

Dissertation Abstract

Burns, Gavin (2012) Gene expression and regeneration in ophiuroids: A study of transcriptional activity and regeneration in the temperate *Amphiura filiformis* and Antarctic *Ophionotus victoriae*.

Regeneration, in which lost or damaged tissues are re-grown, is a common phenomenon amongst animals and especially so within the ophiuroids (brittle stars) of the phylum echinodermata. The process of regeneration begins with the injury event followed by a period of cell and tissue reorganization which results in wound healing and tissue re-growth. The genetic programme of regeneration is complex and poorly understood, however the role of gene expression is becoming increasingly well characterised.

The goal of this thesis was to identify genes that are transcribed during the process of regeneration in ophiuroids to determine the pathways and gene families active during the various stages of regeneration. By developing and adapting high throughput genomic techniques for use with ophiuroids the investigation of ophiuroid regeneration was taken from the single gene to whole transcriptome studies. This effort was carried out in two diverse ophiuroid species; the Antarctic *Ophionotus victoriae* and temperate *Amphiura filiformis*. Initially the levels of natural arm damage and rate of regeneration was investigated in *O. victoriae*. Subsequently, the genomic techniques required to explore the transcriptomes of these organisms were developed or adapted. Using these techniques the scale of gene expression and gene networks active during regeneration in both *O. victoriae* and *A. filiformis* were surveyed. Determining the extent of gene expression and identifying gene involvement in regeneration in two diverse ophiuroid species facilitates a deeper understanding of the conservation of this important survival and potentially clinically important mechanism.

Investigation of natural arm damage and the rate of regeneration in *O. victoriae* demonstrated that this stenothermic Antarctic ophiuroid has a slow rate of regeneration. This is preceded by an unexpected and unprecedented delay (approximately 5 months) followed by a long period of regeneration (>1 year) at a reduced rate that is expected for an organism living at low temperatures. Additionally this study discovered that *O. victoriae* has a very high level of natural arm damage with 97% of sampled animals showing some arm damage or signs of previous arm regeneration.

A DNA microarray was constructed to determine the extent of gene expression and the genes and gene networks involved in regeneration in *A. filiformis*. The DNA microarray analysis allowed the measurement of the activity of several thousand genes in each sample to construct a dataset describing transcriptional activity during the early, mid and late stages of regeneration. It was demonstrated that the early stage of regeneration, in which the stem cell rich blastema is formed, is the most active in terms of gene expression followed by the intermediate stage, in which approximately half of the cells are differentiated. The later stage, in which most cells are terminally differentiated, had the fewest differentially expressed clones of the three stages measured. From this dataset genes involved in energy production, structural proteins, cell polarity, segmentation, stem cell maintenance and inhibitors of cell proliferation were putatively identified.

Further measurements of gene expression in amputated arms (explants) of *A. filiformis* that had been double amputated to form a wound site at the proximal and distal end further confirmed the specific regenerative activity of several genes, including SoxB1 and DSP-1 homologues. The results demonstrated a polarity of gene expression in double amputated arm explants and that the mechanisms for forming and maintaining a pool of undifferentiated cells are present in the distal part of the explant.

The genes present during regeneration in *O. victoriae* were identified using next generation sequencing to produce approximately 18,000 contiguous sequences, of which 19% were identified using similarity searching. Few sequences were synonymous between *O. victoriae* and *A. filiformis* but the 111 that were included the SoxB1 and DSP-1 orthologues previously described. The increased depth of sequence data gained compared to that achieved for *A. filiformis* allowed the identification of more gene families and pathways including several Hox gene family members, four SOX genes and members of the Notch, TGF-beta and Wnt signalling families. All of these genes and gene families have been previously shown to be involved in the regenerative process of non echinoderms, providing important comparators that give an insight into the processes occurring during ophiuroid regeneration.

Presented in this thesis is the first large scale study of gene activity in ophiuroids. The data described provides an insight into the genetic control of regeneration in ophiuroids and will facilitate the further characterisation of regeneration in these organisms. On a wider scale, this investigation of the

extraordinary regenerative ability of ophiuroids could contribute to the overall understanding of this clinically important process.

Keywords: echinoderm, regeneration, ophiuroid, gene expression, Antarctica.

TO MY WIFE, FAMILY AND COLLEGUES

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List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals. The papers are appended at the end of the thesis.

- I** **Clark, M.S., Dupont, S., Rossetti, H., Burns, G., Thorndyke, M.C. & Peck, L.S.** (2007) Delayed arm regeneration in the Antarctic brittle star *Ophionotus victoriae*. *Aquatic Biology*, 1, 45-53.
- II** **Burns, G., Ortega-Martinez, O., Thorndyke, M.C., Peck, L.S., Dupont, S. & Clark, M.S.** (2011) Dynamic gene expression profiles during arm regeneration in the brittle star *Amphiura filiformis*. *J. Exp. Mar. Biol. Ecol.*, 407, 315-322.
- III** **Burns, G., Ortega-Martinez, O., Dupont, S., Thorndyke, M.C., Peck, L.S. & Clark, M.S.** (2012) Intrinsic gene expression during regeneration in arm explants of *Amphiura filiformis*. *J. Exp. Mar. Biol. Ecol.*, 413, 106-112.
- IV** **Burns, G., Thorndyke, M.C., Peck, L.S. & Clark, M.S.** (2012) Transcriptome pyrosequencing of the Antarctic brittle star *Ophionotus victoriae*. *Marine Genomics*, *In Press*.

Introduction

When an organism suffers injury or loss of tissue through damage or predation it can be repaired through wound healing or replaced by regeneration. The processes of wound healing and regeneration involve large scale cellular events which are controlled by genes and their expression in the form of mRNA and subsequent protein production. Regenerative mechanisms by which lost or damaged tissues are reformed by regrowth or repair following traumatic injury or self induced amputation (autotomy) as a predator avoidance mechanism are complex. Many metazoans possess regenerative ability (reviewed extensively in Maginnis, 2006 and Bely & Nyberg, 2010) but the mechanism used to regenerate can vary both within and between organisms and species (Alvarado & Tsonis, 2006). Regeneration is generally achieved through the alteration of current tissue (morphallaxis) or proliferation and differentiation of stem cells from a blastema (epimorphosis) resulting in the reformation of lost tissue. The process of regeneration is perhaps executed to its greatest extent in echinoderms (Candia Carnevali, 2006) in which it is used as both a survival and reproductive mechanism.

Regeneration and gene expression

The genetic control of the regenerative process has only recently begun to be explored, facilitated by advances in the fields of genetics and genomics, such as the advent of genome sequencing and large scale gene expression profiling, in addition to techniques including Q-PCR, transgenic animals and gene knockout. These tools have already been applied to investigate regeneration in a number of organisms such as zebrafish, amphibians and planarians (for a wider review of the organisms and tools available for determining the genes involved in regeneration see Alvarado & Tsonis, 2006). From studies in a diverse range of organisms it has become clear that there is a high level of conservation of genes, gene families and pathways involved in the process of regeneration. These included the homeobox containing genes, bone morphogenetic proteins (BMP's) and TGF gene families and the Wnt and Notch signalling pathways.

For echinoderms the large scale measurement of gene expression during regeneration has only been carried out in holothurians (Ortiz-Pineda et al., 2009) with other investigations being single gene/protein investigations of homeobox genes and BMP's in asteroids and crinoids (Thorndyke et al., 2001; Thorndyke et al., 2000; Patruno et al., 2002; Patruno et al., 2003) and TGFb and BMP in ophiuroids (Bannister et al., 2008; Bannister et al., 2005). A limiting factor in

the study of echinoderm regeneration has been the paucity of genome or transcriptome sequencing in this phylum. At present only the sea urchin *Strongylocentrotus purpuratus* has undergone whole genome sequencing (Sodergren et al., 2006).

It is clear that more detailed analyses of gene expression during regeneration in ophiuroids is required to enable the characterisation of the genes involved in the various aspects of regeneration from wound healing through to terminal differentiation of stem cells to form the structures of a new appendage.

Ophiuroids, brittle stars

Ophiuroids, commonly known as brittle stars, are pentaradially symmetric members of the phylum echinodermata, are deuterostomes and are therefore in the same superphylum as chordates. In common with all echinoderms, ophiuroids have a calcium carbonate (calcite) skeleton and a water vascular system. They consist of a central disc and, generally, five segmented arms which are used for both locomotion and food capture. The central disc contains the mouth on the underside in addition to the digestive system, bursae for gas exchange, gonads and main nerve ring. Connected to the main nerve ring is the radial nerve that runs the length of each of the long, articulated arms.

Regeneration in ophiuroids is most frequently observed after the loss or partial loss of an arm through predation, external perturbation such as rock movements, hydrodynamic effects etc. or autotomy. They also possess the ability to regenerate large parts of the central disc after damage or after asexual reproduction by fission (Wilkie et al., 1984). The ability to readily sacrifice an arm by autotomy is an important survival mechanism for ophiuroids and their arm structure aids this. The ophiuroid arm contains intervertebral muscles which aid the process of autotomy by allowing the detachment of the arm at any point between the vertebral ossicles (reviewed in Wilkie, 2001). Once the autotomised arm has been detached the process of wound healing begins, in which the wound site is covered by epithelial cells followed by the appearance of a small regenerative bud or blastema consisting of mostly undifferentiated cells which have been formed from migratory stem cells (Biressi et al., 2010). After the formation of a blastema a period of growth and differentiation follows during which the regenerating arm increases in size and the structures of the arm are formed from the pool of undifferentiated cells moving into a differentiated state. The histological process of regeneration has only recently been characterised in ophiuroids (Biressi et al., 2010). The rate of growth and

differentiation during regeneration in ophiuroids can vary within an organism, with intrinsic (e.g. length of arm loss, Dupont & Thorndyke 2006) and extrinsic factors (e.g. temperature, food availability) and between species (discussed further in **Paper I**).

Ophiuroids offer a useful model in which to study regeneration for the following reasons. Firstly, the regenerative ability of ophiuroids is both extensive and reproducible, facilitating rapid and repeatable study. Secondly, members of the echinoderm phyla have been shown to share 70% of their genes with humans (Sodergren et al., 2006) offering an intriguing comparator of gene function. Finally, they are evolutionary diverse from other common regenerative models such as planarians, cnidarians, amphibians and the zebrafish thus presenting an alternative view of regeneration. The following organisms were chosen for these studies due to their evolutionary and geographic distance offering the potential opportunity of a comparative investigation of the conservation of the regenerative process and the genes involved.

Ophionotus victoriae

O. victoriae (Fig. 1) has a circumpolar distribution in Antarctic waters and is one of the most common ophiuroids along the Antarctic Peninsula (Fig. 2) (Arnaud et al., 1998). It is found from the coastal shallows to depths of 1266 m and in a temperature range at the sampling site of -1.9°C - $+1.0^{\circ}\text{C}$. *O. victoriae* reaches a disc diameter of ≤ 35 mm with legs ≤ 150 mm in length and has a lifespan of up to 22 years (Dahm & Brey, 1998; Clark et al., 2007). It is known to have a varied diet, not only being a predator but also scavenger, cannibal, deposit feeder and a detritivore (Fratt & Dearborn, 1984; McClintock, 1994). Prior to experiments detailed in this thesis regeneration in *O. victoriae* had not been studied, with previous investigations being based on distribution, feeding habit and age (Fratt & Dearborn, 1984; Dahm & Brey, 1998; Moya et al., 2003). Few studies producing DNA/cDNA sequences for *O. victoriae* have previously been carried out. Indeed, at present, there are fewer than 70 DNA sequences on NCBI Genbank as of September 2012. This paucity of sequence information offers an opportunity to discover what genes are present in this organism, particularly during the process of regeneration.



Figure 1.

The brittle star *Ophionotus victoriae* displaying several regeneration scars (highlighted) and a whole regenerating arm.



Figure 2.

Antarctica and the Antarctic Peninsula with Adelaide Island midway along the Peninsula. Map courtesy of MAGIC Department, British Antarctic Survey.

Amphiura filiformis

A. filiformis (Fig. 3) is an infaunal temperate brittle star found at depths of $\leq 200\text{m}$ in the North Sea and Mediterranean and in temperatures of $4\text{-}15^\circ\text{C}$ (Bowner, 1982; Muus, 1981). This brittle star has a maximum disc diameter of $\leq 10\text{ mm}$, arm length of $\leq 85\text{ mm}$ and can live up to 16 years (Dupont & Thorndyke, 2006; Duineveld et al., 1987; Ryland & Tyler, 1989). It is a burrowing filter feeder that can be found in densities of $>1000\text{ individuals/m}^2$ (Duineveld et al., 1987).

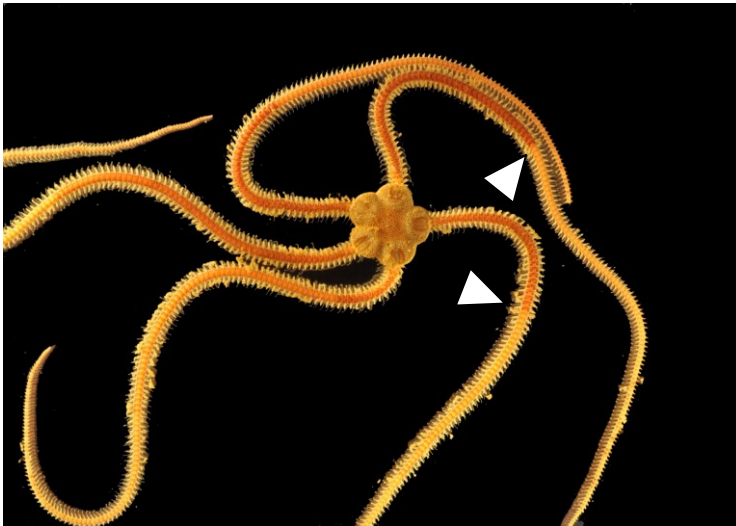


Figure 3.

The brittle star *Amphiura filiformis* displaying several regeneration scars (highlighted).

The regenerative process in *A. filiformis* has been previously investigated to a greater extent than any other ophiuroid. Such studies include its contribution to secondary production (Sköld et al., 1994) through adaptive regeneration according to the position or length of arm lost (Dupont & Thorndyke, 2006) to the activity of genes and proteins during regeneration (Bannister et al., 2008 and Bannister et al., 2005). These investigations have resulted in a greater amount DNA/mRNA sequence available for *A. filiformis* than *O. victoriae* but only for tens of nuclear genes, compared to the mainly mitochondrial sequences of *O. victoriae*.

A. filiformis and *O. victoriae* have an estimated divergence time of 200 million years before present (lower Jurassic) (Smith et al., 1995). Combined with their different habitats, feeding behaviour and size they offer diverse models in which to study regeneration, particularly in terms of gene expression. As stated by Brockes et al (2001), 'A compelling account of regeneration must encompass examples throughout metazoan phylogeny, and the importance of the invertebrate perspective should be evident from this account'. By beginning to characterise the genes involved in regeneration in two diverse ophiuroid species an understanding can be gained into the conservation of these processes within this order and potentially to the wider knowledge of regeneration.

Aim of the thesis

The goal of this thesis was to identify genes that are active during the complex process of regeneration in ophiuroids. The identification of regeneration associated genes will provide an insight into this remarkable ability by determining the pathways and gene families active during the various stages of regeneration. With the advent of high throughput genomic techniques, such as DNA microarrays and next generation sequencing, it is now possible to interrogate the whole transcriptome of an organism or tissue sample. A significant part of this project involved extensive work in developing and adapting these genomic tools and associated techniques for use with ophiuroids. Ultimately, the tools developed during this project were utilised to take the investigation of ophiuroid regeneration from single gene studies to whole transcriptome studies. By determining the genes involved during ophiuroid regeneration an insight will be gained into the conservation of regeneration associated gene pathways and families.

Specifically for each paper the aims were:

- Paper I** Investigate the extent of arm regeneration in natural populations of the Antarctic ophiuroid *O. victoriae*.
- Paper II** Construct a cDNA microarray for and determine the genes involved in arm regeneration in *A. filiformis*.
- Paper III** Investigate genes active in the polarity of regeneration in arm explants of *A. filiformis*.
- Paper IV** Use next generation sequencing (NGS) to construct a library of genes/pathways present in arm samples during the prolonged regeneration process of the Antarctic ophiuroid *O. victoriae*.

Methodology

Animal collection and sampling

Two species of brittle star were used in this study: the temperate *Amphiura filiformis* (Papers **II** and **III**) and the Antarctic *Ophionotus victoriae* (Papers **I** and **IV**). Due to the nature of gene expression studies, the sampling and RNA extraction regime used can have a significant impact on the results obtained in terms of RNA quality and abundance. This consideration is particularly important for long term experiments such as those described in Papers **II** and **IV** and for the stability of transporting samples from collection sites back to the laboratories at the British Antarctic Survey, Cambridge, UK where all gene expression experiments were carried out.

A. filiformis

A. filiformis were collected by Peterson mud grabs at a depth of 25-40m from near the Sven Lovén Centre for Marine Sciences, Kristineberg, on the Gullmar fjord, Sweden (58° 15' N, 11° 25' E). Once sorted from mud, animals were maintained in a flow through aquarium at 14°C until used. Tissues for RNA extraction and gene expression analysis from regenerating arms (Paper **I**) were frozen using liquid nitrogen and kept at -80°C until the sampling was completed and RNA extraction was carried out. Explants of *A. filiformis* (Paper **II**) were sampled and placed in Tri-reagent (Life Technologies) for immediate RNA extraction. All extracted RNA was transported to the British Antarctic Survey either on dry ice or using a dry shipper which maintains a temperature of -80°C for long periods.

O. victoriae

Animals were collected by SCUBA divers from locations adjacent to the British Antarctic Survey research station at Rothera Point, Adelaide Island, West Antarctic Peninsula (67° 34.5' S, 68° 07.0' W, Fig.4). Animals were kept in flow through aquaria at -0.5°C ±0.4°C at Rothera Research Station for the duration of the experiment. Measurements (Paper **I**) and tissue sampling (Paper **IV**) were taken on a monthly basis for a period of one year (carried out by the Rothera Marine Assistant) or weekly for four weeks (conducted by myself during a visit to Rothera in 2007) over a period of 1 year. Due to the long duration of this experiment and the lack of consistent liquid nitrogen supply in the Antarctic it was decided that the tissue samples collected would be stored in

RNAlater (Life Technologies). RNAlater is a highly concentrated solution of salts that dehydrates tissues by osmosis and thus denatures any nucleases that could degrade RNA. Tissues stored in RNAlater are stable for long periods at either -20°C or -80°C and as the samples were to be returned to the U.K. (in a -80°C freezer) by ship, this minimised the impact of the variation in temperature associated with long distance transport.

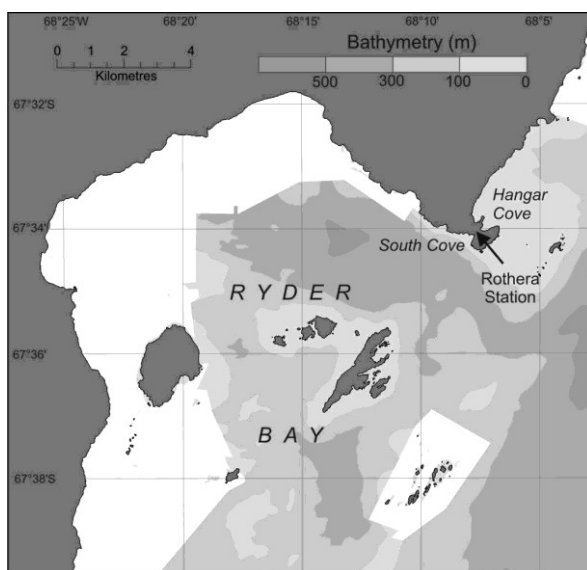


Figure 4.

Rothera Station, Adelaide Island, Antarctica showing the sampling sites used in Paper I and IV. Map courtesy of MAGIC department, British Antarctic Survey.

RNA extraction

O. victoriae: RNA extractions were carried out using a standard Tri reagent (TRIsure, Bioline) method. This method often produced high quality RNA, as determined by gel electrophoresis and spectrophotometry but was not consistent. In addition, RNA samples that were seemingly high quality could not be consistently reverse transcribed. Attempts were made to improve RNA quality with different extraction methods such as altering the precipitation stages, the addition of chelating agents, the addition of acids and alkalis and the use of Qiagen RNeasy cleaning columns. Despite the extensive testing of alternative total RNA extraction methods, no consistent reverse transcription

could be performed on *O. victoriae* total RNA. More reproducible results were achieved by using an extraction method in which the mRNA contained within the total RNA was extracted with Qiagen Oligotex beads. Using this method the polyadenylated mRNA is attached to latex beads that contain polythymine strings on their surface which are then isolated by centrifugation. This physical separation method vastly improved the success of reverse transcription but was more costly and labour intensive. This method also led to a reduced amount of mRNA from each sample thus requiring the pooling of samples for Paper **IV**. None of these issues were observed with RNA samples of *A. filiformis* which were successfully extracted using a standard Tri reagent protocol, however pilot experiments with another Antarctic brittle star, *Ophiura crassa*, encountered similar issues with total RNA and reverse transcription. The reason for these RNA extraction and reverse transcription difficulties is not clear, although a possible explanation could be co-purification of metal cations (Ca^{2+}) formed from the calcareous skeleton present in ophiuroids. Reverse transcription reactions are metal cation driven, usually by Mg^{2+} , and the addition of extra cations could be disruptive. Subsequent to the completion of this study, the issues of calcium ion co-precipitation with RNA held in bioceramics have been highlighted in relation to RNA extraction (Tsz Yan Lee et al., 2011). In this investigation the authors suggest improvements to the TRIzol RNA extraction methods that reduce the co-precipitation of metal cations, some of which had already been unsuccessfully attempted for *O. victoriae*. However the information provided by Tsz Yan Lee et al. (2011) could assist the future development of further optimized RNA extraction protocols for ophiuroids.

DNA Microarrays

DNA microarrays were used extensively in **Papers II** and **III** to measure the relative expression of genes between regenerating arms or regenerating explant arms and control, non-regenerating arms. Microarrays for model organisms (mouse, human, fruit fly, yeasts) that have undergone genome sequencing can often be purchased commercially and are generally supplied with a high level of annotation of the genes present and well optimised protocols for the associated procedures. Microarrays for non-model organisms, in which there has been little or no genome/transcriptome sequencing, are more labour intensive to produce, annotate and develop protocols for. To annotate a cDNA microarray for a non-model organism there are two strategies open to the investigator: either sequence all of the cDNA clones present and annotate them through sequence similarity searches (e.g. BLAST) or sequence and annotate only those clones that demonstrate a difference of expression between samples. The former of these

options is labour intensive and more expensive as it requires large scale sequencing, but gives a greater depth of information, not only for the genes that are undergoing expression changes but also those transcripts which are not changing in abundance. The latter option is more cost effective as the amount of sequencing required is dependent upon the number of differentially expressed genes that are detected but the results are biased in that no information is obtained about the genes that are not changing in expression.

As *A. filiformis* had undergone neither genome nor transcriptome sequencing at the time of initiating this project, a novel DNA microarray had to be manufactured and annotated. In an attempt to limit the costs and labour involved only those transcripts that were found experimentally to be differentially expressed from the full complement present on the microarray would be selected for sequencing. A large cDNA library consisting of >60,000 clones had previously been prepared from mixed samples of regenerating arms of *A. filiformis* and was utilised in this project (made available by M. Thorndyke). From the cDNA library 9,216 clones were amplified by PCR using amine terminated M13 primers, designed to enable the resulting cDNA to covalently bind to the amine binding surface of the glass printing substrate. From these, a subset of amplified clones was chosen to construct a small test microarray consisting of 768 clones. Using the microarray printer (Genetix Q-array2) at the British Antarctic Survey the printing process was optimized and a number of pilot microarrays were printed. These test microarrays were hybridized with amplified and labelled *A. filiformis* cDNA to confirm the binding efficiency of the amplified PCR products to the surface of the microarray. This was also an opportunity to test the PCR based SMART mRNA amplification (Petalidis et al., 2003, Fig. 5) and fluorescent labelling methods (Purac et al., 2008) on mRNA from *A. filiformis* as this was the first time such techniques had been used with this organism. Once the quality of the initial microarray was confirmed and the associated protocols were developed and proven to work reproducibly, production of the full microarray consisting of all 9,216 amplified clones was carried out.

Subsequent to the hybridizations for Papers **II** and **III** attempts were made to utilize the *A. filiformis* cDNA microarray to measure gene expression during regeneration in *O. victoriae* using cross hybridization. Amplified and labelled *O. victoriae* cDNA was hybridized to the *A. filiformis* cDNA microarray using standard conditions and processed with standard stringency washes as described in Purac et al. (2008). These initial hybridizations resulted in poor fluorescent signal in comparison to the previous *A. filiformis* hybridizations and resulted in a

large proportion of data being rejected during the quality control of the data. Attempts were made to increase the signal by reducing the stringency of the hybridization and wash protocols specifically, by decreasing the temperature of the overnight hybridization incubation and decreasing the stringency of post hybridization washes. Despite comprehensive exploration of all technical options, no satisfactory increase in signal could be achieved. After the results of pyrosequencing of *O. victoriae* mRNA were obtained (Paper **IV**) a comparison was made to equivalent pyrosequencing data of *A. filiformis* (unpublished, access kindly provided by O. Ortega-Martinez). Blastn comparison of the 18,003 contigs constructed in Paper **IV** for *O. victoriae* and 35,742 contigs of *A. filiformis* demonstrated that there were only 510 sequences present in the *O. victoriae* data that were >40 bases in length and with a pairwise identity >78% (data not shown). It has previously been demonstrated that cross hybridization between different species using DNA microarrays requires a minimum of 70% sequence homology for signal to be reliably detected with greater homology corresponding to greater increase in signal (Lyne et al., 2003). This lack of sequence homology between the two species could be explained by their divergence time of 200 million years before present (Smith et al., 1995).

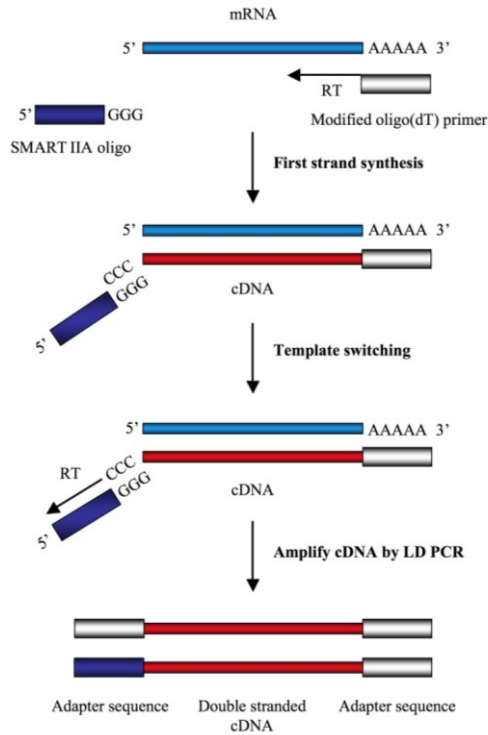


Figure 5.

SMART amplification procedure used to amplify mRNA for Papers **II** and **III**. Figure after Burns (2008).

Quantitative PCR

The measurement of mRNA levels by cDNA microarrays is an established and robust way to simultaneously detect the expression levels of thousands of genes. Quantitative PCR (Q-PCR), which targets individual genes, is often used as a verification of gene expression modulation following microarray experiments. Q-PCR measurements of gene expression can be comparative between samples through the use of a control gene that does not change in expression or, less commonly, via absolute quantification using a known concentration standard curve as a measure of abundance. In model organisms the choice of control gene for Q-PCR is straightforward with several genes being known as good 'housekeeping' genes that have consistent expression levels and well designed

primer pairs, for example GAPDH or ACTB (β -actin). For non-model organisms, in which there has been limited genome/transcriptome sequencing, the use of a ‘housekeeping’ gene is more challenging, particularly for a physiological event such as regeneration in which cells and tissues are undergoing large structural re-arrangements and therefore corresponding extensive modulation of gene expression. For Paper **II** a commercial set of QuantumRNA Universal Internal Standard 18S primers (Life Technologies) that had been designed to amplify from a wide range of organisms was used as a control ‘gene’ in *A. filiformis*. Initial tests on RNA from different stages of regeneration and control non-regenerating arms showed moderate consistency in the detection level using these 18S primers. However this consistency was not seen between the explant samples of Paper **III** and control non-regenerating arms. Despite further tests using other potential control genes (for example actin) no gene could be identified that did not have significant variability in abundance between explant samples and control non-regenerating arms. Subsequently a standard curve approach was applied in which the expression level of selected *A. filiformis* genes in control and explant samples were absolutely quantified against a standard curve produced from 10-fold serial dilutions of purified kanamycin mRNA (Promega). From this standard curve the relative masses of *A. filiformis* transcripts could be interpolated.

Next generation sequencing

Papers **II** and **III** utilized DNA microarrays to measure gene expression in regenerating arms of *A. filiformis* resulting in the identification of hundreds of differentially expressed genes. To better enable the identification of transcripts that could potentially be involved in regeneration in *O. victoricae* a next generation sequencing (NGS) approach was taken in Paper **IV**. NGS enables a vastly greater number of sequences to be produced from an individual sample, far more than could be obtained from Sanger sequencing, thus dramatically increasing the ability to identify the transcripts present. Of the several NGS technologies available, 454 pyrosequencing from Roche was chosen as this method currently produces the longest read length of 400-600 bases compared to the ~150 bases read length of other NGS methods available at the time of this work (for a review of NGS see Glenn (2011)). A greater read length allows for an increased accuracy in *de novo* sequence assembly (sequence assembly in an organism in which a reference genome is unavailable). This ability to accurately assemble sequences without the use of a reference genome/transcriptome is central to the choice of NGS system used. The computational requirements of assembling short sequences and the potential for the production of chimaeric

assemblages limit the choice of NGS platforms available for organisms lacking whole genome sequence.

Results and Discussion

The characterisation and comparison of the regenerative process in different species of ophiuroids is important as these organisms display variety in their mode and rate of regeneration both within an organism, depending upon the damage received, and between species depending upon their habitat. The exploration of regeneration in diverse species could provide information on the fundamental biology of this important process. *A. filiformis* is a well studied brittle star with a number previous investigations of its regenerative ability (Dupont & Thorndyke, 2006; Bannister et al., 2008; Bannister et al., 2005; Dupont et al., 2009; Nilsson & Sköld, 1996; Munday, 1993; Nilsson & Sköld, 1996). In contrast, the process of regeneration in *O. victoriae* was a completely uncharacterised with the previous work on this organism focusing on its distribution, feeding biology and reproduction (Frat & Dearborn, 1984; Grange et al., 2004). Hence it was essential to characterise the levels of arm damage and subsequent rates of regeneration in natural populations (Paper I) before progressing further with molecular analyses (Paper IV).

Natural levels of arm regeneration in O. victoriae (Paper I)

To enable subsequent molecular investigations the measurement of natural arm damage and regeneration rate in *O. victoriae* was required. The level of arm damage, determined by the number of visible regeneration scars, in surveyed *O. victoriae* in South Cove and Hangar Cove at Rothera Research Station (Fig. 4) was high, with more than 97% of animals sampled showing arm damage; indeed 50% of animals displayed damage to all five arms. This level of arm damage is not unprecedented (Munday, 1993; Stancyk et al., 1994) but is much higher than has previously been reported for a non-suspension feeding ophiuroid (Sköld & Rosenberg, 1996). The amount of damage present correlated with the size of the brittle star with larger (therefore more likely to be older) specimens having a greater number of arms damaged. The reason for this high level of arm damage was unclear with high levels of sub-lethal predation being unlikely due to local lack of known ophiuroid predators. We therefore hypothesised that an abiotic factor such as iceberg scouring may have a significant local effect on these ophiuroid communities.

O. victoriae is a slow growing brittle star (Dahm & Brey, 1998) and this growth rate is also reflected in its rate of regeneration (0.22-0.68 mm/week) as

measured over a 12 month period. The low growth rate significantly correlates with temperature when compared to temperate brittle stars and is in line with the general low growth rate observed in Antarctic ectotherms (Peck, 2002). At the time of publication (Paper I, 2007) the rate of regeneration of *O. victoriae* was the slowest recorded for an ophiuroid but has now been superseded by the temperate *Ophioderma longicaudum* at 0.17 mm/week (Biressi et al., 2010) and the Antarctic *Ophiura crassa* at 0.16 mm/month (Clark and Souster, 2012). As a comparator the ophiuroid used in Papers II and III, *A. filiformis*, has a regeneration rate of between 0.6 and 3.3 mm/week depending upon the length of arm lost (Dupont & Thorndyke, 2006).

In addition to the slow rate of regeneration in *O. victoriae* an unusual delay in the onset of the regenerative process was identified. The lag phase of regeneration, the period of time after injury or autotomy under which wound healing and blastema formation occurs but before any noticeable regrowth is observed, has been observed to be 3-6 days (Biressi et al., 2010, pers. obs. S. Dupont in Paper I) to 1 month (Stewart, 1996). The lag phase observed in *O. victoriae* was, on average, 4.7 months. Again, until recently (Clark and Souster, 2012), this was the longest lag phase recorded for a brittle star and unlike the previously described slow regeneration rate is unlikely to be a function of the low temperatures (0°C) in which this organism lives (Q_{10} rates described in Paper I). This unusual delay in the onset of regeneration is of interest in relation to the adaptation of organisms to life at low temperatures. It could indicate a particular challenge at low temperatures on the processes taking place during the initial stages of regeneration.

Gene expression during A. filiformis arm regeneration (Paper II)

Gene expression during regeneration in *A. filiformis* was explored using a custom produced 9,216 clone cDNA microarray. Regeneration was demonstrated to be very dynamic, with a large number of clones showing differential expression when compared to control non-regenerating arms. During arm regeneration (Paper II) gene expression was measured at three stages as previously defined by Dupont and Thorndyke (2006): blastema (7 days post autotomy (p.a)), 50% DI (3 weeks p.a. in which 50% of the regenerate arm is differentiated) and 95% DI (5 weeks p.a. in which 95% of the regenerate arm is differentiated). The most active stage for gene expression was the blastema stage in which 3,458 clones were measured as being differentially expressed compared to control arms. The absolute numbers of differentially expressed clones was reduced in the 50% DI stage (2,290) and further again in the 95% DI

stage (1,671). This demonstrates that the blastema stage, in which undifferentiated cells predominate, was the most transcriptionally active stage and that a lower number of genes were involved as the regenerate arm extends and differentiates.

The strategy of sequencing selected differentially expressed clones resulted in 817 cDNA sequences being selected containing representatives from each the 11 clusters of clones with similar expression patterns produced. cDNA sequences were assembled into 89 contigs and 505 singletons which after sequence similarity searches (blastx against the NCBI non-redundant database) resulted in matches for 313 clones (38%). Subsequent Gene Ontology searches matched 23% of these sequences to morphogenesis and cellular, organ and embryonic developmental processes, categories which would be expected to be present in regenerating arms.

As mentioned previously, extensive differential gene expression was detected in the blastema stage of regeneration. Of the 3,458 clones differentially expressed at this stage, 227 were sequenced and identified. The sequence matches demonstrated an accumulation of transcripts involved in energy production, transcription and translation (Paper I, Table 2). The inferred increase in energy production in the blastema was further supported by the expression patterns of the mitochondrial genes cytochrome b and NADH dehydrogenase, both of which had an elevated expression only in the blastema and 50% DI stages. In addition to the suggested increase in energy production in the blastema stage, transcripts putatively identified as encoding structural proteins (actin and collagen) and those involved in protein degradation (polyubiquitin and proteasome sub-units) had raised expression levels. These results suggested an increase both in protein turnover and abundance of cellular structural proteins that would be found in the early stages of regeneration in which apoptotic and large scale tissue cellular remodelling events would be taking place.

Several clones identified as having sequence similarity to known regeneration associated genes were found to be expressed ubiquitously during regeneration in arms of *A. filiformis*. Two clones forming Af_Contig_30 matched the high mobility group box containing protein DSP1 of *Drosophila melanogaster* (e-value $5.86E^{-52}$) and had increased expression throughout the regenerating samples, although less so in the 95% DI stage. This expression profile was confirmed with Q-PCR (Paper II, Figure 4). DSP1 is involved in the regulation of Hox genes (Decoville et al., 2001; Lamiable et al., 2010; Rappailles et al., 2005). Hox developmental associated genes are involved in the polarity and

segmentation of developing embryos and tissues (reviewed in Hueber & Lohmann, 2008). Their involvement in echinoderm regeneration has been previously identified (Thorndyke et al., 2001) and their role in regeneration in other animals has been extensively reported (Cameron et al., 2005; McClure & Schubiger, 2008; Monaghan et al., 2009; Ortiz-Pineda et al., 2009; Schebesta et al., 2006). No Hox genes were directly detected in the *A. filiformis* regeneration study but further evidence of their activity was present with the identification of clone (Af_126D24) demonstrating sequence similarity to a CREB binding protein which, like DSP1, has been shown to be an activator of Hox genes (Saleh et al., 2000). As with the DSP1 associated clones, Af_126D24 also had increased expression in the blastema and 50% DI stages of regeneration. The number and timing of expression of Hox genes during arm regeneration in *A. filiformis* remains unknown and is an area of interest for future work.

The blastema stage of regeneration mostly consists of undifferentiated cells, recruited by migration of coelomic cells and dedifferentiation of the surrounding muscle cells (Biressi et al., 2010). The maintenance of cells in an undifferentiated state is crucial to the regenerative process as early terminal differentiation could cause stunted growth in the regenerate appendage (Yan et al., 2011; Quinlan et al., 1997). A single clone with sequence similarity to a SoxB1 transcription factor was detected (Af_127P7, e-value $5.19E^{-71}$) with increased expression in the blastema and 50% DI stages. Sox1 transcription factors are involved in neuronal determination by preventing undifferentiated cells from terminally differentiating into neurons (Bylund et al., 2003). The expression pattern of the SoxB1 like Af_127P7 is in line with its putative role of maintaining cells in an undifferentiated state, with expression reduced in the 95% DI state in which the majority of cells have become terminally differentiated.

The 50% DI stage of regeneration provided only 55 uniquely differentially expressed genes (compared to the 1,181 and 541 gained for blastema and 95% DI stages respectively), sharing most similarity with the blastema stage (Paper II, Figure 2) including the differential expression of the previously mentioned DSP1 and SoxB1 genes. This similarity of expression between the two stages is unsurprising given the extensive level of cell proliferation in present in both stages.

The 95% DI stage of regeneration was notable for its comparative general decrease in both the levels and number of differentially expressed clones reflecting the impending cessation of the regenerative process and return to the

regular expression patterns of the control, non-regenerating arms. The near completion of the regenerative process in the 95% DI results in an increase in the number of cells that were terminally differentiated and therefore cell proliferation was reduced. The most notable transcript detected at this stage was contig Af_Contig_74 which showed sequence similarity to bax inhibitor 1 (BI-1, e-value $2.05E^{-18}$). This contig was strongly down regulated in blastema and 50% DI samples with expression in the 95% DI stage returning to levels observed in control non-regenerating arms (Paper II, Figure 4). In mice, *BI-1* knockout mutants have been shown to have accelerated liver regeneration due to an increase in the proliferation of hepatocytes, indicating that BI-1 inhibits cell proliferation (Bailly-Maitre et al., 2007). The expression pattern of Af_Contig_74 correlated with the expected modulation of a gene that would inhibit cell proliferation, being strongly down regulated during the early stages of regeneration (blastema and 50% DI) in which cell proliferation was potentially at its maxima.

Gene expression in arm explants of A. filiformis (Paper III)

In both crinoids and ophiuroids arm explants can survive independently from the main body for several weeks and, when double amputated to form wound sites at the proximal and distal ends, can undergo partial regeneration at the distal end (Candia Carnevali et al., 1998; Dupont & Thorndyke, 2006). This demonstration of the polarity of regeneration in explants offers a unique opportunity to investigate the intrinsic regenerative linked transcriptional activity present in ophiuroid arms. In Paper III gene expression in arm explants of *A. filiformis* was determined using the *A. filiformis* microarray described in Paper II. Gene expression was measured in double amputated arm explants of *A. filiformis* that were sampled after seven days of culturing in a flow through aquarium. Analyses were conducted by separating each explant into three sections: proximal, medial and distal (Paper III, Figure 1). By comparing the expression present in the explant samples to the same control non-regenerating arms as used in Paper II, a direct comparison of gene expression over the three explants sections was made.

The extent of regeneration observed in the double amputated arm explants was similar to that of regeneration in normal (attached) arms after a similar period of time (Biressi et al., 2010) as determined by the size of the extended blastema. A similarly limited formation of an extended blastema was observed as had previously been reported for crinoid explants (Candia Carnevali et al., 1998).

A total of 1,733 clones were determined to be differentially expressed between the explant samples and control non-regenerating arms. This was less than half the number detected during normal (attached) regenerating arms, but indicates that explants are remarkably active, in terms of gene transcription, for tissue that has been separated from the donor body for seven days. As in Paper II a subset of differentially expressed clones were chosen for sequencing (in addition to those already sequenced for Paper II) resulting in a total of 356 clones receiving a BLAST hit.

Almost half of the clones detected (46%) were differentially expressed throughout the explant (Paper III, Figure 2) and the clones detected indicated an increase in translational machinery (ribosomal proteins and eukaryotic translation initiation factors) and protein production (chaperonins). The increase in translation and subsequent protein production detected throughout the explant could be expected of tissue that is undergoing extensive tissue remodelling associated with wound healing (proximal and distal ends) and regeneration (distal end).

A total of 342 clones were differentially expressed only in the distal end of the explant compared to 31 clones only in the proximal end when compared to control non-regenerating arms (Paper III). This clearly demonstrates a greater level of gene expression present in the regenerating distal end. Included in the 342 clones that were uniquely differentially expressed in the distal part of the explant was the previously discussed Af_127P7 with sequence similarity to a SoxB1 protein. This distally specific expression pattern of Af_127P7, along with its increased expression in the blastema and 50% DI stages in normal regenerating arms further supports this putative SoxB1 being involved in the early part of regeneration when undifferentiated cells predominate. In addition to those previously identified, three clones with distally specific up-regulation with sequence similarity to the receptor protein Notch1 were discovered. Notch signalling also has the ability, like SoxB1, to maintain neuronal stem cells in an undifferentiated state and actually requires SoxB1 activity to do so (Holmberg et al., 2008). Holmberg et al. (2008) further demonstrated that SoxB1 can prevent neuronal stem cells from differentiating independently of Notch signalling.

In conjunction with the transcriptional evidence of the maintenance of a pool of undifferentiated neuronal stem cells in the distal part of the explants, an indication of muscle cell differentiation was identified. A single clone, Af_141E14 was putatively revealed as being selenoprotein W and having increased expression in the distal part of the explant. Selenoprotein W, in

addition to having a role in oxidative stress, has been shown to have increased expression in early differentiating myocytes (Loflin et al., 2006; Noh et al., 2010). Biressi et al. (2010) describe the involvement of differentiating myocytes during early regeneration in *A. filiformis* and Candia Carnevali et al. (1998) noted the increased utilization of dedifferentiated myocytes as a source of stem cells in crinoid explants compared to the normal regenerative process.

As in normal attached regenerating arms, clones showing sequence similarity to the *D. melanogaster* HMG box containing protein DSP-1 were found to be differentially expressed in the blastema containing distal regenerating part of the explant. These clones were also differentially expressed in the proximal part of the explant. In addition to the previously described role of DSP-1 in Hox gene regulation, HMG box containing proteins have been indicated in migration of mesodermal stem cells (Palumbo & Bianchi, 2004; Palumbo et al., 2004) and tissue damage signalling (Rovere-Querini et al., 2004). The precise identity and role of this HMG box containing gene is an excellent target for further study in the early stages of arm regeneration in ophiuroids. The results described show that the mechanisms for forming and maintaining a pool of undifferentiated cells are present in the distal part of the explant and that expression initiation and activity is intrinsic to the amputated arm.

The proximal part of the explant, which seven days post amputation displayed a healed wound site similar to that described in crinoid explants (Candia Carnevali et al., 1998), contained 1,109 clones that were differentially expressed when compared to control non-regenerating arms (Paper III). Although this large number of differentially expressed clones demonstrates high levels of transcriptional activity in the proximal part of the explant, there were few clones that were unique to this list (Paper III, Figure 2). A comparison of gene expression between the proximal and distal parts was performed (based on Significance Analysis of Microarrays analysis of the microarray data Tusher et al., (2001)). Only three clones were found to be two-fold and significantly differentially expressed between the two parts of the explant. The only blast hit obtained for one of these clones matched tropomyosin and was one of several clones showing sequence similarity to this protein that had differing expression profiles. The lack of differential expression between the two ends of the explant demonstrated the fine scale of changes in gene expression seen throughout the explant. Future work on the precise location of gene expression within the tissue structures of the explant (e.g. *in situ* hybridizations of transcripts) rather than the current whole sections based levels of gene expression could be more informative.

The comparison of gene expression data of the blastema stage of normal attached regenerating arms gained from Paper **II** and the distal part of the explant (Paper **III**) showed that the levels of differential gene expression were 58% lower in the explant (3,458 and 1,454 differentially expressed clones respectively). A total of 959 differentially expressed clones were common to both groups and included several previously discussed genes (Notch1, Sox1, DSP-1). This result was informative as it indicated that the genetic mechanisms for certain parts of regeneration (wound healing and blastema formation) were intrinsic to the brittle star arm and did not rely upon activation factors from the main ophiuroid body. Investigating the differences in gene expression between the two blastema sites identified clones involved in the inhibition of apoptosis being up-regulated only in the explant. These included a serpin peptidase and prosopodin both of which have been shown to have anti-apoptotic activity (Suminami et al., 2001; Vidalino et al., 2009; Sun et al., 2010). This may be a consequence of the isolation of the explant from the nutritional and respiratory support of the main ophiuroid body.

The role of dedifferentiating myocytes in regeneration of arms of *A. filiformis* and their involvement in regeneration in arm explants of crinoids has been previously described (Biressi et al., 2010; Candia Carnevali et al., 1998). Two clones with sequence similarity to proteins with muscle related functions were uniquely up-regulated in all explant samples when compared to normal regenerating arms. These were Af_132K8 which was similar to myoferlin and Af_134H7 matched neprilysin. Myoferlin is involved in muscle fibre production during growth and regeneration (Demonbreun et al., 2010; Doherty et al., 2005) and neprilysin has been shown, amongst other functions, to be involved in myoblast differentiation (Broccolini et al., 2006; Carmeli et al., 2004). The increased expression of these two clones only in the explant could be an indication of the increased recruitment and activity of muscle cells in the regenerative process than in normal attached regenerating arms.

The major difference in the regenerative process between explants and attached regenerating arms is that the blastema explants do not grow and differentiate into identifiable structures. It is perhaps the genes that are expressed in normal regenerating arms and not in explants that could provide an insight into the switch from growth to differentiation. Clone Af_133N6 showed sequence similarity to the beta-thymosin repeat containing *Hydra* protein thypedin which has been shown to be involved in the differentiation of foot tissue (Hoffmeister, 1996). Blast sequence similarity of the four beta-thymosin repeat containing Af_133N6 also showed similarity to homologues of the *D. melanogaster* protein

ciboulot which also contained four beta-thymosin repeats. In *Drosophila* ciboulot is essential for active cytoskeleton re-arrangement in brain morphogenesis (Boquet et al., 2000) and the termite homologue of ciboulot is involved in neural reorganisation in the formation of termite soldiers (Koshikawa et al., 2010). Af_133N6 did not show any differential expression in explants but was up-regulated in the blastema of normal attached regenerating arms (Paper III). The expression pattern and similarity to genes involved in tissue differentiation make Af_133N6 an excellent candidate for a cellular differentiation associated gene.

Papers II and III indicated that the regenerative process in *A. filiformis* involves large scale gene expression, particularly during the early stages in which undifferentiated cells predominate. The genes identified in this previously uncharacterised brittle star transcriptome include several classic regeneration associated genes and pathways that have been identified from organisms in different phyla thereby demonstrating the high levels of conservation of these regenerative mechanisms.

Pyrosequencing of the regeneration transcriptome of O. victoriae (Paper IV)

The identification of genes and pathways which are active during regeneration of ophiuroid arms had been carried out using the cDNA microarray of *A. filiformis* (Papers II and III). Due to the previously described inability of *O. victoriae* cDNA to successfully cross hybridize to the *A. filiformis* cDNA microarray an alternative approach of 454 pyrosequencing was taken to investigate the genes present during arm regeneration in *O. victoriae*. Selected samples representing the entire regeneration process of *O. victoriae* (58 regenerating samples in total) that were produced in Paper I were pooled and used in 454 pyrosequencing (Paper IV). Sequencing on a ¼ of a picotitre plate resulted in 169,990 high quality sequence reads which, after assembly, produced 18,003 contigs with an average length of 606 bp. The remaining singleton sequences (those that could not be accurately assembled), totalling 31,947 reads, were not used in the rest of the study. Annotation of the contig sequences by blast similarity searches against the NCBI non-redundant (nr) database resulted in 3,340 matches (19%). This low level of annotation is not unexpected for a non-model species and was a limiting factor in the analysis of genes involved in regeneration.

A comparison of the *O. victoriae* contigs to the *A. filiformis* sequences produced in Papers II and III demonstrated that the majority of the 111 matches were

structural, ribosomal and energetic genes. Significantly two matches were made to the putatively annotated and previously discussed HMGB1 and SOX1 transcripts that were significantly up-regulated during the early stages of regeneration in *A. filiformis*.

Analysis of blast results against the NCBI non-redundant database and the KEGG Automatic Annotation Server (Moriya et al., 2007) resulted in the identification of gene products forming several known regeneration associated pathways and gene families.

Members of the Hox gene family have a significant role in body patterning and tissue specification during development and have previously been identified during regeneration in a sea cucumber (Ortiz-Pineda et al., 2009) and a starfish (Thorndyke et al., 2001; Thorndyke et al., 2000). Contigs demonstrating similarity to Hox genes included an *Aristaless*-like homeobox protein which is involved limb axis specification and patterning in *Drosophila* (Campbell & Tomlinson, 1998) and to *Even-skipped* which is expressed during zebrafish fin regeneration and is involved in fin ray specification (Borday et al., 2001). Two further putative Hox genes were identified as *Meis1* and *Pitx* with both having roles in other organisms that are pertinent to the regenerative process in ophiuroids. *Meis1* is involved in the proximodistal identity in the regenerating arms of *Axolotl* (Mercader et al., 2005) and *Pitx* has an asymmetrical left-right expression pattern during deuterostome development (Yasui et al., 2000).

As mentioned previously in the comparison between *A. filiformis* and *O. victoriae* sequence data, members of the SOX gene family were identified in the transcriptome of regenerating arms of *O. victoriae*. SOX genes play an important and diverse role in the cell transition from an undifferentiated state through to terminal differentiation during development and regeneration (Wegner, 1999; Wegner, 2011). The identification of four SOX genes in *O. victoriae* (*Sox1*, *Sox9*, *Sox11* and *Sox17* in Paper IV) provide an excellent target for further study of the expression pattern of these genes in particular, their association with nerve development.

Perhaps the most studied development and regeneration associated pathways to be identified in Paper IV were members of the Notch, TGF-beta and Wnt signalling pathways. These pathways are known to have significant crosstalk and interactions with each other during the developmental process (Guo & Wang, 2008). The timing, mechanisms, interactions and impact of these pathways on the regenerative process can only be elucidated in ophiuroids once

the tools to manipulate gene expression (such as gene silencing) are developed in these organisms.

Six members of the Notch signalling pathway were identified using sequence similarity searching of public databases: Delta, Jagged, Notch, Deltex, E1A/CREB-binding protein and CBF1 interacting corepressor. Notch signalling is central to the processes of stem cell maintenance, cell proliferation and differentiation both in the developing embryo and during neural regeneration (Kishimoto et al., 2011). Notch signalling can act in a similar manner to the previously described SoxB1 signalling (Paper II and III description of increased expression of SoxB1 and Notch1 in early regeneration process of *A. filiformis*) in maintaining a pool of neuronal progenitor cells, indeed the activity of Notch can be attenuated by SoxB1. The data described in Papers II and III regarding the expression of Notch1 and SoxB1 and the timing of expression of these markers of stem cell maintenance in *O. victoriae* will allow the determination of the point at which a pool of stem cells are formed after the wound healing process. Given the delay in the onset of regeneration in *O. victoriae*, the activity of these and other proneural genes may give an indication of the regenerative stage at which this delay takes place.

The importance of the Wnt signalling pathway in regeneration has been highlighted both in *Hydra* in which it is essential for the process of head regeneration (Galliot & Chera, 2010; Guder et al., 2006) and in *Holothurian* intestinal regeneration (Ortiz-Pineda et al., 2009). Within the sequence reads obtained from mixed regenerating arms of *O. victoriae* the Wnt signalling pathway was well represented with homologues to eight members of the canonical pathway being identified (Paper IV, Table 3 and Figure 3).

Members of the TGF beta family are the most studied asterozoan regeneration associated genes, particularly in relation to neuronal regeneration (Patrino et al., 2001; Patrino et al., 2002; Patrino et al., 2003; Bannister et al., 2005; Bannister et al., 2008). In Paper IV homologues to ten members of the TGF beta family were identified including the dorsalizing protein chordin and the BMP inhibitor SMAD6.

The genes, gene families and pathways identified in the first large scale sequencing of the transcriptome of any ophiuroid further illustrated the conservation of regeneration associated genes. The sequencing of the transcriptome of regenerating arms of *O. victoriae* provides a tool set for the

further study of regeneration in this organism with particular attention focussing on the unusual delay in the onset of regeneration.

Conclusions

The microarray based investigation in *A. filiformis* demonstrated that the early stages of regeneration were the most active in terms of gene expression. These stages require further analysis and a greater depth of sequencing to fully characterise the presence and activity of regeneration associated genes. The discovery of a delayed onset of regeneration in *O. victoriae* was intriguing and could afford an insight into the adaptations or limits to life in low temperature environment. The cataloguing of gene expression during arm regeneration in *O. victoriae* provided evidence for the presence of several classical regeneration associated gene pathways that have not previously been described in any ophiuroid. The data presented here has moved the study of transcriptional control of regeneration in ophiuroids from single gene studies to a position in which the activities of hundreds of genes are now described. Furthermore there is a large database of unannotated transcripts, which represent a source of novel genes for functional studies. Hence, in addition to the data described in this thesis, this work will enable future targeted studies of the detailed functional activity and interactions of genes and pathways active during ophiuroid regeneration. The activity of regeneration associated genes during early regeneration in *O. victoriae* will perhaps determine the exact stage and reasoning for the delay in the onset of regeneration in this Antarctic brittle star.

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