DEVELOPMENT OF DYSTROPHIN-BASED BIOMARKER FOR PENAEID SHRIMPS

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UNIVERSITY OF MALAYA
KUALA LUMPUR

2019

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DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2019

UNIVERSITY OF MALAYA

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Matric No: **SGF150020**

Name of Degree: **MASTER OF BIOTECHNOLOGY**

Title of Dissertation: **DEVELOPMENT OF DYSTROPHIN-BASED BIOMARKER FOR PENAEID SHRIMPS**

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DEVELOPMENT OF DYSTROPHIN-BASED BIOMARKER FOR PENAEID SHRIMPS

ABSTRACT

The shrimp aquaculture industry is constantly hounded by diseases, which cause an astounding loss of crops and money. Lack of early disease detection and diagnostic methods, both in the past and currently, for emerging diseases, contributes greatly to this. Great strides have, however, been made in developing detection and diagnostic methods for the oldest and most common shrimp viruses, which include White Spot Syndrome Virus (WSSV). DNA-based methods are commonly used for detection, diagnosis and surveillance, these days. The dystrophin gene is a muscle gene which plays a crucial role in maintaining muscle rigidity. Mutations in this gene give rise to dystrophies which are characterized by muscle wasting, and are, eventually, fatal. Dystrophin exists in all vertebrates and has been found and characterized in only a few invertebrates. It has been characterized in two shrimp species, *Macrobrachium rosenbergii* and *Penaeus monodon*. In one of those studies, its expression was found to be affected in WSSV infected *M. rosenbergii*. This study sought to identify the dystrophin gene in *Litopenaeus vannamei* and then, design a biomarker for disease detection, based on the dystrophin gene. Total RNA was extracted from the muscles of healthy *L. vannamei* and then, reverse transcribed to cDNA which was then used for PCR, using primers designed from dystrophin sequences of different organisms. The biomarker was then designed from a conserved region of the gene, and was used in quantitative real-time PCR, to quantify the expression of the gene in WSSV and AHPND- challenged *P. monodon*. The expression of dystrophin was found to be altered in the challenged shrimps, following different patterns for both infections. This finding makes the dystrophin gene a suitable diagnostic biomarker and a possible predictive biomarker for penaeid shrimps.

Keywords: Dystrophin; Penaeid shrimps; Biomarker, WSSV; AHPND

PEMBANGUNAN PENANDA BIOLOGI BERASASKAN DISTROFIN BAGI UDANG *PENAEID*

ABSTRAK

Industri akuakultur udang selalunya diancam dengan penyakit yang menyebabkan kerugian yang sangat besar dalam pengeluaran produk tersebut. Hal ini terus melarat disebabkan kelewatan dalam mengesan penyakit jangkitan udang pada peringkat awal melalui kaedah diagnostik pada masa lalu sehingga kini. Pelbagai usaha dalam mengesan penyakit jangkitan udang melalui kaedah diagnostik telah dilakukan untuk mengenal pasti virus yang kebiasaannya menyerang populasi udang, termasuk *White Spot Syndrome Virus (WSSV)*. Kaedah menggunakan *DNA* sering kali digunakan pada masa kini dalam mengesan, mendiagnosis, dan mengawas penyakit jangkitan udang. Gen *dystrophin* merupakan suatu gen otot yang memainkan peranan penting dalam mengekalkan keutuhan otot. Mutasi dalam gen ini menyebabkan penyakit *dystrophies* yang digambarkan melalui penyusutan otot dan seterusnya mengakibatkan kematian. Gen *dystophin* terdapat dalam semua vertebrat dan juga dijumpai dalam sebilangan kecil invertebrat. Setakat ini, gen tersebut telah dikenal pasti dalam dua spesies udang, iaitu *Macrobrachium rosenbergii* dan *Penaues monodon*. Melalui kajian terdahulu ungkapan gen *dystrophin* telah terjejas semasa serangan *WSSV* dalam *M. rosenbergii*. Kajian yang dijalankan kini telah menemui sebahagian gen *dystrophin* dalam udag putih, *Litopenaeus vannamei*. Jujukan gen tersebut sangat terpelihara di kalangan spesies vertebrat dan invertebrat. Pencetus telah direka berdasarkan jujkan tersebut dan telah diguna pakai dalam Reaksi Polimerase Berantai Kuantitatif (*qPCR*) untuk menghitung ungkapan gen dalam udang sihat dan udang yang terjangkit. Ungkapan gen tersebut telah terjejas dalam udang yang terjangkit seperti yang dijangka. Hasil penyelidikan ini menunjukkan gen distrofin sesuai dijadikan penanda

biologi diagnostik dan berkemungkinan sebagai penanda biologi ramalan untuk udang penaeid.

Kata kunci: Gen *dystrophin*; Udang *penaeid*; Penanda biologi; WSSV; AHPND

ACKNOWLEDGEMENTS

My utmost gratitude goes to Allah for making all this possible and for keeping me. I would also like to express my gratitude to my parents, who sponsored me and have always been very supportive. To my wonderful supervisor, Associate Prof. Dr. Subha Bhassu, I say a very big Thank You; thank you for your guidance.

I would also like to thank my colleagues in AGAGEL laboratory for being amazing and supportive. I appreciate all my friends, home and abroad, who have been supportive throughout this journey. To my special man, Ilyas, thank you for being consistent and highly supportive and always ready to help. Thank you for being my rock.

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LIST OF SYMBOLS AND ABBREVIATIONS

α	:	Alpha
β	:	Beta
γ	:	Gamma
µl	:	Microliter
AHPND	:	Acute Hepatopancreatic Necrosis Disease
BLAST	:	Basic Local Alignment Search Tool
BMD	:	Becker Muscular Dystrophy
BMN	:	Baculoviral Mid-gut gland Necrosis
BP	:	Baculovirus penaei
bp	:	Base pairs
C. elegans	:	*Caenorhabditis elegans*
CDD	:	Conserved Domain Database
cDNA	:	Complimentary Deoxyribonucleic Acid
CT	:	Threshold Cycle
D. melanogaster	:	*Drosophila melanogaster*
DMD	:	Duchenne Muscular Dystrophy
DNA	:	Deoxyribonucleic Acid
DPC	:	Dystrophin-Associated Protein Complex
EHP	:	Enterocytozoon hepatorenal
ELF	:	Elongation Factor
EMS	:	Early Mortality Syndrome
F. chinensis	:	*Fenneropenaeus chinensis*
g	:	Gram
GC	:	Guanine Cytosine
HPV	:	Hepatopancreatic Parvovirus

hr	:	Hour
ICES	:	The International Council for the Exploration of the Sea
IHHNV	:	Infectious Hypodermal and Hematopoietic Necrosis Virus
IMNV	:	Infectious Myonecrosis Virus
kb	:	Kilobases
kDa	:	Kilodalton
L. vannamei	:	*Litopenaeus vannamei*
LvDys	:	*Litopenaeus vannamei* dystrophin
M. rosenbergii	:	*Macrobrachium rosenbergii*
MBV	:	Monodon Baculovirus
MEGA	:	Molecular Evolutionary Genetics Analysis
mg	:	Milligram
min	:	Minute
mL	:	Millilitre
mLAMP	:	Multiplex Loop- mediated isothermal Amplification
mRNA	:	Messenger Ribonucleic Acid
MSA	:	Multiple Sequence Alignment
NCBI	:	National Centre for Biotechnology Information
NHP	:	Necrotizing Hepatopancreatitis
OIE	:	Office International des Epizooties
P. chinensis	:	*Penaeus chinensis*
P. monodon	:	*Penaeus monodon*
P. stylirostris	:	*Penaeus stylirostris*
PCR	:	Polymerase Chain Reaction
*Pemo*NPV	:	*Penaeus monodon* Nucleopolyhedrovirus
*Pm*DNV	:	*Penaeus monodon* Densovirus

*Pm*Dys	:	*Penaeus monodon* dystrophin
*Pv*NV	:	*Penaeus vannamei* nodavirus
qPCR	:	Quantitative Polymerase Chain Reaction
RDS	:	Runt Deformity Syndrome
RNA	:	Ribonucleic Acid
RT-PCR	:	Reverse Transcription Polymerase Chain Reaction
sec	:	second
SPF	:	Specific Pathogen-free
TEM	:	Transmission Electron Microscope
TSV	:	Taura Syndrome Virus
USMSFP	:	U.S. Marine Shrimp Farming Program
V. parahaemolyticus	:	*Vibrio parahaemolyticus*
WSD	:	White Spot Disease
WSSV	:	White Spot Syndrome Virus
YHV	:	Yellow Head Virus

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

The shrimp aquaculture industry is a very lucrative one, providing food and employment for a huge percentage of the human population, especially in Asia. A report showed that the Far East (East Asia and Southeast Asia), including India and the rest of Asia, have the highest aquaculture production. The shrimp industry also provides the highest employment (both direct and indirect) rate in aquaculture, accounting for 92% of total employment and 91% percent of total production in aquaculture, worldwide (Valderrama *et al.*, 2010).

Shrimp aquaculture started as an industrial activity in the 1970s; it quickly developed, and there was an increase in the number of farms and hatcheries. Farmed shrimps were contributing about 30% to the total quantity of shrimps supplied worldwide. By the middle of the 1970s, hatcheries were providing large amounts of post-larvae shrimp. Global production of cultured shrimp started to rapidly increase, and reached around 22,600 metric tonnes in 1975 (Briggs *et al.*, 2004). At that time, Thailand's *Penaeus monodon* industry was just starting, and the Taiwan Province of China and Mainland China were semi-intensively producing *Penaeus chinensis*. Over the next decade, production grew, to 200,000 metric tonnes; and was exceeding 560,000 metric tonnes by 1988. Seventy-five percent (75%) of this was from Southeast and Eastern Asia. In 2009, farmed shrimp produced globally weighed about 3.5 million metric tons with an estimated value greater than USD$14.6 billion (Moss *et al.*, 2012). However, there was a sudden, unfortunate crash in production, due to the emergence of viral and bacterial diseases. Some of these diseases include Yellow Head Virus (YHV) disease, Taura Syndrome Virus (TSV) disease and White Spot Syndrome Virus (WSSV) disease. Production dropped drastically from 207,000 metric tonnes in 1992, to 64,000 metric tonnes in 1993-1994, an aftermath of WSSV infection, in Mainland China. Similar problems were observed in the Philippines, Indonesia, and Thailand (Briggs *et al.*, 2004).

It was clear that a disease-resistant species of shrimps was needed if sales were to be boosted again, and the industry was to be saved. This was when an alien shrimp species, known as *Litopenaus vannamei,* which is non-native in Asia, was introduced. *Litopenaeus vannamei* was introduced into the Asian aquaculture industry because it is believed to be resistant to a wide range of pathogens. It is also very easy to culture and can grow in diverse environmental conditions. Sure enough, profit and production increased exponentially. However, they eventually got infected by some of these pathogens and this crashed production and led to losses in billions of dollars. This was referred to as the "boom and bust" phenomenon.

Another concern when it comes to shrimp diseases is food biosecurity and food safety. Diseases pose a problem to food production and food security in countries where aquaculture products are a key source of dietary protein. Therefore, to maintain the sustainability of aquaculture and ensure food security, it is important to be able to diagnose diseases early and prevent their spread.

The product of the dystrophin gene is a protein which is found on the cytoplasmic surface of the cell membranes of skeletal muscle; it binds to the sarcolemma to protect the muscle from contraction-induced injury (Petrof *et al.*, 1993). It also targets other proteins to the sarcolemma (Thomas, 2013). It is the biggest gene complex in humans. Mutations in the dystrophin gene or loss of the gene cause several types of dystrophies which are characterized by muscle wasting and loss of rigidity in the muscle.

The dystrophin gene has been established to exist in vertebrates, but the same has not been done for invertebrates. Dystrophin homologues have been identified in the round worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and in the sea urchin. There was no report of the dystrophin gene in crustaceans, until recently, when a portion of a dystrophin-like gene was identified in *Macrobrachium rosenbergii* and

Penaeus monodon (Noor *et al.*, 2017). It has been shown that the expression of the gene was found to be altered during WSSV infection.

There are cases of asymptomatic infections where the shrimp appear to be healthy; hence, they are not tested for infections until it is too late, and mortality has occurred. This raised the idea and hypothesis that dystrophin is present in other crustaceans, and the question whether its expression is always affected during an infection. This especially, since the muscle is essentially the majority of the shrimp body, and it is what would be affected when a disease results in stunted growth. Thus, this study hypothesized that the dystrophin gene contains a conserved region which can be used as a biomarker for disease detection.

To answer the above question, this study identified and characterized a portion of the dystrophin gene in another very popular shrimp, *Litopenaeus vannamei* and compared it with dystrophin sequences from many other species, both vertebrates and invertebrates alike. A conserved region was then identified across all the species, and primers were designed based on this region, to be used to monitor real-time expression of dystrophin, both in healthy and infected shrimps; for comparison. The region from which the primers were designed can be used as a biomarker in disease diagnosis. This would help in the early detection of diseases and also in propagating Specific Pathogen-free (SPF) broodstock.

Objectives of the Study

1. To identify the dystrophin gene in *Litopenaeus vannamei*.
2. To establish a biomarker for penaeid disease detection, based on the dystrophin gene.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to *Litopenaeus vannamei*

Litopenaeus vannamei, known also, as the Pacific white shrimp or white leg shrimp, is native to the western Pacific coast of Latin America, from Peru to Mexico. It was experimentally introduced into Asia from 1978 to 1979, but only commercially introduced into Taiwan and China, since 1996 (Briggs *et al.*, 2004). It was introduced afterwards, to several southeast and south Asian countries. *L. vannamei* made up 67% of the 2008 production of cultured penaeid shrimp worldwide. This was attributed to an exponential increase in production in Asia; Asia alone accounted for 82% of the total world production of *L. vannamei* (Liao & Chien, 2011). The introduction of *L. vannamei* was commercially successful because of its superior aquaculture traits, in comparison with the widely cultured Asian penaeid, *Penaeus monodon*.

These traits include high larval survival, higher growth rate and lower dietary protein requirement. Most important of the traits is its lower susceptibility to grave viral pathogens which infect *P. monodon* (Liao & Chien, 2011). Also, closing of the life cycle of *L. vannamei* is easy, for the production of broodstock in culture ponds. This eliminates the need to return to the wild for broodstock or postlarvae stock and allows for domestication and genetic selection for preferred traits.

L. vannamei has the potential to grow rapidly, up to 20 g, at 3g per week, under intensive culture conditions. They are compliant to culture at very high stocking densities, going up to 150/m^2 in pond culture. They even go as high as 400/m^2 in controlled recirculated tank culture. However, intensive culture systems such as these, do require a lot more control over environmental parameters. On the plus side, however, it allows for the production of large quantities of shrimp in limited areas. This results in more output per unit area, than that which was achievable with *P. monodon* in Asia.

L. vannamei is also easier to culture because of its low protein requirement, as opposed to *P. monodon* and *P. stylirostris* (earlier cultured penaeid shrimps). They require 20-35% less protein feed than *P. monodon*, and 36-42% less than *P. stylirostris*, which can also be aggressive, and may demand higher water quality, making them difficult to culture as intensively as *L. vannamei*. In addition to all this, *L. vannamei* is tolerant of a wider range of salinities, ranging from 0.5- 45ppt. Low-temperature tolerance is another characteristic of *L. vannamei* that give them higher preference above other (penaeid) shrimp. They are tolerant to temperatures as low as 15ºC, permitting them to be cultured in cold seasons. Their larvae also have a higher survival rate of about 50 to 60%, hatcheries, compared to *P.monodon* with 20 to 30%.

Harvesting *L. vannamei* is very easy. They tend to stay more within the water column, and not burrow to the bottom, and this makes it possible to harvest them without completely draining the pond. This technique helps to avoid the stirring up of bottom sediments of poor quality. Harvesting using this non-draining method also provides an opportunity to avoid discharging harvesting effluent that is high in organic matter and nutrients, which is important, as a discharge of waste from aquaculture farms has been a big issue. They also have a high meat yield, at 66 to 68%, compared to *P. monodon* with 62% (Briggs *et al.*, 2004).

Also contributing to the large production of *L. vannamei* and the industry's explosive growth is the adoption of the Specific Pathogen-Free (SPF) concept to the domestication of *L. vannamei*. The use of SPF *L. vannamei* has given rise to improved survival, less disease and crop predictability, in almost all the places that were previously dominated by *P. monodon* and *P. chinensis* (FAO, 2006). The development of SPF stocks of *L. vannamei* and other penaeid species has become vital to the sustainability of modern shrimp farming.

Starting from 1999, significant amounts of SPF *L. vannamei* were introduced into East Asia and were found to perform well. By 2006, almost half of the shrimp supply in the world came from marine penaeid shrimps that were being produced from farms. These shrimps were nearly 3 million metric tonnes, of which about 57% was *L. vannamei*. Oddly enough, more *L. vannamei* was produced in 2006, in Asia than in the Americas where it is actually native. In FAO's 2006 publication, "State of world Aquaculture", this paradigm shift in shrimp farming was credited to the development and export of SPF *L. vannamei* (FAO, 2006).

The culture of *L. vannamei* generally produces more profit, as it is seen as luxury food, being a marine shrimp. Studies have shown that white shrimp such as *L. vannamei* and *P. stylirostris* are the preferred shrimp for consumption in the USA, and the USA consumers also prefer the taste of *L. vannamei*, particularly those cultured in freshwater (Liao & Chien, 2011). In Mainland China and Taiwan Province of China, there is also a high demand for *L. vannamei*. Approximately, 75% of the production in Mainland China is sold locally, while 100% of the production in Taiwan Province of China is sold locally.

L. vannamei, however, served as a carrier of various viral pathogens that were new to Asia, and this led to further losses. Something had to be done to expel these pathogens. Strategies had to be devised to ensure that seed stock was free from pathogens and safe to be cultured. The concept of propagating Specific Pathogen-Free stocks was birthed, and rapid and sensitive detection methods were developed and employed in order to screen stock and culture only certified pathogen-free ones.

2.2 The Dystrophin gene

The dystrophin gene codes for the protein, dystrophin, which plays an important role in maintaining muscle rigidity. It is a sub-sarcolemmal structural protein that

provides a link between the actin cytoskeleton and a complex of proteins linked to the extracellular matrix (Wilton *et al.*, 2014). It is a rod-shaped protein which is 2.6 mb long in sequence. The exons, however, only cover 14 kb. These code for 3685 amino acids. The amino acids of dystrophin have been divided into four domains. The first is the 240-amino acid N-terminal domain which has been shown to be conserved, together with the actin-binding domain of α-actinin. The second domain, which is the central domain, is predicted to be rod-shaped; it is formed by the succession of 25 triple helical segments which are similar to the spectrin repeat domains. This segment is then followed by a cysteine-rich domain which has parts that are similar to the entire COOH domain of the Dictyostelium α-actinin. The last domain of dystrophin is a 420-amino acid C-terminal domain which did not show any similarity to other previously reported proteins (Koenig *et al.*, 1988). Figure 2.2 below shows a schematic representation of the organization of the human dystrophin gene, and the dystrophin-associated protein family.

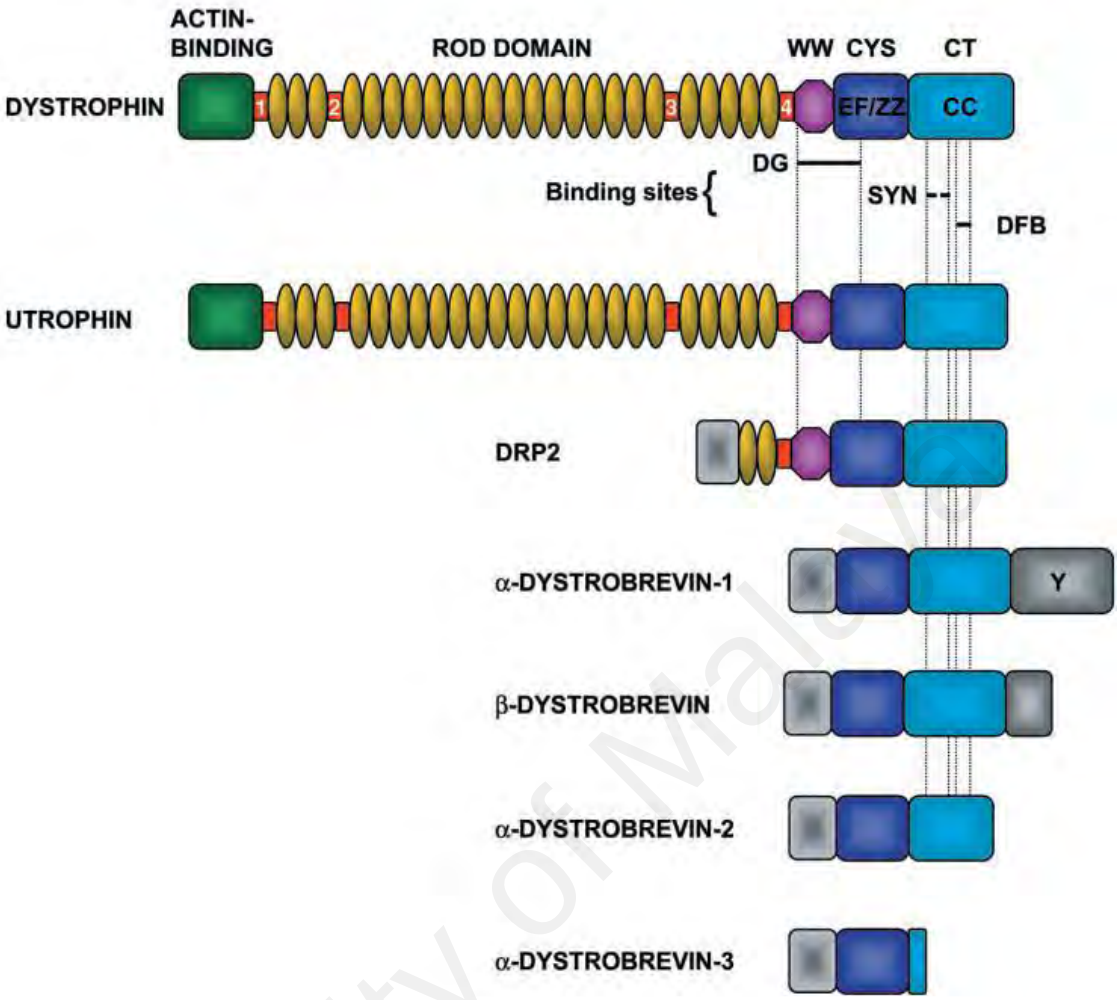


Figure 2.1: Schematic diagram showing the organization of the human dystrophin gene, and the dystrophin-associated protein family. Adapted from (Blake *et al.*, 2002)

The consensus is that dystrophin works together with other proteins, called the Dystrophin-Associated Protein Complex (DPC); and that dystrophin is linked to the sarcolemma of normal muscle, by this complex (Blake *et al.*, 2002). This complex consists of at least ten proteins, which can be grouped into Dystroglycan and the Dystroglycan complex, Sarcoglycan complex, Syntrophins and Dystrobrevin.

Dystrophin exists in all vertebrates and homologues have been found in some invertebrates as well. The gene appears to retain high conservation across metazoans, and the dystrophin-like counterpart in invertebrates support this, albeit weakly, sometimes

(Roberts & Bobrow, 1998). However, the remarkable similarity that has been observed between dystrophin sequences of organisms from different species and even phyla has generated interest in investigating the viability of invertebrates as model organisms for studying the dystrophin gene.

A dystrophin homolog was found in *Caenorhabditis elegans*, which is 31kb in size and codes for a 3,674 amino acid protein. The protein's C-terminal end possesses a 37% similarity with the human dystrophin (Bessou *et al.*, 1998). A 98kDa protein was identified in the sea urchin, which has a high homology with the C-terminal of dystrophin, although the region of the gene in sea urchin, that encodes the protein, is significantly smaller than the matching region in human dystrophin (Wang *et al.*, 1998). Another homologue of the dystrophin gene was also found in the fruitfly, *Drosophila melanogaster* and has proved to be just as complex as the dystrophin gene in mammals, due to the presence of large introns which contribute to its large size (Neuman *et al.*, 2001).

So far, there has been only one report on the discovery of a dystrophin-like gene in crustaceans. (Noor *et al.*, 2017) identified a 1246 base pair long dystrophin-like sequence in the giant freshwater prawn, *Macrobrachium rosenbergii* and a 1082 base pair long dystrophin-like sequence in the tiger shrimp *Penaeus monodon*. It was also observed that during a WSSV-infection in *M. rosenbergii*, the expression of dystrophin was altered, together with an increase in intracellular calcium concentration.

2.3 Impact of Disease on Shrimp Muscle

It can be said that all diseases affect the muscle of the shrimp since these diseases eventually affect the growth and development of the shrimp, and the muscle is essentially

about 80% of the size of the shrimp. Hence, when the shrimp stops growing, it means the muscle has stopped growing. This is also supported by the findings from some research.

It has generally been reported that muscle deterioration occurs during WSSV infection. It was recently discovered during a WSSV infection in *M. rosenbergii*, that the expression of dystrophin was altered (Noor *et al.*, 2017). In 24 hours, the expression of dystrophin in *M. rosenbergii* muscle had reduced to less than what it was in the control samples, and then, at 36 and 48 hours, it rose higher than the expression in the control samples. The immune system of the shrimp may have played a role in this, with the shrimp trying to fight the infection, but it was clear that the infection affected the expression of dystrophin in the muscle.

A **B**

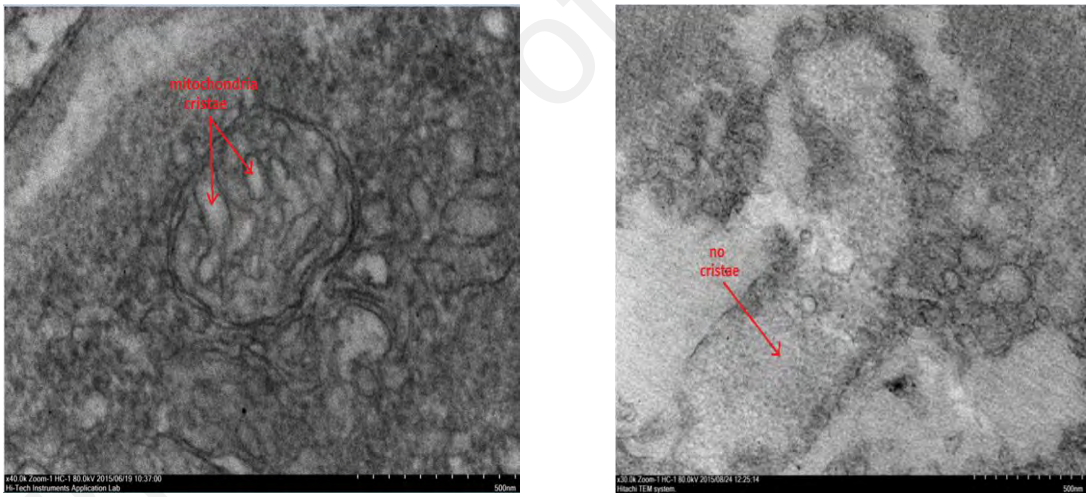


Figure 2.2: Transmission Electron Microscope (TEM) images of *M. rosenbergii* muscle during a WSSV infection. (A) Shows the mitochondrion organelle in the muscle at 24 hours post-infection. The mitochondria of cristae can be seen to be swollen. (B) shows the mitochondria organelle at 48 hours post infection with no cristae. The cristae presumably burst from having swollen too much. This shows the disintegration of the muscle during the infection. Adapted from (Noor *et al.*, 2017)

In a study conducted by (Durand *et al.*, 2003), it was discovered that the viral load of WSSV in infected *L. vannamei* was similar in the tail and the head, even though there are more organs contained in the head. This shows that the muscle is just as affected as

any other part of the shrimp during this viral infection; and during this particular one, it was greatly affected. In a similar study by (Nunan *et al.*, 2004), viral load of Taura Syndrome Virus (TSV) was quantified in *L. vannamei*, in different parts of the shrimp. The samples which were infected by injection, which was the more virulent infection, were all consistently in the 10^9 range, for all the parts examined. Again, this shows that infection is uniform.

Apart from pathogen copy numbers in shrimp body parts, another indicator of disease effect on muscle is the obvious gross signs of disease. Signs of Infectious Myonecrosis Virus (IMNV) infection include focal to extensive necrosis in areas in skeletal muscle tissues and the appearance of white discolouration of affected muscle (Chaivisuthangkura *et al.*, 2014; D. Lightner *et al.*, 2004). *Penaeus monodon* nucleopolyhedrovirus (*Pemo*NPV), formerly known as Monodon Baculovirus (MBV) has been connected to stunted growth, with the mean length of *Pemo*NPV-infected shrimp being notably shorter than the length of uninfected shrimp from the same pond (Flegel *et al.*, 2004). *Penaeus monodon* Densovirus (*Pm*DNV) causes infected shrimp to grow very slowly and eventually stop growing at approximately 6cm (Flegel, 2006; Flegel *et al.*, 2004).

Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) infection causes cuticular deformities and growth retardation, collectively termed Runt Deformity Syndrome (RDS) in *L. vannamei*. Shrimp infected with Necrotizing Hepatopancreatitis (NHP) show lethargy and abdominal muscle atrophy (Poornima & Alavandi, 2014). Acute Hepatopancreatic Necrosis Disease (AHPND)- infected shrimps also exhibit lethargy and inactivity, which can be associated with muscle strength, or lack thereof.

2.4 Impact of Diseases on Shrimp Aquaculture

At a time, it used to be that some of the most important diseases were limited to either the Western or Eastern hemisphere. These diseases were transferred between countries and between continents, together with the transfer of live shrimp stock, even before their aetiology was understood (Lightner, 2003). Infectious agents are mostly responsible for cultured shrimp diseases which have economic impacts. From literature, it can be gathered that approximately 60% of shrimp production losses, as a result of diseases, is due to viral diseases and 20% is due to bacterial diseases. The remaining 20% of the loss can be credited to other pathogens, including fungi and parasites (Chaivisuthangkura *et al.*, 2014). Among all these infectious agents, certain viral diseases stand out; an example is the White Spot Disease (WSD), which is caused by the White Spot Syndrome Virus (WSSV). WSSV, which has been described as a thorn in the flesh of global shrimp aquaculture (Stentiford *et al*., 2012), was introduced into Asia in 1992, and between then and 2001, it caused the industry to lose 4 to 6 billion dollars (Lightner, 2003; Lightner, 2005). It was estimated that in the year 2000 alone, WSSV caused the industry to lose approximately 200,000 metric tonnes in production, which was worth more than $1 billion.

The pandemics resulting mainly from the penaeid viruses, Taura Syndrome Virus (TSV) and WSSV have had profound impacts on the industry. They have left the loss of jobs and export revenue in their wake, in addition to the billions of dollars that are lost in crops. In Asia, first, the Yellow Head Virus (YHV) from 1992, and afterwards, from 1994, WSSV, caused continued direct losses of about US$ 1 billion a year to the local cultured shrimp industry (Briggs *et al.*, 2004; Moss *et al.*, 2003). Mortalities associated with TSV alone, were as high as 80% in three days, in Taiwan Province of China in 1999. Runt Deformity Syndrome (RDS) which is usually due to Infectious Hypodermal Hepatopancreatic Necrosis Virus (IHHNV) was also a common occurrence. Acute

Hepatopancreatic Necrosis Disease (AHPND) is another disease of shrimps which is caused by a bacterial pathogen, *Vibrio parahaemolyticus*. AHPND first emerged in Asia in 2009 and has also been referred to as Early Mortality Syndrome (EMS). AHPND-infected shrimps usually exhibit clinical symptoms of shrunken hepatopancreas, empty stomachs and midguts, and stunted growth. The *V. parahaemolyticus* strain that causes AHPND is extremely virulent and can cause mortality in as early as three days (Linda *et al*., 2014). This, of course, leads to great economic losses.

The first major crash in production happened in the Taiwan Province of China, from 1987 to 1989, with the sudden decline in *P. monodon* production, when it went from 78,500 metric tonnes to 16,600 metric tonnes. This was widely thought to be due to stress, pollution and increased susceptibility to pathogens, especially viruses. Subsequent crashes are largely related to viral diseases. The first of these occurrences were in Mainland China when production fell to 64,000 metric tonnes in 1993-1994 from 207,000 metric tonnes in 1992, due to WSSV outbreak. Similar problems were observed in the Philippines, Thailand and Indonesia, first with YHV, and then, with WSSV, since the early 1990s. As a result of these diseases and the associated losses, farmers began to utilize cheaper, pond-reared broodstock indiscriminately, without even considering biosecurity or the genetic makeup. This, of course, led to more problems, including inbreeding and increased introduction of diseases, through hatchery-produced post-larvae. With the introduction of diseases came a loss of money and decreased food production. This is especially the case for those countries which depend heavily on aquaculture products for their protein.

2.5 Food Biosecurity

If governments and industries had known about the risks involved in the transfer of shrimps across countries, the introductions of pathogens could have been prevented. The time between the first recognition of these diseases and the development of diagnostic methods has also contributed to the international transfer of these diseases. Perhaps, if appropriate pathogen detection and disease diagnostic methods had been readily available, the devastating impacts of the first disease transfers could have been avoided.

Biosecurity simply refers to the prevention of disease by excluding specific pathogens from cultured shrimp stocks in broodstock facilities, farms and hatcheries; and even from regions and countries. Concepts central to this practice, are stock control and pathogen exclusion, with stock control receiving more attention. Stock control simply means the development and rearing of stocks that are specific pathogen-free. This does not mean that they are free from all pathogens; just some specific ones of concern, like all living organisms can never be completely free from pathogens. Facility design and geographic location are also taken into consideration when developing SPFs (Lightner, 2005).

The pathogens to be excluded from the selected stock are determined, based on a working list of infectious, diagnosable, and excludable pathogens. This list changes over time as new diseases emerge, which show potential to cause serious pandemics (Lightner, 2011; Lightner, *et al.*, 2009). The most current working list includes nine viruses or virus groups, which include WSSV, TSV, IHHNV, the YHV group, Hepatopancreatic Parvovirus (HPV), Baculovirus penaei (BP), baculoviral mid-gut gland necrosis (BMN), monodon baculovirus (MBV), infectious myonecrosis (IMNV) and Penaeus vannamei nodavirus (PvNV); certain classes of parasitic protozoa (gregarines, haplosporidian, and microsporidians), and the bacterial agent of necrotizing hepatopancreatitis, or NHP (Lightner, 2011).

The first SPF stocks developed by the U.S. Marine Shrimp Farming Program (USMSFP) were developed in the spirit of the ICES Code (The International Council for the Exploration of the Sea; Code of Practice to Reduce the Risks of Adverse Effects Arising from the Introduction on Non-indigenous Marine Species 1973), as reviewed in (Sindermann, 1988; Sindermann, 1990; Lightner, 2005). Below is a table showing the recommended steps in the ICES guidelines to reduce risks in the introductions of aquatic species (modified from Sindermann, 1988; Sindermann, 1990; Lightner, 2005), which have been adapted to the development of SPF shrimp (Lightner, 2011).

Table 2.1: Recommended steps in the ICES guidelines for risk reduction in aquatic species introductions.

Original ICES Guidelines	**Adapted to SPF shrimp development**
Conduct comprehensive disease study in native habitat	Identify stock of interest (i.e., cultured or wild).
Transfer founder stock system in the recipient area	Evaluate stock's health/disease history
Maintain and study closed system population	Acquire and test samples for specific listed pathogens (SLPs) and pests
Develop broodstock in a closed system	Import and quarantine founder (F0) population; monitor F0 stock
Grow isolated **F1** individuals; destroy original introductions	Produce F1 generation from F0 stock
Introduce small lots to natural waters - continue disease study	Culture F1 stock through the critical stage(s); monitor general health and test for SLPs
	If **SLPs,** pests, other significant pathologies are not detected, F-1 stock may be defined as SPF and released from quarantine

With the practice of the above, SPF shrimp stock means that the stock in question have been free from the disease agents listed on its working list of specific pathogens for

at least two years and would have been cultured in biosecure facilities and environments. The stock would have been either cultured in conditions under which the listed disease agents would have caused recognizable disease if they were present and/or that the stock would have been subjected to routine surveillance and testing for the listed pathogens (Lightner, 2005; Lightner, 2011; Lightner *et al.*, 2009). The pathogens should have also met certain criteria which include: (a) strict excludability of the pathogen(s); (b) availability of adequate pathogen detection and diagnostic methods, and (c) the pathogen(s) must pose the substantial threat of causing disease and leading to production losses. These criteria are also among those required for disease listing by the Office International des Epizooties, OIE (OIE, 2003a; OIE, 2003b).

Not all potential causes of diseases can be excluded because shrimps have a diverse and large microbial population as part of their natural aquatic environment and microbial flora. Some of these microorganisms are facultative and can strike whenever there are any stressors or enhancers. Hence, a list of specific pathogens, to be excluded, needs to be developed. This is one of the essential elements of a good aquaculture biosecurity plan. There should always be one, even for a single culture facility, or a group of farms. It also goes as far as for a country or a region which consists of many countries. Some pathogens share a common characteristic which makes them excludable. Being limited to geographic distribution, having a limited host range, and being an obligate parasite/pathogen which requires a suitable host for replication, are some of these characteristics (OIE, 2003a; OIE, 2003b). For an SPF program to be fully functional, a surveillance program must be incorporated, which includes both routine, scheduled specific and general surveillance components.

2.6 Current Methods of Diagnosis

In addition to the absence of pathogens, successful application of the SPF concept depends greatly on the availability of accurate and sensitive methods of diagnosis and pathogen detection. Molecular diagnostic procedures have become just as important as the classical methods like microbiology and routine histopathology. They are especially important to routine surveillance which is essential to the establishment and declaration of stock freedom from disease, and also, to the monitoring of shrimp stock in farms (Lightner, 2011; Subasinghe *et al.*, 2004).

Molecular procedures, which make use of gene probes and gene amplification methods that utilize PCR, have been noted to offer accurate and standardisable means of disease diagnosis and pathogen detection in the penaeid shrimp culture industries, especially for certain penaeid viruses (Lightner, 2005; OIE, 2003a). The first reports on using DNA-based technologies for diagnosis were made over two decades ago. Among others, (Vickers *et al.*,1992) were the first to report on the employment of PCR in the detection of Monodon-type Baculovirus (MBV). Today, DNA-based diagnostic tests are regularly used to detect most of the major shrimp viruses and several bacterial and parasitic diseases. Molecular diagnostic tests have become the "gold standard'' for diagnosing many of the penaeid shrimp viral diseases, and for detecting their etiological agents. Gene amplification methods, like reverse transcription PCR, RT-PCR and PCR are recommended for surveillance (screening) for six of the seven currently listed shrimp viruses by OIE, for crustacean diseases, (Lightner, 2005).

The use of DNA-based diagnostic methods has been reported severally, with the high degree of success. One-step nested PCR has been used in the diagnosis of WSSV and IHHNV (Pazir *et al*., 2011). Two other researchers, (Nunan & Lightner, 2011) optimized a PCR assay for the detection of WSSV, somewhat as a criticism, and also as an improvement to what (Lo *et al.*, 1996) had done. They compared (Lo *et al.*, 1996)'s

method with a modified version of (Lo *et al.*, 1996)'s method, and their optimized PCR. The method used in 1996 by Lo *et al.* was a two-step PCR which was not very sensitive and specific, and also took a lot of time. Nunan and Lightner created a new one-step PCR, using the primers for the second step of Lo *et al.*'s two-step PCR, because the primers had been routinely used, and could detect all geographical isolates. Hence, specificity would not be a problem. Their own new PCR is faster and also cheaper.

Real-time PCR was also used for the detection of hepatopancreatic parvovirus (HPV) by Yan and his collaborators in 2010. They selected a pair of primers and TaqMan probe, which were based on an HPV sequence that had been previously obtained from samples of *Fenneropenaeus chinensis* from Korea. These primers and probe were used to amplify a 92bp fragment of the HPV DNA sequence. This real-time PCR was found to be specific to HPV as it did not react with other shrimp viruses. They also constructed a plasmid, containing the target HPV sequence, which was used in determining the sensitivity of the assay. They were able to detect a single copy of plasmid DNA, using the assay, and it was successfully used in finding HPV in shrimp samples from Taiwan, the China-Yellow Sea region, Thailand, Korea, New Caledonia, Madagascar and Tanzania (Yan *et al.*, 2010).

In another study, in situ hybridization and PCR assay were developed for the detection of Enterocytozoon hepatorenal (EHP) in infected *Penaeus stylirostris* from Brunei, and *Litopenaeus vannamei* from Vietnam. The researchers, (Tang *et al*., 2015) also used PCR to amplify 18S rRNA gene from EHP. They generated a digoxigenin-labeled probe and used it to identify the EHP infection within the cells by *in-situ* hybridisation. They also developed a specific PCR for detecting EHP in faeces, water and shrimp tissues. The *in-situ* hybridisation gave specific results; the probe only reacted to the EHP within the cytoplasmic inclusions, and not to another Pleistophora-like microsporidium which is associated with cotton shrimp disease. The PCR was also

specific to EHP and did not react to 2 other parasitic pathogens, the cotton shrimp disease microsporidium and amoeba, nor to genomic DNA of various other crustaceans including crabs, squids, polychaetes and krill.

PCR is not only used to detect viral pathogens and parasites, but also bacterial pathogens. A PCR assay has been developed for the detection of *Vibrio parahaemolyticus*, the AHPND-causing bacteria, which targets a unique sequence that is present in only AHPND isolates. The PCR test can differentiate between pathogenic AHPND-causing *V. parahaemolyticus* isolates and the non-pathogenic isolates. The study was undertaken to confirm the presence of AHPND in Mexico (Nunan *et al.*, 2014).

Another DNA-based diagnostic test is the development of a multiplex Loop-mediated Amplification (mLAMP) assay for the simultaneous detection of WSSV and IHHNV. The mLAMP method was able to distinguish between IHHNV and WSSV because of the subsequent restriction enzyme analysis that was carried out. The method showed high sensitivity and specificity as well (He & Xu, 2011).

It can generally be seen that DNA-based diagnostic methods have been used successfully in the detection and diagnosis of different kinds of shrimp diseases. They have proven to be very specific and sensitive and time-saving; even cheap. PCR and real-time quantitative PCR have especially been used time and time again and can be trusted. However, they have only been used to detect the pathogenic agents themselves, alone; this is usually done when symptoms have been observed. In cases where diseases are asymptomatic, they are not screened for the pathogenic agents until it is too late, and mortality has occurred already. Perhaps, it is possible to use something from the host itself to detect such conditions; say a gene whose expression is affected by infections. In this case, it will not be just one infection that the gene responds to, but different infections.

Also, the effect of disease on shrimp cannot be overlooked, seeing as diseases eventually affect the feeding and activity, and eventually, the growth of the shrimps. The part of the shrimp which is most affected in the condition of stunted growth and runt deformity is the muscle of the shrimp. Consequently, it makes sense to study the expression of a muscle gene during pathogenic infections. This is a new angle that has not been investigated, except in cases where muscle deformity is obvious. The involvement of the muscle in different pathogenic infections is worthy of some attention.

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample Collection

Thirty live and healthy *Litopenaeus vannamei* shrimps were collected from a farm in Sepang, Malaysia. They were kept in tanks which contained water from the farm pond. Oxygen was provided via the use of oxygen pumps, and they were transported to the laboratory. They were dissected as soon as they reached the laboratory, and the muscle tissues were removed and stored at -80^{o}C for use in future procedures.

3.2 Total RNA Isolation

The workbench was sterilized with 10% Chlorox, 70% ethanol, and RNAse Zap, prior to the procedure. Following the manufacturer's (TransGen Biotech, Beijing) instructions,100mg of muscle tissue was ground thoroughly in liquid nitrogen to powder, in a pre-sterilized mortar. The tissue powder was transferred to a microcentrifuge tube, and 1ml of *TransZol* Up (TranGen Biotech, Beijing) was added to it. It was homogenized and repeatedly pipetted up and down. It was then incubated at room temperature for five min. Afterwards, 200 µl of chloroform was added, and the tube was vigorously shaken for 30 seconds, after which it was then incubated at room temperature for 3 min. The sample was centrifuged at 10000×g for 15 min at 4^{o}C. The mixture separated into a lower pink organic phase, interphase, and a colourless upper phase which contained the RNA. This colourless upper phase was transferred into a fresh RNase-free tube, and equal portion of absolute ethanol was added. Then the tube was inverted gently to mix.

The resulting solution was then transferred into a spin column and was then centrifuged at 12000×g for 30 sec at room temperature. The flow-through was discarded. After this, 500µl of clean buffer was added to the spin column then, centrifuged at 12000×g for 30 sec at room temperature. The flow-through was discarded, and the step

was repeated once more. Then, 500µl of wash buffer with added ethanol was then added to the spin column and centrifuged at 12000×g for 30 sec at room temperature. The flow-through was discarded, and the step was also repeated one more time. Then, the spin column was centrifuged at 12000×g for 2 min at room temperature, to completely remove the remaining ethanol. The column matrix was then allowed to air-dry for several minutes.

Then, the spin column was placed into a clean 1.5ml RNase-free tube. Next, 50µl of RNase-free water was added into the spin column matrix and incubated at room temperature for 1 min. It was then centrifuged at 12000×g for one min to elute RNA. The isolated RNA was then stored at -80ºC.The quantity of the resulting RNA was checked using a Nanodrop 2000 Spectrometer (Thermo Scientific, USA) and only those with a 260/280 value of more than 2.00 were used for the next step.

During this procedure, special care was taken to prevent cross-contamination between samples and from surrounding materials. The tools and work bench were decontaminated in between samples and, talking was avoided. Also, movement from work bench to other parts of the laboratory was avoided.

3.3 Reverse Transcription and First Strand cDNA Synthesis

According to the manufacturer's (Promega, Germany) instructions, 4µl of total RNA (200ng/µl) was combined with 1µl of random primer in microcentrifuge tubes; the tubes were placed in a preheated 70°C heat block for 5 min. Immediately after this, the tubes were chilled in ice-water for 5 min. Each tube was then centrifuged for 10 sec in a microcentrifuge to collect the condensate and maintain the original volume. The tubes were kept closed and on ice until the reverse transcription reaction mix was added. The reverse transcription reaction mix was prepared by combining the components of the GoScriptTM Reverse Transcription System (Promega, Germany) in a sterile

microcentrifuge tube on ice. Sufficient mix was prepared to allow 15µl for each cDNA synthesis reaction to be performed. The volume needed for each component was determined, and combined, as shown in Table 3.1 below. All components were vortexed gently to mix and kept on ice before dispensing into the reaction tubes.

Next, 15µl aliquots of the reverse transcription reaction mix were added to each reaction tube on ice. Great care was taken, to prevent cross-contamination. Then, 5µl of RNA and primer mix was added to each reaction for a final reaction volume of 20µl per tube. The tubes were placed in a controlled-temperature heat block equilibrated at 25°C and incubated for 5 min. They were then incubated in a controlled-temperature heat block at 42°C for one hour. The extension temperature may be optimized between 37°C and 55°C. The reaction tubes were then incubated in a controlled-temperature heat block at 70°C for 15 min, to inactivate the reverse transcriptase.

The quality of the cDNA was checked by running it on 1% Agarose gel, and then, the quantity was checked, using a Nanodrop 2000 Spectrometer (Thermo Scientific, USA). Only the cDNA samples which had a 260/280 of 1.80 or above were used for the next step of the experiment. All 30 samples were used; the ones that did not pass the absorbance QC test were repeated until they met satisfactory criteria. The cDNA was stored at -20^{o}C for further use.

Table 3.1: Components of the transcription reaction mix and their corresponding volume.

Component	Volume
Nuclease-Free Water	7.8µl
GoScriptTM 5X Reaction Buffer	4.0µl
$MgCl_2$ (final concentration 1.5–5.0mM)	1.2µl
PCR Nucleotide Mix (final concentration 0.5mM each dNTP)	1.0µl
GoScriptTM Reverse Transcriptase	1.0µl
Final volume	15.0µl

3.4 DNA Extraction from the muscle of *L. vannamei*

Using TransGen EasyPure Marine Animal Genomic DNA Kit (TranGen, Beijing), and following the enclosed instructions, 30mg of minced muscle tissue was placed into a sterile microcentrifuge tube, then 200µl of lysis buffer and 20µl of RNase A were added to the tube and the tube was vortexed for 10 sec then incubated at room temperature for 2 min. Then, 20µl of Proteinase K was then added to the tube. It was mixed thoroughly, using a vortex, making sure that the tissue was completely immersed in the solution. This was then incubated at 55^{o}C until lysis was complete. The lysis step took about three hours.

Once complete lysis was achieved, 1.5× volume (360µl) of binding buffer with added ethanol, was added to the tube and mixed thoroughly. The mixture was then added to a spin column and centrifuged at 12,000×g for 30 sec. The flow-through was discarded, and 500µl of cleaning buffer with added ethanol was added and centrifuged at 12,000×g for 30 sec. The flow-through was discarded, and again, 500µl of cleaning buffer with added ethanol was added and centrifuged at 12,000×g for 30 sec. The flow-through was discarded, then 500µl of washing buffer with added ethanol was added and was centrifuged at 12,000×g for 30 sec and the flow-through was discarded. Once again, 500µl

of washing buffer with added ethanol was added and was centrifuged at 12,000×g for 30 sec and the flow-through was discarded.

The spin column was centrifuged at 12000×g for 2 min to completely remove residual washing buffer. The spin column was then placed in a sterile 1.5ml microcentrifuge tube for elution. 25µl of elution buffer which had been preheated to 60ºC was then added to the center of the column. It was incubated at room temperature for 2 min then centrifuged at 12000×g for 1 min to elute the genomic DNA.

The obtained DNA was then run on 1% Agarose gel at 100V and 200A for 30 minutes to check the quality. Only DNA that had 260/280 of >1.80 was used in the next procedure.

3.5 PCR Amplification of L. vannamei Dystrophin (LvDys)

Primers were designed on the Primer 3 software, using the conserved regions across several dystrophin sequences from different species, as the template. Several parameters were taken into consideration, including annealing temperature, hair pin loop, self-complementary, any complementary at all and GC ratio. Optimization of the primers was done by varying the primer concentration, annealing temperature and the number of cycles of the PCR reaction. Once the annealing temperature had been optimized, PCR was then conducted using both the synthesized cDNA and extracted DNA.

The primer sequences are as follows:

Table 3.2: Primer sequences for the amplification of LvDys.

LvD 1.22F:	**5'- GTG AGG TTG CAG CAT TTG G -3'**
LvD 1.2R:	**5'- ACC GCT GAC ACA TAT CAA AGC T -3'**

PCR reagents (TransGen EasyTaq Polymerase) used and their corresponding volumes:

Table 3.3: PCR reagents used for the amplification of LvDys and their corresponding volumes.

dNTP (2.5µM)	**0.8µl**	**2.5µM**
EasyTaq Polymerase (5 unit/µl)	**0.1µl**	**0.5 unit/µl**
10 × EasyTaq Buffer	**1.0µl**	**1×**
Forward Primer	**0.2µl**	**0.2µM**
Reverse Primer	**0.2µl**	**0.2µM**
Template (DNA)	**0.48µl**	**100ng/µl**
Ultrapure water	**7.22µl**	
Total	**10.0µl**	

The following protocol was used for the PCR procedure:

Table 3.4: Thermal profile for the amplification of LvDys.

Initial denaturation	**95°C for 5 minutes**
Denaturation	**95°C for 45 seconds**
Annealing	**51.2°C for 45 seconds**
Extension	**72°C for 30 seconds**
Final extension	**72°C for 5 minutes**

The PCR products were then run on 2% Agarose gel at 80Vand 180A for 40 min.

3.6 Purification of PCR product from Agarose Gel

The PCR product was purified from agarose gel using NucleoSpin® Gel and PCR Clean-Up kit (Macherey-Nagel, Germany). After gel electrophoresis, the band with the expected size was excised and placed into a 1.5ml microcentrifuge tube and weighed. For every 100 mg of 2% agarose gel, 400µl NTI buffer was added. The tube was incubated

at 50ºC until the gel slice was completely dissolved. A filter column was placed in a 2ml collection tube, and 700µl of the gel mixture was loaded. The tube was centrifuged at 11000 x g for 30 sec, and the flow-through was discarded. This step was repeated until all the remaining sample was loaded. Next, 700µl of Buffer NT3 was added into the column, centrifuged at 11000 x g for 30 sec, and the flow-through was discarded. This step was repeated once. A 1-minute centrifugation at 11000 x g was done to completely remove any remaining buffer solution. The column was transferred into a new 1.5ml microcentrifuge tube. 15µl of elution buffer was added to the column and incubated at room temperature for 1 min. Lastly, the PCR product (DNA) was eluted at 11000 x g centrifugation for 1 min. The purified PCR product was stored at -20ºC for further use. It was eventually subjected to Sanger Sequencing, using the LvDys primers. This was done by a company.

3.7 Bioinformatics Analysis

After the PCR products had been sequenced, a BLAST check of the obtained longer sequence was conducted against the nucleotide database at NCBI, for confirmation of the sequence. Most of the parameters were left at the default setting; but it was optimized to search for somewhat dissimilar sequences in "other" databases, as opposed to human or mouse databases. Using the MEGA 7 software, Multiple Sequence Alignment (MSA) was conducted, of the obtained dystrophin sequence and dystrophin sequences from other species, across both vertebrate and invertebrate species. This was conducted using the Muscle Alignment tool. Then, to graphically examine the relationship between the sequences, a bootstrapped Maximum-likelihood phylogenetic tree was constructed, also using the MEGA 7 software. The tree was drawn to scale, with branch lengths showing the percentage of similarity between sequences.

3.8 Quantification of *Penaeus monodon* Dystrophin (PmDys) gene

Primers were designed, based on the conserved region observed from the Multiple Sequence Alignment of all the dystrophin sequences. The primer sequences used are presented in Table 3.5 below.

Table 3.5: Primer sequences used for the quantification of PmDys. The expected amplicon size is 94bp.

qLvD 2F:	**5'- CAGGCTGTACACTTCCTAACA -3'**
qLvD 2R:	**5'- GAATGTTCTCAGAGGCAGCTA -3'**
ELF1_qPCR_F:	**5'- TAT GGT TGT CAA CTT TGC CCC -3'**
ELF1_qPCR_R:	**5'- AAC CTC GCT TCA GAT CCT TTA C -3'**

The primers were first used in a standard PCR, to optimize the primers. The optimum annealing temperature was determined to be 50^{o}C. Promega GoTaq qPCR MasterMix was then used for the quantitative real-time PCR (qPCR). SYBR Green was used as the fluorescent dye. The reaction mixture for the qPCR analysis is presented in Table 3.6 below.

Table 3.6: Reagents used for the quantification of LvDys and their corresponding volume and a final concentration.

Components	Volume	Final Concentration
Template	4.0µl	100ng/µl
Forward primer (2µM)	2.0µl	200nM
Reverse primer (2µM)	2.0µl	200nM
2× GoTaq qPCR MasterMix	10.0µl	1×
Passive Reference Dye II (50×)	0.4µl	
RNase-free Water	1.6µl -α	
Total	20.0µl	

Three technical replicates were made per sample, to normalize the result. Same was done for the negative control. The procedure was done in a biosafety cabinet, and

special care was taken, to avoid contamination. Air bubbles were also avoided in the tubes. The content of the tubes was spun down in a microcentrifuge and tapped to mix.

The strip was placed in the qPCR thermal cycler. The top part of the tubes' cover was cleaned with 70% ethanol, to remove further any contamination that may have occurred during micro centrifuging. The wells, in which the tubes were placed, were noted.

For plate setup, the boxes corresponding to the wells of the tubes were selected, on the computer screen. ROX and FAM were selected for fluorescence dyes, and ROX was selected as the reference dye. Next, the thermal profile was set up. The thermal profile was set for: Initial denaturation at 95^{o}C for 5 min; Amplification (Denaturation at 95^{o}C for 15 sec, Annealing and extension at 50^{o}C for 35 sec). Amplification was done for 40 cycles. The melting curve was determined at 95^{o}C for 1 min, 55^{o}C for30 sec and then, 95^{o}C for30 sec.

The primers were used in a quantitative real-time PCR, using cDNA from muscle tissues of both healthy and AHPND and WSSV-infected shrimps. Cycle threshold (Ct) values were calculated by the inbuilt ABI 7500 SDS software. The specificity of the qPCR amplification was verified through a melt curve analysis by generating a dissociation curve. An internal control gene, elongation factor 1-alpha ELF-1(Dhar *et al.*, 2009), was quantified using the same reaction mixture as above, with ELF-specific primers. The relative gene expression of the dystrophin gene compared to the internal control gene was calculated, using the comparative CT method ($2^{-\Delta\Delta CT}$) (Livak & Schmittgen, 2001).

CHAPTER 4: RESULTS

4.1 Identification of *Litopenaeus vannamei* Dystrophin gene

Polymerase Chain Reaction (PCR) was conducted, using the primers (Table 3.2) designed from different dystrophin species, and the products were run on 2% agarose gel for inspection. The result of the agarose gel electrophoresis showed successful amplification of the gene. A band was observed at about 600bp for each of the products. Additionally, another band at 300bp was discovered for each of the products; both bands were very vivid (Fig 4.1). The products were subjected, afterwards, to Sanger sequencing to verify the sequences (Fig 4.2).

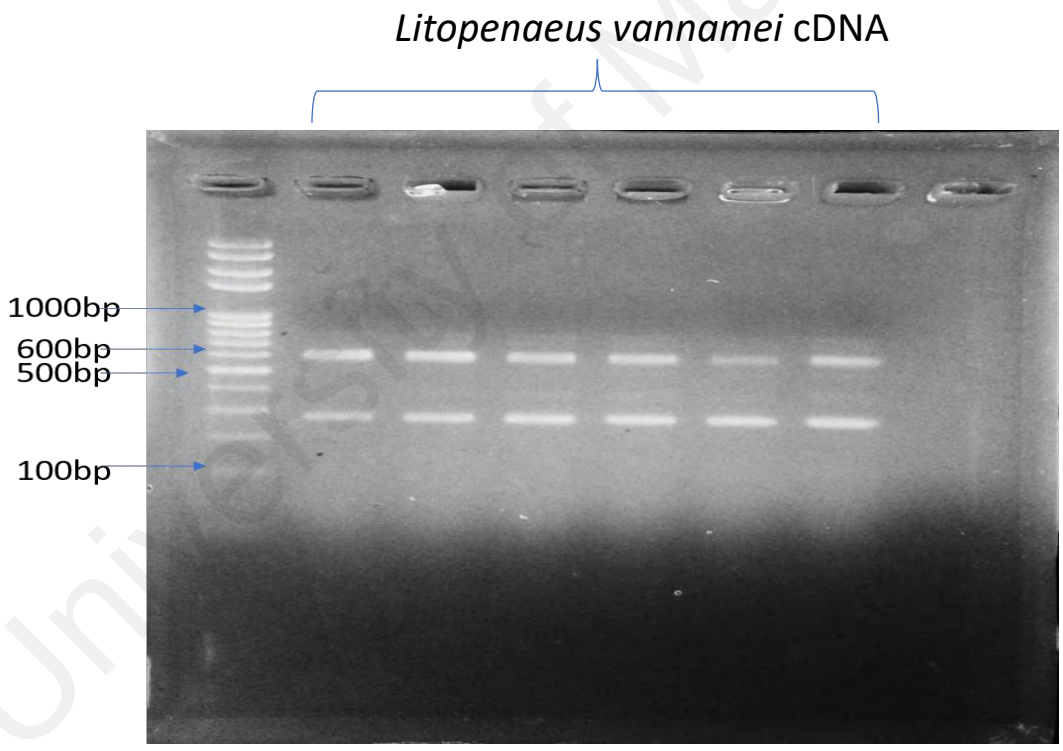


Figure 4.1: Gel electrophoresis image was showing nucleic acid bands at about 300bp and about 600bp. The content of the wells is: Lane 1: 100bp ladder; Lanes 2 to 7: *Litopenaeus vannamei* muscle cDNA

LvDys: 5'- NTTACTAAGAGCTGGCAAGGATAGAGAAACAATTGAGGTA

ATTGTCTAAGATAACAAAGATCTGAAAACATATTAAGGTTATT

TTATTATTGACTTCGGATTATTTTCTCATTTGTTTTATTCTGTG

TATTTGTATATATATTTATATATAAATGTATGTATGATATAGTA

TAATATAATATGTAATATAACATATTCAGTAATGTTATAAATGC

ATTCTCTTTTTCAGGCTGTACACTTCCTAACATGGGTACAGCA

AGAACCACAGTCCCTTGTGTGGTTGGCCGTTTTGCACCGAGTA

GCTGCCTCTGAGAACATTCAGCATCAGGTTAATATCTATTTTA

TAGTTAAAACTTGTTTGAAGTGTACAGATGTATATGTTTATTGT

GTAACATAATCCCTAATTATTACAAACATCTTTATTTTCATACA

CAAAAAGAAAAATCACAACTTATGTCGCAATTTCTTCCTCATCT

TCAGGTGAAGTGCAACATCTGTAAGGCTTACCCAATTGTAGGC

CTGCGCTACCGTTGCCTCAAGTGCCTCAGCTTTGA -3'

Figure 4.2: The 572bp- long nucleotide sequence of the obtained portion of LvDys

LvDys: 5'-

AAGAGCATCTGTTCGTACTGCTNCTAAGGCTGGCAGGATAGAG

AACAATTGAGGCTGTACACTTCCTAACATGGGTACAGCAAGAA

CCACAGTCCCTTGTATGGTTGGCCGTTTTGCACCGAGTAGCTG

CCTCTGAGAAATTCAGCATCAGGTGAAGTGCAACATCTGTAAG

GCTTACCCAATTGTAGGCCTGCGCTACCGTTGCCTCAAGTGCC

TCAGCTTTGATATGGGTCAGCGGTAA -3'

Figure 4.3: The 241bp region of the L.vannamei dystrophin gene that was identified

Figure 4.4: Pairwise alignment of the two sequences obtained. Regions of similarity are observed at the beginning, in the middle and at the end

4.2: Bioinformatics Analysis of the obtained sequence

The longer obtained sequence was subjected to a BLAST check, against the NCBI database. The result showed that it was similar to the dystrophin sequences of a few *Drosophila* organisms (*Drosophila hydei*, *Drosophila eugrasilis*, *Drosophila kikkawai*, *Drosophila takahashii* and *Drosophila elegans*), by 71.6% to 82.4%. Afterwards, the sequence was aligned with dystrophin sequences of individuals belonging to vertebrate and invertebrate species. A somewhat conserved region was observed from this alignment. A table (Table 4.1) is provided below, showing the names of the organisms used for the alignment, and their NCBI Accession number.

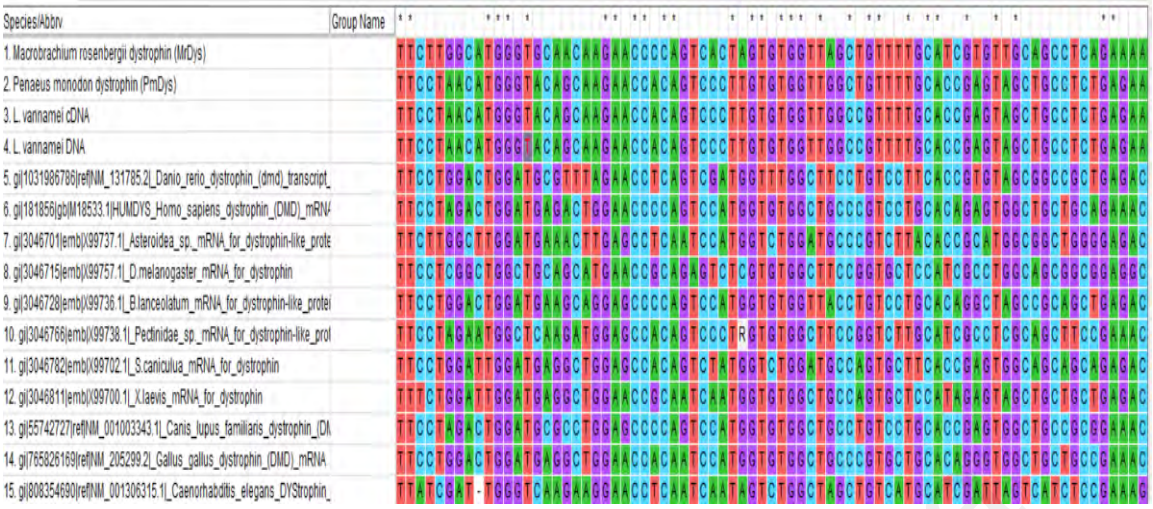


Figure 4.5: The conserved region that was observed from the Multiple Sequence Alignment (MSA)

Table 4.1: Organisms used in the MSA and their corresponding NCBI Accession number.

Species name	Common name	NCBI Accession Number
Asteroidea sp	Starfish	X99737.1
Branchiostoma lanceolatum	Lancelet	X99736.1
Caenorhabditis elegans	Round worm	NM_001306315.1
Canis lupus	Dog	NM_001003343.1
Danio Rerio	Zebrafish	NM_131785.1
Drosophila melanogaster	Fruit Fly	X99757.1
Gallus gallus	Chicken	X13369.1
Homo sapiens	Human	M18533.1
Pectinidae sp	Scallop	X99738.1
Scyliorhinus canicular	Dogfish	X99702.1
Xenopus laevis	African Frog	X99700.1

A phylogenetic tree was constructed to examine and graphically view the relationship between *Litopenaeus vannamei* dystrophin and the dystrophin of the other organisms used in the MSA. It was seen that *L. vannamei* dystrophin is closely related to *P. monodon* and *M. rosenbergii* dystrophin and they are all closest to the *C. elegans* dystrophin.

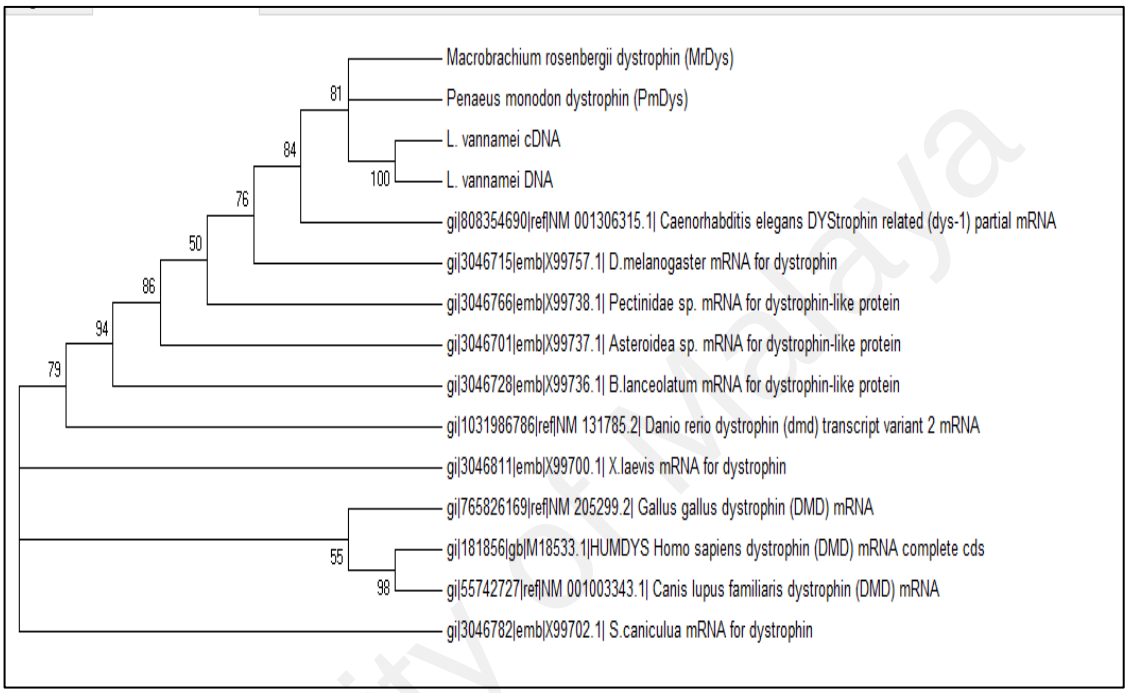


Figure 4.6: Bootstrapped Maximum Likelihood tree is showing the relationship between *L. vannamei* dystrophin and dystrophin from 13 other species. The tree is drawn to scale, with branch lengths showing the percentage of similarity

4.3 Conserved Domain Analysis

The two sequences were queried against the Conserved Domain Database (CDD) on the NCBI database. Two conserved domains were identified: the EF-hand superfamily conserved domain and the ZZ superfamily conserved domain, which are both found in the dystrophin-dystrobrevin family. The two conserved domains were identified in both the short and the long sequence (Fig 4.7 and 4.8).

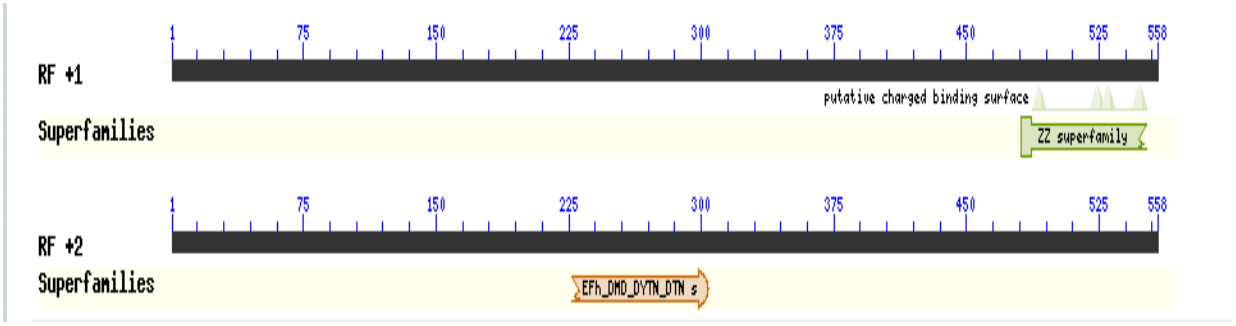


Figure 4.7: Conserved protein domains identified in the longer portion of the *L. vannamei* dystrophin

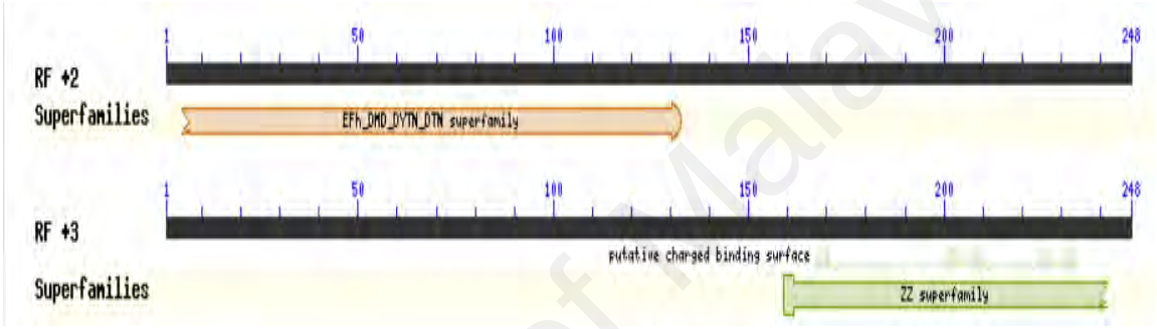
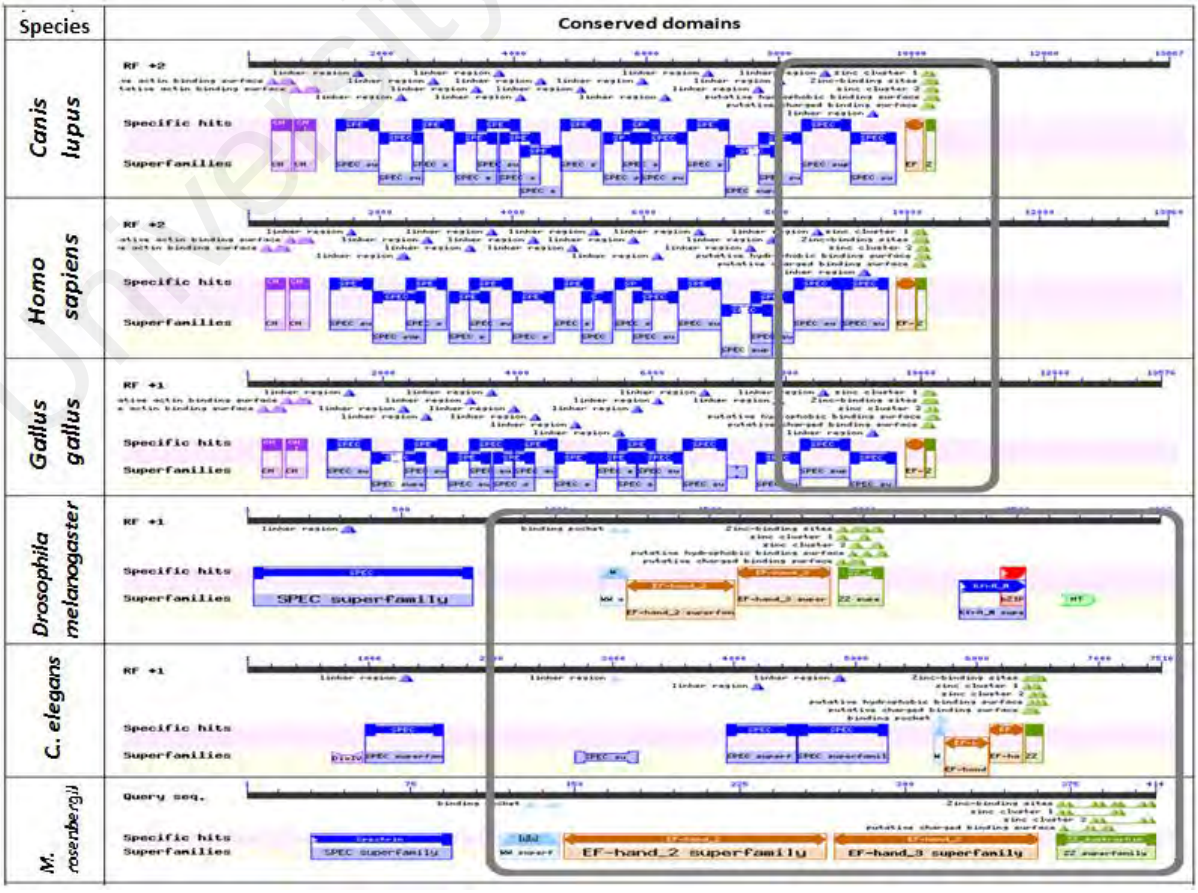


Figure 4.8: Conserved protein domains identified in the shorter portion of the L. vannamei dystrophin. The domains are much closer than they are, in the longer portion



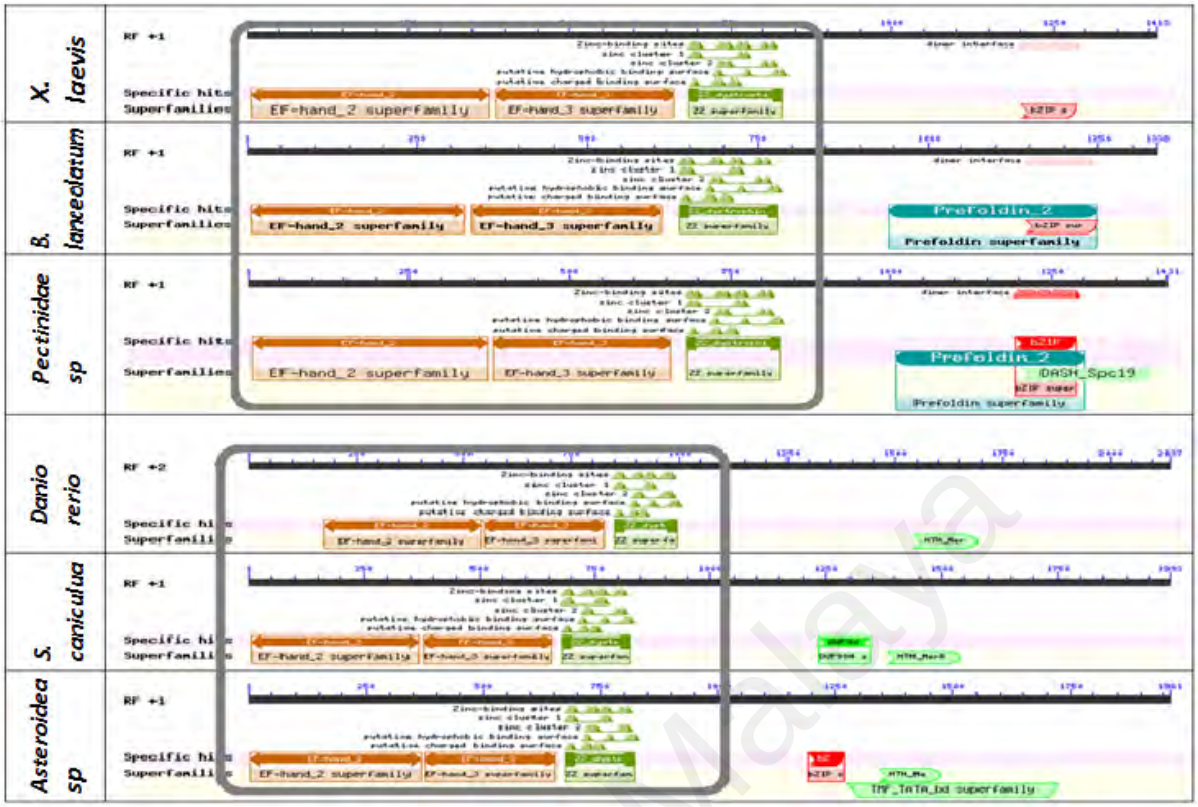


Figure 4.9: Image of the different conserved domains identified in the dystrophin of different organisms. The highlighted parts show the arrangement of the domains which is similar to what was found in *L. vannamei* dystrophin

4.4 Quantification of Dystrophin in shrimp muscle

Primers were designed from the EF-hand conserved domain and used in a quantitative real-time PCR experiment to quantify dystrophin expression in *P. monodon* and *L.vannamei*. These primers were also used to quantify dystrophin in the muscle of WSSV and AHPND-infected *P. monodon*. Quantification of dystrophin was successful and compared between healthy and infected individuals.

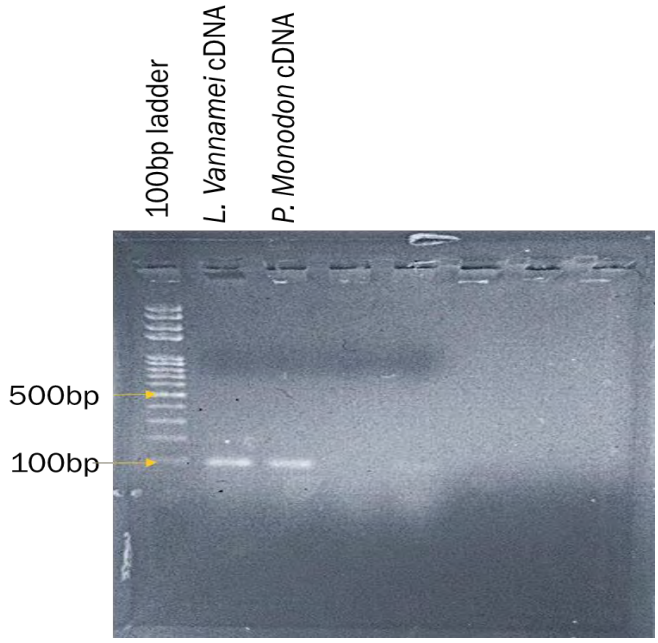


Figure 4.10: Gel electrophoresis image showing the successful quantification of dystrophin in both *P. monodon* and *L. vannamei*, using the same primers. Lane 1 contains 100bp ladder, lane 2 contains *L. vannamei* muscle cDNA, and lane 3 contains *P. monodon* muscle cDNA

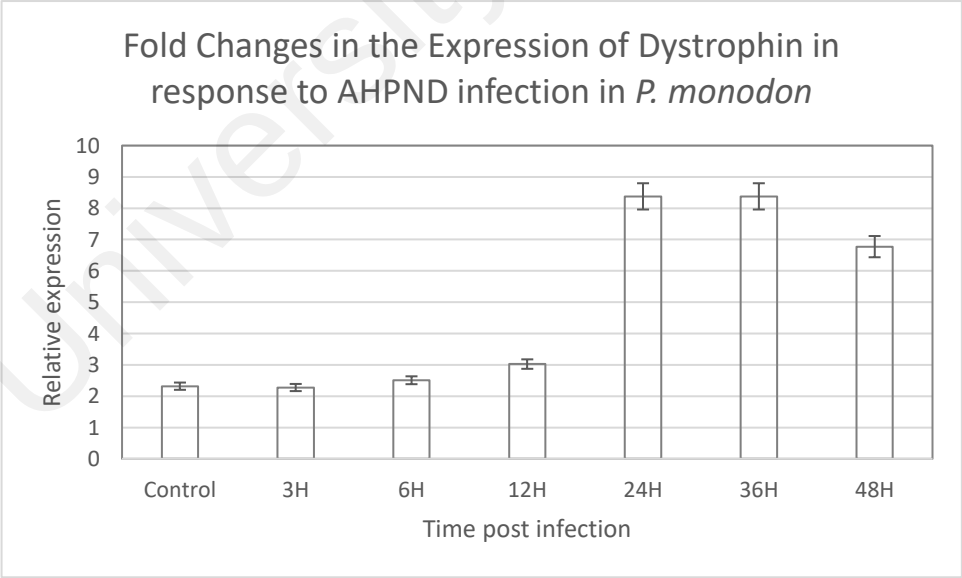


Figure 4.11: Fold changes in the expression of dystrophin in AHPND-infected *P. monodon*. Quantification was conducted in triplicates, and the values presented on the graph are mean values of the obtained data

The graph shows that dystrophin expression was already high, at over 2.0. Upon infection with AHPND, dystrophin expression did not change much at 3 hours and 6 hours post infection. There was only a slight variation at this point. At 12 hours, dystrophin expression was higher; not too high but still significant. However, there was a much more significant increase in the expression of dystrophin at 12 hours post-infection. Expression was more than three times what it was in the control group. The same was maintained at 36 hours post-infection. It dropped at 48 hours post infection but was still significantly higher than the expression in the control group (more than two times the expression).

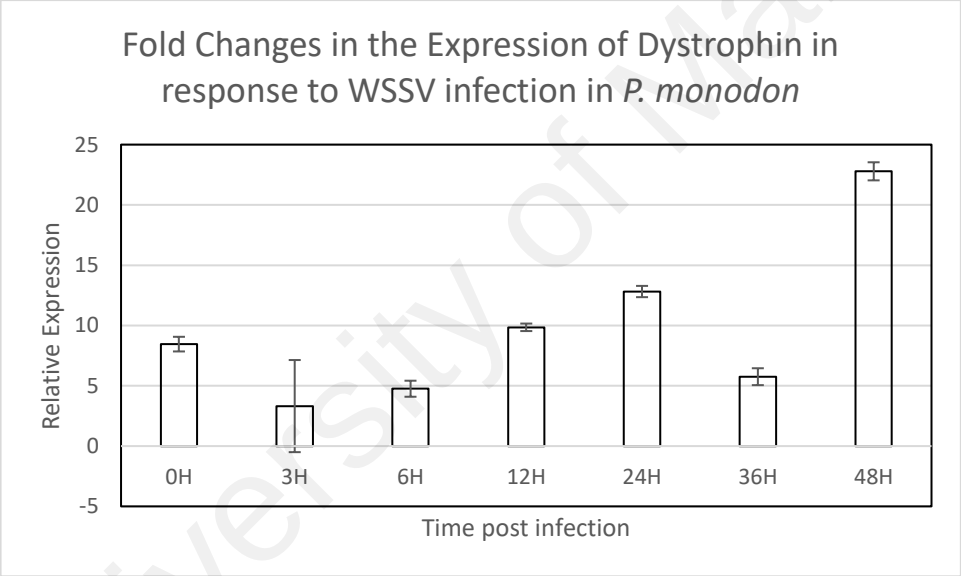


Figure 4.12: Fold changes in the expression of dystrophin in WSSV-infected *P. monodon*. Quantification was conducted in duplicates, and the values presented on the graph are mean values of the obtained data

The graph shows that the expression of dystrophin during WSSV infection fluctuated wildly, between upregulation and down regulation. At 0 hours post infection, the expression was already high, and it dropped 3 hours after infection, to less than half of what it was, at 0 hours post infection. It rose again at 6 hours post infection and rose even higher at 12 and 24 hours post infection. At this point, dystrophin expression was

already significantly higher than it was at the beginning of the experiment. However, it dropped at 36 hours post infection, to what it was at 6 hours post infection. At 48 hours post infection, the expression shot up to over double of what it was, at the beginning of the experiment. The experiment was cut off at this point.

4.5 Biomarker Validation

To validate the biomarker, its sensitivity and specificity had to be tested. To test for specificity, products form standard PCR conducted on muscle cDNA from *L. vannamei* and *P.monodon* were sequenced. The obtained sequences were then compared between the two species for similarity. The sequences were identical; the sequence is presented below. For sensitivity, the cDNA was diluted to 10µl and then used for qPCR. The primers were able to detect the template still, and a similar result was obtained with the 100µl cDNA assay.

5'- CAGATTCAATTGTGTGGTTGGCCGTTTTGCACCGAGTAGCTGCCTCTGAGAACATTCA -3'

Figure 4.13: Sequence amplified by the qPCR primers

CHAPTER 5: DISCUSSION AND CONCLUSION

5.1 Identification of *Litopenaeus vannamei* Dystrophin

A portion of the dystrophin gene in *L. vannamei* was successfully identified. This portion appears to have two isoforms, as there were two distinct bands on the Agarose gel, for each sample, after electrophoresis was conducted. After bioinformatics analysis (Fig. 4.4), this was confirmed as several exons were missing for the smaller isoform, which were present in the bigger. This is not surprising, as the dystrophin gene in humans is known to have several isoforms with several functions (Muntoni *et al.*, 2003). Identified dystrophin-like genes in invertebrates such as *Drosophila melanogaster* have also been shown to have isoforms. An interesting finding is the fact that, when the same primers were used on DNA obtained from the muscle of *L. vannamei*, instead of mRNA, the same sequence was obtained. This could mean that this is a continuous region of the gene, with no introns.

The protein was not characterized, as there are still parts of it left to be identified. A full characterization and structure prediction at this point would be counterproductive, as it would have to be updated in the future. However, two conserved protein domains were identified; the EF-hand conserved domain and the ZZ superfamily conserved domain which are usually found in the dystrophin-dystrobrevin family. The arrangement follows the same pattern as it does in the dystrophin of other organisms. This does confer some level of function on the region that was identified. These functions would include Calcium/ metal binding (Johnson *et al.*, 2000) and binding to dystrophin-related proteins, specifically to β-dystroglycan (Vulin *et al.*, 2014).

The EF-hand region can bind Calcium and Magnesium and its properties have even been adopted by bacteria when they are useful (Johnson *et al.*, 2000). The ZZ domain is one of the two known binding sites of the central rod of dystrophin; the other being the

WW domain. It coordinates zinc finger motifs and mediates the binding of dystrophin to β-dystroglycan (Vulin *et al.*, 2014).

From what was observed (Fig. 4.7, Fig.4.8), the region that was obtained is downstream of the gene and so, if a longer portion is to be identified in the future, the target would be upstream. More of the EF-hand motifs are expected to be found upstream, together with the WW conserved domain and the spectrin repeats which have been found in other vertebrate and invertebrate dystrophin. Only a portin of the ZZ-superfamily conserved domain is left to be identified downstream. On further analysis, the shorter region that was obtained, possessed both conserved domains that were observed. The portions that were missing in the shorter region, but present in the longer region, were analysed and did not contain any conserved domain. It can be inferred from this that the shorter region of the gene is just as functional as the longer region. The reason for this event could be the existence of repeated identical sequences in the gene, which also happens to be the target of the primers used.

The phylogenetic tree that was constructed shows the relationship between the obtained region and the dystrophin from other species/organisms. The *L. vannamei*, *P. monodon* and *M. rosenbergii* dystrophin(s) are in the same clade and do not seem to have diverged too much from one another. The closest in relationship to them is the *D. melanogaster* dystrophin, followed by the *C. elegans* dystrophin. Much cannot be discussed from an evolutionary point of view since the full sequence has not been obtained, and the tree itself was not constructed using all the full sequences of the other organisms. The tree was constructed using the conserved region across all the species. This region happens to be in the EF-hand conserved protein domain, so it can be said that this domain, which is a superfamily of many EF-hand motifs, is pretty conserved. This is supported by literature, which shows that the EF-hand conserved domain maintains almost the same length in arthropods as in mammals (Noor *et al.*, 2017); hence, this makes

it a suitable domain to design the primers from, since it is intended to be used for more than one species.

The primers for the biomarker were specific (Table 3.5), and so far, have worked for the penaeid shrimps, *L. vannamei* and *P. monodon*. It remains to be seen if they can work for other shrimp species and even other crustacean species. It would be interesting to research the possibility of using this biomarker across both vertebrate and invertebrate species. This would also lead to the possibility of using shrimps as model organisms for the study of dystrophy. This possibility is not too far-fetched, since the *C. elegans* dystrophin, which is closely related to the shrimps' dystrophin, has been extensively studied and is being used to study muscular dystrophy (Gieseler *et al*., 2000). Also, *C. elegans* is being used widely as a model organism for genetic diseases (Kaletta & Hengartner, 2006). *D. melanogaster* is another invertebrate that is being used as a model organism for human diseases (Lloyd & Taylor, 2010).

Similar to *C. elegans*, shrimp are easy to culture and also grow fast. While they do not always reproduce fast, they produce a large number of eggs per spawn. Their anatomy is also quite simple and this makes them easy to use as model organisms for human diseases. Of course, extensive work has to be done to make that possible; and the entire genome would have to be known and compared to the human genome.

5.2 Quantification of Dystrophin in *Vibrio parahaemolyticus*-infected *Penaeus monodon*

The shrimp had been infected with *Vibrio parahaemolyticus* Strain KS17.S5-1, the causal agent of acute AHPND. The obtained result (Fig. 4.11) clearly shows that the expression of dystrophin was upregulated during the AHPND infection. Initially, the upregulation was not pronounced, up until about 12 hours after infection. 24 hours post

infection was when the expression really shot up to almost three times what it was at 12 hours post infection. This exponential increase was maintained at 36 hours post infection, and only dropped about a fifth, at 48 hours.

During this study, the normal gross clinical signs of AHPND infection were not observed in the experimental shrimps. They were active all through the study, although the infection was confirmed. This could explain the increase in expression of dystrophin; they thrived despite the infection, so it is possible that they had compensated for that with over-expression of dystrophin. The experimental shrimps had survived up to ten days, but eventually died, albeit showing slow mortality. It can be said that the infection caused a lot of stress on the muscle, as evidenced by the expression observed and it could also be related to the death of the shrimps. It looked unlikely to recover from such stress. This is a classic example of an asymptomatic infection, in which the shrimps showed no symptoms of the infection but eventually died as a result of the infection.

The activity of the shrimps is not very surprising, since similar reports have been made of *C. elegans* showing defective behaviour, like hyperactive locomotion and exaggerated bending of the head, while having a null mutation in its dystrophin-like gene (Gieseler *et al.*, 2000). No muscle degeneration was observed and it was postulated that this was because of *C. elegans* short life span. Usually, phenotypes take weeks to appear in mammals and since the animals did not have such time, muscle degeneration was not observed till they had used up their lifespan. Although the current study is not on mutations in the dystrophin gene, this bit of literature shows that muscle degeneration may be delayed, if dystrophin is indeed affected.

On the other hand, diseases and infections do not necessarily lead to dystrophy; just overactivity in the muscle. This is also supported by studies that have shown upregulation of dystrophin homologues during isolated cardiomyopathy (Chan *et al.*, 2018). Hence, upregulation or downregulation of dystrophin expression can be in

response to a disease; and this makes it suitable as a diagnostic biomarker. Again, this only supports the hypothesis of the study, that diseases and infections can be asymptomatic, and there are markers that can detect other responses to infection. This dystrophin marker is such a marker.

Furthermore, fluctuation in the expression of dystrophin could be predictive of an underlying problem. Studies have shown that similar muscle gene expression patterns were observed in DMD patients, and in the muscles of healthy people who underwent endurance exercise training (Timmons *et al.*, 2005). The findings show clearly that stress takes its toll on the expression of muscle genes. Therefore, if shrimps are under stress, whether as a result of an infection or not, the pattern in the expression of dystrophin could be used as an indicator of this.

Also, muscle degeneration is not dependent on dystrophin mutations alone. Other mechanisms are involved, and mutations in other genes could worsen the phenotype. Although overexpression of dystrophin has not been shown to lead to muscle wasting, which is supported by the results in this study, over expression of related proteins have been shown to lead to lethal muscle wasting. In one study, overexpression of γ-sarcoglycan, triggered by mutations in the dystrophin gene in *mdx* mice, led to rapid muscle wasting, so fast that another generation of the mice could not be propagated (Zhu *et al.*, 2001). If the dystrophin in invertebrates/ crustaceans is more closely related to the sarcoglycan in vertebrates, there could be the possibility of overexpression of dystrophin leading to muscle wasting, in invertebrates. However, so far, this is not the case.

5.3 Quantification of Dystrophin in WSSV-infected *Penaeus monodon*

WSSV infection in this study, is a clear case of a symptomatic infection, in which all of the expected symptoms were observed. The expression of dystrophin in the WSSV-

infected *P.monodon* fluctuated between upregulation and down regulation. Taking into consideration, (Noor *et al.*, 2017)'s findings, it was expected that dystrophin expression would be altered during a WSSV infection. The pattern was just not quite as anticipated. It is possible that the pattern is different because different shrimp species were used for both studies. Nevertheless, the result was not disappointing because the expression was altered and pronounced. Upregulation and downregulation were quite pronounced during the course of this infection, and this gives rise to the possibility of muscle fibre regeneration. The exact mechanism of this is not understood, especially not from this study, but it is expected that dystrophin would play a key role.

Muscle cell regeneration has generated quite some interest, and it has been discovered that muscle fibres do regenerate by the activation of muscle stem cells, called satellite cells, in response to myofiber damage (Dumont *et al.*, 2015). It was also reported that dystrophin is expressed in these satellite cells, which gives credibility to the impression that dystrophin plays a role in muscle fibre regeneration. The discovery that mice which lack dystrophin have worse DMD phenotype if their satellite cells are dysfunctional (Sacco *et al.*, 2010), shows just how important these satellite cells are, in maintaining muscle integrity. Satellite cells have also been implicated in the regeneration of muscle fibers in denervated mammal muscles (Carraro *et al.*, 2015). It has also been suggested that satellite cells are eventually depleted or have a decrease in their regenerative capacity, and this can be linked to the progressive loss of muscle function in DMD patients. This is of course, only the case for mammals and vertebrates, but it may explain why the experimental shrimps eventually succumbed to their infection and muscle weakness.

Although it has been said that satellite cells do not exist in *C. elegans*, we cannot say the case would be the same in shrimp, since such a study has not been conducted in

them. It is possible that shrimp have satellite cells or similar cells, or a mechanism that makes cell regeneration possible.

5.4 Conclusion

In this study, dystrophin expression was measured during a WSSV infection and an AHPND infection in *P. monodon*. The primers used for this were identified from *L. vannamei*, simply because the dystrophin gene had been identified in *P. monodon* already, and the primers are meant to be used for both species, and possibly more. The expression of dystrophin in both cases was altered, but followed different patterns. There are some possible reasons for this. One could be the fact that it was different infections, so the shrimps could have reacted differently. Another reason could be just that individual shrimps react differently, irrespective of the infection. Whatever the case, the expression of dystrophin was altered in both disease states, and the primers have proven to be specific.

What is certain is, the expression of dystrophin is altered during infections, both of viral and bacterial source; although, only one of each type was examined in this study. This, therefore, makes dystrophin a suitable diagnostic biomarker. Whether the difference in expression pattern is dependent on the pathogen or type of infection and species of the shrimp, is left to be determined, in future research. The viability of dystrophin as a predictive biomarker, though somewhat established here, can be further investigated in future studies. The biomarker can be used as the biological component of a biosensor, for disease detection and prediction. This would be even easier to use in the field and by the layman.

It has been discovered that half of the genes which are involved in human diseases also exist in *C. elegans* and *Drosophila*, and this makes them good candidates for studying such genes and their functions, and the resulting diseases. The *L. vannamei* full genome

has not been sequenced yet, so it is hard to tell if the case is the same for it. Therefore, if the entire genome is sequenced and similarities are observed, it could be an addition to the arsenal of genetic diseases models.

There seemed to be a wide variation between biological replicates. On closer inspection, there was very low variation between technical replicates, which tells us that individual shrimps are affected differently. Furthermore, more individuals would be required to validate the actual acceptable expression of dystrophin gene, especially if it is to be used in a biosensor. It did not seem really necessary to take test animals at 0 hours post infection, since they had not started showing symptoms at that point, and there was already an uninfected control group. However, for the sake of studying the virulence of a pathogen, and determining if it is asymptomatic or not, it should be considered. An alternative to this, is to relate the expression of dystrophin at different time points to the expression at the first time point (T1= 3H).

Characterization of the full length of *L. vannamei* dystrophin and extensive study of it would give further insight into how this gene works. With this information, it would be possible to explore the viability of crustaceans as model organisms for the study of dystrophy and muscle function.

This study has been able to contribute to the ongoing investigation into the involvement of muscle and muscle genes in pathogenic infections in shrimps, and the effects thereon. It has been able to achieve this by showing direct effect of infections on the expression of a muscle gene, and providing preliminary data for future research; especially ones in which diagnosis will be done, using host materials.

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