods. Low abundant proteins may be hidden among the larger mass, but with further development of the method, including more separation steps, it may be possible to analyze several more, low abundant proteins (Gygi et al, 2000).

Screening the protein pattern of sea star coelomocytes showed that a large portion of the total protein content was in the range 6-50 kDa (**Paper IV**). The gel gradient was also chosen to separate smaller proteins with a gel of 10-20%, and this might lead to an underestimation of larger proteins. Proteins found in the fractions from the coelomic fluid are likely to be found also in the coelomocytes, the source of many humoral immune components, but among the large mass of proteins a 2-DE of total protein content provides, together with interactions of proteins during a 2-DE run, it is difficult to make firm conclusions about specific proteins without more specific markers for identification.

The method development of 2-DE for sea star coelomocytes, the MS analysis and possible matched homologs, is only the beginning, but demonstrates the enormous potential for

further research in this area. It is possible to obtain huge amounts of information, which should also be useful in search for the identification of new and novel differentiating factors in wound healing and regeneration among many organisms, including man.

Conclusions

For many years, several tissues of the sea star have been suggested to harbour haematopoietic stem cells. This is the first quantitative study of proliferation rate in sea star tissues, and it can now be concluded that cells from the coelomic epithelium respond with a significant proliferation when triggered with mitogenic factors, and have a pattern of protein expression very similar to that of circulating coelomocytes. Furthermore, the cells migrating out of the coelomic epithelium show high similarities in both morphology and behavior compared to coelomocytes. The significant increase in proliferation following stimulation, together with a shared protein expression pattern with coelomocytes, could also be found for cells from Tiedemann body and axial organ, lead to the proposal that these too represent haematopoietic tissues. This identification of haematopoietic tissues is in addition to solving the question of the source of the coelomocytes, important for future studies on stem cells, cell plasticity, the wound healing process as well as for further understanding of the extensive regenerative capacity of sea stars.

The rapid increase in coelomocyte numbers (TCC) in response to injection of non-self molecules as well as to stressors such as wounding and hypoxia, indicate that coelomocytes might be stored in tissues, that there are tissue reservoirs for coelomocytes. The observation of cells released from the coelomic epithelium suggests this tissue to be both a haematopoietic tissue and acts as a storage site for mature coelomocytes. The response in terms of TCC seems to be related to the magnitude of the stress. When stressed with only wounding or hypoxia the increasing trend of TCC was interpreted as a mild stress. In contrast, the combined stress, wounding together with hypoxia, produced a strong synergistic effect, evidenced by both highly elevated TCC and increased levels of HSP70 in the cells. At this stage is it not possible to say if regeneration in sea stars is affected during hypoxia or if there are other consequences of this stress. There remains a possibility that the increase in cell numbers and HSP70 production have a protective role, sufficient to maintain homeostasis and metabolism within the normal range. However, considering the growing problems with hypoxia on the sea floor, and the reduced capacity of regeneration reported for brittle stars under hypoxic conditions, there might be significant consequences also for sea star regeneration.

Cells from different species responded differently to proteins fractionated from cell free coelomic fluid of wounded sea stars. The protein fractions affected tissue from wounded individuals stronger than non-wounded individuals. The results confirmed the previous findings of up-regulation of proteins due to wounding, including not only proliferative and adhesive, but also cytotoxic molecules. The cell response and protein pattern found could be

linked to molecules involved in vertebrate wound healing, and previously described in either *A. rubens* or other echinoderms. As a molecular approach for future studies of the wound healing process in sea stars, the coelomic proteins should be further separated and purified, for investigation of specific functions and identification.

The development of proteomic methods for sea star coelomocytes, has increased the knowledge about the protein expression of these cells. With Western blotting it is possible to quantify the expression of evolutionary conserved proteins, as HSPs, and with two dimensional gel electrophoresis it is possible to search for and discover proteins earlier not described. The overall protein expression of the sea star coelomocytes together with up- and down-regulation of proteins in these cells after injury was demonstrated and the protocol developed will be useful in future proteomic studies, maybe also for other marine organisms. Surprisingly, the database search for protein homologies with the sea urchin *S. purpuratus* indicated some distance from Asteroids, showing no closer relationships to the sea urchin than to other invertebrates or even vertebrates within the database. Future proteomic studies on coelomic fluid and the coelomocytes of *A. rubens*, may give valuable insights of the underlying mechanisms and molecules involved in wound healing and regeneration.

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References

- Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2004), *Essential cell biology*, Second edition, Garland Science, New York, USA
- Anteunis, A., Leclerc, M., Vial, M., Brillouet, C., Luquet, G., Robineaux, R. and Binaghi, R. A. (1985) Immunocompetent cells in the starfish *Asterias rubens*. An ultrastructural study. *Cell biology international reports* 9:663-670
- Bannister, R., McGonnell, I. M., Graham, A., Thorndyke, M. C. and Beesley, P. W. (2005) *Afuni*, a novel transforming growth factor-beta gene is involved in arm regeneration by the brittle star *Amphiura filiformis*. *Dev Genes Evol* 215:393-401
- Beck, G. and Habicht, G. S. (1986). Isolation and characterization of a primitive interleukin-1-like protein from an invertebrate, *Asterias forbesi*. *Proc. Natl. Acad. Sci.* 83, 7429-7433.
- Beck, G. and Habicht, G. S. (1991) Purification and biochemical characterization of an invertebrate interleukin 1. *Mol Immunol* 28:577-584
- Beck, G. and Habicht, G. S. (1996). Characterization of an IL-6-like molecule from an echinoderm (*Asterias forbesi*). *Cytokine* 8, 507-512.
- Beck, G., Ellis, T. W. and Truong, N. (2000). Characterization of an IL-1 receptor from *Asterias forbesi* coelomocytes. *Cellular Immunology* 203, 66-73.
- Becker, J. and Craig, E. A. (1994). Heat-shock proteins as molecular chaperones. *Eur. J. Biochem.* 219, 11-23.
- Bond, J.-A. and Bradley, B. P. (1995). Heat-shock reduces the toxicity of malathion in *Daphnia magna*. *Marine environmental research* 39, 209-212.
- Braun, S., Keller, U., Beer, H.-D., Krampert, M., Müller, M., Werner, S., Dickson, C. and Werner, S. (2002). Meeting report. Growth factors in development, repair and disease. *European Journal of Cell Biology* 81, 375-382.
- Brockes, J. P. and Kumar, A. (2005) Appendage regeneration in adult vertebrates and implications for regenerative medicine. *Science* 310:1919-1923
- Browne, C. L., Swan, J. B., Rankin, E. E., Calvert, H., Griffiths, S. and Tytell, M. (2007). Extracellular heat shock protein 70 has novel functional effects on sea urchin eggs and coelomocytes. *The journal of experimental biology* 210, 1275-1287.
- Bruemmer-Smith, S., Stüber, F. and Schroeder, S. (2001). Protective functions of intracellular heat-shock protein (HSP) 70-expression in patients with severe sepsis. *Intensive care med.* 27, 1835-1841.
- Brändén, H. (2001) *Molekylärbiologi*, Second edition, Studentlitteratur, Lund, Sweden
- Buchner, J. (1996). Supervising the fold: functional principles of molecular chaperones. *The FASEB journal* 10, 10-19.
- Candia Carnevali, M. D. (2006). Regeneration in echinoderms: repair, regrowth, cloning. *ISJ* 3:64-76
- Candia Carnevali, M. D., Bonasoro, F., Welsch, U. and Thorndyke, M. C. (1998A). Arm regeneration and growth factors in crinoids. *Echinoderms: San Francisco, Mooi and Telford*, 145-150.
- Candia Carnevali, M. D., Bonasoro, F., Patruno, M. and Thorndyke, M. C. (1998B). Cellular and molecular mechanisms of arm regeneration in crinoid echinoderms: the potential of arm explants. *Dev Genes Evol* 208, 421-430.
- Candia Carnevali, M. D. and Bonasoro, F. (2001A) Introduction to the biology of regeneration in echinoderms. *Microscopy research and technique* 55:365-368
- Candia Carnevali, M. D. and Bonasoro, F. (2001B) Microscopic overview of crinoid regeneration. *Microscopy research and technique* 55:403-426
- Cheng, W., Liu, C-H., Hsu, J-P. and Chen, J-C. (2002). Effect of hypoxia on the immune response of giant freshwater prawn *Macrobrachium rosenbergii* and its susceptibility to pathogen *Enterococcus*. *Fish and shellfish immunology* 13, 351-365.

- Chong, K.-Y., Lai, C.-C., Lille, S., Chang, C. and Su, C.-Y. (1998). Stable overexpression of the constitutive form of heat shock protein 70 confers oxidative protection. *Journal of molecular and cellular cardiology* 3, 599-608.
- Coico, R., Sunshine, G. and Benjamini, E. (2003) *Immunology, a short course*. Fifth edition. John Wiley & sons, Inc. Hoboken, New Jersey, USA
- Coteur, G., Corriere, N. and Dubois, Ph. (2004). Environmental factors influencing the immune responses of the common European starfish (*Asterias rubens*). *Fish and shellfish immunology* 16, 51-63.
- Coteur, G., DeBecker, G., Warnau, M., Jangoux, M. and Dubois, Ph. (2002). Differentiation of immune cells challenged by bacteria in the common European starfish, *Asterias rubens* (Echinodermata). *European Journal of Cell Biology* 81, 413-418.
- Cowan, C. R. and Hyman, A. A. (2007) Acto-myosin reorganization and PAR polarity in *C. elegans*. *Development* 134:1035-1043
- Dahl, E. (1972) *Evertebratzoologi*, Almquist & Wiksells Boktrycker AB, Stockholm, Sweden
- Diaz, R. J. and Rosenberg, R. (1995). Marine benthic hypoxia: a review of its ecological effects and the behavioural responses of benthic macrofauna. *Oceanogr. Mar. Biol. Ann. Rev.* 33, 245-303.
- Diaz, R. J. and Rosenberg, R. (2008). Spreading dead zones and consequences for marine ecosystems. *Science* 321, 926-929.
- Diz, A. P. and Skibinski, D. O. F. (2007) Evolution of 2-DE protein patterns in a mussle hybrid zone. *Proteomics* 7:2111-2120
- Ferguson, J. C. (1984). Translocative functions of the enigmatic organs of starfish- the axial organ, hemal vessels, Tiedemann's bodies, and rectal caeca: an autoradiographic study. *Biological bulletin* 166, 140-155.
- Grinnell, F., Billingham, K. E. and Burgess, L. (1981). Distribution of fibronectin during wound healing in vivo. *J Invest Dermatol* 76, 181.
- Gurtner, G. C., Werner, S., Barrandon, Y and Longaker, M. T. (2008). Wound repair and regeneration. *Nature* 453, 314-321.
- Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y. and Aebersold, R. (2000). Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *PNAS* 97, 9390-9395.
- Hallare, A. V., Pagulayan, R., Lacdan, N., Köhler, H.-R. and Triebkorn, R. (2005). Assessing water quality in a tropical lake using biomarkers in zebrafish embryos: developmental toxicity and stress protein responses. *Environmental monitoring and assessment* 104, 171-187.
- Hamer, B., Pavicic Hamer, D., Müller, W. E. G. and Batel, R. (2004). Stress-70 proteins in marine mussel *Mytilus galloprovincialis* as biomarkers of environmental pollution: a field study. *Environment international* 30, 873-882.
- Hernroth, B., Baden, S. P., Holm, K., André, T. and Söderhäll, I. (2004). Manganese induced immune suppression of the lobster, *Nephrops norvegicus*. *Aquatic Toxicology* 70, 223-231.
- Holman, J. D., Burnett, K. G. and Burnett, L. E. (2004). Effects of hypercapnic hypoxia on the clearance of *Vibrio campbellii* in the Atlantic blue crab, *Callinectes sapidus* Rathbun. *Biological bulletin* 206, 188-196.
- Hughes, T. K., Smith, E. M., Chin, R., Cadet, P., Sinisterra, J., Leung, M. K., Shipp, M. A., Scharrer, B. and Stefano, G. B. (1990). Interaction of immunoactive monokines (interleukin 1 and tumor necrosis factor) in the bivalve mollusc *Mytilus edulis*. *Proc. Natl. Acad. Sci.* 87, 4426-4429.
- Jollès, J. and Jollès, P. (1975) The lysozyme from *Asterias rubens*. *Eur. J. Biochem.* 54:19-23
- Katow, H., and Hayashi, M. (1985). Role of fibronectin in primary mesenchyme cell migration in the sea urchin. *The journal of cell biology* 101, 1487-1491.
- Kerlin, R. L., Cebra, J. J., Weinstein, P. D. and Prendergast, R. A. (1994). Sea star factor blocks development of T-dependent antibody secreting clones by preventing lymphokine secretion. *Cellular Immunology* 156, 62-76.

- Kiang, J. G. and Tsokos, G. C. (1998). Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol. Ther.* 80, 183-201.
- Kozlova, A. B., Petukhova, O. A. and Pinaev, G. P. (2006). The analysis of cellular elements in coelomic fluid during early regeneration of the starfish *Asterias rubens* L. *Tsitologiya* 48, 175-83.
- Kumar, A., Godwin, J. W., Gates, P. B., Garza-Garcia, A. A. and Brockes, J. P. (2007). Molecular basis for the nerve dependence of limb regeneration in an adult vertebrate. *Science* 318, 772-777.
- Lawrence, L. M. (2001). Function of eponymous structures in echinoderms: a review. *Can. J. Zool.* 79, 1251-1264.
- Leclerc, M., Arneodo, V. J., Legac, E., Bajelan, M. and Vaugier, G. L. (1993). Identification of T-like and B-like lymphocyte subsets in sea star *Asterias rubens* by monoclonal antibodies to human leucocytes. *Thymus* 21, 133-139.
- Lee, S.-M., Lee, S.-B., Park, C.-H. and Choi, J. (2006). Expression of heat shock protein and hemoglobin genes in *Chironomus tentans* (Diptera, chironomidae) larvae exposed to various environmental pollutants: A potential biomarker of freshwater monitoring. *Chemosphere* 65, 1074-1081.
- López, J. L. (2007). Two-dimensional electrophoresis in proteome expression analysis. *Journal of chromatography B* 849, 190-202.
- Maginnis, T. L. (2006). The cost of autotomy and regeneration in animals: a review and framework for future research. *Behavioral Ecology* 17, 857-872.
- Martin, P. (1997). Wound healing- aiming for perfect skin regeneration. *Science* 276, 75-81
- Matranga, V., Bonaventura, R. and Di Bella, G. (2002). HSP70 as a stress marker of sea urchin coelomocytes in short term cultures. *Cellular and molecular biology* 48, 345-349.
- Matranga, V., Pinsino, A., Celi, M., Di Bella, G. and Natoli, A. (2006). Impacts of UV-B radiation on short-term cultures of sea urchin coelomocytes. *Marine Biology* 149, 25-34.
- Matranga, V., Toia, G., Bonaventura, R. and Müller, W. E. G. (2000). Cellular and biochemical responses to environmental and experimentally induced stress in sea urchin coelomocytes. *Cell stress and chaperones* 5, 113-120.
- Millot, N. (1969) Injury and the axial organ of echinoids. *Experientia* 25:756-757
- Miya, T., Morita, K., Ueno, N. and Satoh, N. (1996) An ascidian homologue of vertebrate BMPs-5-8 is expressed in the midline of the anterior neuroectoderm and in the midline of the ventral epidermis of the embryo. *Mech Dev* 57:181-190
- Moss, C., Hunter, A. J. and Thorndyke, M. C. (1998). Patterns of bromodeoxyuridine incorporation and neuropeptide immunoreactivity during arm regeneration in the starfish *Asterias rubens*. *Phil. Trans. R. Soc. Lond. B* 353, 421-436.
- Muñoz-Chápuli, R., Carmona, R., Guadix, J. A., Macías, D. and Pérez-Pomares, J. M. (2005). The origin of the endothelial cells: an evo-devo approach for the invertebrate/vertebrate transition of the circulatory system. *Evolution & development* 7, 351-358.
- Nilsson, H. C. and Sköld, M. (1996). Arm regeneration and spawning in the brittle star *Amphiura filiformis* (O.F. Müller) during hypoxia. *Journal of Experimental Marine Biology and Ecology* 199, 193-206.
- Ottaviani, E., Franchini, A. and Franceschi, C. (1993). Presence of several cytokine-like molecules in molluscan hemocytes. *Biochemical and biophysical research communications* 195, 984-988.
- Oweson, C., Sköld, H., Pinsino, A., Matranga, V. and Hernroth, B. (2008). Manganese effects on haematopoietic cells and circulating coelomocytes of *Asterias rubens* (Linnaeus). *Aquatic Toxicology* 89, 75-81.
- Oweson, C. A. M., Baden, S. P. and Hernroth, B. E. (2006). Manganese induced apoptosis in haematopoietic cells of *Nephrops norvegicus* (L.). *Aquatic Toxicology* 77, 322-328.

- Parcellier, A., Gurbuxani, S., Schmitt, E., Solary, E. and Garrido, C. (2003). Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. *Biochemical and biophysical research communications* 304, 505-512.
- Passey, S., Pellegrin, S. and Mellor, H. (2004) What is in a filopodium? Starfish versus hedgehogs. *Biochemical Society Transactions* 32:1115-1117
- Patruno, M., Thorndyke, M. C., Candia Carnevali, M. D., Bonasoro, F. and Beesley, P. W. (2001). Growth factors, heat-shock proteins and regeneration in echinoderms. *The journal of experimental biology* 204, 843-848.
- Patruno, M., McGonnell, I., Graham, A., Beesley, P., Candia Carnevali, M. D., and Thorndyke, M. (2003) Anbmp2/4 is a new member of the transforming growth factor- β superfamily isolated from a crinoid and involved in regeneration. *Proc. R. Soc. Lond. B* 270:1341-1347
- Patruno, M., Smertenko, A., Candia Carnevali, M. D., Bonasoro, F., Beesley, P. W. and Thorndyke, M. C. (2002) Expression of transforming growth factor β -like molecules in normal and regenerating arms of the crinoid *Antedon mediterranea*: immunocytochemical and biochemical evidence. *Proc. R. Soc. Lond.* 269:1741-1747
- Pechenik, J. A. (2000). *Biology of the invertebrates*. Fourth edition. The McGraw-Hill Companies Inc. USA.
- Pinsino, A., Thorndyke, M. C. and Matranga, V. (2007). Coelomocytes and post-traumatic response in the common sea star *Asterias rubens*. *Cell stress and chaperones* 12, 331-341.
- Prendergast, R. A. and Liu, S. H. (1976). Isolation and characterization of sea star factor. *Scand. J. Immunol* 5, 873-880.
- Prendergast, R. A. and Suzuki, M. (1970). Invertebrate protein stimulating mediators of delayed hypersensitivity. *Nature* 227, 277-279.
- Quiñones, J. L., Rosa, R., Ruiz, D. L. and García-Arrarás, J. E. (2002). Extracellular matrix remodeling and metalloproteinase involvement during intestine regeneration in the sea cucumber *Holothuria glaberrima*. *Developmental biology* 250, 181-197.
- Quintana, F. J. and Cohen, I. R. (2005). Heat shock proteins and endogenous adjuvants in sterile and septic inflammation. *Journal of immunology* 175, 2777-2782.
- Raftos, D. A., Cooper, E. L., Habicht, G. S. and Beck, G. (1991). Invertebrate cytokines: Tunicate cell proliferation stimulated by an interleukin 1-like molecule. *Proc. Natl. Acad. Sci.* 88, 9518-9522.
- Redd, M. J., Cooper, L., Wood, W., Stramer, B. and Martin, P. (2004). Wound healing and inflammation: embryos reveal the way to perfect repair. *Phil. Trans. R. Soc. Lond. B* 359, 777-784.
- Roccheri, M. C., Sconzo, G., Di Bernardo, M. G., Albanese, I., Di Carlo, M. and Giudice, G. (1981). Heat shock proteins in sea urchin embryos. Territorial and intracellular location. *Acta Embryol. Morphol. Exper.* 2, 91-99.
- Roitt, I. (1997) *Essential immunology*, Ninth edition, Blackwell Science Ltd.
- Ruppert, E. E. and Barnes, R. D. (1994) *Invertebrate Zoology*, Sixth edition, Harcourt, Inc., Orlando, Florida, USA.
- Santella, L., Kyojuka, K., Hoving, S., Munchbach, M., Quadroni, M., Dainese, P., Zamparelli, C., James, P. and Carafoli, E. (2000). Breakdown of cytoskeletal proteins during meiosis of starfish oocytes and proteolysis induced by calpain. *Experimental cell research* 259, 117-126.
- Sea Urchin Genome Sequencing Consortium, et al. (2006). The genome of the Sea Urchin *Strongylocentrotus purpuratus*. *Science* 314, 941-952.
- Sewell, M. A., Eriksen, S. and Middleditch, M. J. (2008). Identification of protein components from the mature ovary of the sea urchin *Evechinus chloroticus* (Echinodermata: Echinoidea). *Proteomics* 8, 2531-2542.

- Sizemore, R. C., Miller, N. W., Cuchens, M. A., Lobb, C. J. and Clem, L. W. (1984). Phylogeny of lymphocyte heterogeneity: the cellular requirements for *in vitro* mitogenic responses of channel catfish leukocytes. *The journal of immunology* 133, 2920-2924.
- Sköld, M., Loo, L.-O. and Rosenberg, R. (1994). Production, dynamics and demography of an *Amphira filiformis* population. *Marine ecology progress series* 103, 81-90.
- Smith, V. J. (1981). The echinoderms. In *Invertebrate Blood Cells*, (ed. Ratcliffe, N. A. and Rowley, A. T.) pp. 513-562: Academic Press, London.
- Smith, V. J. (1991). Invertebrate immunology: phylogenetic, ecotoxicological and biomedical implications. *Comparative haematology international* 1, 61-76.
- Stolen, J. S., Fletcher, T. C., Smith, S. A., Zelikoff, J. T., Kaattari, S. L., Anderson, R. A., Söderhäll, K. and Weeks-Perkins, B. A. (1995). Immunological and pathological techniques of aquatic invertebrates. In *Techniques in fish immunology*: SOS Publications.
- Söderhäll, I., Bangyeekhun, E., Mayo, S. and Söderhäll, K. (2003). Hemocyte production and maturation in an invertebrate animal; proliferation and gene expression in hematopoietic stem cells of *Pacifastacus leniusculus*. *Developmental and comparative immunology* 27.
- Tedengren, M., Olsson, B., Reimer, O., Brown, D. C. and Bradley, B. P. (1999). Heat pretreatment increases cadmium resistance and HSP 70 levels in Baltic Sea mussels. *Aquatic Toxicology* 48, 1-12.
- Theede, H., Ponat, A., Hiroki, K. and Schlieper, C. (1969). Studies on the resistance of marine bottom invertebrates to oxygen-deficiency and hydrogen sulphide. *Marine Biology* 2, 325-337.
- Thorndyke, M. C., Chen, W.-C., Beesley, P. W. and Patrino, M. (2001). Molecular approach to echinoderm regeneration. *Microscopy research and technique* 55, 474-485.
- Van De Braak, C. B. T., Botterblom, M. H. A., Liu, W., Taverne, N., Van Der Knaap, W. P. W. and Rombout, J. H. W. M. (2002). The role of the haematopoietic tissue in haemocyte production and maturation in the black tiger shrimp (*Penaeus monodon*). *Fish and shellfish immunology* 12, 253-272.
- Wilkie, I. C. (2001). Autotomy as a prelude to regeneration in echinoderms. *Microscopy research and technique* 55, 369-396.
- Voronkina, I., Sakuta, G., Sharlaimova, N., Blinova, M., Pinaev, G. (2001). Factors of sea star *Asterias rubens* coelomic liquid and their influence on mammal cells in culture. *Abstr. of 43rd International Meeting of the European Tissue Culture Society, Granada*, 115.
- Wu, R. S. S. (2002). Hypoxia: from molecular responses to ecosystem responses. *Marine Pollution Bulletin* 45, 35-45.