INCLUSION OF SEAWEED IN DIET ON GROWTH, IMMUNE PROFILE AND ECTOPARASITE INFESTATION IN CULTURED CRIMSON SNAPPER (*Lutjanus erythropterus*) AT JEREJAK ISLAND, PENANG

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by

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LIST OF ABBREVIATIONS

H	Hepatocyte
Hb	Haemoglobin
HIS	Hepatosomatic index
Ig	Immunoglobin
LC	Leukocyte count
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
N	Necrorosis
NCC	Nonspecific cytotoxic cells
PCV	Packed cell volume
PV	Portal vein
RBC	Red Blood Cell
VSI	Viscerosomatic Index
V	Vacuolization
WG	Weight Gain
WBC	White Blood Cell

PENAMBAHAN RUMPAI LAUT DALAM DIET KE ATAS TUMBESARAN, PROFILE IMUN DAN INFESTASI EKTOPARASIT DALAM KULTUR IKAN MERAH (*Lutjanus erythropterus*) DI PULAU JEREJAK, PULAU PINANG

ABSTRAK

Kaedah penyelesaian masalah ektoparasit ikan seperti pengunaan bahan kimia dan rawatan fizikal pada ikan ialah hanya penyelesaian sementara. Kajian ini telah menerangkan masalah ektoparasit di tapak akuakultur dan menangani masalah ektoparasit dengan penambahan rumpai laut ke dalam diet ikan bagi meningkatkan imunisasi. Penyelidikan ini dilakukan keatas Ikan Merah, *Lutjanus erythropterus* kultur, dari sangkar laut di Pulau Jerejak, Pulau Pinang melalui beberapa eksperimen bersiri. Pertamanya, kelimpahan ektoparasit direkod pada tapak akuakultur tersebut. Seterusnya, eksperimen morfologi dan molekular DNA dilakukan untuk mengenalpasti ektoparasit hingga ke tahap spesis. Keberkesanan diet ikan yang mempunyai rumpai laut dikaji berdasarkan ujian pemakanan dengan tiga jenis rumpai laut iaitu produk komersial, Ocean Harvest Technology, OHT dan dua spesis tempatan, iaitu *Ulva reticulata* dan *Gracilaria changii*. Sebelum ujikaji pemakanan ikan, kesemua rumpai laut diperiksa melalui kaedah HPLC untuk mengenalpasti kompaun fukosa. Kajian rawatan ektoparasit ini telah dikendalikan selama 12 minggu dengan integrasi rumpai laut ke dalam pelet ikan dan bertempat di tapak akuakultur industri. Sasaran nilai protein dan rumpai laut dalam pelet ikan, ialah 45% dan 15% masing-masing. Kesemua eksperimen diet ikan ini dinilai untuk kelimpahan ektoparasit, pertumbuhan ikan, haematologi, histologi dan ungkapan gen *NCCRP1*. Kajian morfologi ektoparasit dapat mengenalpasti tiga spesis

ektoparasit iaitu *Neobenedenia melleni*, *Caligus clemensi* dan *Zeylanicobdella arugamensis*. Ungkapan DNA parasit diterima oleh GenBank dengan nombor kesertaan KU843501-KU843504 dan KY441717-KY441721. Seterusnya, 200 ekor ikan dikaji untuk kelimpahan ektoparasit merekodkan keputusan tertinggi (mean±SD) bagi *C. clemensi* iaitu 7276 (36.38 ± 12.20) dengan kelaziman 99%, manakala *N. melleni*, 5390 (26.95±2.90), 98% dan jangkitan minimum oleh *Z. arugamensis*, 34 (0.17 ± 0.38), 11.5%. Kajian HPLC untuk kepekatan ekstrak rumpai laut 3mg/mL menunjukan kompaun fukosa sebanyak 0.79mg/mL untuk OHT, 0.37 mg/mL untuk *Ulva reticulata* dan 0.12 mg/mL untuk *Gracilaria changii*. Eksperimen diet ikan, OHT telah menunjukan kadar kemandirian tinggi iaitu 93%, malah diikuti oleh *Ulva reticulata*, 88%, *Gracilaria changii*, 84% dan kawalan (tiada tambahan rumpai laut) terendah iaitu 75%. Untuk, kadar pertumbuhan ikan (SGR) ialah 0.5% bagi OHT manakala *Ulva reticulata* dan *Gracilaria changii* tidak menunjukan kesan ketara daripada kawalan. Seterusnya, tindak balas parameter haematologi di kajian ini menunjukan perbezaan antara kadar sedimentasi eritrosit (ESR) dan kiraan nombor sel darah putih (WBC) semasa jangkitan ektoparasit diantara diet ikan rumpai laut apabila dibandingkan dengan kawalan. Walaubagaimanapun, kajian histologi daripada hati ikan tidak menunjukan kesan yang ketara untuk kesemua eksperimen diet ikan. Akhirnya, ungkapan gen *NCCRP1* menunjukan naikkawal bagi kesemua eksperimen diet ikan rumpai laut diantara julat 2 hingga 3.4 kali ganda perbezaan dalam ungkapan normalisasi. Kesimpulannya, diet ikan rumpai laut yang digunakan dalam kajian ini lebih berkesan untuk pengurusan ektoparasit dan mungkin akan digunakan untuk jangka masa yang panjang sebagai aplikasi teknologi hijau berbanding kaedah rawatan lain di industri akuakultur Ikan Merah, *L. erythropterus.*

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ABSTRACT

The commonly used treatments such as chemical and physical remedies for ectoparasite are only temporary solutions. This study has elucidated the ectoparasitic problems in the aquaculture industry and addressed these parasitic infestations through seaweeds inclusion on diets as an immunostimulant. The investigation was conducted using cultured Crimson snapper, *Lutjanus erythropterus* at an aquaculture marine farm, in Jerejak Island, Penang through a series of experimental procedure. Firstly, the abundance of ectoparasites was recorded at the aquaculture site. This was followed by morphological and molecular DNA analyses to identify the ectoparasites up to species level. The efficacy of seaweeds inclusion diet was tested based on feeding trials with three different types of seaweeds namely a commercial product, Ocean Harvest Technology (OHT) and two locally cultivated species, *Ulva reticulata* and *Gracilaria changii*. Before the feeding trials, all seaweeds were screened using high pressure liquid chromatography (HPLC) for fucose compound. The feeding trials were conducted for 12 weeks by incorporating these seaweeds into fish pellets at the aquaculture rearing site. Protein and seaweed requirements in all diets were targeted at 45% and 15% respectively. All seaweeds diet experiments were evaluated for ectoparasite abundance, growth, haematology, histology and *NCCRP1* gene expressions. Morphology investigation identified three species of ectoparasites namely *Neobenedenia melleni*,

Caligus clemensi and *Zeylanicobdella arugamensis*. The DNA sequences for each parasite species were accepted by GenBank with accession numbers of KU843501-KU843504 and KY441717-KY441721. Meanwhile, 200 fishes examined for ectoparasite abundance resulted higher (mean±SD) for *C. clemensi* a total of 7276 (36.38±12.20) with prevalence 99%, followed by *N. melleni* at 5390 (26.95±2.90), 98% and lowest by *Z. arugamensis* at 34 (0.17±0.38), 11.5%. HPLC screening on 3mg/mL seaweed extracts revealed fucose compound of 0.79mg/mL for OHT, 0.37 mg/mL for *Ulva reticulata* and 0.12 mg/mL for *Gracilaria changii*. The OHT resulted in the highest survival rate at 93%, followed by *Ulva reticulata*, 88%, *Gracilaria changii*, 84% and lowest in the Control diet experiment (no seaweed added) at 75%. For growth parameters, specific growth rate (SGR) was 0.5% with OHT, while *Ulva reticulata* and *Gracilaria changii* diet experiments did not show any significant effects from Control. In addition, haematological parameters showed alterations for blood erythrocyte sedimentation rate (ESR) and number of white blood cells (WBC) during ectoparasite infestations between seaweed diets compared to Control diet. Meanwhile, histological assessment of the liver showed no severe effects due to the seaweeds diets. Lastly, expression profile of *NCCRP1* gene was up regulated during the seaweeds diet experiments as it ranged from 2 to 3.4 fold differences in normalized expression. In summary, inclusion of seaweeds into fish diet for ectoparasite management is effective and may be used as long term sustainable ‘go green’ applications compared to other existing treatments in the aquaculture industry for Crimson snapper, *L.erythropterus.*

CHAPTER ONE

INTRODUCTION

1.0 General Introduction

Diseases in aquaculture are caused by a complex interaction between fish host, pathogens and environment (Snieszko, 1974). Diseases in cultured fishes can become an outbreak if certain conditions are fulfilled (Olivier, 2002). Examples of some outbreak conditions are like presence of pathogens, parasites in water source, presence of susceptible suitable host and viability in terms of fish stocking density (Olivier, 2002). Furthermore, once disease becomes firmly established into the natural farming environment, there is no possibility for eradication and only control measures can be implemented (Olivier, 2002). Some of the dominant ectoparasites found in Southeast Asian region cultured marine fishes include various species of caligid parasitic copepods, marine leeches and monogeneans (Chong and Chao, 1986; Kua *et al.*, 2010; Main and Rosenfeld, 1995; Seng, 1998; Snieszko, 1974). Numerous studies on ectoparasite fauna have indicated that the dominant species of parasites in every fish species are the same despite the cultured or wild types (Leong and Wong, 1990). However, the main differences between the wild and cultured fishes are the number of parasites in both groups, where cultured fishes have more parasites than the wild fishes

(Leong and Wong, 1990). In natural farming conditions, the level of ectoparasite infection in a host is expected to be considerably low for a healthy Crimson snapper, *Lutjanus erythropterus*. However, at intensive over stocking densities, temperature and salinity conditions favorable to the reproduction of ectoparasites would increase the infestation rate (Seng *et al.*, 2006). Thus, when the interrelation stability bond between parasite and fish is broken, it will lead to parasite outbreaks (Seng *et al.*, 2006). In relation to this phenomenon, when a parasite infection weakens the fish immunity, it will further contribute to increased bacterial and viral diseases (Seng *et al.*, 2006).

Parasitic copepods such as caligids are widespread throughout the marine culture facilities of snapper (*Lutjanus* spp.) and other fish species (Maran *et al.*, 2009). Previous studies have recorded five species of caligid infestations consisting *Caligus chiastos C. epidemics, C. longipedis, C. punctatus* and *C. rotundigenitalis* from Penang and Langkawi Island, Malaysia (Maran *et al.*, 2009). These copepods are an important threat to the marine aquaculture industry due to its high infestation capabilities (Maran *et al.*, 2009). Leaw *et al.* (2012) recorded parasitic outbreak from *C. rotundigenitalis* with high infestation rate of 81% prevalence after a 10 months culture period involving Crimson snapper, *L. erythropetrus* from floating cages in Penang Island. Besides that, another type of ectoparasite that has been regularly reported in cultured marine fishes is marine leech, *Zeylanicobdella arugamensis* (Kua *et al.*, 2006). Kua *et al.* (2010) recorded that on May 2006, there was an outbreak of 60% mortality in marine cage culture at Bukit Tambun, Penang Island due to heavy *Z. arugamensis* infestations. Furthermore, from this outbreak, a secondary infestation from

bacteria *Vibrio alginolyticus* was also recorded at that marine aquaculture farm site (Kua *et al.*, 2010). In addition to that, another type of commonly reported ectoparasite is from capsalid monogenean. Seng (2014) reported that parasite infestations of *Benedenia epinepheli* with 70% prevalence, *B. lutjani* and *Neobenedenia girellae* as 100% prevalence in Crimson snapper, *L. erythropetrus* marine cage culture at Bukit Tambun, Penang. Additionally infestations of capsalid monogenean predisposed to the secondary infestation from bacteria *Streptococcus iniae*, further causing the red boils diseases in its farm (Seng, 2014).

1.1 Problem Statements

Despite knowing the outbreaks from this parasite threats in marine fish farm cultures, only several publications have explained the accurate species identification of parasites. As an example, the morphological observations for *C. rotundigenitalis* and *C. clemensi* copepods were similar and it cannot be conclusive on accurate species (Kabata, 1972). The daunting tasks of these identifications were due to parasites direct, fast and rapid proliferation life cycles from stages of nauplius, copepodid and chalimus (Kabata, 1972). Approximately in 40 years of research, there are only about five papers mentioning about the complete development stages and morphological descriptions of *C. clemensi* with no claims to be laid for absolute certainty of observations presented in their papers.

Besides that, capsalid monogenea, *Neobenedenia melleni* has also similar problems in morphological identification. Past studies have recorded that *N. girellae* and *N. melleni* were similar in morphological identifications due to inadequate and inconsistent morphological records (Whittington and Horton, 1996). In addition to that, all the morphological diagnosis for marine leech, *Z. arugamensis* are more certain compare to caligids and monogeneans. However, the lacks of pulsatile vesicle from *Z. arugamensis* in its morphological classification records deserves a further investigation. According to Nagasawa and Uyeno (2009), the lack of pulsatile vesicle in *Z. arugamensis* compared to other marine leech species was due to evolutionary process and suggested that morphological diagnosis of its species needs to be supported with other methods for absolute certainty. In summary, lack of uniformities in parasite identification studies, absence of identified parasite types and materials are regarded as species inquirenda.

Despite the limitations, advancement of technology has made molecular evidences as essential supporting tools for parasite identifications. Following this conception, several studies have recorded the DNA sequences from some caligids, capsalid monogenea and marine leech species (Jones *et al.,* 2006; Olson and Littlewood, 2002; Williams and Burreson, 2006). The contribution of knowledge in understanding parasites up to its species level is important in aquaculture parasite controls. Upon understanding the parasites species than, one can implement its accurate control measures due to each and every parasite species would adapt differently in its life cycles and environment.

Traditionally, the parasite infestation problems in marine cage culture system have been addressed with several approaches. One of the simplest approaches is to stop the production cycle in fish farms. The reason for this is to address the stocking density problems, but however this approach have major disadvantages in aquaculture due to its major profit reduction and lack of cooperation between aquaculture companies. Major problems in implementing control measures to prevent parasitic infection in net-cage fish farming in Asia is the overlapping generations and species of fishes (Johnson *et al.*, 2004). There is no break in their production cycle or following before the next batch of fish is stocked (Johnson *et al.*, 2004). As a consequence, naive fish introduced into the farm would likely be infected with one or more species of parasite which already exist on the farm (Woo and Buchmann, 2012). Therefore, the fish farm itself is a reservoir for parasites. Given the above scenario, how could one then, be able to control parasitic infections in a farm, it is not possible to have a parasite free environment (Woo and Buchmann, 2012). However, one can implement fish immunostimulant control measures to limit the populations of parasites in fish and to minimize the associated diseases.

Another parasite treatment method was freshwater bath systems that have been investigated against copepod and monegenean parasite infestations although the exposure time differs among parasite and fish species (Pironet and Jones, 2000). Studies have shown that the freshwater bath treatment on *Caligus* spp. have been effective for pre adult and adult stages with short exposure time duration (Fajeravila *et al.*, 2008). However the chalimus stage of parasite could not be removed from fish with short exposure time in freshwater bath system

(Fajeravila *et al.*, 2008). Similarly, Stone *et al.* (2000), suggested that the water bath treatment was only effective against pre adult and adult stages of *Caligus* spp., but cannot eliminate the chalimus stage which would continue the infestation. Effectiveness of freshwater bath on vulnerability of different monogenean species to treatments could be influenced by either the biology of the species or environmental factors such as temperature and salinity (Ernst *et al.*, 2005). Previous study had shown that a 60 minute bath treatment could remove 99% immature and adult monogeneans in marine cage fishes (Fajeravila *et al.*, 2008). However, this long duration in freshwater bath would be fatal for the cultured snapper (*Lutjanus* spp.) (Fajeravila *et al.*, 2008). In addition, *N. melleni* in seawater cultured Florida red tilapia was also found to be resistant to hyposaline conditions in commercial scale fish farm showing that ineffectiveness of these measures in controlling this type of parasite infections on cultured fishes (Ellis and Watanabe, 1993).

The use of other methods like formalin bath system have also been utilised in parasite treatments (Cruz-Lacierda *et al.*, 2000). The use of a 60 minute, 50 ppm formalin bath was recommended as treatment for leech infections (Leatherland and Woo, 2006). However this method fails to address the deposition of marine leech cocoons, which are resistant to chemical treatments (Leatherland and Woo, 2006). It has also been reported the use of formalin and freshwater bath systems with aeration on cultured snapper (*Lutjanus* spp.) would increase the host mortality after 30 minutes of treatment (Liang and Leong, 1992). Thus, a better and efficient parasite treatment method is necessary in fish farming industry.

Another major problem in the current parasite disease management in open marine cage culture is that it has only provided temporary solutions such as removing the attached parasites, however there are no effective methods to increase fish resistance or immune stimulant against further parasite infestations (Woo and Buchmann, 2012). The water or chemical bath treatments are often ineffective in killing developing embryos within eggs of parasites, are labour intensive, stressful to the fish and causes mortalities due to non optimal bath solution concentrations (Williams *et al.*, 2007). Furthermore, although bath treatments can be used to eliminate the parasites, re-infection from untreated eggs and larvae in the open marine culture environment may occur immediately following to the treatments (Fajeravila *et al.*, 2008). In contrast, in closed systems such as brood stock facilities, land based nurseries, aquaria, effective parasite control only requires methods to eliminate viable parasite and eggs from tanks and equipment (Ernst *et al.*, 2005).

Reliability of natural compounds have been identified as an emerging alternative as immunostimulant in controlling diseases in aquaculture industry (Blunt *et al.*, 2015). Within this framework, seaweeds have been found to have potential as immunostimulant for controlling fish diseases in the aquaculture industry (Blunt *et al.*, 2015). Seaweed is rich in biological active natural product with antibacterial, anti-inflammatory and anthelmintic properties (Smit, 2004). Eventhough seaweed posses potential compounds against parasite treatments, it still do have some antinutritional properties that could affect the growth of carnivorous fish. According to Omnes *et al.* (2017), *Ulva* sp. seaweed inclusion of 100g in diet for European seabass, *Dicentrarchus labrax L.* only contain a

total of 1.6g kg $^{-1}$ tanin, which did not affect the digestibility and growth. The anti-nutritional properties from seaweed can be controlled by lower inclusion levels on carnivorous fish diets.

A study has shown the efficacy of seaweed treatment in inhibiting the larval development of *N. melleni* and almost entirely, prevented the hatching and attachment of these parasites to host fishes (Hutson *et al.*, 2012). However, this study was conducted in-vivo to fish and is still debatable for sustainable long term usage. This is due to the resistance capabilities of *N. melleni* against the chemical and hyposaline bath treatments which could be similar situations for seaweed extracts. Besides that, in another study El-Ghany and Alla (2008), used seaweed, *Fucus vesiculosus* extracts (2g kg^{-1} body weight) inclusion on Nile tilapias, *Oreochromis niloticus* diet for immunostimulant experiments againts protozoan endoparasite *Ichthyophonus hoferi* and found reduced mortalities. The effects of seaweed against parasites on fish were proven from both of their previous studies. However the applicable and sustainable seaweed approach would be in production of feed inclusions for immunostimulant against parasites. Thus, based on previous studies result, it can be hypothesized that seaweed natural products can be used not only against *I. hoferi* and *N. melleni* but also on other species of parasites in marine aquaculture systems. Moreover, the application in industrial farm conditions will be only accessible and economical through feed inclusion as an immunostimulant property. Furthermore, the biological properties from seaweed will be able to stimulate the fish immune systems in order to sustain its survival rate against parasite infestations.

1.2 General Objectives

The general objectives of this study are as follows:

1. To elucidate the ectoparasite species, infestations abundance and to conduct detail abiotic factors analysis on parasite infestations in cultured Crimson snapper, *Lutjanus erythropterus* from an industrial aquaculture farm.

2. To investigate the immunostimulant fucose compounds in seaweeds and its effectiveness on fish feed application for managing the ectoparasite infestations, survival rate and growth performances.

3. To evaluate the haematological response and gene expression of *NCCRP-1* gene in cultured Crimson snapper, *Lutjanus erythropterus* during the seaweed feed inclusions against ectoparasite infestations.

CHAPTER TWO

LITERATURE REVIEW

2.1 Aquaculture of Crimson Snapper (*Lutjanus erythropterus*)

Producers and researchers have focused their attention towards cage culture because of its high yields, cost affectivity and fish cultured in cages are under similar conditions to their natural environment (Kongkeo *et al.*, 2010). However, in any fish production system, cage culture has advantages and disadvantages that should be considered before choosing a production system (Hussain and Khatoon, 2000). Several advantages of snapper (*Lutjanus* spp.) farming in floating cages are like the water renewal factor which is higher in oceans and solid waste is not accumulated near the cages resulting in low variations of physicochemical variables (Alongi *et al.*, 2003). Furthermore, major disadvantages of floating cages are the requirements to use areas with depth and protected shorelines from severe wind, water currents, storms other problems include poor bio security, and water supply cannot be filtered (Huguenin, 1997). Floating cage farms in Malaysia are comparatively medium and large-scale operations (80-120 cages). Farms are well constructed with wooden walkways, sheltered house (accommodating 6-10 workers) and equipped with freshwater supply, electricity and high-pressure pumps for net cleaning (Kechik, 1995). Previous studies have

recorded that the growth rates of snapper (*Lutjanus* spp.) are faster in floating marine cages (Castillo-Vargasmachuca *et al.*, 2007). Furthermore, in comparison with snapper (*Lutjanus* spp.), raised in floating net cages with hatchery reared are with almost same mean specific growth rate, SGR of 1.0% till 1.2% (Hernandez *et al.*, 2015). However the economic apparent food conversion ratio, AFCR of fish fed in floating net cages were substantial lower values compared to fish fed in tanks, thereby floating net cages techniques reduce the cost of feed and increase the economic benefits to producers (Hernandez *et al.*, 2015).

In Malaysia, the Crimson snapper, *L. erythropterus* species are cultured in hatchery and later upon maturity, transferred to floating marine cages (Kechik, 1995). Brood stock practises are usually from wild caught fishes with short stimulation period of maturation and ovulation with hormones (Emata *et al.*, 1999). Nevertheless, not all wild female brooders will be able to spawn when captured and transferred to concrete pond hatcheries (Cai *et al.*, 1997). Alternative method to overcome these spawning problems is to cultivate broodstocks in sea cages for improving gonadal development in most of aquaculture species (Li *et al.*, 1996). However according to earlier studies, for Crimson snapper, *L. erythropterus* brood stock was easily adapted to captivity hatchery and the gonad can be developed easily (Hong and Zhang, 2003). Some studies showed similarities to natural spawning condition during monsoon season from November to February which are most suitable for captivated broodstocks (Bolong and Munafi). Moreover the study also demonstrated successfully, the induced spawning of this Crimson snapper, *L. erythropterus*, with human chorionic gonadotropin HCG as an effective agent for inducing final maturation and ovulation leading to spawn (Bolong and Munafi). Spawning will occur after 24 hours of injection with suggested dose, water

temperature about 28.5°C and salinity, 30 ppt respectively (Emata *et al.*, 1999). The eggs for snapper (*Lutjanus* spp.), has a mean diameter of 0.80 mm at the time of spawning (Hong and Zhang, 2003). First hatching of fertilised eggs occurs at 14 hours after spawning and newly hatched larvae measured 1.66 ± 0.15 mm in total length (Bolong and Munafi). The larvae had yolk sac (capacity of 0.00663 ± 0.0013 mm^3) that extended forward of the snout and the oil globule (capacity of 0.0060 ±0.0010 mm^3) was situated at the mid ventral to posterior side of the yolk sac (Emata *et al.*, 1999). The mouth will began to develop at 28 hours upon hatching but the function will start at around 35-57 hours and the yolk sac will be fully absorbed at 75 hours (Doi *et al.*, 1994). Based on mouth size development, which starts from 50 hours after hatching, the snapper larvae were willing to accept the exogenous nutrient (Doi *et al.*, 1994). Earlier studies for snapper fish showed that the first feeding for the larvae are phytoplankton in green water and upon 3 days of completed mouth development, zooplankton will be its further feed supply (Wan-shu and Qi-yong, 2002). However, for snapper (*Lutjanus* spp.) larvae, it cannot take the normal rotifers due to its small mouth size compared with other types of fish larvae and there are higher probability for mortalities around 71 hours after hatching due to feed starvation (Wan-shu and Qi-yong, 2002). The growth duration in floating cages for Crimson snapper, *L. erythropterus* is 8 to 12 months, whereby the fish will be transferred from the hatchery on 1 month to 3 month duration based on its breed growth developments (Kechik, 1995).

2.2 Seaweeds Inclusion in Fish Diets

Economic losses and disease outbreaks can be avoided by drugs which are commonly used in aquaculture. These drugs and antimicrobials are administered frequently as additives in fish feeds, water baths treatments, therapeutics injections. However the application of drugs and chemical treatments are becoming restricted since they cause various harmful side effects to the environment (Rico *et al.*, 2013). Examples, as been reported in previous studies on the massive use of antibiotics that have resistant bacterial strains or the presence of residual antibiotics in the muscle of commercialized fish and thus has contribute to negative consequences on human health (Romero *et al.*, 2012; Seyfried *et al.*, 2010). Furthermore, the use of antibiotics would be resistant against parasites and it cannot be implemented for sustainable aquaculture. Another method which is vaccination method is the potential treatment against disease outbreaks in aquaculture, however commercial vaccines are too expensive for a widespread use by fish farmers and the effectiveness of vaccine is only specific to single type of pathogen (Harikrishnan *et al.*, 2011). Considering the potential harm of drugs, chemicals and limitation factors of vaccine, disease management in aquaculture should be focused on harmless, preventative and sustainable methods.

In previous studies the methods to manage fish disease in aquaculture are often associated with fish health and immunity, thus many researchers have applied plant products to elevate the immune system in fish. Moreover, with the application of plant extracts in fish feed, it also contributes to the interest of consuming organic, “go green” and environmental friendly food products (Reverter *et al.*, 2014). Following this

conception, some recent studies reveal that seaweed have the potential source of antimicrobial and parasitic products. A previous study showed that the infection rate for monogenean parasite in *Lates calcarifer* decreased upon seaweed, *Asparagopsis taxiformis* (51%) and *Ulva* sp. (54%) extracts in seawater application compared with the control (71%), (Hutson *et al.*, 2012). Hutson *et al.* (2012) suggested that seaweed extracts could be only successful in either closed or integrated farming systems. However no assessment has been done on the applicability of these methods under inclusion of feeds in farming conditions. Besides that, another report when seaweed fucose extract (2 g kg^{-1} body weight) was applied for 3 months in an experiment with infected protozoan endoparasite *Ichthyophonus hoferi*, exhibited reduced mortality in Nile tilapias, *Oreochromis niloticus* (El-Ghany and Alla, 2008). Moreover, it should be noted that in Nile tilapias, *O. niloticus* previous research did not provide adequate information on the characteristic, production and extraction of compounds from seaweed. Yang *et al.* (2014) have shown that fucoidan compound from seaweed, *Sargassum horneri* influenced the blood characters and non specific immune response in Yellow catfish, *Pelteobagrus fulvidraco*. Similarly, Peixoto *et al.* (2016) have shown that seaweed consist of *Ulva* sp. and *Gracilaria* sp. with 7.5% and 2.5% inclusion in diets for European seabass, *D. labrax* have shown increase respiratory burst and innate immune system stimulations. Both of their studies are from carnivorous fish species, which is similar to this current study and the innate immune system stimulation properties from seaweed would work against ectoparasite infestations. This is because the pathway mechanisms of ectoparasite responses in fish are from the skin response mechanisms in its innate immune system. Activation of skin mucosal mechanisms as a response to monogeneans is from activation and productions of cytokines (IL-1 and

possibly TNF and INF) (Buchmann, 1999). Cyctokines direct the leucocytes to the monogeneans inflammation sites and noxious substances including reactive oxygen metabolites will be released by leucocytes to affect the monogeneans (Buchmann, 1999). Thus, seaweed inclusions in fish diet would stimulate and increase the production of cytokines in innate immune system for its responses towards ectoparasitic infestations.

Besides those immune properties, alternative use and development research for growth enhancement in aquaculture remains high priority. Extensive studies have been conducted to check the nutritional values of alternative protein sources and growth enhancers especially from plant sources to solve these problems (Venou *et al.*, 2003). Seaweeds are rich in proteins, minerals, vitamins, in soluble dietary fibers, antioxidants, and polyunsaturated fatty acid (Zhu *et al.*, 2016). Varieties of seaweed species, such as the red alga *Porphyra* (Araujo *et al.*, 2016), brown algae *Ascophyllum nodosum* (Nakagawa *et al.*, 1997), *Gracilaria cornea*, *Gracilaria vermiculophylla* (Valente *et al.*, 2006), green Algae, *Ulva rigida* (Valente *et al.*, 2006), have been examined for incorporation into aquaculture feeds. Most of these studies have reported the potential of seaweed as feed ingredient and partial inclusion in fishmeal or protein in aquaculture feeds. Furthermore, in recent studies a low level of seaweed meal inclusion have been utilized as a successful feed binder, additive substance for enhancement of digestibility resulting in higher fish growth (Shapawi *et al.*, 2015). In addition, a previous study recorded that the inclusion of 15% level of *Ulva* sp. seaweed in fish diet produced a higher growth performance in Red tilapia, *Oreochromis* sp. (El-Tawil, 2010). The level of seaweed inclusion varies from every studies and it has species specific responses for its growth effects. These factors can be also due to anti-nutritional properties from seaweeds. Seaweeds contain appreciable amounts of polyphenolic substances, broadly

referred to as tannins. Polyphenolic tannins show both positive and negative effects, they possess anti-nutritional properties, but are also beneficial for health due to their role as antioxidants and their ability to stimulate the immune system and various effectors (Omnes *et al.*, 2017). Tannic compound has also been reported to have antimicrobial activity against fish pathogens (Omnes *et al.*, 2017). The incorporation of green algae 50g and 100g, (Valente *et al.*, 2006) or seaweed 25g and 75g, (Peixoto *et al.*, 2016) in the European seabass, *D. labrax* diets, and the inclusion of seaweed up to 100g, (Guroy *et al.*, 2013), in the Rainbow trout, *Oncorhynchus mykiss* diets also did not adversely affect the growth. However, incorporation of *Ulva* sp. meal 100g to 300g that brought tannin levels to 1.6g to 2.2 g, to replace soybean meal in the diet of Nile tilapia, *O. niloticus*, impaired growth (Azaza *et al.*, 2008). Thus, differences in nutrient digestibility appear to be species specific, and depend on the tolerance of the fish to tannin containing plant protein sources and tannin supplements. In accordance to a previous study, the nutrient requirement for Crimson snapper, *L. erythropetrus* is 43% of protein, 11% of crude lipid, 1.5% of crude fiber, 22% carbohydrates, 3.5% to 5% of vitamin and mineral premix (Liao *et al.*, 2007). However, the feeding strategy of Crimson snapper, *L. erythropetrus* is influenced by ration level and feeding frequency in farms. The feeding frequency is important for maximum growth, high feed utilization and stable body composition in fish (Booth *et al.*, 2008). In another study with snapper (*Lutjanus* spp.), it was shown that feeding level of 1% to 4.5% of body weight and feeding frequency 3 to 4 times daily had the highest growth performance with significantly higher weight gain and better feed conversion ratio (Abbas *et al.*, 2015).

2.3 Fish Parasite Disease

The natural environment in ocean will have ectoparasites which co-exist in wild fishes, however only cultured fishes are prone for ectoparasite infestation problems. Mortality outbreaks from large number of fishes are rarely experienced in the wild and, when they do occur, it is most likely due to unexpected environmental deterioration (Liang and Leong, 1992). However, in restricted net-cage environment, mortalities are often seen (Liang and Leong, 1992). Signs of such abnormalities in fishes can be detected from its behavior, darkened body, exophthalmia (pop-eye) and ulcerations on the fish body (Liang and Leong, 1992). There are many causes of fish mortalities in the confined net-cage environment but highly possible causes for these disease outbreaks are pathogenic parasites (Seng, 1997). Parasites are invertebrate organisms, some are free-living and can become opportunistic parasites, others require hosts for their survival and reproduction, and these are referred to as obligate parasites (Ravi and Yahaya, 2015). Both opportunistic and obligate parasites are found in fish hosts but most parasitic diseases in fish are generally caused by obligate parasites (Ravi and Yahaya, 2015).

The parasites which exist may not or may cause illness, and this attribute is governed by diverse factors. Parasites have arisen by development from free living animals, whereby some of them have developed special organs to be able to live in host organisms (Ravi and Yahaya, 2015). Most healthy fish usually tend to harbor various parasites at low numbers, either on or in their bodies (Ravi and Yahaya, 2015). The low number of these parasites generally causes little or no harm to the fish. However, when the number of parasites per fish increases significantly (the natural parasite-host

equilibrium becomes unstable) due to changes in water temperature, overstocking or salinity that are positive to the growth, reproduction of parasites, or that cause a decrease in fish immunity, parasitic disease outbreaks often occur (Buchmann and Lindenstrøm, 2002). Besides that, there are also factors like fish length which will influence the number of fish parasite infestations (Ravi and Yahaya, 2015). Eventually, both parameters that closely correlated are abiotic and biotic factors in parasitic infestations (Ravi and Yahaya, 2015). A large diversity of parasites has been reported in cultured marine fish whereby some of these parasites have caused serious disease outbreaks in farmed fish resulting in significant financial losses to fish farmers (Seng, 1997). Parasites either cause major disease outbreaks in cultured fish or rather contribute to chronic sub-clinical effects (Liang and Leong, 1992).

In general, fishes are most susceptible at the early stages, particularly at the hatchery and juvenile stages of the culture cycle when fish are newly introduced to cage culture system (Nowak, 2007). Parasite organisms affecting cultured fish can be grouped into namely plathyhelminthes, such as monogenean, caligus and leech (Nowak, 2007). At each stage of the culture cycle, the pathogenicity of a particular pathogen on a fish host may differ (Liang and Leong, 1992). For example, the protozoa *Amyloodinium ocellatum* is very pathogenic to fry and fingerlings at the hatchery and nursery stages (Wang *et al.*, 2005). At the initial stage of the grow-out phase of net-cage culture, the newly stocked fish become increasingly susceptible to parasitic diseases caused by monogeneans (Johnson *et al.*, 2004). Many of these parasites can cause disease outbreaks and significant financial losses (Johnson *et al.*, 2004). At the nursery stage, fishes are placed in ponds, cement tanks or net cages for some time, before being transferred to grow-out cages and it is very unlikely that fish nursed in cement tanks

would have monogenean infections but they could be infected with protozoa (Wang *et al.*, 2005). If the fry are nursed in ponds or open cages, they could acquire protozoa and monogeneans, as well as other pathogens (Wang *et al.*, 2005). Moreover, at the initial grow out cage in an open sea environment, parasites such as leech, *Z. argumensis*, may cause cage infestations as stated recently in cultured Crimson snapper, *L. eryhtopterus* fish (Kua *et al.*, 2014). Furthermore, net-cage farming is an open system whereby fish reared in the cages are in close proximity with wild fish which may transfer pathogens to those inside the cages (Jithendran *et al.*, 2005). Also, in this Asian region, trash fish are commonly used as feed which can act as a source of parasites (Jithendran *et al.*, 2005). In summary there are many various factors which will influence the pathogenicity of parasites.

2.3.1 *Caligus clemensi*

The subclass caligid parasitic copepods consist of over 250 described families, 2,600 genera and 21,000 described species (Gonzalez and Carvajal, 2003; Hogans and Trudeau, 1989; Kabata, 1965). Sea lice infestation was first recorded in Malaysia on Golden snapper, *Lutjanus johni* (Leong and Wong, 1988). Furthermore, five species of copepods belonging to genus *Caligus* spp. were reported in marine farmed fish from Penang and Langkawi island in Malaysia (Maran *et al.*, 2009). Parasitic copepod Caligid are known as an economically important parasite in marine culture as it has a low host specificity and prone to fast replication in nature for infestations (Johnson *et al.*, 2004). Moreover, there is no data of host specificity in *C. clemensi* and the latter authors opinionated that the parasite will infect any species of fish inhabiting surface waters.

In past studies, the complete development of life cycles from *C. clemensi*, descriptions suggest that comparison of five *Caligus* spp. species involved differences in their ontogeny much more widely than expected in view of their close taxonomic relationship (Kabata, 1972). The complete development in life cycle for *C. clemensi* is from first nauplius, second nauplius, copepodid, first chalimus, second chalimus, third chalimus, fourth chalimus, pre-adult and adult stage (Kabata, 1972). The time duration in its life cycle remains unknown from all data records (Jones *et al.*, 2006). The damaged caused by copepods on the skin surface mainly due to its feeding activities. Microscopic observations was done on *C. clemensi* by Kabata (1974), as its moving over the surface of the fish, *C. clemensi* hold their mouth tubes folded along the ventral surface of the body, their buccal orifices pointing backwards. The mouth tube consisting tip of labium on *C. clemensi* differs from other species namely *C. curtus* and *C. rotundigenitalis* (Kabata, 1974).

2.3.2 *Neobenedenia melleni*

The next commonly found ectoparasite in marine aquaculture system is from the class monogenea, which also causes major losses to the fisheries industry. In taxonomic classification, class monogenea is divided into two subclasses namely polyopisthocotylea and monopisthocotylea (Cone and Woo, 1995). The main differences between the two subclasses are one is blood feeding (polyopisthocotyleans) and the other mucus, epithelium feeding (monopisthocotyleans) (Cone and Woo, 1995). However, these subclasses are united by various morphological synapomorpic larvae with three ciliated zones, adults and larvae with two pairs of pigmented eyes, one pair of

ventral anchors (hamuli) and one egg filament (Bentz *et al.*, 2003). Within the taxonomic subclass of monopisthocotyleans, the most commonly found parasites from Asian countries are from genus *Neobenedenia* spp. (Deveney and Chisholm, 2001). It is a widespread pathogen of many aquaculture fish species (Deveney and Chisholm, 2001).

Many teleost species in aquaculture are known to have widespread pathogen of capsalid monogenean from the subfamily *Benedenia* spp., known as *N. melleni* (MacCallum, 1927) Yamaguti, 1963 (Littlewood *et al.*, 1998). This parasite feeds on epithelial cells mucus of host fish, which gives increased effects towards irritation and mucus hyperproduction of their hosts (Leong and Wong, 1995). Like most of other monogenean groups, *Benedenids* sp. has traditionally been identified to species on the basis of morphological characters such as the shape of posterior hamuli, the type of anterior attachment organ, and the length of uterus, vitelline reservoir, and the type and relative size of testes (Whittington and Horton, 1996). Though it has been argued for a long time that morphological characters based identification of parasite can be affected, to a large extent by extrinsic factors such as the age of parasite, environmental temperature, and even artifacts caused by various dealings for specimen processing, most of monogeneans can be identified based on species to host specifications.

As discussed by Li *et al.* (2005), most monogeneans could be appropriately distinguished because of their high level of host specification. However, *N. melleni* does not obey the rule because it has been reported from more than 100 fish species belonging to more than 30 families with worldwide distributions (Whittington and Horton, 1996). Several monogenean species exhibit short life cycles in warm temperatures, accelerated parasitic life cycles will increase the metabolic and development rate associated with warm conditions (Tubbs *et al.*, 2005). As a summary presently, the reason for the

unpredictable and irregular nature of *N. melleni* infection is unknown from many studies.

2.3.3 *Zeylanicobdella arugamensis*

Besides that, another commonly found ectoparasite in marine floating cages is the marine leech species *Z. arugamensis* from the family Piscicolidae (Leong and Wong, 1988). *Z. arugamensis* (*Ottoniobdella stellata* Moore, 1958) was originally described from Sri Lanka, however, is now known to be widely distributed throughout the Indian Ocean (Moore, 1958). It has been found as an ectoparasite on a wide range of demersal and benthic hosts from different fish families (Cruz-Lacierda *et al.*, 2000; De Silva and Fernando, 1965; Sanjeeva Raj *et al.*, 1977). The first report of *Z. arugamensis* from Malaysian waters was from the seahorse, *Hippocampous kuda* and unidentified eel species by De Silva and Fernando (1965). Host mortality usually occurs within a 3 day period following infestation due to secondary infections by pathogenic bacteria such as *V. alginolyticus* (Kua *et al.*, 2014). Ectoparasites can represent stressors for fish and have been associated with decreased food intake, anti-predator behaviour and reduced growth (Cruz-Lacierda *et al.*, 2000).

A previous study found that juvenile leeches were able to hatch at temperatures ranging from 25°C to 35°C but unable to hatch at 40°C (Kua *et al.*, 2014). The survival periods of adult and juvenile leeches ranged from 11 to 16 days at 25°C, which was comparatively longer than the 5 to 13 days and 10 h to 5 days observed at 27°C to 30°C and 35°C to 40 °C, respectively (Kua *et al.*, 2014). Hence, shorter life cycle duration

may lead to decreases in infestation levels compared to the infestation levels of other parasites types (Kua *et al.*, 2014). The most frequently identified physical effect of leeches on fish has been determined to be a localized effect of bleeding resulting from feeding activity and ulceration at attachment sites (Kua *et al.*, 2014). The morphological description of *Z. arugamensis* was first described by Desilva and Fernando, (1965) with its pulsatile vesicles. According to Nagasawa and Uyeno (2009) and Moore (1958), the the absence of pulsatile vesicles were reported in their morphological studies. Furthermore, the body pigmentation of *Z. arugamensis* is as dark brown and deep olive green with oral and caudal suckers (Nagasawa and Uyeno, 2009). The total body length of *Z. arugamensis* is known to be at ranged of 9mm to 19mm (Nagasawa and Uyeno, 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Fish Farm Conditions

This study was conducted in a floating sea cage station at Jerejak Island, Penang, Malaysia. Location of the fish farm coordinates are as 5º18’08.7’’N 100º18’52.1’’E (Plate 3.1, 3.2 and 3.3). The fish farm operations are conducted by an aquaculture company called GST group of companies, in which the study site has a total of 145 sea cages. The total numbers of cages used in this farm for Crimson Snapper, *L. erythropterus* are 60 cages, making up to 41% total farming in the area. Next, the total number of cages used for Asian seabass, *Lates calcarifer* is 40 cages, 28% of total fish farming in their area and followed by 25 cages for Cobia, *Rachycentron canadum* with 18% of farming area. Lowest usage is for Giant grouper, *Epinephelus lanceolatus,* 20 cages and 13% of farming area.

According to the GST group of company aquaculture settings, all the cages was separated in equal size of 5 meter in length, 7 meter in width, netting depth of 3 meters below sea surface. While the condition for wind velocity was less than 10 knots and height of waves less than 1 meter in that area. The criterion for water exchange in this study area is acceptable due to sufficient depth measures of each net cage which bound to maximize water exchange (Loka *et al.*, 2012; Mustafa and Shapawi, 2015). During