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Molecular assessment of spawning cues in temperate abalone *Haliotis laevigata*

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Dedication:

In memory of the 43 Mexican students who disappeared at the hands of the Mexican government on the 26th of September, 2014 in Ayotzinapa, Guerrero, Mexico.

Since I was a kid I learnt that:

Nothing like the ocean to put your feet in the ground.

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SUMMARY

Abalone are edible marine snails distributed throughout the world in tropical and temperate waters. Abalone are highly valued for their palatability, particularly in Asian countries where consumption is inherent to traditions. Wild caught abalone supplies some of the global market, however to meet increasing commercial demand abalone are also farmed. In fact, the majority of abalone supplied on the global market is produced on farms. Despite the advances in abalone farming, many issues remain unresolved such as prolonged feeding requirements, disease management and inefficient spawning all of which have downstream affects on production. In temperate greenlip abalone *Haliotis laevigata*, inefficient spawning has limited pair-wise breeding of selected broodstock that would otherwise produce highly marketable abalone. Understanding the mechanisms that control spawning and identifying the molecule(s) associated with abalone spawning are key to solving this issue.

This research utilised proteomics coupled to mass spectrometry (MS) to profile the gonad proteome in sexually mature abalone and further to identify proteins that were differentially expressed in gonads during artificially induced spawning.

Mass spectrometry spectral datasets were searched against two publicly available molluscan databases (UniProt and NCBI) and a custom-built database comprising genomic information from closely related molluscan species to profile the gonad proteome. The results from the multi-database approach were collated and protein redundancy manually removed. A total of 110 and 162 proteins were identified in males and females respectively. From those proteins, 47 were involved in sperm and egg structure, sexual maturation, acrosomal reaction and fertilisation. Utilisation of the custom-built database increased the protein identification rate by more than 60%

compared to searches employing public protein databases alone.

To identify differentially expressed proteins, male and female abalone gonads from different physiological states (spawning, post-spawning and failed-to-spawn) were studied using two-dimensional difference in gel electrophoresis coupled to MS. The gonads were compared as follows: spawning versus failed-to-spawn; spawning versus post-spawning and post-spawning versus failed-to-spawn. Proteins from these gonads were fluorescently labelled and separated according to isoelectric point and relative molecular weight. The change in protein expression level was assessed for the three pair-wise comparisons facilitating the location of differentially expressed proteins. The selected gel spots were excised and identified using tandem MS. The corresponding MS/MS datasets were searched against UniProt and NCBI protein databases. Gene ontology of the identified proteins revealed roles in gametogenesis, egg and sperm structure, acrosomal reaction, fertilisation, energy metabolism and oxidative stress. The differentially expressed proteins were quantified by multiple reaction monitoring mass spectrometry (MRM-MS) across all physiological states. Significant differences in the abundance of reproductive and anti-oxidant proteins were observed across the physiological states. These differences correlated with propensity or failure to spawn. For example the reproductive protein vitelline receptor for lysin (VERL) was significantly higher in gonads from spawning abalone relative to gonads from post-spawning abalone when assessed in female abalone. In males, sperm protein, atrial natriuretic peptide receptor A (ANF) and chaperonin containing tcp1 (CCT) showed significant differences in gonads from spawning abalone in comparison to gonads from post-spawning abalone. Additionally, significant differences in abundance of antioxidant proteins were observed in both abalone sexes. For female and male ferritin was rendered significantly different in gonads from post-spawning abalone compared to both spawning and failed-to-spawn

abalone. Superoxide dismutase (SOD) and peroxiredoxin 6 (Prx6) were significantly different in gonads of spawning and post-spawning of female abalone, whereas thioredoxin peroxidase 2 (TPx2) was significantly different in male gonads of spawning compared to post-spawning abalone. Overall, the majority of significant differences were detected when gonads from spawning and post-spawning abalone were compared.

This research revealed the identity of proteins differentially expressed during spawning that may prove to be key molecules involved in *H. laevigata* spawning. The combined findings of this research provide a greater understanding of the protein expression in abalone gonads following artificial induction of spawning. This fundamental knowledge will inform future research programs that aim to develop new techniques to improve artificial spawning rates on farms. This research also provides the identity of proteins that will assist in performing accurate assessments of sexual maturity in selected abalone broodstock to better predict spawning propensity. In conclusion, genomic and proteomics tools have been used to perform a comprehensive study of abalone gonad protein composition, revealing protein identities and enabling measurement of protein expression levels in abalone gonads following artificial spawning induction.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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Omar Mendoza Porras

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STATEMENT OF THE CONTRIBUTIONS OF JOINTLY AUTHORED MANUSCRIPTS

CHAPTER 2

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CHAPTER 3

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CHAPTER 4

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preparation. Reverter A, provided statistical methods. Colgrave ML, provided expertise in mass spectrometry, bioinformatics and manuscripts preparation.

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..... Cook, MT

..... Harris, JO

..... Wijffels, G

..... Colgrave, ML

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1 CHAPTER: LITERATURE REVIEW

1.1 Introduction

Abalone are edible marine gastropods highly appreciated for their palatability. Wild-caught abalone stocks in Australia are under strict government surveillance to avoid depletion. Abalone aquaculture has emerged as a way to fulfill market demands. Despite steady growth, abalone aquaculture has not been free of obstacles. Farmed abalone are susceptible to diseases and are vulnerable to variations in water quality that affect production [1, 2]. In addition, temperate farmed abalone take up to four years to reach market size and therefore financial investment is high due to prolonged feed requirements. Another major concern in abalone production is inefficient reproduction. A lack of full control over both gonad conditioning and artificial induction of spawning results in suboptimal efficiency in on-farm reproduction [3]. Abalone aquaculture has implemented selective breeding programs (SBPs) to improve physical characteristics in cultured abalone [4, 5]. The application of SBPs to abalone production is highly desirable, but is dependent on the efficient control of spawning in specific broodstock. Full control over spawning would allow highly marketable abalone with desirable characteristics to be farmed. This is not currently available for abalone.

In this regard, there is a need to investigate the mechanisms that regulate spawning in abalone. Proteins play important roles in a range of biological functions including motility, feeding, regulation of sensory organs, osmoregulation, growth, mating and reproduction. Key to improving spawning rates is to understand which proteins are directly involved in spawning. In proteomics, proteins are studied using a range of tools that include one and two-dimensional polyacrylamide gel electrophoresis to separate and visualise proteins and in-gel digestion to yield peptides suitable for

downstream analysis. In recent years mass spectrometry (MS) has improved the power of proteomics by allowing large scale data analysis enabling protein identification and quantification. Proteomics has been applied to studies of reproduction in molluscs facilitating identification of proteins crucial to gametogenesis, mating behavior and fertilization. Moreover, MS is a reliable tool to rapidly identify and quantify peptides and proteins, such as hormones. In molluscs, surveys based on proteomics and MS have identified known and novel proteins and peptides from abalone and other molluscan model species including *Aplysia spp* and *Lymnaea stagnalis* [6-10]

1.2 Abalone fisheries and aquaculture

Abalone are edible marine snails distributed worldwide. Wild abalone populations worldwide have been negatively impacted by over-exploitation and illegal fishing [11, 12]. More recently climate change, ocean warming and alteration of ocean chemistry may have contributed to further decline in abalone numbers by increasing the destruction of abalone habitats [13, 14]. These issues combined have resulted in a decrease in the global catch from 20,000 t in the 1970s to less than 8,000 t in 2011 [11, 12]. Parallel to this the abalone demand has increased, and in the year 2010, the overall worldwide supply of abalone (wild-caught and farmed) was 77,000 t of which ~85% was cultured abalone [11]. In 2012 China contributed with 90,694 t from a total international production of 100,496 t of farmed abalone [15]. For Australia the predicted aquaculture production of abalone for the financial year 2013-14 is of 910 t [Pers. Comm. Australian Abalone Growers Association (AAGA), 2012].

From an economic viewpoint, the most important abalone species in Australia are *H. roei, H. asinina, H. laevigata* and *H. rubra*, however, the latter two are considered the most valuable species due to their significance to both the wild fishery and

aquaculture in temperate Australian waters [3]. Their natural distribution is in the southwest and southeast waters of Australia with aquaculture production mainly occurring on coastal land in southern Australian states and southern Western Australia (Figure 1-1). Most Australian abalone is exported to Asian markets where it is highly appreciated as part of their traditional diet [16].

Figure 1-1. Geographical distribution of Australian commercial abalone species.
Adapted from [17].

Many governments have implemented fishing quotas on the fishing industry and recreational fishers to prevent over-exploitation of the wild population. Aquaculture assists in the sustainable management of the wild fishery by aiming to fulfill the shortfall between the wild harvest quota and consumer demand for product. In some countries, aquaculture efforts are directed to restocking purposes with some farms working solely to replenish depleted wild stocks [12].

In the wild, abalone feed on seaweed, microalgae and diatoms depending on the stage of their life cycle. In some countries farmed abalone rely on harvested natural foods such as *Macrocystis pyrifera* [12]. Farmers feed newly settled abalone on diatoms (*Naviculata incerta*) and algae (*Ulvella lens*), which are grown on settlement plates until the abalone are large enough to feed on pellets or seaweed [18, 19]. Some cultured abalone are given manufactured feed in the form of pellets in place of

seaweed for juvenile and adult stages. The use of manufactured food or harvested algae depends on local availability of technology and natural resources. Manufactured and enriched feed have been developed to promote faster growth rates, and there is also substantial research effort to develop enriched algae for use in cultured abalone production [20, 21]. However, feeding costs and slow growing rates in the cultured abalone industry are still major concerns for the industry.

Water temperature is the most critical parameter in abalone aquaculture as it is associated with stress, slow growth, and disease outbreaks causing mortality [22-24]. Summer mortality is a condition that affects older (> 3 years) *H. laevigata* when steadily exposed to higher (>22°C) temperatures. This condition is characterized by a general disruption of the abalone homeostasis resulting in death [25-27]. Water flow is required to maintain water quality and to provide a prophylactic environment for sustained growth throughout all stages of abalone farming. Water exchange is needed to avoid the buildup of harmful gases and noxious compounds, such as ammonia and nitrite, and to maintain optimal pH. Ammonia and nitrite concentrations appear to adversely affect *H. laevigata* growth and health [28, 29]. To reduce the harmful effects of decaying organic matter in flow-through systems, water exchange can be up to 800% per day for abalone cultured at high density [30].

In aquacultured systems abalone are susceptible to infection from parasites, viruses and bacteria. Poor water quality is the major factor contributing to microorganism growth resulting in disease outbreaks and subsequently, mortalities. These microorganisms occur naturally in the water and because farms are open systems they enter the farm via direct water intake from the sea. Microorganism growth is promoted by degradation of organic matter and decomposition of uneaten food [31, 32]. The most severe abalone mortalities were caused by a herpes-like virus between 2005 to 2010 in the coast of Victoria, Australia [1, 33]. The abalone viral

ganglioneuritis (AVG) outbreak caused mortalities on both land-based farms and the wild fishery along the coast of Victoria. In the affected areas, the AVG outbreak caused mortalities up to 95% in the following two weeks after the onset of infection [1, 33]. This resulted in the closure of several farms and production stock was destroyed to prevent further spread of the disease [1].

1.3 Abalone biology and geographical distribution

Abalone are molluscs in the class Gastropoda of the genus *Haliotis*. Currently, there are 56 extant species distributed worldwide in temperate and tropical waters [34]. Australia has nine endemic abalone species with six temperate species found in the cooler waters of southern Australia: *H. cyclobates*, *H. laevigata*, *H. scalaris*, *H. roei*, *H. rubra* and *H. coccoradiata* [34]. Australia also has three tropical species: *H. varia*, *H. asinina* and *H. ovina* that are found in northern waters of Australia [35]. Abalone are herbivorous marine snails found in intertidal waters up to 40 m in depth, usually on rocky coastlines and reefs [36, 37]. Depending on the species they inhabit clefts and small caves as refuges, settling on both horizontal and vertical surfaces. Abalone possess one shell which in conjunction with a strong adductor muscle (foot) protects them from potential predators. During the larval state abalone feed on their egg yolk until they detect the appropriate environmental signal(s) or conditions for metamorphosis and settling [38]. Juvenile abalone prefer to ingest microalgae and diatoms growing on rock surfaces, while adult abalone feed mainly at night on seaweed. The temperate abalone *H. laevigata* and *H. rubra* reach sexual maturity at about three years of age at a length of ~75.0-120.0 mm, while the tropical wild abalone *H. asinina* attain sexual maturity within 12 months and at ~23.1-40.6 mm [5, 39-41].

1.4 Spawning control and reproduction

Fertilization in abalone occurs externally after both sperm and egg are simultaneously expelled into the ocean (broadcast spawning). Ocean dynamics and chemotaxis are determinants for gamete recognition [42-44]. Natural spawning of temperate adult abalone occurs over only a few months of the year when location and optimal environmental conditions promote peak gonad conditioning. Water temperature plays a major role in gonad development, oogenesis and abalone spawning [41, 45-47]. However, photoperiod and environmental cues such as salinity, food availability and ocean dynamics also influence the reproductive cycle in abalone (Figure 1-2) [48-50].

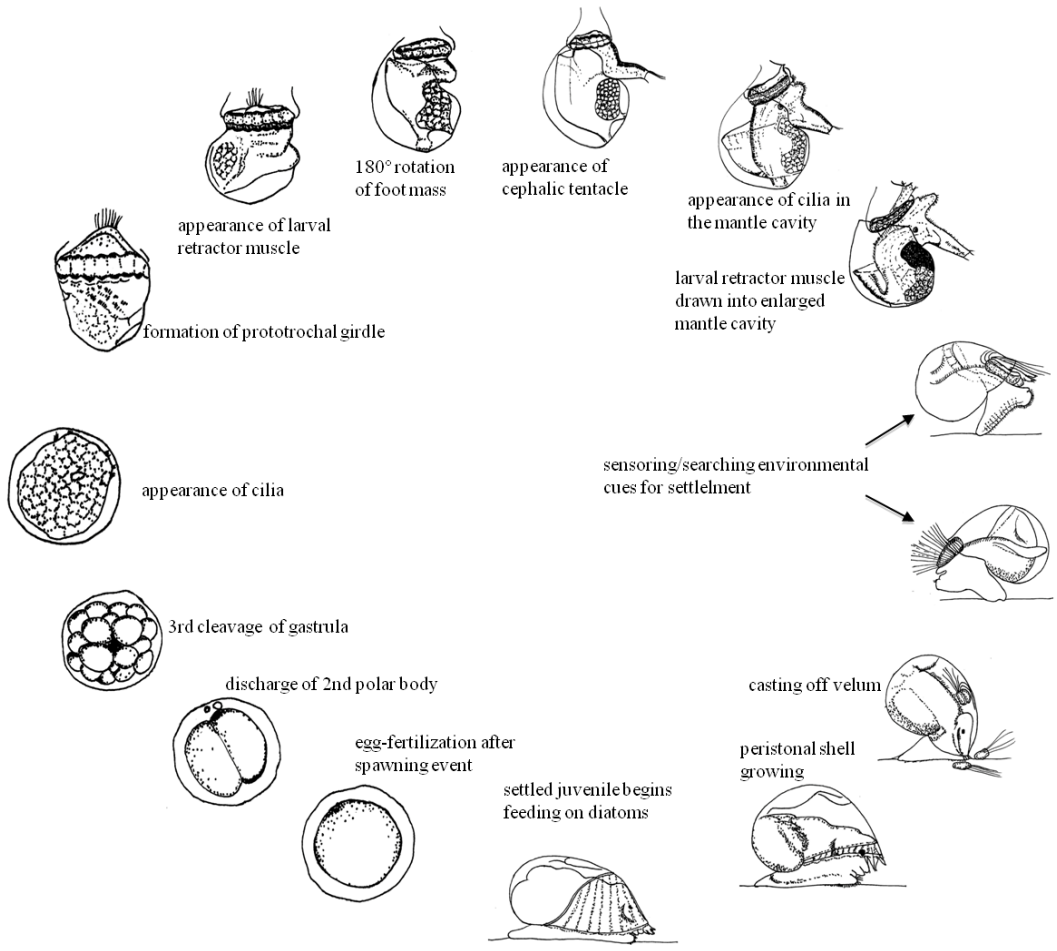

Figure 1-2. Abalone life cycle.
Abalone have two stages: one as a planktonic larvae and another sessile stage that begins under specific settling environmental conditions. Figure edited and text modified from [51, 52].

Abalone spawning can occur at different times according to the species, for example *H. laevigata* spawning may occur during spring and summer whilst *H. rubra*, spawning depends on local environment conditions resulting in variations in the spawning seasons at different locations [41]. On cultured abalone farms, broodstock conditioning is often required out of their natural spawning season for re-stocking purposes.

Unsynchronized spawning in both abalone sexes is currently a major concern for the Australian cultured abalone industry and current practices yield spawning rates in less than 40% of the induced broodstock [3]. To compensate for low spawning indices mass spawning is used but this practice results in a reduced rate of genetic improvement and the potential of developing unwanted genetic traits.

1.4.1 Gonad conditioning

Gonad conditioning is the process during which abalone gonads are stimulated to grow in preparation for spawning induction. During gonad conditioning, the processes of gametogenesis and vitellogenesis take place [45, 53].The current methods used within the industry for gonad conditioning varies from farm to farm but in essence is based on the effects of water temperature over the gonad development which is called accumulative effective temperature (AET). The AET technique requires the broodstock to be placed in holding tanks where they are subjected to specific temperatures for an extended period and it is expressed as degree days (°C-days) [45, 46, 53].The temperature threshold at which no gonad maturation occurs is called the biological zero point (BZP). During gonad conditioning the BZP is subtracted from the daily water temperature and summed over the treatment time to build the AET.

The AET varies from species to species. In the case of tropical abalone *H. discus*

hannai the AET for gametogenesis was determined at 600-900°C-days. The onset of vitellogenesis is triggered at 900°C-days and the temperature at which abalone are ready to spawn is at an AET of 1500°C-days [53]. For wild-caught *H. laevigata* and *H. rubra* the best temperature for gonad conditioning and spawning is 16°C [45]. The AET to guarantee gametogenesis and successful spawning in *H. laevigata* is 1700°C-days for male and 1930°C-days for female [45]. In the case of *H. rubra* the AET values are 1540 and 1350°C-days for male and female respectively [45]. The conditioning time for *H. laevigata* and *H. rubra* is about four weeks starting with a temperature of 16°C and 15°C respectively and spawning occurring at 20°C for both species (D. Connell, KIAB manager, pers. comm. 2011). It is important to mention that AET and consequent spawning are strongly dependent on abalone species and the influence of local environmental parameters.

Despite the effects of the AET on gonad conditioning and successful spawning, providing the optimum water temperature on farms is a difficult and time-consuming task with no guarantee of a successful spawning with the selected broodstock.

To determine the stage of sexual maturation and therefore readiness to spawn, a gonad condition assessment is performed by scoring the gonad according to the visual gonad index (VGI) that ranges from 0 to 3. This method was developed as a tool to predict ripeness in *H. discus hannai* where a VGI of 3 was associated with a high propensity to spawn [46] and the gonad denotes a swollen-round conical appendage. A VGI of 0 is assigned to a "flat/even" shaped gonad. Over the years, the VGI has undergone variations and a more extensive scale to assess VGI in *H. laevigata* has been developed [54] (Table 1-1).

Table 1-1. VGI scale for gonad development in *H. laevigata*.

VGI	Criteria
0	No gonad visible
1	Gonad membrane apparent, beginnings of gonad on underside of conical appendage
2	Some gonad membrane on proximal side of stomach and visceral spire
3	Gonad tissue apparent on conical appendage
4	Gonad tissue apparent on visceral spire
5	Gonad tissue thickening around visceral spire and proximal side of stomach
6	Gonad tissue thickening on conical appendage
7	Thick gonad tissue on proximal side of stomach
8	Approximately 50% of visceral spire covered in gonad tissue
9	Visceral spire covered in gonad tissue
10	Approximately 50% of conical appendage covered in gonad tissue
11	Thick gonad tissue covering entire visceral spire, stomach and conical appendage

Footnote; **VGI**, visual gonad index. Modified from [54]

Gametogenesis can also be assessed by cross-sectional measurements of the conical appendage. Linear measures of the complex gonad-digestive gland provide an estimate of gonad volume (EGV) which is then divided by the wet weight of the shucked abalone gonad to yield the modified gonad bulk index (MGBI). The gonad area can then be used instead of linear measurements to estimate the EGV (Figure 1-3) [47, 55], however, this is a destructive method and not useful in a practical sense on farm as an assessment of abalone propensity to spawn.

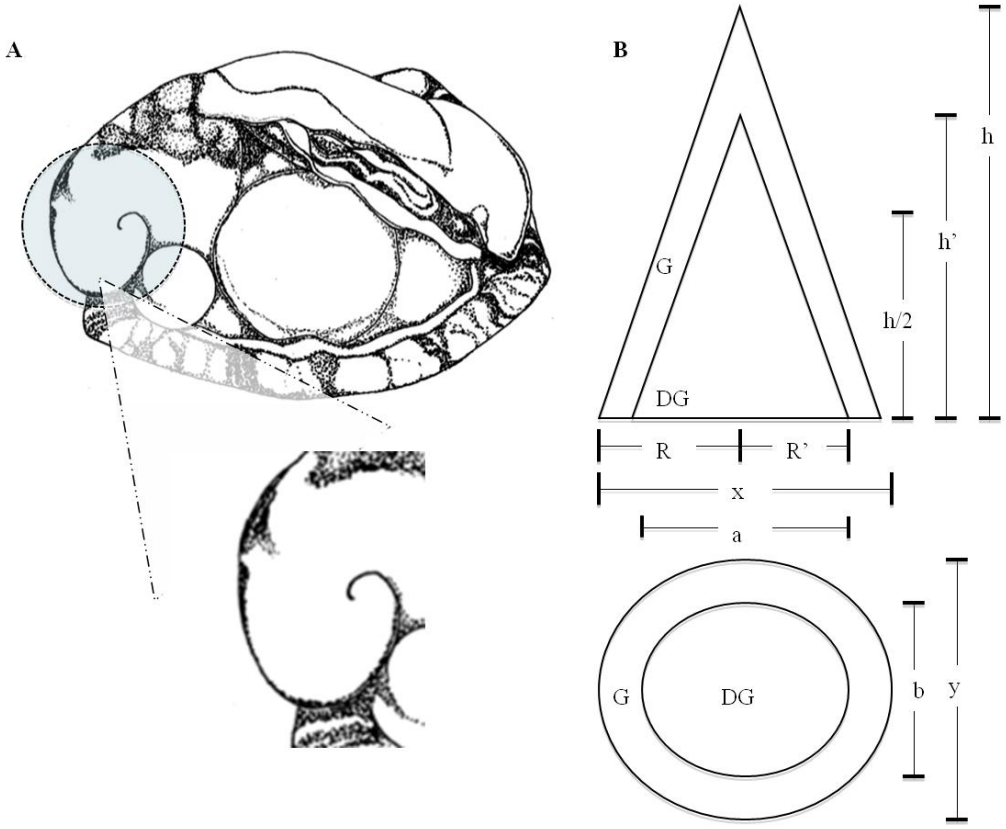

Figure 1-3. Top: schematic representation of an abalone without shell and conical appendage A, blue shade indicates the conical appendage complex. Bottom: magnified conical appendage complex. B, Conical appendage complex showing gonad (G) and digestive gland (DG) used to estimate gonad volume (EGV). Top: h, height of conical appendage; h' height of digestive gland; h/2, midpoint of conical appendage; R, radius of conical appendage; R' radius of digestive gland. Bottom: transversal section of conical appendage at midpoint; a, width of digestive gland; b, height of digestive gland; x, width of conical appendage; y, height of conical appendage. Replicated from [55].

Histology has been reported as a reliable tool for the accurate assessment of gametogenesis development in abalone. In *Haliotis varia*, oogenesis was divided into five stages including oogonia, pre-vitellogenic, early vitellogenic, late vitellogenic and mature oocytes [56]. Recently in another abalone species (*Haliotis midae*) oogenesis was comprised by nine stages where the progression from oogonia to mature egg is apparent in microphotography (Appendix 1-1) [57]. The same study reported six stages for spermatogenesis in the same abalone species (Appendix 1-1) [57]. Although destructive, histology is so far the most reliable technique to evaluate gonad development. As a consequence, there is a need for a non-destructive technique that allows accurate assessment of gonad development in abalone.

1.4.2 Spawning

On abalone farms, following assessment of abalone "ripeness", spawning is induced using artificial induction methods. There are two main methods used on farms to promote spawning: 1) addition of hydroxide peroxide (H_2O_2) at a concentration of ~ 0.25-5 mM with increase of tank water pH to 9.1 [58, 59] and/or 2) UV irradiation of seawater (50 – 253.7 nm) combined with an increasing thermal shock to a specific temperature, usually higher than the biological optimal [60]. Addition of H_2O_2 generates hydroxyl radicals in the water that have been suggested to activate prostaglandin pathways believed to be involved in the spawning process [58]. In several species including humans, prostaglandins E and F mediate reproductive processes such as ovulation, fertilization, pregnancy and birth [61]. The precursors of prostaglandins are prostaglandin peroxides whose endoperoxide and hydroperoxy moieties are catalyzed by the fatty acid enzyme cyclooxygenase [62]. Since biosynthesis of prostaglandin depends on previous synthesis of prostaglandin peroxide, the pathway can be blocked by addition of acetylsalicylic acid which is known to inhibit cyclooxygenase activity [58]. Irradiation of seawater with UV light also produces hydroxyl radicals [63]. The efficiency rates on spawning success of these methods vary across abalone species as it depends on adequate degree of gonad condition, environmental signals, and intrinsic species requirements [58-60, 64].

To compensate for uncontrolled spawning rates, alternative techniques such as cryopreservation of abalone gametes has been tested successfully for several species of abalone including *H. laevigata* with fertilization rates as high as 95% after 20 days of storage and 89% after one year [65-70]. Cryopreservation may solve the spawning inefficiencies and could be particularly useful for spawning abalone out of season but despite its potential, cryopreservation still relies on efficient abalone spawning for gamete collection.

Full control of spawning would benefit the Australian abalone industry in many ways, including reduction of the labour during mass spawning induction required in farm restocking and assisting the SBPs to reach expected results by producing abalone with desirable market characteristics. In this regard, identifying the molecular mechanisms that regulate abalone spawning is a prerequisite to improve the spawning success of farmed abalone.

1.5 Molecular analysis and selective breeding programs in abalone

Several molecular studies have been applied to genomic and mitochondrial DNA as well as proteins in many abalone species. These molecular techniques include random amplification of polymorphic DNA (RAPDs), microsatellites, restriction fragment length polymorphism (RFLP); single nucleotide polymorphism (SNPs) and allozymes studies (Table 1-2) [2, 4, 35, 71-83]. Next generation sequencing (NGS) has elucidated the transcriptomes for some tissues of *H. rufescens* and *H. midae* [10, 75]. These studies have used this data to establish phylogenetic relationships and determine evolutive divergences on abalone species and populations. Moreover, these studies have identified traits and predicted gains that have assisted in building selective breeding programs (SBPs) for abalone [4, 73, 75, 77, 83, 84].

Table 1-2. Molecular analysis performed on genus *Haliotis*.

Organism	Analysis	Contribution	Reference
Genus *Haliotis*	Allozymes, isozymes	Phylogenetic relationships	[72]
H. laevigata; *H. rubra*	Hybridisation	Hybrid identification	[73]
Genus *Haliotis*	DNA satellite characterization	Phylogenetic relationships	[80]
H. asinina	Gene expression on ganglia	Gene function & phylogeny	[85]
Genus *Haliotis*	Phylogeny based in ITS	Clade differentiation	[74]
H. asinina, H. varia, H. ovina	16S, 18S, RAPD, RFLP	Genetic diversity, species identification	[77]
H. discus hannai	Microsatellites	Gynogenesis, inheritance	[78]
H. rubra	Mitochondrial DNA analysis	Genome composition and organization	[79]
H. asinina	RAPD and Microsatellites	Population structure, isolation of transcripts important for biological processes	[81]
Genus *Haliotis*	Mitochondrial DNA, Cytochrome oxidase III	Clade differentiation	[35]
H. rubra	Microsatellites, RFLP	Population genetics	[71]
Hybrid (*H. rubra* x *H. laevigata*)	Abalone herpes-like virus identification	Molecular assay for diagnosis	[2]
H. rufescens, H. fulgens	Microsatellites	Hybridization and allotriploidization	[76]
H. laevigata	Phenotype	Growth gain in analysed families	[4]
Hybrid *(H. rubra x H. laevigata)*	Computer modeling	Production cost reduction	[83]
H. rubra	Computer modeling	Disease resistance and fast growth	[84]
H. tuberculata tuberculata	18S, ITS1, sperm lysin	mtDNA paternal inheritance	[82]
H. midae	Next generation sequencing	Transcriptome	[75]

Footnote: **RAPD**, Randomly amplified polymorphic DNA. **ITS**, Internal transcribed spacer regions. **RFLP**, restriction fragment length polymorphism.

The need for SBPs arose from demand to reduce costs and decrease production time for market size abalone. The aim of SBPs is to enhance specific desirable traits though the genetic improvement and manipulation of gene diversity within a species. These traits may include colour, texture, size, tenderness, foot appearance, disease resistance, adaptability to growth environments and higher survival rates [3]. To highlight the importance and usefulness of SBPs, one remarkable success in Australian aquaculture is the development of SBPs for the tiger prawn which improved the wet weight on progeny of selected broodstock by 200% [86]. Abalone

SBPs can be based on genetic or phenotypic selection [71, 73, 77, 84]. An important additional benefit of SBPs is the reduction of inbreeding rates. Phenotypic selection is based on genetic expression of a desired behaviour or physical feature. SBPs based on molecular markers, explore and quantify genetic diversity within a species. Due to genetic compatibility and geographic convergence, *H. laevigata* and *H. rubra* cross to produce a reproductively functional hybrid with backcrosses occurring naturally on occasion [73]. This hybrid has been suggested to more easily adapt to farming conditions than its progenitors [87]. However, given that the productivity of SBPs relies upon pair-wise crossing and synchronized spawning, current farming practices limit breeding opportunities due to uncontrolled gonad conditioning and spawning.

1.6 Approaches to identify egg-laying and spawning cues

Currently, efforts have been made to develop methods to manipulate spawning in a convenient and pragmatic way to improve the global abalone industry. Research to date has focused on understanding the influence of external factors involved in triggering abalone spawning, including temperature, chemical stimuli, specialized diets and correlations between astronomical factors and their effects on ocean hydrodynamics [46, 47, 49, 58, 88]. The influence of these factors on the endocrine system pathways and associated molecules that stimulate abalone spawning are yet to be elucidated.

The molluscan endocrine system is less complex, except for cephalopods, than that present in higher organisms such as vertebrates in both the number of cells and intercellular connections, but the functional principles are similar. Neurosecretory cells are located at the periphery of the ganglion, which is an aggregation of nerve regulatory cells equivalent to a brain [89-91]. The neurosecretory material is stored in the equivalent molluscan neurohaemal organ and then released to the perineurium.

This is subject to axonal processes and is in contact with connective tissue irrigated by haemolymph [89, 92].

Delivery of neurosecretory material is believed to occur in the perineurium upon electrical stimulation. It is then diffused through the connective tissues to the haemolymph where it travels to the target site (Figure 1-4) [92, 93]. In molluscan ganglia, the low number of cells and neuronal connections is compensated by a high degree of peptide diversity due to post-translational processes, alternative splicing and differential expression from precursors [94-96]

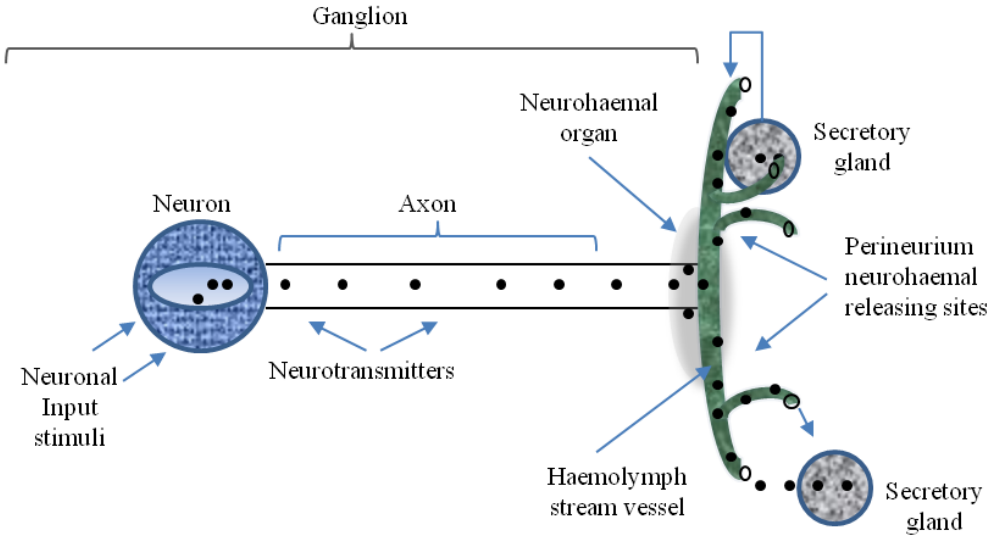

Figure 1-4. Schematic representation of a molluscan endocrine system
Adapted from the description of synapses in vertebrates [91, 97].

Research on gastropods and cephalopods has revealed substantial information about structure and performance of their endocrine system. It has elucidated the location of neurosecretory cells, neurohaemal organs and axonal transport, and their relevance in reproductive processes such as egg deposition and egg release. Using lead citrate and uranyl acetate staining and microscopy the sheath of the infraoesophageal ganglion was deemed to be a neurohaemal organ in the garden snail *Helix aspersa* [98]. Monoaminergic and peptidergic associations to the stroma were observed. The

peptidergic associations were specialized for the release of neurosecretory substances [98]. Another study on *Helix aspersa* reported an increase of oocytes and sperm transport from the ovotestis duct (hermaphroditic organ of some gastropods) to the fertilization complex. This gamete transport was promoted by contractions in response to administration of acetylcholine, serotonin and electrical stimulation [99]. Contractions in sexual organs are required for the release of gametes. Identifying the hormones peptides and/or proteins that control spawning in abalone would provide a better understanding of the molluscan endocrine system.

1.6.1 Molluscan molecules involved in reproductive processes

Analogous hormones and peptides to those known to stimulate reproduction and associated behaviors in vertebrates have been identified in molluscs. Three isoforms of gonadotropin-releasing like hormone (GnRH-like) have been identified in the sword squid *Loligo edulis*, common octopus *Octopus vulgaris* and sea hare *A. californica* [100]. Injection of the octopus GnRH-like has been shown to stimulate gonad development and partial spawning in *H. asinina* when used as an interventional method to promote egg deposition and reproductive behaviors [101]. In contrast, when *A. californica* was injected with a synthetic version of its own GnRH it showed no induction of reproductive mechanisms [102]. In the case of the Pacific oyster *C. gigas*, administration of mammalian, salmon, chicken and lamprey GnRHs promoted DNA synthesis in gonial cells whilst in *Mytilus edulis* mammalian GnRHs promoted mitotic activity in mantle cells [103].

Egg-laying hormone (ELH) is a neuropeptide that promotes reproductive behaviors such as change in body pace and egg deposition in *Aplysia* [104]. Experimentation using recombinant ELH has shown potential in inducing efficient spawning, egg deposition and associated behaviours in gastropods and crustaceans. When injected in *A. californica*, ELH stimulated mating behaviors and spawning [105]. In 1998, an

abalone egg-laying hormone (aELH) isoform was identified in black lip *H. rubra* [106]. Three years after its elucidation, aELH was detected by immunolocalization in neurons and reproductive tissues in *H. rubra* [107]. When recombinant aELH was injected in *H. asinina*, it influenced early sexual differentiation towards female gonad development and spawning [101]. Furthermore, the effect of aELH on *H. asinina* spawning was dose dependent. In prawns (*Macrobrachium rosenbergii*), aELH was shown to induce ovarian maturation and spawning [108]. The aELH had a 95.4% nucleotide sequence homology with the ovulation hormone (CDCH) of the pond snail *L. stagnalis* [106], which has been demonstrated to cause sexual maturation and spawning when injected into *L. stagnalis* [93, 109, 110]. Table 1-3 shows the known peptides and hormones of molluscan species associated with physiological functions, such as growth, contraction required for spawning, mating behaviors and sexual reversion.

Table 1-3. Peptides that mediate physiological events in several molluscs.
Modified from [111] enriched from [112].

Name	Sequence	Event	Species
APGWa	APGWa, TPGWa, KPGWa	Relax sperm ducts, reproduction antagonist	*Crassostrea gigas* *Thais clavigera*
CDCH-I	LSITNDLRAIADSYLYDQHKLRERQEENL RRRFLELa	Ovulation, control	*Lymnaea stagnalis*
CDCH-II	SITNDLRAIADSYLYDQHKLREQQEENLR RRFYELSLRPYPDNL	Shell turning	*L. stagnalis*
ELH	ISINQDLKAITDMLLTEQIRERQRYLADLR QRLLEKa	Egg-laying	*Aplysia californica*
aELH	LSITNDLRAIADSYLYDQHKLRERQEENL RRRFLRL	Spawning	*Haliotis rubra*
ERH	ISIVSLFKAITDMLLTKQIYANYFSTPRLRF YPI	Egg-releasing	*A. californica*
Califin A (L)	ISINQDLKAITDMLLTEQIQARRRCLDALR QRLLDLa	Neural modulation	*A. californica*
Califin A (S)	DSDVSLFNGDLLPNGRCS	Neural modulation	*A. californica*
α-CDCP	EPRLRFHDV	Egg-laying	*A. californica*
α-BCP	APRLRFYSL	Egg-laying	*A. californica*
TEP-1	KSCGKWAIHACWGGNa	Contractions penial complex	*T. clavigera*
TEP-2	KSYGKWAMHACWGGNa		*T. clavigera*
FMRFa	FMRFa	Myoactive	*Fusinus perplexus ferrugineus*
Has-SLP	FSCDPECFSYLDGPFCISNGSVVCDNCLRD RMLCEDMSLTSKDCSAPCD	Growth	*H. asinina*
Has-HGAP	MKVLCILLVVVGTALSREVGKVARRDED FVCPPQEESFGYMSFCAWDPCDIFADQPC ADIPEAVCKFDACPNHACIPMFFVDKQW VECHDLVYRDK	Growth	*H. asinina*
FVa	GAPRFVa	Increase K+ current in pleural sensory neurons	*Aplysia* spp.
Novel peptide	HF (FH) FYGPYDVFQRDVa	Cease reproduction depending on age	*L. stagnalis*
F(X)RIa	ASSFVRIa	Unknown	Unspecified

Footnote: **a**, amide.

Additional aquaculture research has provided insights into molecules potentially involved in sexual maturation and spawning in abalone. The presence of neurosecretory cells was determined in cerebral ganglia of *H. discus hannai* at different stages of reproductive cycle promoted by AET [89]. These neurosecretory cells were stained with different dyes and the cells affinity for the dye was correlated

with the abalone reproductive stage [89]. The more advanced the reproductive cycle, the higher the cell staining affinity for the dyes. The cell staining affinity for the dyes decreased after abalone spawning [89]. In a different study *H. discus* female were induced to spawn by injecting crude homogenates of pleural-pedal ganglia previously collected from sexually mature females [113] These injections also caused swelling of the gonads [113]. However, spawning did not occur when cerebral ganglion homogenates were injected [113]. These results indicated that the pleural-pedal ganglia could produce a molecule(s) responsible for the initiation of abalone spawning [113].

Other studies have focused on identifying the genes participating in biological processes such as reproduction. For example, in *H. asinina*, differentially expressed genes have been suggested to participate in gametogenesis and cell proliferation [114, 115]. The gene expression of tektin A1 and axonemal protein 66.0 were quantified in testes of *H. asinina* and positively correlated to spermatogenesis [114]. These transcripts were deemed as sperm-specific in *H. asinina* as they were undetected in ovaries. These observations have been corroborated in *H. rufescens* [10]. Lysin has been extensively studied in evolutive approaches and functional interaction with proteins from the vitelline envelope zona pellucida and its relevance for acrosomal reaction and fertilization in abalone [116-120]. With regards to females, substantial research has been undertaken to characterize the vitelline environment of abalone eggs. To date, up to 30 vitelline envelope zona pellucida domain proteins have been reported in Californian abalone species and inferences have been made about their possible roles in the abalone acrosomal reaction and fertilization [118, 119]. With respect to identification of molecules with involvement in reproductive processes, an EST coding for the putative 17 beta-hydroxysteroid dehydrogenase type 12 (17 β-HSD-12) was identified in digestive gland of *Haliotis*

diversicolor supertexta. Further functional analysis revealed that 17 β-HSD-12 transfected into HEK-293 cells converted estrone (E1) into estradiol (E2). Subsequent qPCR showed that 17 β-HSD-12 was differentially expressed across gonads of pre-spawning, during-spawning and post spawning abalone with high expression during-spawning, intermediate in pre-spawning and low in post-spawning abalone. Therefore, 17 β-HSD-12 was suggested to be involved in the steroid synthesis during the reproductive season [121].

1.7 Proteomic strategies applied to identification of molecules in marine invertebrates

Proteomics, whether solely based on mass spectrometry (MS) or coupled to two-dimensional gel electrophoresis (2DE), is an efficient means to identify and characterize both known and novel proteins. In the case of molluscs, proteomics has the potential to assist in understanding how proteins may be involved in regulation of crucial biological process such as spawning and reproduction. The basic principle of this technique is to separate proteins in a gel based on their isoelectrical point (p*I*) in the first dimension and in the second dimension the proteins are resolved according to their molecular weight by SDS-PAGE [122]. This technique allows separation and identification of diverse classes of proteins and protein isoforms, including those that contain post-translational modifications. Additionally, by assessing the staining intensity of the gel spots information may be inferred about protein quantity.

An illustrative example of the application of 2DE coupled to MS applied to molluscs is the high number of protein identifications from the eggs and sperm of mussels. [123-125]. Furthermore, the staining intensity of the proteosomes was correlated with an unusual process named double uniparental inheritance that occurs in certain molluscan species, where both progenitors provide mitochondrial genetic information to their offspring [123]. In abalone *H. diversicolor*, 2DE coupled to MS

was employed to validate and characterize haemocyanin-like proteins in virus-infected abalone [126]. In a recent study using a similar approach, the heterosis of Japanese and Taiwanese abalone (and their hybrid) *H. diversicolor* was assessed in muscle [127] using 2DE and MS. A suggested genetic improvement was observed in the hybrid with regards to a higher expression of proteins involved in energy metabolism [127].

In toxicological studies the effects of endocrine disrupting compounds (EDCs) in molluscs has been significantly assisted by 2DE coupled to MS to identify and characterize differentially expressed proteins. The effects of the EDC, diallyl phthalate and bisphenol A, upon protein expression were assessed in hepatopancreas of *H. diversicolor supertexta*. Eight proteins were identified as differentially expressed. Moreover, these proteins were involved in cell protective process such as detoxification, hormone and immune regulation as well as against oxidative stress [128]. This approach has identified several molluscan species as biomarkers in ocean zones of high anthropogenic impact [128-130].

Difference-in-gel-electrophoresis (DIGE) is a modification to traditional 2DE. This complementary technique incorporates different dyes that are used to fluorescently label the proteins prior to isoelectrical focusing [131], allowing pair-wise comparison of proteins under different treatments or physiological states. The two samples are labeled with different dyes and a third dye is used to label a 50:50 mixture of the two samples. This pooled sample is used as an internal reference to compare treatments. The three samples are then pooled and resolved in a single 2DE gel. Assisted by specific software, the changes in protein content for each focused protein spot of each sample can be rapidly quantified by comparing the fluorescence intensity (corresponding to the protein abundance ratio) measured at the wavelength of each dye. This method facilitates localisation of differentially expressed proteins by

difference in color of the protein-spots in the 2D-DIGE gel image where red and green protein-spots indicate a down- and up-regulated protein respectively, whereas a yellow spot-protein indicates no change in protein expression. A study using 2D-DIGE evaluated the EDC's effects in reproductive organs of *L. stagnalis* [8]. Several proteins involved in reproductive processes such as gametogenesis and egg deposition were identified as differentially expressed. Specifically, EDCs negatively influenced the expression patterns of yolk ferritin (the equivalent of vitellogenin) and ovopostatin [8].

The power of proteomics relies in the availability of comprehensive protein and genomic databases. Identification of proteins in non-model organisms has been further improved by transcriptome assisted proteomics. To exemplify this point, a comprehensive transcriptomics study identified 569 shell matrix proteins in the owl limpet *Lottia gigantea* [132] whilst only 14 shell matrix proteins were identified in a *H. asinina* study that relied on a UniProt protein database [133]. In *H. rufescens* sperm, 975 proteins were identified from a large transcript library.

Peptidomics arose from the need to separately study bioactive peptides and proteins of a relative low molecular weight (0.5 -15 kDa) and low abundance [134]. MS analysis, when coupled with liquid chromatography, has the potential to resolve and detect extremely low quantities of small peptides. The present use of MS approaches in molluscs has focused on the isolation and characterisation of peptides of the FMRFa family including several FMRF amide-related peptides (FaRPs), FMRFa-like peptides (FLPs) and amidated tetrapeptides (APGWa). These peptides act as cardio-excitatory molecules, neurotransmitters, neuromodulators and muscle relaxants [9, 99, 135-139]. The FVRIa peptides involved in muscle modulation during mating and feeding of *L. stagnalis* were identified through electron impact mass spectrometry (EI-MS) and matrix-assisted laser desorption/ionization mass

spectrometry (MALDI-MS) [96]. The cardio-active peptides, Ocp-1 (G-D-FGD) and Ocp-3 (GSWD), were isolated from brain of *Octopus minor* using time-of-flight mass spectrometry (TOF-MS) [90]. In *Octopus vulgaris*, the peptide ENYHFSNGWHPGa which has sequence similarity to GnRH, was identified through TOF-MS and suggested to be involved in octopus reproduction [100]. In *A. californica*, the well known molluscan peptides FMRFa, FLRFa and APGWa were identified in a 430-5000 Da range neuropeptide mapping experiment [140]. The peptide dynamic pattern and peptide identity of *A. californica* bag cells were profiled after electrophysiological stimulation *in situ* employing of MALDI-TOF and tandem MS (MS/MS) [141]. Buccalins have also been identified in *A. californica* [140]. Figure 1-5 shows the signaling pathways for reproduction and spawning in *A. californica* and *L. stagnalis*.

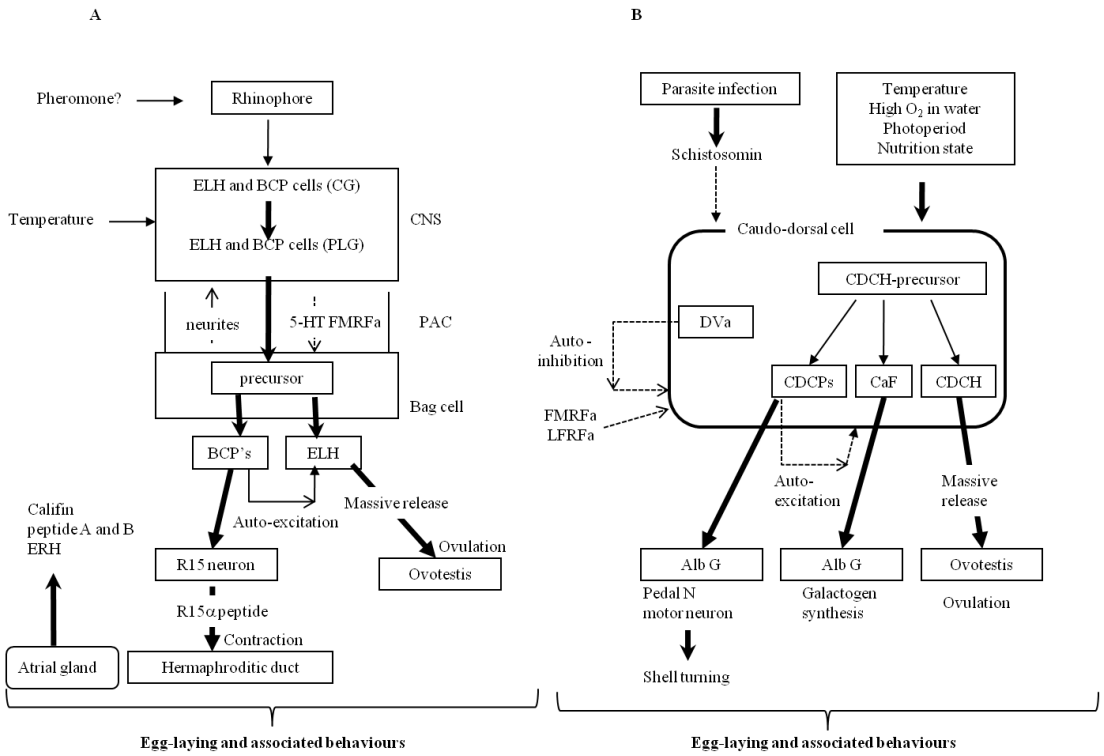

Figure 1-5. Egg-laying systems
A, ELH or BCP egg-laying systems of *Aplysia californica*. Solid arrows indicate excitatory action and dashed ones indicate inhibition. BCPs, bag cell peptides; CG, cerebral ganglion; CNS, central nervous system; ELH, egg-laying hormone; FMRFa, FMRFamide; 5-HT, serotonin; PLG, pleural ganglion; and PAC, pleuro-abdominal connective. B, Egg-laying system of *Lymnaea stagnalis*. Solid arrows represent excitatory action and dashed arrows indicate inhibition. Alb G, albumen gland; CaF, calflaxin; CDCH, caudo-dorsal cell hormone; CDCPs, caudo-dorsal cell peptides; DVa, HFFYGPYDVFQRDVamide; FMRFa, FMRFamide; and LFRFa, LFRFamide. Pathways replicated from [111].

Aside from the studies performed on lysin and vitelline envelope zona pellucida protein domains (VEZPs) by the Vacquier and Swanson laboratories, limited research has been directed at elucidating the molecular mechanisms that regulate reproduction in abalone. The VEZPs domains have been extensively described in several species of Californian abalone. Roles in fertilization, gamete co-evolution and sexual biomarkers have been suggested for VEZPs in different species [119, 142, 143]. Vitelline envelope receptor for lysin (VERL) have been demonstrated to assist fertilization by interacting with lysin [117].

Relevant progress has been made on the peptides involved in abalone gonad conditioning and spawning. In the gonad, hepatopancreas, cerebral and pleura-pedal

ganglia of *H. rubra*, an alpha-CDCP-like peptide with 94% sequence homology to *L. stagnalis* ELH was identified [144]. This peptide is known to be involved in egg laying behavior in *L. stagnalis* in the same way as the alpha-BCP peptide acts in *A. californica* [109, 145]. Administration of serotonin (5-HT) caused contractions on gonadic tissue of male *H. rubra* that resemble those movements that naturally occur during abalone spawning [137]. Consequently, 5-HT was proposed to participate in molluscan reproduction [137]. In another study on *H. asinina*, an attractin-like pheromone was identified in hypobranchial gland, gill and foot that is thought to influence mating behavior in *Aplysia* [146]. The peptide APGW influenced growth rate of juvenile *H. asinina* also as evidenced by a doubling of body weight when injected over a period of 14 weeks [147].

In summary, the methods utilised to promote abalone spawning have not been subjected to substantial modifications since their development in 1970's. There has been significant research in other important areas such as physiology, nutrition, culturing systems, genetic improvement and elucidation of neuropeptides involved in growth and mating behaviour. This research has positively contributed increasing our understanding of many aspects of abalone aquaculture, however, full control over abalone spawning under farming conditions remains an unsolved problem. The advent of high-throughput techniques (transcriptomics, genomics and proteomics) will aid in the discovery of molecules intrinsically involved in reproductive pathways in non-model animals such as abalone.

The aim of this thesis is to make a substantial contribution to understanding the proteins involved in abalone spawning and its regulation during spawning. The objectives of this thesis are: i) increase the knowledge of the constituting proteins of female and male abalone gonads and ii) perform a differential protein expression assessment in gonads of both sexes of abalone after artificial spawning induction.

High-throughput tools including mass spectrometry and genetic information will be the foundation of this research.

The first objective will use liquid chromatography mass spectrometry (LC-MS/MS). In this instance, the MS generated datasets from abalone gonads in basal state will be searched against an extensive custom-built protein database made from genomic information from a diversity of molluscan species. In addition, two protein databases publicly available (UniProt, NCBI) will be also employed. The second objective will assess the differential protein expression using 2D-DIGE coupled to a further MS analysis to identify and quantify differentially expressed proteins in both sexes of abalone. To achieve this, ripe *H. laevigata* will be artificially induced to spawn and gonadic tissue will be sampled in three different physiological states: spawning, post-spawning and failed-to-spawn. The levels in protein expression will be correlated with spawning and failure to spawn.

2 CHAPTER: EXPLOITING GENOMIC DATA TO IDENTIFY PROTEINS INVOLVED IN ABALONE REPRODUCTION

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Abstract

Aside from their critical role in reproduction, abalone gonads serve as an indicator of sexual maturity and energy balance, two key considerations for effective abalone culture. Temperate abalone farmers face issues with tank restocking with highly marketable abalone owing to inefficient spawning induction methods. The identification of key proteins in sexually mature abalone using proteomics will serve as the foundation for a greater understanding of reproductive biology. Addressing this knowledge gap is the first step towards improving abalone aquaculture methods. Proteomic profiling of female and male gonads of greenlip abalone, *Haliotis laevigata*, was undertaken using liquid chromatography-mass spectrometry. Owing

to the incomplete nature of abalone protein databases, in addition to searching against two publicly available databases, a custom database comprising genomic data was used. Overall, 162 and 110 proteins were identified in females and males respectively with 40 proteins common to both sexes. For proteins involved in sexual maturation, sperm and egg structure, motility, acrosomal reaction and fertilization, 23 were female specific, 18 were male specific and 6 were common. Gene ontology analysis revealed clear differences between the female and male protein profiles reflecting a higher rate of protein synthesis in the ovary and higher metabolic activity in the testis.

Significance

A comprehensive mass spectrometry-based analysis was performed to profile the abalone gonad proteome providing the foundation for future studies of reproduction in abalone. Key proteins involved in both reproduction and energy balance were identified. Genomic resources were utilised to build a database of molluscan proteins yielding >60% more protein identifications than in a standard workflow employing public protein databases.

Keywords

Haliotis laevigata, abalone, mass spectrometry, LC-MS/MS, gonad, proteome

2.1 Introduction

Abalone are marine gastropods that can be found worldwide in temperate and tropical waters from intertidal zones and to a depth of 40 meters [34]. Abalone are highly valued for their palatability in Europe, the United States and most Asian countries. In the wild, abalone resources are strictly regulated to prevent depletion of natural stocks which in turn has promoted artificial cultivation to fulfill market

demands. Abalone are cultivated in many countries including Australia, New Zealand, South Africa, Japan, China, Korea, Chile, Mexico, the United States and Thailand [16]. In Australia, temperate black lip *Haliotis rubra*, greenlip *H. laevigata* and their hybrid are the predominant aquaculture species farmed onshore in the coastal areas of Victoria, Tasmania, South Australia and Western Australia. In 2011-12, the value of wild-caught and farmed abalone production was estimated to be AUD$178 million [148].

Australian abalone farmers face significant challenges. Financial commitments are high as it can take up to four years to bringing temperate abalone to market. Aquatic animal health is a major concern in the abalone industry. Massive mortalities can occur due to pathogens and variations of temperature that compromise the immune systems of abalone. Another issue in temperate abalone farming is the inefficient control over spawning which hinders the application of selective breeding programs to improve production and allow highly marketable abalone with desirable characteristics to be farmed. Selection of sexually mature abalone on farms relies upon the visual assessment of the gonad diameter and state of engorgement. While this technique is not entirely accurate it allows the farmer to select potential broodstock without sacrificing the animal to evaluate its sexual maturity.

Considerable research has been performed on *H. rubra* and *H. laevigata*, however this has focused on physiological studies to improve culture conditions and on genetic studies to develop selective breeding programs and improve animal health. There has been some progress in the use of proteomic approaches in gastropod molluscs, such as the sea slug *Aplysia californica*, the limpet *Lottia gigantea*, and pond snail *Lymnaea stagnalis*, where substantial genomic information is available [6, 132, 149]. In contrast, the successful application of proteomics to studies of abalone is limited by the poor availability of genomic information in public databases (e.g.

UniProt, NCBI). A study employing proteomics and a NCBI *H. asinina* database identified only 14 proteins from abalone shell matrix, two of which had previously been reported [133]. This is a low identification rate compared to similar studies in well characterised species. For example, a similar approach reported the identification of 569 proteins from limpet shell matrix [132]. Owing to the limitations of the public protein databases (34 reviewed proteins exist in Uniprot-KB), characterization of abalone proteins relies on either examination of homology to orthologous proteins in other gastropod species or predictions based on the use of genomic resources from other molluscan species. To illustrate this point, a recent study employing transcriptomics to generate a database specific to *H. rufescens* was coupled to proteomics and facilitated the identification of 975 proteins in abalone sperm [10].

Reproduction must be efficient to ensure continuity of any specie. In broadcast spawning organisms, efficient reproduction seems slightly more complex due to the random nature of their ecological niche. Abalone rely upon numerous environmental cues and chemotaxis for gametes to coincide and for fertilization to occur. While reproduction is well documented in model organisms, control of synchronized spawning which is a prerequisite for efficient abalone reproduction on farms remains unsolved.

In this study, a molluscan protein database was assembled using protein sequences predicted by EST and genomic resources, which facilitated a more in-depth proteomic analysis of abalone gonad. This approach was applied to the comprehensive proteomic profiling of mature female and male *H. laevigata* gonads and provides the basis for ongoing studies of reproduction in farmed abalone.

2.2 Material and Methods

2.2.1 Protein preparation and digestion using Filter Aided Sample Preparation (FASP)

Sexually mature *H. laevigata* with a visual gonad index (VGI) of 3 were selected from production tanks at the Kangaroo Island Abalone (KIAB) farm (35°35'42.2" S; 137°25'52.5" E). The VGI is a non-destructive technique for the assessment gonad development during spawning season [46] and consists of four categories (0-3) that relate to changes in the size and shape of the gonad wherein VGI 3 refers to animals with a swollen gonad with rounded tip and are classified as sexually mature. The selected abalone were anaesthetized by injecting 1 ml of magnesium chloride (360 mM) into the haemolymph duct using a 30 gauge and 8 mm ultra fine syringe. For each animal the outer membrane of the gonad was excised and care was taken to avoid disrupting the underlying digestive gland. Approximately 500 mg of gonadal tissue (eggs or sperm) was snap-frozen in liquid nitrogen and stored at -80°C prior to analysis.

Samples were prepared as described in [150]. Briefly, aliquots of 50 mg of abalone gonad were homogenised in 350 μL 4% SDS, 100 mM Tris-HCl (pH 7.6) and reduced with 100 mM dithiothreitol (DTT). Five abalone gonads of each sex were independently treated. The tissue disruption was performed on ice using a homogeniser (Ultra-turrax T8, IKA) for 5-10 s and then sonicated at 30% (amplitude) for 5 s. Preparations were incubated with agitation at room temperature for 2 h at 700 rpm. A clear lysate was obtained after centrifugation at 14,000 *g* for 30 min at 4°C.

A total of 100 μg of protein as estimated by the Bradford protein assay were incubated at 95°C for 3 min and then sonicated for 10 min. Protein was solubilised in 200 μL of 8 M urea (in Tris-Cl pH 8.5) and then transferred to a 10 kDa MWCO

filter unit (Millipore) and centrifuged at 14,000 *g* for 15 min at room temperature. A further 200 μL of 8 M urea was added to each tube and centrifuged again. Cysteine residues were alkylated for 20 min by incubation at room temperature with 50 mM iodoacetamide (IAM). Excess IAM was removed by repeated washing of the filters with 100 μL 8 M urea and centrifugation. The filter unit was equilibrated with 50 mM ammonium bicarbonate. Tryptic peptides were generated using 1 μg trypsin per 100 μg of protein in 50 mM ammonium bicarbonate and overnight incubation at 37°C. The peptides were recovered in a fresh tube by centrifugation. An additional elution step involving addition of 40 μL 50 mM ammonium bicarbonate and centrifugation was performed. The two eluates were pooled. The samples preparations were vacuum-dried and resuspended in 0.5% formic acid.

2.2.2 Mass spectrometry identification

Two injections of tryptic peptides from each protein preparation were chromatographically resolved using a Shimadzu Prominence LC20 HPLC system with a C18 Vydac column (75 μm x 15 cm, 300 Å, 5 μm). Protein digests were reconstituted in 20 μL of 0.5% formic acid and 2 μL of each tryptic digest was injected on-column. A linear gradient at a flow rate of 300 nL/min from 2-40% solvent B over 56 min was utilised where solvent A was 0.1% formic acid and solvent B was 0.1% formic acid in 90% acetonitrile. The eluate was directed into the nanoelectrospray ionisation source of the TripleTOF™ 5600 system (AB/Sciex, Foster City, CA, USA). Data were acquired in information dependent acquisition (IDA) mode using Analyst TF1.6 software (AB/Sciex). The IDA method consisted of a high resolution TOF-MS survey scan with an accumulation time of 0.5 s followed by 20 MS/MS in a second with a maximum accumulation time of 50 milliseconds. First stage MS analysis was performed in positive ion mode over the mass range 300-1800. The ionspray voltage was set to 2400 V, the curtain gas was set to 25, the

nebuliser gas to 12 and the heated interface was set to 180°C. Tandem mass spectra were acquired over the mass range 80-1800 using rolling collision energy for optimum peptide fragmentation. Precursor ion masses of charge states 2 to 5 that exceeded 120 counts per second were acquired and then excluded for 8 s after two occurrences.

2.2.3 Molluscan Database construction

Genetic information from several molluscan species (Appendix 2-1) (provided in CD) was used to build the customised database utilised in this study. Contigs, mRNA, SRA reads (Sequence Read Archive), EST sequences and transcriptome assemblies were downloaded from a range of public sources. SRA reads were assembled de novo into contigs using MIRA 3.4.0 http://sourceforge.net/p/mira-assembler/wiki/Home/. EST, mRNA or transcriptome assemblies were used directly to detect open reading frames (ORFs) from six reading frame translations using the getorf application. EMBOSS suite default parameters were applied to obtain the ORFs (minimum ORF 30 nt, translation between stop codons, standard codons) http://emboss.sourceforge.net/. The longest ORF for each sequence was collated into the customised molluscan database. This strategy was chosen to ensure that the least number of false and/or corrupted ORFs were compiled into the database.

Figure 2-1 shows a schematic representation of the workflow employed to obtain improved identification rates in abalone gonads. Following tryptic digestion, the extracts were subjected to LC-MS/MS analysis. Protein identification was performed by searching the resulting spectral datasets against two public and one custom-built (cDNA and EST-based) protein databases. The proteins identified in each search were compared at the spectral level and only the minimum number of confident proteins after manual verification and curation are reported

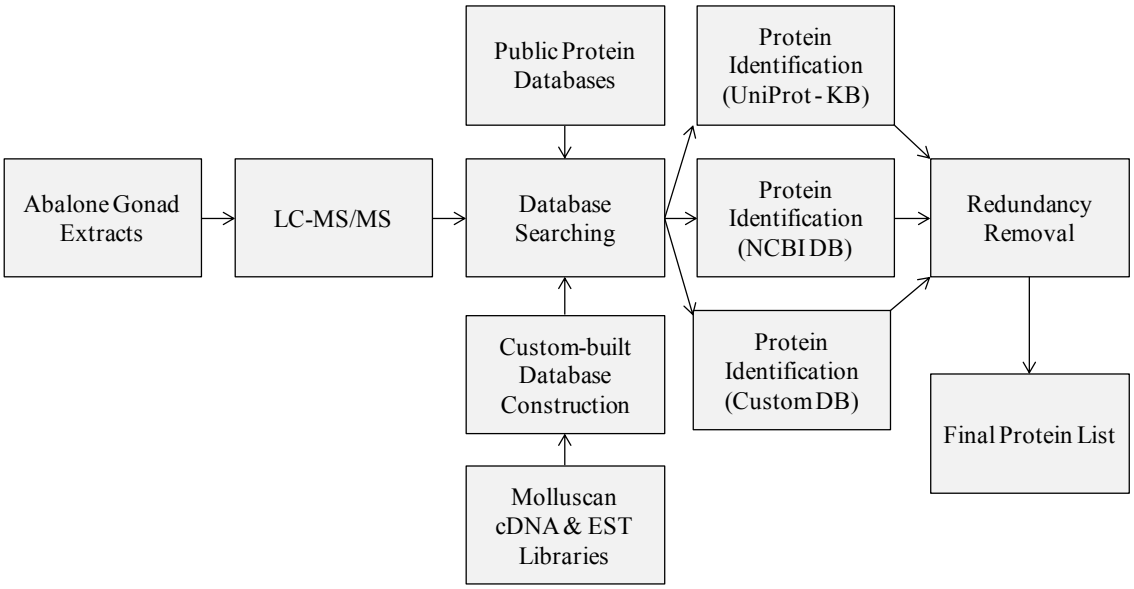

Figure 2-1. Proteomics approach employed to profile the gonad proteome of male and female *Haliotis laevigata*. Tryptic digest of total protein preparations from gonads of mature abalone were subjected to LC-MS/MS analysis. Protein identification was performed by searching the resulting spectral datasets against two public and one custom-built (cDNA and EST-based) protein databases. The redundancy was manually removed. Proteins with 99% confidence are reported.

2.2.4 Protein identification

The ten spectral datasets for each sex were pooled to perform a combined protein search. ProteinPilot™ 4.0.1 software (Applied Biosystems), with the Paragon Algorithm and integrated false discovery rate analysis [151], was used for protein identification. The MS/MS spectra were searched initially against all molluscan proteins present in the UniProt database (Mollusca: version 20130201; 130,756 proteins); NCBI databases (Mollusca: version 20130101; 246,968 proteins) and then widened to a customised database (Mollusca: version 20120529; 1,757,464 proteins) to include ESTs and cDNA libraries from other molluscan species not present or annotated in standard databases. Search parameters were defined as cysteine alkylation with iodoacetamide, trypsin as the digestion enzyme and no restrictions were placed on taxonomy. Modifications were set to the "generic workup" and "biological" modification sets provided with this software package, which consisted of all modifications listed in Unimod, for example, acetylation, methylation and phosphorylation. The generic workup modifications set contains 59 potential modifications that may occur as a result of sample handling, for example, oxidation, dehydration and deamidation. The criteria for positive protein identification were proteins with ≥99% confidence, with one or more unique peptides ≥95% confidence using the false discovery rate (FDR) analysis provided within the ProteinPilot™ software wherein $p = 0.05$. High confidence protein identifications were confirmed by manual inspection of the spectra and search results. Identified proteins from the three MS workflows were combined and manually curated for annotation redundancy in the UniProt and NCBI databases and tabulated (Appendix 3-2 for identifications in female abalone and Appendix 3-3 for identifications in male abalone) (provided in CD). Gene names were sourced from UniProt, NCBI, HUGO Gene Nomenclature Committee http://www.genenames.org/ and gene ontology

analysis were carried out in http://amigo.geneontology.org/cgi-bin/amigo/go.cgi and http://www.uniprot.org/.

2.3 Results and discussion

A total of 162 and 110 proteins were identified in female and male gonads respectively using a proteomic approach employing high resolution MS and database searching utilising both protein and DNA sequences housed in public resources. The benefit of using genomic resources in poorly characterised species is highlighted by the application of the custom-built database described here. Additional gains of 60% (female) and 72% (male) in the identification rates (from only 101 and 64 proteins identified using public databases) were obtained using genomic resources (contigs, mRNA, SRA reads, ESTs and cDNA sequences). A full description of the identified proteins (including fragment evidence, *m/z*, charge, mass error and peptide sequence for proteins reported based on single peptide identifications) can be found in Appendices 2-2; 2-3; 2-4 and 2-5 (provided in CD). Many proteins were identified only in one gender (122 female and 70 male) whilst an additional 40 proteins were common to both sexes (Figure 2-2A) [152]. Gene ontology revealed that 34 of the common proteins were involved in structural, ribosomal, signaling, transport and energy metabolism processes while the functions of the remaining six were in sexual reproduction (Figure 2-2B). The proportion of proteins involved in ribosomal activity was higher in females than in males, perhaps due to the high demand for protein synthesis (vitellogenin, vitelline and vitelline zona pellucida domain proteins) during oogenesis. In the case of testis, gene ontology revealed a large proportion of proteins involved in metabolic pathways in accordance with the increased energy demands, specifically for the flagellar movement of newly produced sperm. Additionally, an increased proportion of proteins involved in signaling in males compared to females (5% versus 1%) was observed and is consistent with the diversity of acrosomal and

axonemal proteins found in abalone sperm that have critical roles in flagellar structure and motility. No peptide hormones or endogenous peptides less that 10 kDa were expected to be identified using our methodology. The use of 10 kDa filters during sample preparation would have excluded such small molecular species.

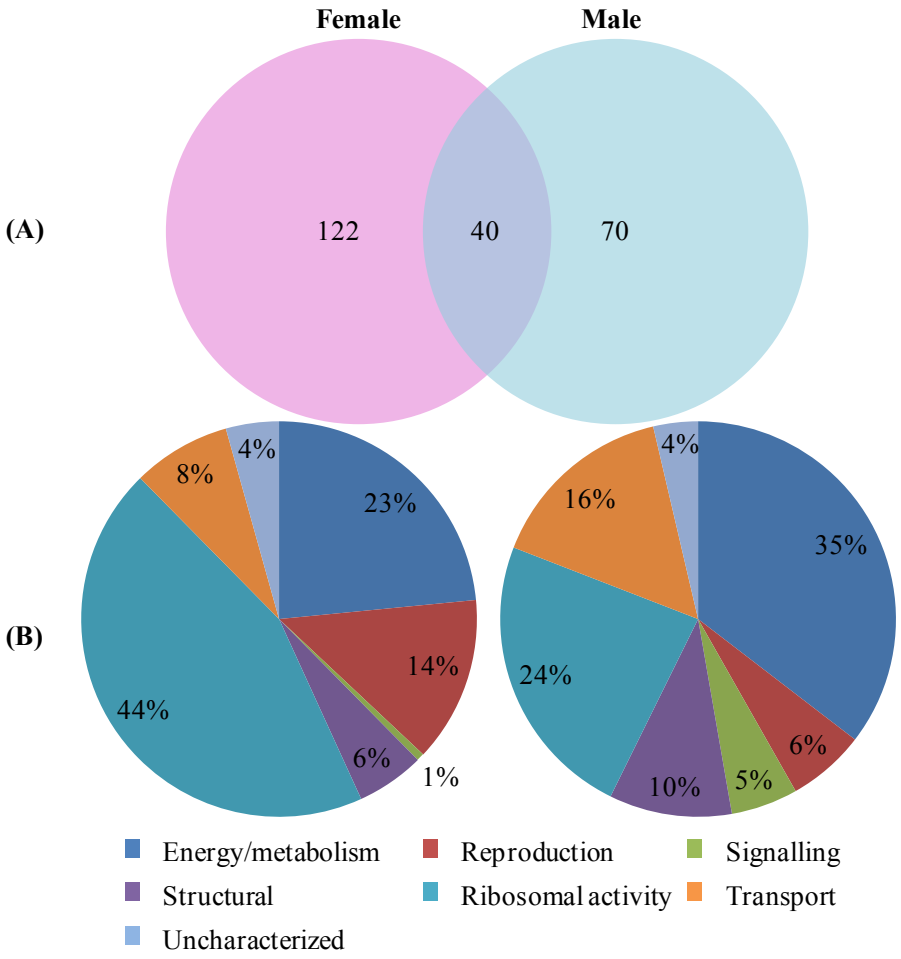

Figure 2-2. Distribution and functional classification of abalone gonad proteins from both females and males.
(A) Distribution of common and sex-specific proteins identified in abalone gonads using LC-MS/MS. (B) Comparison of functional characterization between male and female abalone. Sources of gene ontology analysis: UniProt, HUGO, AmiGO. Venn diagram analysis from [152].

Owing to the incomplete nature of *Haliotis* protein databases, multiple-peptide identifications were possible only with the most abundant or well characterised proteins. As a number of protein identifications resulted from single peptide-

spectrum matches (PSMs), manual verification and *de novo* sequencing was employed for validation of PSMs (Appendices 2-6 and 2-7) (provided in CD). Additionally, the identification of proteins based on a single peptide does not strictly equate to a single ORF used for its identification. For example, zona pellucida domain A is a single peptide identification that was found in seven ORFs from two abalone species (*H. discus and H. diversicolor*).

Below, we describe the identified proteins from both male and female gonads that according to the literature have direct and indirect roles in reproduction and sexual maturation processes (Table 2-1). A table showing details of peptide sequence and what database contained such peptides is given in Appendix 2-8 (provided in CD). The identification of proteins in an automated database search is a multi-step process in which peptides are first sequenced from spectra and subsequently these peptides are mapped back to proteins with protein inference an important step to ensure that the number of proteins reported is not over-inflated. In many instances, peptides were matched to predicted proteins from EST or cDNA reads and as such it was necessary to perform homology searches using the NCBI BLASTp tool to obtain the protein name and/or family. Furthermore, each PSM was assessed at the spectral level to ensure that it had only been used once for protein identification.

Table 2-1 Abalone gonadic proteins involved in the structure of spermatozoa and egg, sexual maturation and fertilization

Male proteins	Peptides	Accession	Species	Reference
Lysin	10	gi|538394	*H. laevigata*	[142, 153]
Ropporin-1-like protein	8	gi|556094603	*L. gigantea*	[154-156]
Tektin-3	6	gi|556096297	*L. gigantea*	[10, 114, 142, 157, 158]
Sperm-associated antigen 6	5	gi|524882912	*A. californica*	[10, 125, 159, 160]
Axonemal dynein light chain p33 (Fragment)	4	gi|405957088	*H. discus discus*	[10, 161, 162]
Tektin-2	4	gi|405950079	*C. gigas*	[10, 125, 159, 160]
Histone H3 (Fragment)	4	gi|405962350	*C. gigas*	[163-165]
Atrial natriuretic peptide receptor A	4	gi|405958266	*C. gigas*	[10, 166-168]
Dynein heavy chain 5, axonemal	2	gi|405957466	*C. gigas*	[10, 162]
71kDa heat shock protein	2	gi|109689150	*H. tuberculata*	[10]
Tektin A1	2	gi|211998646	*H. asinina*	[10, 142]
Radial spoke head protein 9-like protein	2	gi|524897833	*A. californica*	[10, 169, 170]
Enkurin	1	gi|405973830	*C. gigas*	[10, 143, 171, 172]
Parkin coregulated gene protein homolog-like	1	gi|556097114	*L. gigantea*	[10, 173, 174]
Nucleoside diphosphate kinase homolog 5-like	1	gi|524910230	*A. californica*	[10, 175]
Sperm surface protein Sp17	1	gi|405961240	*C. gigas*	[10, 159, 176, 177]
Radial spoke head protein 4 homolog A-like	1	gi|524898469	*A. californica*	[10, 169, 170]
Cytosolic heat shock cognate protein 70	1	gi|77023193	*M. galloprovincialis*	[124, 178, 179]

continued

Female proteins	**Peptides**	**Accession**	***Species***	**Reference**
Vitellogenin	19	gi|164604844	*H. discus hannai*	[180, 181]
Vitelline envelope zona pellucida domain 10	8	gi|91992398	*H. fulgens*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 26	6	gi|260408284	*H. rufescens*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 23	4	gi|260408278	*H. rufescens*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 18	3	gi|260408268	*H. rufescens*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 2 type 1	2	gi|315441238	*H. asinina*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 2 type 7	2	gi|315441240	*H. asinina*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 28	2	gi|260408288	*H. rufescens*	[118, 119, 142, 143]
Zona pellucida domain protein D	2	gi|260408298	*H. rufescens*	[118, 119, 142, 143]
Nuclear autoantigenic sperm protein-like	1	gi|556116977	*L. gigantea*	[182, 183]
Vitelline envelope zona pellucida domain 5	1	gi|91992350	*H. corrugata*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 6	1	gi|91992364	*H. rufescens*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 7	1	gi|91992374	*H. rufescens*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 8	1	gi|91992378	*H. discus hannai*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 9	1	gi|91992390	*H. fulgens*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 13	1	gi|260408258	*H. fulgens*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 16	1	gi|260408264	*H. rufescens*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 20	1	gi|260408272	*H. rufescens*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 21	1	gi|260408274	*H. rufescens*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 25	1	D0EL63	*H. rufescens*	[118, 119, 142, 143]
Vitelline envelope zona pellucida domain 29	1	gi|260408290	*H. rufescens*	[118, 119, 142, 143]
Heat shock protein 60	1	gi|218683627	*B. glabrata*	[124, 184]
Zona pellucida domain A	1	gi|91992358	*H. diversicolor*	[118, 119, 142, 143]
Common proteins	**Peptides**	**Accession**	***Species***	**Reference**
84kDa heat shock protein	6	gi|109689148	*H. tuberculata*	[10]
Voltage-dependent anion channel 2-like	5	gi|298108443	*H. diversicolor*	[156, 172, 185, 186]
Histone H2A	5	gi|126697370	*H. discus*	[163-165]
Histone H4	4	gi|51315709	*M. chilensis*	[163-165]
Histone H2B	4	gi|89520692	*C. farreri*	[163-165]
Ubiquitin	1	gi|388571222	*O. edulis*	[187-191]

2.3.1 Proteins related to sexual maturation and reproduction in female abalone

2.3.1.1 Vitellogenin

Vitellogenin (Vg) is the precursor of the yolk protein vitelline (Vn). Both proteins have been linked with gonad conditioning and sexual maturation in clam and oyster [159, 180, 181]. Here, Vg was identified only in female tissue consistent with the identification elsewhere of Vg as a female-specific protein [159, 180, 181]. Vitellogenin had the highest number of peptides (20) relative to any other protein detected in our study. However, it should be noted that Vg is comprised of 2391 amino acids (MW ~ 264 kDa) and it would be expected to generate a large number of peptides compared to smaller proteins. Analysis of spectral redundancy and precursor signal intensity (Table 2-2) suggested a higher relative abundance of Vg compared to the other proteins described in Table 2-1.

Table 2-2 Analysis of spectral redundancy and precursor signal intensity of vitellogenin peptides

Peptide sequence	Precursor intensity (Mean)	Spectral redundancy
ILEIIAEGK	42615	407
MMLAPYLGPYLPTTCK	100187	16
YEFEAPDGSFFNTPGPFVASYIIK	346044	3
MAATTTGATLSVNGDPITVTR	130800	4
IAVFTVIMK	13028	117
LAATAAIK	4393	70
LAFQLFAR	17382	72
LFGPEGVLTNR	197982	585
LGLIATIDASSPTTPR	5108	4
MLLDPSYHTQIQHDLQFMTR	160966	284
NFVILSAVGESYVSFYPYSAER	8250	90
TAAQSMTGPAVLAEGALFPVIAVTMMR	207124	72
TALPAYPMPWEAITQVMLQYPNR	208523	69
TCGLCSNMDGQQR	4291	73
TEYTVEDPEFNAELPEGTMFVTK	142320	12
TFDGVEYTLPNLGSCPAVLAMDCSPSK	178321	111
TPENEAYWSVNIK	60119	822
TSETIDLDIVPVLTK	260709	337
VTPALFESR	34307	105
YMAQTVTGIPGASK	297950	284
	121021[a]	**3527**[b]
	76728[c]	**11648**[d]

Footnote. a: average precursor intensity for vitellogenin; **b**: sum of spectra acquired for vitellogenin; **c**: average precursor intensity of all proteins (excluding vitellogenin); **d**: sum of spectra acquired for all proteins (excluding vitellogenin)

Biochemical assessment of both Vg and Vn in invertebrates has been extensive. For example, in female geoduck clam *Panopea globosa* Vg and Vn in haemolymph were quantified by ELISA. Increased concentrations of Vg and Vn directly correlated with histological changes observed in oocyte diameter during oogenesis and concentrations of Vg and Vn in haemolymph were proposed as critical indicators of ripeness [180]. Similarly, in oysters *Crassostrea corteziensis* and *C. gigas* increased levels of these proteins correlated with ovarian maturation [181].

Both in the wild and on farms, water temperature plays a major role in gonad development, oogenesis and spawning in abalone [45, 46]. However, multiple other environmental factors such as photoperiod, salinity, ocean dynamics, atmospheric

pressure and food availability also influence spawning [49]. Developing an antibody based diagnostic tool to monitor *H. laevigata* Vg levels could inform farmers of the progress of reproductive development in specific cohorts to accurately select broodstock for spawning. This would also allow educated decisions regarding the timing of harvest when production is threatened by adverse environmental phenomena or by energy shifts related to gonad conditioning.

2.3.1.2 Vitelline Envelope Zona Pellucida domain proteins

The vitelline envelope zona pellucida (VEZP) domain proteins are an external component of the egg coat. VEZP domain proteins form a barrier around the egg nucleus, however disruption occurs during fertilization due to interaction between the lysin vitelline envelope receptor and sperm lysin [153]. The VEZP family in invertebrates is highly diverse and to date, 32 VEZP domain proteins have been reported for a range of species of temperate and tropical abalone, but not specifically for greenlip *H. laevigata* [119, 142]. Previous research has identified the genes that encode abalone VEZPs, where they have been proposed to evolve alongside with sperm proteins as a result of positive Darwinian selection [118, 119]. In this study we report protein evidence for 20 VEZP domain proteins in *H. laevigata*.

In abalone, VEZP domain 2 type 1 and type 7 have been confirmed as sexual biomarkers in *H. asinina* where decreased levels are observed in abalone in the later stages of gonad development than those in previtellogenic stages [142]. The proteins VEZP10, VEZP26 and VEZP23 were identified from the highest number of peptides (9, 6 and 4 respectively), suggesting that these may be abundant members of the VEZP domain proteins in *H. laevigata* zona pellucida.

In molluscs the biological relevance of VEZP proteins is yet to be fully understood due to a lack of detailed information on the interacting mechanisms amongst VEZP proteins and/or with sperm proteins. Therefore, in addition to their suggested roles in

gamete co-evolution [119], sperm acrosomal reaction [143] and as sexual biomarkers [142] further investigations are required to precisely determine the factors (e.g. VEZP abundance, VEZP localisation) that influence sexual maturation and/or gamete release in abalone.

2.3.1.3 Nuclear Autoantigenic Sperm Protein (NASP)

NASP is a histone binding protein expressed in the nucleus of spermatocytes during spermatogenesis and present only in the peri-acrosomal section of the mature germ cell. It has been suggested that NASP transfers histones into the nucleus during cell division and also participates in fertilization [182, 192]. NASP has been reported as a sperm-specific protein in male vertebrates, but has also been reported in females Pacific oyster *C. gigas* and giant tiger prawn *Penaeus monodon* [161, 183]. The presence of NASP in bivalves might be expected due to their hermaphroditic nature [193]. In crustaceans, the increased levels of NASP transcripts were correlated with late stages of ovarian development suggesting a role in female sexual maturation [183]. In this study NASP was present in the gonadal tissue of female abalone but not detected in males. Previous studies in invertebrates have reported the presence of NASP in males, but its abundance in testes was noted to be significantly lower than that of ovaries [161, 183].

2.3.2 Proteins related to sexual maturation and reproduction in male abalone

A total of 110 proteins were identified in testes. Gene ontology analysis revealed that many of these proteins are involved in metabolic functions as opposed to reproduction and sexual maturation (Figure 2-2). With regard to reproduction, a smaller but more diverse set of proteins was observed in the testis compared with the ovary (Table 2-1). Several proteins related to fertilization including signaling-pathway proteins, recognition proteins for fusion between spermatozoa and the zona pellucida, ion channel proteins and chaperones for the migration of the spermatozoon

acrosomal section into the cytoplasm were identified. Excluding lysin, there were 16 novel protein reports (14 sperm and two acrosomal proteins) for *H. laevigata* (Table 2-1).

2.3.2.1 Lysin

Lysin is an acrosomal protein that has been studied in many molluscan species. In abalone lysin has been reported in 30 species (mostly in phylogenetic studies) where its amino acid sequence varied by up to 51% among species. Lysin has been extensively used in abalone speciation studies where a Darwinian extreme positive selection has been established. It has also been deduced that abalone lysin mutates faster than mitochondrial cytochrome oxidase subunit 1 in closely related species [194]. Lysin is regarded as one of the principal acrosomal proteins because of its involvement in the fusion of the spermatozoa to the egg zona pellucida during fertilization [153]. In this study lysin in testes was identified through ten peptides and based on spectral redundancy and precursor signal intensity (Table 2-3), lysin appeared to be the most abundant of the reproductive proteins in male gonad. A study in *H. asinina* assessed the correlation between lysin transcript expression and gonad maturation, but no significant differences were seen [142].

Table 2-3 Analysis of spectral redundancy and precursor signal intensity of lysin peptides

Peptide sequence	Precursor intensity (Mean)	Spectral redundancy
APTVNDYSR	5282	476
DLIAKPVQDIPIR	95435	5018
HLSAIQK	1912	144
IGAEIGR	2635	336
IPLEVTYSFLVR	33185	1534
LVAWLQR	30800	28
SLYFVNR	6314	239
VQIIAGFDR	21795	5922
WHFVPHTHVAR	2396	32
QLASWLQR	62285	147
	26204[a]	**13876[b]**
	92618[c]	**17716[d]**

Footnote. a: average precursor intensity for lysin; **b**: sum of spectra acquired for lysin; **c**: average precursor intensity of all proteins (excluding lysin); **d**: sum of spectra acquired for all proteins (excluding lysin)

2.3.2.2 Sperm surface protein 17

It has been proposed that the sperm surface protein 17 (Sp17) interacts with VEZP during the acrosomal reaction and is also a component of the spermatozoon flagellum [176, 177]. Sp17 is regarded as testis-specific in both vertebrate and invertebrate males, however, its transcript has also been detected in the ovaries of Pacific oyster *C. gigas* [161]. In this study a single peptide mapping to Sp17 was observed only in male gonads. Sp17 has also been reported in *M. edulis* [125]. This peptide also matched a sequence recently reported in red abalone sperm [10]. In order to correlate the identity of the protein with its known functionality in other species, a sequence alignment of the abalone Sp17 identified in this study with human (UniProt ID: Q15506) and oyster Sp17 (UniProt ID: K1PZL4) was performed. Sequence comparison showed the expected higher identity to oyster where Sp17 transcripts have been correlated to spermatozoid maturation [159].

2.3.2.3 Sperm-associated antigen 6

The sperm-associated antigen 6 (SPAG6) protein is known to be involved in flagellar structure and the motility of spermatozoa. The absence of this gene leads to sterility in male mice [160]. In *C. gigas*, differentially expressed transcripts of SPAG6 were quantified across spermatogenesis where it was correlated with spermatozoid maturation [159]. In this study, five peptides contributed to the identification of SPAG6, where four peptides mapped to the protein region encoding the conserved 40 amino acid armadillo repeat (Appendix 2-9) (provided in CD). SPAG6 was also recently reported in red abalone sperm and mussel [10, 125]. Armadillo repeats are present in several proteins with different functions, therefore BLASTp searches were performed to detect peptides to confirm the identity of SPAG6. The complete EST sequence that encoded the peptide (YYSPGYSDALLER) was subject to BLAST analysis retrieving several molluscan SPAG6 isoforms and most notably 96% sequence homology was observed with the oyster ortholog. Other proteins involved in male reproduction that also contain armadillo repeats include Gudu and β-catenin. Gene knockdown studies of Gudu in *Drosophila* resulted in malformations that impaired the production of viable sperm [195]. In the gastropod snail *Crepidula fornicata*, β-catenin has been reported to be involved in the process of gastrulation [196].

2.3.2.4 Enkurin

Enkurin is an acrosomal protein that interacts with the transient receptor potential-canonical cation (TRPC) channels that mediate calcium influx into cells. In mouse sperm TRPC channels are found around the acrosomal region near the crescent and/or equatorial sections where they are expected to be involved in the acrosomal reaction [143, 171]. The entry of calcium into mouse sperm during the acrosomal reaction is understood to be adjusted by TRPC2 upon mammalian zona pellucida 3 stimulation [143]. Enkurin has been proposed as an adaptor in the pathway that leads

to the late stages of the mouse acrosomal reaction by coupling phosphatidylinositide 3-kinase to a TRPC, which in turn regulates the calcium influx into the cytosol [171]. Previous research examining the mussels *M. edulis*, *M. galloprovincialis* and *M. trossulus* has reported enkurin that showed homology with mammalian, vertebrate and molluscan enkurins including that of *H. discus* [172].

In this study enkurin was identified in the search of the custom database search by a single peptide predicted to be encoded by an unnamed EST contig (GenBank ID: CX726831) from *H. discus.* Homology searching revealed the protein identity as an enkurin and sequence alignment showed similarity to molluscan enkurins from sea hare (GenBank ID: XP_005111346.1), Pacific oyster (GenBank ID: EKC38521.1), urchin (GenBank ID: XP_784094.1) and the mussel enkurins (GenBank ID: CCI39191.1, GenBank ID: CCI39204.1 and GenBank ID: CCI39209.1). Enkurin was also recently identified in *H. rufescens* [10].

2.3.2.5 Tektin

Tektins are proteins located in the axonemal cytoskeleton of ciliated and flagellated cells. The axonemal cytoskeleton is a complex protein structure that participates in protein transport and provides support for flagellar motility. Invertebrates tektins were identified as a component of the microtubules of sea urchin sperm flagella [157]. In mice the absence of tektin 4 has been correlated with decreased fertility [158]. Tektin A1 has been previously identified in male abalone where its gene expression was age-dependent and differentially expressed in testes compared to other somatic tissues of adult abalone [183]. In another study involving *H. asinina*, tektin A1 was proposed to participate in spermatogenesis [142]. In this study the tektins A1, tektin-2 and tektin-3 were identified only in male gonad tissue but not in female suggesting sperm localization as they were also identified in sperm of *H. rufescens* [10]. In our study the *H. laevigata* tryptic decapeptide TYRPNVELCR was

identified for tektin-2. Interestingly, this peptide spans a region shown to be highly conserved, specifically the nonapeptide RPNVELCRD is conserved across a wide range of species including echinoderms, molluscs and mammals [157, 161, 197]. A similar nonapeptide from tektin A1 RPGVDLCRD from *H. asinina* has been identified [183].

As mentioned previously, peptide and hence protein identification relies upon the presence of the peptide/protein in the database searched. In the specific example of the genus *Haliotis* only 34 reviewed proteins exist in the UniProt-KB database. In order to maximise the number of peptides identified, searches were performed against a higher taxonomic level that included all molluscan proteins in public sources. This strategy yielded peptide identifications from orthologous proteins. The identification of tektins clearly illustrates the benefit of the search strategy utilised in this study. The databases NCBI and UniProt enabled in the identification of tektin A1 and tektin-2 using two and four peptides respectively. Tektin-3 was absent from both of these databases and hence failed to yield its identification. A search of the custom database comprising additional EST and cDNA information resulted in the identification of tektin-3 using six peptides (Figure 2-3). This is just one example of several in this study that resulted in either an increased number of peptides mapping to an identified protein or the identification of a protein not found in the searches of publicly available protein databases.

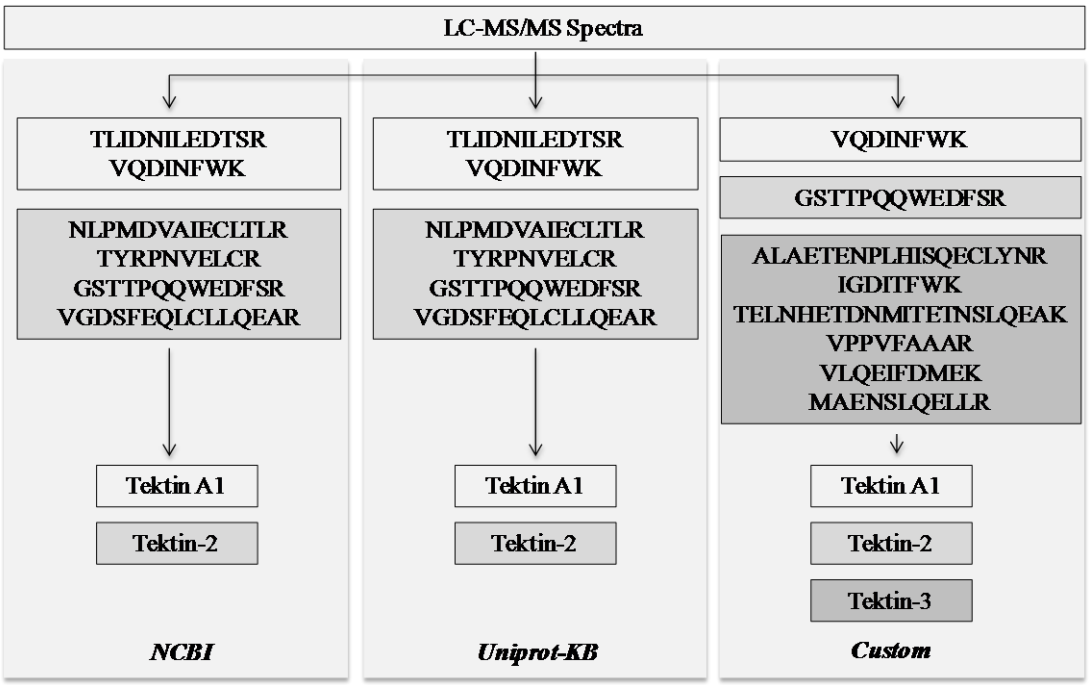

Figure 2-3. Tektin identification using complementary database searches
Tektin A1 and tektin-2 were both identified from NCBI and UniProt databases. Tektin-3 was identified using two converted molluscan EST sequences from *H. discus* (4 peptides) and *C. gigas* (2 peptides) exclusively present in the custom DB. Additionally, one peptide for tektin A1 and tektin-2 were identified in the custom DB.

2.3.2.6 Axonemal dynein light chain p33 and dynein heavy chain 5 axonemal

The axoneme comprises nine microtubule doublets in a ring-shaped structure around a central doubled microtubule core in ciliated and flagellated cells [198]. This structure is the cytoskeleton that supports motility and is also the platform for protein interactions and intra-ciliar or flagellar transport. The axoneme is composed of more than a hundred structural proteins [199]. In the present study tryptic peptides from *H. laevigata* mapped the axonemal proteins dynein light chain p33 (p33) and dynein heavy chain 5 previously reported in *H. discus* (UniProt ID: B6RB89), *H. rufescens* and *C. gigas* [10, 161]. Four peptides for p33 and two peptides for dynein heavy chain 5 were identified only in male gonads (Table 2-1). In the case of the sea urchins *Lytechinus pictus* and *Stronglyocentrotus purpuratus,* p33 was identified in axonemes and suggested to be crucial for sperm motility [162]. In order to infer a similar function in *Haliotis*, homology searches against sea urchin and Pacific oyster p33 were performed and yielded values of 90% and 85% respectively. For axonemal

dynein heavy chain 5 77% homology to both *C. gigas* (GenBank ID: EKC23674.1) and *A. californica* (GenBank ID: XP_005103646.1) was observed.

2.3.2.7 Radial spoke head protein 4-like protein A and Radial spoke head protein 9-like protein

In this study two members of the radial spoke head proteins (RSP4A and RSPH9) were identified. The radial spoke head proteins (RSP) are also structural components of the axoneme which extend from the outer doublets towards the central pair of single microtubules and are suggested to be involved in producing flagellar patterns through regulation of the dynein arms [169]. The peptides used to identify RSP in this study matched proteins sequences reported in *H. rufescens* [10]. The effects of RSP on sperm motility were studied in the urchins *Paracentrotus lividus* and *L. pictus*, where a p63 homolog of RSP was proposed to be responsible for an observed three dimensional flagellar movement instead of the common two-dimensional movement [170].

2.3.2.8 Parkin co-regulated gene protein homolog

The parkin co-regulated gene protein homolog (PACRG) protein is an axonemal protein important for sperm production and male fertility in mice [173, 174]. Non-vertebrate PACRG was first reported in the green alga *Chlamydomonas reinhardtii* [200]. Here, a single PACRG peptide was identified in male abalone gonad and mapped to an ortholog from *L. gigantea*, a protein recently reported in sperm from *H. rufescens* [10].

2.3.2.9 Nucleoside diphosphate kinase-like protein 5

The family of nucleoside diphosphate kinases (NDK) is extensively involved in the cell life cycle during proliferation, development and differentiation. The mouse nucleoside diphosphate kinase Nm23-H5 has been proposed to play a critical role in spermiogenesis as it regulates cellular glutathione peroxidase 5 [175]. NDK

enzymatic activity in different tissues in molluscs was higher in tissues exposed to anaerobic conditions, such as a phasic adductor and pedal retractor in the gastropod *Monodonta lineata* and bivalve *Ostrea edulis* [201]. Here, a single peptide mapping to *H. diversicolor* nucleoside diphosphate kinase-like protein 5 was observed only in abalone testes. In support of this finding, the protein was also reported in abalone sperm [10]. Gene ontology analysis indicates a role in spermatid development and function.

2.3.2.10 Atrial natriuretic peptide receptor A

The atrial natriuretic peptide (ANP), also known as atrial natriuretic factor (ANF), and its receptor are important polypeptides involved in ion transport and cardiac rhythm regulation [202]. Other research has suggested that ANP participates in the acrosomal reaction, testosterone production and gamete physiology across mammal species [166, 167]. A study based on gradual application of a related ANP precursor in mice suggested that ANP was responsible for the chemotaxis between spermatozoa and the fertilization site [168].

In molluscs the sensitivity of the systemic heart of the cephalopod *Octopus vulgaris* to ANF was evidenced by an increased coronary stroke volume after ANF administration [203]. The ANP receptor A has been reported at the transcriptional level in *C. gigas* and at the protein level in *H. rufescens* [10, 161]. This study identified three male gonad peptides mapping to the ANP receptor A protein. Gene ontology analyses indicate signalling activity for ANP receptor A, however further research is needed to ascertain its testicular function in molluscs.

2.3.2.11 Ropporin

Ropporin is a sperm-specific protein component of the principal and end pieces of the flagellum. It was firstly identified using a cDNA library of mouse testis where it formed complexes with rhophilin, a protein which is highly expressed in testis [154]. Ropporin is suggested to be responsible for asthenozoospermia as low expression levels have been correlated with infertile men when compared to normozoosperic men [155]. This is further corroborated by studies showing decreased expression of ropporin-1 in bulls with low fertility rates [156]. In public databases the reports of ropporin in invertebrates are scarce, and for molluscs there are only a transcriptome and a protein publication for oyster and red abalone [10, 161]. The present study identified eight peptides mapping to ropporin in the gonads of male abalone. While homology exists between closely related species (oyster 85% homology; UniProt ID: K1Q4X4; red abalone 97%), the same conservation is not observed with vertebrates species such as human (52%; UniProt ID: Q96C74) and bull (47%; GenBank ID: 115495919).

2.3.3 Common proteins in both sexes involved in reproduction

2.3.3.1 Heat Shock Proteins: HSP60, HSC70, HSP71 and HSP84

Heat shock proteins (HSPs) are a large family of proteins expressed by cells in response to changing environmental conditions such as increased temperatures and variations in oxygen concentration [204]. In female mammals, several HSPs have been suggested to be involved in reproduction, embryo development and regulation of sex steroid functions [205, 206]. In the case of spermatogenesis, HSPs are also expressed. In mouse HSC70, the constitutive form of HSP70, is accumulated during spermatogenesis whilst low levels of HSP60 were suggested to contribute to low spermatogenic indexes in sterile men [178, 179]. In oogenesis, HSC70 was differentially expressed in mice, although once the egg was fully developed HSC70 expression was undetectable [184]. Most molluscan species are sessile and during

their life cycle endure challenging conditions due to ocean and atmospheric dynamics, thus heat shock proteins play an important role in adaptation. Due to their niche distribution wild abalone are exposed to thermal stress and variations in oxygen concentration. The majority of farmed abalone are reared in shallow water tanks and are subject to similar stressors. In our study we report HSP60 and HSP84 in female gonad and HSP71, HSP84 and HSC70 in male gonad. Our findings correlated with the detection of HSC70 and HSP71 in red abalone sperm [10]. HSP60 and HSP70 have also been reported in eggs and sperm of mussel [124]. The levels of molluscan HSPs have been linked to reproductive status. In the apple snail *Pomacea canaliculata* HSPs were evaluated in kidney and foot. In the kidney HSC70 and HSP70 showed no expression changes in organisms undergoing aestivation. In the foot, expression of HSP70 was high during periods of activity and aestivation, but decreased after arousal [207]. Expression levels of HSPs were evaluated in albumen glands and eggs of the land snails *Sphincterochila cariosa* and *S. zonata* during aestivation and after arousal. High expression of HSP70 and HSP25 was detected in freshly laid eggs and albumen glands of *S. Cariosa*, while HSP70 was the most expressed HSP in the albumen gland of *S. zonata* [208]. A study in *C. gigas* evaluated the expression of HSP69 and HSP72 in pre-spawning and post-spawning oysters after thermal challenge. After subsequent sub-lethal and lethal heat shocks, high expression of these HSP in gills was observed in pre-spawning oysters and correlated with decreased mortality (<10%), while low expression of these HSPs was observed in post-spawning oysters that suffered from 80% mortality rates suggesting that HSP protein synthesis was inhibited after spawning [209].

2.3.3.2 Ubiquitin

A peptide consisting of 16 amino acids (TITLEVEPSDTIENVK) was identified for ubiquitin a highly conserved 76 amino acid domain, and may have derived from

several ubiquitin isoforms. Ubiquitin is known to be involved in proteolysis during the cell life cycle in all cell lineages where ubiquitin is covalently tagged via one of its eight lysine residues to proteins destined for processing by proteosomes. However, it is now evident that ubiquitin tagging alters the function of many proteins especially those involved in cell signalling [210]. The main source of ubiquitin is poly-ubiquitin B, a 300 amino acid protein which possesses three tandemly repeated ubiquitin domains. Gene knock-out experiments have shown that the *Ubb* gene, which encodes poly-ubiquitin B, is essential for meiosis and gonad development, however, it is still unclear at what level of reduced expression ubiquitin causes sterility in mice [187]. It has also been demonstrated that sperm mitochondria are selectively ubiquitinated during spermatogenesis and then destroyed after sperm decondensation [188]. A recent review addressed the importance of ubiquitin in different aspects of sperm development and its effect on the function and structure of sperm organelles [189]. Additionally, a combined transcriptomics-proteomics study of red abalone sperm identified ubiquitin [10]. Further highlighting its role in reproduction a study involving chickens revealed that poly-ubiquitin B was preferentially expressed in testis compared to other tissues during spermatogenesis [190].

Additionally, two ribosomal proteins, also highly conserved across species, the fusion proteins ubiquitin/ribosomal S27 (UBS27) and ubiquitin/ribosomal L40 (UBL40) each contain a single ubiquitin domain. In the mitten crab *Eriocheir sinensis*, both proteins have been associated with seasonal gametogenesis and reproduction [191].

2.3.3.3 Voltage-dependent anion-selective channel 2-like protein (VDAC2)

VDAC (1, 2 and 3) are small pore-forming proteins, also known as porins, present in the outer membrane of mitochondria. VDAC are involved in transport pathways of

energy molecules and apoptosis [211]. They have also been reported in the structure of the outer dense fiber of the sperm flagellum of mammals and structurally present in a myriad of other organisms [10, 212]. In mouse VDAC2 and VDAC3 proteins were identified in the acrosomal region and tail of spermatozoa and were found to be crucial for sperm motility, acrosomal reaction and fertilization [185]. In a comparative study between high and low fertility bulls VDAC2 under-expression was associated with individual infertility [156].

The present study reports VDAC2 in both sexes of abalone. The presence of VDAC2 in females contrasts with mammalian studies suggesting that it is testes-specific [186]. In molluscs, VDAC proteins have been reported in red abalone [10]; Chinese scallop *Chlamys farreri*, *C. gigas* and two species of mussels from the genus *Mytilus*. VDCA2 has been proposed as a potential antivirus biomarker in the Chinese scallop and a reproductive biomarker for *Mytilus* [172, 213]. Amino acid sequence comparison with *C. gigas* (UniProt ID: A5LGH1) showed 70% sequence identity, whilst 75% and 76% identity for was shown for *M. californianus* (GenBank ID: ES4402029) and *M. edulis* (GenBank ID: HE663490) respectively.

2.3.3.4 Histones

Histones are proteins involved in DNA wrapping that together with other proteins comprise chromatin [214]. Histones have also been correlated with changes occurring during spermiogenesis. In the pulmonate snail *Helix aspersa*, histones were evaluated and compared with salmon protamine in their ability to stain with several dyes as a means to characterize basic nuclear proteins during sperm development, fertilization and embryo development [215]. Extensive histone characterization was performed in the sperm of several molluscan species including *Haliotis tuberculata* [163]. Recently, Histones H2A, H2B and H4 were identified in sperm of red abalone [10]. In *H. asinina* a full pattern of basic nuclear proteins was

established that described the presence of protamine-like proteins and histones H1 and H4 during sperm development [164]. In this study the histones H2A, H2B and H4 were identified in both male and female gonads in searches against the UniProt and NCBI databases. Interestingly, six peptides mapping to H4 (4 peptides) and H2B (2 peptides) were identified as mapping to a single *L. gigantea* EST (jgi|Lotgi1|180742) present in the custom-built database. BLASTp analysis of the EST (GenBank ID: 556098074) yielded a number of related proteins termed histone clusters, which contained regions of both H4 and H2B and showed conservation across diverse phyla.

Moreover, a histone H3 was identified in male abalone gonad. Histone H2B was reported with the highest number of tryptic peptides, perhaps due to its lysine content of (15.6%) which would yield more tryptic peptides. Protein alignment of the *H. laevigata* H2A with *H. discus discus* H2A (GenBank ID: ABO26642.1) showed 98% homology. Histone H3 was compared to *H. asinina* H3 (UniProt ID: Q58PA8) revealing 100% identity. In this study H4 was identified through peptides mapping to H4 from the Chilean blue mussel (UniProt ID: Q6WV74) [165]. More recently, reports of H4 in red abalone have been made [10]. This protein is highly conserved across the species with 99% sequence identity when compared to human H4 (UniProt ID: P62805) and mouse H4 (UniProt ID: B2RTM0). Further research is needed to assess the relevance of H3 in testis.

2.4 Conclusion

This study has revealed the identity of 232 proteins, the majority of which are reported for the first time in *H. laevigata*. The most significant aspect in this study is the identification of 47 proteins related to sexual maturity, fertilization and reproduction. Inconsistent spawning in the Australian abalone aquaculture industry has hindered the application of selective breeding programs. The identification of key proteins with roles in reproduction provide the foundation for research to elucidate the mechanisms that underlie spawning. For example, understanding which proteins are expressed immediately prior to spawning may allow interventions to promote spawning in a reliable and reproducible way. Additionally, these proteins may have value as biomarkers of reproductive development in future research programs. The enzymatic and metabolic proteins identified in this study may also have potential as biomarkers for deleterious physical and chemical changes occurring in the production tanks to prevent disease occurrence and decrease mortality rates during culture. In summary, genomic resources were employed to build a custom protein database that was utilised in conjunction with public databases to comprehensively profile the proteome of *H. laevigata* gonads.

3 CHAPTER: DIFFERENTIALLY EXPRESSED REPRODUCTIVE AND METABOLIC PROTEINS IN THE FEMALE *HALIOTIS LAEVIGATA* GONAD UPON ARTIFICIAL INDUCTION OF SPAWNING.

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3.1 Abstract

Inefficient control of temperate abalone spawning prevents pair-wise breeding and production of abalone with highly marketable traits. Traditionally, abalone farmers have used a combination of UV irradiation and application of temperature gradients to the tank water to artificially induce spawning. Proteins are known to regulate diverse and crucial processes in living organisms such as respiration, muscle contraction, feeding, growth and reproduction. Spawning as a pre-requisite of abalone reproduction is likely to be regulated, in part, by endogenous proteins. A first step in elucidating the mechanisms that regulate spawning is to identify which proteins are directly involved during spawning. The present study examined protein expression following traditional spawning induction in *Haliotis laevigata*. Gonads were collected from abalone in the following physiological states: 1) spawning; 2) post-spawning; and 3) failed-to-spawn. Differential protein abundance was initially

assessed using two-dimensional difference in-gel electrophoresis coupled with mass spectrometry for protein identification. A number of reproductive proteins such as vitellogenin, prohibitin, vitelline envelope receptor for lysin, and metabolic proteins such as thioredoxin peroxidase, superoxide dismutase and heat shock proteins were identified and subsequently quantified using scheduled multiple reaction monitoring mass spectrometry. Differences in protein abundance levels were determined between physiological states. Positive correlations were observed between the abundance of specific proteins such as heat shock cognate 70, vitelline envelope zona pellucida 29 and peroxiredoxin 6, and the propensity or failure to spawn in abalone.

Significance

The present study reveals the identity of proteins that are differentially expressed during spawning. These findings will allow new hypotheses to be formed including deeper analysis of the mechanisms triggered during thermal and oxidative stress that may promote spawning. These findings will also assist in the development of methods to provide the abalone optimal environmental cues to initiate spawning and improve on-farm pair-wise breeding.

3.2 Introduction

Abalone are edible marine snails highly appreciated for their palatability. In abalone aquaculture, selective breeding of specific individuals with desirable traits is essential to produce offspring with highly marketable characteristics. In the wild, successful reproduction of broadcast spawning molluscs, such as abalone, relies on a myriad of integrated environmental factors comprising: tides, moon cycle, food availability, atmospheric pressure, photoperiod, temperature and salinity [41, 45-49]. Farmed abalone rely on many, if not all, of these factors and inability to artificially reproduce these cues prevents successful spawning on demand.

In vertebrates, reproduction is under the direct control of hormones produced in the gonads, primarily estrogen, progesterone and testosterone. For example, oestradiol (E_2) and progesterone regulate ovulation in chordates in response to gonadotropin release in the hypothalamus [216, 217]. For example, gonadotropin-releasing hormone (GnRH) and kisspeptin stimulate the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland, promoting ovulation. In molluscs, many attempts to elucidate the reproductive mechanisms have been made, especially in model organisms such as the pond snail *Lymnaea stagnalis* and the sea hare *Aplysia californica* [6, 104, 145, 218]. Nevertheless, despite the importance of these contributions to understanding vertebrate reproduction, similar depth of knowledge in molluscan reproduction has not yet been achieved.

To date a large repertoire of neuropeptides related to reproduction and spawning have been discovered in molluscs. In gastropods and cephalopods, several analogues of GnRH have been identified [7, 100, 144, 219, 220], but even though invertebrate and vertebrate GnRHs are thought to evolve from a common ancestor, GnRH function in molluscs is yet to be fully elucidated [100, 102, 219, 221, 222]. The following experiments in two gastropod species illustrates the discrepancies in GnRH functions in molluscs: injections of GnRHs from mammals, lamprey and octopus successfully induced spawning and sexual maturation in donkey's ear abalone, *H. asinina*, whereas, injections of *H. asinina* GnRH did not stimulate reproductive behaviour in *A. californica* [101, 102]. Several molecules with reproductive functions have also been identified in other molluscs. For example, the ovulation hormone caudodorsal cell hormone (CDCH) was shown to stimulate reproductive behaviours such as sexual maturation, mating and spawning in *L. stagnalis* [109, 139, 218, 223]. Analogues of CDCH have also been found in other

molluscan species, for instance, egg-laying hormone (ELH) stimulated reproductive patterns and induced spawning in *A. californica* [7, 105, 144]. Subsequently, an orthologue of *L. stagnalis* CDCH termed aELH was identified in the blacklip abalone *Haliotis rubra* [144]. Intramuscular injection of aELH induced sexual maturation and spawning in *H. asinina* and in the giant prawn *Macrobrachium rosenbergii* [101, 108].

Additional peptide hormones with the ability to stimulate sexual maturation and/or spawning have been identified in abalone and other closely related terrestrial and marine gastropods. In *H. asinina*, injections of the peptide APGWamide into both sexes stimulated spawning within 2-4 h exclusively in males [224]. In both sexes of *H. asinina* a pheromone termed "attractin-like" was identified and predicted to play a role in mating behaviour in abalone [146]. The exogenous administration (in the tank water) of the neurotransmitter serotonin (5-HT) stimulated contractions in gonadic tissue of *H. rubra* that resembled those occurring naturally during abalone spawning [137]. Prohormone convertase 1 (PC1) was identified in *H. diversicolor supertexta*. PC1 is responsible for cleaving neuropeptide precursors into the mature forms that function to regulate reproductive processes. Following qPCR of PC1 transcripts in abalone gonads, it was demonstrated that PC1 expression was particularly high in pre-breeding and during-breeding stages. Post-breeding exhibited a low PC1 expression. As a result, PC1 expression was suggested to participate in abalone reproductive process [225]. In the marine snail *Thais clavigera*, the ovaries expressed an estrogen receptor that correlated with seasonal sexual maturation [226]. Other studies have reported the expression of gonadic proteins or morphological changes in gonads that vary with the stage of sexual maturation and gonad conditioning in several molluscan species. For example, vitellogenin (Vg) and vitelline have been deemed as sexual biomarkers in clam *P. globosa* and oyster *C. corteziensis* [180,

181].

Other approaches focusing specifically on spawning control have modified physical and chemical parameters in seawater to efficiently induce gonad conditioning and spawning in abalone [46, 58-60]. These studies employed addition of hydrogen peroxide and ultraviolet light irradiation in seawater promoting abalone spawning in several abalone species where *H. laevigata* is not included [58-60]. These spawning techniques generated hydroxyl radicals that are known to generate an oxidative environment in the abalone holding tank [63, 227]. From these reports is evident that the presence of reactive oxygen species (ROS) and consequent oxidative stress plays a role in abalone spawning. To date, the molecular mechanisms underlying abalone spawning oxidative-stress dependent remain unsolved. Therefore, a more comprehensive analysis of the effects of the current spawning methodology is needed to understand the biological basis for spawning.

This study employed two-dimensional difference in-gel electrophoresis (2D-DIGE) and scheduled multiple reaction monitoring mass spectrometry (MRM-MS) quantification to examine differential protein expression in abalone that had successfully spawned compared to those that failed-to-spawn and at the time of artificial spawning induction. In this study, we focused on proteins with roles in abalone gametogenesis and fertilisation (termed reproductive proteins) and those with metabolic function (termed metabolic proteins) such as oxidative stress that are relevant for abalone spawning.

3.3 Materials and Methods

3.3.1 Abalone spawning induction and gonad dissection

Sexual maturation assessment of female *H. laevigata* (100-130 mm; 36 months old) (50 males; 50 females) was performed using the visual gonad index (VGI). The VGI

is a non-invasive technique that evaluates gonad shape and size addressed in four categories (0-3), wherein VGI 3 denotes a swollen-round conical appendage [46]. Sexually mature abalone, with a VGI of 3, were collected from production tanks at Kangaroo Island Abalone (KIAB) (35°35'42.2" S; 137°25'52.5" E). On the day of spawning induction, abalone were divided into groups of 10 to 12 and placed in resting tanks for ~7 h before artificial spawning induction was performed as previously described [60] with modifications. Filtered seawater was irradiated with ultraviolet light while the water was simultaneously heated until the temperature increased from 18 to 22°C (approximately within 1 h from the start of the experiment) (Figure 3-1). The circulation of the UV irradiated water supply was stopped in the holding tanks as the abalone started to spawn to allow gamete recovery for farm restocking purposes. Consistent with previous observations, males released gametes earlier than females. As such, 50 - 100 mL of water from the male tank was added to each female tank to further stimulate spawning. Following these induction methods, abalone were given 12 h to spawn. Sampled gonads were classified according to the response to the spawning induction. Abalone started spawning within four hours of spawning induction. Abalone releasing gametes were promptly taken during gamete release for dissection and classified as spawning (SP). Post-spawning (PSP) were those that were left to spawn *ad libitum* and sampled approximately two hours after they finished spawning. Abalone that failed to spawn over the duration of the experiment were classified as failed-to-spawn (FSP) and sampled at the conclusion of the experiment. Figure 3-1 depicts the chronology of abalone gonads sampling.

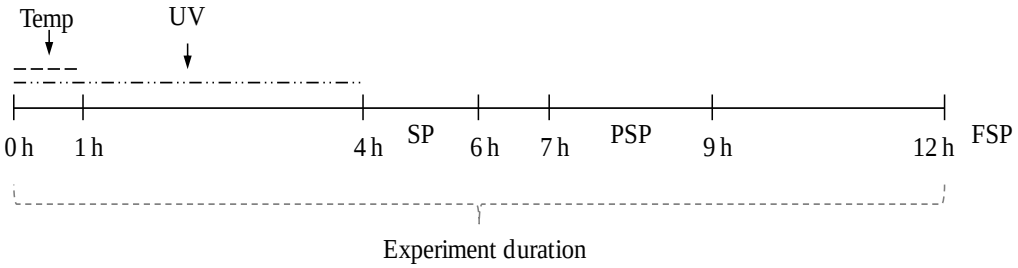

Figure 3-1. Timeline of abalone gonad sampling
Temp refers to an increase of temperature from 18 to 22°C. UV refers to irradiation of sea water with ultraviolet light. Gonads from spawning abalone (SP) were collected between the fourth and sixth hour of induction. Gonads from post-spawning abalone (PSP) were collected between the seventh and ninth hours within the experiment. Gonads from abalone that failed-to-spawn (FSP) were collected at the completion of the experiment (at 12 hours).

For dissection, the selected abalone were anaesthetized by injecting 1 mL of magnesium chloride (360 mM) into the haemolymph duct using a 30 gauge and 8 mm ultra fine syringe. Gonadic tissue (possibly containing eggs) was snap-frozen in liquid nitrogen and stored at -80°C prior to analysis.

3.3.2 Two-dimensional difference in-gel electrophoresis (2D-DIGE)

3.3.2.1 Protein preparation

Proteins were extracted from tissues using standard protocols recommended by GE Healthcare for 2D-DIGE utilising the Ettan-Dalt system. In brief, 100 mg of gonad tissue was homogenized (Ultra-turrax T8, IKA) using 1 mL of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM dithiothreitol (DTT)) followed by sonication at 30% (amplitude) on ice. Preparations were then incubated at room temperature for 3 h. A clear lysate was obtained by centrifugation at 14,000 x g at 4°C for 30 min. Protein extracts were prepared for isoelectrofocusing (IEF) using the 2D-Clean-up kit and quantified using the 2D-Quant kit (GE Healthcare). One abalone gonad was used per physiological state. Overall, three 2D-DIGE experiments were performed in which each physiological state was compared in a pair-wise manner (Table 3-1).

3.3.2.2 CyDye Labelling

Prior to IEF, 50 µg of protein was labelled with Cy fluorescent dyes according to manufacturer's directions (Amersham). Briefly, CyDyes were prepared by addition of 5 µL of fresh dimethylformamide (DMF) to give a final dye concentration of 400 pmol/µL. Cy3 and Cy5 dyes were used to assess the biological differences between gonads of different physiological status. The Cy2 dye was used to prepare the internal standard by pooling half of each gonad (25 µg of each) under analysis. The experimental design required all gonads to be contrasted against each other as follows: SP versus PSP; SP versus FSP; and PSP versus FSP (Table 3-1). Protein labelling was carried out as follows: 1 µL of CyDye (400 pmol) was added to 50 µg of protein (pH 8.5) for minimal labelling and incubated on ice for 30 min in the dark. The reaction was stopped by addition of 1 µL of 10 mM lysine followed by 10 min incubation in the dark.

Table 3-1. 2D-DIGE experimental design.

Experiment	Gonad physiological status comparison	Cy3 (50µg)	Cy5 (50µg)	Cy2 (25µg each)
1	Spawning (SP) vs Failed-to-spawn (FSP)	SP	FSP	SP + FSP
2	Spawning (SP) vs Post-spawning (PSP)	SP	PSP	SP + PSP
3	Failed-to-spawn (FSP) vs Post-spawning (PSP)	FSP	PSP	FSP + PSP

3.3.2.3 Isoelectrofocusing

For IEF, proteins were solubilised in 100 µL of loading buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1.3% DTT, 0.5% IPG buffer 3-11 NL (GE Healthcare) and 0.002% bromophenol blue). Proteins were cup loaded into 24 cm strips (3-11 NL, GE Healthcare) that had been previously rehydrated (DeStreak Solution, GE Healthcare). Proteins were separated with 40,850 Vh at 20°C (IPGphor3, GE Healthcare). Following IEF, the equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 0.002% bromophenol blue) containing either 1% DTT or 2.5% iodoacetamide (IAM) was used for protein reduction and alkylation in

two separate processes with 15 min incubation at room temperature. For the second dimension precast 12.5% polyacrylamide matrix gels suitable for DIGE were used (GE Healthcare). Protein migration was performed at 10°C, employing 10 mA per gel and 80 V for 1 h. A further step of 50 mA per gel and 500 V was utilised until the tracking dye reached the bottom edge of the glass plate.

3.3.2.4 Image acquisition and analysis

Glass plates were rinsed with ultrapure water and cleaned with 70% ethanol. Acquisition of fluorescent protein patterns were obtained in a Typhoon scanner variable mode imager (GE Healthcare) by setting the photomultiplier tube (PMT) voltage to 500 and resolution quality to 100 μm using the default laser wavelength settings for each corresponding CyDye. The module Differential In-Gel Analysis (DIA) embedded in the software DeCyder 2D version 6.5 (GE Healthcare) was used for detection of differentially expressed protein-spots. The DIA module utilises algorithms to quantify abundance, dependent on fluorescent intensity, between two corresponding protein-spots in a CyDye linked single gel. The abundance values are then converted to ratios allowing visualisation of up- and down-regulated protein-spots. As significant variation may arise from the stage of sexual maturity of each sampled abalone, the threshold for differential protein expression (fold-change in protein expression level) was set to a ratio of ≥ 4. It should be noted that this value is expressed either as a positive value (up-regulated protein expression) or a negative value (down-regulated protein expression). Figure 3-2 shows a typical example of the output of the DeCyder software clearly depicting a protein that is differentially expressed.

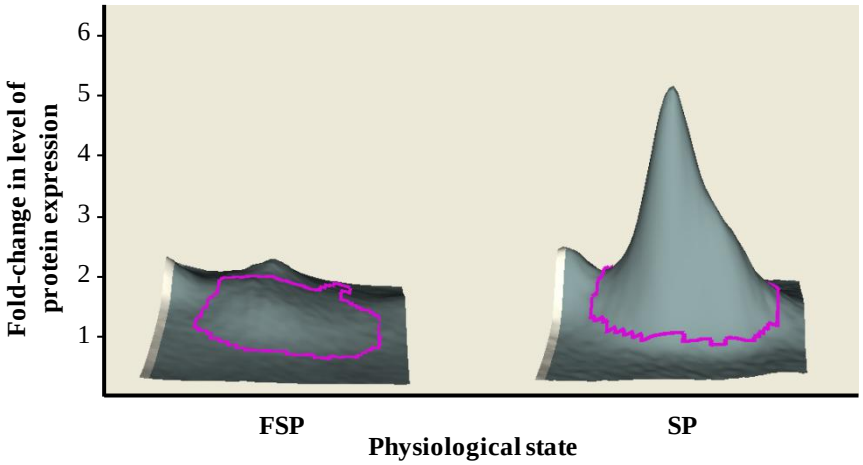

Figure 3-2. Differential expression of thioredoxin peroxidase 2 (TPx2) in gonadic tissue from abalone that failed-to-spawn (FSP) compared to spawning abalone (SP).
The differential expression of TPx2 (fold-change -4.2) in gonads of FSP abalone relative to SP abalone is apparent.

By choosing this strategy, we ensured that a maximum of 2.1% of the proteins were detected as being differentially expressed as shown in the histogram from Appendix 3-1. Gel spots with 4-fold or higher ratios based on their fluorescence intensity were selected for identification by mass spectrometry. Protein-spot picking maps were generated for each experiment in the DIA module of DeCyder software (Results section).

3.3.3 Gel staining and In-gel digestion

After images were acquired, the gels were stained using silver nitrate as described previously [228]. Briefly, gels were soaked in 30% ethanol and 10% acetic acid for 30 min to remove excess of sodium dodecyl sulphate (SDS) before a 30 min wash in 30% ethanol completed protein fixation. Gels were then submerged in sodium dithionite for 1 min and then washed in ultrapure water for 30 min. After removal of excess sodium dithionite, incubation steps in silver nitrate and formaldehyde for 30 min were performed. Gels were then washed quickly in ultrapure water and developed by adding a solution of sodium carbonate and formaldehyde. The reaction was stopped with 10% acetic acid. Gels were flooded with ultrapure water for

washing and stored at 4°C prior to spot excision and in-gel digestion and mass spectrometry (MS) analysis.

The peptide preparation for mass spectrometry identification was carried out as described previously [229]. Briefly, the selected spots were excised from the gel with silver nitrate removed using 30 mM potassium ferricyanide and 100 mM sodium thiosulphate. Gel spots were washed successively with ultrapure water, 50 mM ammonium bicarbonate and 100% acetonitrile. Proteins within gel pieces were reduced for 30 min at room temperature using 10 mM DTT dissolved in 100 mM ammonium bicarbonate. Protein alkylation employed 100 mM IAM in 100 mM ammonium bicarbonate. Protein digestion was accomplished by addition of 1.2 µg of trypsin in cold 50 mM ammonium bicarbonate per gel piece and overnight incubation at 37°C. Tryptic peptides were passively recovered by addition of a dehydrating buffer containing 1% formic acid in 50% acetonitrile followed by 20 s sonication and 10 min incubation at room temperature. A brief centrifugal pulse followed by micropipetting enabled collection of the eluate. To maximise peptide recovery the gel pieces were subsequently treated with 1% formic acid and 80% acetonitrile and a second eluate collected as described. Recovered peptides were pooled, lyophilized and then resuspended in 0.5% formic acid for liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) identification.

3.3.4 Mass spectrometry identification of differentially expressed proteins

The tryptic peptide solution (10 µL) from each in-gel digestion was chromatographically separated using a Shimadzu Prominence LC20 HPLC system with a C18 Vydac column (75 µm x 15 cm, 300 Å, 5 µm). A linear gradient at a flow rate of 300 nL/min delivered 2-40% solvent B over 6.8 min where solvent A was 0.1% formic acid and solvent B was 0.1% formic acid in 90% acetonitrile. The

column eluate was directed into the nanoelectrospray ionisation source of the TripleTOF™ 5600 system (AB/Sciex, Foster City, CA, USA). Data were acquired in information dependent acquisition (IDA) mode using Analyst TF1.6 software (AB/Sciex). The IDA method consisted of a high resolution TOF-MS survey scan with an accumulation time of 0.5 s followed by 20 MS/MS per second with a maximum accumulation time of 50 milliseconds. First stage MS analysis was performed in positive ion mode over the mass range 300-1800 Da. The ionspray voltage was set to 2500 V, the curtain gas was set to 25, the nebuliser gas to 12 and the heated interface was set to 150°C. Tandem mass spectra were acquired over the mass range 80-1800 Da using rolling collision energy for optimum peptide fragmentation. Precursor ion masses of charge states 2 to 5 that exceeded 120 counts per second were acquired and then excluded for 8 s after two occurrences.

3.3.5 Protein identification

ProteinPilot™ 4.0.1 software (Applied Biosystems), with the Paragon algorithm [151], was used for protein identification. The MS/MS spectra were searched initially against all molluscan proteins present in the UniProt database (Mollusca: version 20120229; 36,718 proteins). Search parameters were defined as cysteine alkylation with IAM, trypsin as the digestion enzyme with no restrictions placed on taxonomy. Modifications were set to "generic workup" and "biological" modification as provided with this software package, which consisted of all modifications listed in Unimod, for example, acetylation, methylation and phosphorylation. The generic workup modifications set contains 59 potential modifications that may occur as a result of sample handling, for example, oxidation, dehydration and deamidation. The criteria for positive protein identification were proteins with ≥99% confidence, with one or more unique peptides ≥95% wherein $p = 0.05$. High confidence protein identifications were confirmed by manual inspection of the spectra and search

results.

3.3.6 Protein preparation and digestion using filter aided sample preparation (FASP) for scheduled multiple reaction monitoring mass spectrometry (MRM-MS).

Protein preparation for MRM-MS was carried out using the filter-assisted sample preparation (FASP) protocol with minor modifications [150]. In brief, 50 mg aliquots of abalone gonad were homogenised in 350 μL 4% SDS, 100 mM Tris-Cl pH 7.6 and reduced with 100 mM DTT. The tissue disruption was performed on ice using a homogeniser (Ultra-turrax T8, IKA) for 5-10 s and then sonicated at 30% (amplitude) for 5 s. These preparations were incubated at room temperature for 2 h in an orbital shaker at 700 rpm. A clear lysate of reduced proteins was obtained by centrifugation for 30 min at 14,000 x g at 4°C. In order to evaluate biological variation and variation due to physiological status, 18 gonads were treated independently. Five gonads were from spawning abalone (SP), four from post-spawning abalone (PSP) and the remaining nine from abalone that failed-to-spawn (FSP). Four technical replicates for each gonadic protein extract were prepared.

A total of 100 μg of protein (estimated using the Bradford assay) was incubated at 95°C for 3 min and then sonicated for 10 min to shred DNA. Protein was solubilised in 200 μL 8 M urea (in 100 mM Tris-Cl pH 8.5) and then transferred to a 10 kDa MWCO filter unit (Millipore) and centrifuged at 14,000 x g for 15 min at room temperature. A further 200 μL of 8 M urea was added to each tube and centrifuged again as above. Cysteines were alkylated for 20 min using 50 mM IAM. Filter units were washed twice to remove excess IAM using 100 μL 8 M urea and centrifugation as before. The filter unit was equilibrated twice with 50 mM ammonium bicarbonate and centrifugation to allow buffer exchange. Trypsin (1 μg) in cold 50 mM ammonium bicarbonate was added to each sample followed by an overnight

incubation at 37 °C. The next day, the tryptic peptides were recovered from the filter by centrifugation at 14,000 x g for 15 min. The filter was washed with an addition of 40 μL of 50 mM of ammonium bicarbonate with centrifugation at 14,000 x g for 15 min. The two filtrates were pooled and vacuum dried. The lyophilised tryptic peptides were resuspended in 20 μL of 0.5% formic acid for MRM-MS assessment.

3.3.7 MRM method development

The reproductive and metabolic proteins identified as being differentially expressed in the 2D-DIGE approach were further assessed using larger set of samples (n = 18) by MRM-MS. Peptides previously identified with ≥ 95% confidence were used to build the MRM method. Manual inspection of the (PSMs) was undertaken to select the peptide *m/z* and fragment ion *m/z* to be used as Q1 and Q3 MRM transitions. Where available, three peptides per protein were used with three MRM transitions per peptide.

A 4000 QTRAP (AB/Sciex. Foster City, CA, USA) mass spectrometer with a TurboV source in positive ionisation mode was utilised for the scheduled MRM experiments. The peptides from each protein preparation (2 μL injection) were chromatographically separated on a Phenomenex Kinetex C18 (2.1 mm x 10 cm) column controlled by Shimadzu Nexera UHPLC (Shimadzu. Rydalmere, NSW, Australia) delivering a linear gradient of 5–45% of solvent B over 10 min with a flow rate of 400 μL/min. The HPLC solvents were 0.1% formic acid in water (solvent A) and 0.1% formic acid in 90% acetonitrile (solvent B). The UHPLC eluate was directed into the mass spectrometer. Analyst 1.5 software™ was used for data acquisition and processing. A preliminary MRM assay was performed to determine the retention time of each peptide to be used in subsequent scheduled MRM acquisition. Peptides were fragmented in the collision cell with nitrogen gas. The collision energy was determined based on the size and charge of the precursor ion. A

dwell time of 15 ms was used.

Peptide quantification was achieved using MultiQuant software v3.0 (AB/Sciex). Peak integration was manually assessed by examining each of the three transitions per peptide ensuring retention time alignment. The peak areas were exported to an Excel file (Microsoft) and the peak area of the two most intense MRM transition for each peptide were selected and summed as described in Figure 3-3 and subjected to statistical analysis. Each peptide was individually analysed across all samples (n=18) and physiological states (n=3).

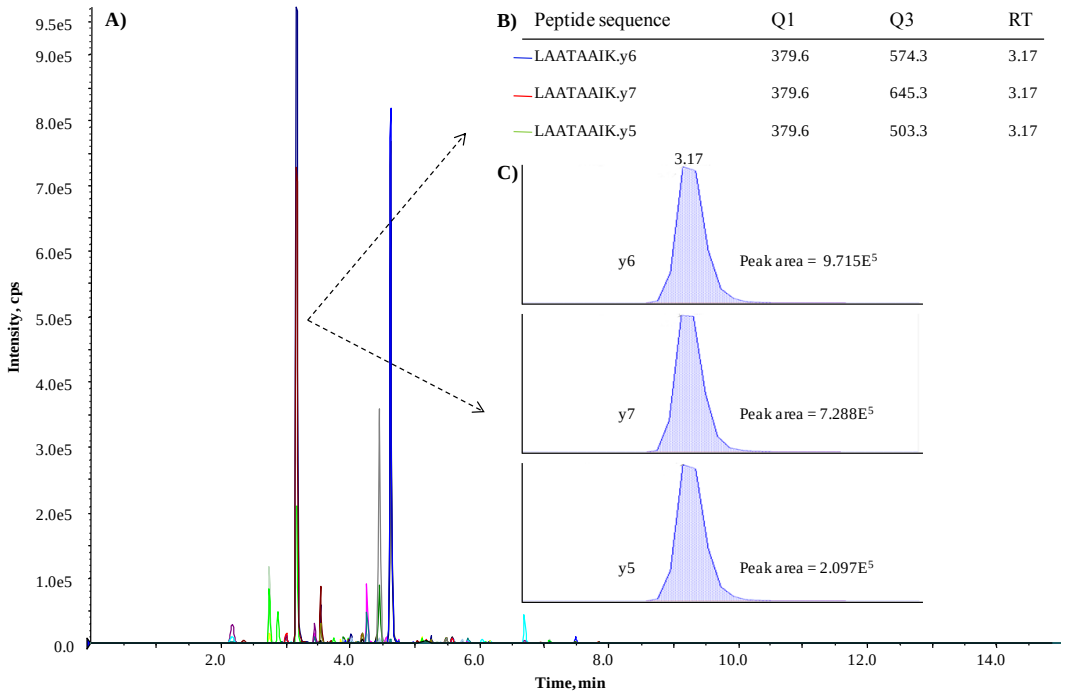

Figure 3-3. Integration of peak area to quantify proteins using scheduled MRM-MS.
A) Typical MRM trace showing the total ion chromatography (TIC) revealing all peptides targeted in the analytical method. B) Details of transitions of vitellogenin peptide LAATAAIK detected at 3.17 minutes. C) Extracted ion chromatograms (XICs) showing the transitions y6, y7 and y5 for LAATAAIK indicating the integrated peak area under the peak (blue). Image generated in MultiQuant software 3.0. As indicated for the peak area in panel C, the two most intense transitions y6 and y7 were summed and subjected to statistical analysis to estimate peptide and therefore protein abundance. Where available, this selection process was applied to all proteins quantified in this study. Q1, precursor *m/z*. Q3, fragment ion *m/z*. RT, retention time.

3.3.8 Statistical analysis

The variation in protein abundance between abalone gonads with different physiological state was accomplished using analysis of variance over the least-square means. This statistical model incorporated aspects such as variation within and between physiological groups to determine differences. This model was appropriate to this study because the treatment groups contained different numbers of samples.

Initially, the samples were normalized using the reference protein proteasome sub alpha unit 5 that did not show significant differences in expression levels across all the physiological states of abalone gonads. The selection of this reference protein is

further discussed in Results section 4.4.2. Following normalization, the statistical software SAS 9.3 (SAS Institute Inc., Cary, NC, USA) was used to identify proteins that were differentially expressed between physiological stages.

In detail, the expression abundance of each protein was analysed by fitting an analysis of variance (ANOVA) model that incorporated the within protein across physiological status component of variance. The model was as follows:

$$A_{ijkm} = \mu + P_i + S_j + A_k(S_j) + P*S_{ij} + e_{ijkm}$$

Where A_{ijkm} is the abundance in the m-th technical replicate (m = 1, ..., 4) of the i-th protein measured during the j-th physiological status in the k-th animal; μ is the overall mean; P_i is the main effect of the i-th protein; S_j is the main effect of the of the j-th physiological status; $A_k(S_j)$ is the main effect of the k-th animal nested within the j-th physiological status; $P*S_{ij}$ is the effect of the interaction between the i-th protein and the j-th physiological status; and e_{ijkm} is the effect of the random measurement error associated with A_{ijkm}. For statistical significance analysis, the difference between the least squares means for pre-planned comparisons in the P*S interaction was assessed for significance using a two-tailed t-test. Differences were deemed significant when the p-value < 0.05. A second model was explored by averaging the four technical replicates in each animal. The model was the same as above except with the main effect of animal nested within physiological status. The reason to explore this more parsimonious model was a concern that the four technical replicates might not represent true within animal variation because the technical replicates were all from the same gonad biopsy. Additionally, statistical adjustments employing corrective methods such as Bonferroni may yield discrepant results to the ones presented in this thesis. Instead we choose to report the individual p-values of each test and advise the reader to use caution when interpreting the results.

3.4 Results

3.4.1 2D-DIGE. Differential protein expression in female abalone gonads.

Two dimensional gel images were obtained for each experiment. The proteins were separated based on their isoelectric point (*pI*) in the first dimension and then according to their relative molecular weight in the second dimension. Protein-spots showing at least a 4-fold difference in abundance were selected and identified.

A number of other proteins were designated as proteins of interest due to their relevance in reproduction and metabolism while these proteins showed a lower fold change in the 2D-DIGE experiments they were targeted in the MRM-MS experiment. These proteins of interest are described in the tables and text of the manuscript, however they were not included in determining the number of identifications in each experiment nor were they incorporated in the functional characterisation charts. As some protein redundancy was seen across the experiments, the results will be briefly introduced and followed with extensive discussion of significant proteins involved in reproduction and relevant metabolic processes.

3.4.1.1 Experiment 1: Comparison of gonadic proteins from failed-to-spawn versus spawning female abalone.

The 2D-DIGE image for the assessment of differential expression of proteins in failed-to-spawn (FSP) and spawning (SP) female abalone gonads is shown in Figure 3-4A. The location of the selected proteins (showing ≥ 4-fold change in differential expression) in the gel is indicated in Figure 3-4B. The highest number of differentially expressed proteins was identified in this experiment. Table 3-2 lists the 23 proteins identified with fold-changes ≥4. As a consequence this experiment produced the largest spectrum of functional characterisation as shown in Figure 3-5. Gene ontology analysis revealed that 52% of the identified proteins were involved in

energetic and metabolic processes. It was also observed that the majority of these proteins are involved in the metabolism/processing of reactive oxygen species (ROS) which will be discussed further. The abundance of such proteins was decreased in gonads from FSP abalone. Four proteins (17%) involved in transcriptional processes were also identified. Additionally two proteins (9%) involved in reproduction were identified as vitellogenin (Vg) and vitelline envelope zona pellucida domain 29 (VEZP29). Two proteins (9%) involved in calcium transport were also identified. The remaining identified proteins (13%) were of unknown function.

A slight discrepancy was observed for the experimental and theoretical MW of the 60S acidic ribosomal protein P0 and 40S ribosomal protein S15 proteins, both of which were identified as partial sequences (or fragments) from the UniProt database. For 60S acidic ribosomal protein P0 the full sequences of an orthologue from *C. gigas* (UniProt: K1QWX2) exhibited a MW of 34.1 kDa which is similar to the observed MW (32 kDa) in this study. In a similar manner, the full sequence of an orthologue of 40S ribosomal protein S15 from *Lineus viridis* (UniProt: B0Z9Q6) produced a MW of 17.3 kDa, similar to the experimental MW (17 kDa) observed. The discrepancy observed for Vg is further explained in the discussion section. In the cases of the proteins ferritin; Williams-Beuren syndrome chromosomal region 27 protein-like and 40S ribosomal protein SA orthologues with similar MW were identified. An orthologue of ferritin in *Lymnaea stagnalis* (UniProt: P42577) produced a MW of 20.1 kDa which is in closer agreement to the observed MW (24 kDa) reported here. In the case of 40S ribosomal protein SA the MW discrepancy described for this protein may be due to post-translational modification (PTM) of the protein (as the MW is higher than that expected from the mature protein). This is a relatively common phenomenon in protein identification employing two-dimensional electrophoresis, where the increased MW of proteins are due to PTMs [230].

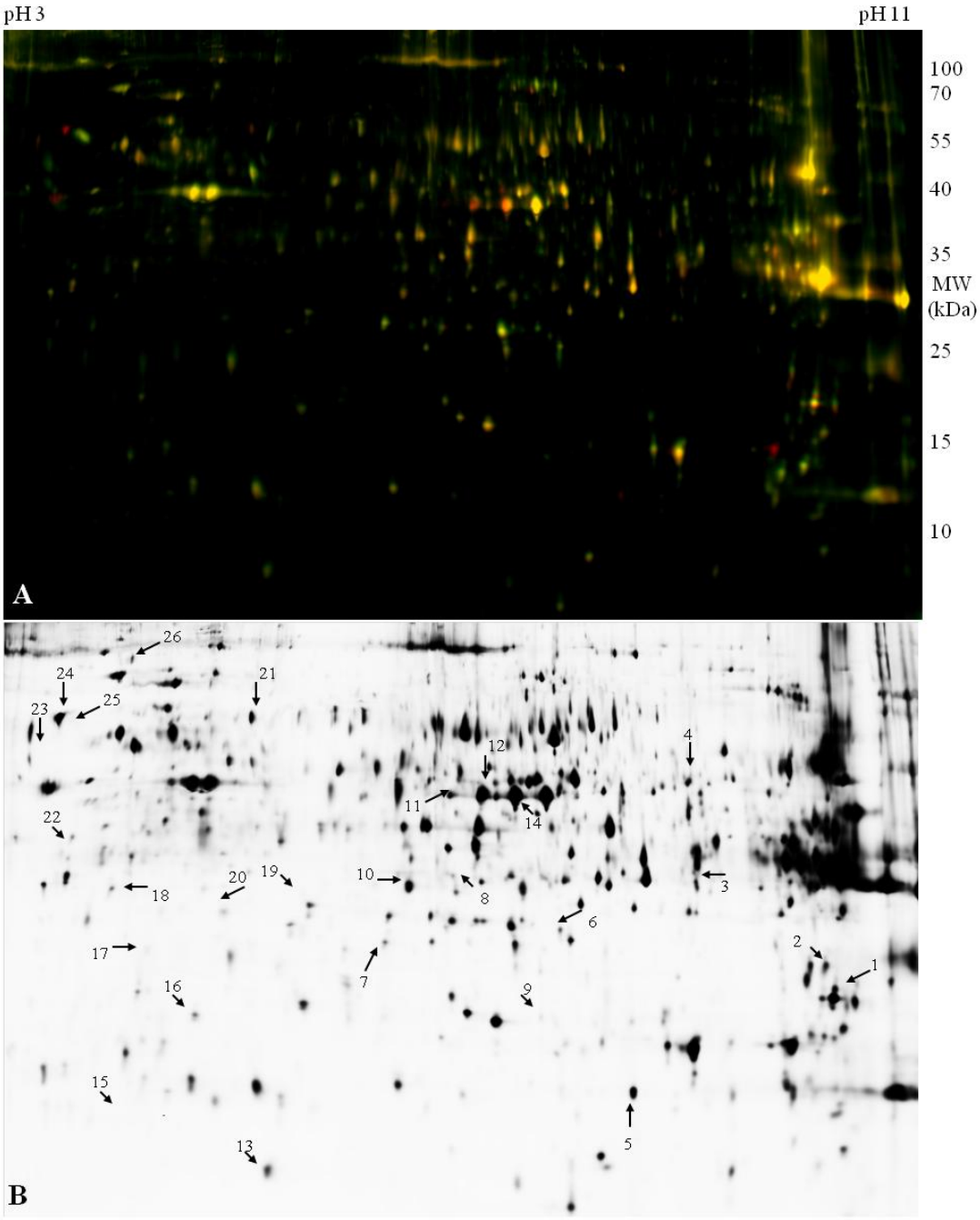

Figure 3-4. 2D-DIGE image comparing the expression of proteins from failed-to-spawn (FSP) versus spawning (SP) female abalone gonads.
A): 2D-DIGE analysis of protein expression in the gonads from FSP and SP abalone. Proteins with increased and decreased abundance are respectively indicated in green and red. Yellow spots are proteins with no change in abundance. Image acquisition was accomplished with a Typhoon scanner (GE Healthcare). B): Two-dimensional map indicating proteins of interest. Image generated by DIA module of DeCyder 6.5^{TM}.

Table 3-2. Differentially expressed proteins in the gonads of failed-to-spawn and spawning female abalone.

Spot	Protein name	Accession	Species	Theor p*I*	Exp p*I*	Theor MW	Exp MW	Pept	Fold change
1	*Peptidyl-prolyl cis-trans isomerase*[a]	D3WFS3	*Conus novaehollandiae*	8.79	10.30	22614	21000	9	1.14
2	Putative mitochondrial ATP synthase	B6RB53	*Haliotis discus discus*	8.96	10.10	19973	23000	6	-5.63
3	60S acidic ribosomal protein P0 (Fragment)	B3TK58	*Haliotis diversicolor*	9.47	9.20	28534	32000	7	-5.17
4	*Vitellogenin*	A9ZQ48	*Haliotis discus hannai*	6.66	9.00	264151	41000	3	-5.57
5	*Nucleoside diphosphate kinase*	B6RB47	*Haliotis discus discus*	8.58	8.50	18639	14000	2	-12.44
6	*Glutathione-S-transferase*	B3TK24	*Haliotis diversicolor*	6.84	7.90	24726	26000	4	9.03
7	*Thioredoxin peroxidase 2-like*	gi|556103891	*Lottia gigantea*	7.56	6.10	24004	24000	3	-4.12
8	Thioredoxin peroxidase 1	B1N693	*Haliotis discus discus*	5.97	7.00	27961	30000	8	-4.67
9	40S ribosomal protein S15 (Fragment)	Q8T699	*Aplysia californica*	9.07	7.70	8870	19000	3	-4.57
10	*Prohibitin*	gi|524879616	*Aplysia californica*	5.62	6.30	29817	30000	7	-1.84
11	*Vitelline envelope zona pellucida domain protein 29*	D0EL67	*Haliotis rufescens*	6.82	6.80	38025	37500	2	-6.69
12	*Glyceraldehyde-3-phosphate dehydrogenase*	B6RB30	*Haliotis discus discus*	6.13	7.30	31477	41000	9	-5.63
13	PREDICTED: 10 kDa heat shock protein, mitochondrial-like	gi|524881473	*Aplysia californica*	8.05	5.20	13612	10000	2	4.33
14	Arginine kinase	P51544	*Haliotis madaka*	5.73	7.50	39814	37000	10	-4.48

Footnote. **Theor**, Theoretical. **p*I***,isoelectric point. **MW**, molecular weight. **Obs**, observed. **Pept**, number of peptides identified at ≥95% confidence. Observed MW and pI are approximate values. *Italics*: proteins selected for MRM-MS analysis. ***a***: Protein of interest. **Fold-change**, the values shown represent fold increases (+) and decreases (-) in protein expression levels in gonads from FSP abalone when compared to gonads from SP abalone.

Continued Table 3-2

15	60S acidic ribosomal protein P2	B3TK87	*Haliotis diversicolor*	4.57	4.00	11427	13000	1	11.34
16	Ca2+ sensor, putative-like	gi|556116518	*Lottia gigantea*	5.05	4.70	21841	19000	3	-16.44
17	*Ferritin (Fragment)*	D3WGD7	*Conus novaehollandiae*	4.99	4.30	17784	24000	2	4.54
18	*Proteasome subunit alpha type-5-like*[b]	B5X9F8	*Salmon salar*	4.75	4.10	26449	28000	9	-1.14
19	Light organ C8 alpha proteasome subunit	Q6IWX9	*Euprymna scolopes*	5.34	5.30	28204	29000	3	6.98
20	Williams-Beuren syndrome chromosomal region 27 protein-like	gi|556107222	*Lottia gigantea*	5.50	4.80	27030	29000	2	5.55
21	*Chaperonin containing tcp1*	B6RB18	*Haliotis discus discus*	5.65	5.10	59257	52000	9	-4.7
22	Tropomyosin 1	Q7YZR4	*Haliotis asinina*	4.57	3.70	32850	35000	6	-8.51
23	40S ribosomal protein SA	B6RB17	*Haliotis discus discus*	4.84	3.50	35225	45000	3	8.69
24	Calreticulin	Q26268	*Aplysia californica*	4.36	3.60	46738	50000	8	-9.18
25	Protein disulfide isomerase	B6RB63	*Haliotis discus discus*	4.56	3.80	55235	52000	8	18.15
26	*Heat shock protein 90A*	A6N8F4	*Haliotis asinina*	4.85	4.20	84012	77000	12	4.49

Footnote. **Theor**, Theoretical. **p*I***,isoelectric point. **MW**, molecular weight. **Exp**, experimental. **Pept**, number of peptides identified at ≥95% confidence. Observed MW and pI are approximate values. *Italics*: proteins selected for MRM-MS analysis. ***a***: Protein of interest. ***b***: Protein selected as a reference for normalization. **Fold-change**, the values shown represent fold increases (+) and decreases (-) in protein expression levels in gonads from FSP abalone when compared to gonads from SP abalone.

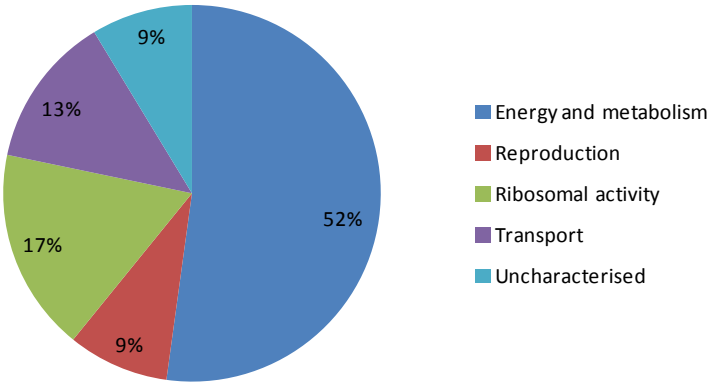

Figure 3-5. Functional characterisation of proteins identified in gonads from FSP and SP female abalone using 2D-DIGE technology

3.4.1.2 Experiment 2: Comparison of gonadic proteins from post-spawning abalone versus spawning female abalone

In experiment 2, six proteins with a fold-change in protein expression level ≥ 4 were identified. Figure 3-6A shows the 2D-DIGE image for protein extracts from post-spawning (PSP) and spawning (SP) female abalone. The locations within the gel of the identified proteins are shown in Figure 3-6B. Details of the identified proteins are given in Table 3-3. Gene ontology analysis determined only two types of functions to the identified proteins. Five proteins were involved in energy and metabolic processes and one in proton transmembrane transport (Figure 3-7). The proteins nucleoside diphosphate kinase (NDK), thioredoxin peroxidase 2 (TPx2), glutathione-S-transferase (GST) and chaperonin containing tcp1 (CCT) were also identified in experiment 1. In this experiment NDK had a fold-change less than 4, but it was deemed as a protein of interest; in experiment 1 NDK was observed to have undergone a 4-fold increase expression in spawning abalone (relative to failed-to-spawn abalone). ATP synthase subunit beta was initially selected as a potential reference protein, however MRM data exhibited significant differences across the studied physiological states (section 3.5.2.3), therefore it was deemed as a protein of interest in this experiment. Superoxide dismutase (SOD) was only identified in this experiment and its abundance was higher in the gonads from SP abalone than in

those from PSP abalone. The majority of the antioxidants proteins were decreased in gonads from PSP abalone.

A discrepancy between the observed and expected MW of superoxide dismutase was noted, however this disagreement was not unexpected as the theoretical MW shown in Table 3-3 was calculated from a partial sequence of SOD. A full-length orthologue of SOD in *H. discus discus* (UniProt: Q19BK4) yielded a MW of 24.9 kDa that is in closer agreement with the observed MW of 23 kDa. The observed MW (12 kDa) for NDK was lower than the expected MW (18.6 kDa) possibly due to protein degradation or turnover.

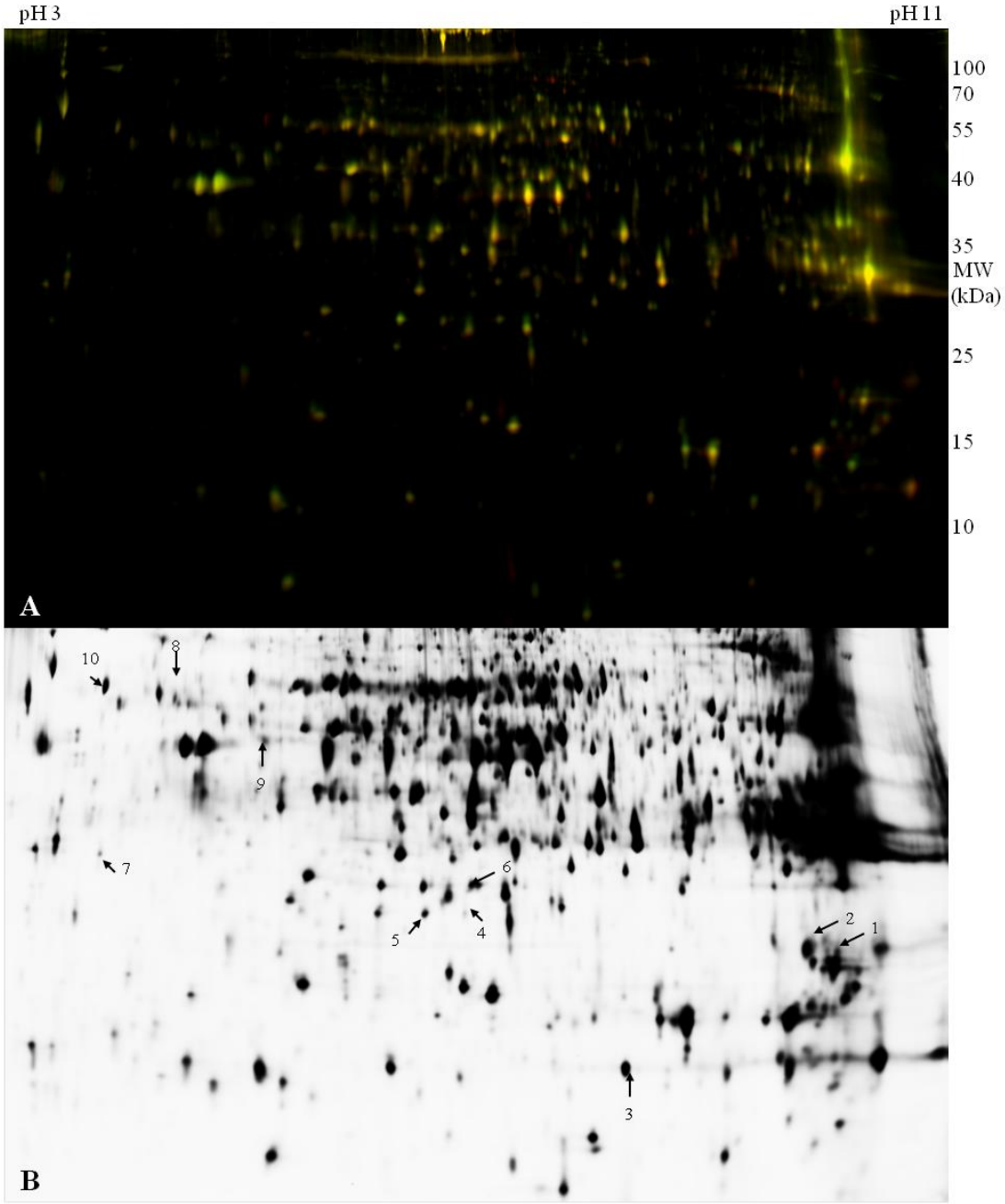

Figure 3-6. 2D-DIGE image comparing the expression of proteins from post-spawning (PSP) versus spawning (SP) female abalone gonads.
A): 2D-DIGE analysis of protein expression in the gonads from PSP and SP abalone. Proteins with increased and decreased abundance are respectively indicated in green and red. Yellow spots are proteins with no change in abundance. Image acquisition was accomplished with a Typhoon scanner (GE Healthcare). B): Two-dimensional map indicating proteins of interest. Image generated by DIA module of DeCyder 6.5™.

Table 3-3. Differentially expressed proteins in the gonads of post-spawning abalone and spawning female abalone.

Spot	Protein name	Accession	Species	Theor p*I*	Exp p*I*	Theor MW	Exp MW	Pept	Fold change
1	*Peptidyl-prolyl cis-trans isomerase*	D3WFS3	*Conus novaehollandiae*	8.79	10.20	22614	21000	9	-4.44
2	Putative mitochondrial ATP synthase	B6RB53	*Haliotis discus discus*	8.96	10.00	19974	22000	6	-9.09
3	*Nucleoside diphosphate kinase*[a]	B6RB47	*Haliotis discus discus*	8.58	8.40	18640	12000	8	-1.98
4	*Thioredoxin peroxidase* 2	B1N694	*Haliotis discus discus*	6.73	7.00	22258	24000	2	4.20
5	*Superoxide dismutase (Fragment)*	B3TLB3	*Haliotis diversicolor*	5.95	6.60	18770	23000	4	-6.58
6	*Glutathione-S-transferase*	B6RB03	*Haliotis discus discus*	5.73	7.10	26152	26000	2	-4.26
7	*Proteasome subunit alpha type-5-like*[b]	B5X9F8	*Salmo salar*	4.75	3.90	26449	27000	10	-1.11
8	*Chaperonin containing tcp1*	B6RB18	*Haliotis discus discus*	5.65	4.50	59257	57000	12	4.45
9	Actin 2	Q8TA69	*Crassostrea gigas*	5.30	5.20	41743	39000	12	-3.07
10	*ATP synthase subunit beta*[a]	Q45Y90	*Haliotis rufescens*	4.89	4.20	46210	50000	70	-1.23

Footnote. **Theor**, Theoretical. **p*I***, isoelectric point. **MW**, molecular weight. **Exp**, experimental. **Pept**, number of peptides identified at ≥ 95% confidence. Observed MW and pI are approximate values. *Italics*: proteins selected for MRM-MS analysis. ***a***: Protein of interest. ***b***: Protein selected as a reference for normalization. **Fold-change**, the values shown represent fold increases (+) and decreases (-)in protein expression levels in gonads from PSP abalone when compared to gonads from SP abalone.

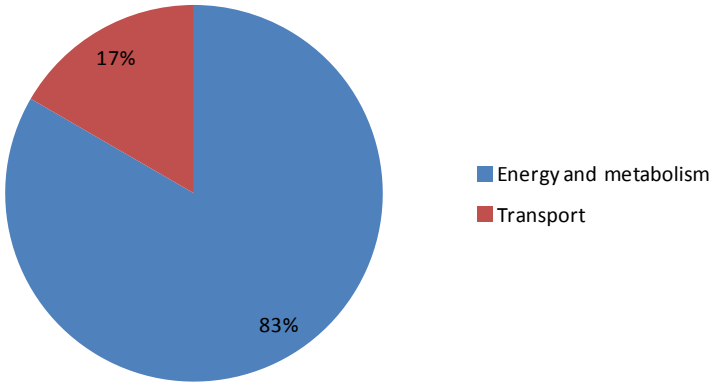

Figure 3-7. Functional characterisation of proteins identified in gonads from PSP and SP female abalone using 2D-DIGE technology

3.4.1.3 Experiment 3: Comparison of gonadic proteins from failed-to-spawn versus post-spawning female abalone

Five proteins showing differences in protein expression level (fold-change ≥4) were identified in this experiment. Figure 3-8A-B show the 2D-DIGE image for this experiment and the location of the identified proteins. As in the previous experiments, gene ontology revealed that the majority of the identified proteins (83%) were involved in energy and metabolism (Figure 3-9). Their abundance was higher in gonads from PSP abalone. Peroxiredoxin 6 (Prx6), another enzyme involved in protection against oxidative stress, was also identified. TPx2 was identified with a fold-change of -3.65 and deemed as a protein of interest due to its previous identification in experiments 1 and 2. Both Prx6 and TPx2 showed decreased abundance in gonads from FSP abalone. The same expression pattern was observed in experiment 1 with TPx1 and 2 showing decreased abundance in gonads of FSP abalone. Overall, there was a correlation between increased abundance of antioxidant proteins in the gonads of SP and PSP abalone and ability to spawn. FSP in the other hand exhibited down-regulation of antioxidant proteins. Heat shock cognate 70 (HSC70) was identified with increased expression in gonads of PSP abalone. Peptidyl-prolyl cis-trans isomerase (PPIB) was identified in experiments 2

and 3 and appeared to be down-regulated against SP gonads, but up-regulated in FSP gonads. PPIB showed a MW discrepancy between the observed (10 kDa) and theoretical MW (22.6 kDa) in this experiment that could be explained by protein degradation during protein preparation or protein turnover *in vivo*.

Additionally, Vg and VEZP29 were identified in the same spot where enolase was identified. However, after analyzing values of relative molecular weight, isolectric point (p*I*) and the number of peptides (Table 3-4) used for their identification, it was concluded that Vg and VEZP29 were found in that location due to protein-protein (Vg-enolase; VEZP29-enolase) interactions. Occasionally, during protein preparation for two-dimensional electrophoresis the proteins would undergo modifications such as degradation that would affect both their isoelectric point and relative molecular weight causing them to be misplaced in the final gel image. As a consequence, 2DE-DIGE is unable to address which protein (enolase, Vg or VEZP29) has been differentially expressed. However, based on previous findings and literature, Vg and VEZP29 were deemed as proteins of interest for their relevance in the sexual maturation in abalone.

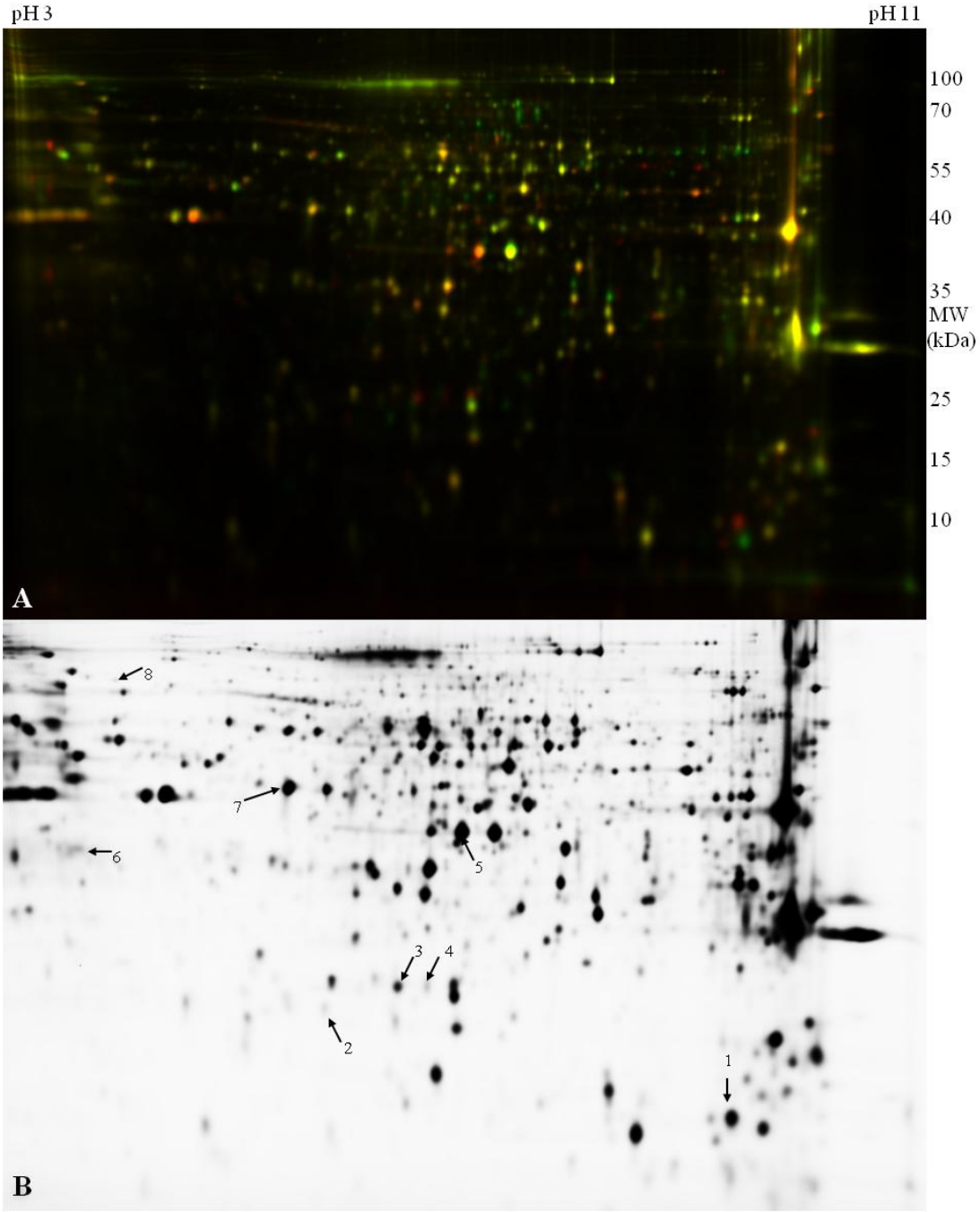

Figure 3-8. 2D-DIGE image comparing the expression of protein from failed-to-spawn (FSP) versus post-spawning (PSP) female abalone gonads.
A). 2D-DIGE analysis of protein expression in the gonads from PSP and FSP abalone. Proteins with increased and decreased abundance are respectively indicated in green and red. Yellow spots are proteins with no change in abundance. Image acquisition was accomplished with a Typhoon scanner (GE Healthcare). B): Two-dimensional map indicating proteins of interest. Image generated by DIA module of DeCyder 6.5^{TM}.

Table 3-4. Differentially expressed proteins in the gonads of failed-to-spawn and post-spawning female abalone.

Spot	Protein name	Accession	Species	Theor p*I*	Obs p*I*	Theor MW	Obs MW	Pept	Fold change
1	*Peptidyl-prolyl cis-trans isomerase B*	D3WFS3	*Conus novaehollandiae*	8.79	9.50	22614	10000	2	-4.54
2	*Thioredoxin peroxidase 2*[a]	B1N694	*Haliotis discus discus*	6.73	6.00	22257	20000	3	-3.65
3	Proteasome subunit beta type-2	gi|405954435	*Crassostrea gigas*	7.73	6.50	23570	23000	4	6.41
4	*Peroxiredoxin 6*	F8SQY0	*Sepiella maindroni*	6.16	6.80	24422	24000	4	-6.21
5	Arginine kinase	Q9BLC7	*Aplysia kurodai*	6.77	7.20	39328	38000	8	-5.33
6	*Proteasome subunit alpha type-5-like*[b]	B5X9F8	*Salmo salar*	4.75	3.90	26449	35000	10	-1.22
7	Enolase[c]	O02654	*Loligo pealeii*	5.78	5.70	47426	47000	19	-1.31
8	*Heat shock cognate protein 70*	C1KC83	*Haliotis diversicolor*	5.19	4.30	71330	69000	15	-19.57

Footnote. **Theor**, Theoretical. **p*I***, isoelectric point. **MW**, molecular weight. **Exp**, experimental. **Pept**, number of peptides identified at ≥ 95% confidence. Observed MW and pI are approximate values. Italics: proteins selected for MRM-MS analysis. ***a***: Protein of interest. ***b***: Protein selected as a reference for normalization. ***c***:Vitellogenin and vitelline envelope zona pellucida domain 29 were also found in same gel spot. **Fold-change**, the values shown represent fold increases (+) and decreases (-) in protein expression levels in gonads from FSP abalone when compared to gonads from PSP abalone.

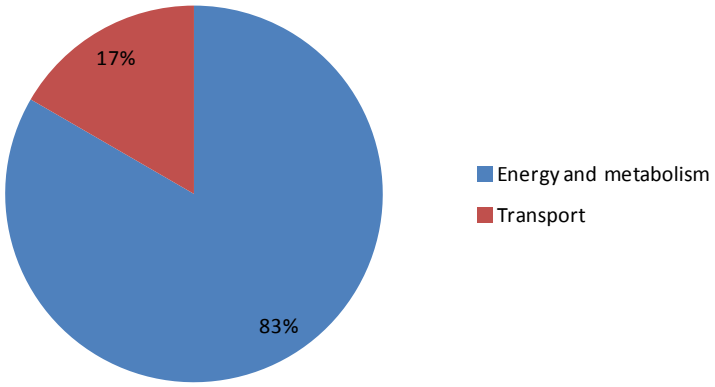

Figure 3-9. Functional characterisation of proteins identified in gonads from FSP and PSP female abalone using 2D-DIGE technology

A summary of the differential expression across the three 2D-DIGE experiment is detailed in Figure 3-10. Panels A to C show the expression and identity of the proteins described in experiments 1, 2 and 3.

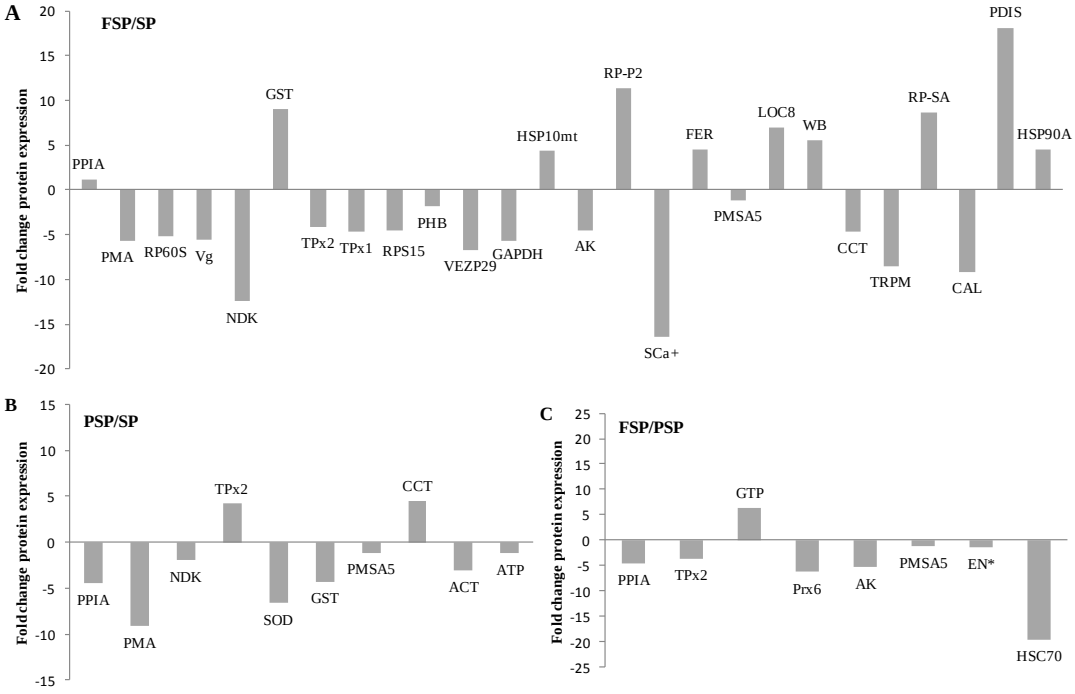

Figure 3-10. Fold changes in protein expression in female abalone gonads in different physiological state.

The values shown represent fold increases (+) and decreases (-) in gonads from abalone with unique physiological background. The fold-change in protein expression level was determined from 2D-DIGE gels using the DIA module of the DeCyder Software 6.5. A, Fold-changes are indicated for gonads of FSP abalone when compared to gonads of SP abalone in 2D-DIGE experiment 1. B, Fold changes are indicated for gonads of PSP abalone when compared to gonads of SP abalone in 2D-DIGE experiment 2. C, Fold changes are indicated for gonads of FSP abalone when compared to gonads of PSP abalone in 2D-DIGE experiment 3. PPIB, Peptidyl-prolyl cis-trans isomerase. PMA, Putative mitochondrial ATP synthase. RP60S, 60S acidic ribosomal protein P0. Vg, Vitellogenin. NDK, Nucleoside diphosphate kinase. GST, Gluthatione-S-transferase. TPx1, TPx2, Thioredoxin peroxidase 1 and 2. RPS15, 40S ribosomal protein S15. VEZP29, Vitelline envelope zona pellucida domain 29. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. HSP10mt, 10 kDa heat shock protein, mitochondrial-like. ATP, ATP synthase subunit beta. SOD, superoxide dismutase. AK, Arginine kinase. RP-P2, 60S acidic ribosomal protein P2. SCa+, Hypothetical protein LOTGIDRAFT_180935 (Ca2+ sensor, putative-like). FER, Ferritin. PMSA5, proteasome subunit alpha type-5-like. LOC8, Light organ C8 alpha proteasome subunit. WB, hypothetical protein LOTGIDRAFT_175063 (Williams-Beuren syndrome chromosomal region 27 protein-like). CCT, Chaperonin containing tcp1. TRPM, Tropomyosin 1. RP-SA, 40S ribosomal protein SA . CAL, Calreticulin. PDIS, Protein disulfide isomerase. HSP90A, Heat shock protein 90A. ACT, actin. HSC70, heat shock cognate protein 70. Prx6, Peroxiredoxin 6. GTP, GTP-binding nuclear protein Ran, EN*, Enolase; Vg, and VEZP29 were co-identified in this spot

3.4.2 MRM-MS quantification

In MRM-MS, the mass spectrometer is set to monitor only specific *m/z* pairs that represent the peptide precursor mass and diagnostic fragment ion mass in what is termed an MRM transition. Following tryptic digestion the peptides are chromatographically resolved. In the first stage of mass analysis occurring in quadrupole 1 (Q1) the specific mass of the targeted peptide is selected and transmitted to the collision cell where the peptide is fragmented by collision induced dissociation (CID) to yield fragments (or product) ions resulting from cleavage of the peptide amide bond(s). The product ions are then transmitted to the third quadrupole (Q3) and only specific *m/z* values are selected and transmitted to the detector. Protein quantification is then derived from the MRM peak area as described in Figure 3-3 of section 3.3.7.

Following identification of differentially expressed proteins in abalone gonads under defined physiological status, validation of the 2D-DIGE results was undertaken using MRM-MS in conjunction with a larger sample set (n=18). The threshold for significant difference was set at a p-value of <0.05. Aside from the proteins identified in the previous section, this analysis was extended to include other proteins identified in Chapter 2 [231] such as vitelline envelope receptor for lysin (VERL), prohibitin, heat shock cognate protein 70 and heat shock protein 90A that are known to participate in reproductive processes in abalone and closely related molluscs. A total of 17 proteins were analysed by MRM-MS and compared between animals to give the relative peptide abundance. The peak area of the MRM transitions were integrated for each peptide and therefore inferences were made with respect to protein abundance. For a reference protein, the DIA module of the DeCyder 6.5 software facilitated the selection of a protein that remained unaltered across all the physiological states examined in this study and that was subsequently used as a

reference protein for normalisation of total protein content. The reference protein was an ortholog of the proteasome subunit alpha (PMSA5) from *Salmo salar* (UniProt: B5X9F8). Two-tailed t-test analysis (n=18) of PMSA5 MRM-MS data confirmed that there was no significant change in abundance in either of the PMSA5 analysed peptides (Figure 3-11). The PMSA5 peptide LFQVEYAIEAIK was selected to normalise the apparent abundance of all other proteins as it exhibited a lower coefficient of variance (CV=7.3%) compared to LGSTAIGIQTGEGVVLAVEK (CV=7.8%).

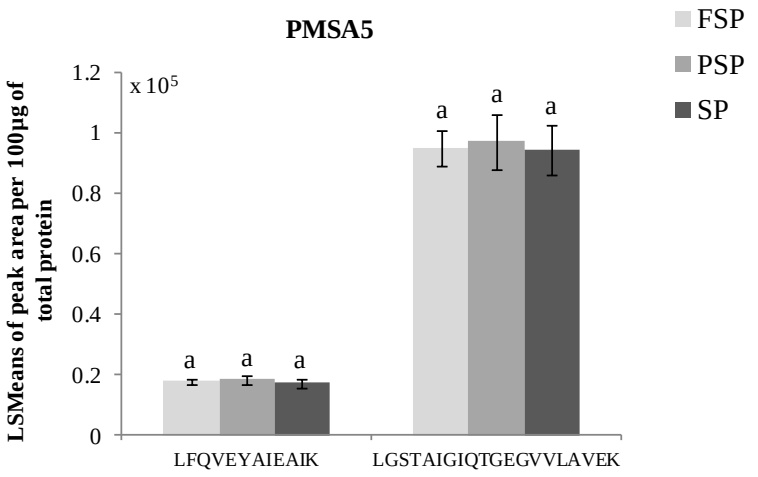

Figure 3-11. Evidence for the invariable expression of proteasome subunit alpha type 5 in gonads of failed-to-spawn, post-spawning and spawning female abalone.
Plotted values are least square means of summed peak areas for each peptide. A two-tail t-test was performed in the statistical software SAS 9.3. The letter a on top of bars denote lack of statistical significance ($p>0.05$) in peptide abundance between physiological states. The peptide LFQVEYAIEAIK was utilised as a reference to normalise the remaining proteins FSP, gonads from abalone that failed-to-spawn. PSP, gonads from post-spawning abalone. SP, gonads from spawning abalone

Differential protein expression was assessed using two peptides per protein where each peptide was assessed individually. The relative abundance of both peptides for a given protein should follow the same trends. That is, peptides from the same protein should display the same expression pattern and should be consistent across the

physiological states. Table 3-5 and Table 3-6 show the least squares means and p-values determined for MRM-MS in the ANOVA analysis for each of the 17 targeted proteins (including the reference protein). The interpretation of MRM-MS results will refer as peptide 1 and peptide 2 to each peptide in the left and right side of each graphic in Figure 3-12.

Table 3-5. Least squares mean and p-values of proteins involved in reproduction

Protein	Peptide sequence	Least squares mean			p-value		
		FSP	PSP	SP	FSP-SP	PSP-SP	FSP-PSP
Vitellogenin (Vg)	LAATAAIK[a]	1.6141	1.7305	1.8049	0.1732	0.6496	0.4306
	VTPALFESR[a]	1.6567	1.7567	1.8595	0.1527	0.5348	0.5012
Vitelline envelope zona pellucida domain protein 29 (VEZP29)	AGCGDGVVFGQK	1.8099	1.9828	1.8166	0.9588	0.2889	0.2214
	VVLTASTDGK	3.7223	3.1297	2.5158	0.0024*	0.1447	0.1180
Vitelline envelope receptor of lysin (VERL)	AGCGDGIVFAK	8.7078	5.7406	10.3686	0.2956	0.0240*	0.0924•
	NDTVVLYTVTAR	0.0940	0.1218	0.1429	0.2050	0.6405	0.4960
Prohibitin (PHB)	AFGEAGEGLVELR	2.1981	2.0181	1.9530	0.1289	0.7275	0.2906
	DLQNVNITLR	1.3025	1.2697	1.2162	0.4390	0.6877	0.7830
Heat shock cognate protein 70 (HSC70)	VEIIANDQGNR	6.1170	7.2211	5.2168	0.1914	0.0230*	0.1402
	VSIHDIVLVGGSTR	1.6101	1.7129	1.0915	0.0011*	0.0011*	0.4698
Heat shock protein 90A (HSP90A)	ADLVNNLGTIAK	3.0652	3.6598	2.9589	0.7224	0.0658•	0.0797•
	DSVQNSAFVER	1.7795	2.0963	1.6925	0.6137	0.0652•	0.1020

Footnote: Superscript indicate that values were divided by a specific number to maintain data within scale: **a,** divided by 100. SP, gonads from spawning abalone. FSP, gonads from abalone that failed-to-spawn. PSP, gonads from post-spawning abalone.***** indicates significant differences ($p<0.05$). • indicates differences, but not deemed to be significant ($p<0.1$).

Amongst the proteins involved in reproductive processes the expression pattern of the Vg peptides monitored was lowest in gonads from FSP abalone, intermediate in PSP and highest in SP abalone (Figure 3-12A). Nevertheless, this difference in expression was not significant across the physiological states.

The lack of correlation between DIGE and MRM can be explained by the fact the 2D-DIGE showed differential expression for a fragment of Vg and not the intact molecule. The protein MW detected was ~41 kDa compared to the theoretical MW of 264 kDa.

Vg plays an important role in gametogenesis, sexual maturation and spawning in oyster and clams. Therefore, this lack of difference indicated that abalone that failed-to-spawn had not reached the optimal gonad conditioning to undergo spawning.

The relative abundance of peptide 1 from VEZP29 did not show any difference across the three physiological states, whilst peptide 2 exhibited a significantly decreased difference between the SP and FSP groups (Figure 3-12B). However, both peptides did not follow the same abundance pattern. For VERL, a significant higher relative abundance was observed in peptide 1 with regards to the SP group relative to the PSP group. Peptide 2 did not show any difference across the groups (Figure 3-12C). The two peptides of VERL did not show the same pattern of expression.

The relative abundance of both peptides derived from HSC70 followed the same expression pattern: highest in gonads of PSP abalone, intermediate in FSP abalone and lowest in SP abalone (Figure 3-12E). In peptide 1 a significantly lower abundance was observed for the SP group relative to the PSP group whilst peptide 2 exhibited a significant lower abundance in SP with respect to both the FSP and PSP groups (Figure 3-12E). For two other proteins prohibitin (PHB) and HSP90A the pattern of relative abundance was consistent between the two peptides for each

protein but the differences were not deemed to be significant across the physiological states (Figure 3-12D, F).

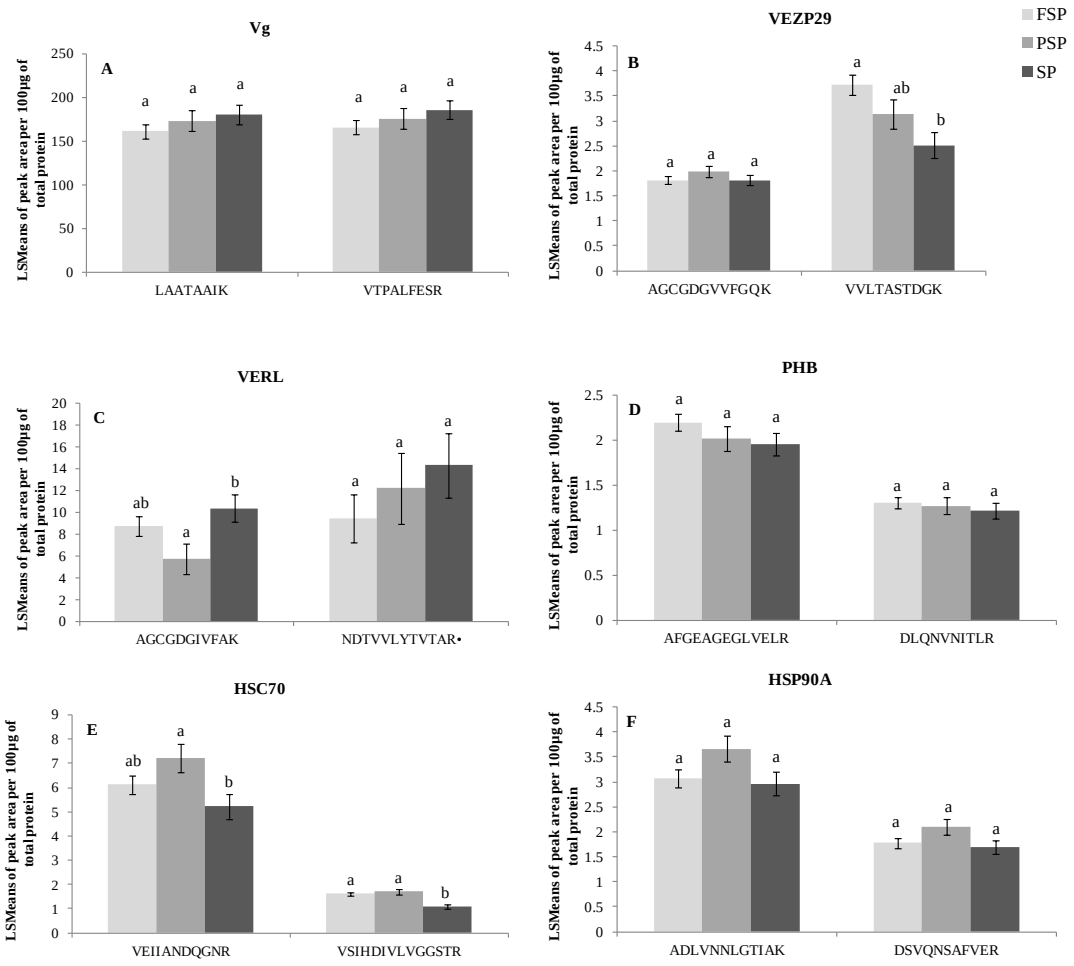

Figure 3-12. Differential protein expression of proteins involved in abalone reproduction. Least square means of peak area per 100 µg of total protein normalised against proteasome subunit alpha type 5. A two-tail t-test was performed in the statistical software SAS 9.3. Error bars (expressed as standard error) are shown. The different letters (a, b) denote statistically significant differences ($p<0.05$) in peptide abundance between physiological states. Symbol in VERL peptide indicates division of the original value by (• 200) to present data on the same scale. FSP, gonads from abalone that failed-to-spawn. PSP, gonads from post-spawning abalone. SP, gonads from spawning abalone. A, Vitellogenin (Vg). B, Vitelline envelope zona pellucida domain 29 (VEZP29). C, Vitelline envelope receptor for lysin (VERL). D, Prohibitin PHB. E, Heat shock cognate 70 (HSC70). F, Heat shock protein 90A (HSP90A).

Table 3-6 describes the abundance and statistical significance of proteins involved in metabolic processes. This table also includes the MRM-MS results of the reference protein PMSA5.

Table 3-6. Least squares mean and p-values of proteins involved in metabolism.

Protein	Peptide sequence	Least squares mean			p-value		
		FSP	PSP	SP	FSP-SP	PSP-SP	FSP-PSP
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AGIALTDNFVK[a]	1.1099	1.1913	0.9564	0.0899•	0.0358*	0.3869
	LVSWYDNEYGYSNR	2.9110	2.6804	2.3005	0.0062*	0.1205	0.2821
Peptidyl-prolyl cis-trans isomerase B (PPIB)	FPDENFK	0.3982	0.5166	0.3580	0.2004	0.0005*	0.0024*
	HYGAGWLSMANSGK	0.2225	0.3234	0.1965	0.6441	0.0750•	0.1108
ATP synthase subunit beta (ATP synthase)	AHGGYSVFAGVGER	3.5544	3.5870	3.0229	0.0139*	0.0090*	0.4553
	FLSQPFQVAEVFTGSEGK	1.6845	1.7947	1.3130	0.0578•	0.0899•	0.9087
Ferritin (FER)	IVLQDIK	1.5538	2.4940	1.2316	0.2860	0.0026*	0.0090*
Thioredoxin peroxidase 2 (TPx2)	IPLLADK	2.3485	2.8735	2.6803	0.3365	0.6378	0.1654
	GLFIIDDK	1.5115	1.7805	1.5086	0.9915	0.4145	0.3686
Peroxiredoxin 6 (Prx6)	AVFIIGPDK	1.8729	2.0367	1.7394	0.4345	0.1576	0.3746
	VIDSLQLTATK	1.9172	2.9066	2.1342	0.3478	0.0117*	0.0009*
Glutathione-S-transferase (GST)	GLAQPIR	5.6960	6.4708	5.1404	0.7606	0.5460	0.6936
	LTQSNAILR	4.2943	4.3805	3.4950	0.5171	0.5503	0.9479
Superoxide dismutase (SOD)	NDINTIISLQPALR	1.8014	2.0217	1.4741	0.0365*	0.0060*	0.1720
	NVRPDYVK	0.8160	0.8334	0.7168	0.0843•	0.0911•	0.7685
Nucleoside diphosphate kinase (NDK)	TFIAVKPDGVQR	6.1510	5.9645	6.7863	0.1724	0.1441	0.7017
Chaperonin containing tcp1 (CCT)	ALEFLPDLTIGEVK[a]	1.1561	1.3051	1.1579	0.9907	0.4343	0.3784
	FASSAACTVLK	1.8195	1.7705	1.7082	0.5844	0.7983	0.8223

Footnote: Superscripts indicate that values were divided by a specific number to maintain data within scale: **a,** divided by 10. **b** divided by 10,000. SP, gonads from spawning abalone. FSP, gonads from abalone that failed-to-spawn. PSP, gonads from post-spawning abalone.***** indicates significant differences ($p<0.05$). **•** indicates differences, but not deemed to be significant ($p<0.1$).

The interpretation of MRM-MS results will refer as peptide 1 and peptide 2 to each peptide in the left and right side of each graphic in Figure 3-13.

For proteins involved in metabolic processes, glyceraldehyde-3- phosphate dehydrogenase (GAPDH) showed significantly decreased abundance between SP and PSP gonads for peptide 1. Peptide 2 exhibited a decreased abundance in SP compared to FSP gonads (Figure 3-13A). Both GAPDH peptides displayed a slightly different pattern of abundance, but for both peptides the SP group showed the lowest levels. Both peptidyl-prolyl cis-trans isomerase (PPIB) peptides showed the same pattern of relative abundance, but only peptide 1 exhibited significant higher abundance in the PSP group with respect to the SP and FSP groups (Figure 3-13B). For ATP synthase subunit beta the lower relative abundance of peptide 1 was significant in gonads from SP abalone with respect to the other two groups while peptide 2, despite exhibiting the same expression pattern, did not show any significant difference across the groups (Figure 3-13C). Ferritin was only quantified with one peptide where gonads from PSP abalone exhibit a significant higher abundance compared to the SP and FSP groups (Figure 3-13E).

The relative abundance of the antioxidant proteins thioredoxin peroxidase 2 (TPx2) and peroxiredoxin 6 (Prx6) displayed a similar pattern of expression where gonads from PSP abalone showed the highest abundance, followed by an intermediate abundance in SP and the lowest abundance in FSP gonads, except in Prx6 peptide 1 where FSP had an intermediate and SP a lower abundance (Figure 3-13E, F). Both peptides of TPx2 did not show any significant difference across the physiological states (Figure 3-13F). Prx6 peptide 2 showed a significant higher abundance in PSP in comparison to SP and FSP gonads (Figure 3-13G).

Both monitored peptides for glutathione-S-transferase displayed the same pattern of

expression (Figure 3-13H), but no significant differences were detected across the physiological groups. For superoxide dismutase (SOD) the pattern of expression of both peptides was consistently high in PSP, intermediate in FSP and low in SP gonads. A significantly lower abundance was only observed in SOD peptide 1 for SP compared to the PSP and FSP groups (Figure 3-13I). For nucleoside diphosphate kinase (NDK) there were no significant differences observed for the single monitored peptide across the physiological states (Figure 3-13J). The relative abundance of peptide 1 and 2 for chaperonin containing tcp1 (CCT) did not show significant differences across the physiological states and neither peptide displayed a similar pattern of expression (Figure 3-13K).

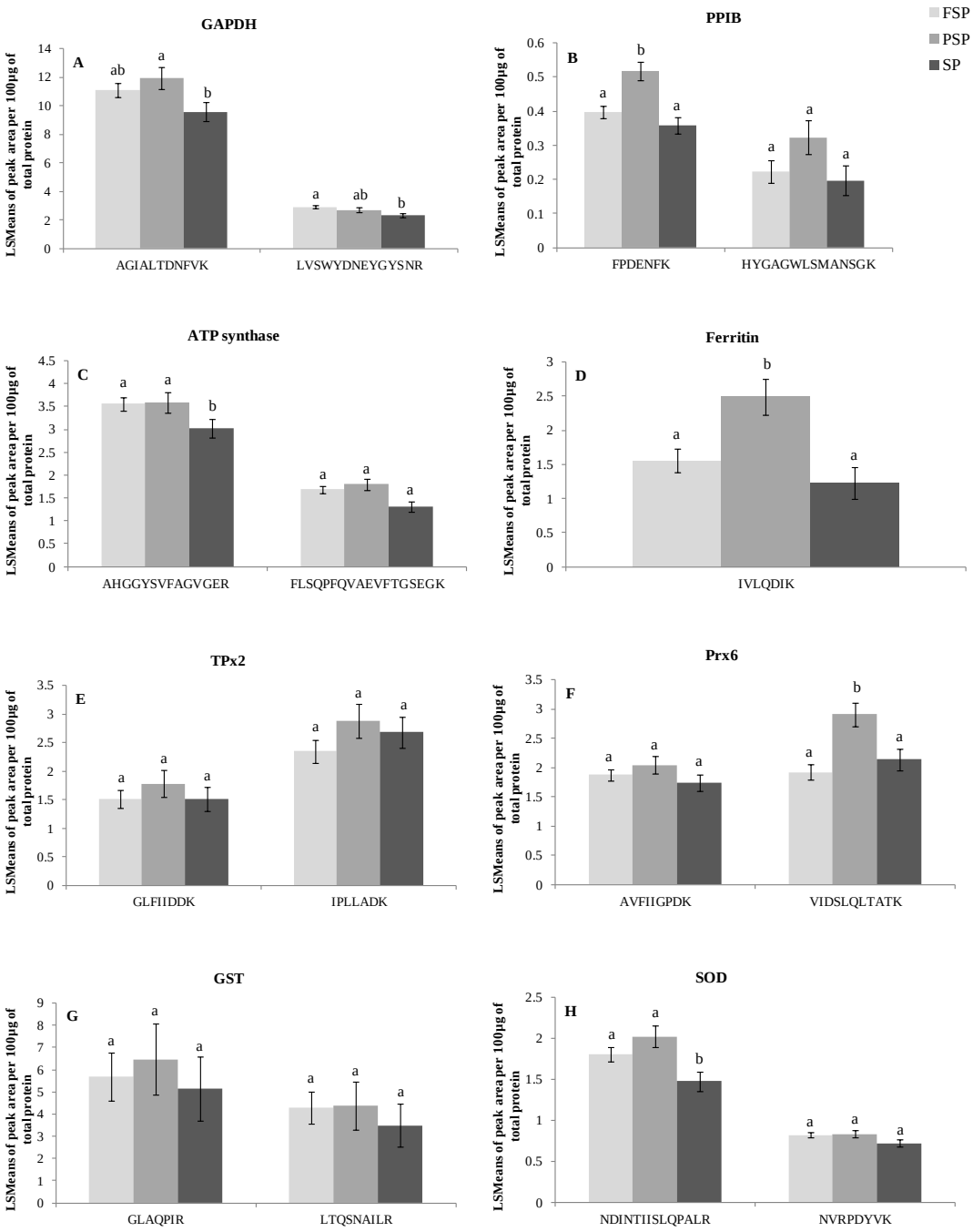

Figure 3-13. Differential expression of proteins involved in metabolism.
Least squares means of peak area per 100 µg of total protein normalised against proteasome subunit alpha type 5. A two-tail t-test was performed in the statistical software SAS 9.3. Error bars (expressed as standard error) are shown. The different letters (a, b) denote statistically significant differences ($p<0.05$) in peptide abundance between physiological states. FSP, gonads from abalone that failed-to-spawn. PSP, gonads from post-spawning abalone. SP, gonads from spawning abalone. A, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). B, Peptidyl-prolyl cis-trans isomerase (PPIB). C, ATP synthase subunit beta (ATP synthase). D, Ferritin. E, Thioredoxin peroxidase (TPx2). F, Peroxiredoxin 6 (Prx6). G, Gluthatione-S-transferase (GST). H, Superoxide dismutase (SOD).

Continued Figure 3-13

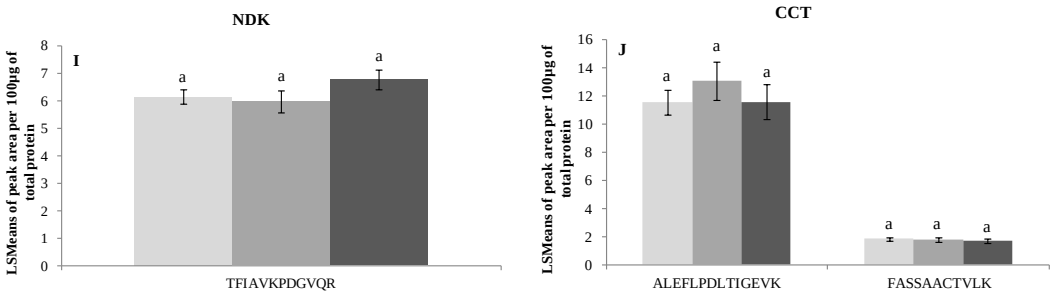

Figure 3-13. Differential protein expression of proteins involved in metabolism.
Least squares means of peak area per 100 µg of total protein normalised against proteasome subunit alpha type 5. A two-tail t-test was performed in the statistical software SAS 9.3. Error bars (expressed as standard error) are shown. The letter a on top of bars denote lack of statistical significance ($p>0.05$) in peptide abundance between physiological states. FSP, gonads from abalone that failed-to-spawn. PSP, gonads from post-spawning abalone. SP, gonads from spawning abalone. I, Nucleoside diphosphate kinase (NDK). J, Chaperonin containing tcp1 (CCT).

Overall, application of 2D-DIGE allowed the identification of proteins differentially expressed in abalone gonads in different physiological states and mass spectrometry monitoring of peptides from proteins involved in both reproductive and metabolic processes enabled the detection of significant differences that correlated with abalone ability to undergo spawning.

3.5 Discussion

Reproduction is a complex multi-step process of crucial importance for any species. In wild abalone, an organism that reproduces via broadcasting spawning, reproduction relies on environmental cues to initiate synchronised spawning followed by aquatic chemotaxis to facilitate gamete fusion. These environmental signals are not easy to ascertain due to the dynamic nature of its ecological niche. Despite its economic relevance in fisheries and aquaculture, abalone is one of the least studied commercial molluscs. Therefore, characterising the key proteins differentially expressed as a result of artificial spawning induction will deliver new insights into the mechanisms that underlie abalone reproduction.

2D-DIGE analysis provided a list of candidate proteins that were differentially expressed in the gonads of individual abalone of a defined physiological status. A subset of these proteins were then quantitatively assessed across 18 individual abalone gonads wherein four were from post-spawning abalone, five from spawning and nine from abalone that failed-to-spawn. Table 3-5 and Table 3-6 show the comparison of p-values from the MRM-MS data across all physiological states. Where available, the consistency in relative protein abundance between both 2D-DIGE and MRM-MS approaches is shown in Table 3-7. The relevance of metabolic and reproductive proteins is discussed herein.

Table 3-7. Comparison of the consistency between the protein abundance from 2D-DIGE compared to their corresponding peptides monitored in MRM-MS.

Protein	Peptide sequence	Ratio change of LSM			2D-DIGE fold-change		
		FSP/SP	PSP/SP	FSP/PSP	FSP/SP	PSP/SP	FSP/PSP
Vitellogenin (Vg)	LAATAAIK[a]	0.8943	0.9587	0.9327	-5.57♦	-	-
	VTPALFESR[a]	0.8909	0.9446	0.9430			
Vitelline envelope zona pellucida domain protein 29 (VEZP29)	AGCGDGVVFGQK	0.9963	1.0914	0.9128	-6.69○	-	-
	VVLTASTDGK	1.4795*	1.2440	1.1893			
Vitelline envelope receptor of lysin (VERL)	AGCGDGIVFAK	0.8398	0.5536*	1.5168•	-	-	-
	NDTVVLYTVTAR	0.6578	0.8523	0.7717			
Prohibitin (PHB)	AFGEAGEGLVELR	1.1254	1.0333	1.0891	-1.84x	-	-
	DLQNVNITLR	1.0705	1.0439	1.0258			
Heat shock cognate protein 70 (HSC70)	VEIIANDQGNR	1.1725	1.3842*	0.8471	-	-	-19.57♦
	VSIHDIVLVGGSTR	1.4751*	1.5693*	0.9399			
Heat shock protein 90A (HSP90A)	ADLVNNLGTIAK	1.0359	1.2368•	0.8375•	-	-	-
	DSVQNSAFVER	1.0514	1.2385•	0.8488			
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AGIALTDNFVK[b]	1.1606•	1.2456*	0.9317	-5.63♦	-	-
	LVSWYDNEYGYSNR	1.2653*	1.1651	1.0860			
Peptidyl-prolyl cis-trans isomerase B (PPIB)	FPDENFK	1.1125	1.4433*	0.7708*	1.14♦	-4.44x	-4.54♦
	HYGAGWLSMANSGK	1.1326	1.6461•	0.6880			
ATP synthase subunit beta (ATP synthase)	AHGGYSVFAGVGER	1.1758*	1.1866*	0.9909	-	-1.23x	-
	FLSQPFQVAEVFTGSEGK	1.2829•	1.3668•	0.9381			
Ferritin (FER)	IVLQDIK	1.2616	2.0250*	0.6230*	4.54♦	-	-
Thioredoxin peroxidase 2 (TPx2)	IPLLADK	0.8762	1.0720	0.8172	-4.12○	4.20♦	-3.65♦
	GLFIIDDK	1.0019	1.1803	0.8489			
Peroxiredoxin 6 (Prx6)	AVFIIGPDK	1.0767	1.1709	0.9195	-	-	-6.21♦
	VIDSLQLTATK	0.8983	1.3619*	0.6596*			
Glutathione-S-transferase (GST)	GLAQPIR	1.1080	1.2588	0.8802	9.03♦	-4.26x	-
	LTQSNAILR	1.2286	1.2533	0.9803			
Superoxide dismutase (SOD)	NDINTIISLQPALR	1.2220*	1.3714*	0.8910	-	-6.58x	-
	NVRPDYVK	1.1383•	1.1626•	0.9791			
Nucleoside diphosphate kinase (NDK)	TFIAVKPDGVQR	0.9063	0.8789	1.0312	-12.44○	-1.98○	-
Chaperonin containing tcp1 (CCT)	ALEFLPDLTIGEVK[b]	0.9984	1.1271	0.8858	-4.70○	4.45♦	-
	FASSAACTVLK	1.0651	1.0364	1.0276			

Footnote: Superscripts indicate division of value by specific number to maintain data within scale: **a,** (100). **b,** (10). **c,** (10,000). SP, spawning abalone. FSP, failed-to-spawn abalone. PSP, post-spawning abalone.***** indicates significant differences ($p<0.05$). • indicates differences but not significant ($p<0.1$). ♦ indicates that observed abundance in both 2D-DIGE and both MRM-MS peptides was consistent. ○indicates that observed protein abundance in 2D-DIGE was consistent with abundance of one MRM-MS peptide. **x** indicates lack of consistency between 2D-DIGE and MRM-MS results.

3.5.1 Reproductive proteins and sexual biomarkers

3.5.1.1 Vitellogenin (Vg).

Vitellogenin is the precursor of the yolk protein vitelline (Vn). Both proteins have been biochemically quantified in several molluscan species and positively correlated with oogenesis and gonad conditioning [159, 180, 181]. In the oysters *Crassostrea gigas* and *C. corteziensis*, sexual maturation was correlated with increased concentrations of Vg and Vn in the gonads [159, 181]. Similarly, in the clam *Panopea globosa*, high concentrations of both Vg and Vn in haemolymph were directly correlated with histological changes in developing oocytes in clams of different age cohorts [180]. In the tropical abalone *H. asinina*, Vg was quantified using semi-quantitative RT-PCR. In contrast to gonad maturation in oyster, Vg expression in abalone increased in the early stages of gonad maturation and decreased in the later stages [142]. Analysis of spectral redundancy and precursor signal intensity for Vg as a mean of relative protein expression deemed Vg as the most abundant protein in gonads of female *H. laevigata* [231]. This result also correlated with the MRM-MS analysis outcome of this study. In 2D-DIGE experiment 1, the expression of Vg was decreased in gonads from FSP abalone which had retained eggs compared to gonads of SP abalone (Table 3-2). However, it should be noted that the protein identified as Vg in DIGE experiment 1 was from a gel spot of MW ~41 kDa, whereas the intact Vg protein has a theoretical MW of 264 kDa. As such the decreased expression observed was of a fragment of Vg present in higher abundance in the SP group, possibly the result of protein degradation occurring during sample preparation or of protein turnover occurring within the eggs of spawning abalone. In the latter scenario, higher rates of protein turnover are associated with an energy cost to the organism as protein deposition that affects growth rates is essentially the difference between protein synthesis and protein degradation or turnover. During spawning, when energy is being directed from

growth to reproductive processes, protein turnover rates may increase, explaining the increased level of the Vg fragment observed in the spawning abalone relative to the abalone that failed to spawn, wherein available energy may be directed towards protein deposition and growth. A similar protein expression pattern was observed for the MRM-MS (Figure 3-12A) where gonads from FSP exhibited lower abundance than SP gonads, although, no significant differences were observed amongst all the physiological states. The MRM experiments were conducted on gonad extracts with no protein-level separation and as such measurement of Vg-derived peptides would include both intact Vg as well as Vg-derived fragments. If the Vg fragment comprised only a small fraction of total Vg content, then any biological difference in the fragment of the Vg detected in the DIGE experiment would be dwarfed by the total Vg content.

The abundance of Vg is not a response to the spawning induction as Vg is a structural yolk protein relevant for sexual maturation in molluscs. Furthermore, the relative abundance observed across the different physiological states is indicative of abalone gonad conditioning (sexual maturation) and perhaps an indirect estimation of the abalone propensity to spawn. Optimal gonad conditioning refers to fully developed oocytes that are ready to be released. The eggs are the main Vg source in abalone gonads, then we can infer that gonads from SP and PSP abalone were "more capable" to spawn than gonads from FSP abalone. Abalone that failed-to-spawn did not release their eggs. Despite the lack of significance, the lower Vg content in the FSP gonads might indicate that the eggs had not completed vitellogenesis or that vitellogenesis was interrupted or stalled at some stage of development. This observation suggested that the gonads from FSP abalone were not mature enough to undergo spawning. Vg appears to be a potential biomarker of abalone gonad conditioning that is worthy of future investigations as it has also been reported

previously for clams and oyster [159, 180, 181, 232].

3.5.1.2 Vitelline Envelope Zona Pellucida domain 29 (VEZP29) and Vitelline Receptor of lysin (VERL).

The vitelline envelope zona pellucida domain (VEZP) proteins are constituents of the external egg coat. To date, 32 VEZPs domains have been described for the Californian abalone species and six in the Thai abalone *H. asinina* [118, 119, 142]. Protein evidence for 20 VEZPs has been reported elsewhere for greenlip abalone in Chapter 2 of this thesis [231]. The VEZPs have been proposed to play relevant roles in acrosomal reaction [143], as sexual biomarkers [142] and in gamete co-evolution in abalone [119]. VERL is located in the vitelline envelope (VE) region and together with lysin mediate fertilization where lysin creates a hole in the VE and VERL assists in the passage of the sperm into the egg [117]. In another study lysin and the 18 kDa protein (known as 18 kDa) from green abalone, *Haliotis fulgens*, were bound to a resin to test their affinity for VEZPs. Lysin and 18 kDa were found to bind tightly to VERL and VEZP14 respectively, however, the presence of VEZP29 was also reported in the eluates. The presence of VEZP29 in the eluates was attributed to interactions with other VEZPs rather than binding directly to lysin or 18 kDa [118].

In this study, a decreased relative abundance of VEZP29 was observed in gonads from FSP abalone relative to SP abalone in 2D-DIGE experiment 1. MRM-MS analysis did not reveal significant differences in the levels of peptide 1 between the biological groups (Figure 3-12B, Table 3-5). VEZP29 peptide 2 [VVLTASTDGK] presented a different and highly variable pattern of expression: gonads from FSP abalone showed a significant higher abundance ($p = 0.0024$) compared to SP abalone (Figure 3-12B, Table 3-5). The discrepancy between the expression patterns of the monitored peptides could be explained by differences in the trypsin digestion efficiency of the two peptides. Peptide 2 has a dibasic site immediately preceding the

N-terminus. This dibasic motif is otherwise known as a "ragged ends" (indicated by bold letters) in the amino-terminus side of the sequence of peptide 2 [**RK**VVLTASTDGK]. Ragged ends refer to the presence of lysine and/or arginine (RR, KK, KR or RK) flanking a trypsin target peptide [233]. Usually, trypsin cleaves in the carboxyl-terminus of lysine (K) or arginine (R), therefore, when a ragged end is present, trypsin can produce a semi-tryptic [**K**VVLTASTDGK] and/or a fully tryptic peptide [VVLTASTDGK] which would have different masses. The MRM-MS analysis was only conducted using the fully tryptic peptide VVLTASTDGK and as such the levels of this peptide could have been underestimated. In order to assess whether this explanation could have caused the discrepancy observed for the two peptides from VEZP29, the technical variation for each abalone was considered. For each individual gonad, four replicates were taken and subjected to parallel processing and analysis. A 2.1% higher variation was determined for peptide VVLTASTDGK (9.4%) compared to peptide AGCGDGVVFGQK (7.3%) (Appendix 3-2) although this level of variation would not be expected to account for the discrepancy observed for the two VZP29 peptides monitored. The absence of a complete genome and thus a protein database comprising all proteins in Haliotids confounds the correct identification of isoforms and the quantification of peptide products. It is possible that either of these two peptides could be found in a VEZP isoform that would account for the discrepancy observed. However, the lower abundance of VEZP29 in FSP gonads is indication of immaturity of the eggs for ovulation and fertilization.

In 2D-DIGE experiment 3, the VERL peptide [NKGFTTKTIAGGNEK] was identified through *Mytilus edulis* VERL sequence (UniProt: B9WPN6), but due to missed cleavages in lysine positions 2 and 7, discordant isoelectric point and relative molecular mass, VERL was regarded as low confidence identification and not included in 2D-DIGE Table 3-4. Since lysine (K) and glutamine (Q) share a similar

molecular weight (K = 128.17 kDa; Q = 128.14) the possibility of a N**Q**GFTT**Q**TIAGGNEK peptide existing in abalone species was considered. This would have circumnavigated the missed cleavages as trypsin cleavages in lysine. However, further BLASTp analysis of both NKGFTTKTIAGGNEK and NQGFTTQTIAGGNEK did not retrieve any abalone VERL sequence containing either of these peptides. Given the relevance of VERL in acrosomal reaction and fertilization [117, 118], VERL quantification was performed using MRM-MS. To ensure that VERL peptides from abalone were monitored during MRM, the hypothetical peptides AGCGDGIVFAK and NDTVVLYTVTAR derived from *H. discus hannai* VERL (UniProt: A0MCR5) were utilised for MRM-MS monitoring.

In 2D-DIGE experiment 3, VERL was decreased (fold-change of -6.10) in gonads from FSP abalone in comparison with the PSP group. The relative abundance of peptide 2 [NDTVVLYTVTAR] was consistent with the 2D-DIGE results, lower in gonads from FSP abalone compared to the PSP group. However, the difference in abundance was not significant across all groups, owing to a high degree of biological variation (Figure 3-12C, Table 3-5, Appendix 3-3). Peptide 1 [AGCGDGIVFAK] showed significant difference between gonads from PSP and SP abalone ($p = 0.024$), where an increased abundance was seen in gonads from SP abalone (Figure 3-12C, Table 3-5). The groups FSP and PSP showed some differences, but were beyond the cut-off of $p = 0.05$. BLASTp analysis of peptide 1 revealed sequence conservation with VEZP25. As a consequence it is uncertain if the significant variation observed for this peptide between PSP and SP is due to VERL, VEZP25 or a combination of both VERL and VEZP25. The peptide conservation between VERL and VEZP25 could also explain the dissimilar expression pattern between both VERL peptides.

The interaction of VERL with lysin to promote fertilization is well documented in abalone [117, 118]. Despite the inconclusive results, it is worth questioning if the

higher abundance of VERL (represented by its unique peptide NDTVVLYTVTAR) observed in SP and PSP abalone, enabled these abalone to undergo spawning. The vitelline envelope environment is constituted by > 30 VEZP domain proteins (including VERL) that forms a barrier around the egg where VERL and lysin are crucial for abalone fertilisation [118, 119, 153]. VERL is the receptor to which sperm lysin binds subsequently initiating dissolution of the VE fibers allowing the sperm to enter the egg. Moreover, in *M. edulis* high levels of VERL have been correlated with seasonal gametogenesis in preparation for spawning [234]. The findings in *M. edulis* and the lack of significance observed between abalone that had expelled eggs and abalone that did not, would support the hypothesis that VERL could account for sexual maturation and spawning propensity in abalone.

How important is VERL abundance in triggering spawning? Is there any specific abundance that would increase the likelihood of undergoing spawning? It has been suggested that parental energy provision (in the form of specific proteins) to offspring (through the eggs) can increase survival rates in early developmental stages [208, 235]. It is not unreasonable to hypothesize that perhaps specific levels of VERL are required during fertilization to facilitate the binding of lysin and the entry of the sperm through the vitelline envelope and as a result spawning was possible in SP and PSP abalone. Therefore, VERL is a strong candidate for further experimentation.

3.5.1.3 Prohibitin (PHB).

Prohibitin was originally identified in a subtracted cDNA library of normal versus regenerating rat liver [236]. The library clones were screened for anti-proliferative activity by microinjection of the cDNA into human diploid fibroblasts. In the case of prohibitin, inhibition of DNA synthesis was observed, hence its name [236]. Roles in senescence, tumor suppression and development have also been attributed to

prohibitin [237].

Using a proteomic approach similar to that employed in this study, prohibitin was observed to be differentially expressed in chicken oviducts during the egg-laying period [238]. Additionally, prohibitin was preferentially expressed in the magnum which is the site where up to 40% of the albumen for the developing egg is produced [238]. In giant tiger shrimp *Penaeus monodon* a prohibitin was proposed as a biomarker for sexual maturation where high prohibitin expression in testes of domesticated broodstock was correlated with low fertilization and low larval survival rates compared to wild broodstock [239]. Prohibitin has also been expressed as a response to hormone injection. In the snail *Potamopyrgus antipodarum*, increased expression of PHB-2 was directly associated with administration of 17β-oestradiol [240] which regulates fertility and pregnancy in females. Prohibitin is also known to interact with the repressor of estrogen receptor activity [241], which in turn has been suggested to regulate decidualization (that is, changes in a pregnant uterus) [242]. In the breast cancer cell line MCF-7, transcriptional activity of estrogen receptor alpha (ERα) was reportedly inhibited by increased expression of PHB and conversely the expression of ERα target genes was increased when PHB is down-regulated [241].

In this study, prohibitin was identified with a fold-change of less than 4 in experiment 1. Given its hypothesized interactions with molecules relevant for sexual processes PHB was deemed as a protein of interest and was subjected to MRM-MS monitoring. The relative abundance of PHB peptide 1 and 2 [AFGEAGEGLVELR and DLQNVNITLR] followed the same expression pattern. Gonads from FSP abalone showed higher abundance in comparison with SP and PSP groups, but no significant differences were observed across the physiological states (Figure 3-12D, Table 3-5). Despite the lack of significance PHB should be subject to further

investigation to evaluate if regulation of sexual maturation or spawning pathways in abalone depends on endogenous PHB expression, as highly expressed PHB influences fertility rates in shrimp and in snails, it is highly expressed in response to estrogen administration [239, 241].

3.5.1.4 Heat shock cognate 70 (HSC70); Heat shock protein 90A (HSP90A)

Heat shock proteins (HSPs) are a large group of proteins expressed in cells as a response to environmental changes such as variations in oxygen concentrations and temperature [243]. Aside from their chaperone function several HSPs have been proposed with roles in embryo development, regulation of sex steroids and reproduction in mammals [205, 206]. In mouse oocytes HSC70, the constitutive form of HSP70, is intensely expressed during the dictyate stage and plays an important role in follicle maturation [184].

In molluscs, the HSPs have been studied in the association with environmental responses and other stressors. For example HSPs expression have been correlated with chemical administration [207]. In the apple snail *Pomacea canaliculata,* HSC70 and HSP90 were measured after administration of thiobarbituric acid reactive substances. These HSPs were assessed in kidney and foot of active, aestivating and aroused snails. The levels of HSC70 significantly decreased in the one-day aroused group, however no differences were seen in kidney during the tested cycles. HSP90 decreased in kidney in aestivation, but increased after arousal, while in foot tissue, it deceased after arousal. Both proteins were then referred to as determinants in the snail aestivation-arousal cycle [207]. In the land snails *Sphincterochila cariosa* and *S. zonata*, the expression of HSP70 and HSP90 following temperature challenges were correlated with maturation cycle and survival. In both species the levels of HSPs were decreased in foot before the egg-laying period compared to the post-egg-laying state. The decreased expression of HSPs in foot correlated with increased

expression of HSPs in albumen gland (reproductive organ) and freshly laid eggs of both species. The authors concluded that HSPs parental provision increases offspring survival in the early stages [208].

Intertidal molluscan species are particularly vulnerable to changes in temperature and oxygen availability. Therefore, HSPs play an important role in maintaining homeostatic balance in such sessile species enabling them to withstand the dynamism of their ecological niche. In pre- and post-spawning oyster (*C. gigas*) challenged by thermal stress, the levels of HSPs correlated with survival. Oysters with such gonadic states were treated with sub-lethal (37°C) and lethal (44°C) thermal challenges. The mortality rate significantly decreased after a 44°C heat shock in oysters that had a preventative 37°C treatment. However, this was only consistent for pre-spawning oysters. It was suggested that loss of energy (glycogen in mantle) in post-spawning oysters impaired their ability to maintain a homeostatic balance when challenged by heat shock [244].

In the 2D-DIGE experiment 3, the relative abundance of HSC70 was decreased in gonads of FSP abalone in comparison to gonads from PSP abalone (Figure 3-10C, Table 3-5). Both HSC70 peptides monitored in MRM-MS were consistent with the 2D-DIGE observations. The relative levels of both peptides were lower in gonads from FSP compared to the PSP group, however, the differences were not significant. The gonads from SP abalone exhibited a significant lower abundance only in comparison to PSP ($p = 0.023$) for the peptide 1 [VEIIANDQGNR] (Figure 3-12E). With regards to peptide 2, [VSIHDIVLVGGSTR], a highly significant lower abundance ($p = 0.0011$) was observed in SP compared to the both PSP and FSP (Figure 3-12E).

For HSP90A, the relative abundance in 2D-DIGE experiment 1 was higher in gonads

of FSP abalone in comparison to SP gonads. The same correlation was observed for both HSP90A monitored peptides in the MRM-MS assessment, however none of the peptides exhibited significant differences between any physiological group. The pattern of expression of HSP90A peptides was similar to the pattern displayed by HSC70 peptides. For both proteins the relative abundance was higher in gonads from PSP gonads, intermediate in FSP and lower in the SP group.

During artificial spawning induction, the water temperature is gradually increased from 18°C to 22°C, over approximately 1 h and the tank water is allowed to cool over the next 12 h. As a consequence, the pattern of expression of both HSPs could be explained by the abalone exposure time to the thermal shock. The SP group was sacrificed first, followed by PSP abalone. The FSP group was sampled at the end of the experiment which lasted 12 h allowing recovery from the thermal shock. This would explain lower levels in HSPs expression in FSP in comparison with PSP. It is evident that both HSC70 and HSP90A are required in a similar fashion by abalone to maintain homeostasis and withstand the effects of thermal stress during artificial induction of abalone spawning. This mechanism has also been observed in *C. gigas* where up-regulation of HSPs enhanced survival during summer mortalities [244]. Furthermore, a study of the HSP response to thermal shock in the Antarctic clam *Laternula elliptica* showed increased expression of HSP70 reaching a peak after 12 h, with close to complete recovery after 48 h [245].

3.5.2 Proteins involved in metabolism

In the literature many of these proteins have been previously identified in several aquatic species associated with responses to oxidative stress and heat stress caused by environmental or anthropogenic conditions. As a consequence many of these proteins and/or organisms have been proposed as biomarkers of polluted areas to monitor the presence of harmful specific substances. Here, the discussion is limited

to proteins involved in energy and metabolism in the molluscan phyla.

3.5.2.1 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

At the transcriptional level GAPDH has been previously reported in several bivalve species as a biomarker and housekeeping gene [246-249]. In larvae of Pacific Oyster *C. gigas* infected with ostreid herpesvirus 1 GAPDH was tested as an internal control, however, gene expression assessment using RT-PCR ruled out GAPDH as a reliable internal control due to unstable gene expression [247]. In contrast, expression of GAPDH was unaltered in haemocytes from the flat oyster *Ostrea edulis* challenged with the protozoan *Bonamia ostreae* compared to a control group [246]. When determining the behaviour of GAPDH expression in reproduction, expression was unaltered in gonads of both sexes of the king scallop *Pecten maximus* over different stages of gonad conditioning (recovering-filling, half full-full and spent) [248]. A similar approach in the scallop *Patinopecten yessoensis* reported GAPDH as a reliable housekeeping gene due to its stable expression throughout embryo/larval stages [249].

In addition to bivalves, GAPDH expression has been assessed in both sexes of disk abalone as internal control in quantitative PCR assays. After exposure to tributyltin chloride or 17-beta estradiol, GAPDH was differentially expressed in gill and hepatopancreas and therefore deemed as an unreliable internal control [250]. Similarly, a recent study in *H. rufescens* showed that GAPDH expression was not stable in the gonad/digestive gland complex and the authors concluded that GAPDH stability as a housekeeping gene depends on the tissue type subject to analysis [251].

In this study, the relative abundance of GAPDH in 2D-DIGE experiment 1 was higher in gonads from SP abalone when compared to gonads from FSP abalone, nonetheless the MRM-MS results for both GAPDH monitored peptides were

opposite to the 2D-DIGE findings. In MRM-MS the relative abundance of GAPDH was higher in gonads from FSP abalone in comparison to SP (Figure 3-13A, Table 3-6). This lack of consistency between both approaches was likely due to the fact that multiple proteins were identified in the gel-spot containing GAPDH in 2D-DIGE. To determine which protein contributed to the differential expression, an extensive quantification analysis of all the identified proteins in this gel-spot would be recommended. With regards to the relative expression of GAPDH, a significant lower abundance ($p = 0.0358$) was observed for the relative abundance of peptide 1 [AGIALTDNFVK] between SP and PSP gonads whilst differences beyond the cut-off ($p = 0.0899$) were seen with respect to the FSP (Figure 3-13A). For peptide 2 [LVSWYDNEYGYSNR], SP gonads exhibited a significant lower abundance ($p = 0.0062$) in comparison to the FSP groups, however no difference were observed compared to the PSP group (Figure 3-13A, Table 3-6).

GAPDH is a tetrameric enzyme important in the central metabolism of all living cells, it converts glucose into pyruvic acid in the glycolytic pathway [252]. Based on the findings of this study, it is proposed that GAPDH expression is indicative of its involvement in fulfilling the extensive energy requirements to withstand the oxidative conditions and during abalone spawning.

3.5.2.2 Peptidylprolyl cis-trans isomerase B (PPIB).

Peptidylprolyl cis-trans isomerases (PPIases) were first identified in pig kidney and subsequently characterised as enzymes that catalyse the conformational trans and cis arrangements in proline [253-255]. A protein folding function has also been associated to PPIases in plants [256]. PPIases have also been isolated and in the sea snail *Conus novaehollandiae* where PPIases showed chaperone folding activity of conotoxins possessing proline residues [257]. PPIB was identified across all DIGE experiments, but only in experiments 2 and 3 returned fold-changes in protein

expression level greater than 4. In DIGE experiment 1, the relative abundance of PPIB was increased in FSP gonads in comparison with the SP group which agreed with the relative abundance of both PPIB peptides monitored in MRM-MS (Figure 3-10A; Figure 3-13B, Table 3-6). There were agreements in the relative abundance of PPIB between experiment 3 and MRM-MS where gonads from FSP abalone exhibited a lower abundance than gonads from PSP (Figure 3-10C; Figure 3-13B, Table 3-6). In experiment 2, the relative abundance of PPIB was higher in SP gonads compared to PSP, however in the MRM-MS the results were opposite (Figure 3-10B; Figure 3-13B). For PPIB peptide 1 [FPDENFK], a highly significant increased abundance was observed in gonads from PSP abalone in comparison to the SP ($p = 0.0005$) and FSP ($p = 0.0024$). The differences observed in PPIB peptide 2 [HYGAGWLSMANSGK] followed the same trend for PSP gonads in comparison to SP ($p = 0.075$) and FSP gonads ($p = 0.118$), but were not significant. Further BLASTp analysis showed that both peptides utilised in the MRM-MS approach were conserved in both PPIA and B. Consequently, it is uncertain if the significant relative abundances reported in this study are in fact the expression of both PPIases A and B.

Mutations in the gene that codes PPIB, also known as cyclophilin B, have been associated with deficiencies in collagen biosynthesis that affect bone structure and strength [258]. Tissue specific expression of PPIB in the testis of the dogfish *Scyliorhinus canicula* suggested that PPIB participates during stages of spermatogonial differentiation [259]. PPIA is also known as cyclophilin A [254] and has been reported to bind the immune-suppressor drug cyclosporin A that is widely utilised to avoid organ rejection [260]. Cyclosporin A has also been associated with adverse effects in Sertoli cells from rats that leads to impaired spermatogenesis [261]. In LβT2 gonadotroph cells, the normal reproductive functions of the GnRH pathway (that needs intracellular calcium and calcineurin and nuclear factor of

activated T cells) were altered by treatment with cyclosporin A that inhibited calcineurin therefore causing a deficient expression in the downstream hormonal responsive genes regulated by GnRH [262].

The relative high expression of PPIB in the PSP group could have been due to the chaperone function of PPIases which could have been employed during synthesis of proteins required for the production of antioxidant proteins to assist abalone withstanding the oxidative conditions. Given the roles of PPIases in reproductive pathways, it is worth considering implementation of research designed to elucidate reproductive pathways in molluscs.

3.5.2.3 ATP synthase subunit beta.

ATP synthase subunit beta is a mitochondrial protein, critical to energy generation by conversion of ADP to ATP. As with other metabolic and energy proteins identified in this study, ATP synthase subunit beta has been associated with responses to pathogenic infections and homeostasis disruption by exogenous chemicals. In the Mediterranean bream *Sparus aurata*, where 2D-DIGE was also employed, down-regulation of ATP synthase subunit beta was observed in the liver after a 10 days exposure to the parasiticide ivermectin. Additionally, in shrimp *Litopeaneus vannamei* infected with the white spot syndrome virus, the expression of recombinant ATP synthase subunit beta attenuated the noxious effects of the virus [243]. With regards to oxidative stress, ATP synthase subunit beta has been proposed to play an important role in the electron transport chain of the mitochondria during microcystin poisoning caused by cyanobacteria algal blooms [263].

In this study ATP synthase subunit beta was initially selected to be a reference protein to normalise data based on comparison of its expression across the gonad physiological states of this study (as described for the control protein PMSA5). The

relative abundance of ATP synthase subunit beta in the 2D-DIGE was less than 4 in fold-change of protein expression, however, its expression was slightly higher in gonads from SP abalone in comparison with the PSP group. The relative levels of both peptides monitored in MRM-MS, returned contrasting results. Whilst the expression profile for both peptides was similar, only peptide 1 [AHGGYSVFAGVGER] displayed a significant lower abundance in gonads from SP abalone in comparison to PSP ($p = 0.009$) and FSP (0.0139) gonads (Figure 3-13C, Table 3-6).

As ATP synthase is involved in generation of energy for several cellular functions, the higher abundance of ATP synthase subunit beta in gonads from PSP and FSP abalone could be due to the increased energy demands required for a longer spawning step, in the first case, and to withstand the oxidative environment of the experiment in both cases. Therefore, the lower relative abundance seen in gonads of SP abalone may be due to the shorter exposure to the oxidative stress prevailing in the holding tank.

3.5.2.4 Ferritin.

Free iron is highly cytotoxic, however it plays an important role in all living cells mediating electron transfer and DNA synthesis as well as being a component of metabolic enzymes such as metalloproteases and haemoglobin [264-266]. To prevent free iron cell damage, ferritin is able to store up to 4,500 iron atoms (as ferrihydrite) in its central core [267, 268]. Ferritin has been reported in four species of abalone including *H. discus discus, H. tuberculata, H. rufescens* and *H. diversicolor supertexta*. The expression of ferritin has been associated with a response to administration of xenobiotics, pathogen exposure, thermal and oxidative stress [269-273]. In *H. discus discus* two types of ferritin with iron chelating activity were isolated and characterized and found to be ubiquitously expressed in gill, mantle,

gonad, foot and digestive gland [269]. In *H. tuberculata* and *H. diversicolor supertexta* ferritin expression was stimulated by pathogen challenge [270, 271], while in *H. rufescens* ferritin was expressed as a response to thermal stress [272]. Expression of ferritin has been shown to be dose-dependent under stimulation of pro-oxidant agents such as hydrogen peroxide and tert-butylhydroquinone in mouse cells lines [273].

In 2D-DIGE experiment 1 the relative abundance of ferritin was increased in gonads from FSP abalone compared to SP abalone (Figure 3-10A). The relative abundance of ferritin in the MRM-MS approach was accomplished by monitoring the peptide IVLQDIK which exhibited a higher relative abundance in FSP gonads with regards to the SP group in agreement with the 2D-DIGE observation, however, this difference was not significant (Figure 3-13D). Significant differences were only observed in PSP abalone compared to FSP ($p = 0.009$) and SP ($p = 0.0026$).

The current methodology to artificially induce spawning in *H. laevigata* relies on UV irradiation of sea water and an increase in water temperature from ~18 to 22°C. UV light irradiation of water produces residual hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH) [63]. This in turn creates more toxic hydroxyl radicals through the interaction of H_2O_2 with iron (Fenton's reaction) available in seawater [63], therefore an oxidative environment is produced in the abalone holding tank. The higher the UV irradiation dose, the more H_2O_2 is produced in the water with its consequent production of cytotoxic hydroxyl radicals [63]. It has been shown that thermal and oxidative stress stimulates ferritin expression as observed in *H. rufescens* where ferritin reached its highest expression peak at 22°C [272]. Additionally, an excess of H_2O_2 has been reported to decrease life span and egg deposition rates in tick *Ricephalus microplus* [209]. Considering the spawning method used in this study, the relative higher expression of ferritin exhibited by the PSP group, indicates a response

to exposure to thermal and oxidative stress.

3.5.2.5 Thioredoxin peroxidases 1, 2 and peroxiredoxin 6 (TPx1, TPx2, Prx6).

In Haliotids, thioredoxins were first isolated in disk abalone *H. discus discus* where their expression was determined to be a response to oxidative stress induction by addition of xenobiotics [274]. Both abalone TPxs were differentially expressed in the gill and digestive tract, however, expression of TPx2 was higher than TPx1[274]. Activity assays demonstrated that removal of H_2O_2 was directly proportional to increasing recombinant abalone TPx1 and TPx2 [274]. Additionally, the expression of recombinant abalone TPx1 and TPx2 increased the life span of *Escherichia coli* against oxidative stress caused by addition of H_2O_2 into the culture [274]. Peroxiredoxin 6 (Prx6) was also isolated from disk abalone. As observed for TPx1 and 2, Prx6 was preferentially expressed in gill and digestive tract of *H. discus discus* [275]. Expression of the recombinant abalone Prx6 prevented apoptosis driven by H_2O_2 in human leukaemia THP-1 cells [275].

In this study, the relative abundance of TPx2 was decreased in gonads from FSP compared to SP abalone in 2D-DIGE experiment 1(Figure 3-10A). The abundance of peptide 2 [IPLLADK] was consistent with this observation with a decreased abundance in gonads from FSP abalone (Figure 3-13E). For peptide 1 [GLFIIDDK], the levels were equivalent between FSP and PSP and no significant differences amongst the groups were observed. BLASTp analysis of IPLLADK and GLFIIDDK showed that both were conserved in TPx1, TPx2 and Prx1, thus the MRM-MS results could have been derived from the quantification of all three proteins.

In 2D-DIGE experiments 2 and 3, TPx2 exhibited a relative higher abundance in gonads from PSP abalone compared to SP and FSP gonads (Figure 3-10B, C). This was later confirmed in the MRM-MS assessment with both monitored peptides

displaying a higher abundance (not significant) in gonads from PSP abalone with regards to SP and FSP (Figure 3-13E, Table 3-6).

The relative abundance of Prx6 was consistent between both approaches, Prx6 was always higher in gonads from PSP abalone in comparison to the FSP group (Figure 3-10C and Figure 3-13F). Prx6 Peptide 1 [AVFIIGPDK] did not show significant differences across the groups and exhibited a different expression pattern with respect to peptide 2 [VIDSLQLTATK]. For peptide 2, a significantly higher abundance was observed in PSP gonads in comparison to SP ($p = 0.0117$) and FSP ($p = 0.0009$). Examining the technical variation for both Prx6 peptides revealed a variation of 8.3% peptide 1 [AVFIIGPDK] and 11.2% for peptide 2 [VIDSLQLTATK]. However, this difference (2.9%) in digestion efficiency does not explain the slight difference in expression pattern for both Prx6 monitored peptides (Figure 3-13F). In addition, when examining the biological variation (within groups) a higher coefficient of variance for both Prx6 peptides was exhibited by the PSP group (22.7% and 19.5% respectively) compared to the SP (9.1%; 15.6%) and FSP (14.8; 18.5%) groups (Appendix 3-3). These data highlight the effect that the biological variation has in masking significant differences between physiological groups. The physiological group exhibiting the highest variation for peptides from Prx6 and across the majority of monitored peptides, in female, was the group with the lowest number of biological replicates (PSP group).

Overall, the measured levels of both TPx2 and Prx6 appeared to associate with the exposure to the oxidative stress caused by the spawning induction method.

3.5.2.6 Glutathione-S-transferase (GST)

GST is another protein involved in protecting cells from oxidative stress caused by xenobiotics. Transfection of a GSTA1 expression vector into retinal pigment

epithelium (EPR) cells protected against mitochondrial DNA damage and cell death induced by addition of H_2O_2 [276]. A similar role for GSTs has been demonstrated in yeast cultures subjected to H_2O_2 [277]. There are several examples where GST activity or expression has been evaluated in molluscs as a biomarker for the persistent presence of environmental pollution [278-280]. GST activity in the digestive gland of snail *Littorina littorea* was directly correlated with the highest dose of administered xenobiotics [278]. GST expression in the gill and hepatopancreas of the mussel *Perna viridis* was used to monitor the effects of prolonged exposure to pesticides such as organochlorine and polycyclic aromatic based hydrocarbons [279]. In a different study, GST activity was measured in digestive gland and gills of several molluscan species. While there was minimal change in GST activity in the gills, the activity was 260-fold increased in digestive glands amongst the treated species. The authors attributed this result to the ecological niche and to the fact that in invertebrates the digestive gland has liver-like functions [280]. Similarly, in the digestive gland of the mussel *Mytilus galloprovincialis* transcript expression of GST showed significant differences after exposure to nickel and/or heat stress [281].

In this study, the relative abundance of GST expression was higher (9-fold) in gonads from FSP abalone in comparison to SP gonad as observed in 2D-DIGE experiment 1(Figure 3-10A). The relative abundance of both GST peptides in MRM-MS monitoring was also observed to be slightly higher in gonads from FSP abalone compared to SP abalone (Figure 3-13G), however, no significant differences were observed amongst them or with the PSP group.

GST was also identified in 2D-DIGE experiment 2 where its relative expression was higher in gonads from SP abalone (Figure 3-10B). However, the results from the MRM-MS approach were in opposition (Figure 3-13G, Table 3-6) where gonads

from PSP abalone showed a relatively higher abundance for peptide 1 that was not significant. BLASTp of both GST peptides revealed that peptide 1 [GLAQPIR] was common to several isoforms of GSTs while peptide 2 [LTQSNAILR] is unique for the GST targeted in this study.

3.5.2.7 Superoxide dismutase (SOD)

SOD is another antioxidant enzyme that provides protection by converting superoxide (O_2^-) into oxygen and H_2O_2, the latter is subsequently converted into water by the action of catalase or scavenged by peroxiredoxins [227, 274, 275, 282]. In molluscs SOD has also been used as a biomarker for heavy metal pollution and to evaluate the effects of pesticides and endocrine-disrupting chemicals. In the digestive gland of *M. galloprovincialis* the activity and mRNA expression of SOD showed increased significant differences with the severity of challenge with nickel and/or heat stress [281]. Another study on the gill and mantle of *M. galloprovincialis* collected from heavy metal polluted sites were found to have increased differences in SOD activity over the course of three years when compared to non-polluted ecological niches [283]. Likewise the hepatic and gill SOD activity increased in response to pesticides in a time-dependent fashion in snail *L. littorea* [279]. In the *Haliotis* genus there are several reports of increased SOD activity in response to xenobiotic exposure. The SOD activity in haemolymph and hepatopancreas of *H. diversicolor supertexta* was measured as a response to concentrations of malondialdehyde arising from the anti-fouling tributyltin (TBT). The conclusions were that SOD did not participate in detoxification of TBT in the hepatopancreas but there was increased activity in the haemolymph after 24 and 96 hours of exposure [284]. A similar dietary approach in *H. discus hannai* utilising zinc at different concentrations showed that the transcript expression of SOD was dose-dependent and that total antioxidant capacity in hepatopancreas decreased with excessive zinc

concentration in the diet [285].

In this study, SOD was only identified in 2D-DIGE experiment 2 with a decreased abundance (6-fold) in gonads of PSP abalone in comparison to the SP group (Figure 3-10B, Table 3-3), however this was not consistent with the observations in MRM-MS where SOD relative abundance was increased in PSP in comparison to SP (Figure 3-13H). This disagreement could be explained by the fact SOD isoforms from *H. diversicolor* (UniProt: B3TLB3), *H. rufescens* (UniProt: D1M8P4) and *Tegillarca granosa* (UniProt: D3JCC4) were identified in the same gel-spot. Monitoring peptides for all the isoforms present in that gel-spot could have identified which SOD isoform was contributing to the differential expression in the 2D-DIGE approach.

In MRM-MS analysis peptide 1 [NDINTIISLQPALR] showed a significantly lower abundance was observed in gonads from SP abalone in comparison to the PSP ($p = 0.006$) and FSP ($p = 0.036$) groups. This peptide was found to be unique to Mn-SOD from *H. diversicolor* by pBLAST homology searching. The relative abundance of peptide 2 [NVRPDYVK] did not exhibit significant differences, however, this peptide matched to four protein isoforms as judged by pBLAST homology searching and the quantitative results could thus be affected by the presence of multiple isoforms in the extract

Despite the presence of multiple SOD isoforms in the MS/MS analysis following 2D-DIGE and the use of at least one peptide that is common to multiple isoforms utilised in MRM-MS, the relative abundance of SOD follows the same expression pattern as other antioxidant proteins reported here, where a higher abundance was observed in the PSP group followed by an intermediate abundance in the FSP group which correlated with the increased time of exposure to oxidative stress conditions in

the experiment.

3.5.2.8 Nucleoside diphosphate kinase (NDK)

NDK plays a role in central metabolism in the conversion of guanosine triphosphate into adenosine triphosphate [286]. In the fish *Oryzias melastigma* NDK concentration was increased after exposure to neurotoxins. This increase was correlated with neuron development as NDK is known to participate in neural recovery and proliferation [287]. NDK was found to be differentially expressed in the gill of *M. galloprovincialis* challenged by *Vibrio anguillarum* [288]. In *O. edulis* the levels of transcript expression for NDK were high in haemolymph compared to other tissues when affected with heavy bonamiosis and disseminated neoplasia. The levels of NDK were also assessed in *O. edulis* gonads, but no significant differences in expression were observed [289].

In this study, the relative abundance of NDK was higher in gonads from SP abalone in comparison to PSP and FSP gonads as observed in 2D-DIGE experiments 1 and 2 (Figure 3-10A, B; Table 3-2, 3). In the MRM-MS assessment, quantification of NDK was accomplished using only a single peptide. The relative expression of this peptide was consistent with the observations in 2D-DIGE, however, this difference was not significant with PSP or FSP (Figure 3-13I, Table 3-6).

3.5.2.9 Chaperonin containing tcp1 (CCT).

This type of two-ring shape chaperonin has been suggested to play an important role in newly synthesized protein folding in the cytosol [290]. In mouse, CCT has also been reported as one the first proteins to be expressed after fertilization where it was proposed to participate in the rearrangement of actin and tubulin filaments of mouse oocytes [291]. Expression of CCT has also been observed as a response to oxidative stress in abalone *H. discus hannai*. In this species the CCT mRNA expression correlated with the oxidative stress caused by a zinc-enriched diet in a dose-

dependent manner [292]. Moreover, expression of CCT transcripts was found to be higher in abalone gonad compared to other tissues [292]. In the gill of *C. gigas* subjected to prolonged thermal stress, up-regulation of the CCT transcript was observed in the temperature-treated group (25°C) compared to the control group [293].

In this study CCT was more abundant in gonads from SP abalone in comparison to FSP in 2D-DIGE experiment 1 (Figure 3-10A, Table 3-2). In experiment 2, abundance of CCT was higher in PSP gonads compared to the SP group (Figure 3-10B, Table 3-3). The relative abundance of both peptides in the MRM-MS approach were consistent with experiment 2 in 2D-DIGE where gonads from PSP abalone exhibited the highest abundance in comparison to SP gonads, however, this difference was not significant because of the biological variation within each group (Figure 3-13J, Table 3-6; Appendix 3-3).

In comparison to the 2D-DIGE experiment 1, the relative abundance of CCT peptide 1[ALEFLPDLTIGEVK] was higher in PSP gonads compared to FSP gonad consistent with 2D-DIGE. Peptide 2 [FASSAACTVLK] showed an opposite result where gonads from FSP showed a subtle, but higher abundance (LSM = 1.819) in comparison to the abundance in SP (LSM = 1.708). However, neither peptide showed significant differences across the gonad groups.

It is implied that several transcriptional and translational processes occurred during artificial induction of spawning enabling abalone to withstand thermal and oxidative stress and to undergo spawning. The detection of CCT in this study could have been due to CCT functioning as a protein-folding chaperone for newly synthesized proteins directed toward reproductive and/or homeostatic functions.

In this thesis, the expression of metabolic proteins, specifically ROS scavengers, highlights the role of antioxidant systems during artificial spawning induction in abalone. It must be noted that there was a pattern of expression of the antioxidant proteins with the highest abundance occurring in gonads from post-spawning abalone followed by a median value in gonads of abalone that failed-to-spawn. Gonads from spawning abalone had the lowest value (often statistically significant). In this regard, the differential protein expression observed across the physiological states could possibly be explained by the exposure time to oxidative conditions and the sampling chronology. In other words, the exposure time to oxidative stress conditions dictates the effects on abalone metabolism. In previous studies, it has been found that the residual effects of oxidative stress diminish with time. As described in the Material and Methods section, UV irradiation of seawater ceased when the first abalone started spawning. Therefore, the effects of UV irradiation on abalone metabolism are finite and should decrease with time and correlate with variations in the expression of antioxidant proteins. To understand this, one must consider the sampling chronology as well. Gonads from spawning abalone were sampled as soon as they started releasing eggs; whilst gonads from post-spawning abalone were sampled after the abalone finished spawning. Gonads from abalone that did not spawn over 12 h (duration of experiment) were classified as failed-to-spawn. The intensity of oxidative stress (decaying with time) must then be correlated with gonad expression of antioxidant proteins (depending on oxidative stress intensity). Artificially added H_2O_2 is known to decrease over time in seawater and therefore its noxious effects also decay with time [59]. With that in mind, the metabolism from spawning abalone (with less exposure time) should be less affected by oxidative stress and the expression of antioxidant proteins would be expected to be lower compared to post-spawning and failed-to-spawn gonads. As such, the abalone that failed-to-spawn

remained in the holding tank for the longest time, allowing recovery from the oxidative stress conditions incurred in the first half of the experiment. This would explain the higher expression of antioxidant proteins in gonads of post-spawning abalone that were still in the tank when the intensity of oxidative stress conditions were at the expected peak.

3.6 Conclusions

In this study the novel approach used to assess the effects of artificial spawning induction has identified differentially expressed proteins involved in both reproduction as well as the response to oxidative and thermal stress. The reproductive proteins identified do not appear to be expressed as a direct effect of the spawning method, but are a good indicator of abalone gonad condition and propensity to spawn at the time of induction. More importantly, this study revealed the physiological changes in abalone gonads during and after spawning induction: they are spawning followed by recrudescence of reproductive tissues, and response to the oxidative shock of the artificial induction methodology. Many studies have manipulated physical and chemical parameter to induce spawning, however, metabolic assessments of the effects of spawning methods across abalone species or other molluscs are limited or nil.

In summary, the present study has elucidated the identity of proteins differentially expressed in gonads of female abalone H. *laevigata* in response to artificial spawning induction. Reproductive and metabolic proteins with roles in oogenesis, gonad conditioning, acrosomal reaction, fertilization and antioxidant activity were amongst the identified proteins. The current study has contributed to the understanding of abalone spawning and the industry in two ways:

- Indicators for reproductively immature abalone females that should not be subjected to the stress of spawning induction; and
- Indicators of the response to the oxidative shock and measures that can direct investigation into less stressful induction methods.

If the industry is ambitious in making genetic gain through genetically elite brood

stock, it is imperative that it preserves its elite individuals by developing more controlled and less stressful spawning induction and regimes to assist recovery of the post-spawning brood stock.

4 CHAPTER: DIFFERENTIALLY EXPRESSED REPRODUCTIVE AND METABOLIC PROTEINS IN THE MALE *HALIOTIS LAEVIGATA* GONAD UPON ARTIFICIAL INDUCTION OF SPAWNING

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4.1 Abstract

Abalone are gastropod snails from the genus *Haliotis*. The abalone adductor muscle is edible and highly appreciated for its palatability. Due to strict government control over wild stock and fishing quotas, farmed abalone is currently supplying the majority of the global market to fulfil market exigencies. Despite steady growth of the abalone farming industry and importance for local economies, many farming issues remain unsolved. Incomplete control of abalone spawning by artificial induction methods and insufficient knowledge of abalone reproductive processes have prevented on-demand production of abalone with highly marketable features. Proteins regulate a wide range of biological processes; therefore identifying the proteins expressed during artificial spawning induction could provide the molecular tools to better understand the mechanisms underpinning initiation of abalone reproduction. This study identified proteins differentially expressed in male abalone

following artificial induction of spawning. Gonads from abalone under three distinct physiological states: spawning, post-spawning and after failure to spawn were compared using a two staged proteomics approach. In the first stage, specific combinations of gonads were compared and proteins involved in metabolic and reproductive processes identified using fluorescent two-dimensional electrophoresis combined with mass spectrometry. Consequently, sperm-specific proteins such as lysin, tektin-3, enkurin, ropporin and sperm surface protein 17 and antioxidant proteins such as superoxide dismutase and thioredoxin peroxidases were quantified using multiple reaction monitoring mass spectrometry. The results showed significant differences between physiological states. Positive correlations were determined between these differences in protein abundance and abalone spawning propensity or failure to spawn.

Significance

These findings provide new insights into the metabolic processes that occur in abalone gonads when subjected to artificial spawning induction. The identities of sperm-specific proteins were revealed. These proteins will allow accurate measurements of proteins involved in sexual maturation to increase spawning efficiency in abalone farming.

4.2 Introduction

Abalone are marine snails from the class Gastropoda. Abalone are highly appreciated for the palatability of the edible foot. Highly regulated by government quotas, abalone harvests from wild stock have decreased. Abalone aquaculture has increased to produce more than 80% of the current global market, mostly provided by extensive culturing in China and Korea [11]. In Australia, the production value of fisheries and farmed abalone was estimated at AUD 178 million for the year 2011-12

[148]. Despite the commercial importance of abalone, its reproduction is a poorly understood process.

The current methods utilised by the abalone industry to induce spawning were developed in the 1970's. These techniques subject abalone to an oxidative environment stimulated by the addition of hydrogen peroxide [58] or by the irradiation of sea water with ultraviolet (UV) light [60]. The use of UV light is usually accompanied by thermal shock. The success of these techniques greatly depends upon the degree of abalone gametogenesis, which can be rapidly assessed by observations of the diameter of the abalone gonad. The more rounded and engorged the gonad, the higher the degree of sexual maturation [46, 53]. Other approaches to induce abalone spawning have employed injections of hormones or crude homogenates from abalone body parts [101, 113]. The egg-laying hormone, a neuropeptide known to stimulate ovulation in sea hare *Aplysia californica* and pond snail *Lymnaea stagnalis,* was injected into male and female *Haliotis asinina* and resulted in induction of spawning of 70-80% of selected broodstock within 6-8 hours of injection [101]. A lower spawning efficiency (20%) was observed when octopus gonadotropin-releasing hormone was injected in *H. asinina* [101]. A different study in *H. asinina* injected the tetra-peptide APGW into the foot of males stimulating spermiation and spawning within 3-5 h from injection [224]. In a different study crude homogenates from pleural-pedal ganglia of mature female abalone were successively injected into *H. discus* causing swelling in the conical appendage and promoting spawning in all of the animals [113]. In *H. asinina*, mRNA transcripts abundance of flagellar and acrosomal proteins from sperm have been associated with sexual maturation [114, 142]

Generally speaking research involving male abalone is limited, however, the abalone sperm protein lysin has attracted significant research attention. A strong Darwinian

positive selection has been determined for lysin in several species of Californian abalone [294], as such it has also been established that lysin mutates at a faster rate than mitochondrial cytochrome oxidase subunit 1 [194]. Additionally, the structure, sequence and role of lysin have been well established in several abalone species [117, 153, 295-298].

Other approaches focused at boosting reproduction rates have applied cryopreservation techniques to abalone eggs and sperm aiming to circumnavigate the problems of low fertilisation rates and to increase gains from selective breeding programs [65, 66, 70]. This approach could also be useful for farm restocking out of natural spawning season. Despite its usefulness, cryopreservation still relies on efficient spawning for sperm and egg collection.

In this study, a proteomics workflow was utilised to evaluate differential protein expression in male abalone gonads after artificial spawning induction using UV light irradiation and increase in the water temperature of the holding tanks. Differentially expressed proteins were identified in abalone gonads from different physiological states employing fluorescent two-dimensional electrophoresis coupled to mass spectrometry. Proteins involved in abalone male reproductive processes and metabolism such as lysin, sperm surface protein, superoxide dismutase and glutathione-S- transferase were quantified in a larger set of gonad samples using multiple reaction monitoring mass spectrometry (MRM-MS). The results are discussed in this chapter.

4.3 Materials and Methods

Collection of male abalone gonads was performed as described for female abalone in Chapter 3. Gonads were also classified as SP for spawning abalone; PSP for gonads from post-spawning abalone and FSP for gonads from abalone that failed-to-spawn.

Protein preparation to assess the differential protein expression using 2D-DIGE and subsequent mass spectrometry identification were carried out as described in Chapter 3 for female abalone. In this chapter three abalone gonads were used for the SP and PSP groups respectively and four abalone gonads constituted the FSP group.

Multi-peptide protein identification was possible for the majority of the proteins identified in this study. However, a small number resulted from single peptide-spectrum matches (PSMs) that required manual verification and *de novo* sequencing to validate each PSM (Appendix 4-1) and to ensure that each spectrum had been used only once for protein identification. Furthermore, details of peptide sequence, theoretical and observed mass, charge and observed mass error (< 50 ppm) are provided in Appendix 4-2.

The MRM-MS quantification of reproductive and antioxidant proteins identified in gonads of male abalone was accomplished as described in Chapter 3. However in the analysis of reproductive proteins in male abalone, peptides and transitions specific to each of the proteins of interest derived from the discovery proteomics approach were utilised. In addition, a 6500 QTRAP mass spectrometer (AB/Sciex, Foster City, CA, USA) equipped with a TurboV ionisation source operated in positive mode was utilised in Chapter 5. The MS parameters for the 4000 QTRAP (Chapter 3) were ionspray voltage (IS) 5300 V, curtain gas 35, GS1 and GS2 set to 50, source temperature 500ºC, declustering potential (DP) set to 70 and entrance potential (EP) set to 10. The only parameters that differed on the 6500 QTRAP were IS (5500 V) and GS1 (40). The statistical analysis was performed as described for female abalone in Chapter 3.

4.4 Results

4.4.1 2D-DIGE. Differential protein expression in male abalone gonads

The proteins were separated based on their isoelectric point (*pI*) in the first

dimension and then according to their relative molecular weight (MW) in the second dimension. Protein-spots showing at least a 4-fold difference in abundance were selected for further analysis. Selected proteins with a lower fold-change were also subjected to MRM-MS due to their relevance to reproduction and metabolism. Two dimensional gel images were obtained for each experiment.

4.4.1.1 Experiment 1: Comparison of gonadic proteins from failed-to-spawn versus spawning male abalone

In this experiment seven differentially expressed proteins were identified. Panel A in Figure 4-1shows the 2D-DIGE gel image obtained for the comparison of gonads from spawning (SP) abalone versus abalone that failed-to-spawn (FSP). Panel B in Figure 4-1shows the location of the identified proteins. Six proteins were down regulated in gonads from FSP abalone and one protein up-regulated with respect to SP abalone. Details of the identified proteins are summarised in Table 4-1. Gene ontology analysis determined the functional characterisation of the identified proteins (Figure 4-2). Four proteins were involved in metabolic processes including response to oxidative stress. Two proteins were involved in reproduction specifically in sexual maturation and fertilization, and as sperm constituents. The remaining protein was identified as having a role in the immunological response.

Lysin, a protein known to be crucial in abalone fertilisation by interacting with the vitelline envelope receptor for lysin (VERL) [153], was identified in this experiment. Tektin-3 was also identified amongst the reproductive proteins. Tektin-3, is an axonemal protein that has been reported as important for spermiogenesis in *H. asinina* [142]. Some discrepancies in observed and theoretical molecular weight (MW) and isoelectric point (p*I*) were seen in Table 4-1 for the proteins interferon-induced protein 44-like, triosephosphate isomerase (fragment) and superoxide dismutase (fragment). To clarify this disparity, a BLASTp analysis was carried out

on the sequences of the three proteins to assist in determining the isoelectric point and molecular weight in homologues (two proteins from the same species related by descent from the same gene) or orthologues (related proteins from a common ancestral gene in different species) of these proteins. For interferon-induced protein 44-like, the theoretical MW is 12 kDa, however, the gel spot rendering the identification appeared at ~67.0 kDa. This identification may have resulted from non-specific binding between interferon-induced protein 44-like, however, the binding partner responsible for the increased MW was not identified. The identification of triosephosphate isomerase was based on a fragment (the complete sequence was absent in the UniProt database). Homology searching revealed the complete sequence of an orthologue from *L. gigantea* (UniProt: V4AUY5) with 76% sequence homology with a MW of 27.2 kDa and p*I* of 6.0 which was a closer match to the observed MW and p*I* (31.0 kDa, 5.8). Likewise, in the case of superoxide dismutase, a fragment was matched in the database search, however, a full-length orthologue in *H. discus discus* (UniProt: Q19BK4) with 92% sequence homology showed a theoretical MW of 24.9 kDa and p*I* of 6.5. These examples further highlight the incomplete nature of the protein databases wherein even in the presence of matching protein sequences, these are often incomplete.

Arginine kinase and cellulase were identified in the same gel spot. Based on molecular weight (arginine kinase 39,840 Da; cellulase 64,601 Da), it was inferred that cellulase was in the correct location (observed MW = 65.0 kDa). The detection of arginine kinase in this gel spot could have been due to protein-protein interactions with cellulase. Without further analysis of their relative abundance, the identity of the protein responsible for the fold-change in that specific spot remains undetermined.

A strong correlation was observed between the altered expression of antioxidant

proteins such as superoxide dismutase (SOD) and spawning in female abalone in Chapter 3. As such, SOD has also been deemed relevant for further investigation. Overall, from this experiment two reproductive and one metabolic protein involved in oxidative stress responses have been selected for quantification by MRM-MS analysis.

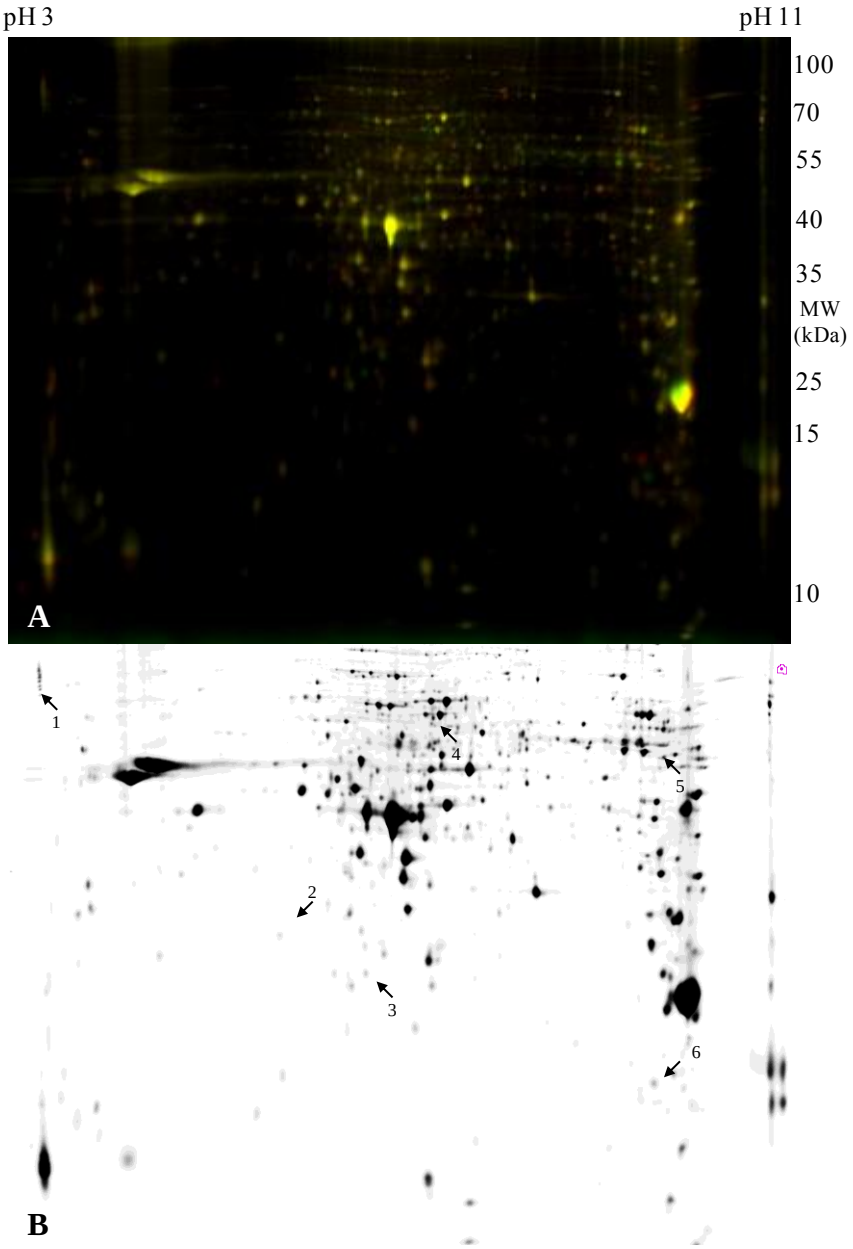

Figure 4-1. 2D-DIGE image comparing the expression of proteins from failed-to-spawn (FSP) versus spawning (SP) male abalone gonads.
A): 2D-DIGE analysis of protein expression in the gonads from FSP and SP abalone. Proteins with increased and decreased abundance are indicated in green and red respectively. Yellow spots are proteins with no change in abundance. Image acquisition was accomplished with a Typhoon scanner (GE Healthcare). B): Two-dimensional map indicating proteins of interest. Image generated by DIA module of DeCyder 6.5^{TM}.

Table 4-1. Differentially expressed proteins in the gonads of failed-to-spawn and spawning male abalone

Spot	Protein name	Accession	Species	Theor p*I*	Obs pI	Theor MW	Obs MW	Pept	Fold change
1	Interferon-induced protein 44-like protein	K1RBB3	*Crassostrea gigas*	5.22	3.20	12049	67000	1	-4.62
2	Triosephosphate isomerase (Fragment)	Q45Y86	*Haliotis rufescens*	5.20	5.80	22629	31000	14	-4.11
3	*Superoxide dismutase (Fragment)*	B3TLB3	*Haliotis diversicolor*	5.95	6.70	18770	24000	4	-5.88
4	Arginine kinase	O15989	*Turbo cornutus*	6.18	7.20	39840	65000	3	-6.97
4	Cellulase (Fragment)	Q86M37	*Haliotis discus*	5.95	7.20	64601	65000	2	-6.97
5	*Tektin-3*	gi|524899863	*Aplysia californica*	7.66	9.60	51842	48000	6	6.40
6	*Lysin*	Q25078	*Haliotis laevigata*	11.39	9.50	17769	14000	1	-6.72

Footnote. **Theor**, Theoretical. **p*I***, isoelectric point. **MW**, molecular weight. **Obs**, observed. **Pept**, number of peptides identified at ≥ 95% confidence. Observed MW and pI are approximate values. *Italics*: proteins selected for MRM-MS analysis. **Fold-change**, the values shown represent fold increases (+) and decreases (-) in gonads from FSP abalone when compared to gonads from SP abalone.

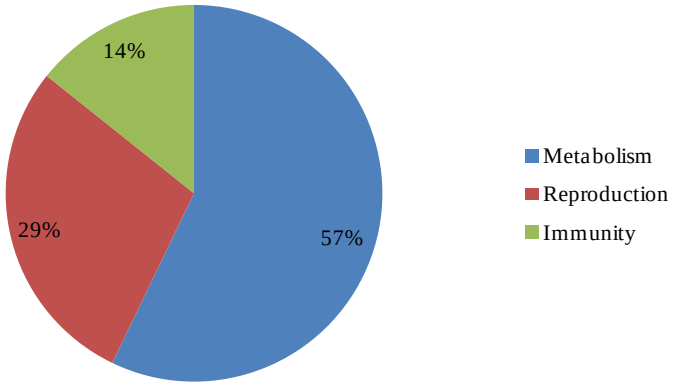

Figure 4-2. Functional characterisation of differentially expressed proteins identified in gonads from FSP and SP male abalone using 2D-DIGE technology

4.4.1.2 Experiment 2: Comparison of gonadic proteins from post-spawning abalone versus spawning male abalone

Ten differentially expressed proteins were identified in experiment 2 in compliance with the fold-change threshold of ≥ 4. Seven proteins were down-regulated and four were up-regulated in gonads from PSP abalone compared to SP abalone. Two additional proteins of interest were identified that displayed less than a 4-fold change in expression level and included ubiquitin, and tektin-3, however, they were not discussed in the number of differentially expressed protein nor in the analysis of functional characterisation. Panel A in Figure 4-3 shows the 2D-DIGE image comparing proteins from gonads of SP versus PSP abalone. The location of the identified proteins is shown in Figure 4-3 Panel B. Table 4-2 lists the identified proteins.

As was the case in Table 4-1, many of the proteins identified were based on matches to partial sequences (or fragments) found in the protein databases and as such the predicted and observed MW and p*I* values were not close matches. Each protein fragment was subjected to homology searching in an attempt to obtain the true expected MW. In the case of sperm protein, an orthologue with 86% homology in *H. rufescens* (Uniprot: M9WF52) had a theoretical MW of 25.2 kDa in closer agreement

to the observed MW of ~30 kDa. Likewise, the fragment of fascin had a theoretical MW of 36.3 kDa compared to the observed MW of ~55.0 kDa. Two orthologues in *L. gigantea* (Uniprot: V3ZP01) and *C. gigas* (Uniprot: K1QEZ3) revealed expected MW in the range 55.6-56.9 kDa adding weight to the identification of fascin. Proteasome 26S subunit was identified as the fragment (Uniprot: K4INQ5; expected MW of 27.4 kDa) which did not match the observed MW of 41.0 kDa. An orthologue of protein proteasome 26S subunit from *L. gigantea* (Uniprot: V4AE02) yielded an expected MW of 44.9 kDa giving confidence to the identification of proteasome 26S subunit in gel spot 11.

There were other discrepancies though that could not be as easily explained. For example, the predicted MW of the complete sequence of nacrein-like protein P2-like was 14.8 kDa, yet the gel spot revealing this protein identification was at an observed MW of ~35.0 kDa. An orthologue in scallop *Mizuhopecten yessoensis* (Uniprot: A0ZSF4) yielded an expected MW of 36.9 kDa and a p*I* of 6.0, but this protein was also a noted to be a fragment, whereas two other orthologues in pearl oyster (Uniprot: A0ZSF3) and scallop (Uniprot: A0ZSF5) gave expected MW ranging between 47.4-48.1 kDa. In a similar fashion, a discrepancy was noted between the observed MW (36.0 kDa) and expected MW (21.5 kDa) for splicing factor arginine/serine-rich 4. An orthologue in *Branchiostoma floridae* (Uniprot: C3YFE9) yielded an expected MW of 33.2 kDa suggesting that the sequence for *H. diversicolor supertexta* was a partial sequence.

Multiple protein identifications resulted from spot six where actin II and the histones H4 and H2A were co-located at an observed MW of ~28 kDa. The experimental MW of actin II did not match (41.7 kDa) nor did either of the histones (expected MW < 15 kDa). Due to these difficulties it was postulated that actin II and histones H4 and H2A converged in that particular gel spot due to possible protein-protein interactions

of a degraded form of actin II with intact histones H4 and H2A. These proteins were excluded from the analysis of functional characterisation as each of these proteins had different molecular functions and it was not apparent which protein was responsible from the differential expression seen for this gel spot.

Figure 4-4 shows the functional characterisation of the proteins identified in experiment 2. Half of the identified proteins had metabolic functions (50%) followed by two reproductive proteins. One protein was categorized as a structural protein and another was involved in immunity. The remaining protein had no reported function.

From this experiment sperm proteins, histones H4 and H2B, ubiquitin and tektin-3 were selected for further MRM-MS quantification.

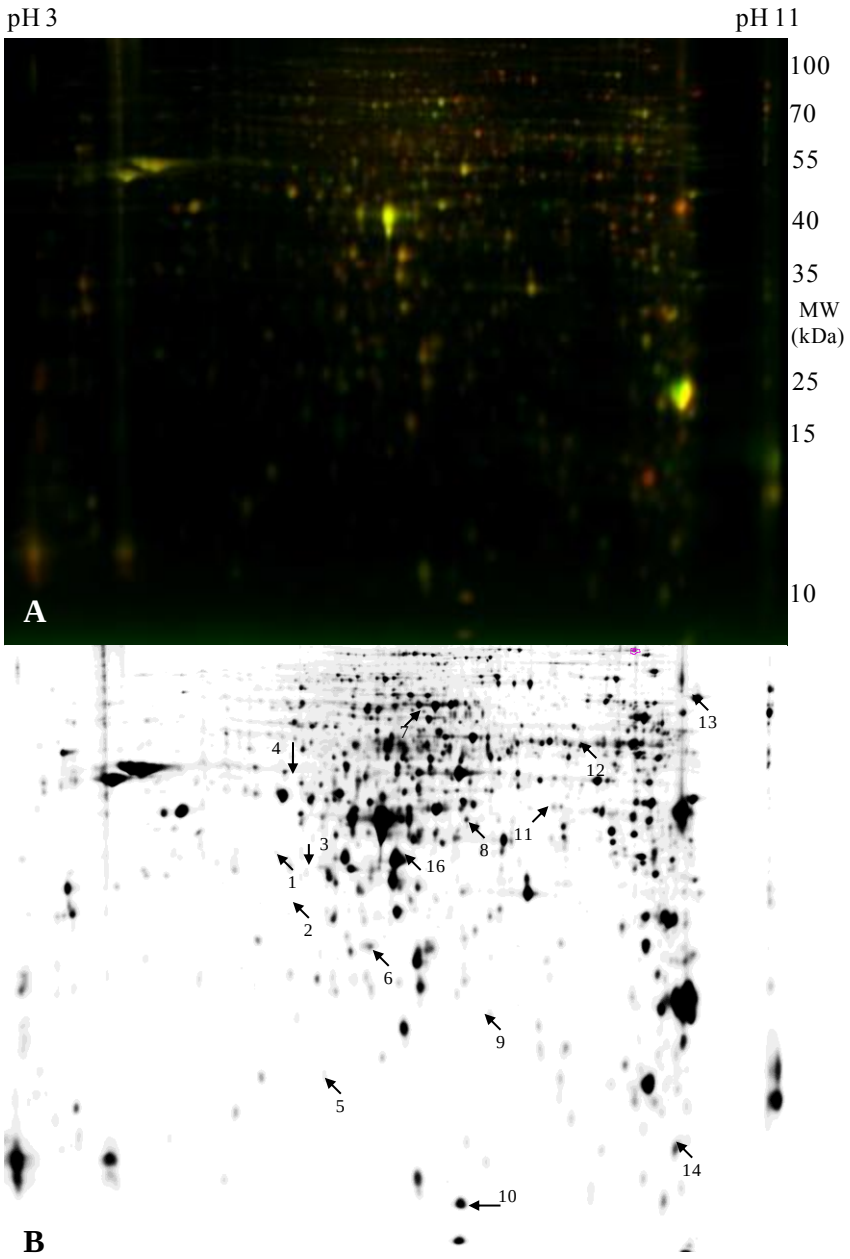

Figure 4-3. 2D-DIGE image comparing of spawning (SP) versus post-spawning (PSP) male abalone gonads.
A): 2D-DIGE analysis of protein expression in the gonads from PSP and SP abalone. Proteins with increased and decreased abundance are indicated in green and red respectively. Yellow spots are proteins with no change in abundance. Image acquisition was accomplished with a Typhoon scanner (GE Healthcare). B): Two-dimensional map indicating proteins of interest. Image generated by DIA module of DeCyder 6.5^{TM}.

Table 4-2. Differentially expressed proteins in the gonads of post-spawning abalone and spawning male abalone

Spot	Protein name	Accession	species	Theor p*I*	Obs pI	Theor MW	Obs MW	Pept	Fold change
1	Nacrein-like protein P2-like	gi|524884525	*Aplysia californica*	5.22	5.90	14801	35000	1	-5.02
2	*Sperm protein (Fragment)*	M9WF07	*Haliotis fulgens*	6.33	6.10	17796	30000	2	-7.35
3	Splicing factor arginine/serine-rich 4	E6Y2Z7	*Haliotis diversicolor supertexta*	9.35	6.30	21490	36000	2	16.23
4	Fascin (Fragment)	B3TK44	*Haliotis diversicolor*	7.1	6.10	36334	55000	2	-4.28
5	Interferon-induced protein 44-like protein	K1RBB3	*Crassostrea gigas*	5.22	6.30	12049	13500	1	6.42
6	Actin II	I0JGU0	*Sepia officinalis*	5.29	6.70	41704	28000	4	-5.47
6	*Histone H4 (Fragment)*	gi|448646149	*Charpentieria lamellata*	11.41	6.70	9599	28000	1	-5.47
6	Histone H2A	K1RXS6	*Crassostrea gigas*	10.72	6.70	14244	28000	1	-5.47
7	Cellulase	Q65Z49	*Haliotis discus discus*	6.14	7.40	64697	67000	1	-15.41
9	*Peptidyl-prolyl cis-trans isomerase B*	V3ZKW7	*Lottia gigantea*	9.69	8.00	22312	19000	1	7.37
10	*Ubiquitin*[b]	K1RA77	*Crassostrea gigas*	9.94	7.60	14744	11000	4	1.38

Footnote. **Theor**, Theoretical. **p*I***, isoelectric point. **MW**, molecular weight. **Obs**, observed. **Pept**, number of peptides identified at ≥ 95% confidence. Observed MW and p*I* are approximate values. *Italics*: proteins selected for MRM-MS analysis. ***a***: Protein selected as a reference for normalization. ***b***: Protein of interest. **Fold-change**, the values shown represent fold increases (+) and decreases (-) in gonads from PSP abalone when compared to gonads from SP abalone.

Continued Table 4-2

11	Proteasome 26S subunit (Fragment)	K4INQ5	*Solen grandis*	7.06	8.80	27409	41000	2	-10.84
12	*Tektin 3-like*	gi|524889528	*Aplysia californica*	8.88	9.20	52929	55000	2	-3.89
13	Piwi-like protein 1	K1QVJ8	*Crassostrea gigas*	9.43	10.10	99952	84000	5	4.94
14	*Histone H2B*	K1QY71	*Crassostrea gigas*	10.67	9.90	13809	12000	1	-10.82

Footnote. **Theor**, Theoretical. **p*I***, isoelectric point. **MW**, molecular weight. **Obs**, observed. **Pept**, number of peptides identified at ≥ 95% confidence. Observed MW and p*I* are approximate values. *Italics*: proteins selected for MRM-MS analysis. ***a***: Protein selected as a reference for normalization. ***b***: Protein of interest. **Fold-change**, the values shown represent fold increases (+) and decreases (-) in gonads from PSP abalone when compared to gonads from SP abalone.

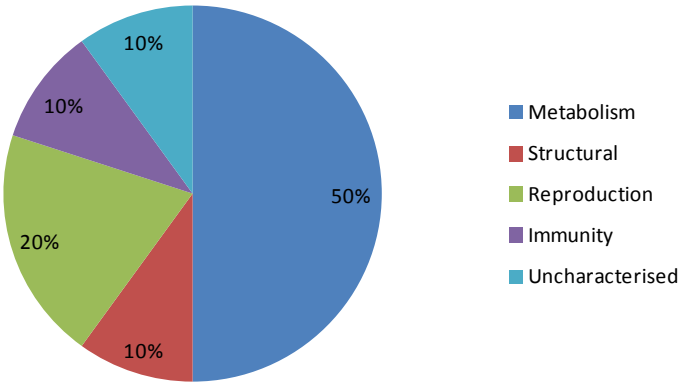

Figure 4-4. Functional characterisation of differentially expressed proteins identified in gonads from PSP and SP male abalone using 2D-DIGE technology.

4.4.1.3 Experiment 3: Comparison of gonadic proteins from failed-to-spawn versus post-spawning male abalone.

In experiment 3, eight proteins were identified as being differentially expressed. A ninth protein, voltage-dependent anion channel 2, was identified with less than a 4-fold change in expression level, however it was deemed a protein of interest as it has been reported as a structural protein in spermatozoa with relevance to fertility in mouse [185]. Panel A in Figure 4-5 shows the 2D-DIGE image comparing proteins from gonads of FSP versus PSP abalone male gonads. The localisation of the identified proteins is shown in Figure 4-5 panel B. Five proteins were down-regulated and three were up-regulated in gonads from PSP abalone. The Table 4-3 details the identified proteins. The functional characterisation is represented in Figure 4-6 and shows that 45% of the identified proteins were involved in metabolism, three proteins (33%) were categorized as reproductive, and the remaining protein (22%) had no reported function.

A couple of discrepancies were observed in the MW and p*I* of the proteins listed in Table 4-3. The first of which was glutathione-S-transferase sigma which was

observed at 25.0 kDa but was identified by a match to a partial sequence from *H. discus hannai*. A closely-related orthologue from *H. discus discus* (UniProt: Q19BK1) revealed a complete sequence with an expected MW of 25.9 kDa correlating with the observed MW for gel spot 1. For transaldolase the expected MW was 62.9 kDa, but the observed MW was 37.5 kDa suggesting that the protein identified may have been the result of protein degradation or turnover. The discrepancy observed in sperm protein was previously explained in section 5.4.1.2, where an orthologue from *H. rufescens* (UniProt: M9WF52) produced a theoretical MW of 25.2 kDa that shows closer agreement with the observed MW.

Chaperonin containing tcp1 (CCT) and tetratricopeptide repeat 25 (TTC25) were co-identified in the same spot. Based on their corresponding MW and p*I* both proteins appeared have to migrated as expected and were selected for MRM-MS quantification along with tektin-3, sperm protein and voltage-dependent anion channel 2.

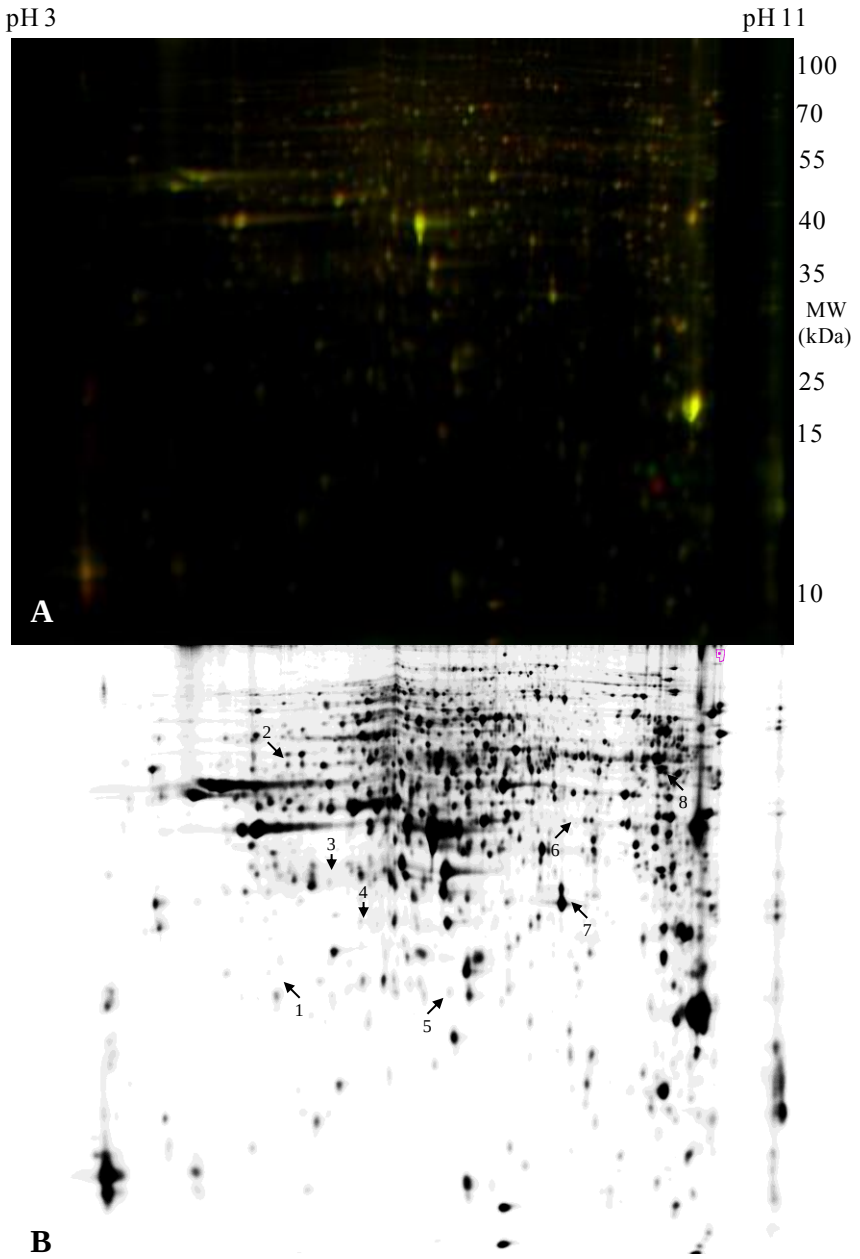

Figure 4-5. 2D-DIGE image comparing the expression of proteins from failed-to-spawn (FSP) and post-spawning (PSP) male abalone.
A): 2D-DIGE analysis of protein expression in the gonads from PSP and FSP abalone. Proteins with increased and decreased abundance are indicated in green and red respectively. Yellow spots are proteins with no change in abundance. Image acquisition was accomplished with a Typhoon scanner (GE Healthcare). B): Two-dimensional map indicating proteins of interest. Image generated by DIA module of DeCyder 6.5™

Table 4-3. Differentially expressed proteins in the gonads of failed-to-spawn and post-spawning male abalone

Spot	Protein name	Accession	species	Theor p*I*	Obs pI	Theor MW	Obs MW	Pept	Fold change
1	Glutathione S-transferase sigma	gi|190576752	*Haliotis discus hannai*	5.15	5.20	18586	25000	5	-6.02
2	*Chaperonin containing tcp1*	B6RB18	*Haliotis discus discus*	5.65	5.30	59257	66000	4	4.59
2	*Tetratricopeptide repeat protein 25*	K1PYM8	*Crassostrea gigas*	5.58	5.30	63229	60000	2	4.59
3	Transaldolase	K1QVK0	*Crassostrea gigas*	5.87	5.90	62888	37500	1	-10.16
4	*Sperm protein (Fragment)*	M9WF07	*Haliotis fulgens*	6.33	6.20	17796	32500	2	-6.37
5	Actin depolymerisation factor/cofilin	gi|157072781	*Haliotis diversicolor*	6.73	6.90	18146	22500	1	5.76
6	Hydroxysteroid dehydrogenase-like protein 2	gi|405963114	*Crassostrea gigas*	6.13	8.50	46266	40000	2	-6.63
7	*Voltage-dependent anion channel 2-like protein*[a]	D7RP02	*Haliotis diversicolor*	6.84	8.40	30632	34000	27	-1.24
8	*Tektin 3-like*	gi|524889528	*Aplysia californica*	8.88	9.80	52929	55000	5	-7.97

Footnote. **Theor**, Theoretical. **p*I***, isoelectric point. **MW**, molecular weight. **Obs**, observed. **Pept**, number of peptides identified at ≥ 95% confidence. Observed MW and p*I* are approximate values. *Italics*: proteins selected for MRM-MS analysis. ***a***: Protein of interest. **Fold-change**, the values shown represent fold increases (+) and decreases (-) in gonads from FSP abalone when compared to gonads from PSP abalone.

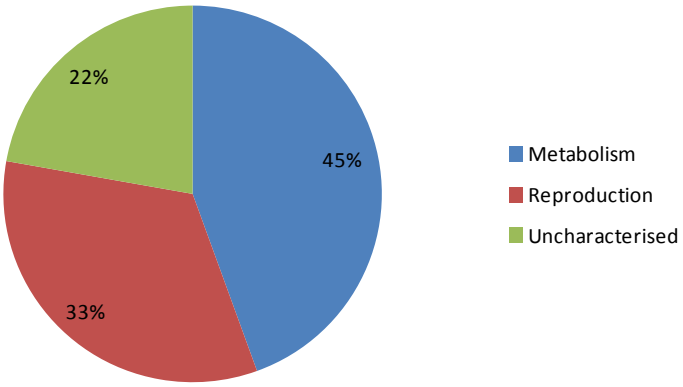

Figure 4-6. . Functional characterisation of differentially expressed proteins identified in gonads from SP and PSP male abalone using 2D-DIGE technology.

Figure 4-7 summarises the fold-changes in protein expression levels observed across all 2D-DIGE experiments. Eleven proteins including superoxide dismutase, tektin-3, lysin, sperm protein, histones 4 and 2HB, voltage-anion dependent channel 2, peptidyl-prolyl cis-trans isomerase B-like, ubiquitin, tetratricopeptide repeat 25 and chaperonin containing tcp1, were selected for further MRM-MS quantification analysis.

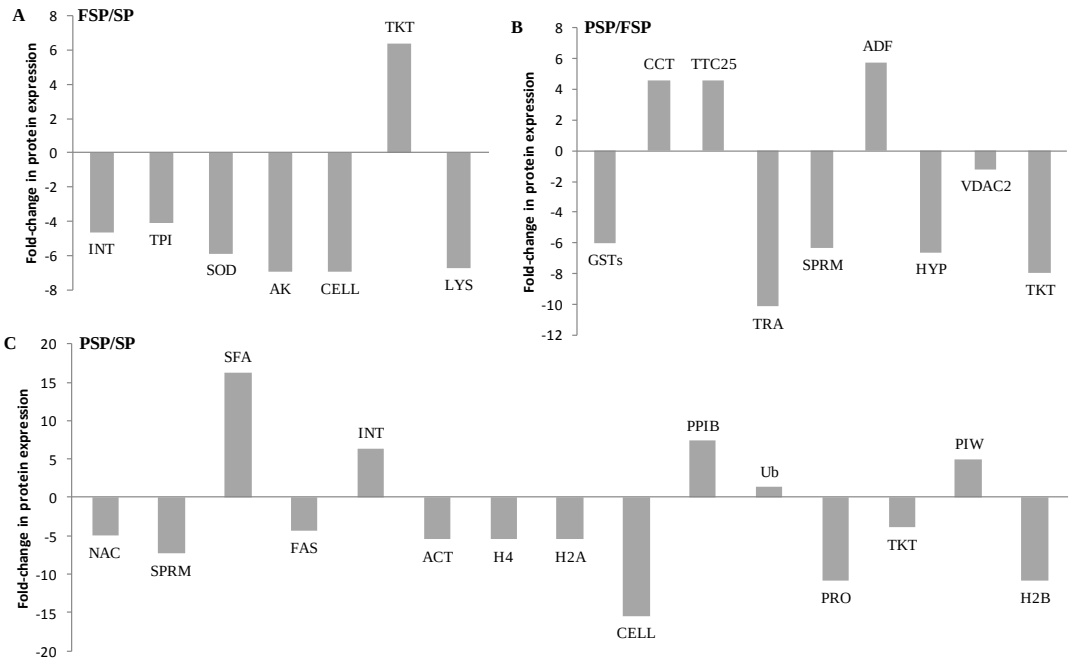

Figure 4-7. Fold-changes in protein expression in male abalone gonads in different physiological state.

The values shown represent fold increases (+) and decreases (-) in gonads from abalone with unique physiological state. The fold-changes in protein expression level were determined from 2D-DIGE gels using the DIA module of the DeCyder Software 6.5. A, Fold changes are indicated for gonads of FSP abalone when compared to gonads of SP abalone in 2D-DIGE experiment 1. B, Fold changes are indicated for gonads of PSP abalone when compared to gonads of FSP abalone in 2D-DIGE experiment 3. C, Fold changes are indicated for gonads of PSP abalone when compared to gonads of SP abalone in 2D-DIGE experiment 2. INT, Interferon-induced protein 44-like protein. TPI, Triosephosphate isomerase (Fragment). SOD, Superoxide dismutase (Fragment). AK, Arginine kinase. CELL, Cellulase. TKT, Tektin-3. LYS, Lysin. GSTs, Glutathione S-transferase sigma. CCT, Chaperonin containing tcp1. TTC25, Tetratricopeptide repeat protein 25. TRA, Transaldolase. SPRM, Sperm protein (Fragment). ADF, Actin depolymerisation factor/cofilin. HYP, Hypothetical protein LOTGIDRAFT_205585. VDAC2, Voltage-dependent anion channel 2-like protein. NAC, Nacrein-like protein P2-like. SFA, Splicing factor arginine/serine-rich 4. FAS, Fascin (Fragment). ACT, Actin II. H4, Histone H4 (Fragment). H2A, Histone H2A. H2B, Histone H2B. PPIB, Peptidyl-prolyl cis-trans isomerase B-like. UB, Ubiquitin. PRO, Proteasome 26S subunit (Fragment). PIW, Piwi-like protein 1.

4.4.2 MRM-MS quantification

Following the identification of differentially expressed proteins in gonads from abalone with unique physiological status, proteins relevant to reproduction and anti-oxidative mechanisms were selected for validation using MRM-MS. Aside from the proteins identified in the previous section (5.4.1), the analysis was extended to include other proteins identified in Chapter 2 [231] including enkurin, ropporin, sperm surface protein 17 and atrial natriuretic peptide receptor A that are known to play roles in gametogenesis and acrosomal reaction. Additionally, ferritin, glutathione-S-transferase, glyceraldehyde-3-phosphate dehydrogenase, heat shock cognate protein 70, heat shock protein 90A, nucleoside diphosphate kinase, peroxiredoxin 6 and thioredoxin peroxidase 2 identified in Chapter 3 were selected for MRM-MS quantification and to assess the relevance of oxidative stress response during male abalone spawning. A total of ten gonads (three spawning; three post-spawning and four that failed-to-spawn) were analysed by MRM-MS. The MRM-MS data was subject to the same statistical analysis as described in Chapter 3. The threshold for significant differences was a p-value <0.05. As described in Chapter 3, the differential protein expression was assessed using two peptides per protein with each peptide assessed individually. The peak area of each MRM transition was integrated for each peptide enabling evaluation of protein abundance.

In order to obtain a reference protein (for protein normalisation) several proteins that exhibited similar staining intensity across the different gels were excised and identified. ATP synthase subunit alpha was selected as a candidate identified using this strategy. ATP synthase subunit alpha was subsequently analysed by MRM-MS and the statistical analysis showed that ATP synthase subunit alpha expression remained unaltered across all of the physiological states examined in this (Figure 4-8). During sample preparation protein extracts were quantified using the Bradford

assay and an equal amount of protein was taken from each sample. However, variation may still occur and as such the protein expression levels were normalised using ATP synthase subunit alpha.

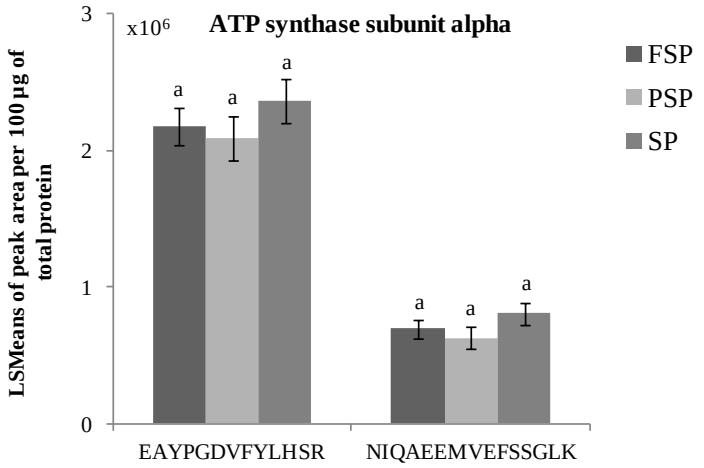

Figure 4-8. Evidence for the invariable expression of ATP synthase subunit alpha in gonads of failed-to-spawn, post-spawning and spawning male abalone.
Plotted values are least square means of summed peak areas for each peptide. A two-tail t-test was performed in the statistical software SAS 9.3. The letter on top of bars denotes lack of statistical significance ($p>0.05$) in abundance between physiological states for each analysed peptide. The peptide EAYPGDVFYLHSR was utilised as a reference to normalise the remaining proteins. FSP, gonads from abalone that failed-to-spawn. PSP, gonads from post-spawning abalone. SP, gonads from spawning abalone.

Peptides from proteins involved in male abalone reproductive processes and from antioxidant proteins were successfully monitored using MRM-MS to infer protein abundance. Table 4-4 shows the LSM obtained from each monitored peptide for proteins associated to reproductive processes. The LSM is provided for each of the physiological states; likewise the p-value comparison between physiological groups is also shown. Figure 4-9 summarises the peptide abundance of proteins involved in reproductive processes. The results refer to the peptide in the left of each graphic as peptide 1 and peptide 2 is the one in the right side of the graphic.

Table 4-4. Least squares means and p-values of proteins involved in reproduction

Protein	Peptide sequence	Least squares mean			p-value		
		FSP	PSP	SP	FSP-SP	PSP-SP	FSP-PSP
Lysin	DLIAKPVQDIPIR	4.87	3.94	3.74	0.5584	0.9232	0.6274
	VQIIAGFDR	15.11	12.18	10.65	0.3996	0.7825	0.5737
Sperm protein	DHNGVIDVDELDR	0.09	0.09	0.12	0.0081*	0.0155*	0.8019
	TNLPDQVK	0.52	0.55	0.47	0.3849	0.2499	0.6910
Tektin-3 (TKT)	VLQEIFDMEK	1.92	2.25	2.15	0.7972	0.9074	0.7041
	VPPVFAAAR	1.64	1.37	2.02	0.0946•	0.0172*	0.2078
Histone (H2B)	AMSIMNSFVNDIFER	16.20	19.67	5.22	0.0532•	0.0244*	0.4879
	LLLPGELAK[a]	26.50	31.70	12.25	0.0791•	0.0343*	0.4777
Histone (H3)	STELLIR[a]	19.30	23.54	6.32	0.1487	0.0839•	0.6125
	SAPATGGVK	5.39	6.66	1.73	0.1607	0.0886•	0.6031
Histone (H4)	ISGLIYEETR[a]	7.59	9.31	3.23	0.1711	0.0870•	0.5666
	VFLENVIR[a]	13.95	17.46	5.58	0.1937	0.0977•	0.5647
Chaperonin containing tcp1 (CCT)	ALEFLPDLTIGEVK	0.02	0.03	0.01	0.0426*	0.0041*	0.0847•
	FASSAACTVLK	0.15	0.23	0.07	0.1086	0.0099*	0.0977•
Ubiquitin (Ub)	ESTLHLVLR	0.55	0.69	0.29	0.0994•	0.0306*	0.3560
	TITLEVEPSDTIENVK	0.27	0.30	0.19	0.1802	0.0863•	0.5404
Sperm surface protein Sp17 (Sp17)	GFQNILEGLAR	1.22	2.87	0.66	0.1585	0.0006*	0.0023*
	SDSQLPSEK	0.15	0.19	0.10	0.2732	0.0581•	0.2584
Atrial natriuretic peptide receptor A (ANF)	YCLFGDTVNTASR	4.41	3.91	5.36	0.0639•	0.0168*	0.2924
	YSSNLEAIVSDR	3.46	3.11	4.27	0.0485*	0.0149*	0.3310
Heat shock cognate protein 70 (HSC70)	VEIIANDQGNR	0.89	1.20	0.47	0.0691•	0.0101*	0.1561
	VSIHDIVLVGGSTR	1.07	1.49	0.54	0.0728•	0.0090*	0.1304
Heat shock protein 90A (HSP90A)	DSVQNSAFVER	0.40	0.57	0.15	0.0619•	0.0091*	0.1541
	GVVDSEDLPLNISR	0.35	0.48	0.14	0.0392*	0.0066*	0.1654

Footnote: Superscripts indicate that values were divided by a specific number to maintain data within scale: a, divided by 10. FSP, gonads from abalone that failed to spawn. SP, gonads from spawning abalone. PSP, gonads from post spawning abalone.* indicates significant differences ($p<0.05$). • indicates some differences but not deemed to be significant ($p<0.1$).

The relative abundance of the two lysin peptides monitored displayed the same pattern of expression: high abundance in gonads from FSP abalone, intermediate in PSP abalone and low in spawning abalone. However, no significant differences were observed in any physiological state owing to both high biological variation (within groups) and high technical variation for these two peptides (Figure 4-9A; Appendices 4-3, 4-4).

While the overall technical variation (derived from four technical replicates of each abalone) was <13%, the variation observed for lysine peptides was significantly higher (peptide 1 = 23.9%; peptide 2 = 29.1%). Peptide 1 [DLIAKPVQDIPIR] was the C-terminal peptide present in the mature lysine and the variation observed for this peptide could be in part due to the action of exopeptidases or due to variable cleavage by trypsin at the KP motif within the peptide. Peptide 2 [VQIIAGFD**R**↓**K**] contains a ragged end (dibasic site at the C-terminus of the peptide indicated in bold type font) which again could have lead to variable trypsin digestion efficiency.

The relative abundance of the peptides monitored for both sperm protein and tektin-3 did not follow the same pattern of expression. For sperm protein, peptide 1 exhibited a subtle, but significant increase in abundance in the SP group compared to PSP and FSP (Figure 4-9B). In the case of tektin-3, peptide 2 showed a significant increased abundance in the SP group with regards to PSP and FSP (Figure 4-9C). The other peptide monitored for both sperm protein and tektin-3 did not show significant differences across the physiological states and greater intergroup variation was noted for these peptides.

All peptides from the histones (H2B, H3 and H4) showed the same pattern of expression: high abundance in PSP abalone, intermediate in FSP abalone and low in the SP group. Significant differences were displayed by the SP group only with

respect to the PSP group for histone H2B (Figure 4-9D). The pattern of expression for histones H3 and H4 was not deemed statistically significant owing to the high degree of biological variation within groups (Figure 4-9E, F). This variation was consistent between all the histones and all physiological states (Appendices 4-3, 4-4).

The relative abundance of peptides from chaperonin containing tcp1 (CCT), ubiquitin (Ub), sperm surface protein 17 (Sp17), heat shock cognate protein 70 (HSC70) and heat shock protein 90 alpha (HSP90A) also exhibited a similar expression pattern in their abundance that was high in PSP abalone, intermediate in FSP and low in SP abalone gonads. For CCT peptide 1 a decreased but significant abundance was observed in the SP group in comparison to PSP and FSP abalone gonads whilst peptide 2 delivered a significant decrease in abundance in SP gonads relative to PSP only (Figure 4-9G). For Ub, peptide 1 exhibited a decreased significant abundance in the SP group compared to the PSP group (Figure 4-9H). In the case of Sp17, peptide 1 exhibited a significant increased abundance in the PSP group when compared to SP and FSP groups (Figure 4-9I).

Both HSC70 peptides showed a significant decreased abundance in the SP group when compared to the PSP group (Figure 4-9K). HSP90A Peptide 1 exhibited a significant decreased abundance in the SP group in comparison to the PSP group while peptide 2 showed a similar expression pattern with abundance of SP significantly decreased compared to PSP and FSP (Figure 4-9L).

The relative abundance of both of the monitored peptides for the atrial natriuretic peptide receptor A (ANF) followed the same expression pattern and both were significantly higher in the SP group compared to the PSP group. Only peptide 2 exhibited significant differences between SP and FSP (Figure 4-9J).

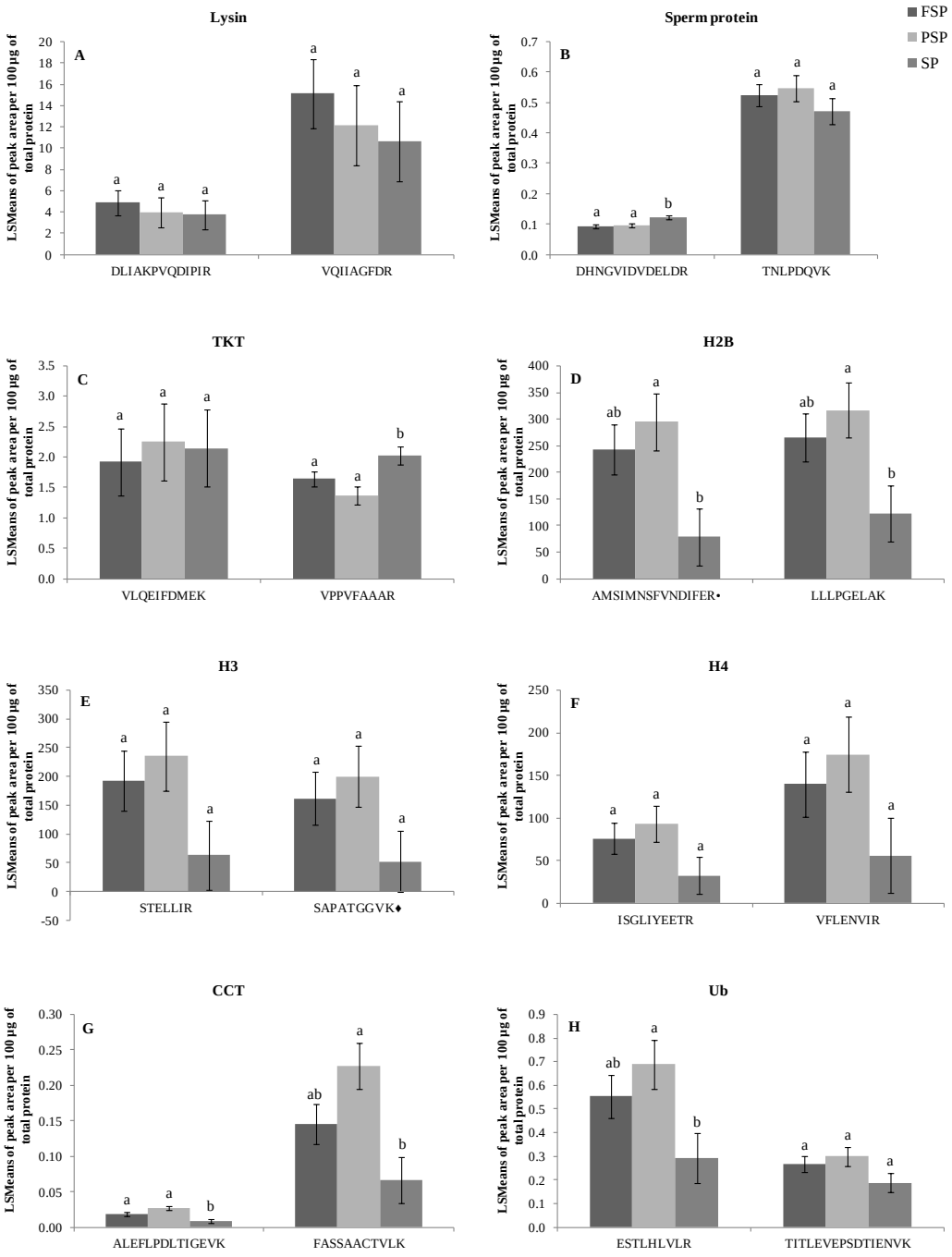

Figure 4-9. Differential protein expression of proteins involved in male abalone reproduction. Least squares means of peak area per 100 µg of total protein normalised against ATP synthase subunit alpha. A two-tail t-test was performed in the statistical software SAS 9.3. Error bars (expressed as standard error) are shown. The different letters (a, b) denote statistically significant differences ($p<0.05$) in abundance between physiological states for each analysed peptide. Symbols in H2B and H3 peptides indicates multiplication of the original values by (• 15) and (♦ 30) to present data on the same scale. FSP, gonads from abalone that failed-to-spawn. PSP, gonads from post-spawning abalone. SP, gonads from spawning abalone. A, Lysin. B, Sperm protein. C, Tektin-3 (TKT). D, Histone H2B (H2B). E, Histone H3 (H3). F, Histone H4 (H4). G, Chaperonin containing tcp1 (CCT). H, Ubiquitin (Ub).

Continued Figure 4-9

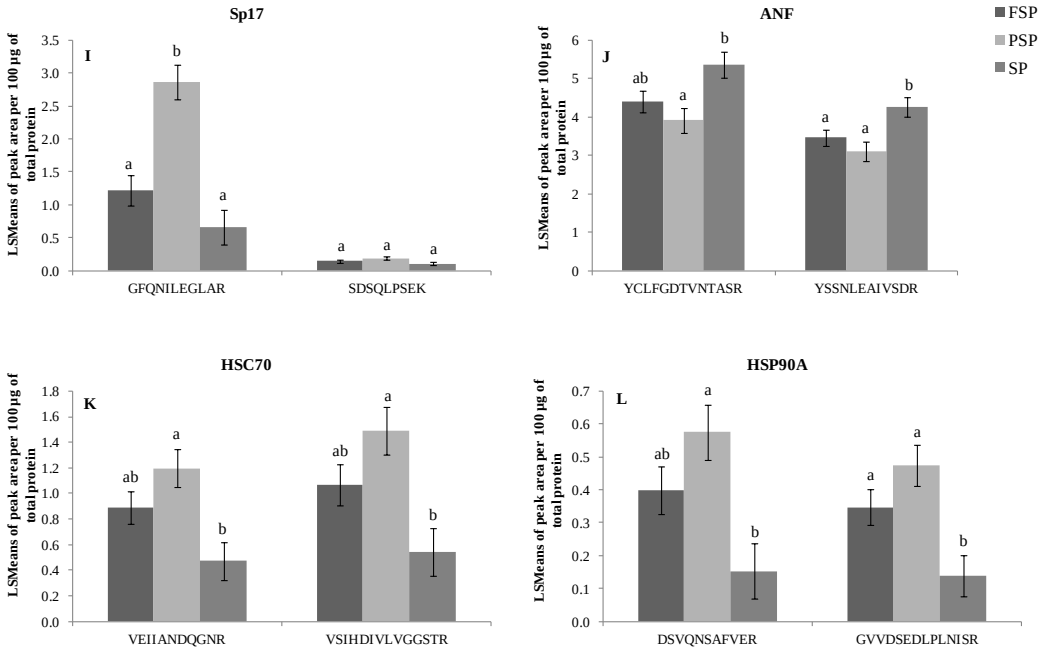

Figure 4-9 **Differential protein expression of proteins involved in male abalone reproduction.** Least squares means of peak area per 100 µg of total protein normalised against ATP synthase subunit alpha. A two-tail t-test was performed in the statistical software SAS 9.3. Error bars (expressed as standard error) are shown. The different letters (a, b) denote statistically significant differences ($p<0.05$) in abundance between physiological states for each analysed peptide. FSP, gonads from abalone that failed-to-spawn. PSP, gonads from post-spawning abalone. SP, gonads from spawning abalone **I**, Sperm surface protein 17 (Sp17). **J**, Atrial natriuretic peptide receptor A (ANF). **K** Heat shock cognate protein 70 (HSC70). **L**, Heat shock protein 90 alpha (HSP90A).

Table 4-5 shows the LSM obtained from each monitored peptide for proteins associated with metabolic processes. The LSM is provided for each of the physiological states; likewise the p-value comparison between physiological groups is also shown.

Table 4-5. Least squares means and p-values of proteins involved in metabolic processes

Protein	Peptide sequence	Least squares mean			p-value		
		FSP	PSP	SP	FSP-SP	PSP-SP	FSP-PSP
Superoxide dismutase (SOD)	NDINTIISLQPALR	0.14	0.18	0.17	0.1514	0.6146	0.0663
	HHNAYVTNLNVAQEK	1.33	1.67	1.45	0.5012	0.2878	0.0937•
Glutathione-S-transferase (GST)	GLAQPIR	7.41	8.42	7.99	0.6713	0.7613	0.4608
	KPTDPLQEGLELR	4.51	5.67	5.45	0.0297*	0.5810	0.0124*
Ferritin (FER)	IVLQDIK	0.44	0.50	0.28	0.0902•	0.0414*	0.5063
	VGPGLGEYMFDK	0.40	0.55	0.38	0.6983	0.0041*	0.0047*
Thioredoxin peroxidase 2 (Tpx2)	IPLLADK	1.63	1.90	1.19	0.0730•	0.0148*	0.2262
	GLFIIDDK	1.29	1.47	0.82	0.0456*	0.0163*	0.3812
Nucleoside diphosphate kinase (NDK)	GDFCIDVGR	0.49	0.63	0.38	0.0936•	0.0049*	0.0486*
	YMASGPVVAMVWEGK	0.11	0.15	0.07	0.0292*	0.0008*	0.0130*
ATP synthase subunit alpha (ATP synthase)	EAYPGDVFYLHSR[a]	2.18	2.08	2.36	0.4277	0.2669	0.6679
	NIQAEEMVEFSSGLK[b]	6.93	6.26	8.06	0.3162	0.1510	0.5403

Footnote: Superscripts indicate that values were divided by a specific number to maintain data within scale: a, divided by 100,000. b, divided by 10,000. SP, gonads from spawning abalone. FSP, gonads from abalone that failed to spawn. PSP, gonads from post spawning abalone.* indicates significant differences ($p<0.05$). • indicates some differences but not deemed to be significant ($p<0.1$). ♦ indicates consistency in protein abundance between 2D-DIGE and both peptides monitored in MRM-MS.○ indicates consistency in protein abundance between 2D-DIGE and one peptide monitored in MRM-MS. x indicates lack of consistency in both approaches.

Figure 4-10 summarises the expression of antioxidant proteins in gonads from male abalone. The proteins superoxide dismutase (SOD) and glutathione-S-transferase (GST) displayed the same pattern of expression: high in PSP abalone gonads, intermediate in SP and low in FSP abalone gonads. Whilst none of the SOD peptides showed significant differences (Figure 4-10A), peptide 2 from GST showed a significant lower abundance in the FSP group in comparison to SP and PSP (Figure 4-10B). The relative abundance of the monitored peptides for proteins ferritin (FER), thioredoxin peroxidase 2 (TPx2) and nucleoside diphosphate kinase (NDK) exhibited the same pattern of expression amongst each other, but differed to that of SOD and GST. The PSP group showed the highest abundance as was also observed for SOD and GST, however, FSP gonads showed an intermediate abundance and the lowest abundance was noted for gonads from SP abalone. Both ferritin peptides displayed significant differences when comparing the SP and PSP groups (Figure 4-10C), however only peptide 2 displayed significant differences when the PSP and FSP

groups were compared (Figure 4-10C). For TPx2, only peptide 2 showed reduced levels that were significant in the SP group when compared to the PSP and FSP groups (Figure 4-10D). TPx2 peptide 1 showed a similar expression pattern exhibiting significant differences between the SP and PSP groups, however, the FSP group could not be differentiated from either the SP or PSP group.

The expression of NDK peptide 1 was found to be significantly higher in gonads from PSP abalone in comparison to the other two groups (Figure 4-10E). The second peptide displayed significant differences across all physiological states with very low intra-group variation (Figure 4-10E).

Overall, all peptides from the five antioxidant proteins monitored by MRM-MS were observed to be present at increased levels in PSP abalone. Moreover, the difference relative to the FSP and SP groups was deemed to be significant in half of the pair-wise comparisons for PSP. In contrast, comparison of the levels of the peptide abundance between the FSP and SP groups showed similar levels with only three (of ten) pair-wise comparisons showing significant differences.

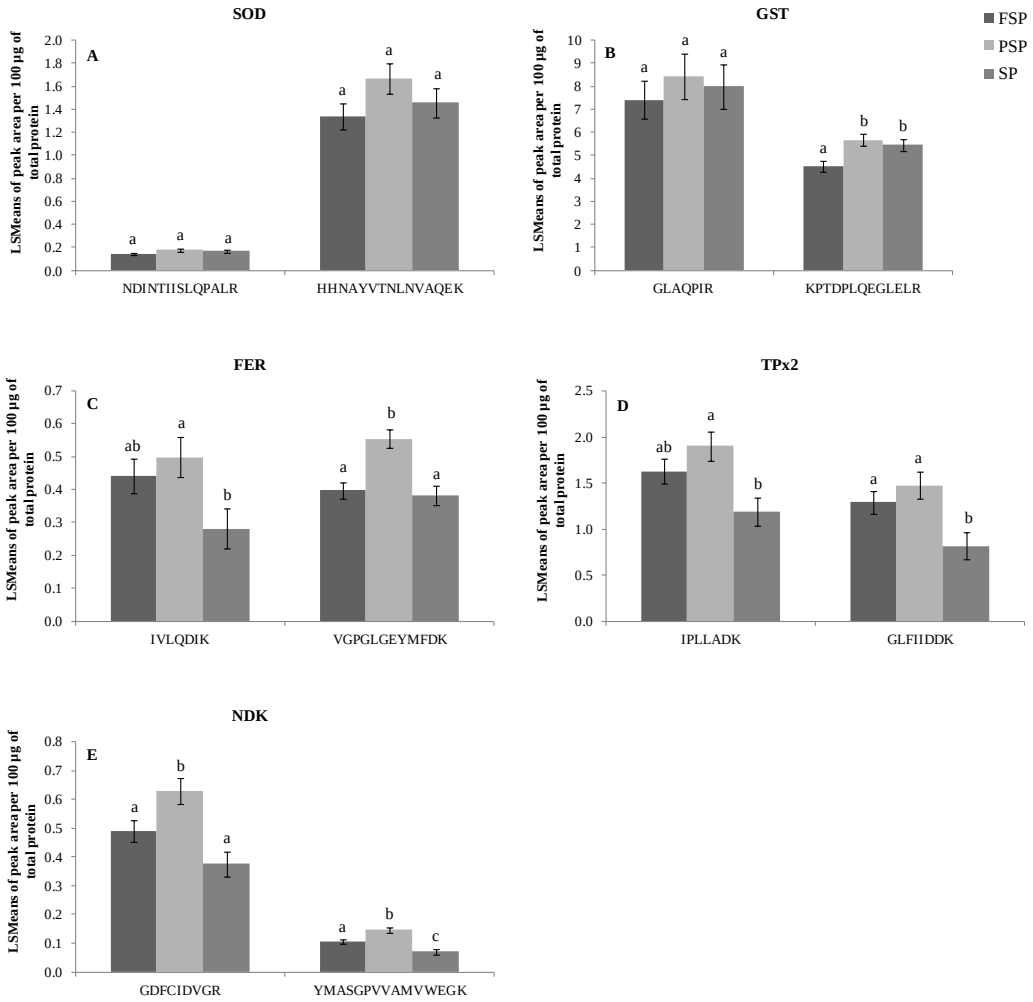

Figure 4-10. Differential protein expression of proteins involved in metabolism
Least square means of peak area per 100 µg of total protein normalised against ATP synthase subunit alpha. A two-tail t-test was performed in the statistical software SAS 9.3. Error bars (expressed as standard error) are shown. The different letters (a, b, c) denote statistically significant differences ($p<0.05$) in abundance between physiological states for each analysed peptide. FSP, gonads from abalone that failed-to-spawn. PSP, gonads from post-spawning abalone. SP, gonads from spawning abalone. A, superoxide dismutase (SOD). B, glutathione-s-transferase (GST). C, ferritin (FER). D, thioredoxin peroxidase 2 (TPX2). E, nucleoside diphosphate kinase (NDK).

2D-DIGE analysis allowed the identification of candidate proteins that exhibited differential expression in gonads of individual abalone of a defined physiological status. Proteins with relevance in metabolism and reproduction then quantitatively assessed across ten individual abalone gonads wherein four were from abalone that failed-to-spawn and three were from post-spawning abalone and spawning respectively. Where available, the consistency in relative protein abundance between both 2D-DIGE and MRM-MS approaches is shown in Table 4-6.

Table 4-6. Comparison of the consistency between the protein abundance from 2D-DIGE compared to their corresponding peptides monitored in MRM-MS.

Protein	Peptide sequence	Ratio change of LSM			2D-DIGE fold change		
		FSP/SP	PSP/SP	FSP/PSP	FSP/SP	PSP/SP	FSP/PSP
Lysin	DLIAKPVQDIPIR	1.3014	1.0524	1.2365	-6.72x	-	-
	VQIIAGFDR	1.4185	1.1431	1.2409			
Sperm protein	DHNGVIDVDELDR	0.7561*	0.7735*	0.9775		-7.35○	-6.37♦
	TNLPDQVK	1.1107	1.1602	0.9573			
Tektin-3 (TKT)	VLQEIFDMEK	0.8960	1.0501	0.8532	6.4x	-3.89○	-7.97○
	VPPVFAAAR	0.8126•	0.6781*	1.1983			
Histone (H2B)	AMSIMNSFVNDIFER	3.1060•	3.7698*	0.8239	-	-10.82x	-
	LLLPGELAK[a]	2.1624•	2.5869*	0.8358			
Histone (H3)	STELLIR[a]	3.0519	3.7223•	0.8199	-	-	-
	SAPATGGVK	3.1181	3.8529•	0.8092			
Histone (H4)	ISGLIYEETR[a]	2.3507	2.8832•	0.8153	-	-5.47x	-
	VFLENVIR[a]	2.4983	3.1281•	0.7986			
Chaperonin containing tcp1 (CCT)	ALEFLPDLTIGEVK	2.1510*	3.0849*	0.6978•	-	-	4.59x
	FASSAACTVLK	2.1720	3.3900*	0.6407•			
Ubiquitin (Ub)	ESTLHLVLR	1.8926•	2.3572*	0.8029	-	1.38♦	-
	TITLEVEPSDTIENVK	1.4221	1.6046•	0.8862			
Sperm surface protein Sp17 (Sp17)	GFQNILEGLAR	1.8436	4.3277*	0.4260*	-	-	-
	SDSQLPSEK	1.3953	1.8042•	0.7734			
Atrial natriuretic peptide receptor A (ANF)	YCLFGDTVNTASR	0.8218•	0.7296*	1.1264	-	-	-
	YSSNLEAIVSDR	0.8112*	0.7286*	1.1134			
Heat shock cognate protein 70 (HSC70)	VEIIANDQGNR	1.8834•	2.5379*	0.7421	-	-	-
	VSIHDIVLVGGSTR	1.9673•	2.7528*	0.7146			
Heat shock protein 90A (HSP90A)	DSVQNSAFVER	2.6115•	3.7717*	0.6924	-	-	-
	GVVDSEDLPLNISR	2.5201*	3.4502*	0.7304			
Superoxide dismutase (SOD)	NDINTIISLQPALR	0.8473	1.0533	0.8044	-5.8♦	-	-
	HHNAYVTNLNVAQEK	0.9167	1.1444	0.8010•			
Glutathione-S-transferase (GST)	GLAQPIR	0.9283	1.0546	0.8801	-	-	-
	KPTDPLQEGLELR	0.8276*	1.0391	0.7965*			
Ferritin (FER)	IVLQDIK	1.5670•	1.7692*	0.8857	-	-	-
	VGPGLGEYMFDK	1.0406	1.4508*	0.7172*			
Thioredoxin peroxidase 2 (Tpx2)	IPLLADK	1.3683•	1.6001*	0.8551	-	-	-
	GLFIIDDK	1.5820*	1.8061*	0.8759			
Nucleoside diphosphate kinase (NDK)	GDFCIDVGR	1.3025•	1.6745*	0.7778*	-	-	-
	YMASGPVVAMVWEGK	1.4739*	2.0473*	0.7199*			
ATP synthase subunit alpha (ATP synthase)	EAYPGDVFYLHSR[b]	0.9238	0.8834	1.0458	-	3.52x	-
	NIQAEEMVEFSSGLK[c]	0.8596	0.7759	1.1078			

Footnote: Superscripts indicate that values were divided by a specific number to maintain data within scale: a: 10. b:100,000. c: 10,000. SP, gonads from spawning abalone. FSP, gonads from abalone that failed to spawn. PSP, gonads from post spawning abalone.* indicates significant differences ($p<0.05$). • indicates some differences but not deemed to be significant ($p<0.1$). ♦ indicates consistency in protein abundance between 2D-DIGE and both peptides monitored in MRM-MS.○ indicates consistency in protein abundance between 2D-DIGE and one peptide monitored in MRM-MS. x indicates lack of consistency in both approaches.

4.5 Discussion

The current practice of artificial induction of abalone spawning subjects the abalone to thermal and oxidative stress. However, it appears that the induction treatments do not solely promote spawning. Based on the protein abundance determined in this study, there appears to be a relationship between the degree of gonad conditioning and the abalone's ability to undergo spawning. Additionally, a correlation was observed between the expression of metabolic proteins (including antioxidant proteins and proteins involved in energy metabolism) and time of exposure to oxidative stress conditions during spawning induction. The relevance of the observed significant differences in abalone reproduction is further discussed herein for individual proteins.

4.5.1 Proteins involved in male abalone reproduction

4.5.1.1 Lysin

In the 2D-DIGE experiment 1, lysin levels appeared to be decreased in gonads from FSP abalone when compared to the SP group (Figure 4-7A). However, the MRM-MS quantification of both lysin peptides contrasted with the 2D-DIGE results as lysin relative abundance was higher in gonads from the FSP group in comparison to the SP group (Figure 4-9A). No significant differences were observed in the MRM-MS approach in any physiological state owing to both high technical variation as well as high biological variation (Appendices 4-3, 4-4). In addition to the presence of possible cleavage sites within the lysin peptides by MRM-MS, lysin is also known to exist as a dimer [299] which may further preclude the efficient action of trypsin.

Lysin is an acrosomal protein in abalone sperm that has been extensively utilised in phylogenetic studies [116, 120, 296, 298, 300]. Lysin is known to interact with VERL mediating transit of spermatozoid into the egg during fertilisation [117, 153]. In fact, lysin does not act as an enzyme but it forms a hole in the vitelline envelope

by dissolution of the VE fibers in a concentration-dependent manner [57]. In this way, the success of fertilisation is dependent on the concentration of lysin in the sperm. Analysis of spectral redundancy and precursor signal intensity in *H. laevigata* revealed that lysin and vitellogenin were the most abundant protein in testes and eggs of abalone respectively [231]. As reported in chapter 3, vitellogenin is a structural protein crucial for yolk and egg development during gametogenesis of female abalone. Does lysin have a similar role in gametogenesis in male abalone? Further assessment employing other approaches such as quantification of transcripts combined with quantitative proteomics and substantial sample size may assist in obtaining a more favorable answer to this question.

4.5.1.2 Sperm protein

The relative abundance of the sperm protein peptide 1 [DHNGVIDVDELDR] shown in Figure 4-9B, was significantly higher in gonads from SP abalone in comparison to gonads from FSP ($p = 0.008$) and PSP ($p = 0.0155$) abalone. The high abundance of sperm protein in SP gonads correlated with the results observed in 2D-DIGE experiment 2 (Figure 4-7C). In experiment 3 (Figure 4-7B) the gonads from PSP showed lower abundance compared to FSP gonads, but in the MRM-MS the results were opposite and PSP gonads showed a subtle higher abundance compared to FSP gonads, however no significant differences were observed between these groups (Figure 4-7B). Being a structural protein, sperm protein would have been expected to be highly abundant in the FSP group (as they did not release any sperm, provided that they were sexually mature), intermediate in the SP group (that expelled some sperm) and low levels would be expected for the PSP group as they expelled most of their sperm. The higher abundance in the SP group for peptide 1 [DHNGVIDVDELDR], relative to PSP abalone, was consistent with the expected pattern of Sp17 abundance, however, disagreed with the abundance observed in the

FSP group unless the failure to spawn was due to a lower degree of sexual maturation in comparison with a more advanced stage of sexual maturity of the SP and PSP groups.

Peptide 2 [TNLPDQVK] did not follow the same abundance pattern showed by peptide 1 in gonads from SP abalone. Although both peptides are unique to sperm protein, the discrepancy in the MRM results may be attributed to a particular amino acid arrangement preceding the amino-terminus of the peptide [TNLPDQVK]. Trypsin was used in this study to generate peptides for MS analysis (LC-MS/MS and MRM-MS) and cleaves proteins at the carboxyl-terminus of lysine (K) and arginine (R). In this case, peptide [TNLPDQVK] is preceded by two arginine residues in the native sequence [**RR**TNLPDQVK]. This sequence condition is called a "ragged end" [301]. In this scenario trypsin can cut at either arginine producing the semi-tryptic peptide [**R**TNLPDQVK] and/or the fully tryptic peptide [TNLPDQVK]. MRM-MS analysis was conducted only on the latter peptide and as such could represent an underestimation of the peptide [TNLPDQVK]. To assess whether this condition was responsible, the technical variation was assessed and while peptide 1 showed variation of 9.7%, peptide 2 (containing the ragged end) showed slightly higher variation (12.1%) showing slightly decreased digestion (Appendix 4-3). It should also be noted that greater intra-group variation (biological variation 13.9%) was observed for peptide 2 (Figure 4-9B, Appendix 4-4). The effects of the biological variation within a group are magnified by the limited number of samples employed in this study: PSP and SP (three gonads) and FSP (four gonads). The number of samples in this study was restricted by the sexually mature abalone obtained from the industry and from the spawning response during the spawning induction. Therefore, in some instances was difficult to accurately detect significant differences between physiological groups.

The sperm protein has been previously reported in *H. rufescens* and *H. fulgens* in a study that identified 975 proteins from abalone sperm [10]. However no reports have discussed the relevance of this protein in abalone reproduction, spawning or fertilisation. Sperm protein is a structural sperm component in abalone that is likely to have a role in male abalone gametogenesis. This is the first time that an attempt has been made to correlate this protein with reproduction in abalone.

4.5.1.3 Tektin-3

The abundance of the tektin-3 was quantified by MRM-MS using the peptides [VLQEIFDMEK] and [VPPVFAAAR]. The first peptide did not show any significant difference across the physiological groups and showed high variation within groups. The second peptide was significantly higher in gonads from SP abalone compared to PSP ($p = 0.0172$) and was higher than in FSP albeit above the p-value threshold ($p = 0.0946$) (Figure 4-9C). The challenges with the quantification of tektin-3 here are numerous. Tektin-3 was identified by peptide mapping to an orthologous protein in *A. californica* with no Haliotid sequences identified. In fact, only tektin A1 sequences exist in Uniprot (from *H. rufescens* and *H. asinina*). The absence of the true protein sequence in the database brings with it questions as to whether the targeted peptides are unique to a single tektin isoforms or are they common to multiple isoforms? Homology searching using the targeted peptides reveals at least two isoforms of tektin-3 in *A. californica* implying that multiple protein isoforms might also exist in Haliotids. Thus the variation observed between the protein expression pattern at a peptide level may be at least in part due to the presence of multiple isoforms.

In 2D-DIGE experiments 2 and 3, tektin-3 showed decreased expression in gonads from PSP in comparison to SP and FSP gonads Figure 4-7B, C). These results were in agreement with the protein abundance observed in the MRM-MS for peptide

[VPPVFAAAR] (Figure 4-9C). In 2D-DIGE experiment 1 (Figure 4-7A), tektin-3 showed decreased abundance in gonads from SP abalone compared to FSP abalone in contrast to the in MRM-MS. However, since tektins are a structural sperm component it is expected that a lower abundance of tektin-3 would be present in the SP and PSP groups (that had expelled sperm) compared to abalone that failed-to-spawn (that did not expel sperm).

Tektins participate in protein transport and provide support for flagellar motility. The absence of tektin 4 has been correlated with reduced sperm motility and fertility in mice [158]. In *H. asinina*, transcripts of tektin A1 were quantified in four different stages (I, II, III and IV) of testis development where abundance was associated with testis development [142]. From the *H. asinina* results it was observed that the levels of tektin A1 transcripts were significantly lower in stage I, however no significant differences can be seen from stage II to IV [142]. Given that tektin-3 is a structural component of sperm, the decreased levels in PSP abalone relative to the SP group (for peptide VPPVFAAAR) may be explained by the completion of expulsion of sperm and the constitutive proteins The decreased levels of tektin-3 in the FSP group relative to the SP group may account for the animals failure to spawn if tektin-3 is considered an indicator of gonad ripeness or readiness of the abalone to spawn as evidenced by the role of tektin-1 in spermatogenesis [302] and the age dependent increase in expression observed in murine embryonic development [197].

4.5.1.4 Histones H2B, H3 and H4

In the 2D-DIGE approach, the histones H2B and H4 were only identified in experiment 2 (Figure 4-7C) and found to have decreased expression in gonads from PSP abalone compared to the SP group. Only histone 3 (H3) was previously identified in males in Chapter 2, therefore, H3 was selected also for MRM-MS analysis aiming to determine its importance in the testis of abalone.

The pattern of abundance, PSP>FSP>SP, was the same for all histones in the MRM-MS approach, however the only significant differences were found for histone H2B with significantly lower levels in SP compared to the PSP and FSP groups (Figure 4-9D). This trend was opposite to that found in the 2D-DIGE experiment 2 where SP gonads showed the highest abundance. Despite the significant difference of H2B in gonads from SP abalone, it must be mentioned that there was an extremely high variation (>65%) in histone abundance between gonads of the same physiological group, particularly with both H3 peptides [SAPATGGVK] and [ISGLIYEETR] (Appendix 4-4).

Histones are involved in DNA wrapping and in conjunction with other proteins comprise chromatin. Chromatin is a nucleoprotein complex that regulates a range of cellular processes including transcription, cell division and cellular repair [303]. Chromatin is organised in two main components denominated euchromatin and heterochromatin. The first is less condensed and allows for transcription to occur whereas the latter does not [304]. For transcription to occur the chromatin integrity needs to be altered and the histone-DNA complexes dismantle to make DNA accessible to transcription factors [305]. Histones are highly amenable to a large number of PTMs, in particular the amino acids arginine and lysine (the targets of trypsin digestion) as well as serine and threonine [306]. These PTMs include acetylation, mono- and deubiquitination and sumoylation of lysine, variable degrees of lysine and arginine methylation as well as ADP-ribosylation of arginine [303, 304, 306]. Table 4-7 describes the potential sites of PTMs and the ragged ends identified in the histone target peptides utilised in this study.

Table 4-7. Residues involved in "ragged ends and potential PTMs in the sequence of histones.

Protein	Tryptic peptides	
H2B	S*K*AMSIMNSFVNDIFER	LLLPGELAK
H3	*RK*SAPATGGV**KK**	KSTELLI**RK**
H4	KVFLENVIR	**KR**ISGLIYEETR

Footnote: Underlined text indicates the tryptic peptide sequence utilised to quantify the proteins. *Italics* indicate possible PTMs. Black bold indicates ragged ends.

This variation in histone abundance may be explained by three scenarios. The first explanation considers the "ragged ends" observed with sperm protein in section 5.5.1.2. As depicted in Table 4-7 both peptides in H3 and peptide 2 [VFLENVIR] in H4 have "ragged ends" at the trypsin cleavage sites. A second scenario involves post-translational modifications (PTMs) as a source of variation in histone abundance. PTMs result in modifications of the peptide mass (and fragment ion mass) deriving in the modified peptide not being measured by MRM-MS. Furthermore modifications of lysine or arginine can result in failure of trypsin to cleave the protein leading to variation in the reported peptide abundance. However, assessment of the technical variation revealed that all six peptides were reproducibly extracted, digested and analysed (CV range 7-16%) ruling out the first two scenarios as the cause of the observed variation. The third scenario would involve intra-group variation caused by different degrees of sexual maturation between abalone of the same physiological state.

The expression pattern of histones (PSP>FSP>SP) in the MRM-MS approach could be explained by the longer exposure time that PSP and FSP endured during spawning induction compared to the SP group. This would have required more translational processes to assist abalone in maintaining homeostasis (PSP and FSP groups) and to

undergo spawning (PSP group). Likewise, SP abalone had a shorter exposure to the prevailing oxidative stress and spawning conditions resulting in less transcriptional activity for both processes.

The lack of statistical significance may be explained by the intra-group variation caused by having abalone with different degrees of sexual maturation (related to the abundance of sperm) and exacerbated by the limited number of samples utilised in this study. This variation appeared to be particularly high for proteins with structural roles in abalone sperm such as tektin and the histones. The location of the protein source within the mature spermatozoa should also be considered. To extract tektin-3, several cellular barriers must be hydrolysed to reach the axonemal region in the cytoskeleton that provides support to the spermatozoa flagellum [157]. A similar scenario is faced when extracting histones that comprise the chromatin complex [304]. The universal protein extraction method utilised in this study may not be efficiently in extracting these structural proteins thus affecting the accurate quantification of both axonemal (tektin-3) and tightly packed proteins (histones). As a consequence greater within-group variation in the abundance of histones (and tektin) could have resulted masking significant differences potentially relevant to sexual maturation or spawning.

Histones have been previously identified in other Haliotid species including *H. asinina*, *H. rufescens* and both sexes of *H. laevigata* (Chapter 2). Histones H3, H4, H2A and H2B have been previously observed in SDS-PAGE of testicular cells from *H. asinina* [164]. Additionally in the same study, immunolocalization of histones also detected H1 and H4 in all stages of male germ development where a decrease in histone abundance in the stages of spermatid and spermatozoa development was observed [164]. In a transcriptome study in sperm from *H. rufescens*, EST sequences coding for H2A, H2B and H4 were also identified [10]. From this study, due to the

high variation observed, it could not be concluded whether histones have a role in sexual maturation or are relevant for spawning in male *H. laevigata*. In order to determine the relevance of histone expression in sexual maturation and spawning in male abalone, it is suggested to perform an exclusive chromatin extraction from abalone sperm followed by extraction of histones that could be used for further MRM-MS analysis using fully tryptic peptides (if available) to rule out potential variation and to effectively determine the histone role in abalone sexual maturation and artificial spawning induction.

4.5.1.5 Chaperonin containing tcp1 (CCT)

Chaperonin containing tcp 1 showed increased expression levels in 2D-DIGE experiment 3 in gonads from PSP abalone (Figure 4-7B). This result agreed with the MRM-MS approach where both CCT peptides, showed higher levels in gonads from PSP abalone. For peptide 1 [ALEFLPDLTIGEVK], gonads from SP abalone exhibited a significant decreased abundance with respect to FSP ($p = 0.0426$) and PSP ($p = 0.0041$). The increased abundance of this peptide in the FSP group compared to the PSP group was not deemed significant ($p = 0.0847$).The abundance of peptide 2 [FASSAACTVLK] was significantly lower in gonads from SP abalone in comparison to PSP gonads ($p = 0.0099$) and showed decreased levels compared to FSP but was not significant ($p = 0.108$). No significant differences for peptide 2 were observed between PSP and FSP ($p = 0.0977$) (Figure 4-9G).

CCT is a cytosolic two-ring shape chaperonin that folds newly synthesized proteins [290]. It has also been detected as the first protein to be expressed upon fertilization of mouse oocytes where it is hypothesized to participate in rearrangement of actin and tubulin filaments [291]. In mice, high abundance of CCT has been identified in all stages of cyto-differentiation of spermatids into mature spermatozoa [307]. In somatic and germ cells from mice and rats, high abundance of CCT has been

associated with chromatin packing and remodeling of heterochromatin during spermiogenesis [308].

In mammalian systems CCT appears to be associated with spermatid development, however it is possible that the CCT levels measured in this study reflected the exposure time to oxidative stress during the artificial spawning induction method. The effect of oxidative stress on expression of CCT in this study agrees with the results in *H. discus hannai* where CCT mRNA expression increased in response to oxidative stress caused by a zinc enriched diet in a dose-dependent manner [292]. SP abalone were exposed to the least amount of oxidative stress as their gonad were sampled first (within the first six hour from commencement of the experiment), providing an explanation for the low CCT levels. Conversely the PSP (sampled between the seventh and the ninth hour from spawning induction) and FSP (sampled after the experiment concluded) abalone underwent more oxidative stress exposure as their gonads were sampled after the SP abalone, possibly resulting in the increased CCT levels observed.

4.5.1.6 Ubiquitin

Ubiquitin was selected for MRM-MS because it is associated with reproductive processes. Reduced expression of poly-ubiquitin B (which has several ubiquitin sequence repeats) has been correlated with sterility in mice [187] whereas increased expression was observed in chicken testis during spermiogenesis [190]. Ubiquitin has also been associated with mitochondrial tagging during spermatogenesis as a pre-requisite for destruction after sperm decondensation [188]. In this study, ubiquitin was identified in 2D-DIGE experiment 2 with a fold-change in protein expression level of less than 4 (PSP/SP = -1.38) (Table 4-2). The abundance of peptide 1 [ESTLHLVLR] was significantly lower in gonads from SP abalone compared to PSP abalone ($p = 0.0306$), whereas a non-significant decrease was observed in the SP

group with regards to FSP abalone (p = 0.0994) (Figure 4-9. Differential protein expression of proteins involved in male abalone reproduction.

H). PSP and FSP gonads did not show any difference. Peptide 2 [TITLEVEPSDTIENVK] did not significantly vary across the physiological states, however the levels in the SP group were lower than that observed in the PSP group (p = 0.0863). A homology search revealed that both peptides utilised in this study are conserved across several ubiquitin isoforms including poly-ubiquitin and other fusion proteins as ubiquitin/ribosomal protein complexes. The differences observed between SP and PSP abalone gonads could indicate participation of ubiquitin in spermiogenesis and gonad conditioning. However, we are unable to accurately identify the exact protein isoform (containing these peptides) that is responsible for the differences observed or if it is a combined effect of multiple ubiquitin isoforms.

4.5.1.7 Sperm surface protein Sp17

Sperm surface protein Sp 17 was selected for the MRM-MS assessment due to its relevance in the acrosomal reaction and gametogenesis. Sp17 was previously identified in abalone testes (Chapter 2). For peptide 1 [GFQNILEGLAR], the gonads from PSP abalone showed an excess of two-fold increase in abundance. These levels were significantly different to both SP (p = 0.0006) and FSP (p = 0.0023) whereas no differences were detected between SP and FSP groups (Figure 4-9. Differential protein expression of proteins involved in male abalone reproduction.

I). For peptide 2 [SDSQLPSEK], the same expression trend was observed with the PSP group showing the highest level of peptide abundance, however these differences were not found to be significant in comparison to the SP group (p = 0.05810) (Figure 4-9. Differential protein expression of proteins involved in male abalone reproduction.

I).

In male reproduction Sp17 has been proposed to interact with proteins from the vitelline envelope zona pellucida (VEZP) during the acrosomal reaction [177]. Sp17 has also been reported as a structural protein in the sperm flagellum [176]. Sp17 has previously been identified in molluscs where an increased expression of Sp17 transcripts correlated with late stages of spermatogenesis in *C. gigas* [159]. Additionally, Sp17 has been identified in *H. rufescens* [10], Portuguese clam *Ruditapes decussates* [232] and mussel *M. edulis* [125] where it was suggested to have a role in molluscan reproductive processes. In this study, the lower abundance of Sp17 in gonads from FSP and the high abundance observed in abalone that successfully spawned (PSP) would suggest that Sp17 participates in abalone gonad conditioning as it has been reported that Sp17 participates in spermatogenesis in other molluscs.

4.5.1.8 Atrial natriuretic peptide receptor A

The atrial natriuretic peptide receptor A is also known as atrial natriuretic factor (ANF). This protein was previously identified in Chapter 2 and due to its relevance in reproductive processes was included in the MRM-MS assessment. The ANF has been proposed to participate in testosterone and gamete production, sperm chemotaxis, acrosomal reaction and fertilization in mammals [166-168]. On the basis of these biological roles, ANF was also selected for MRM-MS quantification. In the MRM-MS assessment, both ANF monitored peptides were more abundant in SP abalone. For peptide 1 [YCLFGDTVNTASR], the levels in SP abalone gonads were significantly higher relative to the PSP group ($p = 0.0168$) but not significantly different relative to the FSP group ($p = 0.0639$) (Figure 4-9J). There were significant differences also between SP and FSP ($p = 0.0485$) and SP and PSP ($p = 0.0149$) abalone gonads for peptide 2 [YSSNLEAIVSDR]. There were no differences

between FSP and PSP groups for either peptide.

In this study the abundance of ANF in gonads from SP abalone appeared to directly correlate ANF with the propensity of abalone to spawn further implicating ANF in sperm production and function. It is also suggested that high levels of ANF could be an intrinsic cue, sensed by male abalone prior to spawning, for a better performance during fertilization.

4.5.1.9 Heat shock cognate protein 70 (HSC70) and heat shock protein 90A (HSP90A)

As previously discussed in Chapter 3, HSC70 and HSP90A are involved in protecting cells from thermal stress and oxidative stress. Due to their ecological niche, abalone are constantly exposed to thermal stress. The peptides [VEIIANDQGNR] and [VSIHDIVLVGGSTR] were used for quantification of HSC70 in the MRM-MS approach. Abundance of both peptides was significantly lower in gonads from SP abalone compared to PSP (peptide [VEIIANDQGNR], $p = 0.0101$; peptide [VSIHDIVLVGGSTR], $p = 0.0090$) and lower but not-significant with respect to the FSP group (peptide [VEIIANDQGNR] $p = 0.0691$; [VSIHDIVLVGGSTR] $p = 0.0728$) (Figure 4-9K).

A similar expression pattern was determined for HSP90A peptides [DSVQNSAFVER] and [GVVDSEDLPLNISR] where significant decreased levels for the SP group relative to the PSP group for both peptides (peptide [DSVQNSAFVER], $p = 0.0091$; peptide [GVVDSEDLPLNISR], $p = 0.0066$) were observed. When comparing SP and FSP groups there were significantly lower levels of peptide 2 [GVVDSEDLPLNISR] in SP ($p = 0.0392$) and a decrease in peptide 1 [DSVQNSAFVER] that was not significant ($p = 0.0619$) (Figure 4-9L).

Overall, HSC70 and HSP90A followed a similar trend of protein expression during

the thermal and oxidative stress associated with artificial spawning induction in a way that is consistent with the varying length of exposure to the stresses experienced by the different physiological states of abalone gonads. The abundance pattern for both proteins was higher in the PSP group; intermediate in the FSP group and low SP group. As addressed previously, whilst all groups were subjected to the same level of thermal and oxidative conditions during the first four hours of the experiment, the timeline of abalone gonads collection explains the observed difference in abundance between the groups. Gonads from the SP group were collected between the fourth and the sixth hours of the experiment, therefore having the shortest exposure time to thermal stress. The FSP and PSP groups were exposed over a longer duration and thus developed a higher response to the stressor. The lower abundance observed in the FSP group correlates with a partial recovery from the thermal stress, compared to the PSP group that was sampled between the seventh and ninth hour of the experiment.

Other studies also implicate heat shock proteins in spermatogenesis, as is the case for HSC70 where high expression was observed during mouse and rabbit spermatogenesis and sperm storage [179, 309, 310]. In another study aimed at understanding the mechanisms of flagellar movement in mice spermatozoa, HSP90A was identified as a protein of mice sperm flagella [311]. In addition, the testis-specific complex serine/threonine phosphatase PP1γ2 has been found to be responsible for flagellar motility; HSP90 has been associated with regulating its function [312]. Moreover, HSP90A has also been referred to as a spermatogonia biomarker [313, 314].

4.5.2 Proteins involved in metabolism

The current method utilised in farms to artificially induce abalone spawning involve the use of chemicals or physical processes that promote an oxidative environment in the water of the abalone holding tanks. In the 2D-DIGE approach, only two proteins with known antioxidant functions were identified. At first glance the oxidative environment did not appear to stress male abalone to the same extent as female abalone wherein eight antioxidant proteins were identified with >4 fold-change in differential protein expression. In order to further evaluate the effects of oxidative stress in abalone spawning the group of antioxidant proteins identified from female in Chapter 3 were included in the MRM-MS assessment of gonads from male abalone.

4.5.2.1 Superoxide dismutase (SOD)

Superoxide dismutase was identified as being differentially expressed in 2D-DIGE experiment 1 (Figure 4-7A) where it showed decreased expression in gonads from FSP abalone. In the MRM-MS approach the abundance of SOD, represented by the peptides [NDINTIISLQPALR] and [HHNAYVTNLNVAQEK], was also decreased in FSP abalone gonads compared to the PSP group (Figure 4-10A). However, these differences were beyond the p-value cut-off ($p<0.05$) between gonads from PSP and FSP abalone for both peptides (peptide [NDINTIISLQPALR] $p = 0.063$; peptide [HHNAYVTNLNVAQEK] $p = 0.0937$). No differences were observed between gonads from SP and FSP abalone.

Superoxide dismutase converts superoxide (O_2^-) into oxygen and hydrogen peroxide. The H_2O_2 is then converted into water by catalase or eliminated by peroxiredoxins [227, 274, 275, 282]. Undeveloped germ cells and spermatozoa may be vulnerable to reactive oxygen species (ROS) as was observed in Japanese eel where high levels of SOD correlated with providing protection and improved survival of spermatogonia

when exposed to an oxidative agent [315]. The high abundance of SOD in gonads from PSP abalone may be due to the effects of the spawning induction as well as the induced expression of SOD in the testes during spermatogenesis to protect mature spermatozoa. Moreover, gonads from FSP abalone had the lowest level of SOD suggesting a reduced antioxidant capacity for maintaining a functional redox status during spawning with a consequent inability to engage in spawning.

4.5.2.2 Glutathione-S-transferase (GST)

The peptides [GLAQPIR] and [KPTDPLQEGLELR] were employed to quantify GST in male abalone gonads (Figure 4-10B). Peptide 2 [KPTDPLQEGLELR] was detected in lower abundance in the FSP group compared to the SP and PSP groups ($p = 0.0297$ and $p = 0.0124$ respectively). Peptide 1 [GLAQPIR] did not reveal any significant differences across the physiological states. BLASTp analysis revealed that peptide [GLAQPIR] is common to three GST isoforms and the WD repeat-containing protein 19 (GenBank: 405968088), thus accounting for the lack of differences for [GLAQPIR].

GST is an antioxidant protein found to be expressed in response to the oxidative environment created during spawning induction. Some studies have reported that an increase in expression of antioxidant proteins, including GST, helps to prevent cellular damage and deformities in sperm and testes of different organisms when challenged with chemicals [316, 317]. In sperm of *C. gigas*, high expression of GST occurred after administration of the herbicide metolachlor and the associated oxidative stress caused by the herbicide [316]. In rat testes, the antioxidant activity of GST was enhanced by addition of fruit extract where the combined effects prevented damage to germ and Leydig cells as well as deformations during spermiogenesis [317]. In our study, high GST abundance in gonads from SP and PSP abalone may correlate with maintenance of homeostasis thereby allowing the spawning processes

to occur, whereas the low GST expression in FSP gonads could indicate a failure to achieve a functional redox state.

4.5.2.3 Ferritin

The ferritin peptides [IVLQDIK] and [VGPGLGEYMFDK] were utilised in the MRM-MS assessment (Figure 4-10C). The abundance of both peptides followed the trend: SP<FSP<PSP. Significant differences were observed in gonads from SP abalone compared to PSP abalone ($p = 0.0414$) but for peptide 1 [IVLQDIK], differences between SP and FSP abalone ($p = 0.0902$) were not significant. In the case of peptide 2 [VGPGLGEYMFDK], significant differences were observed in gonads from PSP abalone with both SP ($p = 0.0041$) and FSP abalone ($p = 0.0047$). As described in Chapter 3, the function of ferritin is to capture free Fe^{2+} that can be toxic to cells. Expression of ferritin has been associated with H_2O_2. The interaction of Fe^{2+} with H_2O_2 leads to production of harmful hydroxyl radicals (Fenton's reaction) [63].

In this study, it is suggested that expression of ferritin was a direct response to the effects of the spawning induction methodology. This is supported by the fact that for peptide 1 [IVLQDIK] gonads with a longer exposure (FSP and PSP) to the oxidative conditions showed a higher ferritin expression. As addressed in Chapter 3, ferritin has been previously reported for other abalone species including *H. discus discus, H. tuberculata*, *H. rufescens* and *H. diversicolor supertexta* where ferritin expression was stimulated by thermal and oxidative stress, pathogen exposure and the addition of chemicals [269-273].

4.5.2.4 Thioredoxin peroxidase 2 (TPx2)

As addressed in Chapter 3, the main function of TPx2 and other peroxiredoxins is to remove H_2O_2 than can lead to lipid peroxidation by hydroxyl radicals. Peptide 1 [IPLLADK] was significantly lower in gonads from SP compared to PSP ($p =$

0.0148) and lower (non-significant) than in FSP gonads (p = 0.0730) (Figure 4-10D). The same expression trend was observed for peptide 2 [GLFIIDDK], however, in this case the levels observed in SP abalone were significantly lower when compared to both PSP (p = 0.0163) and FSP abalone (p = 0.0456) (Figure 4-10D). Research has reported the expression of thioredoxins during spermatogenesis. The mammalian testes thioredoxin system comprises three thioredoxins specific for spermatocytes and spermatids (Sp-Trx1, 2, 3) and one that is specific for sperm flagellum (Txl-2) [318]. Over-expression of a spermatocyte and spermatid specific thioredoxin (Sp-Trx3) was detected in infertile men and proposed as a marker for sperm and testes pathologies [319]. However, it is unknown whether over-expression of the thioredoxin system is the cause or the effect of infertility in men.

In this study it is postulated that expression of TPx2 is a metabolic response to protect the cells from the toxic effects of oxidative stress where expression of antioxidant proteins enables homeostasis and a redox environment suitable for spawning. The higher expression of TPx2 in both PSP and FSP relative to SP gonads is suggested to be the result of a longer exposure to the oxidative environment in the abalone holding tank.

4.5.2.5 Nucleoside diphosphate kinase (NDK)

The NDK peptides [GDFCIDVGR] and [YMASGPVVAMVWEGK] were used in the MRM-MS assessment. For peptide 1, the levels were highest in the PSP group compared to the SP group (p = 0.0049) and the FSP group (p = 0.0486). Higher levels of this peptide were observed in the FSP group relative to the SP group (p = 0.0936) (Figure 4-10E). The apparent abundance of peptide 2 was significantly different across all gonad states FSP versus SP (p = 0.0292); FSP versus PSP (p = 0.0486) and SP vs PSP (p = 0.0008) (Figure 4-10E) with the following trend PSP>FSP>SP.

NDK is involved in energy metabolism by converting guanosine triphosphate into adenosine triphosphate [286]. In prawn *Macrobachium rosenbergii*, abundance of a NDK transcript (from the Nm23 family, identified from a cDNA library) in developing spermatids suggested a role in prawn spermiogenesis [320]. Due to NDK involvement in energy metabolism and localization of NDK in head and spermatozoa, it has been suggested that NDK aids in the production of energy required for flagellar movement [321]. In this study, NDK as an intermediate in energy production followed a pattern of expression that is consistent with the protein abundance of other metabolic proteins across the physiological states: high abundance in the PSP group; intermediate in the FSP and low in the group SP. The SP group developed a lower response to stress conditions as a result of an earlier sampling time whilst PSP and FSP developed a more intense response. The FSP group (sampled after the conclusion of the experiment) exhibited a lower abundance (relative to the PSP group) as FSP had sufficient time for a partial recovery from the stressors.

4.5.3 Proteins involved in structure, reproduction and metabolism with no change in expression

Other proteins identified in the 2D-DIGE approach and reported in Chapter 4 did not show any significant difference when assessed in MRM-MS. For example, there was no significant differences across the reproductive states in apparent abundance of the tetratricopeptide repeat protein 25 (TTCP25) that participates in cilliogenesis [322, 323] (Figure 4-11A) nor the voltage-dependent anion channel 2-like protein (VDAC2) which is a structural component of sperm flagellum that has been associated with acrosomal reaction and fertilization in mouse [185, 211, 212] (Figure 4-11B). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been reported as a suitable reference gene in quantitative PCR assays in abalone and other close-related species [246, 249, 251]. However, this thesis has revealed that a significant

decreased abundance of GAPDH ($p < 0.05$) was observed in female gonads from SP group compared with the FSP and PSP groups for both peptides (Figure 4-13A). For males, no significant differences were observed for SP versus PSP ($p = 0.0796$) and FSP (0.0661) (Figure 4-11C).

Peroxiredoxin (Prx6), involved in removal of H_2O_2, only showed minor and not significant differences between gonads from SP and FSP abalone for both peptides [AVFIIGPDK] ($p = 0.0808$) and [VIDSLQLTATK] ($p = 0.112$) (Figure 4-11D). It should be mentioned that the pattern of expression of Prx6 was different compared to TPx2 expression (Figure 4-11D) whose function is to eliminate H_2O_2. Gonads from SP abalone had the higher Prx6 expression (Figure 4-11D) despite the shorter exposure to oxidative conditions whereas TPx2 expression in SP abalone gonads was the lowest of all. This could indicate that Prx6 is expressed earlier as a protective mechanism against oxidative conditions. It is also suggested that gonads from abalone that failed-to-spawn were also unsuccessful in expressing sufficient Prx6 and TPx2 to maintain proper homeostasis to facilitate spawning.

Peptidylprolyl cis-trans isomerase B (PPIB) is part of a family of PPIases that are involved in conformational changes of proline from cis to trans conformation [253-255]. There were no significant differences with PPIB (Figure 4-11E), nor with enkurin or ropporin (Figure 4-11F-G). Enkurin and ropporin are acrosomal proteins with roles in spermatozoa structure, fertility and roles in acrosomal reaction [143, 154, 155, 171, 172]. As addressed previously for other structural sperm-related proteins, a significantly higher abundance would be expected in the FSP group wherein no sperm was expelled (relative to the SP and PSP groups), however no differences were observed. This lack of significant difference in abundance of enkurin and ropporin across the physiological states suggest that gonads from FSP abalone were at a state of sexual maturity insufficient to undergo spawning.

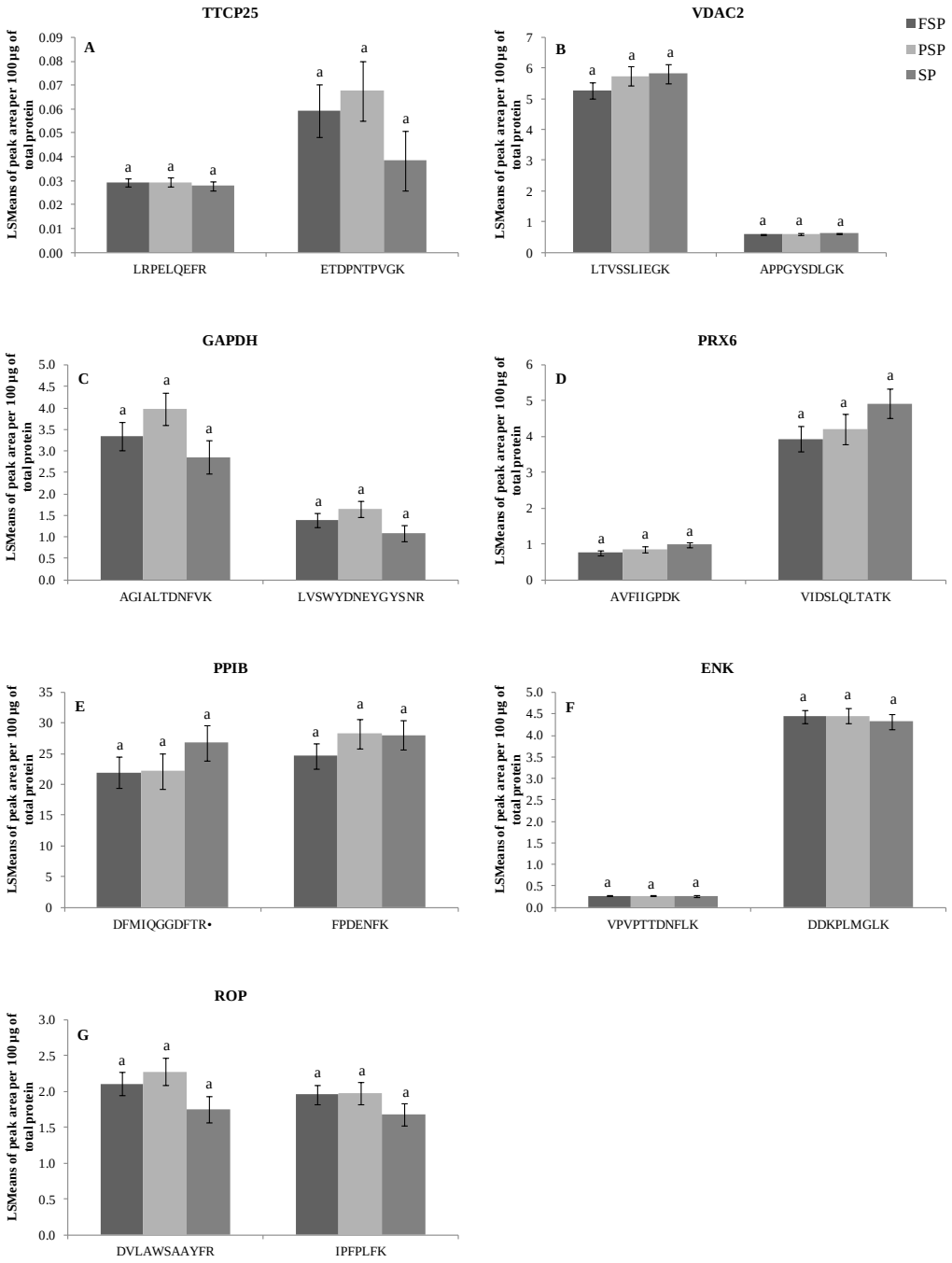

Figure 4-11. MRM quantification of proteins involved in structure, reproduction and metabolism

Least square means of peak area per 100 µg of total protein normalised against ATP synthase subunit alpha. A two-tail t-test was performed in the statistical software SAS 9.3. Error bars (expressed as standard error) are shown. The same letter on top of bars denote lack of statistical significance ($p<0.05$) in peptide abundance between physiological states. The symbol (•) annotating the PPIB peptide indicates multiplication of the original value by (200) to present data on the same scale. FSP, gonads from abalone that failed-to-spawn. PSP, gonads from post-spawning abalone. SP, gonads from spawning abalone. A, Tetratricopeptide repeat protein 25 (TTCP25). B, Voltage-dependent anion channel 2-like protein (VDAC2). C, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). D, Peroxiredoxin 6 (PRX6). E, Peptidylprolyl cis-trans isomerase A (PPIA). F, Enkurin (ENK). G, Ropporin (ROP).

Using a proteomic approach, the identities of proteins differentially expressed following artificial spawning induction were revealed in gonads from male *Haliotis laevigata*. The majority of differentially expressed proteins were involved in metabolism with a fewer number known to participate in reproductive processes. From the results of this study, it is suggested that the degree of abalone sexual maturity is key for spawning to occur. Despite the fact that highly significant differences could not be observed between the physiological groups with regards to sperm-specific proteins such as enkurin, lysin, Sp17, sperm protein, tektin-3 and the histones, valuable insights were obtained with regards to the state of sexual maturation in abalone that failed-to-spawn in comparison to the successful spawners (SP and PSP groups).

As indicated previously, failure to engage in spawning would have resulted in the FSP group possessing a higher abundance of protein constituents of sperm, however, this was not observed for proteins such as lysin, enkurin and ropporin that are involved in acrosomal reaction and fertilization. The lack of significant differences between the gonad states, with regards to the afore-mentioned proteins, supports the possibility that abalone that failed-to-spawn were sexually immature. From a comparative view point, and based on only two antioxidant proteins being identified in the male 2D-DIGE approach, it appears that oxidative stress does not have as pronounced effect on males as for females wherein eight antioxidant proteins were identified as being differentially expressed in the 2D-DIGE approach. However, the high level in protein expression of metabolic proteins observed from MRM-MS in gonads from PSP abalone suggests that male abalone experienced higher levels of oxidative and thermal stress (as occurred in female) that correlates with spawning. Nevertheless, it is unclear the extent of this association, perhaps it is only a matter of maintaining abalone homeostasis with the remaining likelihood of spawning

governed by the degree of sexual maturity and temperature stimuli during spawning induction.

As described in the timeline of abalone gonad sampling (Figure 4.1 from Chapter 4), the time of gonad collection varied throughout the experiment. During the experimental sampling it was expected that the oxidative and thermal conditions would decrease in the abalone holding tank with a consequent effect on abalone physiology. This could explain the expression pattern of metabolic proteins being low in gonads from SP (collected between the fourth and sixth hours of the experiment); high in gonads from PSP abalone (collected between the seventh and ninth hour of the experiment) and intermediate in gonads from FSP abalone (collected after the experiment concluded). Thus, the SP group was exposed to oxidative and thermal stress over the shortest period and as such did not reach the same expression levels observed for FSP and PSP. The PSP and FSP groups were exposed to both oxidative and thermal stress for the same period of time. The levels of expression of metabolic proteins were higher in the PSP group as they were sampled without sufficient time to recover from the stressors. The FSP group, however, were sampled at 12 hours allowing at least partial recovery and thus the levels of thermal and oxidative effects would have been decreased reflected by a lower expression level for metabolic proteins expression than seen for the PSP group.

Parental provision may contribute to offspring survival [208, 235]. Abalone are broadcast spawners; the spermatozoa and eggs are released into the ocean where they withstand adverse conditions (such as temperature, oxidative conditions and ocean dynamics). So it is plausible to suggest that expression of these metabolic proteins, aside from playing a direct role in maintaining homeostasis, may be inherited by the offspring as a mechanism to increase the survival rates of larvae that will also face the same environmental challenges.

4.6 Conclusions

Differentially expressed proteins with roles in metabolism and reproduction were identified in gonads from male *H. laevigata* after artificial spawning induction. The outcomes of this study will aid research targeted to increasing the understanding of reproduction in abalone by performing research on specific processes such as acrosomal reaction and fertilization. The present study also delivers the identity of proteins that could assist in developing new methods to accurately perform assessments of abalone sexual maturity to improve spawning efficiencies.

5 GENERAL DISCUSSION AND CONCLUSION

Abalone are broadcast spawners that release gametes into the ocean in response to different sensorial cues. Chemotaxis and hydrodynamics aid gamete localisation enabling fertilisation to occur. Whilst temperature is the only parameter known to initiate gametogenesis, the exact cues that promote abalone spawning remain unknown. Several authors have proposed that fluctuations in chemical and physical ocean parameters, as well as atmospheric factors, influence spawning. Under farming conditions, spawning is artificially induced by exposing abalone to thermal and oxidative stress.

All abalone selected for analysis were judged to be sexually mature using the widely accepted visual gonad index assessment method. Despite this, the results presented in this thesis revealed that successful spawning appeared to correlate with the stage of sexual maturation of abalone. Abalone that failed to spawn (FSP group), if sexually mature, were expected to display a significantly higher abundance of constitutive proteins from eggs (female) or spermatozoa (male) as they had retained all of the eggs/sperm. This was not the case and the abalone within the FSP group generally showed equivalent levels of proteins involved in reproduction or that were constitutive of gametes to abalone in the SP and PSP groups.

Significant variation was observed in the expression of antioxidant proteins when comparing the different physiological states associated with abalone spawning, highlighting a link between artificial spawning and oxidative stress. Literature is emerging in this field, however it is still unknown to what extent oxidative stress influences the initiation of spawning.

5.1 Effects of oxidative and thermal stress over reproduction

The methods to artificially induce abalone spawning were developed 37 years ago and are still being employed as initially prescribed. Whilst these methods have achieved success in Californian abalone species [58], spawning induction efficiency is far from ideal in other species from the *Haliotis* genus. Thermal shock and hydrogen peroxide (H_2O_2), either added to abalone tank water, or generated by UV irradiation of tank water, have been proposed as being responsible for initiating spawning in abalone by artificial methods. However, to date, the precise mechanism by which H_2O_2 triggers spawning in abalone remains unknown. It has been proposed that H_2O_2 promotes abalone spawning by inducing oxidative stress and stimulating the prostaglandin pathway [58] that is known to influence several stages of reproduction in other species, including humans [61]. Further research demonstrated that the prostaglandin pathway could be blocked in abalone by addition of acetylsalicylic acid thus preventing spawning [58]. There is abundant evidence that changes in temperature have a developmental effect over maturation of abalone gonads and increase in spawning rates in several abalone species including *H. laevigata* [45-47, 88]. However, despite its reliability gonad conditioning employing temperature is a time consuming and costly process. Interestingly, sudden temperature changes have also been associated to generation of oxidative stress as observed by the expression of antioxidant proteins such as ferritin, thioredoxins and HSPs in tissues of several species of abalone and other molluscs [272, 274, 324].

Since 1977, no further molecular approaches have been employed to determine the relevance of reactive oxygen species (ROS) or oxidative stress in abalone spawning. However, oxidative stress as a reproduction cost (diversion of energy for basic functions) has been investigated in flies, birds and oysters. Additionally, it has been reported that naturally or artificially induced changes in temperature also promotes

oxidative stress in molluscs deriving in the expression of antioxidant proteins [272, 325, 326].

In the fruitfly *Drosophila melanogaster* it was demonstrated that flies producing eggs had less capacity to tolerate oxidative stress conditions than flies that were not undergoing oogenesis [327]. A similar comparison was made between non-breeding and breeding zebra finch *Taeniopygia guttata* where results showed a significant decrease in antioxidant capacity in breeding birds [328]. Furthermore, the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were significantly decreased in zebra finches rearing larger brood sizes compared to zebra finches rearing smaller brood sizes [329].

Although a link between reduced antioxidant capacity and reproduction has been suggested in birds and flies, recent discoveries in oyster show a different trend. In studies involving *C. gigas,* reproduction did not significantly affect antioxidant capacity. In fact, higher transcript levels of SOD, GPx and Mn-SOD were determined in the gills that directly correlated with advanced gonad development in *C. gigas*. Additionally, low transcriptional levels of these antioxidant proteins were observed in *C. gigas* at an earlier stage of gonad development [330]. Thus it was postulated that an increase in the transcription of antioxidant genes maintained antioxidant capacity in an increasingly oxidative environment often associated with reproduction in a marine environment that is exposed to fluctuations on oxygen level. It is possible that the cost of reproduction in terms of decreased antioxidant capacity, might not apply for organisms that are naturally and constantly challenged by thermal and oxidative stress, as is the case for all intertidal molluscs.

In this thesis, the molecular response of abalone to oxidative and thermal stress during spawning induction has been demonstrated by the measurement of the

expression of several antioxidant and thermal shock proteins. In both sexes of abalone the highest abundance of antioxidant proteins was observed in post-spawning (PSP) abalone, whilst the group of abalone that failed-to-spawn (FSP) showed an intermediate value in expression of antioxidant proteins. Expression of antioxidant proteins remained consistently low in spawning abalone (SP) that were sampled as soon as they began to spawn. The expression of thermal shock proteins followed the same pattern as the antioxidant proteins. It is possible that these differences in protein expression were associated with both sample chronology and reproductive status [299, 325, 326, 328].

Sample chronology pertains to the period of time the abalone were exposed to the artificial spawning method including thermal and oxidative stress stimulated by increased temperature and UV irradiated water. The effects and concentration of ROS produced by UV irradiation or by addition of H_2O_2 in seawater are known to slowly decrease with time [59, 63]. Gonads from spawning abalone were sampled from the time they started spawning (typically between the fourth and sixth hours from initiation of spawning induction). As such these abalone were exposed to less oxidative stress compared to PSP and FSP abalone that remained in the holding tank for longer periods of time (as long as nine hours for PSP and twelve hours for FSP). From the abundance of antioxidant proteins in the PSP group, it appeared that a maximum peak of antioxidant protein expression was reached for this group. The lower expression observed in the FSP group (relative to PSP) could be explained by the abalone having sufficient time to recover from the oxidative stress resulting from UV irradiation over the first four hours of the experiment (Figure 4-1).

The abalone gonad samples utilised in this thesis were taken during the natural spawning season for abalone. The experiments were designed to assess three defined physiological states with a focus on proteins directly involved with reproductive

processes. The results of the experiments revealed clear differences in these physiological states for proteins with antioxidant function. It was not possible to determine the precise cost of reproduction with respect to antioxidant capacity because the initial experimental design did not include sexually immature abalone or abalone at a basal state, that is, abalone sampled prior to the induction of spawning. Although juvenile (sexually immature) abalone are known to have increased antioxidant capacity compared to adult abalone [331], the sampling of abalone in a basal state could have provided valuable information about the levels of antioxidant proteins in sexually mature abalone. This information would have provided a baseline level allowing comparison of abalone that were subjected to oxidative stress to those that were not and would allow elucidation of the molecular differences and changes to redox status after spawning induction for *H. laevigata*.

5.2 Reproduction

This thesis has demonstrated that another important factor to successfully promote abalone spawning was the degree of sexual maturity. As observed by the relative abundance of reproductive proteins especially those important constituents of abalone eggs (vitellogenin, VERL, VEZP29) and sperm (sperm protein, Sp17, tektin, lysin, ropporin, enkurin), there were indications that gametogenesis was incomplete or arrested in the FSP group. Specifically, it would be expected that releasing or retaining eggs/sperm would lead to a significant difference in abundance of these proteins amongst the different physiological states. As such a higher abundance of proteins that comprise the eggs and/or sperm would be expected in the FSP group that did not release the reproductive material. However, significant differences in abundance for these egg/sperm-related proteins (vitellogenin, lysin, ropporin and enkurin) between the physiological states were not observed. This suggests that the level of reproductive proteins (normalised to total protein content) was lower in the

FSP group supporting the hypothesis that abalone in the FSP group were not ready to spawn.

Promoting gametogenesis is a costly and time consuming task that increases the spawning rates [45], however it requires the abalone to be placed in specific facilities with controlled temperature where gradual gametogenesis will occur. In the farms, abalone undergo gametogenesis in the production tanks only during the natural spawning season. The current method (VGI) to evaluate abalone sexual maturation relies on a quick and non-destructive visual assessment of the gonad where gametogenesis is correlated with the engorgement and swelling of the gonad [46]. However, this technique has deficiencies in enabling the selection of abalone with optimal degree of sexual maturity that in turn would result in efficient spawning. The result of applying VGI is a large number of "possible" spawners that could also perish due to stress by handling, and by thermal and oxidative stress imposed by artificial spawning induction. As a consequence, there is a need to re-evaluate the way gametogenesis is assessed on farms. As such, the proteins identified in this study will provide the opportunity to perform further research directed toward the development of non-invasive biochemical kits that could provide reliable measurements of the sexual maturity of abalone, therefore, increasing the likelihood of abalone spawning.

5.3 Discrepancies between 2D-DIGE versus MRM-MS

Both 2DE and 2D-DIGE have been widely employed in a range of molluscan species, however, MRM-MS is a relatively new approach that has not been often utilised to facilitate investigations into mollusc reproduction.

In this thesis, there were instances where the relative protein expression from the 2D-DIGE approach was not consistent with the relative abundance observed in the

MRM-MS for the peptides of a particular protein. In part, this may be explained by the fact that the DIGE results relied upon the pair-wise comparison of individual abalone. The MRM-MS experiments were conducted on a larger sample size, with a minimum of three abalone assessed, for each physiological state. Based on the findings of this thesis, in particular considering the variation in the stage of sexual maturation, future experiments employing DIGE should utilise pooled samples from a larger number of abalone to ensure that the protein composition is representative of the group. The biological variation (within groups) could then be assessed by MRM-MS which allows a greater number of samples to be processed and analysed. There were limiting factors that contributed to the observed biological variation. Firstly, the limited number of potentially "ripe" abalone available at the time of the experiment and secondly, the reduced number of abalone obtained to constitute the physiological groups. The number of samples in the PSP and FSP groups (four and nine individuals respectively) in the female experiment demonstrated the impact of sample number upon biological variation. In such instance, the PSP group consistently exhibited the high biological variation across the majority of peptides monitored in MRM-MS, whereas the group with lowest variation was the FSP group which had the higher number of samples (Appendix 3-3). As a consequence of the increased biological (intra-group) variation potentially significant differences between the physiological states could have been masked. In order to overcome this, future research should increase the number of abalone in each experiment such that proteins potentially relevant to abalone sexual reproduction and induction of spawning can be clearly identified.

In 2D-DIGE, the proteins are labelled with fluorescent dyes and then resolved on two-dimensional gels. The gels are scanned at the excitation wavelength of each dye to generate three separate gel images for a single protein gel. The fluorescence

intensity is compared between samples and converted to fold-changes in protein abundance for specific gel-spots. Subsequently, the proteins present in the gel spots that show fold-changes above a specified threshold are identified. In this thesis, this threshold was set to a conservative value of ≥ 4, however, there may have been additional relevant proteins excluded by using such a conservative value.

As addressed in chapters 4 and 5, the co-localisation of several proteins in the same gel spot in 2D-DIGE prevented the identification of the precise protein responsible for the differential expression. Proteins for further evaluation by MRM-MS were selected based on the results of the identification wherein those proteins giving the higher number of peptides were presumed to be the major contributor to the fold-change in gel spot intensity. Developing an MRM-MS method for quantification of each of the co-localised proteins would have assisted with the identification of which protein was responsible for the differential expression.

In MRM-MS experiments specific arrangement of the amino acid sequence may lead to incomplete digestion of some of the proteins targeted for MRM-MS. An example of this phenomenon is the presence of "ragged ends" which are adjacent basic residues (KR, KK, RR and RK) within a peptide sequence. Trypsin is commonly utilised as it is a highly specific enzyme cleaving proteins carboxyl-terminal to Arg and Lys residues and yielding peptides that are suitable for downstream MS/MS analysis. However, when ragged ends are present in a protein sequence, trypsin may preferentially cleave only one of the sites leaving terminal basic residues not expected in a fully processed peptide product. Trypsin, while being a highly specific endoprotease, exhibits poor exopeptidase activity.

For this reason, there were noted discrepancies between two peptides of a particular protein. As explained, the presence of ragged ends can generate either semi-tryptic

peptides or fully tryptic peptides that would affect the quantification ratio for two peptides from the same protein. In a similar manner, post-translational modifications (PTMs) to proteins can also affect the quantification ratio by altering the peptide mass (and fragment ion mass) thus making the modified peptide invisible to MRM-MS analysis. We included peptides with expected modifications, such as oxidation of methionine and carbamidomethylation of cysteine, however, without prior knowledge of PTMs, it is difficult to anticipate all forms that are required. Moreover, modification to either lysine or arginine will affect the ability of trypsin to cleave at these residues leading to greater variation peptide and protein quantification.

When employing targeted quantitative proteomics, peptides containing "ragged ends" or those that could be subject to variable modifications should be avoided where possible. In this study, additional peptides for each targeted protein were included despite not having spectral data arising from discovery proteomic experiments. The peptide MRMs were designed hypothetically, but it is hard to judge if a peptide will be produced by trypsin digestion and will subsequently be optimal for downstream MS/MS analysis. In the majority of cases, a minimum of two peptides were successfully detected for each targeted protein.

In automated bottom-up proteomic workflows, protein identification relies on databases comprised of known or predicted proteins. It is common, especially in poorly sequenced genomes, that a protein database may not contain all proteins present in a sample. In these cases the best protein match to the spectral evidence is given. Furthermore, a peptide may map to more than one protein. In the case where a specific protein is targeted for quantification by MRM-MS based on the peptides previously identified, it is important to know if the peptides used are unique to the protein isoforms or are shared/common to multiple protein isoforms. In this thesis each peptide target was subjected to homology searching by BLASTp analysis,

however, the absence of a true protein in the database searched may preclude the determination of the uniqueness of a given peptide. MRM-MS analysis will quantify the total amount of each peptide irrespective of its protein precursor. In the case where two peptides are selected for a given protein, of which one is truly unique and the other is common, a dissimilar pattern of peptide expression will be observed for the same protein.

All of these considerations must be taken into account to efficiently perform protein quantification when a targeted proteomics workflow is employed.

5.4 *Certus mortem (impending death)*

Species preservation is a driving force for any organism. During artificial abalone spawning induction, the changes in temperature and the UV irradiation generate oxidative conditions in the abalone tank. It is possible that the abalone sense the changed environmental conditions and their deleterious effects as an event of certus mortem (impending death) and subsequently release the gametes as a last attempt to preserve the species. There are currently no scientific reports in abalone to support this hypothesis, however, ovo-deposition over impending death can be easily appreciated in some insect species of the order *blattodea* in any ordinary day (personal observations).

5.5 Future directions

The future directions from this study would be to undertake a meticulous evaluation of the molecular mechanisms (aside from the cellular expression of antioxidant proteins) that are activated during oxidative stress and determine possible associations with reproductive pathways and spawning.

Further investigation that includes gonads from sexually immature in different

environmental conditions (stress/no stress) would provide valuable insights into the direct effects of oxidative stress caused by (H_2O_2, UV or temperature increases) on abalone when energy has not been directed into sexual maturation. Additionally, analysis of sexually mature abalone in a basal state (not induced to spawn) will provide a baseline for the endogenous expression of antioxidant proteins and provide the level of egg/sperm-constitutive proteins in what are expected to be sexually mature abalone. This would allow measurement of the response displayed by abalone that are subjected to oxidative and thermal stress under artificial spawning conditions. By including the abalone in the basal state (prior to induction) and by assessing sexually immature abalone, the effect of oxidative/thermal stress on protein expression could be delineated from the changes in protein expression resulting from the onset of reproduction/spawning. Both set of samples would also allow for assessment of the effects of ROS over specific tissues and the molecular consequences within abalone, for example, lipid peroxidation and DNA damage. Lastly, implementing monthly experimentation (stress/no stress) to evaluate expression of antioxidant proteins in juvenile abalone over a period of 16 months until they reach sexual maturity could assist in determining the optimal parameters (H_2O_2, UV or temperature increases) to achieve successful abalone spawning. This approach would be carried out using abalone from the same cohort. Simultaneously, quantitative measurement of proteins involved in sexual maturation can be assessed and correlated with gametogenesis and spawning.

This thesis has also provided the identity of sex-specific proteins with specific roles in sexual maturation and reproduction. This could assist in the development of research focused in determining the relevance of these proteins in specific physiological, developmental stages and reproductive events. For example, Vg and proteins from the vitelline environment (VEZPs) that are constituents of abalone

eggs can be utilised to develop antibody-based assays to evaluate sexual maturation in different cohorts of *H. laevigata*. A similar approach could be employed for constituent proteins of abalone sperm, such as the acrosomal proteins lysin, ropporin, Sp17 and enkurin, whose abundance also could be assessed and associated with efficiencies during the acrosomal reaction and/or fertilisation. Although, this thesis focused only in gonads, there are other abalone tissues including cerebral ganglia and haemolymph that can be subjected to quantitative proteomics or genomic approaches for the identification of neurohormones and neuropeptides related to attracting chemotaxis or reproductive processes such as gametogenesis, mating, spawning and reproduction.

5.6 Cumulative Findings

This thesis has made thorough use of molluscan genomic information available in public databases to identify 232 proteins from both sexes of abalone; 60% more compared to protein identifications resulting from the use of standard (public) protein databases (UniProt and NCBI). This thesis has also determined the identity of reproductive and metabolic proteins differentially expressed in gonads of abalone with different physiological states following artificial spawning induction methods. Valuable information was also obtained regarding the molecular mechanisms occurring in abalone gonads during conditions of thermal and oxidative stress that are imposed upon abalone during artificial spawning stimulation.

In conclusion, the application of proteomics has assisted in elucidating the gonadic proteome in both sexes of abalone providing a foundation for future studies of abalone reproduction. Pair-wise comparison of gonads with unique physiological backgrounds has assisted in identifying proteins with relevance in the sexual maturation of abalone. Similarly, the antioxidant response was assessed in both sexes

of abalone that were subjected to both oxidative and thermal stress during artificial spawning induction.

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7 APPENDIX

Appendix 1-1

Histology depiction of abalone gonad maturation

In *H. midae*, the process of gametogenesis has been observed comprising nine and six stages for female (Figure 1) and male (Figure 2) respectively. In both sexes, sexual maturation (visualisation of all gametogenesis stages) was reached at 24 months; however mature oocytes were not abundant in female.

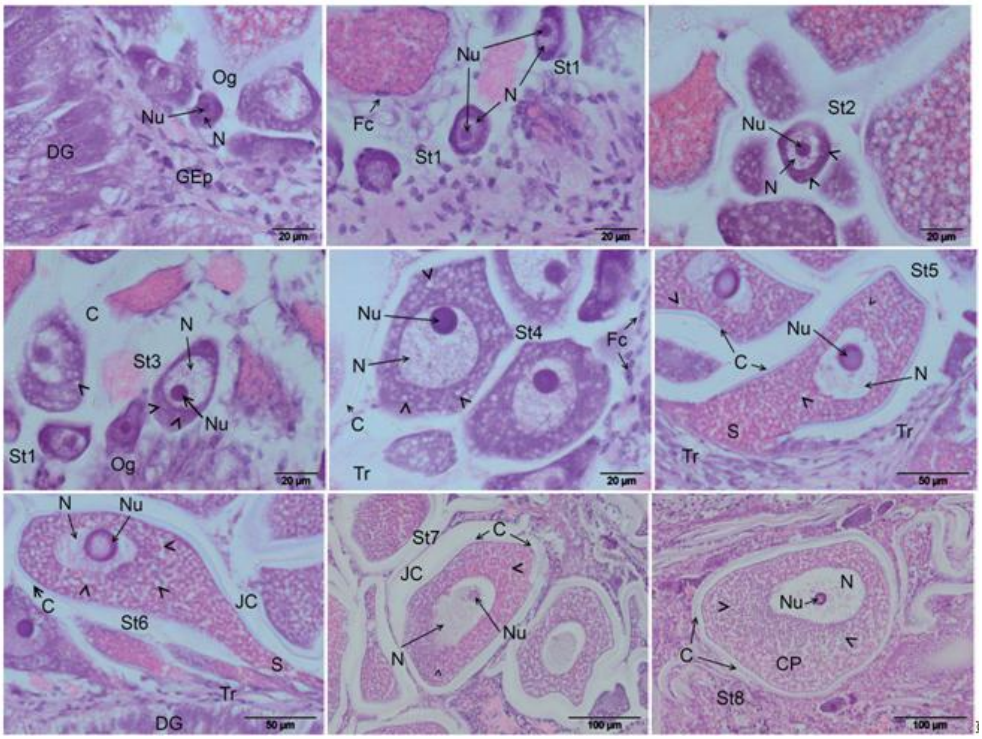


Figure 1. Oogenesis development in *Haliotis midae.*
Histologic micrography showing nine stages of egg development in *H. midae*. DG, digestive gland; GEp, gonad ephitelium; N, nucleus; Nu, nucleolus; Og, oogonia; Fc, follicular cell; C, corion; >, lipid droplets; St1-St8, stages 1 to 8; S, cytoplasmic stalk; T, trabeculae; JC, jelly coat. Microphotos taken from [57].

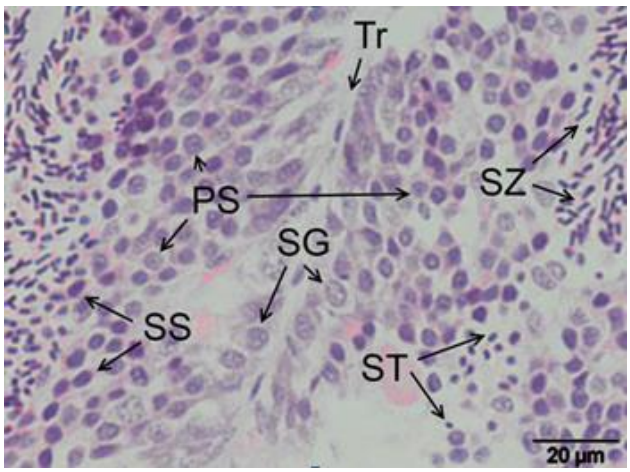
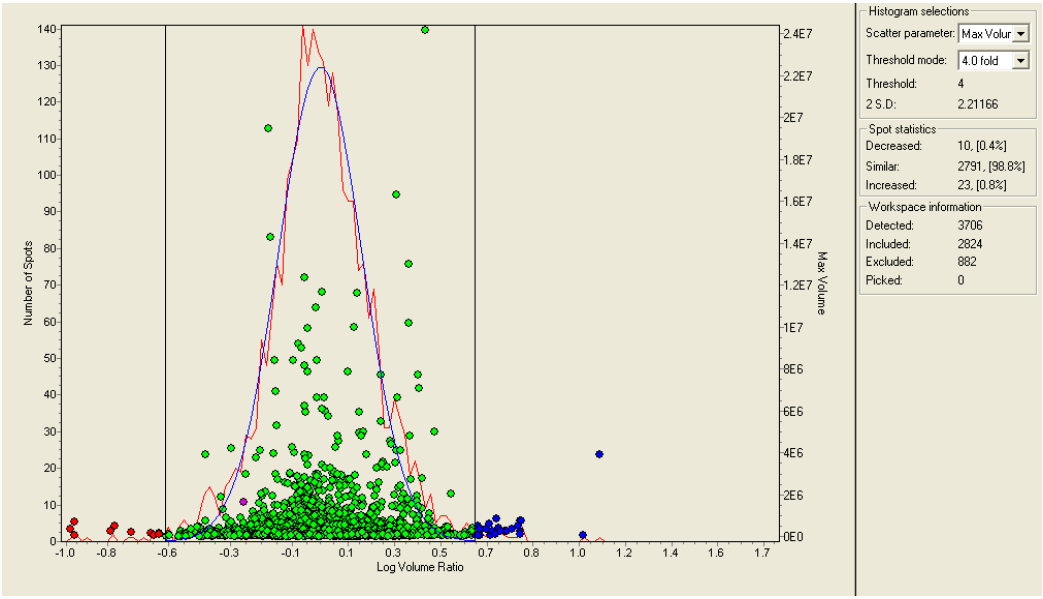


Figure 2. Spermatogenesis in *H. midae*.
Histologic micrography showing six stages of spermatogenesis in *H. midae*. PS, primary spermatocytes; SG, spermatogonia; SS, secondary spermatocytes; ST, spermatide; SZ, spermatozoa; Tr, trabaculae. Microphotos taken from [57].

Appendix 3-1.



Typical histogram of protein distribution in 2D-DIGE experiment. Comparison of proteins from gonads of spawning abalone and post-spawning gonads. Up- and down-regulated proteins are represented by blue and red dots respectively, whereas proteins with no change in expression are indicated in green. Image created in DIA module of DeCyder software 6.5.

Appendix 3-2

Coefficient of variance for technical replicates of each peptide monitored in MRM-MS in gonads of female abalone. Comp Biochem Physiol A Mol Integr Physiol

Protein	Peptide sequence	Coefficient of variance (%)			
		FSP	PSP	SP	Mean
Vitellogenin (Vg)	LAATAAIK	5.9	7.7	7.8	6.8
	VTPALFESR	6.5	14.8	7.2	8.5
Vitelline envelope zona pellucida domain protein 29 (VEZP29)	AGCGDGVVFGQK	6.8	9.7	6.4	7.3
	VVLTASTDGK	8.1	9.0	12.2	9.4
Vitelline envelope receptor of lysin (VERL)	AGCGDGIVFAK	7.2	12.9	8.6	8.8
	NDTVVLYTVTAR	23.4	23.6	27.1	24.5
Prohibitin (PHB)	AFGEAGEGLVELR	7.1	10.3	10.1	8.7
	DLQNVNITLR	9.4	11.6	10.3	10.1
Heat shock cognate protein 70 (HSC70)	VEIIANDQGNR	6.7	9.2	7.8	7.6
	VSIHDIVLVGGSTR	10.1	10.7	12.6	10.9
Heat shock protein 90A (HSP90A)	ADLVNNLGTIAK	7.0	9.2	9.9	8.3
	DSVQNSAFVER	9.1	9.0	11.5	9.8
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AGIALTDNFVK	6.7	9.4	10.5	8.4
	LVSWYDNEYGYSNR	7.0	12.8	9.9	9.1
Peptidyl-prolyl cis-trans isomerase B (PPIB)	FPDENFK	8.8	8.5	16.1	10.8
	HYGAGWLSMANSGK	13.6	15.1	19.6	15.6
ATP synthase subunit beta (ATP synthase)	AHGGYSVFAGVGER	7.2	9.8	11.2	8.9
	FLSQPFQVAEVFTGSEGK	5.9	6.4	7.7	6.5
Ferritin (FER)	IVLQDIK	16.9	16.4	13.8	15.9
Thioredoxin peroxidase 2 (TPx2)	IPLLADK	7.5	6.4	9.3	7.8
	GLFIIDDK	8.4	8.7	8.3	8.5
Peroxiredoxin 6 (Prx6)	AVFIIGPDK	7.6	9.4	8.5	8.3
	VIDSLQLTATK	10.4	12.6	11.6	11.2
Glutathione-S-transferase (GST)	GLAQPIR	7.4	9.9	7.5	8.0
	LTQSNAILR	6.8	8.2	8.9	7.7
Superoxide dismutase (SOD)	NDINTIISLQPALR	7.2	9.2	9.1	8.2
	NVRPDYVK	8.5	16.6	11.0	11.0
Nucleoside diphosphate kinase (NDK)	TFIAVKPDGVQR	7.6	8.4	7.7	7.8
Chaperonin containing tcp1 (CCT)	ALEFLPDLTIGEVK	7.2	10.4	9.4	8.6
	FASSAACTVLK	8.9	11.0	11.2	10.0
Proteasome subunit alpha type 5 (PMSA5)	LFQVEYAIEAIK	7.0	7.8	7.4	7.3
	LGSTAIGIQTGEGVVLAVEK	6.8	8.2	9.5	7.8

Appendix 3-3.

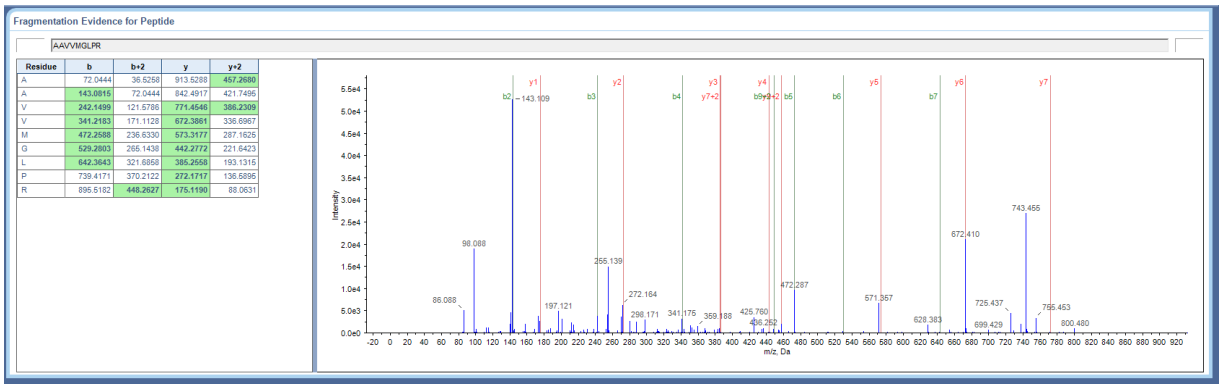
Coefficient of variance for biological variation of each peptide monitored in MRM-MS in gonads of female abalone.

Protein	Peptide sequence	Coefficient of variance (%)			
		FSP	PSP	SP	Mean
Vitellogenin (Vg)	LAATAAIK	14.6	11.3	15.2	13.7
	VTPALFESR	14.2	12.2	14.6	13.7
Vitelline envelope zona pellucida domain protein 29 (VEZP29)	AGCGDGVVFGQK	12.5	7.4	15.0	11.6
	VVLTASTDGK	12.6	31.0	16.8	20.1
Vitelline envelope receptor of lysin (VERL)	AGCGDGIVFAK	29.0	51.4	29.0	36.5
	NDTVVLYTVTAR	64.2	68.2	42.9	58.4
Prohibitin (PHB)	AFGEAGEGLVELR	11.5	21.0	4.2	12.2
	DLQNVNITLR	11.6	27.4	6.3	15.1
Heat shock cognate protein 70 (HSC70)	VEIIANDQGNR	12.7	23.8	25.7	20.8
	VSIHDIVLVGGSTR	8.4	21.1	23.3	17.6
Heat shock protein 90A (HSP90A)	ADLVNNLGTIAK	13.4	21.3	15.9	16.9
	DSVQNSAFVER	10.7	23.4	17.8	17.3
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AGIALTDNFVK	6.2	20.6	18.6	15.1
	LVSWYDNEYGYSNR	5.6	18.7	19.6	14.6
Peptidyl-prolyl cis-trans isomerase B (PPIB)	FPDENFK	12.5	15.8	11.2	13.2
	HYGAGWLSMANSGK	31.8	46.1	51.3	43.0
ATP synthase subunit beta (ATP synthase)	AHGGYSVFAGVGER	13.3	19.3	6.4	13.0
	FLSQPFQVAEVFTGSEGK	14.9	16.9	11.0	14.2
Ferritin (FER)	IVLQDIK	27.5	36.6	65.8	43.3
Thioredoxin peroxidase 2 (TPx2)	IPLLADK	22.4	25.6	23.3	23.7
	GLFIIDDK	29.0	35.3	29.7	31.4
Peroxiredoxin 6 (Prx6)	AVFIIGPDK	14.8	22.7	9.1	15.5
	VIDSLQLTATK	18.5	19.5	15.7	17.9
Glutathione-S-transferase (GST)	GLAQPIR	53.2	49.7	68.8	57.2
	LTQSNAILR	49.6	46.9	66.0	54.2
Superoxide dismutase (SOD)	NDINTIISLQPALR	13.9	15.8	14.1	14.6
	NVRPDYVK	11.3	13.6	12.6	12.5
Nucleoside diphosphate kinase (NDK)	TFIAVKPDGVQR	14.6	12.7	8.5	11.9
Chaperonin containing tcp1 (CCT)	ALEFLPDLTIGEVK	13.1	39.6	16.6	23.1
	FASSAACTVLK	8.5	37.7	18.1	21.4
Proteasome subunit alpha type 5 (PMSA5)	LFQVEYAIEAIK	11.4	29.2	9.7	16.8
	LGSTAIGIQTGEGVVLAVEK	14.0	33.8	8.5	18.8

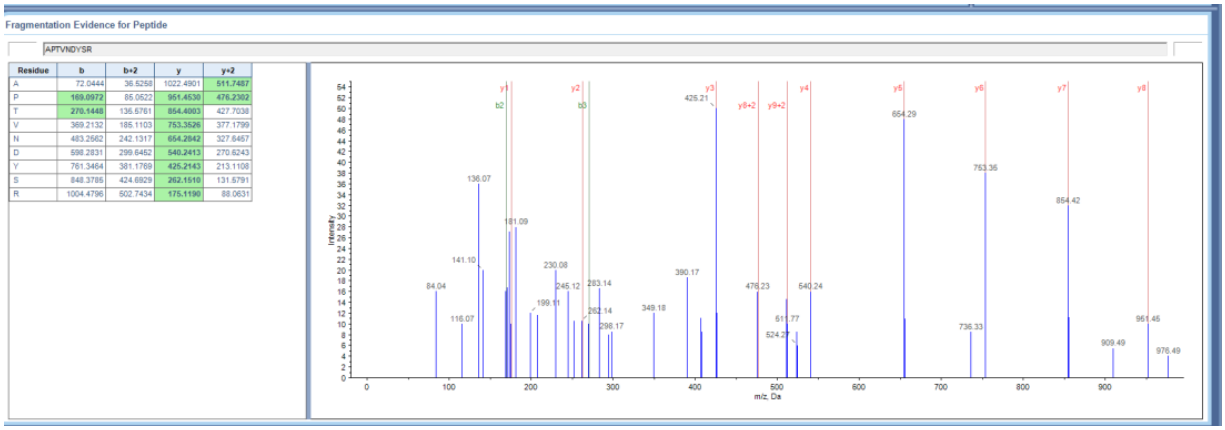
Appendix 4-1.

Peptide-spectrum matches (PSMs) for single-peptide protein identifications in Chapter 5 “Reproductive and metabolic proteins differentially expressed in male Haliotis laevigata after UV and temperature spawning induction”.

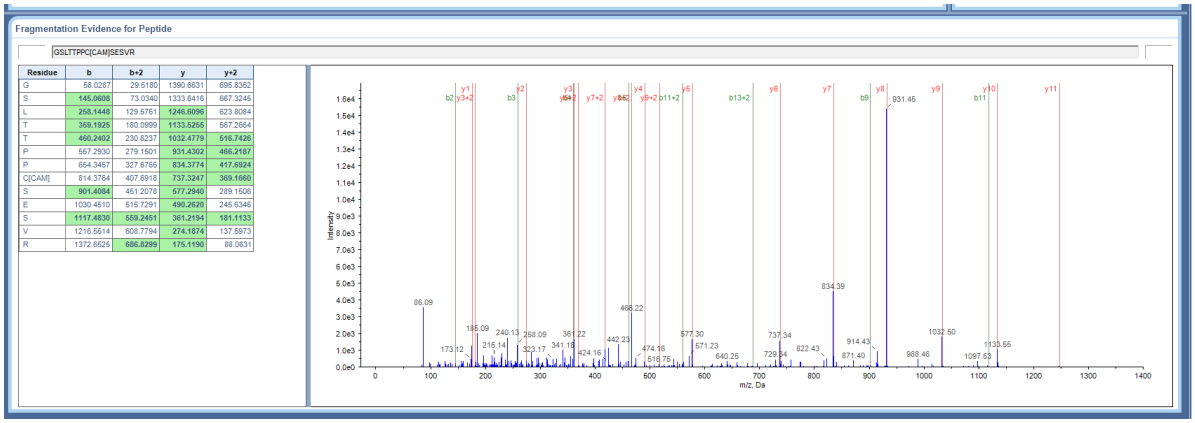
Interferon-induced protein 44-like protein (K1RBB3)



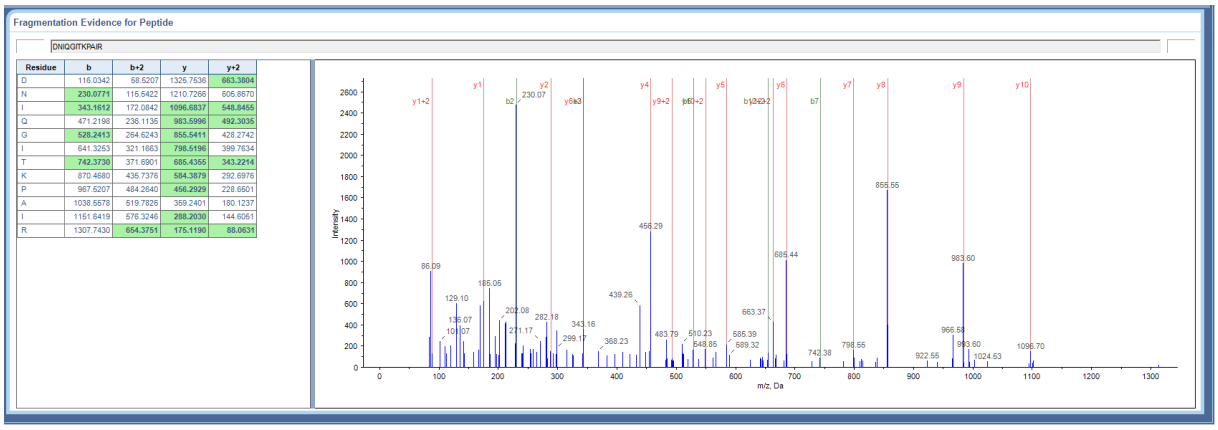
Lysin (Q25078)



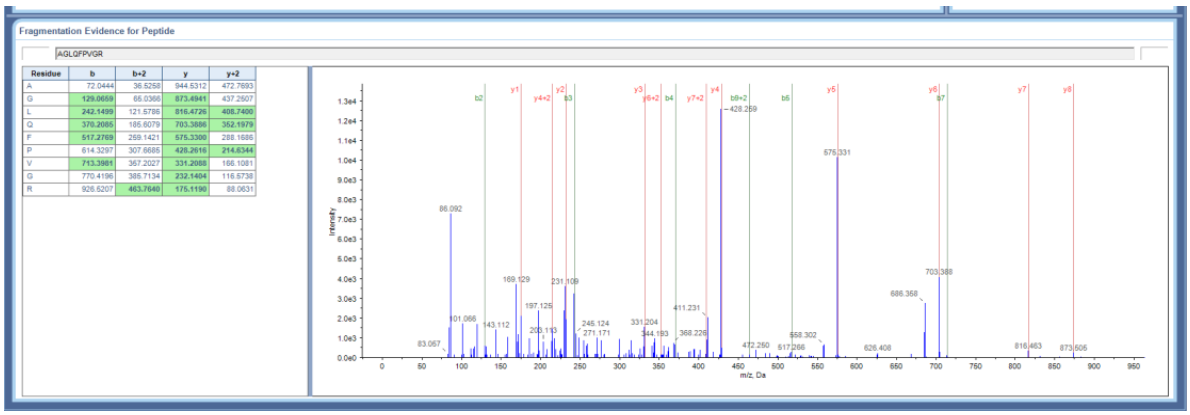
Nacrein-like protein P2-like (gi|524884525)



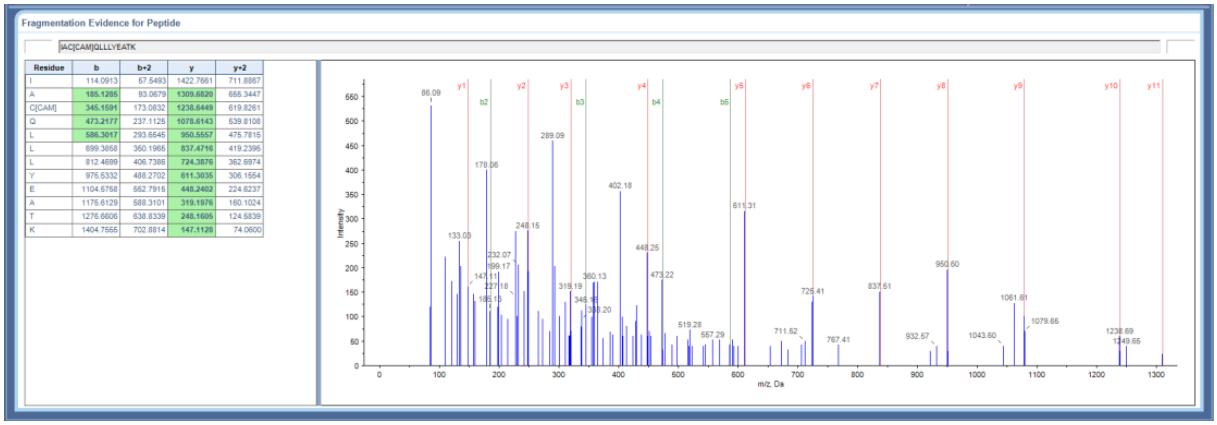
Histone H4 (Fragment) (gi|448646149)



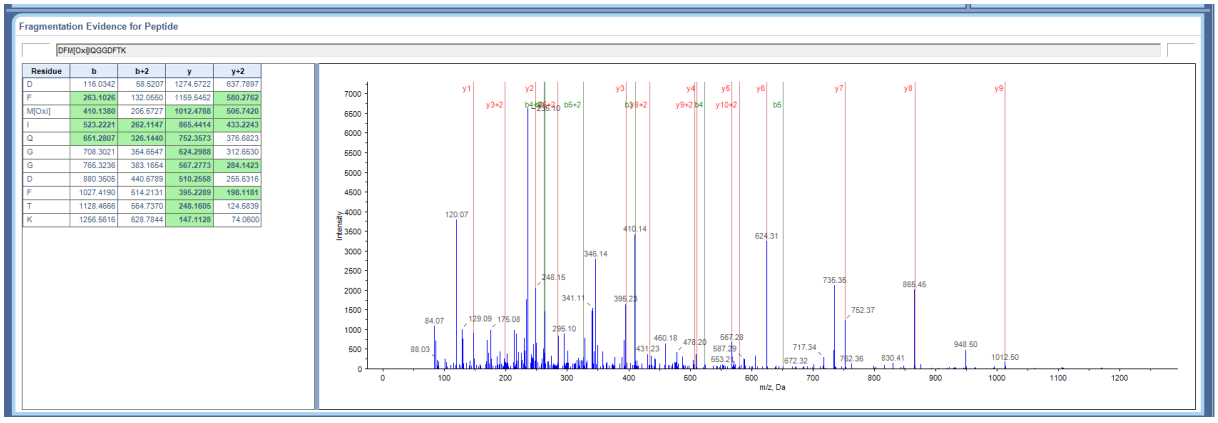
Histone H2A (K1RXS6)



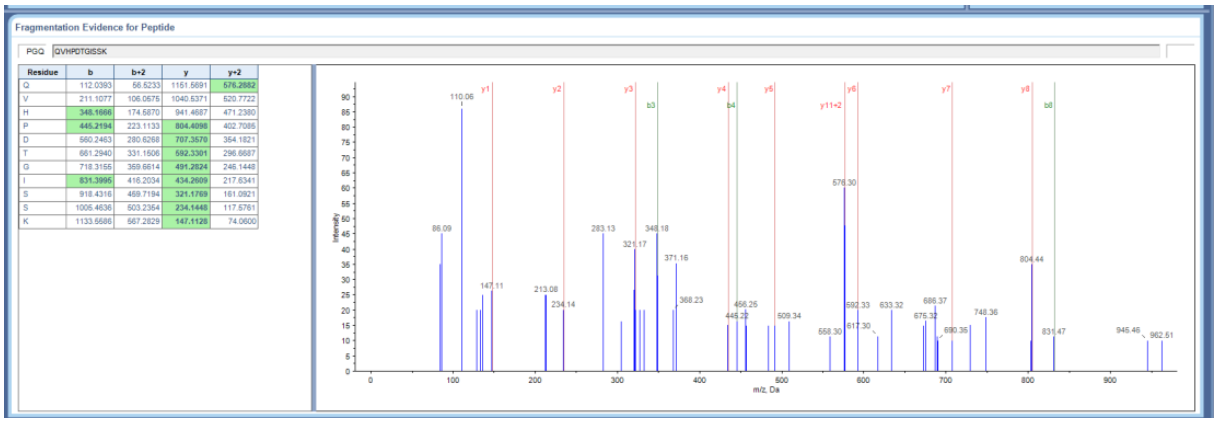
Cellulase (Q65Z49)



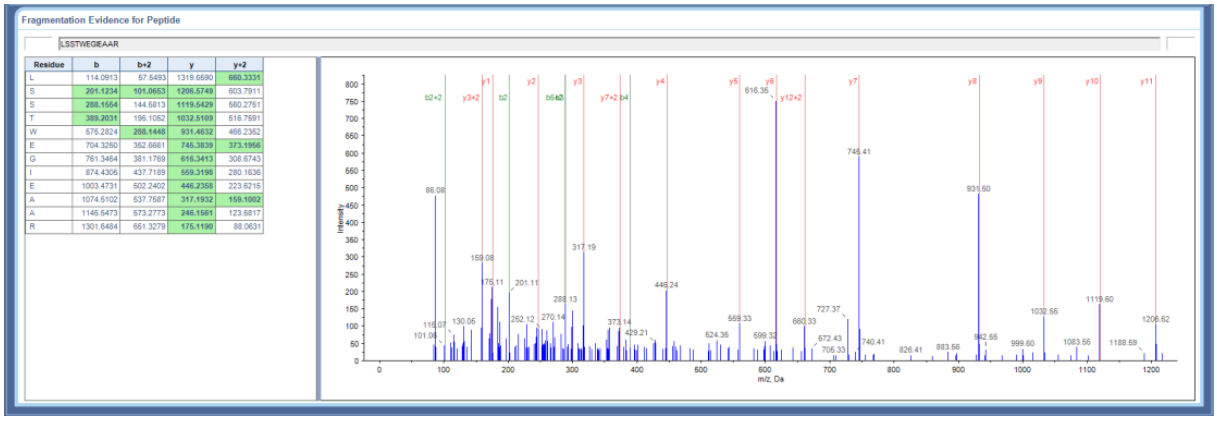
Peptidyl-prolyl cis-trans isomerase B-like (gi|676493403)



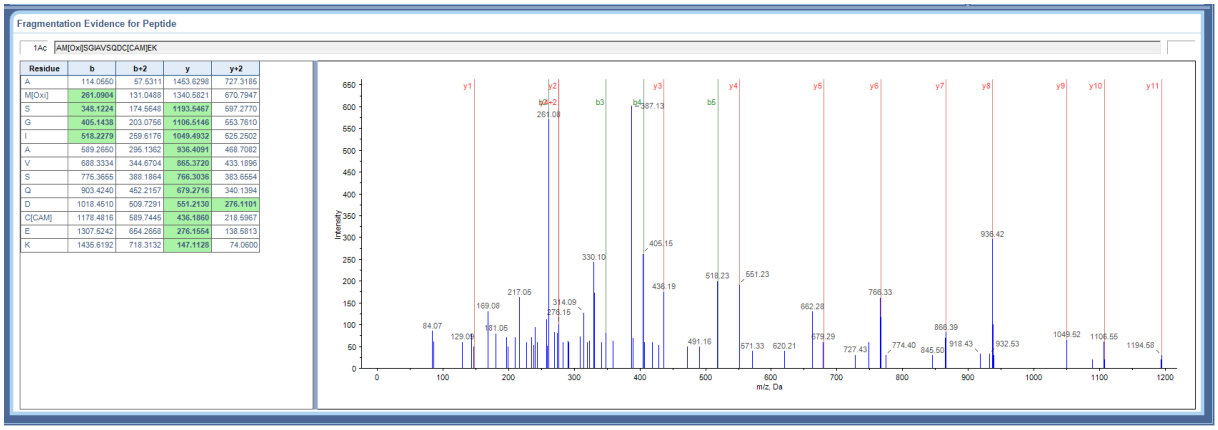
Histone H2B (K1QY71)



Transaldolase (K1QVK0)



Actin depolymerisation factor/cofilin (gi|157072781)



Appendix 4-2.

Details for proteins based of single-peptide identification on gonads of male abalone.

Protein name	Acession	Peptide sequence	Prec MW	Prec m/z	z	Theor MW	Theor m/z	Δmass (ppm)
Interferon-induced protein 44-like protein	K1RBB3	AAVVMGLPR	912.5395	457.277	2	912.5215	457.268	19.73
Lysin	Q25078	APTVNDYSR	1021.4915	511.753	2	1021.4828	511.7487	8.52
Nacrein-like protein P2-like	gi|524884525	GSLTTPPCSESVR	1389.6816	695.8481	2	1389.6559	695.8352	18.49
Histone H4 (Fragment)	gi|448646149	DNIQGITKPAIR	1324.7344	663.3744	2	1324.7463	663.3804	8.98
Histone H2A	K1RXS6	AGLQFPVGR	943.5200	472.7673	2	943.5239	472.7693	4.13
Cellulase	Q65Z49	IACQLLLYEATK	1421.7887	711.9016	2	1421.7588	711.8867	21.03
Peptidyl-prolyl cis-trans isomerase B-like	gi|676493403	DFMIQGGDFTK	1273.5814	637.798	2	1273.5649	637.7897	12.96
Histone H2B	K1QY71	QVHPDTGISSK	1150.5809	576.2977	2	1150.5619	576.2882	16.51
Transaldolase	K1QVK0	LSSTWEGIEAAR	1318.7035	660.359	2	1318.6517	660.3331	39.28
Actin depolymerisation factor/cofilin	gi|157072781	AMSGIAVSQDCEK	1452.6666	727.3406	2	1452.6224	727.3185	30.43

Appendix 4-3

Coefficient of variance for technical replicates of each peptide monitored in MRM-MS in gonads of male abalone.

Protein	Peptide sequence	Coefficient of variance (%)			
		FSP	PSP	SP	Mean
Lysin	DLIAKPVQDIPIR	15.2	32.6	23.9	23.9
	VQIIAGFDR	19.2	43.7	24.4	29.1
Sperm protein	DHNGVIDVDELDR	8.6	9.8	10.7	9.7
	TNLPDQVK	10.8	13.3	12.3	12.1
Tektin-3 (TKT)	VLQEIFDMEK	5.9	6.4	9.4	7.2
	VPPVFAAAR	8.0	8.8	9.3	8.7
Histone (H2B)	AMSIMNSFVNDIFER	14.3	23.6	8.8	15.6
	LLLPGELAK	9.8	10.1	12.5	10.8
Histone (H3)	STELLIR	8.2	7.1	11.3	8.9
	SAPATGGVK	15.1	10.8	16.1	14.0
Histone (H4)	ISGLIYEETR	7.0	6.4	7.9	7.1
	VFLENVIR	7.3	6.6	11.0	8.3
Chaperonin containing tcp1 (CCT)	ALEFLPDLTIGEVK	14.0	19.8	5.0	13.0
	FASSAACTVLK	10.9	13.6	19.8	14.8
Ubiquitin (Ub)	ESTLHLVLR	12.8	6.8	16.1	11.9
	TITLEVEPSDTIENVK	12.3	14.2	14.6	13.7
Sperm surface protein Sp17 (Sp17)	GFQNILEGLAR	5.1	6.2	7.9	6.4
	SDSQLPSEK	7.1	7.2	10.7	8.3
Atrial natriuretic peptide receptor A (ANF)	YCLFGDTVNTASR	7.2	6.4	9.6	7.7
	YSSNLEAIVSDR	5.6	6.1	7.9	6.6
Heat shock cognate protein 70 (HSC70)	VEIIANDQGNR	13.2	12.8	9.4	11.8
	VSIHDIVLVGGSTR	12.7	14.3	19.1	15.4
Heat shock protein 90A (HSP90A)	DSVQNSAFVER	13.1	14.1	10.3	12.5
	GVVDSEDLPLNISR	12.8	15.4	7.9	12.0
Superoxide dismutase (SOD)	NDINTIISLQPALR	10.3	16.2	7.0	11.1
	HHNAYVTNLNVAQEK	12.6	16.7	8.5	12.6
Glutathione-S-transferase (GST)	GLAQPIR	14.1	12.8	12.3	13.1
	KPTDPLQEGLELR	11.5	13.3	11.7	12.1
Ferritin (FER)	IVLQDIK	25.0	16.9	15.7	19.2
	VGPGLGEYMFDK	13.2	24.1	9.1	15.4
Thioredoxin peroxidase 2 (TPx2)	IPLLADK	13.5	11.2	12.6	12.4
	GLFIIDDK	12.9	11.7	12.1	12.2
Nucleoside diphosphate kinase (NDK)	GDFCIDVGR	12.2	16.4	14.7	14.4
	YMASGPVVAMVWEGK	10.9	16.6	13.2	13.6
ATP synthase subunit alpha (ATP synthase)	EAYPGDVFYLHSR	11.3	14.1	16.5	14.0
	NIQAEEMVEFSSGLK	16.0	22.2	17.4	18.5

Tetratricopeptide repeat protein 25 (TTCP25)	LRPELQEFR	10.6	15.6	10.3	12.1
	ETDPNTPVGK	15.0	7.6	18.9	13.8
Voltage-dependent anion channel 2-like (VDAC2)	LTVSSLIEGK	6.9	7.6	11.0	8.5
	APPGYSDLGK	8.5	9.8	14.6	10.9
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AGIALTDNFVK	12.3	13.1	12.2	12.6
	LVSWYDNEYGYSNR	13.8	14.2	8.1	12.0
Peroxiredoxin 6 (Prx6)	AVFIIGPDK	13.9	14.0	13.0	13.6
	VIDSLQLTATK	14.3	12.5	14.3	13.7
Peptidyl-prolyl cis-trans isomerase B (PPIB)	DFMIQGGDFTR	8.2	18.6	13.9	13.6
	FPDENFK	12.0	14.7	10.1	12.3
Enkurin (ENK)	VPVPTTDNFLK	6.9	10.8	10.1	9.3
	DDKPLMGLK	10.2	8.5	7.6	8.8
Ropporin (ROP)	DVLAWSAAYFR	11.1	12.9	27.3	17.1
	IPFPLFK	9.5	9.4	16.8	11.9

Appendix 4-4

Coefficient of variance for biological variation of each peptide monitored in MRM-MS in gonads of male abalone.

Protein	Peptide sequence	Coefficient of variance (%)			
		FSP	PSP	SP	Mean
Lysin	DLIAKPVQDIPIR	45.4	44.8	83.5	57.9
	VQIIAGFDR	35.5	44.9	81.5	53.9
Sperm protein	DHNGVIDVDELDR	14.5	9.9	5.2	9.9
	TNLPDQVK	16.1	11.8	13.7	13.9
Tektin-3 (TKT)	VLQEIFDMEK	49.2	7.9	78.2	45.1
	VPPVFAAAR	20.2	13.1	9.0	14.1
Histone (H2B)	AMSIMNSFVNDIFER	55.4	17.1	28.1	33.5
	LLLPGELAK	41.2	21.5	65.0	42.6
Histone (H3)	STELLIR	69.8	41.7	62.0	57.8
	SAPATGGVK	74.7	39.9	65.5	60.0
Histone (H4)	ISGLIYEETR	61.3	38.5	59.9	53.2
	VFLENVIR	70.3	39.0	63.0	57.4
Chaperonin containing tcp1 (CCT)	ALEFLPDLTIGEVK	35.5	13.7	49.4	32.9
	FASSAACTVLK	46.0	18.4	74.4	46.3
Ubiquitin (Ub)	ESTLHLVLR	44.5	20.0	19.6	28.0
	TITLEVEPSDTIENVK	34.0	22.2	7.6	21.2
Sperm surface protein Sp17 (Sp17)	GFQNILEGLAR	14.7	16.3	13.9	15.0
	SDSQLPSEK	66.4	34.9	65.6	55.6
Atrial natriuretic peptide receptor A (ANF)	YCLFGDTVNTASR	15.6	11.7	8.6	12.0
	YSSNLEAIVSDR	15.2	8.5	10.5	11.4
Heat shock cognate protein 70 (HSC70)	VEIIANDQGNR	35.5	14.9	45.1	31.8
	VSIHDIVLVGGSTR	38.1	16.9	44.7	33.2
Heat shock protein 90A (HSP90A)	DSVQNSAFVER	45.5	20.2	68.0	44.6
	GVVDSEDLPLNISR	37.9	19.1	60.4	39.1
Superoxide dismutase (SOD)	NDINTIISLQPALR	16.2	8.7	13.2	12.7
	HHNAYVTNLNVAQEK	10.7	22.2	6.1	13.0
Glutathione-S-transferase (GST)	GLAQPIR	20.9	29.5	6.8	19.0
	KPTDPLQEGLELR	10.3	9.3	6.2	8.6
Ferritin (FER)	IVLQDIK	30.0	19.3	23.0	24.1
	VGPGLGEYMFDK	18.3	5.3	2.0	8.6
Thioredoxin peroxidase 2 (TPx2)	IPLLADK	23.5	4.6	14.9	14.4
	GLFIIDDK	22.7	7.1	36.8	22.2
Nucleoside diphosphate kinase (NDK)	GDFCIDVGR	22.4	2.2	13.1	12.6
	YMASGPVVAMVWEGK	20.4	1.5	21.0	14.3
ATP synthase subunit alpha (ATP synthase)	EAYPGDVFYLHSR	15.1	14.1	10.1	13.1
	NIQAEEMVEFSSGLK	22.6	14.5	18.6	18.6

Tetratricopeptide repeat protein 25 (TTCP25)	LRPELQEFR	9.9	13.2	11.8	11.6
	ETDPNTPVGK	49.3	27.0	16.1	30.8
Voltage-dependent anion channel 2-like (VDAC2)	LTVSSLIEGK	5.8	8.6	13.4	9.3
	APPGYSDLGK	4.5	8.7	7.8	7.0
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AGIALTDNFVK	37.9	17.4	35.4	30.2
	LVSWYDNEYGYSNR	29.2	7.4	28.1	21.6
Peroxiredoxin 6 (Prx6)	AVFIIGPDK	18.9	23.0	5.2	15.7
	VIDSLQLTATK	16.2	21.4	12.3	16.6
Peptidyl-prolyl cis-trans isomerase B (PPIB)	DFMIQGGDFTR	21.3	24.0	18.8	21.4
	FPDENFK	16.6	20.3	4.9	13.9
Enkurin (ENK)	VPVPTTDNFLK	2.7	15.9	13.1	10.6
	DDKPLMGLK	6.5	9.1	4.2	6.6
Ropporin (ROP)	DVLAWSAAYFR	6.7	19.6	21.5	15.9
	IPFPLFK	6.7	14.0	22.3	14.3