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Editor's Note

We are pleased to have the Malaysian Fisheries Journal (MFJ), Issue No 2, Volume 21, 2022 which serves as a special edition devoted to the the 11th Diseases in Asian Aquaculture (DAA11) Symposium, Kuching Sarawak. MFJ is an annual publication by the Fisheries Research Institute (FRI), Department of Fisheries Malaysia. The idea of this issue arose from the DAA11 National Organising Committee in commemorating the unique DAA11 which is held for the first time after a 5 year gap and as a hybrid (physical and online) event. This special edition seeks to provide platform for the young reseachers from the research community gathered for DAA11 to publish their findings. The DAA11 has received about 141 abstract submissions from 23 countries which cover a wide range of aquatic animal health aspects including biosecurity in aquaculture, epidemiology, detection, prevention, and control of diseases in finfish, crustacean and shellfish and trends in fish and shrimp health management. We are pleased to bring you a compilation of several articles presented in the DAA11. It is hoped that the papers will provide the reader with new and current information on the animal health aspects.

MFJ is freely available with special request to the Library FRIBM, 11960 Batu Maung, Pulau Pinang, Tel No: +6046263925/26

Fish Wear Their Immune System on the Outside – What This Means for Aquaculture and Ecology

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Abstract

Abstrak: Selepas 40 tahun akuakultur secara intensif, kita lebih mengenali ikan sebagai haiwan daripada yang pernah kita lakukan selama beribu tahun dalam perikanan. Perbezaan asas antara haiwan akuakultur dan haiwan pertanian termasuk masa yang dihabiskan dalam persekitaran terkawal yang stabil untuk organogenesis juvana (sehingga 70% daripada jumlah jangka hayat untuk terestrial dan 0-5% dalam akuatik), bilangan peristiwa evolusi penduaan genom keseluruhan yang menyumbang kepada genom semasa (2R untuk daratan dan 3R atau 4R untuk akuatik) dan lokasi mukosa penghalang imun (di dalam untuk daratan dan kedua-dua di dalam dan di luar untuk akuatik). Lapisan berlendir luaran (mukosa kulit, insang, dan usus yang berada di luar dan bahagian dalam) membentuk penghalang imun mukosa ini. Ia mengandungi bahan antiviral, antikulat, antiparasit dan antibakteria dalam dialog berterusan dengan alam sekitar selama lebih setengah bilion tahun. Sel-sel mukosa mempamerkan tindak balas berulang seluruh organ kepada rangsangan seperti terapeutik, tekanan, diet dan persekitaran. Penggunaan kaedah piawai yang tidak berat sebelah, pemetaan mukosa atau Veribarr™, selama 12 tahun dan dengan lebih 100 percubaan dalam ekologi dan akuakultur telah menyumbang kepada pemahaman yang belum pernah berlaku sebelum ini tentang bagaimana mukosa teleost berfungsi. Insang, yang merangkumi kira-kira 50% daripada luas permukaan ikan, telah terbukti sebagai amaran awal yang paling sensitif terhadap disregulasi sistemik. Beberapa hasil daripada pengeluaran berskala komersial, daripada "kerja detektif" dan daripada kajian makmal terkawal akan diserlahkan dalam kertas ini bersama-sama dengan rayuan untuk pembangunan piawaian untuk kesihatan ikan. Skop untuk pertumbuhan dalam akuakultur sebagai industri terletak dalam mewujudkan dan mengekalkan kesihatan stok yang baik.

Abstract: After 40 years of intensive aquaculture, we know the fish as an *animal* better than we ever did from millenia of fishing. The fundamental differences between aquaculture animals and agriculture animals include the time spent in a controlled stable environment for juvenile organogenesis (upto 70% of total lifespan for terrestrials vs 0-5% in aquatics), the number of evolutionary whole genome duplication events contributing to the current genome (2R for terrestrials and 3R or 4R for aquatics) and the body location of the mucosal immune barrier (inside for terrestrials and both inside and outside for aquatics). The external slimy layers (mucosa of skin, gills, and intestines which are the outside of the inside) make up this mucosal immune barrier. They contain antiviral, antifungal, antiparasitic and antibacterial substances in constant dialogue with the environment for over half a billion years. The mucous cells exhibit an organ-wide repeatable response to stimuli such as therapeutics, stress, diet and environment. The application of an unbiased standard method, mucosal

mapping or Veribarr™, over 12 years and over 100 trials in ecology and aquaculture has contributed to unprecedented understanding of how teleost mucosa function. Gills, which comprise about 50% of the surface area of fish, have proven to be the most sensitive early warning of systemic dysregulation. Some results from commercial-scale productions, from "detective work" and from controlled lab studies will be highlighted in the talk along with a plea for development of standards for fish health. The scope for growth in aquaculture as an industry resides in establishing and *maintaining* good stock health.

Keywords: Digital fish health, mucosal immunology, One Health, Mucosal Mapping

Introduction

It is tempting to manage aquaculture as though it is agriculture under water, but the aquatic animals we are rearing are very fundamentally different in 3 aspects: firstly, farmed terrestrial animals may spend upto 60% of their total lifespan from sperm-meets-egg to harvest in the protected stable environment of a maternal womb or large egg, relying on this to develop into a functional small or juvenile animal. By contrast farmed aquatic animals exert little or no maternal stability to make a juvenile and instead may broadcast or lay fertilized eggs externally and let the new organism develop in response to the variable environmental signals such as temperature, light, nutrition and physical enclosure. Secondly, the broad genetic vocabulary needed for aquatic juvenile development in a variable environment exists because of a teleost-specific whole genome duplication about 350 million years ago, or about 200 million years before men got a Y-chromosome, and contrasts to the more limited 2R genome duplication of terrestrial animals (Sato and Nishida 2010). This magnanimity regarding gene function in aquatics means that mammalian correlates for syntenic genes or para- and orthologs of teleosts may be nonexistent or misidentified. Thirdly, the external slimy surfaces of the skin and gills reflect and respond to the higher microbial diversity of eg. inland water vs air (Walters and Martiny 2020) and are the fish's immune system on the outside composed of *living* cells. It has already been shown that wiping off the mucus from the skin leads to higher mortalities than does physically wounding the skin of fish (Svendsen and Bogwald 1997). The external mucosal barriers of skin and gills have thus been protecting aquatic animals for over 500 million years (Xu, Takizawa et al., 2016). These mucosa plus that of the intestines are the interactive user-interface between the organism and the aquatic environment (Minich, Poore et al., 2020, Salinas, Fernandez-Montero et al., 2021) (Fig. 1).

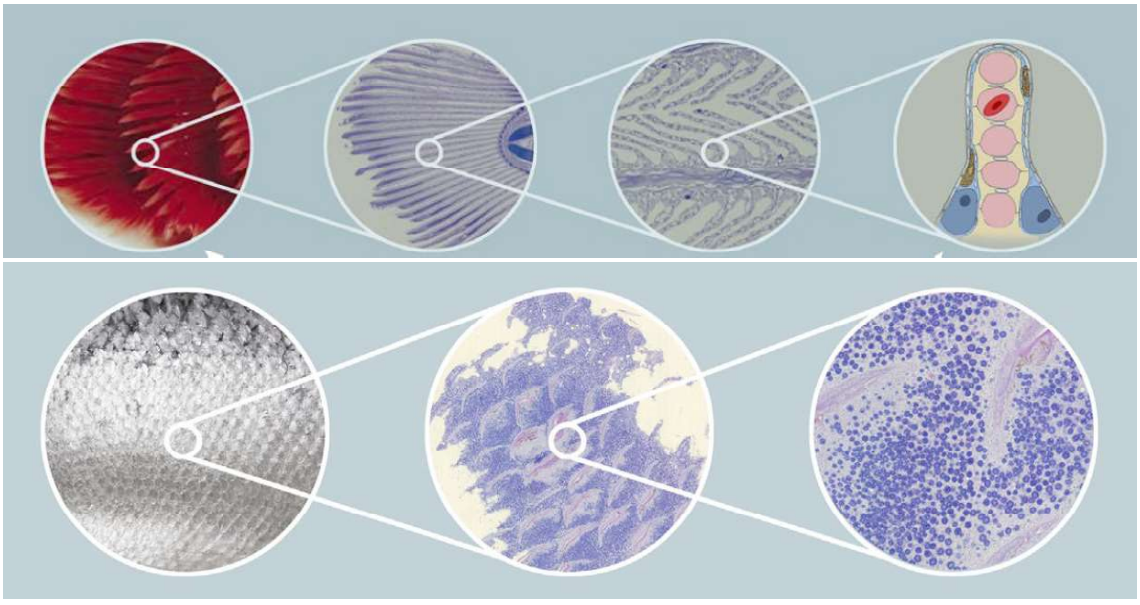

Figure 1: Top: Location of protective mucous cells in the gill lamellae (respiration). Left: macroscopic image of healthy gills; Midleft: histological preparation of gill tissue with Periodic Acid Schiff – Alcian Blue making mucous cells blue dots; Midright: closeup of prepared gill lamellae with a few blue mucous cells on narrow lamellae; Right: illustration of placement of mucous cells (brown) in the double layered membrane between water and blood cell (red). Illustration by K. Moe.

Bottom: Location of protective mucous cells on the skin, over the scales. Left: macroscopic image of healthy skin; Middle: histological preparation of skin tissue with Periodic Acid Schiff – Alcian Blue making mucous cells blue dots and scales pink crescents; Right: closeup of multiple blue mucous cells and 3 pink crescents of scales. All images from "The Robust Fish" (2019) with permission.

A mucosal membrane depends by definition on the presence of mucous cells which form an integral part of the dialogue between fish and environment and show distinct differences according to body site (Pittman, Pittman et al. 2013). The dynamic changes in the size and amount of mucous cells (hyperplasia and hypertrophy) are often cited as key characteristics of treatments and responses to pathogens, and Gjessing *et al*. (2019) state that gill disease complexes are characterized by 3 frequent findings: subepithelial leukocytes, epithelial cell hyperplasia and mucus cell hyperplasia. Foyle *et al*. (2020) highlight the essential role of healthy gills, noting that indicators of gill disease include hyperplasia and hypertrophy of epithelial and mucus cells.

Since 2011, the dynamic morphometrics of the key characteristic of any mucosal epithelia, its mucous or goblet cells (MCs), have been made measurable and statistically reliable through the application of mucosal mapping (Pittman, Sourd et al., 2011, Pittman, Pittman et al., 2013). Key work has looked at the microbia populating various mucous layers on the fish body and the immunoglobulins produced (Salinas, Zhang et al. 2011, Minich, Härer et al. 2022) current thinking suggests that mucosal epithelia produce a wide variety of substances and secretory immunoglobulins "on demand" as a function of the dynamic mucosa (Dang, Pittman et al., 2020, Salinas, Fernandez-Montero et al., 2021). Therefore, rather than focusing on the particular substances released at a chemical or organelle level, we have focused on the level of biological organization above this, the cellular and tissue responses of external mucosal barriers.

The dynamic function of the mucosa is both physical and biochemical and is exquisitely sensitive to changes in the environment. For the sensitive gills, this has been demonstrated both in the wild and in controlled or farmed situations: in thirty wild sculpins sampled at 3 stations along a pollution gradient of heavy metals from an abandoned mine in a Greenland fjord, Dang et al. (2019) found that gill lamellar mucous cells were in significantly higher density when there was a high environmental lead (Pb) load, that the size of the mucous cells in the gill filament were positively correlated with the lead level in the liver and that smaller skin mucous cells were associated with higher parasite loads. The study demonstrated that environmental characteristics induced significantly different morphodynamics of three mucosal tissues (skin, gill lamellae and gill filament) in the same species, agreeing with the concept that body site matters to the mucosal response parameters (Pittman, Pittman et al., 2013). The study also demonstrated nuanced protective abilities of the mucosal epithelia and potentially diagnostic properties.

In a series of controlled trials, salmon were exposed to various doses of peracetic acid (PAA) and repeat exposure after 2-3 weeks (Lazado, Haddeland et al., 2020, Haddeland, Lazado et al., 2021, Lazado, Timmerhaus et al., 2021, Lazado, Strand et al., 2022). Salmon not only showed that lamellar mucous cells were always significantly smaller (less than 70 square microns) and less than 2% of the volume of the epithelium while those in the filament were larger and upto 4 times more abundant, confirming the distinct nature of these two populations. The gill cells also showed an initial transient subacute hypertrophy which was linked to a small, generalized stress response. In the skin, mucous cell sizes marginally increased with increasing dose of PAA during the first exposure whereas the second exposure gave no significant changes with dose (Lazado Haddeland et al., 2020), suggesting not only adaptation or learning by this living layer but also showing the strength of the skin barrier compared to the sensitivity of the gill barrier. Rantty found that salmon gills, esophagus, and skin can take about 2 weeks to recover from one treatment of H_2O_2 and postulated that low post-treatment feeding was also due to irritated esophagus (Rantty 2016).

An application of this therapy to controlled infection with Amoebic Gill Disease made clear that the properties of mucous cell sizes and volumetric densities are a sensitive indicator of health, recovery or vulnerability (Lazado Strand et al., 2022). Four treatments were applied: one Control group was uninfected and untreated while three groups were infected with AGD and one was given no treatment, one treated with 5 ppm PAA for 30 minutes and one with 10 ppm PAA for 15 minutes. Samples collected after 24 hrs revealed that the infected groups had begun their cellular hyperplasia relative to the Control group and oxidative stress was measured in the initial phase. By 4 weeks the treatment differences in cell sizes and abundance were significant – untreated AGD induced the largest cells at the highest density and surprisingly the 10 ppm dose gave significantly larger denser mucous cells than the Control group whereas 5 ppm was closer to the Control group measures, suggesting that twice the dose of PAA was half as effective against AGD. The Control group itself maintained its gill mucosal values near those of healthy wild salmon. The authors found that longterm infections corresponded with dysregulation of systemic reactive oxygen species (ROS), a concomitant elevated antioxidant production and altering inosine and guanosine (Lazado, Timmerhaus et al. 2021) as well as inducing lower cortisol responses. The affected tissues responded differently, with the liver and gill being more sensitive than the skin, and the gills displaying mucous cell hypertrophy after the second and third exposure. Metabolomics showed that in the gills, genes for immunity and for ribosomal functions were significantly affected by oxidants, whereas the liver was the site of genes involved in targets of oxidation-reduction. In the skin, whose mucosal morphodynamics remained relatively unchanged, changes were dependent on the duration of exposure and some ribosomal functions were impacted (Lazado Timmerhaus et al., 2021).

The External Immune system of Fishes and One Health

The gill mucosal barrier is exquisitely sensitive and responsive to its environment. Asking "What is Gill Health?", Foyle and colleagues argue that homeostasis or the ability to maintain physiological function in the face of stressors is key, despite no clear evidence on how far the limits can be stretched before health is compromised and little consensus on its objective measure (Foyle, Hess et al., 2020). However, as this is an external part of the immune system in constant contact with water, particles and pathogens, all the fish in a site are experiencing the same environment and will display much the same response in this evolutionarily conserved barrier. It is impossible to vaccinate against a poor environment. If the fish are healthy it should imply the site is healthy.

But health is more than just the absence of a pathogen or disease, as shown by the ecotoxicological responses of sculpins (Dang, Pittman et al. 2019). Indeed the presence of certain parasites, viruses or bacteria may be unimportant to overall health or may fail to cause the expected disease (Nylund, Roed et al. 2021). Thus we come to the interaction between farming systems and fish, where the technological demands of increasingly industrialized systems seem to exceed the biological adaptability of the fish, leading to "lifestyle" disease and mortalities (Gjessing, Steinum et al., 2019, Sommerset, Walde et al., 2021). The basic levels of stress, noise, background chemistry, feed composition, stocking density, frequency of handling and treatment method all induce changes which impact on mucosal and systemic health, leading to an enormous variety of up- and down regulated genes. However it is necessary to understand the measure of baseline health before selecting the response variables and comparing across incomparable trials: Wiik-Nielsen and colleagues found that the seawater intake for a land-based facility could provide a venue for bacterial infections of the gill which would transmit horizontally within the system which could be exacerbated by elevated temperatures, inadequate hygiene and reduce available oxygen levels (Wiik-Nielsen, Gjessing et al., 2017). Low level algal invasions, eutrophication, increased environmental temperatures also expand the wide number of inputs to which the gills and the skin must react and illustrate the multidimensional complexity of health and homeostasis.

Defining gill or skin health in a way that can be reproduced on other individuals or conditions is crucial to understanding the wide variety of responses found by an even wider variety of authors. The inherent plasticity of developing fish also means that there is as yet no standard "best" size because the organogenesis is determined by the environmental inputs combined with the flexible genetic cascades (Pittman, Yufera et al., 2013). Since the presence or absence of secreted mucus and mucous cell hypertrophy characterize many identified clinical diseases and some histocomplex disorders, the focus on this evolutionarily conserved feature offers some clarity. In general healthy gill lamellae are the site for gas exchange and oxygenation of the blood and the mucous cells are contained within a double membrane between the water and the blood cells. Healthy gill lamellae have little need for mucous cells to offset toxins or pathogens which would occlude respiratory surfaces. As such the larger and denser the lamellar mucous cells are in afflicted gills, the thicker the lamellae and the further the distance for gasses to diffuse (Haddeland, Lazado et al., 2021). That the scope of growth can be impacted by the bacterial interaction in lamellae is evidenced by the negative association between gill microbiome diversity measures and mass of the fish (Minich, Härer et al., 2022), such that faster swimming fishes have lower gill microbiome diversity (Minich, Härer et al. 2022) and healthy fast swimming tuna have few mucus cells on the lamella (Merkin et al, *in prep*).

The dynamic range of mucous cell sizes in fish species indicates vulnerability to many challenges as cell sizes increase and biotensegrity decreases (Ingber 2006, Matthews, Overby et al. 2006). Across

12 fish species the largest densest mucous cells are in the skin, followed by the gill filament and the smallest population resides in the gill lamellae (Merkin et al. *in prep*.). Each of these mucous cell barriers displays a repeatable quantifiable response to challenges and to restitution, circling around a putative homeostasis point for physiological functions. This can then be used to establish a standardized tool of gill health or skin health on which to posit molecular or other results across systems and across species to build a more uniform approach to reporting on fish health.

Practical applications

The fish is always responding to its environment and when we industrialize that environment the demands for response from the fish may exceed the biological scope for homeostasis. New locations, new technologies and even new species or dietary ingredients may pose unexpected challenges to fish production. By measuring the barrier cells rather than the molecules, we see the summary of the effects of many hundreds of genes, both known and unknown, while still being infinitely more sensitive than simple growth and mortality. This has practical application for those seeking to improve their production.

Our data indicates that the skin can be relatively insensitive to things which can impact the gills greatly. Therefore, the skin is the shield and gills are the sentinel guard giving early warning of change. The gills are the "lungs" of the fish, where function can be compromised long before it is macroscopically visible. The skin itself can nonetheless be victim of many "lifestyle diseases" such as becoming dry and actually shield-like (few mucous cells but many epithelial cells) or thin and easily disrupted and much depends on the experienced life history of the fish. Since the mucosa lie above and around the scales, when a fish loses scales it also loses the mucosal barrier. This can however grow back if given sufficient pause and continue to protect the fish (Sveen, Karlsen et al. 2020) but repeated insults (or "scale loss") lead to the documented vulnerability of fish with removed mucus (Svendsen and Bogwald 1997). Therefore, we can begin to consider the skin shield as the two-way interactive user-interface of the fish. This shield responds to surface contacts which confer a higher diversity of microbial population than in the gills, secreting immunoglubulins according to need and reflecting the physical ambience (Minich, Härer et al. 2022). The gills respond to the water and its particles, pathogens and quality, as well as to stress of many kinds. The healthy gill sentinel guard has few and small mucus cells in the lamellae whereas the healthy skin shield has sufficient mucous production to fulfil its normal physiological needs. (The intestines, the foundation of health, are another story for a longer paper.) In general, we have a "Fish Detective" always responding to the farm and its routines, helping identify the sources of impact on the external immune system (Fig. 2).

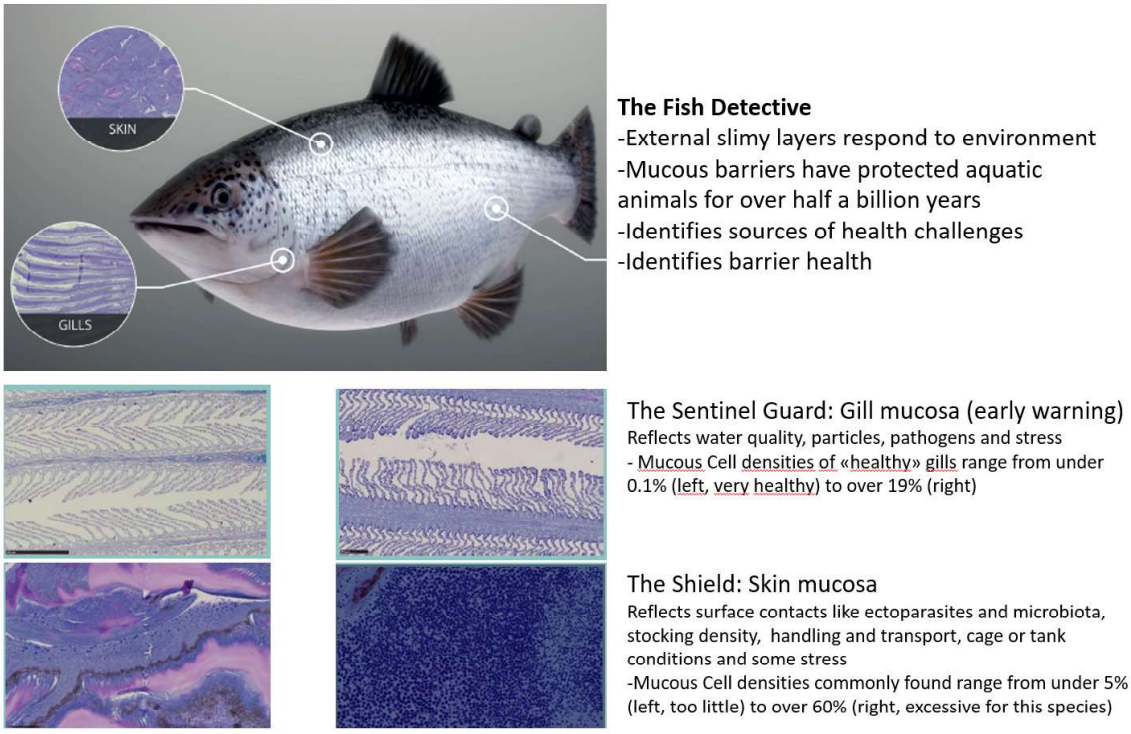

Figure: 2. The Fish Detective: external mucosal barriers respond quantitatively to the environment and can help identify healthy routines on-farm, good sites, and areas for improvement. Images from "The Robust Fish" (2019) available from QuantiDoc with permission.

Conclusion

To accompany the growing need to document One Health across species and systems it is imperative to understand the mechanisms by which mucous cell production influences and responds to immune substances and microbial signals for the maintenance of healthy mucosal barriers (Salinas, Fernandez-Montero et al., 2021). The health of the oceans is the health of our fish and ultimately of us. The interactive mucosal barrier between organism and environment has been slimy for half a billion years. It is time we literally took its measure.

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Gamma Ray Irradiation: A Valuable Tool for Fresh Feed Disinfection

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Abstrak: Akuakultur udang kekal sebagai sektor penting dalam industri akuakultur di Malaysia setakat 2015 hingga 2018 dengan pengeluaran lebih 187,000 MT dengan nilai melebihi RM4, 238 juta. Walau bagaimanapun, kebanyakan pengusaha hatceri bergantung kepada stok induk udang bebas pathogen (SPF) yang diimport dari pusat penggandaan induk (BMC) di luar Malaysia. Induk induk ini mahal dengan harga antara USD 100 - 200 seekor dan biasanya pengusaha akan membelanjakan lebih daripada RM 100,000 dalam setiap penghantaran. Walaupun ia adalah stok induk SPF, wabak penyakit seperti sindrom kematian awal (EMS), virus sindrom bintik putih (WSSV) dan jangkitan Enterocytozoon hepatopenaei (EHP) masih boleh berlaku di peringkat ladang yang mungkin disebabkan oleh isu pengurusan di ladang. Namun begitu, kebersihan makanan segar di hatceri sering diabaikan. Makanan segar telah dilaporkan sebagai sumber atau tuan rumah patogen terutamanya polikit marin dan sotong. Sehingga kini belum ada prosedur khas untuk membasmi kuman sebelum digunakan di hatceri. Di FRI Pulau Sayak, makanan segar dibasmi kuman dengan sinaran gamma sebelum diberi makan kepada udang. Sinaran gamma pada sampel positif terbukti berkesan dalam membasmi patogen. Sinaran gamma memusnahkan DNA patogen secara langsung melalui pengionan air yang menghasilkan radikal bebas. Dos berkesan adalah antara 5 – 10 kGy sinar gamma daripada kobalt (60Co). Walaupun sinaran gamma adalah kaedah baru untuk pembasmian kuman di Malaysia, penggunaannya adalah perlu bagi industri akuakultur tempatan bagi memastikan sumber makanan yang bersih untuk induk udang atau ikan.

Abstract: Shrimps aquaculture remains as an important sector in the aquaculture industry in Malaysia as of 2015 to 2018 with the production of more than 187,000 MT and the value exceeded RM4, 238 million. However, most of the hatchery operators rely on specific pathogen-free (SPF) shrimp broodstock imported from broodstock multiplication centres (BMCs) outside Malaysia. These broodstocks are expensive ranging from USD 100-200 per piece. Hatchery operators spend more than RM 100,000 in a single shipment. Although it is the SPF broodstock, outbreak of diseases such as early mortality syndrome (EMS), white spot syndrome virus (WSSV) and *Enterocytozoon hepatopenaei* (EHP) infections still occur at farm level. It could be due to management issue at farms. Nevertheless, the hygiene of fresh feed at hatcheries is often overlooked. This fresh feed has been documented to be a potential source as host of pathogens particularly for marine polychaetes and squids. There is no procedure yet to disinfect prior to use at private hatcheries. At FRI Pulau Sayak, these fresh feeds are disinfected by gamma irradiation prior to feeding. Gamma ray irradiation on positive sample proved to be effective in disinfecting the potential pathogens. Gamma radiation destroys the pathogen's DNA directly or by the effect of water ionization that produces free radicals. The effective dose is between 5 – 10 kGy of gamma ray from cobalt (^{60}Co). Although gamma irradiation is a new method for food disinfection in Malaysia, the use is crucial for the local aquaculture industry to ensure hygienic feed for the shrimp and fish broodstocks.

Introduction

Marine shrimp is an important aquaculture species for Malaysia with the production of more than 187,000 MT, and the value of more than RM4, 238 million between 2015 and 2018. According to the national Annual Fisheries Statistics by Department of Fisheries Malaysia, the production has increased tremendously from 2,339 MT in 1990 to 48,588 MT in 2020 as shown in Figure 1. The main cultured species are *Penaeus monodon, Penaeus merguiensis* and *Penaeus vannamei*. However, the shrimp aquaculture scenario is not without its problems. In the 90s, the industry was marred by the outbreak of white spot syndrome virus (WSSV) infection that caused huge economical lost with the estimation of US25 million annually (Dacho & Mustafa, 2007). Initially, *P. monodon* was the only cultured species. However, WSSV infection has forced farmers to look for other alternative shrimp species such as *P. mergueinsis* and *P. vannamei*. WSSV was first detected in Japan back in 1992. The disease was then spread to a few countries and subsequently to Southeast Asian countries in 1994 (Walker & Mohan, 2009). The prevalence of white spot disease could be caused by wild broostocks which are detected to be WSSV positive (Remany et al., 2012; Withyachumnarnkul et al., 2013; Orosco & Lluisma, 2017). Currently, *P. vannamei* becomes the most cultured species due to the availability of domesticated broodstock that is specific pathogen-free (SPF) (Lightner, 2011; Alday-Sanz et al., 2018), which boosted the shrimp production globally.

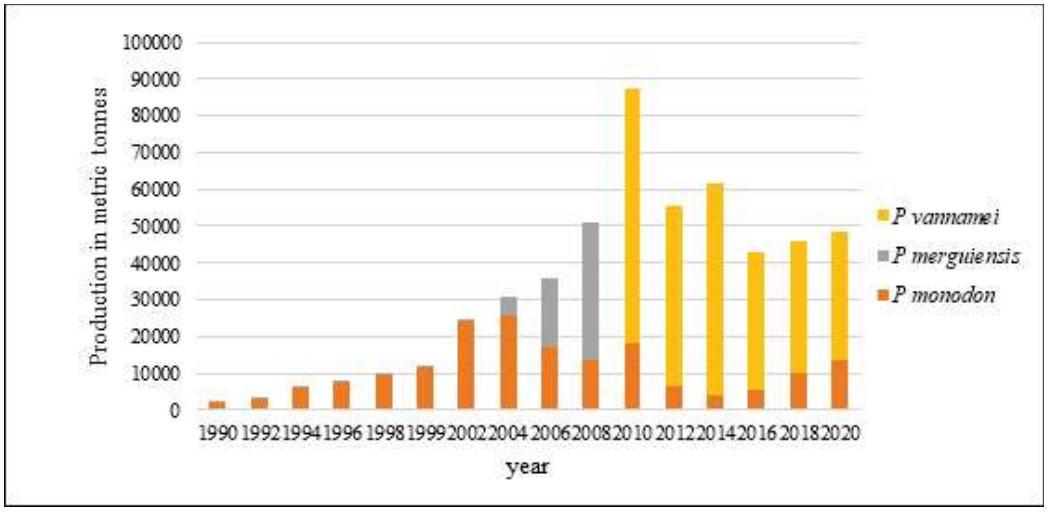

Figure 1: National production of penaeid shrimp culture in Malaysia. The main cultured species are *P. monodon*, *P. merguiensis* and *P. vannamei*. (source: Department of Fisheries Malaysia)

The prevalence of diseases did not end with the introduction of SPF domesticated broodstock. In 2009, early mortality syndrome (EMS) which was renamed as acute hepatopancreatic necrosis disease (AHPND) was detected in *P. vannamei* in China. The disease eventually has spread to Southeast Asian countries and subsequently to South America, Mexico and USA. The AHPND is caused by specific strain of bacteria such as *V. parahaemolyticus*, *V. punensis*, *V. harveyi*, *V. owensii*, *V. campbelli* and *Shewanella* sp. that contain pVA1 plasmid (63–70 kb) encoding the binary $PirA^{VP}$ and $PirB^{VP}$ toxins. The disease has caused the loss of USD 43 billion to the shrimp industry (Kumar et al., 2020; Kumar et al., 2021).

Another pathogen that causes economic loss in the shrimp industry is *Enterocytozoon hepatopenaei*

(EHP). It is estimated to have cause the loss of USD 567.62 M annually in India (Patil et al., 2021). The presence of such microsporidium was first described in *P. monodon* in Malaysia in 1984. However, the presence of such microsporidium in *P. japonicus* was first detected in Australia in 2001 and later, in 2004, it was detected in *P. monodon* in Thailand. It was then formerly described as EHP in 2009 (Chaijarasphong et al., 2021).

One of the most widely used tool to detect WSSV (Flagel, 2002), AHPND and EHP is the PCR test. PCR test screens the presence of pathogens. When the screening test on WSSV comes out negative, consequently the shrimp production will increase due to absence of the virus (Vaseeharan et al., 2003; Seok et al., 2007). Similarly, PCR test is also developed for the detections of APHND (Nunan et al., 2014; Sirikharin et al., 2015) and EHP (Flegel & Sritunyaluucksana, 2018; Hou et al., 2021). Therefore, PCR test has proved that it has subsequently helped to contain the spread of deadly pathogen from shrimp to shrimp and lessen the economic loss.

Many studies have shown that pathogen infections are not limited to penaeid shrimps. It could be found in other aquatic animal as well. Polychaetes have been reported as passive carrier for EHP and WSSV (Desrina et al., 2013; Haryadi et al., 2015; Desrina et al., 2018; Desrina 2020; Krishnan et al., 2021). Apparently, *V. paraheamoliticus* has also been reported in blood clams and squids (Tan et al., 2020). Polychaetes and squids are used as maturation diet for shrimps in hatcheries and blood clamps are being fed occasionally as well. The fresh feeds therefore post a threat to the shrimp industry.

Although hatchery operators can reject the infected broodstock after PCR test by selecting pathogen-free shrimps, when it comes to maturation diet, fresh feeds still pose a threat via vertical transmission. However, by solely relying on formulated maturation diet, it may increase the operational cost. Thus, one of the possible solutions that will enable fresh feed to be used is by gamma ray irradiation treatment. Irradiation of food using ions of beta or gamma rays can inactivate or destroy the food spoilage pests, microorganisms and their toxins (Munir & Federighi, 2020). Besides that, gamma irradiation offers high penetrating power which makes it possible for bulk treatment (Arapcheska et al., 2020).

Materials & Methods

PCR Test

PCR test was conducted at FRI Pulau Sayak facility using Biorad model CFX96 Touch Real-time PCR System. Polychaetes samples were collected from the cultured polychaetes which was purchased from local supplier. Screening was done before and after gamma ray irradiation. Each sample was being tested for AHPND, WSSV and EHP.

Gamma irradiation

The fresh feeds, the squids and the polychaetes were packed inside microwavable food container and frozen prior to packing. Gamma ray irradiation services were done at Malaysian Nuclear Agency, in Bangi, Selangor. For gamma ray irradiation service, the fresh feeds were arranged in a styrofoam box. A block of dry ice was packed together with the frozen fresh feeds. The box weighted 25 kg each. These fresh feeds were then irradiated with 10 kGy gamma ray radiation.

Result & discussion

PCR test result of the fresh feeds showed that polychaetes were positive of EHP while squids were positive of WSSV as in Figure 1 and Figure 3, respectively. After the gamma ray irradiation, WSSV and EHP were not detected in both polychaetes and squids as in Figure 2 and Figure 4, respectively.

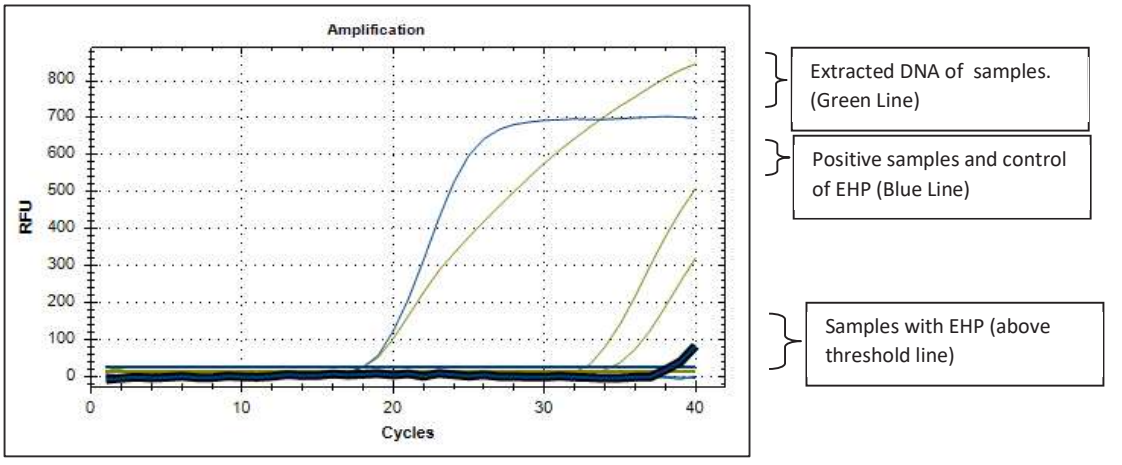

Figure 1: Sample of fresh polychaetes, tested positive for EHP before gamma ray irradiation.

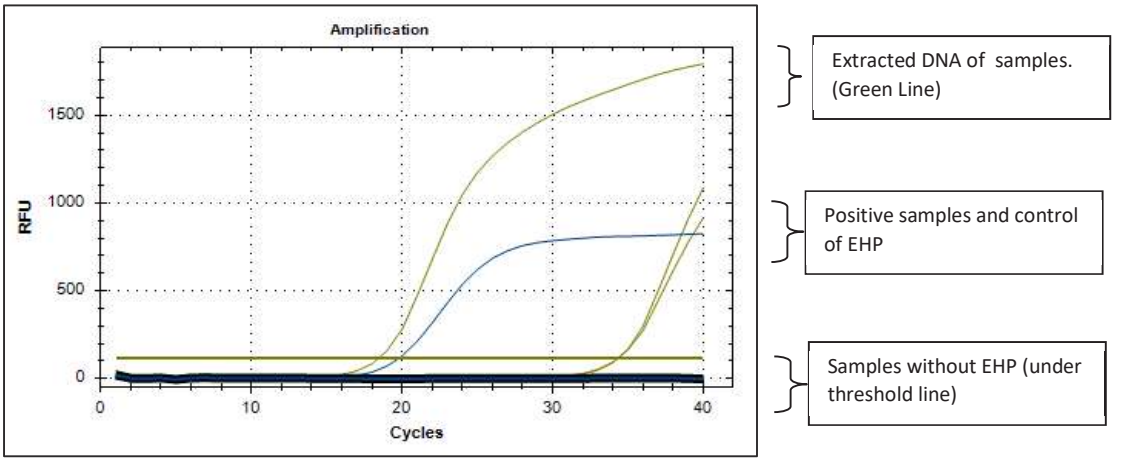

Figure 2: Sample of fresh polychaetes, tested negative for EHP after gamma ray irradiation.

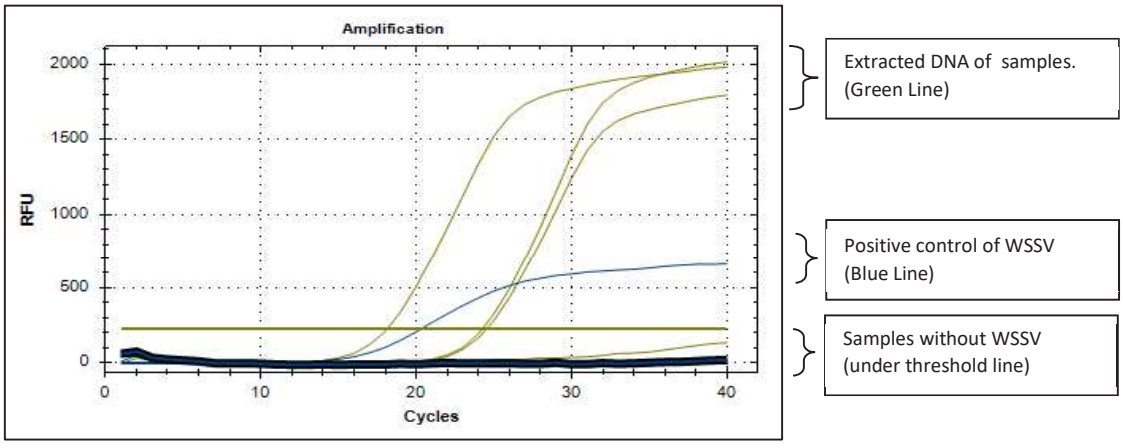

Figure 3: Fresh squids that tested positive for WSSV before gamma ray irradiation

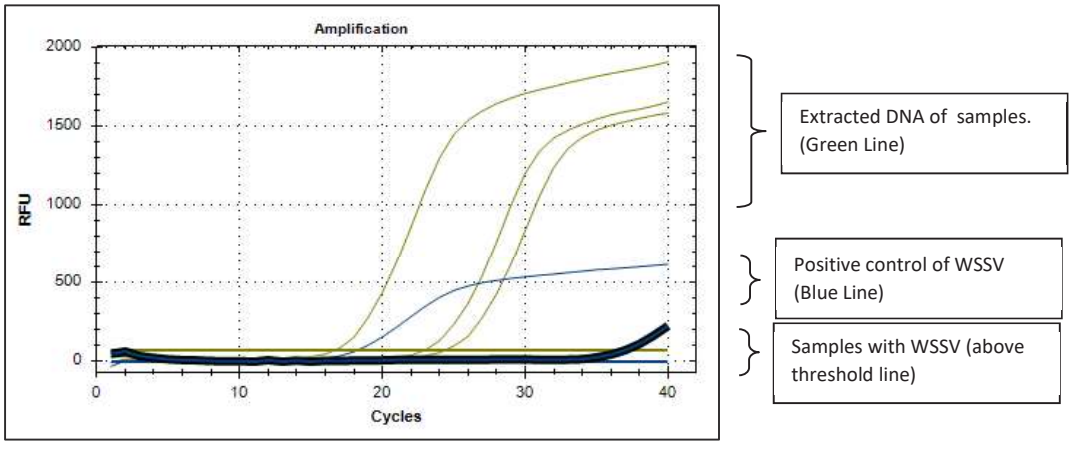

Figure 4: Fresh squids that tested negative for WSSV after gamma ray irradiation.

There is limited study on gamma ray irradiation are limited to reduce storage losses, to extend shelf life, and also to improve microbiological and parasitological safety of foods (Arapcheska et al., 2020). Earlier study on gamma ray irradiation performed on clam was meant for food safety. The study showed that coliform and faecal coliform were 90% eliminated at 1.32 kGy and 1.39 kGy (Harewood et al., 1994). Another study by Chen et al. (2000) found that at 12 kGy, bacteria were not detected in animal feed after irradiation. In the present study, at 10 kGy, EHP and WSSV were not detected after gamma irradiation. The results indicated that gamma ray is capable of disinfecting fresh feeds for the use of shrimp maturation. Thus, gamma ray irradiation will enable locally available polychaetes and squids for the use as maturation diet that are pathogen-free. Consequently, the production cost of having to import fresh feed from other countries can be lowered.

Conclusion

Gamma irradiation has been proven to be a useful tool for disinfecting pathogens in fresh feed especially in the aquaculture maturation diet. The current study was done on the maturation diet for

shrimps. Thus, perhaps it should be extended to the maturation diet for fishes and feeds for ornamental fish as this technique could reduce food-borne disease to the cultured animal.

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Ectoparasites Recovered from Whole Cage Freshwater Treatment on Cultured Marine Fish Disease Outbreak in Floating Cages

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Abstrak: Pelbagai spesies ikan laut diternak dalam sangkar terapung sering mengalami wabak penyakit. Gejala yang biasa diperhatikan ialah calar pada permukaan badan, kejatuhan sisik, reput sirip dan ekor, kawasan kepala tanpa sisik, mata legap dan buta. Penternak di sangkar biasanya melakukan rawatan air tawar untuk ikan yang dijangkiti penyakit yang melibatkan ektoparasit, untuk mengelakkan kematian lanjut. Pemantauan untuk mengenal pasti wabak penyakit ektoparasit di sangkar ikan di Pulau Pinang telah dilakukan antara tahun 2014-2018. Semasa wabak tersebut, keseluruhan ikan dalam sangkar dirawat dengan air tawar dan sampel ektoparasit dikumpul untuk pengecaman dan pengiraan di bawah mikroskop pembedahan. Tiga kumpulan ektoparasit yang ditemui ialah capsalid monogeneans (*Benedenia* spp., *Neobenedenia* spp.), lintah, (*Zeylanicobdella arugamensis*) dan caligid copepods (*Caligus spp*.). Monogenean capsalid, terutamanya *B. lutjani*, adalah ektoparasit dominan yang dipencilkan daripada ikan siakap manakala lintah pula merupakan parasit dominan dalam ikan kerapu. Pemerhatian di awal wabak penyakit menunjukkan kecederaan pada permukaan badan yang disebabkan oleh aktiviti ektoparasit yang meluas. Ektoparasit ini dianggap PATOGEN UTAMA yang memulakan wabak penyakit dalam ekosistem kultur sangkar terapung yang membawa kepada komplikasi fisiologi, jangkitan bakteria sekunder dan kematian ikan. Rawatan air tawar berkesan dalam menghilangkan ektoparasit dalam ikan siakap, tetapi tidak dalam kerapu. Ikan siakap merah kebanyakannya dijangkiti oleh capsalid monogeneans dan menyebarkan parasit kepada spesies lain dalam sangkar. Kerapu, bagaimanapun, adalah penyumbang utama lintah untuk menjangkiti ikan lain. Pemerhatian terhadap rawatan air tawar biasa di sangkar ikan merah selama sembilan bulan menunjukkan tiada wabak penyakit. Pembuangan berterusan dan jangkitan semula ektoparasit dalam ikan ternakan adalah satu proses pemvaksinan semula jadi terutamanya semasa tahun pertama penempatan dalam sangkar. Ikan yang terjejas menjadi kebal terhadap jangkitan ini menyebabkan lebih kurang wabak penyakit apabila ikan membesar.

Abstract: Multi-species marine finfish are farmed in floating cages, with farmers experience frequent fish disease outbreaks. Common symptoms observed are scratches on body surface, scale drop, fin- and tail-rot, head region devoid of scales, eye opaque and blind. Farmers commonly do freshwater treatment for the infected fish in the whole cage when disease outbreak occurs involving ectoparasites, to prevent further mortality. Monitoring study to identify ectoparasites of disease outbreaks in a Penang fish farm was done between 2014 -2018. During the outbreaks, the whole cage of fish was freshwater treated and the ectoparasites samples were collected for identification and counted under dissecting microscope. Three groups of ectoparasites recovered during these monitoring studies were capsalid monogeneans (*Benedenia* spp., *Neobenedenia* spp.), leech, (*Zeylanicobdella arugamensis*) and caligid copepods (*Caligus* spp.). The capsalid monogeneans, especially *B. lutjani*, were the dominant ectoparasites removed from snappers while leech was the dominant parasite in groupers. Observations on initial disease outbreak showed injuries on body surface caused by vast ectoparasites activities. These ectoparasites were considered as the PRIMARY PATHOGENS initiating disease outbreaks in the floating cage culture ecosystem leading to physiological complications, secondary bacterial

infection, and fish dying. Freshwater treatment is effective in removing ectoparasites in snappers, but not in groupers. Crimson snappers was mostly infected by capsalid monogeneans and spread the parasites to other species in the floating cage. Groupers, however, are the major contributor of leeches for infecting other fish. Observations on regular freshwater treatment in the crimson snapper's cage for nine months showed no disease outbreaks. Constant removal and re-infection of the ectoparasites in the cultured fish is a process of natural life vaccination especially during the first year of placement in cages. The affected fish become immune to these infections resulting in fewer disease outbreaks as the fish grow.

Keywords: *Primary pathogens, Benedenia lutjani, Neobenedenia Melleni, Zeylanicobdella arugamensis, Caligus rotundigenitalis.*

Introduction

Floating cage culture system is the most common aquaculture system for culturing marine finfish in Malaysia as well as in Southeast Asia and the Far East. Chua reported mariculture in floating cages began in Jelutong, Penang, Malaysia in 1973. The common finfish culture belongs to the family Serranidae (grouper) and Lutjanidae (snapper). In Peninsular Malaysia, crimson snappers, *Lutjanus erythropterus* and hybrid groupers are the most commonly cultured marine fish.

Fish farmers often claim they encounter disease outbreaks during the first year after the introduction of fingerlings in their cages. Signs of disease outbreak are observed when some moribund fish with scratches on body surface, loss scales with some dead fish appear on the surface or when numerous dead fish are observed at the bottom of the net when the net lifted. When disease outbreak occurs, they treat the whole cage of the affected fish with freshwater. In most cases, they observe mortality ceased after the first freshwater treatment. In severe outbreak cases, they have to repeat the freshwater treatment for the whole cage. After the freshwater treatment with no further mortality, the fish farmers do not proceed with any further investigation.

Numerous published reports on disease outbreaks have exposed fish mortalities that caused by virus, bacteria or concurrent infection with virus and parasites. When disease outbreaks occur, only individual moribund fish is examined for the causative disease agents. Published reports on these moribund fish during disease outbreaks often showed the causative pathogen was either virus (Glazebrook et al., 1990; Chia et al., 1995; Fukuda et al., 1996; Ransangan and Manin, 2010; Gibson-Kueh et al., 2014b; Asrazitah et al., 2014; de Groof et al., 2015; Dong et al., 2017a; Senapin et al., 2018; Nurliyana et al., 2020; Charoenwai et al., 2020), or bacteria (Nash et al., 1987; Zafran, 1998; Dong et al., 2017b; Leong, 1993; 2014a; 2104b; Gibson-Kueh et al., 2014a; Michel, 2017; Nurliyana et al., 2019a; 2019b), or parasites (Leong and Wong, 1985; Supamattaya et al., 1990; Boonyaratpalin et al., 1996; Leong, 2017; 2021), or the concurrent infection of virus and parasites (Charoenwai et al., 2020; Leong 2014a).

Apparently, fish mortality ceased after the fish farmers make freshwater treatment. The question is why fish mortality ceased? Thus, a monitoring study was carried out to determine the external pathogens removed when the whole cage of affected fish was treated with freshwater.

Materials and Methods

A floating cage fish farm at Pulau Jerejak, Penang was selected for this monitoring study. During the disease outbreak, the fish farmer did the freshwater treatment for the whole cage of the affected

fish in a large plastic container. All parasites would be dislodged from the body surface and then, these ectoparasites were collected from the bottom of the container. The ectoparasites were preserved in 90% alcohol for examination, identification and counting under a dissecting microscope.

Although ectoparasites were found infecting the cultured fish, not all of them were included in this study. The gills of fish could also be infected with ectoparasites. However, freshwater treatment is not effective to dislodge the ectoparasites and the fish needed to be killed to obtain the ectoparasites. Therefore, the ectoparasites infecting the fish gills were not included in this study.

Results

During the monitoring study, this farm cultured four species of snapper (*Lutjanus erythropterus, L. johnii, L. sebae* and *L. argentimaculatus*), two hybrid groupers, Asian seabass (*Lates calcarifer*), and pompano (*Trachinotus blochii*), in the floating cages (Figure 1). Crimson snapper, *Lutjanus erythropterus* was the dominant fish species.

Figure 1: Marine finfish cultured in the floating cages during the disease outbreak

Figure 2 shows the observed external symptoms on moribund fish including scratches, scales drop on body surface and forehead, tail- and fin-rot, eye exophthalmia and eye loss. These external symptoms were observed in all species of moribund fish on the cage surface.

Figure 2: Scratches, scales drop on body and forehead, tail- and fin-rot, eye exophthalmia and loss of eye observed on moribund fish

Large numbers of mature and immature leeches were recovered from the spotted hybrid grouper. The body of mature leeches were large and showed reddish colour indicating they were feeding on the blood of the groupers (Figure 3).

Figure 3: Large size leeches full of blood recovered from the freshwater treated groupers

Ectoparasite at the bottom of the container from the three fish species, namely crimson snapper, (*Lutjanus erythropterus*), golden snapper (*L. johnii*) and spotted hybrid grouper (*Epinephelus lanceolatus* × *E. fuscoguttatatus*) were collected and examined. Three major groups of ectoparasites were identified, namely capsalid monogeneans, caligid copepods and a marine leech. The capsalid monogeneans belong to *Benedenia epinepheli*, *B. lutjani*, *Benedenia* spp., *Neobenedenia melleni*, and *Neobenedenia* spp., caligid copepods belong to *Caligus rotundigenitalis*, *C. epidemicus*, *C. minimus* and *Caligu*s spp. and leech, *Zeylenicobdella arugamensis.*

Within the capsalid monogeneans, it is very difficult to separate *Benedenia* spp. from *Neobenedenia* spp. except *Benedenia lutjani*. *Benedenia lutjani* are easily identified and counted separately due to its very small size. All other *Benedenia* spp. and *Neobenedenia* spp. were grouped together and counted as *Benedenia*/*Neobenedenia* spp.. Various caligid species were counted as *Caligus* spp..

The first disease outbreak studied was a 1800 crimson snapper in a floating cage. The average length of the fish was 25.2 cm and the average weight of 282.6 g. The results of ectoparasites recovered from the whole cage freshwater treatment are shown in Figure 4. The capsalid monogeneans (*Benedenia lutjani* and *Benedenia/Neobenedenia* spp.) accounted for 98% of the total numbers of ectoparasites. *Benedenia lutjani* accounted for 89.3% of total number of capsalid monogeneans. The total numbers of caligid copepods and leech accounted for only 2% of total ectoparasites recovered.

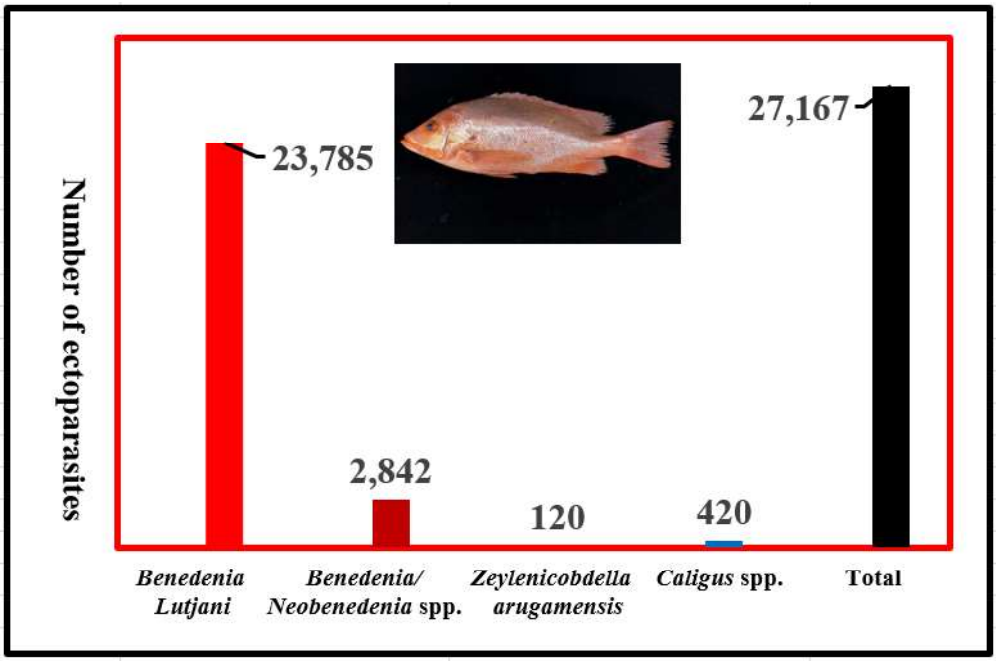

Figure 4: Ectoparasites recovered in the whole cage freshwater treatment of crimson snapper during the disease outbreak

The second disease outbreak study was on golden snappers which observed frequent disease outbreaks soon after their introduction to the cage. During this study, the average length of the snapper was 23 cm and the average weight was 184 g. The results of the whole cage freshwater treatment of golden snapper during a disease outbreak are shown in Figure 5. Three groups of ectoparasites recovered after

the freshwater treatment consisted of *Benedenia/Neobenedenia* spp., caligid copepods and marine leech. The *Benedenia/Neobenedenia* spp. group accounted for 96.9% of total ectoparasites recovered, and *B. lutjani* accounted for 86.2% of the *Benedenia/Neobenedenia* spp. group. Caligid copepods and marine leech accounted for 3.15% of the total ectoparasites.

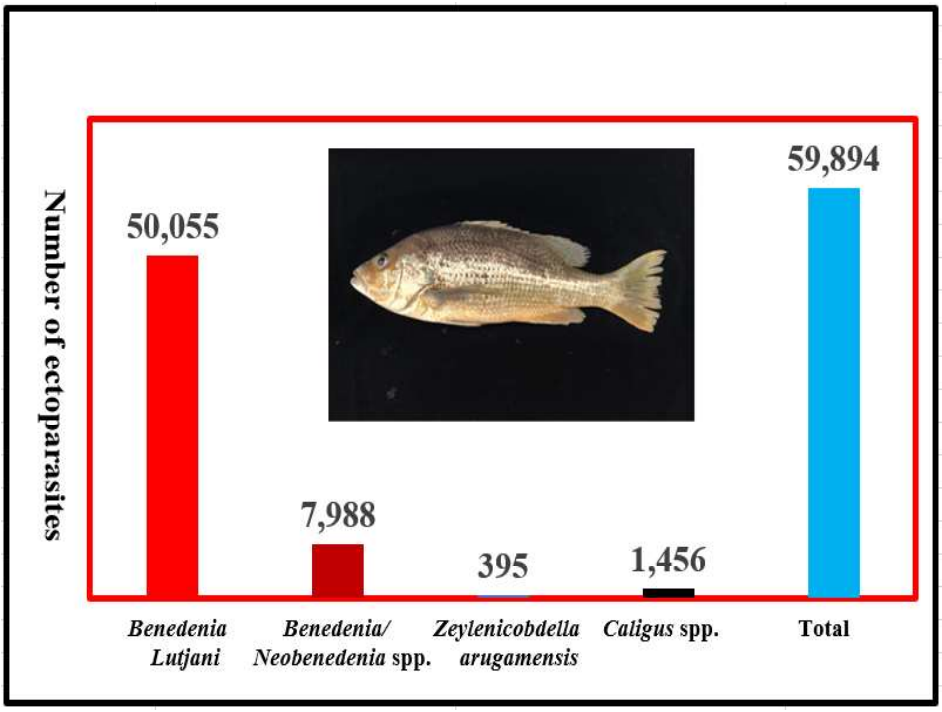

Figure 5: Ectoparasites recovered in whole cage freshwater treatment of golden snapper during disease outbreak

The results of whole cage freshwater treatment of spotted hybrid grouper during disease outbreak are presented in Figure 6. Although the same three groups of ectoparasites, *Benedenia/Neobenedenia* spp., caligid copepods and marine leech were recovered as seen in snappers, but the dominant group of the ectoparasite was the marine leech. The marine leech comprised of 97.8%, caligid copepods comprised 2.18% and capsalid monogeneans was only 0.02% form the total ectoparasites recovered.

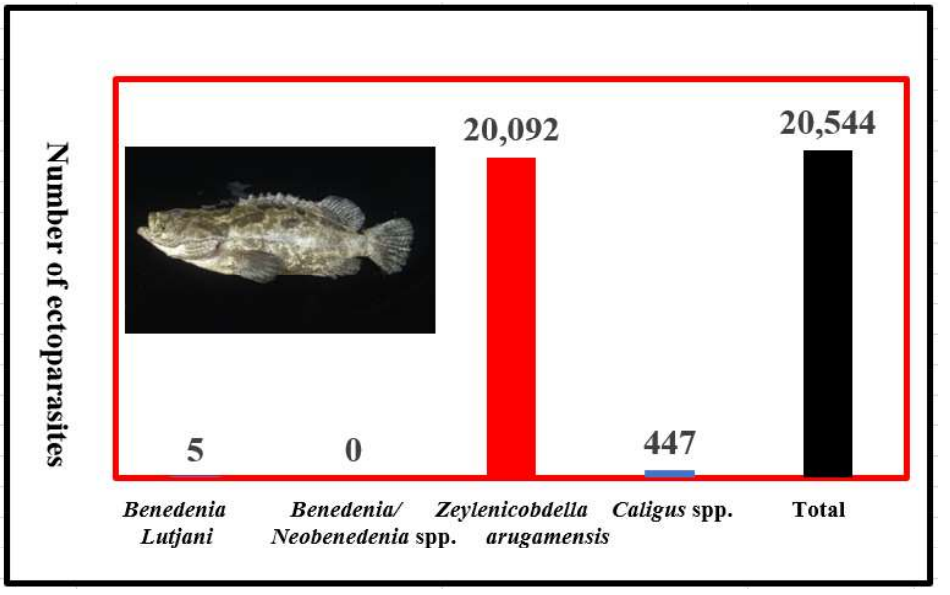

Figure 6: Ectoparasites recovered in the whole cage freshwater treatment of spotted hybrid grouper during Disease Outbreak 1

Disease outbreak occurred in another batch of spotted hybrid grouper and the results are shown in Figure 7. The marine leech was not the dominant ectoparasites, with only 10.3% and caligid copepods with 11.0% of total ectoparasites. The dominant ectoparasites was capsalid monogeneans which comprised 78.6% of the total ectoparasites. However, unlike in the infected snapper, very few *B. lutjani* (0.02%) from *Benedenia/Neobenedenia* spp. group, was recovered.

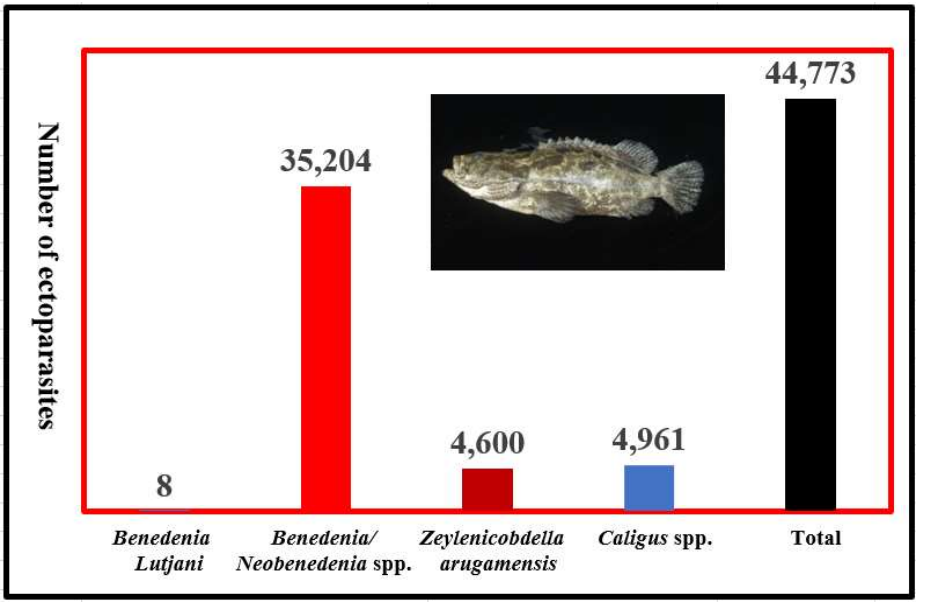

Figure 7: Ectoparasites recovered in the whole cage freshwater treatment of spotted hybrid grouper during Disease Outbreak 2

Discussion

There were eight species of marine fish culture in the floating cages of this fish farms where the present studies were carried out. Crimson snapper, golden snapper and two hybrid groupers – spotted and pearl, were observed with frequent disease outbreaks during the first year of their culture. Apparently, disease outbreak in pearl groupers was not covered in this study.

Three groups of ectoparasites were frequently recovered from the whole cage freshwater treatment during the disease outbreaks in the crimson and golden snappers, and spotted hybrid groupers. The ectoparasites recovered were *Benedenia/Neobenedenia* spp., *Caligus* spp. and a marine leech, *Zeylenicobdella arugamensis*. In both crimson and golden snappers, capsalid monogeneans, *Benedenia lutjani* was the most abundant ectoparasites recovered consisting for slightly less than 90% of total *Benedenia/Neobenedenia* spp.. The dominant ectoparasites recovered from the both disease outbreaks of the spotted hybrid grouper were different, with the first outbreak was dominated by marine leech, *Zeylenicobdella arugamensis* while another outbreak was dominated by *Benedenia/Neobenedenia* spp..

The external symposiums observed in moribund fish showed scratches, scale loss, ulceration damage and blind eyes, and loss of epidermis on forehead. These symptoms were the results of irritation from these ectoparasites crawling over the whole-body surface, feeding on surface mucus as well as on blood. Large numbers of mature leeches and large size of *Benedenia* spp. and *Neobenedenia* spp. showed reddish coloration within the body indicating the ectoparasites were feeding on blood of the fish. Trujillo-Gonzalez et al. (2015) showed the distribution of live fluorescent *Neobenedenia* sp. from the initial infection to maturation on the body surface of *Lates calcarifer* over time. These movements and feeding on body surface would irritate the fish to scratch the body against side of net to get rid of the irritants, resulting in injuries. These injuries provide further entry to other pathogens. Many reported studies have exposed the moribund fish infected with various *Vibrio* species or virus caused mortality of the fish. The results of the present study on the whole cage freshwater treatment of fish at the disease outbreak cage showed that these ectoparasites – *Benedenia* spp., *Neobenedenia* spp., *Caligus* spp., and leech *Zeylanicobdela arugamensis* are the primary pathogens that initiate the disease outbreaks of fish culture in floating cages. Observations on regular freshwater treatment in a cage of crimson snappers over a period of nine months showed no disease outbreaks. This constant removals and re-infections of the ectoparasites pathogens in the cultured fish appear to be a process of natural life vaccination from these infections that act as vaccines, especially during the first year of placement in cages. The affected fish become immune to these infections resulting in fewer disease outbreaks as the fish grow. Fish above 450 g have fewer disease outbreaks.

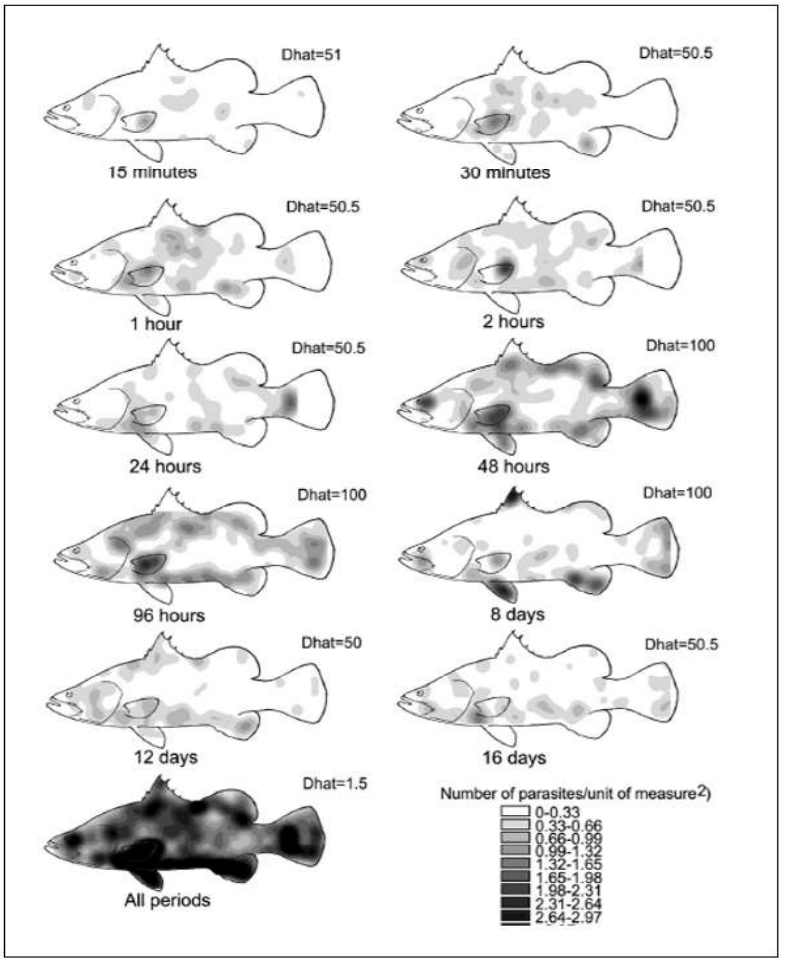

Figure 8: Observation of distribution under live fluorescent Neobenedenia sp. on the body surface of Lates calcarifer over time (Trujillo-Gonzalez et al., 2015).

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An Intelligent Protozoan White Spot Fish Disease Detection

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Abstrak: Penyakit bintik putih protozoa merupakan salah satu punca utama wabak penyakit ikan dalam sektor marikultur di Malaysia. Wabak yang disebabkan oleh parasit aktif, *Cryptocaryon irritans*, adalah berbahaya kerana ia boleh menjejaskan pengeluaran dan ternakan pelbagai spesies lain yang boleh mengakibatkan kesan negatif terhadap perkembangan industri akuakultur. Sebagai sebahagian daripada Revolusi Perindustrian 4.0 di Malaysia, memaksimumkan penggunaan Kecerdasan Buatan (AI) dalam industri boleh menjadi penyumbang utama kepada teknologi pengesanan penyakit yang boleh membendung atau memperlahankan penyebaran jangkitan dalam ikan ternak. Kemajuan pemprosesan imej di bawah air dan teknik pembelajaran mendalam membuktikan adalah mungkin untuk memudahkan pemerhati manusia dalam pengesanan penyakit ikan. Oleh itu, kajian ini menunjukkan prosedur baharu untuk pengesanan penyakit bintik putih menggunakan imej bawah air, berkaitan pembangunan penyepaduan antara kontras terhad penyamaan histogram adaptif (CLAHE) dan rangkaian saraf konvolusi (CNN). Sebanyak 60 imej ikan bawah air normal dan telah sah dijangkiti diuji dalam kajian ini. Teknik yang digunakan mencapai ketepatan 96.67% dalam pengesanan penyakit bintik putih protozoa. Oleh itu, pengesanan penyakit ikan tompok putih protozoa pintar boleh dijadikan alternatif kepada pengesanan awal penyakit ini, sekali gus mencegah jangkitan sekunder pada ikan marikultur. Insentif yang ditawarkan dapat memastikan pertumbuhan industri kerana sektor tersebut menyumbang sumber pendapatan yang signifikan untuk kedua-dua negara dan penternak.

Abstract: Protozoan white spot disease is one of the leading causes in fish disease outbreaks in mariculture secotor in Malaysia. The outbreak induced by an active parasite, *Cryptocaryon irritans*, is hazardous as it could impair the production and the cultivation of diverse species resulting in a negative impact on the expansion of the aquaculture industry. As part of Industrial Revolution 4.0 in Malaysia, maximizing the use of Artificial Intelligence (AI) in the industry could be the key contributor as current technologies in disease detection could contain or slow the spread of infections among cultured fish. The advances of image processing in the underwater environment and deep learning techniques prove it is possible to ease the participation of human observers in fish disease detection. Hence, this study demonstrates a new procedure for white spot disease detection using underwater image, concerning on the development of integration between contrast-limited adaptive histogram equalization (CLAHE) and convolutional neural network (CNN). A total of 60 validated normal and infected underwater fish images have been tested in this study. The techniques used achieved 96.67% accuracy in protozoan white spot disease detection. Therefore, intelligent protozoan white spot fish

disease detection could be served as an alternative to early detection of the disease, thus preventing secondary infection in mariculture fishes. The incentive offered could ensure the industry's anticipated growth as the sector plays a significant economic income for both country and farmers.

Keywords: Protozoan White Spot Disease, Mariculture, Disease Detection, Image Processing, Convolutional Neural Network

Introduction

Aquaculture sector in Malaysia has contributed significantly as source of foods and income to the nation. Since fish is recognized as a great source of protein that is easily digested by the human body, the demand continues to grow as the global population increases (FAO, 2019). According to the National Transformation 2050 vision for Malaysia's smart communities by 2050, this sector has developed into a fast-growing food industry due to the technology revolution, putting Malaysia among the top 15 global producers with an estimated 521,000 tonnes of total aquaculture production annually (Waterland, 2016)hand-holes, dram doors, etc. present very unique sealing and assembly challenges. Unlike other conventional gasketed connections, the majority of the gasket compression forces are developed during operation, and not during assembly. This creates several critical issues that must be understood and overcome in the original gasket selection process and the assembly itself. Even if considered and addressed in the original gasket selection and assembly process, these internal sealing manways will likely still require a post start-up retorque. The timing of the re-torque is of critical importance in ensuring worker safety and continued equipment reliability and uptime. Copyright © 2013 by ASME.","author":[{"dropping-particle":"","family":"Waterland ","given":"A. Fitzgerald","non-dropping-particle":"","parse-names":false,"suffix":""}],"container-title":"FAO, (Food and Agriculture Organization. Besides, fish consumption per head is expected to be 59 kilograms per year, making it one of the world's biggest seafood demand countries (FAO, 2020). Furthermore, Malaysia is heavily reliant on fish production as it is also considered as a way of providing employment and investment opportunities for the nation. Hence, considerable effort should be expanded on aquaculture fish management and production to ensure the sustainability of fish production to meet the country's 30.75 million population demand.

Fish disease has been extensively examined in attempt to establish the reason and the remedy for problems in the country (Sayuthi, 1993). Furthermore, fish disease outbreaks are one of the obstacles to the production and the cultivation of a species, which might limit the sector's development and expansion (Fathi et al., 2018). In Malaysia, a parasitic disease known as Protozoan white spot disease or cryptocaryoniasis caused by active *Cryptocaryon irritans* in saltwater has been a major source of disease outbreaks since mariculture introduced. The parasite attaches to the fish's body and moves beneath the skin, where it feeds on skin, gills and eyes diminishing the functionality of these body parts. Infected fish would suffer small white patches, skin discolouration, mucus hyperproduction and appear thin (Yanong, 2012)reduce "work in progress" and improve the dimensional accuracy of the cast parts and the final products used in the market. Since then chemists, engineers and metal casting experts have developed new cold box (CB. In even serious cases, sloughing skin causes fish to become fatigued and linger just below the water surface, along with corneal haziness and ragged fins. The disease became the primary focus of the research as the disease showed rapid increase in mortality over several days, restricting the ability to cope with regular histopathological analysis. For that reason, there is a need for a robust and immediate early detection approach.

In conjunction with the Fourth Industrial Revolution's emerging technology, intelligent detection of

white spot disease is deemed critical for preventing outbreaks from worsening. The incentives could contain the outbreak by discovering the slight change on the fish bodies shortly. Besides, the strategies of using image processing on underwater images could ease the classification's process, which would effectively detect white spot fish disease in an early stage. It is believed that the integration approach could successfully use Artificial Intelligence (AI) for disease detection, while ease the participation of fish experts in protecting the aquaculture provision.

Artificial Intelligence (AI) has emerged as a vital force in the Industrial Revolution 4.0 in Malaysia. It has the potential to significantly increase global income levels by increasing productivity, adaptability and agility across all industries through intelligent manufacturing (Peres et al., 2020) On that account, to broaden the use of AI in the fisheries sector, research has been made to support the idea of creating an automated detection system for fish disease through underwater images. Therefore, underwater image datasets are critical for the development of underwater detection systems. After all, most of the existing fish studies are focusing on fish identification, fish species classification, fish behaviour detection as well as fish counting. Thus, there is limited dataset specified for fish disease.

Several researchers used their own datasets, which they gathered using self-designed underwater equipment for months (Siddiqui et al., 2018; Villon et al., 2018)yet it remains difficult and time-consuming. In this paper, we present a method to assist the identification of fish species on underwater images, and we compare our model performances to human ability in terms of speed and accuracy. We first tested the performance of a convolutional neural network (CNN. Researchers have also made use of several publicly available databases of real-world underwater images which includes the Fish4Knowledge dataset, the Underwater Image Enhancement Benchmark Dataset (UIEB), as well as ImageCLEF, National Oceanic and Atmospheric Administration (NOAA), LifeCLEF2014 (LCF-14). However, available datasets usually have insufficient data with degradation features characteristics due to limited scenes and poor environment of the water medium (Mohd Azmi et al., 2019).

Image processing is a subfield of AI used to enhance data collected by cameras, x-ray machines, microscopes, radar and satellite sensors (Gonzalez & Woods, 2017). An effective underwater image enhancement is needed as degradation of the underwater images could lead to failure for any visual detection of the images. This action is critical to improve original underwater image quality before training the classification model. Enhancement techniques generate a clearer output image than the original underwater images, making it much easier to visualize the targeted objects in a complicated environment. Subcategories of image enhancement including contrast enhancement, colour correction and hybrid approaches could process the degraded images by restoring the blurred images, enhancing the contrast and removing the unwanted noise (Raveendran et al., 2021).

The advanced approaches for classification that widely used is known as Machine Learning (ML). Yet, in the recent years, revolutionary technology has raised deep learning (DL), a subset of ML that has gained increasing interest due to outstanding performances in various industries. In 2015, research was conducted to detect and to recognize fish species using classification model namely Convolutional Neural Network (CNN). Fast R-CNN (Region-based Convolutional Neural Network) were applied to the domain-specific underwater environment as a faster object detection technique and has successfully proved that CNN detects 80 times faster than the previous technology (Li et al., 2016). Another research has developed CNN-based technique known as YOLO (You Only Look Once) network to detect fish underwater (Sung et al., 2017). The classification accuracy reached 93%. Hence, the great processing capabilities of CNN have demonstrated that this method is effective for problems classification.

Materials and Methods

All the processes involved in the automated protozoan white spot disease detection system are described in detail. Figure 1 demonstrates the system's architecture which consists of the activities for each phase.

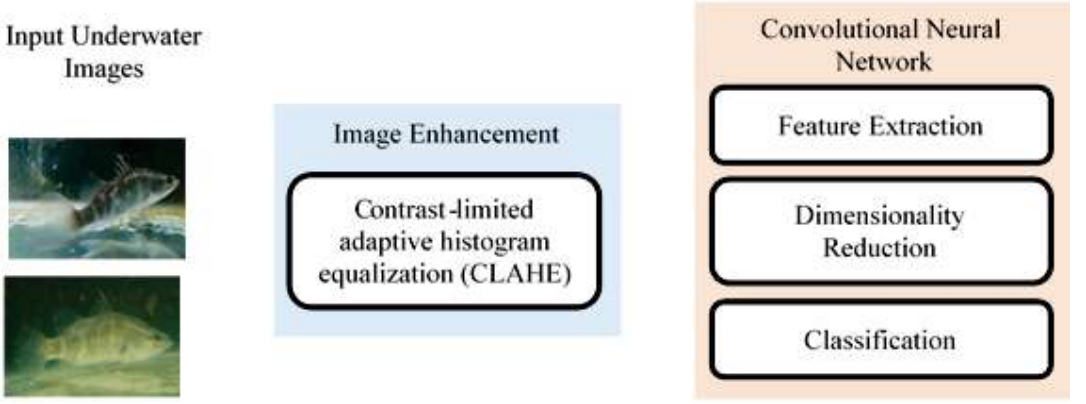
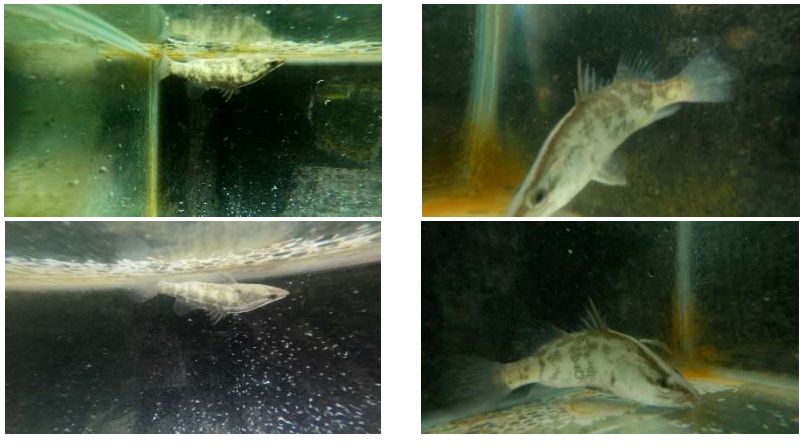


Fig. 1. Architecture of automated protozoan white spot disease detection system

Underwater Image Acquisition

Underwater fish image datasets were prepared from the 4K footage shot taken using GoPro camera at National Fish Health Research Center (NaFisH), Batu Maung Fisheries Research Institute, Penang. Images were extracted from the shot to create the datasets. The extracted images were screened and any images containing no fish were discarded. The datasets contained images of normal and protozoan white spot infected fish in their natural state, without artificial light or filters as displays in Figure 2.

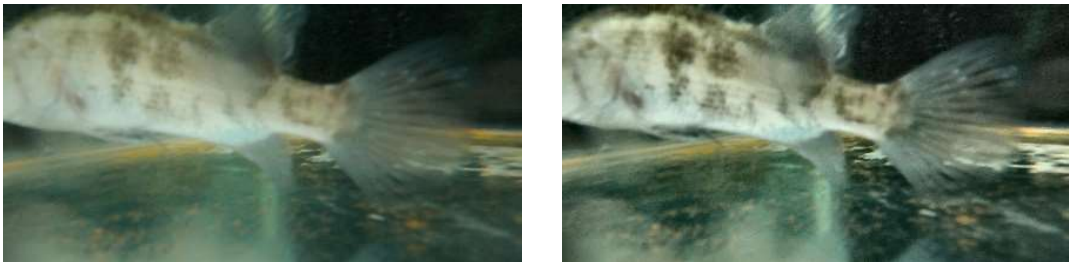


Normal Infected fish

Figure 2: Sample images of normal and protozoan white spot infected fish

Image Enhancement

The detection procedure begins with image processing to enhance the image degradations as an input for the classification to ease the detection process. Contrast enhancement is considered as an important technique for improving image quality and helps recover lost information in images. The vision processing approach often used to improve contrast is the contrast-limited adaptive histogram equalization (CLAHE) algorithm, an extension of adaptive histogram equalization (AHE). CLAHE improves contrast while equalizing the image histogram when compared to the standard histogram equalisation (HE) (Pramunendar et al., 2018)such as varying light intensity levels and varied wavelengths. Low quality of underwater images is one of the major problems in identification of fish species during monitoring of underwater ecosystem. Improving the quality of underwater images is important for accurate fish identification. Some researchers introduce various methods that address colour-correction problem for underwater images. However, previous researches do not consider the noises produced during the implementation of the image processing techniques. To deal with this problem, we propose a novel method called novel contrast-adaptive colour-correction (NCACC. This is because noise amplification issues arising when using HE can be avoided by using CLAHE (Sharma et al., 2019; Suharyanto et al., 2021)besides that light can be absorbed by seawater, as well as the turbidity level of seawater, so special techniques are needed to get clear underwater imagery. In underwater environmental conditions, the images obtained are usually of very poor quality. Backlight and attenuation will occur this is due to water conditions, objects that dissolve easily in water, and other particulate matter so that there is the degradation of the underwater image. Because it is very important if the image is improved in quality to facilitate the process of describing objects. Image matching techniques to determine the key points of image pairs are needed in three-dimensional reconstruction research. Speeded Up Robust Features (SURF. As a result, the enhanced image could highlight the image's important features before moving on to the classification step. Figure 3 shows the conversion of original to enhanced image.



Original Enhanced

Figure 3: Sample images of original and enhanced image of protozoan white spot infected fish

Classification

Enhanced images were processed using CNN to detect the presence of white spot disease. There were two sets of classes to be classified namely normal and infected. The datasets consisted of 60% infected fish images and 40% normal fish images, where 80% would be used for training and 20% for testing. In this study, CNN algorithm was chosen as classification method to classify white spot disease.

During the training process, CNN began by acquiring the enhanced image from the previous stage as the input layer. These pixel values were analysed using multiple connected layers to identify features of white spot disease. Convolution operations were executed in the convolutional layer to extract features such as edges and corners using numerous independent kernels or filters. Each kernel interpreted the input images pixel by pixel and consequently produces a feature map. Then, the rectified linear unit (ReLU) operations were adapted to the feature maps to add nonlinearity in the network. In the pooling layer, the size of input images was reduced using max pooling. After that, the pooled feature map was flattened into a long vector before going into the fully connected layers. During the fully connected layers, the set of features were combined to predict the classes either infected or normal. The error was estimated in each cycle and backpropagated until the network was well-trained. Subsequently, during testing process, CNN was able to generate the output indicating whether the image included the protozoan-infected fish or the normal fish. The overview of classification using CNN is shown in Figure 4.

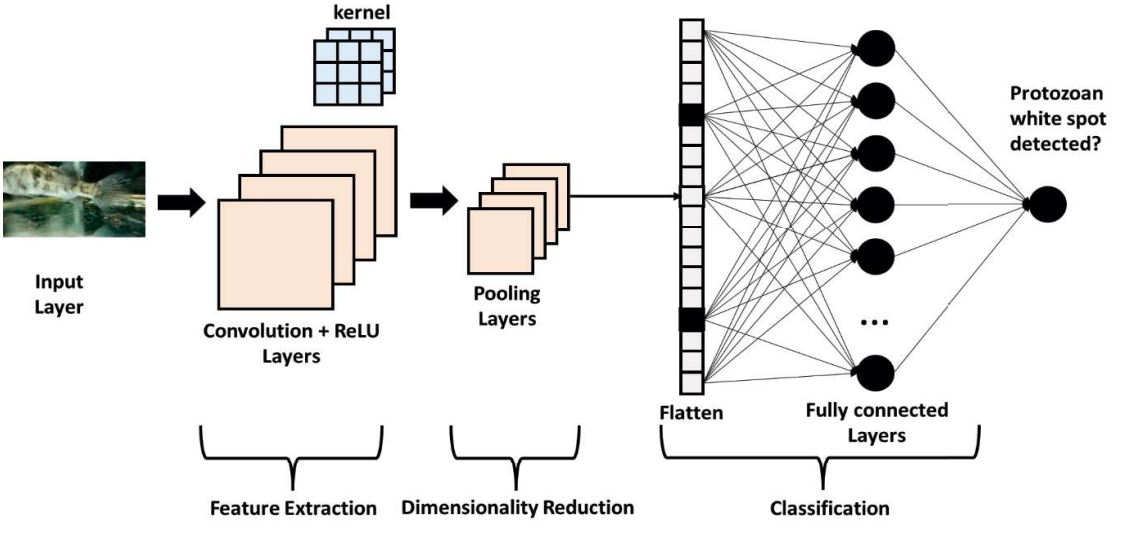


Figure 4: Overview classification process using CNN

Performance Evaluation

The performance of the system would be evaluated according to confusion matrix as presented in Figure 5. A confusion matrix is made up of four components: True Positive (TP), True Negative (TN), False Positive (FP), and False Negative (FN). The terms TP and TN indicate true predictions respectively. Meanwhile, positively false predictions are denoted by FP, whereas negatively false predictions are denoted by FN. Based on the confusion matrix, accuracy of the proposed model was computed by applying the formula as shown in Equation 1. Accuracy measures the proportion of

specifically classified images over the total number of images in the test dataset.

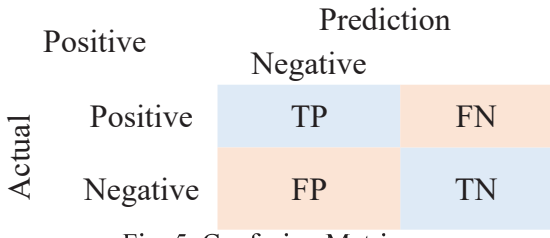


Fig. 5. Confusion Matrix

$$\text{Accuracy (\%)} = \frac{(TP+TN)}{(TP+TN+FP+FN)} \times 100$$

Equation 1. Accuracy

Results

A total of 30 normal fish images and 30 protozoan white spot infected fish images were tested. Table 1 and Table 2 demonstrate the accuracy of all the tested images. These accuracies determine the percentage of the prediction model to classify each tested image. Additionally, Table 3 presents the summary of confusion matrix that was constructed from the results obtained in Table 1 and Table 2.

Table 1: Normal fish detection

Image	Prediction	Accuracy
1	Normal	100
2	Normal	100
3	Normal	100
4	Normal	99.99957275
5	Normal	99.99991608
6	Normal	100
7	Normal	99.99995422
8	Normal	99.49093628
9	Normal	100
10	Normal	99.99997711
11	Normal	100
12	Normal	100
13	Normal	99.99797058
14	Normal	99.99393463
15	Normal	99.99990845

Table 2: Infected fish detection

Image	Prediction	Accuracy
1	Infected	100
2	Infected	99.94576263
3	Infected	99.9947052
4	Infected	99.99806976
5	Infected	99.99807739
6	Infected	99.99807739
7	Infected	99.99954987
8	Infected	99.57289124
9	Infected	99.57289124
10	Infected	99.99967957
11	Infected	99.7928009
12	Infected	100
13	Infected	99.97796631
14	Infected	99.9997406
15	Infected	99.84117889

16	Normal	99.99998474	**16**	Infected	99.99998474
17	Normal	90.63188171	**17**	Normal	96.03152466
18	Normal	100	**18**	Infected	99.91448975
19	Normal	99.9998703	**19**	Infected	99.97936249
20	Normal	99.95469666	**20**	Infected	99.99997711
21	Normal	99.97249603	**21**	Infected	99.83209991
22	Infected	82.51512909	**22**	Infected	97.74310303
23	Normal	99.99908447	**23**	Infected	99.99819946
24	Normal	99.99998474	**24**	Infected	96.77257538
25	Normal	99.72317505	**25**	Infected	95.992836
26	Normal	99.99891663	**26**	Infected	99.96543884
27	Normal	99.99998474	**27**	Infected	99.99993134
28	Normal	99.99982452	**28**	Infected	99.97146606
29	Normal	99.96932983	**29**	Infected	99.99976349
30	Normal	100	**30**	Infected	99.99976349

Table 3: Summary confusion matrix

		Detection	
		Infected	Normal
Fish Condition	Infected	29	1
	Normal	1	29

The values shown in the diagonal pattern of Table 3 accurately detected normal and infected fish images. 29 infected fish images were correctly detected while a single image was wrongly detected as normal. Meanwhile, a single normal fish was incorrectly detected as infected and the rest 29 were correctly detected as normal. According to Equation 1, 96.67% of accuracy has been achieved for protozoan white spot fish disease detection. The results demonstrated the potential of the proposed approach to detect protozoan white spot disease based on underwater images.

Discussion

The ocean covers seventy-one percent of the Earth's surface making it a complex task for diagnosing fish disease due to the challenges of underwater environment and it demands high level of expertise. As an alternative to the traditional method, the demand of fish disease detection study using new approach should be fulfilled to continuously sustain the productivity of aquaculture sector. As part of the efforts, this study strategized the use of image enhancement technique on the degraded underwater images successfully, making the classification process more accurate in detecting the

contagious protozoan white spot disease. Combination of CLAHE and advances technology of CNN has showed the potential to detect the disease based on underwater images. 96.67% accuracy achieved in this study proved that this action could become the key to early prevention of the outbreak and this effort could ease the participation of fish experts in preserving the aquaculture sector.

Conclusion

This research aimed to develop more advanced algorithm for performing detection tasks based on underwater images. Even though most of the past research are not limited to fish disease detection, the major picture of the integration model has been demonstrated in this study as a way to discover fish disease outbreak in an early stage. The results achieved in this study could also become the standard reference for any future studies. Besides maximizing the use of AI in the sector, Malaysia's food security could be secured, which consequently will lead to the sustainable development of aquaculture.

Acknowledgement

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Advances in Technologies towards Enhancing Shellfish Wellbeing for Optimum Aquaculture Production

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Abstrak: Pengeluaran akuakultur telah wujud sejak abad ke-12, walaupun ia tidak begitu popular kerana perkembangan pesat perikanan tangkapan. Sehingga tahun 2000-an, perdebatan mengenai potensi akuakultur sebagai alternatif kepada perikanan artisanal telah menjadi hangat. Apabila jurnal Nature menerbitkan ulasan tentang kemampanan dan sumbangan akuakultur kepada dunia dari 1997 hingga 2017, banyak negara tertarik kepada subsektor itu, yang turut menyaksikan perkembangan pesat dalam akuakultur. Pembangunan akuakultur global tidak pernah tanpa halangan yang berpunca daripada operasinya. Cabaran seperti makanan berkhasiat, perkembangan antibiotik dan vaksin untuk melawan penyakit dan parasit, teknik pembiakan mampan, konflik sosial, kesan alam sekitar, ketersediaan kemudahan air dan budaya yang baik serta isu ekonomi dan pemasaran telah membuatkan para penyelidik berdiri teguh dalam usaha mencari penyelesaian yang mampan. Akibatnya, banyak kemajuan teknologi telah direkodkan dalam beberapa tahun kebelakangan ini. Pengenalan teknologi genetik, Internet of Things (IoT), transkriptom, nanoteknologi, penapis mekanikal dan penapis biologi dalam Recirculation Aquaculture System (RAS), proses pengekstrakan Supercritical Fluid (SCF) dan penjujukan RNA (mRNA) messenger throughput tinggi (RNA-Seq).) kemudahan sememangnya telah meluaskan prospek akuakultur yang lebih mesra alam dan lebih mampan untuk memenuhi matlamat Pertubuhan Makanan dan Pertanian (FAO) 2050.

Abstract: Aquaculture production has been in existence since 12th century, although it was unpopular due to the flourishing of capture fisheries. Up to 2000's, debates on the potentials of aquaculture as an alternative to artisanal fisheries has been intense. When *Nature* journal published a review on the sustainability and the contribution of aquaculture to the world from 1997 to 2017, many countries became attracted to the sub-sector, which saw its rapid development. The global aquaculture development has never been without hurdles emanating from its operations. Challenges such as proper nutritious feed, developments of antibiotics and vaccines to fight diseases and parasites, sustainable breeding techniques, social conflicts, environmental impacts, availability of good water and culture facilities as well as economic and marketing issues have kept researchers on their feet over the years in attempt to find lasting solutions. Consequently, numerous technological advancements have been recorded in recent years. Introductions of gene technology, Internet of Things (IoT), transcriptomes, nanotechnology, mechanical filters and biological filters in Recirculation Aquaculture System (RAS), Supercritical Fluid (SCF) extraction process and high-throughput messenger RNA (mRNA) sequencing (RNA-Seq) facilities have indeed broadened the prospects of eco-friendlier and more sustainable aquacultures to meet the Food and Agriculture Organization (FAO) goal of 2050.

Keywords: Advance aquaculture, gene technology, Internet of Things (IoT), Recirculation Aquaculture System (RAS)

Introduction

The global status and strength of aquaculture must have been birthed from the "Blue Revolution", a replica of the "Grain Revolution" of higher cereal yields from 1950's to date. Since 1974, aquaculture could only boast about 7% contribution to the global fish consumption (O'Donncha & Grant, 2020). Many countries began to consider aquaculture as a viable alternative upon realizing the finite nature of capture fisheries. This sustained attention has led to the exponential growth of cultured fin and shellfish from 10 million tons in 1987 to 29 million tons in 1997. Aquaculture, apart from providing a better alternative to capture fisheries, has shown greater potentials in curbing malnutrition among the ever-expanding population of the world especially the impoverished communities.

Today, the world fish production hits 167 million tons with aquaculture contributing 73.8 million tons (44%) (Jayasankar, 2018)Labeo rohita and Cirrhinus mrigala. It is no longer news that the world capture fisheries cannot sustain the over 7.5 billion people which are projected to grow to 9.8 billion by 2050 (Osmond & Colombo, 2019). Such enormous increasing population poses serious challenge in terms of supply for quality and nutritious food. The threat is further compounded by reports showing that almost 35% of the world fish stock have already been overfished, sustainable fishing only accounts for 55%, while only 10% are being underutilized (Noakes, 2018). With the current pace of development in aquaculture, it is expected to contribute over 60% of the seafood for human consumption by 2030 (FAO, 2018). Rapid aquaculture development, however, is not without its difficulties. It is frequently challenged with water and energy constraints, land rivalry, environmental integrity, disease and parasite outbreaks, as well as climate change (Wang et al., 2018). These issues have prompted farmers and academics to work towards the industry's long-term sustainability by collecting, processing and analyzing huge amounts of diverse data.

Therefore, this article aimed at reviewing the recent activities towards ensuring sustainable development and technologies in aquaculture. In addition, this article identified important technologies in aquaculture production systems and attempts to analyze the major improvement areas regarding previous conventional methods and processes.

Aquaculture in brief

Aquaculture involves breeding, rearing and harvesting of fin, shellfish and algae, as well as other aquatic organisms that are beneficial to man in all kinds of aquatic environments. These water environments vary widely in salinity, and therefore the aquaculture is classified into freshwater, marine and brackish water aquacultures. Freshwater aquaculture entails the rearing of beneficial aquatic organisms in water below 0.5ppt salinity. Such culture environments include ponds, reservoirs, lakes and river. The history of freshwater fisheries can be traced in China back in 12th century (FAO 2018). However, major milestone has been recorded between 1900–1700 during the remarkable expansions of operation and breakthroughs in seed production. Improved communication technology which led to the widespread of information transfer resulted in the rapid global development of freshwater aquaculture. Among the prominent of the new technologies is the introduction of induced spawning cultivable species. This technology consequently has helped to reduce pressure and dependence on wild seed sources (Ed-Idoko et al., 2021). This technology was initially tested on the two major freshwater fish species, Asian carps and Indian major carps. The

period of 1970 to date, has witnessed tremendous developments in aquaculture with emphasis on selection and culture of high value and exportable species (Naylor et al., 2021).

The share of freshwater aquaculture to the global fish production has increased significantly in the past 20 years while relying on compound feed, produced mostly from terrestrial and some marine ingredients (Naylor et al., 2021). Despite its enormous contributions, freshwater fisheries have been under-reported as compared to marine aquaculture. For instance, out of the 11,625 published English articles from year 2000 to 2020, more than half of the articles emphasized on mariculture and 68% on high value mariculture. This data does not include the vast non-English publication from Asia, especially China where freshwater aquaculture begun. Freshwater fish contributes 47,102,391 tons (63.8%) of the global fish production with the major production is from China (Jayasankar, 2018) Labeo rohita and Cirrhinus mrigala. Marine aquaculture encompasses the culture of aquatic species in water bodies up to 35 ppt salinity. Diverse fin and shellfishes such as barramundi (*Lates calcarifer*), grouper (*Mycteroperca* sp. and *Ephinephelus* sp.), mangrove red snapper (*Lutjanus argentimaculatus*), whiteleg shrimp (*Penaeus vannamei),* tiger shrimp (*Penaeus monodon*), oyster (*Crassostrea* sp.) and clam (*Tegillarca granosa).* These species are cultured in diverse facilities like cages, pens, raceways and ponds.

Key development areas and technological advancements in shellfish aquaculture

Shellfish aquaculture like any other venture is confronted with challenges such as species domestication and improvement, production facilities, disease control and epizootics, provision of appropriate feed and development of feeding mechanisms (Li et al., 2020), including water quality issues. These problems have given rise to some technological innovations aimed at providing solutions and making shellfish aquaculture more sustainable (Figure 1) (Maulu et al., 2019). Recent technologies like nanotechnology are being successfully applied to boost shellfish aquaculture production through the discovery and the application of novel nanoparticles as well as nanocomposites. This technology takes advantage of the extremely small size nano-based feed supplements like Nano-863, dietary selenium, zinc and copper nanoparticles. These particles are easily absorbed in the cell and can improve fish growth performance and resistance to disease. In more advanced situations, this technology uses nano emulsions which are made up of immiscible liquids in the coating of surfaces. This is specifically applicable for protecting fishing tools and equipment (Dawit Moges et al., 2020).

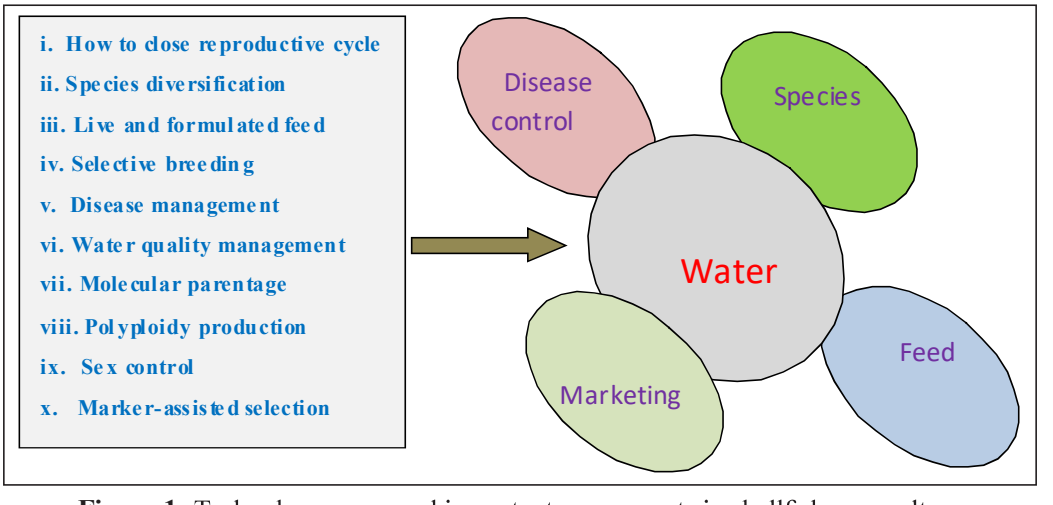

Figure 1: Technology areas and important components in shellfish aquaculture

The technology of Recirculation Aquaculture System (RAS) is no longer considered as new. The system started in the late 1960's with a Japanese biological purification kit static water aquaculture that utilizes gravel as the only medium. This was shortly followed by the European packaged multi-stage aquaculture system (Wu et al., 2018)digestion and nutrients balances of Atlantic salmon (Salmo salar. The application of mechanical filters and biological filters to remove suspended particles has now become a recent technology. This novel approach has been proven to be successful in the elimination of ammonia nitrogen, nitrite, feces, uneaten feed, and at the same time improves dissolved oxygen, removes carbon dioxide and maintains optimum temperature within the culture facility thereby eliminating the possibility of disease outbreaks (Strauch et al., 2018; Hang Pham et al., 2021) inland-based shrimp breeding requires significant inflow of high-quality freshwater. In turn, discharge of substantial loads of poor-quality effluents negatively impacts adjacent water bodies and favors disease outbreaks. This project describes the implementation of a laboratory-based continuous closed recirculation aquaculture system composed of a constructed wetland (CW. Furthermore, farmers will be able to mitigate the effects of climate change such as rainfall variability, floods, droughts, salinity fluctuations, and ocean acidification by constructing an indoor RAS system (Figure 2).

Figure 2: Indoor RAS system will ensure the sustainable operation regardless the effect of monsoon season in tropical country, including Malaysia.

Gene technology is expected to reduce the over-dependence on fishmeal and fish oil by developing genetically engineered feed ingredients for better fish growth performance, higher feed conversion efficiency and improved omega-3 content which helps to boost fish immune system against infectious diseases (Osmond & Colombo, 2019; Yang et al., 2022). Gene technology has also led to the development of transgenic shellfish species with better product quality. In another development, the challenge to find a more sustainable and renewable protein ingredient has also produced a new technology in recent times. Development of single cell proteins (SCPs) - protein meals based on microbial or algal biomass; no doubt has what it takes to cater for this need. This technology, although promising, is still at the novel stage (Alhazzaa et al., 2019; Jones et al., 2020)insufficient n-3 long-chain (≥C20. So far, this review observed that SCPs has already achieved measurable success in Atlantic salmon, rainbow trout and whiteleg shrimp.

One of the recent shellfish aquaculture technologies that is worth of mention is precision aquaculture. This technology operates on a set of interconnected sensors provided within the marine environment

to monitor, analyze and interpret information in order to provide decision support for marine shellfish aquaculture operations (Huan et al., 2020)this article has developed a water quality monitoring system for aquaculture ponds based on the narrow band internet of things (NB-IoT. This framework which is hinged on Internet of Things (IoT) combines sensors, cloud and analytics to enhance real time evidence based on decision making in order to optimize aquaculture operations (Balakrishnan et al., 2019; Donncha & Grant, 2020)particularly in creating nations. Internet of Things (IoT. Undoubtedly, traditional shellfish aquaculture practices have been over-stretched due to continuous increase in fish demand thereby leading to environmental water rate imbalances. This has led to the proliferation of fish pathogens and diseases alike, and ultimately culminating to loss of product quality (Shefat, 2019). The advancement in technology, which has taken a toll on all sectors of the world economy has not left shellfish aquaculture behind. This has led to the concept of intelligent fish farms in recent years (Wang et al., 2021). Studies revealed that intelligent fish farms aim at replacing human labour with machines thereby solving the problem of labour and lack of precision. In shellfish aquaculture, intelligent fish farms are directed at improving disolved oxygen, optimizing feeding and ultimately disease prevention (Shefat, 2019). The system utilizes latest information technologies such as Internet of Things (IoT), 5G network, big data, artificial intelligence, robotic and cloud computing to generate accurate and dependable information where fish wellfare can be monitored and favourable parameters can be maintained within optimum limits (Balakrishnan et al., 2019; Donncha & Grant, 2020; Wang et al., 2021; Yadav et al., 2022).

Similarly, the study of action recognition in fishes has led to the development of a Dual-Stream Recurrent Network (DSRN) in recent times to automatically obtain the spatio-temporal behavior of salmon during swimming. This high technology combines the use of a spatial network with motion-temporal information, a 3D-convolutional motion network as well as a Long Short-Term Memory (LSTM) recurrent classification network. It has been reported that the 80% accuracy of this technology has been validated by the task such as exact prediction of feeding and non-feeding behaviors in salmon in actual farm operations. This technology has consequently prevent wasteful feeding that often results to lose of water quality and disease outbreaks in shellfish culture systems (Halili et al., 2017; Misimi et al., 2017; Måløy et al., 2019).

Disease and parasite have been the critical and very important factors standing in the way of aquaculture development. Diseases of aquatic organisms emanate from bacteria, virus, fungi, nematode, protozoa, and nutritional deficiencies. Nevertheless, technological advancement in vaccinology, biotechnology and immunology over time has led to the development of numerous vaccines such as DNA vaccines, nano vaccines, subunit vaccines, genetically modified vaccines and polyvalent vaccines against many kinds of pathogens causing infectious diseases in aquatic animals and plants (Yue & Shen, 2022). Sadly, since 2000, when modified live *Edwarsiella ictaluri* vaccine – the first licensed bacterial live vaccine in aquaculture was produced, there is yet to be any breakthrough in the development of a potent vaccine to combat the infectious parasitic and fungal diseases even though recent reports have shown that the development of an immobilized adjuvant heat shock protein (Hsp70C) vaccine and cryptocaryonosis which offer better protection against parasitic disease can be potential breakthroughs (Shefat, 2019).

In another development, more research has arose in the transcriptomics of shellfish aquaculture which is believed to be closely related to disease, immunity, reproduction and development, growth and nutrition, toxicology and stress in cultured fish species (Alfaro & Young, 2018). Latest technology has led to the development of high-throughput messenger RNA (mRNA) sequencing (RNA-seq) facilities which have assisted in determining the functional complexity of the overall transcriptome

of an aquatic organism (Robledo et al., 2018)the rapid development and application of sequencing technologies has allowed aquaculture to narrow the gap, leading to substantial genomic resources for all major aquaculture species. While high-density single-nucleotide polymorphism (SNP. Its profiling has been used to identify and analyze the expression of aquaculture potential genes involved in growth, reproduction, development, immunity, illness, stress and toxicity (Abdelrahman et al., 2017; Ye et al., 2018; Chandhini & Kumar, 2019)and its medicinal properties have been attributed to these bioactive compounds. The saponin compounds with diverse structures play a pivotal role in Allium's defense mechanism. Despite numerous studies on the occurrence and chemical structure of steroidal saponins, their biosynthetic pathway in Allium species is poorly understood. The monosomic addition lines (MALs. Farmers can now use a mobile nucleic acid analyzer to undertake disease surveillance on the farm utilizing insulated isothermal polymerase chain reaction technology (iiPCR) (Figure 2).

Figure 3: Farm owners with minimal laboratory skills can use a mobile POCKIT™ nucleic acid analyzer to undertake on-site disease surveillance.

The advent of a new technology called genome editing using CRISPR/Cas9 has demonstrated great potentials for genetic improvement in shellfish aquaculture. The hallmark of this technology is the development and the improvement of production traits such as disease resistance. However, the progress and the success in this area are still slow due to the heritability problem of the improved traits and the generation interval of the species (Gratacap et al., 2019). In addition, surrogate broodstock technology is also slowly taking the center stage recently. This technology involves the production of donor-derived gamete in a surrogate fish (the recipient individual), by transplanting germ cells of the donor into the recipient of the same or individual of another species (Yoshizaki & Yazawa, 2019)and comprises transplanting germ cells of a donor into recipients of a different strain or different species. The following applications of this technology are expected in the field of aquaculture: (1. However, this technology is still developing.

In most recent times, the application of herbs for disease prevention and cure in shellfish aquaculture is gaining popularity. It has been reported that medicinal plants show better potency compared to chemotherapeutic agents due to their wide spectrum activity, low cost and eco-friendliness (Anjusha et al., 2019). To further understand the working principle of these medicinal plants, these herbs have

demonstrated the ability to pose as immunostimulants, thereby modulating the immune response to improve the health status of shellfish.

Over the years, several attempts have been made at improving fish welfare in order to enhance productivity. Such technologies are designed to remove harmful substances that could lead to water deterioration and disease outbreaks. One of such popular systems is called aquaponics (Armenta-Bojórquez et al., 2021; Zhang et al., 2021)including water reutilization, discharge mitigation, and increased profitability by leveraging the symbiotic relationship between organic waste, bacterial mineralization, and plant filtration. The aim of this study was to assess the production of two food items of global socio-economic importance cultivated at different salinities: Pacific white shrimp (Penaeus vannamei. Although aquaponics have proven as a viable technology to improve shellfish aquaculture, the soilless nature of the system as well as the waste utilization by plants have come under intense criticism. However, recently, the replacement of inert media with soil based aquaponics where plants are raised on soil may just be the awaited step towards the organic certification of aquaponic products (Fruscella et al., 2021)

In shellfish aquaculture, fish wellbeing and environmental impacts have been identified as crucial in quantifying the stress level of the fish. Stress levels are associated with fish susceptibility and vulnerability to disease infection and low productivity (Raposo De Magalhães et al., 2020). Over the years, stress monitoring in shellfish aquaculture up until today have depended on a mere measurement of stress level in the study fish. Nevertheless, an emerging technology called proteomics has demonstrated potential for identifying biomarkers in stress monitoring. Some of the biomarkers could be identified as overcrowding, handling and hypoxia, ably utilized for an unbiased evaluation of fish protein-based adaptations.

Conclusion

Aquaculture has proven its worth as the savior of capture fisheries overtime, while providing alternative quality source of protein to combat malnutrition globally. Since the 12th century, aquaculture has developed from contributing only 7% to about 56% of the world fish consumption. It is worthy to note that freshwater aquaculture produces more than 70% of this output. To date, China still at the top in aquaculture production contributing up to 54% of the world supply. Aquaculture development has not been without challenges; ranging from finding suitable feed, combating disease and parasites, genetic improvement, water quality and environmental impacts emanating from the wastewater generated and discharged. These challenges have led to a corresponding technological advancement especially in recent years. Although each technology is designed specifically for a particular area and challenge in aquaculture system, their overall goal is to have more development and better sustainability in aquaculture.

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Mass Mortalities of Golden Pomfret (*Trichinotus blochii*) at Floating Cages Pulau Aman, Penang Associated with Oxygen Crisis, Multiple Infections of Parasites and *Vibriosis*

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Abstrak: Kematian besar-besaran (80%–100%) ikan bawal emas berumur antara satu hingga lima bulan telah dilaporkan oleh penternak ikan di sangkar terapung di luar pantai Seberang Perai, Pulau Aman, Pulau Pinang. Justeru, objektif kajian ini adalah untuk mengenal pasti punca kematian ikan yang berlaku. Penyiasatan di tapak dengan pensampelan ikan (n=13) dan air laut telah dibuat untuk analisis di makmal. Parasit diperiksa di kulit dan insang. Kaedah aseptik dalam inokulasi bakteria dan pengasingan pada agar soya tryptose (TSA+1.5% NaCl) dilakukan daripada organ dalaman buah pinggang, limpa, hati dan otak. Tisu organ terkumpul setiap sampel telah dirawat dalam alkohol 95% untuk pengesanan virus yang menyebabkan Nekrosis Saraf Viral dan penyakit Iridoviral Ikan Siakap Merah menggunakan tindak balas rantai polimerase. Analisis genom menggunakan Penjujukan Generasi Seterusnya spesies bakteria dan capsalid telah dilakukan pada NovaSEQ6000 Illumina. Analisis *in-situ* parameter air menunjukkan kandungan oksigen terlarut yang rendah (3.0–3.5 mg/L) manakala parameter lain berada dalam julat normal untuk aktiviti akuakultur. Prevalen tinggi parasite *Neobenedenia melleni* (92%) dan lintah laut *Zeylanicobdella arugamensis* (31%) ditemui dalam ikan bawal emas dengan ketumpatan purata 5 dan 2, masing-masing. *Vibrio harveyi* berjaya diasingkan dengan enam faktor virulen Sistem Rembesan Jenis III (T3SS) ditemui sebagai pencilan tunggal atau campuran dengan *V. vulnificus*. Kematian tinggi bawal emas yang berlaku di tapak adalah disebabkan oleh oksigen terlarut yang rendah, infestasi daripada serangan parasit *N. melleni*, *Z. arugamensis* dan jangkitan bakteria.

Abstract: Mass mortalities (80%–100%) of golden pomfret aged between one to five months old have been reported by fish farmers at floating cages off the coast of Seberang Perai, Pulau Aman, Penang. Hence, the objective of this study is to determine the cause of fish mortalities. On-site investigation with sampling of fish (n=13) and waters were taken for laboratory analysis. Parasite was examined from skin and gills. Aseptic method for bacterial inoculation and isolation on tryptose soy agar (TSA+1.5% NaCl) were performed from the internal organs of kidney, spleen, liver and brain. Pooled organ tissues of each sample was fixed in 95% alcohol for detection of viruses causing Viral Nervous Necrosis and Red Sea Bream Iridoviral diseases using polymerase chain reaction. Genomic analysis using Next Generation Sequencing of bacterial and capsalid species *were* performed on NovaSEQ6000 Illumina. *In-situ analysis of water parameters* showed low dissolved oxygen (3.0–3.5 mg/L) while other parameters were within normal range for aquaculture. High prevalence of *Neobenedenia melleni* (92%) and marine leech *Zeylanicobdella arugamensis* (31%) were found in golden pomfret with mean density of 5 and 2, respectively. *Vibrio harveyi* were isolated with six virulent factors of Type III Secretion System (T3SS) found as a single or mixed isolates with *V. vulnificus.* High mortalities of golden pomfrets occuring on the site were caused by low dissolved oxygen and multiple infection from parasitic infestations of *N. melleni*, *Z. arugamensis* and bacterial

infection.

Keywords: mass mortalities, low dissolved oxygen, parasite, bacteria

Introduction

Mass mortalities of fish culture are becoming more frequent over the years in Southeast Asia region and worldwide, particularly in countries where aquaculture industry is booming and intensified due to continuous demand of high value protein source. Pollutants, originating from both land and sea as well as increase in fish farms activities add more nutrients to waters, whereas rapid climate change, heavy rain after prolonged dry and warm weather are among many factors in tropical region known to cause lethal and sub-lethal effects on marine life. Fish kills create a concern to general public due to the worries of pollution and the effect to human health by consuming the contaminated fish or sharing common water bodies. Prolonged dry and warm weather followed by heavy rain is a fatal combination that can cause massive fish kills in lakes or open waters within few hours (Svennevig, 2020). When it happens, aquaculture farmers are often badly affected. Mass mortalities of fish culture in Malaysian waters, are often associated with low dissolved oxygen due to algal blooms following heavy rain pour which may last for few days (Lim, 2012; Lim et al., 2014). The runoff through ditches and drainage system that contains high contaminants including suspended solid or garbage materials, inorganic and organic pollutions, bacteria contaminants from industry, animal farms and domestic wastes are commonly been reported as the cause of fish kills (Duraisamy & Latha, 2011; Shah, 2019; Zanuri et al., 2020).

Prolonged dry spell and warm weather predispose fish to stress that weakened the immune system. It is common for the affected fish succumb to bacterial infections as their immune system is weakened when the underlying problem has not been resolved. Stressors, often inevitable in most culture systems, predispose fish to bacterial-borne diseases (Snieszko, 1974). Over time, bacterial or parasitic problems are found to be another problems which enhance the cumulative mortalities percentage in the affected farms. A gradual increase in mortalities caused by *Vibriosis* sp. may be reaching up to 50% (Liao & Leano, 2008; El-Galil & Mohamed, 2012). The actual role of these bacteria may vary from a primary pathogen to an opportunist invader associated with other pathogen involved in the disease process (Richards & Roberts, 1978). Many of these bacteria are usual component of the microflora in aquatic habitat.

The development of cage culture activities has been associated with the emergence of parasitic disease (Kent, 2000). The overlapping generations of fishes in the culture system provide a pool of pathogens for any newly placed fish (Leong, 1997). The common report of parasitic infestation in cage-cultured species in Malaysia are capsalid monogeneans (Kua et al., 2015; Ihwan et al., 2016), marine leech (Rajiv & Shariman, 2017), crustacean isopods (Kumar et al., 2015) and caligus (Maran et al., 2009). Multiple problems are commonly inter-related, associated with stress factors such as rapid climatic change, poor water quality and management practices which may trigger primary or secondary infections that lead to acute disease outbreaks in fish culture system. Hence, the objective of this study is to identify the cause of high fish mortalities occurring at the cage culture farms, off shore Pulau Aman, Penang.

Materials and Methods

Sampling of fish

A total of thirteen (13) specimens of golden pomfret (*Trachinotus blochii*) consisting of apparently healthy, sick and moribund fish from 1–2 months old of age (n=7) and 5 months of age (n=6) were collected for examination and laboratory analysis. Post-mortem examination of fish was performed on-site. *Bacterial inoculation and isolation from the k*idney, spleen, liver and brain samples were taken under aseptic condition, cultured onto tryptose soy agar (Oxoid, UK) with the addition of 1.5% NaCl. Pooled tissues of internal organs and brain of each sample (13) was fixed in 1.5 mL tube containing 95% alcohol for polymerase chain reaction (PCR) test of virus nervous necrosis (VNN) and iridovirus (RSIVD).

Water quality analysis

In-situ analysis of physical water parameters such as temperature (°C), salinity (ppt), dissolved oxygen (mg/L), pH and total suspended solid (TTS, mg/L) were carried out at surface level and water depth of 7 meters using YSI 5908 probe (Yellow Springs, OH, USA) from 3 locations (inside cage, outside cage and outside premise/farm). Water was collected in 500 mL plastic bottles for basic chemical parameters such as total ammonia-N, nitrate, nitrite, sulphide and iron using HACH Kit (Loveland, CO, USA).

Parasitology

Five minutes fresh water dip of each fish sampled was performed in a small container after which the body of fish was gently stripped to remove any external parasites attached on the body for collection, identification and determination of mean density. The collected external parasites were fixed in 96% alcohol, placed on a glass slide followed by Giemsa staining, mounting with DPX and ready for microscopic examination and identification. A small piece of gill sections from the first and third segments of gills branch from both sides were cut off using scissors, placed on the glass slide and dried followed by fixation in methanol, Giemsa staining and microscopic examination.

Bacteriology

Pure culture of the isolate was further tested for characteristic growth on thiosulfate-citrate-bile salt-sucrose agar (TCBS), O/129 vibriostat sensitivity, Gram stain, oxidase, catalase, motility and API 20 NE (Biomerieux, France). Analytical profile index 20 NE Kit was used for biochemical identification of bacterial species with *i*dentification of more than 92.5% similarity to the referred database. Detection of virulent factors haemolysin (vvh) from eight isolates of V. vulnificus and 3 isolates of *V. alginolyticus* (collagenase gene) were tested using PCR method (Brauns et al., 1991; Di Pinto et al. 2005).

Three bacterial isolates (BE8B, BE9K and BE78K) and parasite *N. melleni* was sent to a private laboratory for sequencing and genomic identification. Sequencing was performed on a NovaSEQ6000 (Illumina, San Diego, CA) generating approximately 1 gb of paired-end data (2×150 bp) for each sample. Contigs smaller than 500 bp representing mostly sequencing artifact were removed and the filtered assembly was used for subsequent analysis. Genome assembly statistics were generated using QUAST (Gurevich et al., 2013). ABRicate (https://github.com/tseemann/abricate) for mass

screening of contigs that represent a consensus region of DNA for VFs was employed to perform a BLAST, based on nucleotide similarity search of the assembled genome in the National Center for Biotechnology Information (NCBI) and the Virulence Factor Database (VFDB). The Reference Gene Catalogue facilitates the examination of the genomic links among bacteria and virulent genes (Chen et al., 2005). Determination of VFs gene is based on identity threshold of more than 80% against VFDB.

Virology

DNA/RNA extraction procedures were carried out using taco™ DNA/RNA extraction kit (GeneReach Biotechnology Corp., Taiwan) following protocols outlined by the manufacturer. The PCR program for the detection of RSIVD was modified from the suggested MyTaq Red Mix protocol (Meridian Bioscience, Inc., Cincinnati, Ohio, USA) as follows; initial denaturation at 95°C for one min and 35 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 30 sec and extension at 72°C for 10 sec with final extension at 72°C for 3 min and final hold at 4°C. The size for the amplicon is expected at 570 bp using primer 1F/1R (Kurita et al., 1998). Reverse-transcriptase polymerase chain reaction (RT-PCR) profile for VNN was: reverse transcription 45°C 20 min, initial denaturation 95 °C 1 min, denaturation 95°C 10 sec, annealing 60°C 10 sec, extension 72°C 30 sec at the end of 40 cycles followed by final hold at 4°C. The PCR products were prepared for gel electrophoresis (1.5% agarose gel at 100 V for 30 min) and analyzed under gel documentation system for detection of VNN with expected size base pair of 258 bp (Nishizawa et al., 1995).

Results

Gross observation

Infestation of capsalid *Neobenedenia* sp. and marine leech (*Z. arugamensis*) were identified following the fresh water bath treatment and microscopic examination. The infested fish was badly affected with high mortalities estimated about 80% (**Figure 1**), scale drops and white patches on body, skin wound ranging from inflammation/redness on body in mild cases (46%) to serious skin ulceration (46%) were observed in golden pomfret size between 7–13 cm of total length (1–2 months old) (**Figure 2**). Internal changes observed were congestion of kidney (31%), pale liver (54%) and enlarged spleen (8%) with eodematous fluid in the abdomen.

Figure 1: Accumulative mass mortalities of more than 80% in golden pomfret (*Trachinotus blochii*) aged 1–2 months old of culture.

Figure 2: Skin ulceration on body in 46% of the fish (*Trachinotus blochii*).

Parasitology

Capsalid species at 92% prevalence, mean density of 5 and marine leech with prevalence of 31%, mean density of 2 were found on the body. Microscopic examination of gills tissues showed no other parasites detected from the normal gills. The prevalence of parasites was relatively low as farmers have treated the fish with fresh water bath a day earlier. Capsalid problems was heavy from the previous freshwater treatment carried out by a farmer. Mortalities was reported to continue and increased overnight as fish appeared very weak after treatment especially in golden pomfret aged 1–2 months.

Bacterialogy and PCR for detection of virulent factor

Bacteria isolation from the internal organs of kidney, liver and spleen showed the presence of *V. vulnificus* (69%), *V. alginolyticus* (31%) and *Pasteurella multocida* type 1 (31%) and *P. multocida* type 2 (15%) as mixed of two isolates or a single pathogen in organs of fish. PCR analysis of virulent factor haemolysin (*vvh*) from eight isolates of *V. vulnificus* and collagenase gene from three isolates of *V. alginolyticus* were found to be negative.

Using *in-silico* genome-genome hybridization approach, three isolates BE8B, BE9K and BE78K (identified as *V. alginolyticus* using API 20NE) were all assigned to the species *V. harveyi* with an average ANI value of 98.7%, a value that is much higher than the pairwise ANI against *V. alginolyticus* (86%). Virulent factors Type III Secretion System (T3SS) *vcrH, vcrD, vcrN, vcrI, vcrF* and *VPA0450* were identified from *V. harveyi* bacteria of diseased fish. Results are shown in Table 1.

Table 1: Identification of bacteria from golden pomfret using *in-silico* genome-genome hybridization and virulent factors (VFs) gene with identity threshold of >80% against VFDB.

Sample ID	*Bacteria*	ANI (%)	VFs gene	VFDB accession
1. BE8B	*V. harveyi*	98.7	*vcrH*	NP_798037
2. BE9K			*vcrD*	NP_798041
3. BE78K			*vscN*	NP_798047
			vscI	NP_798070
			vscF	NP_798073
			VPA0450	NP_779960

Virology

VNN and RSIVD were not detected from all sample.

Water quality analysis

pH, salinity, surface temperature, nitrate and phosphate (**Table 2**) were within the permissible limit according to Malaysia marine water quality Class 2 for aquaculture (Department of Environment, Malaysia, 2005). Dissolved oxygen in surface water was low (3.30–3.45 mg/L) at three locations within cages and at 7 metre depth (2.65–3.39 mg/L). Total ammonia level was slightly high (0.03 mg/L) due to an overnight accumulation of dead fish at the bottom of cages. Microscopic examination of water samples taken from three locations (inside and outside cage, outside farm area) showed

undetected to low count of microalgae cells (<10^3 cells/L). The water quality results are shown in **Table 2**.

Table 2. Water parameters at floating cages, Pulau Aman, Penang on 22 September, 2020.

Water parameters	**Cage culture site (3 locations)**		**Class 2 (Aquaculture)**
	Surface (mean±SD)	**7 meter depth** (mean±SD)	Acceptable range
1. Temperature (°C)	30.20±0.00	29.90±0.10	-
2. DO (mg/L)	3.40±0.09	2.99±0.37	5.0–10
3. pH	7.98±0.32	8.14±0.08	6.5–8.5
4. Salinity (ppt)	29.71±0.18	30.31±0.04	-
5. Total Ammonia-N (mg L)	0.022±0.011	-	0.07
6. Nitrite (mg/L)	0.035±0.001	-	0.055
7. Iron (mg/L)	0.02±0.00	-	<5
8. Sulphide (µg/L)	6.00±2.00	-	1–10

Discussion

Mass mortalities of more than 80% of golden pomfret and other fish species at floating cages off shore Pulau Aman, Penang on 22nd September, 2020 were caused by low dissolved oxygen (3.0–3.4 mg/L). Multiple infestations of capsalid *N. melleni*, marine leech *Z. arugamensis* and *V. harveyi* infections led to exacerbation of the existing problems in association with oxygen depletion and hypoxia in fish. Skin injury ranging from scale drop to laceration (46%) and ulceration on the body (46%) were observed in the golden pomfret aged 1–2 months old. Generalised inflammation and haemorrhages on body, fins and tail, congestion of kidney (31%), enlarged spleen (8%) and pale liver (54%) were suggestive of septicaemia.

Poor water quality have been reported by a farmer following the high tide water starting in the afternoon at about 2 pm on 20th September. High tides of 5.6 to 5.7 metre have been forecasted for September 19 and 20 by National Hydrographic Centre in Port Klang (Rajendra, 2020) with an alert of flood in the Selangor coastal areas. As expected, high tide water was also reported by farmers at Pulau Aman, Penang on 20th September, 2020. The water was reported to be fouled smell, milky brownish colour with high debris content. High organic content in waters was believed to have caused algae bloom leading to a decline in dissolved oxygen (DO). The DO level drop further in the early morning or late night leading to high mortalities of fish overnight as observed on a day before of occurance whereby the DO level of surface water (3.3–3.5 mg/L; mean 3.40±0.09) and water at 7 metre depth (2.7–3.4 mg/L; mean 2.99±0.37) were below the acceptable/safe level for aquaculture. Following the incident, fish health started to deteriorate when the appetite was reduced and fish started dying within two days.

Vibriosis affect all stages of fish growth and lead to as much as 50% mortalities in fish culture (Liao & Leano, 2008; El-Galil & Mohamed, 2012). *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus* and *V. harveyi* are the most common bacteria associated with various health problems in marine farm fishes, particularly in many tropical countries (Khouadja et al., 2013; Dong et al., 2017; Nurliyana et al., 2019). *Vibriosis*-infected fish exhibit sluggish movement, fin rots, skin darkness, haemorrhagic patches all over the body particularly at base of fins, detached scales with abscess like lesion and haemorrhagic prolapsed vent (Abdelaziz et al., 2017).

Numerous bacterial products such as extracellular products, proteases, lipase, chitinase, collagenase, haemolysin, siderophore, biofilm, lipopolysaccharide, type I, II and VI secretion systems and quorum-sensing regulating system were described as typical virulence determinants mediating pathogenesis in marine vertebrates (Austin & Zhang, 2006; Ruwandeepika et al., 2012). *Vibriosis* caused by *V. alginolyticus and V. vulnificus in marine fish culture* has been *accounted for less than 45% mortalities (Lopez* et al., *2002;* Yan et al., 2007). *V. alginolyticus* infection causes haemorrhage, skin ulcers, skin darkening and fluid accumulation in the peritoneal cavity and consequently, most fish die within 7 days (Rajan et al., 2001). Haemolysin is a common virulence factor reported in *V. vulnificus* associated with both fish and human diseases (Fouz et al., 2002). *However, our study showed that the virulent factor haemolysin (vvh)* was not detected in eight *V. vulnificus* isolated or collagenase gene from three isolates of *V. alginolyticus* (BE8B, BE9K and BE78K). Instead, whole genome sequence and *in-silico* genome-genome hybridization approach showed that these isolates were *V. harveyi* with multiple VFs of T3SS consisting of *VPA0450, vcrH, vcrD, vcrN, vcrI* and *vcrF.*

More specific and accurate identification of *P. multocida* are based on differences in capsular polysaccaride designated as A, B, D, E and F (Boot et al., 2004; Carter, 1955). Thus, species identification of Pasteurellaceae needs more comprehensive phenotypic, genetic methods such as 16S rRNA gene sequencing and serology test. More importantly, study on pathogenesis of *P. multocida* in fish culture needs to be determined from the isolate obtained before any molecular or serology work. *P. multocida* is a gram negative coccobacillus bacteria normally found in the respiratory tract of a healthy animal. It can act as a primary pathogen causing haemorrhagic septicaemia in cattle, buffalo, fowl cholerae in avian, atrophic rhinitis in pigs and secondary infection of pneumonic pasteurellosis in stressed animals (Khoo et al., 2019). Thus, the isolation of this bacteria from the internal organ of fish showed high possibility of faecal waste contamination from animal farms. However, pathological lesion and post-mortem changes observed were more likely to suggest *Vibriosis* rather than *Pasteurelllosis*.

Conclusion

Low dissolved oxygen (<4 mg/L) was found as the main contributing factors that caused high mortalities in golden pomfret at Pulau Aman cage culture. Mortalities occurred mainly at night or in the early morning whereby the dead fish was found floating in cages the next day. P*arasitic infestations of N. melleni* (92% prevalence) and marine leech *Z. arugamensis* (31%) were observed with secondary infections of *V. harveyi* identified from whole genome sequence analysis of bacteria from the infected fish. These were supported from necropsy examination that showed various lesions on the body such as scale drops, skin wound from mild to severe skin ulceration and haemorrhages on body, based of fins, tail and septicaemia. We believed multiple stress factors such as oxygen crisis and parasitic infestation triggered the secondary bacterial infections of *V. harveyi* in golden pomfret thus causing more than 80% mortalities in juvenile fish and up to 50% mortalities in grow-out stages..

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PvRaf knockdown using RNA interference increased survival of *Litopenaeus vannamei* infected with white spot syndrome virus

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Abstrak: Akuakultur udang merupakan sumber pendapatan utama di negara-negara tropika di rantau Asia Tenggara. Walau bagaimanapun, penurunan besar dalam pengeluaran udang telah disaksikan berikutan vurus virus pathogen utama, Virus Sindrom Bintik Putih (WSSV) yang terus berleluasa walaupun banyak langkah pencegahan dipraktikann untuk menghalang virus itu. Teknologi gangguan RNA (RNAi) telah digunakan untuk mendedahkan fungsi gen tertentu dalam virus dan perumahnya dengan tujuan mengawal WSSV dengan menjelaskan interaksi virus perumah yang kompleks. Kajian ini menentukan penglibatan PvRaf, komponen utama laluan Ras/Raf/MAPK. Raf menggalakkan kemandirian sel melalui interaksi protein-ke-protein dan mekanisme bebas MEK-ERK. Penjujukan DNA mendedahkan bahawa PvRaf mempunyai saiz 1.9 kb. Analisis filogenetik mendedahkan bahawa PvRaf sangat homolog dengan gen Raf organisma lain yang telah dilaporkan. Lebih-lebih lagi, PvRaf dinyatakan di semua organ penting yang menggambarkan ia adalah penting untuk fungsi metabolik udang dan juga boleh memainkan peranan dalam sistem imun semula jadi seperti yang diserlahkan dalam ekspresinya dalam hemosit. Keputusan PCR Kuantitatif *real time* (qRT-PCR) mengesahkan kejatuhan gen *in vivo* PvRaf menggunakan gangguan RNA. qRT menunjukkan bahawa PvRaf knockdown menghasilkan downregulation gen dalam udang yang dirawat PvRaf-dsRNA dan GFP-dsRNA. Analisis ekspresi gen menunjukkan penurunan yang ketara dari hari-0 hingga hari-1 selepas jangkitan dalam rawatan PvRaf-dsRNA berbanding dengan sampel kawalan. Analisis statistik data survival menunjukkan bahawa rawatan PvRaf-dsRNA mempunyai kesan perlindungan yang ketara terhadap WSSV berbanding dengan udang yang dirawat GFP-dsRNA dan PBS.

Abstract: Shrimp aquaculture is a major source of income in the intertropical countries of the Southeast Asian region. However, a great decline in production is observed due to a major viral pathogen, the White Spot Syndrome Virus (WSSV) which continues to prevail despite many preventive measures applied to deter the virus. RNA interference (RNAi) technology has been employed to reveal functions of specific genes in the virus and its host with the aim of controlling WSSV by elucidating complex host-virus interactions. This study determined the involvement of *PvRaf*, a key component of the Ras/Raf/MAPK pathway. Raf promotes cell survival through its protein-to-protein interactions and MEK-ERK independent mechanism. DNA sequencing revealed that *PvRaf* has a resulting size of 1.9 kb. Phylogenetic analysis revealed that *PvRaf* is highly homologous to the reported Raf genes of other organisms. Moreover, *Pv*Raf is ubiquitously expressed in vital organs suggesting that it is essential to metabolic functions of the shrimp and may also play a role in its innate immune system as highlighted

in its expression in the hemocytes. Quantitative Real-Time PCR (qRT-PCR) results confirmed the *in vivo* gene knockdown of *PvRaf* using RNA interference. qRT-PCR showed that *PvRaf* knockdown yields downregulation of the gene in the *PvRaf*-dsRNA and GFP-dsRNA treated shrimps. Gene expression analysis showed significant downregulation from day-0 to day-1 post-infection in *PvRaf*-dsRNA treatment relative to the control samples. Statistical analysis of the survival data indicated that *PvRaf*-dsRNA treatment has a significant protective effect against WSSV compared to GFP-dsRNA and PBS-treated shrimps.

Keywords – *Penaeus vannamei*, *PvRaf*, RNA interference, White Spot Syndrome Virus

Introduction

Shrimp aquaculture is an important source of income in the intertropical countries of the Southeast Asian region. It generates billions of dollars per year in trade and employs millions of people globally. In 2009, the Philippines produced 2204 metric tons of *P. vannamei* but declined to only 1827 metric tons in 2014 (Bureau of Agricultural Statistics Philippines, 2013). It was in early 2000 when several viral pathogens mostly the White Spot Syndrome Virus (WSSV) devastated shrimp farms in the Philippines (Magbanua et al. 2000). Several preventive measures are employed such as surveillance, early detection method, and broodstock selection to deter the WSSV infection in shrimp farms. However, most do not have a significant effect in controlling the virus mainly because shrimps do not have an adaptive immune system.

RNA interference (RNAi) is one of the most important scientific breakthroughs in the modern molecular biology era which allows scientists to observe and to elucidate the functions of specific genes through gene knockdown in shrimps. RNAi is often used in experimental molecular biology studies where it uses double stranded RNAs (dsRNAs) with sequence complementary to a specific gene of interest. In this study, Raf gene was functionally elucidated in *P. vannamei* using RNA interference. RNA interference is an effective biotechnology tool because it targets sequences in the specific host and the viral genes. The White Spot Disease caused by White Spot Syndrome Virus tops the list of diseases in shrimp resulting to 100% mortality within two to ten days post-infection.

Raf is a key component of the Ras/Raf/MAPK pathway. Raf promotes cell survival through its protein-to-protein interactions and MEK-ERK independent mechanism and has an antitumor activity. The interaction of Raf with apoptosis signal-regulating kinase 1 (ASK1) allows it to act independently in the MEK-ERK pathway to inhibit apoptosis (Chen et al. 2001). This study functionally elucidated Raf gene (*PvRaf*) in a penaeid shrimp, *P. vannamei.*

Materials and Methods

Acclimatization and acquisition of P. vannamei

Healthy, WSSV-free, and post-larvae *P. vannamei* at 15–20 days old were reared at the Wet Laboratory of Research Center for the Natural and Applied Sciences, Thomas Aquinas Research Complex, University of Santo Tomas, España, Manila, Philippines. Shrimp samples were acclimatized in sea water at 10 ppt salinity and pH 8.0, and *ad libitum* feeding with Sera™ shrimp feeds until their body weight was 3.0-5.0g.

Bioinformatics analysis of PvRaf

Genomic DNA samples were extracted from shrimp and subjected to PCR using *PvRaf* gene specific primers: (*Pv*RAF-F: 5'-ACGCCTGAACAGCCAGGATCA-3' and *Pv*RAF-R: 5'-GGCTCGGGGTTTTCACGTTCA-3'). DNA extraction and purification was done using Wizard Genomic DNA Purification Kit (Promega, USA). Purified samples were prepared and sent to 1st BASE DNA Sequencing Division (Singapore Science Park II, Singapore) for sequencing. The sequence was analyzed using GENETYX-MAC software. The consensus sequence was subjected to Swiss Institute of Bioinformatics Expert Protein Analysis System (SIB ExPASy) and EMBOSS Sixpack (EMBL-EBI). The functional annotated sequence was analyzed using SIB-SWISS Biozentrum software to reveal its protein structure. The resulting sequence was subjected to Basic Local Alignment Search Tool (BLAST) and aligned with the other reported Raf genes of different organisms using Clustal Omega software. A phylogenetic tree was constructed using Clustal Omega from EMBL-EBI.

Table 1: Primers that were used in the study.

Name	Oligonucleotide Sequence
WSSV-F	5'-GTACGGCAATACTGGAGGAGGT-3'
WSSV-R	5'-GGAGATGTGTAAGATGGACAAG-3'
β actin-F	5'-AACTCCCATGACATGGAGAATCAC-3'
β actin-R	5'-TCTTCTCACGGTTGGCCTTG-3'
GFP-F	5'-ATGGTGAGCAAGGGCGAGGA-3'
GFP-R	5'-TTACTTGTACAGCTCGTCCA-3'
GFP-T7F	5'-TAATACGACTCACTATAGGATGGT GAGCAAGGGCGAGGA-3'
GFP-T7R	5'-TAATACGACTCACTATAGGTTACT TGTACAGCTCGTCCA-3'
*Pv*RAF-F	5'-ACGCCTGAACAGCCAGGATCA-3'
*Pv*RAF-R	5'-GGCTCGGGGTTTTCACGTTCA-3'
*Pv*RAF-T7F	5'-TAATACGACTCACTATAACGCCTGAACAGCCAGGATCA-3'
*Pv*RAF-T7R	5'-TAATACGACTCACTATAGGCTCGGGGTTTTCACGTTCA-3'

Preparation of viral inoculum

Viral inocula were prepared from WSSV-infected shrimps, *P. vannamei.* WSSV-positive status was determined through PCR analysis. DNA was pooled in the virus' known main area of infiltration and replication, the gills. Gills were extracted and homogenized. After homogenization, the gill extracts were centrifuged at 14,000 rpm and 4°C for 5 minutes in a 1.5 ml centrifuge tube containing 1000 µL 1× PBS. The final supernatant solution that was obtained after centrifugation was filtered through a 0.2 µm filter membrane. The filtrate which contained the virus was suspended in an aliquot solution and stored at a -80°C freezer as the experimental viral inoculate.

In vivo viral titration assay

WSSV stock was diluted with 1× PBS ranging from 10^{-1} to 10^{-3} dilution. One dosage higher than the dilution that yielded 50% mortality rate was used. The 10^{-2} WSSV viral dilution dosage was used for the challenge test. Healthy shrimps from the respective tanks were intramuscularly injected with the abovementioned diluted viral inoculums. Shrimps were injected with 1× PBS which served

as negative control. Fifteen (15) shrimps per viral dilution were used and maintained at 10ppt and pH 8.0 in separate tanks. The number of deaths was recorded daily, and cumulative percentage mortality was calculated.

RNA Isolation

Total RNA was extracted from organs of the shrimps using TRIzol™ reagent (Life Technologies, USA) following the manufacturer's protocol. Briefly, the tissue was obtained from samples and were homogenized after adding TRIzol™ reagent proportional to tissue sample (1ml reagent to 50mg tissue sample). Following the homogenization process, the samples were centrifuged at 12,000 rpm and 4°C for 10 minutes. Homogenized samples were incubated for 5 minutes at 25°C. To complete the dissociation of the nucleoprotein complex, 0.2 ml of chloroform per 1ml of TRIzol™ was added and shaken vigorously for 15 seconds. The sample was incubated for 2 to 3 minutes at room temperature before centrifuged at 12,000 rpm and 4°C for 15 minutes. The aqueous phase was transferred into an RNAse free tube and 0.5 mL of absolute isopropanol per 1ml TRIzol™ was added, followed by incubation at 25°C for 10 minutes. The mixture was centrifuged again at 12,000 rpm and 4°C for 10 minutes. The supernatant was removed from the tube leaving only the RNA pellet. The pellet was washed with 1ml of 75% ethanol. The tube was centrifuged for the last time at 7,500 rpm and 4°C for 5 minutes. The final RNA pellet was air-dried for 5 to 10 minutes and resuspended in DEPC-treated water to maintain the integrity of RNA isolate. The quality and the concentration of the RNA were checked through gel electrophoresis and UV-spectrophotometry.

Synthesis of cDNA template

The isolated RNA from the previous procedure were used to synthesize first strand cDNA following the manufacturer's protocol for SuperScript™ III Reverse Transcriptase First-Strand Synthesis System (Invitrogen, USA). Briefly, RNA isolate, oligodT, dNTP mix, and DEPC-treated water were mixed and denatured at 65°C for 5 minutes using a thermal cycler and placed on an ice bath for 1 minute. After cooling the cDNA synthesis mix, the following components were added in specific order: 10× RT buffer, 25mM $MgCl_2$, 0.1M DTT, RNaseOUT™, and SuperScript™ III Reverse Transcriptase. Ten (10) µl of cDNA synthesis mix was added into each RNA primer mixture and gently mixed together. A brief centrifugation was done, and the mixture was incubated for 50 minutes at 50°C. To terminate the prior reaction, the mixture was incubated at 85°C for 5 minutes followed by chilling on ice.

Experimental conditions

Healthy, WSSV-free, and juvenile *P. vannamei* were divided into groups: a) the experimental treatment set-up (*PvRaf*-dsRNA treated) where thirty shrimps were injected with *PvRaf*-dsRNA; b) the unrelated treatment control (GFP-dsRNA treated) where thirty shrimps were injected with GFP-dsRNA; c) the untreated control where thirty shrimps were injected with PBS; and d) the naive treatment control where thirty shrimps were fed with commercial shrimp feeds *ad libitum.* There were four sampling days namely Days 0, 1, 3, and 7 post-infections. Gill tissues, the area where viral particles are more likely to be replicating were extracted from three randomly sampled shrimps and treated independently.

WSSV Challenge Test

Thirty (30) shrimps were used per treatment. WSSV infection was induced by intramuscular injection in the third abdominal segment of 10^{-2} WSSV viral dilution dosage 24 hours after the introduction of dsRNAs to the *PvRaf*-dsRNA treatment, GFP-dsRNA treatment, and PBS treatment. Cumulative mortality rate was recorded up to 7 days post-infection.

Real time quantitative RT-PCR analysis (qRT-PCR)

Time course Real-time quantitative RT-PCR was applied to determine the interaction of *PvRaf*-dsRNA treated shrimps with WSSV pathogen to reveal sequence specific gene silencing by *PvRaf*-dsRNA. Gill tissues, the area where viral particles are more likely to be replicating were extracted from each of the experimental set-ups. The tissues collected were placed in separate 1.5 ml centrifuge tubes containing TRIzol™ reagent (Life Technologies, USA) for RNA isolation, and quantified using UV spectrophotometer to ensure that all samples were of equal concentration when used for qRT-PCR analysis. A volume of 1 µl for each sample was reverse transcribed to produce a single-strand cDNA with the use of Superscript™ Reverse Transcriptase First-Strand Synthesis System (Invitrogen, USA) for qRT-PCR following the protocols recommended by the manufacturer. The following optimized qRT-PCR condition profile for *PvRaf* was used: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of (1) denaturation at 95°C for 30 seconds, (2) annealing at 58°C for 30 seconds, (3) extension at 72°C for 3 minutes and final DNA extension was done at 72°C for 5 minutes. Conditions for qRT-PCR were normalized using housekeeping gene β-actin.

Statistical analysis

Cumulative survival data was subjected to log-rank test and differences were considered significant at $p<0.05$ using software R.

Results and Discussion

Sequence and bioinformatics analysis

Molecular sequencing analysis revealed that *PvRaf* sequence has high sequence similarity to other Raf genes of other species. A resulting sequence was analyzed using GENETYX-MAC. The nucleotide sequences and the deduced amino acid sequences of the full length *PvRaf* consisted of an open reading frame of 1947 nucleotides encoding a predicted protein of 648 amino acids (Figure 1). The start and stop codons are also indicated in the figure respectively. The nucleotide sequence of *PvRaf* showed high homology especially within the conserved regions to known invertebrate and vertebrate Raf genes. Raf gene has three conserved regions namely CR1, CR2 and CR3. The CR1 conserved region of Raf gene consists of two domains, a Ras-binding domain and a cysteine-rich domain. The CR2 conserved region is a serine and threonine rich region where it is also the site of several phosphorylation events. Lastly, the CR3 conserved region is the kinase domain that is regulated by phosphorylation events (Okuda 2013). Based on the amino acid sequence analysis, the target gene elucidated in *P. vannamei* might be characterized as the Raf gene. The nucleotide sequence of *PvRaf* was submitted and published at the DNA Data Bank of Japan (DDBJ) with Accession number LC503538.

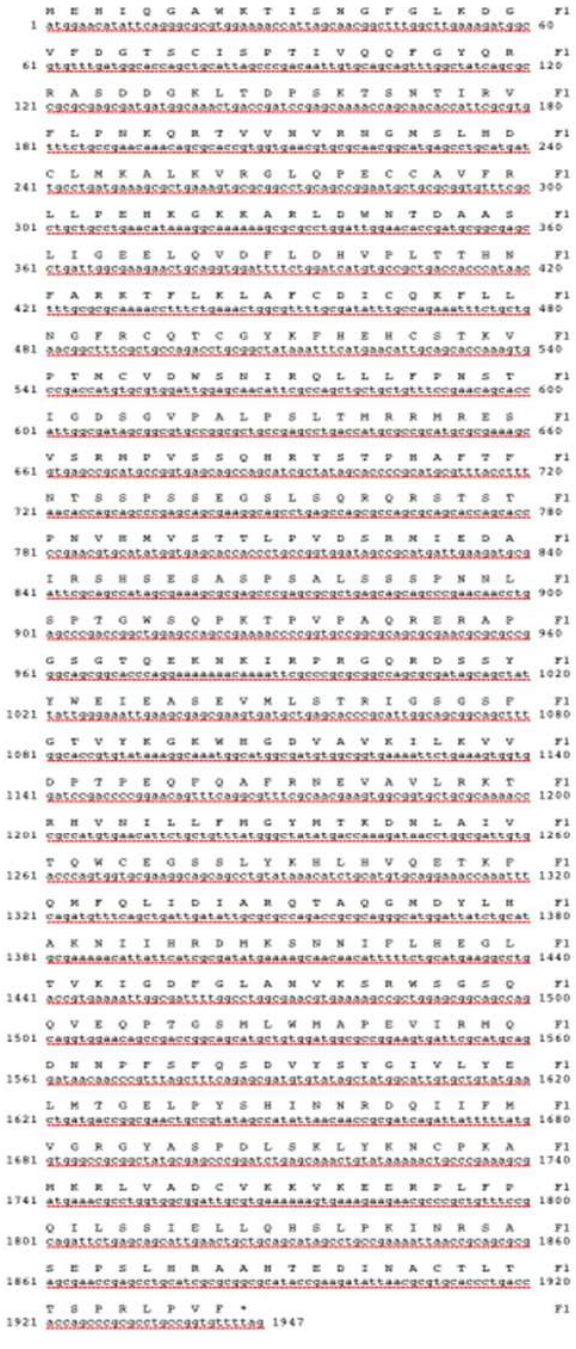

Figure 1: Nucleotide and deduced amino acid sequences of PvRaf as revealed by PCR and nucleotide sequence analysis. The deduced amino acid sequence of PvRaf is shown in a single letter under the respective codon using EMBOSS Sixpack software (EMBL-EBI).

Protein structure and analysis

The total size of the deduced nucleotide sequence of *PvRaf* is 1.947 Kb. The deduced nucleotide sequence was analyzed using SIB-SWISS Biozentrum software to determine the conserved domains for the functional annotation of *PvRaf* protein (Waterhouse et al., 2018). The functional annotated sequence was further studied to reveal the protein structure for *PvRaf* gene. The protein

structure of *PvRaf* was analyzed using SIB-SWISS Biozentrum software. The monomer model result of the protein structure of *PvRaf* was acquired: 1 × (1E)-5-(1-PIPERIDIN-4-YL-3-PYRIDIN-4-YL-1H-PYRAZOL-4-YL)-2,3-DIHYDRO-1H-INDEN-1-ONE OXIME (Figure 2). Ligand 1 is in contact with: Chain A: I355, V363, A373, K375, E393, T421, W423, C424, K431, N472, F475, D486. The *PvRaf* protein model is based on the target template alignment using ProMod3. Conserved coordinates between the target and the template served as the basis for the model building. The geometry of the resulting model was regularized using a force field. The model quality has been assessed using the QMEAN scoring function (Benkert et al., 2011).

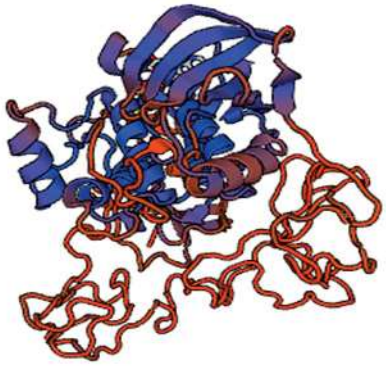

Figure 2: Monomer model of the PvRaf protein revealed by SIB-SWISS Biozentrum software

The result of BLAST analysis revealed that the *PvRaf* protein model has 99% sequence identity with high sequence similarity to RAF proto-oncogene serine/threonine protein kinase. Sequences alignment analysis was done to confirm the alignment between the sequences of different representative organisms (Figure 3) (Barbato et al., 2012). Raf is a family member of serine/threonine protein kinases and is best characterized as Ras effector. Raf is a key effector in the activation of the Ras/Raf/MAPK pathway. The activation of Raf initiates a stimulation of a signaling cascade by phosphorylation of MAPK which then phosphorylates and activates downstream proteins (ERK1 and ERK2). Downstream proteins (ERK1 and ERK2) activation is critical for several Ras induced cellular processes (Molina and Adjei, 2006).

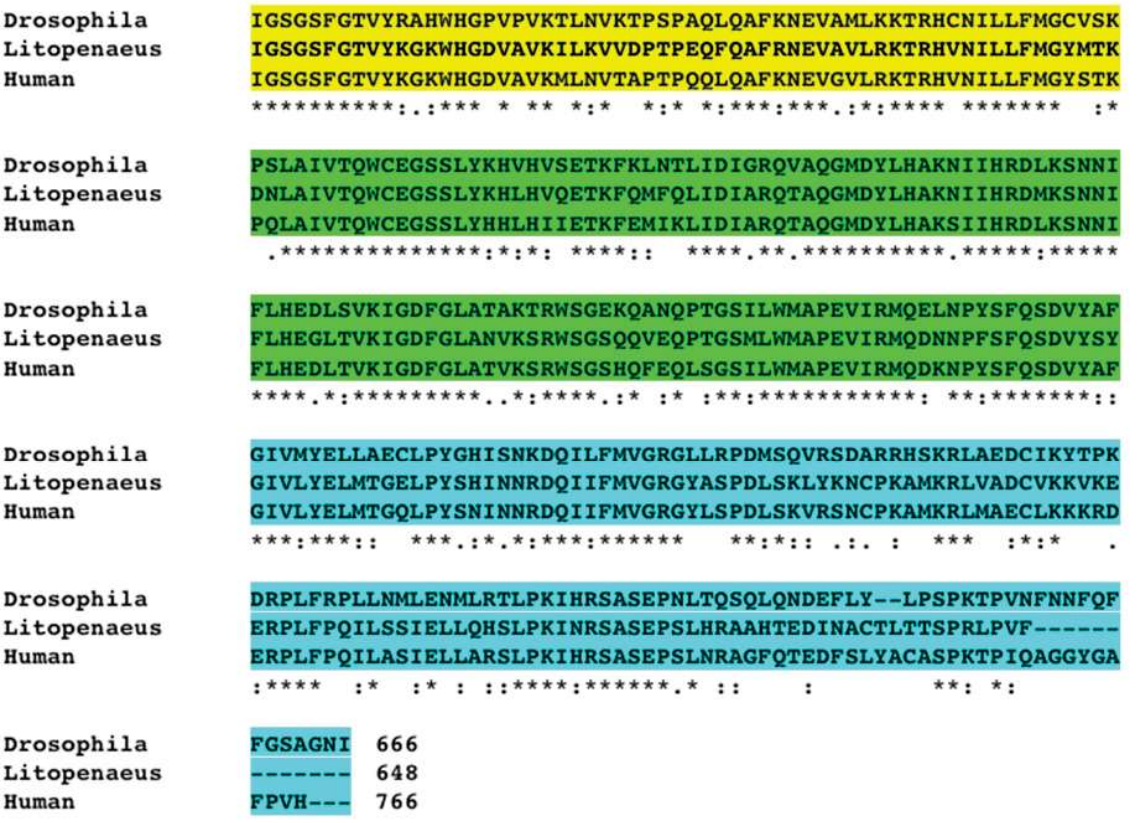

Figure 3: Alignment of the conserved amino acid sequences of Raf in different organisms as revealed by Clustal Omega software. Conserved regions (CR1, CR2, and CR3) of PvRaf are shown.

Molecular phylogenetic analysis

The sequence of *PvRaf* was aligned with the other reported Raf genes of different organisms using Clustal Omega. Multiple sequence alignment has been employed to infer homology and to determine evolutionary relationships between sequences studied. The organisms studied include *Prunus persica* (XP_020419085.1), *Asparagus officinalis* (XP_020262573.1), *Rhizoctonia solani* (CUA67647.1), *Hypsizygus marmoreus* (KYQ41228.1), *Danio rerio* (NP_991307.2), *Homo sapiens* (NP_004324.2), *Oncorhynchus mykiss* (XP_021422388.1), *Xenopus laevis* (NP_001081475.1), *Rattus norvegicus* (NP_036771.1), *Bombyx mori* (NP_001189459.1), *Culex quinquefasciatus* (EDS42440.1), *Aedes aegypti* (XP_001647840.1), *Drosophila melanogaster* (CAA30166.1), *Apis florea* (XP_003698671.1), and *Megachile rotundata* (XP_003699840.1). A phylogenetic tree was constructed employing Neighbor Joining Method using Clustal Omega (Figure 4). The resulting phylogenetic tree grouped into four (4) respective clades namely: (a) plant, (b) fungus, (c) vertebrate, and (d) invertebrate. These findings confirm with other studies on molecular phylogenetic studies (Li et al., 2018).

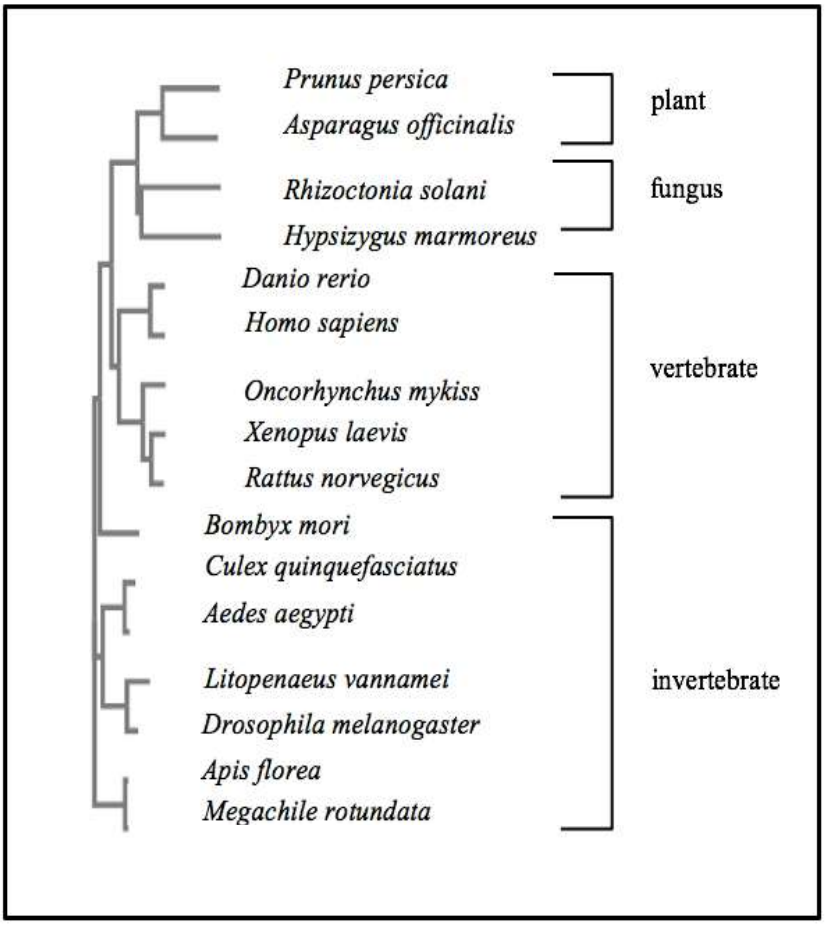

Figure 4: Phylogenetic tree analysis of the deduced Raf amino acid sequences using Neighbor-Joining clustering algorithm in ClustalW2. Distance matrix values are shown next to the organism. Organisms clustered into plant, fungus, vertebrate, and invertebrate groups respectively

Gene expression analysis

Gene specific primers for *PvRaf* were used as follow (*Pv*RAF-F: 5'-ACGCCTGAACAGCCAGGATCA-3') & (*Pv*RAF-R: 5'-GGCTCGGGGTTTTCACGTTCA-3'). Gene expression analysis was done using healthy and WSSV infected organs of the shrimp namely: Gills (G), Heart (HE), Hepatopancreas (HP), Muscle (M), Intestine (I), Lymphoid Organ (LO), and Hemocyte (HM). β-actin served as positive control at 100bp. Interestingly, *PvRaf* was found to be present in both healthy and infected organs of the shrimp at approximately 1.9Kbp (Figure 5).

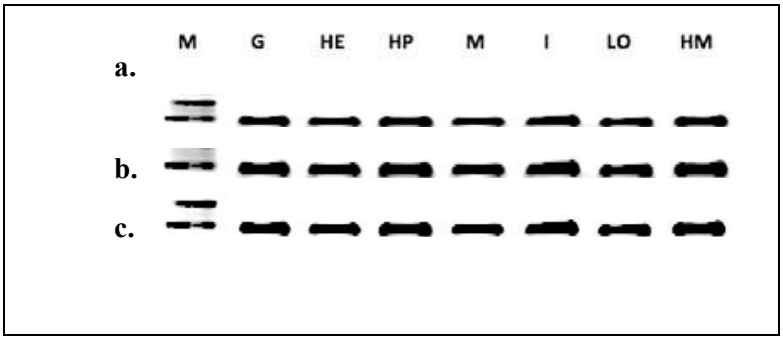

Figure 5: (a) Gene expression analysis using PCR of PvRaf from various healthy organs of shrimp namely Gills (G), Heart (HE), Hepatopancreas (HP), Muscle (M), Intestine (I), Lymphoid Organ (LO), and Hemocyte (HM) at approx. 1.9Kbp. (b) Gene expression analysis via RT-PCR of PvRaf from various infected organs of shrimp namely Gills (G), Heart (HE), Hepatopancreas (HP), Muscle (M), Intestine (I), Lymphoid Organ (LO), and Hemocyte (HM) at approx. 1.9 kb. (c) β-actin served as positive control at 100 bp.

Purified dsRNAs (*PvRaf*-dsRNA and GFP-dsRNA) were introduced to the shrimps. Purified dsRNAs were ran in gel electrophoresis and compared with the positive control. To ensure the purified dsRNAs were of quality state, the absorbance of the purified dsRNAs were measured at wavelength of 260nm. Interestingly, the absorbance of dsRNA positive control at wavelength of 260nm was (3.450). Meanwhile, absorbance values of purified *PvRaf*-dsRNA and GFP-dsRNA were (3.505, 3.507), respectively.

RNA interference and gene knockdown analysis

Consequently, with the increased survival rate of *PvRaf*-dsRNA treated shrimps, Raf may also promote cell survival in shrimps as revealed in its presence in the hemocytes. The hemocytes of shrimp is a key component in its innate immune system. In hemocytes, the Ras/Raf/MAPK pathway is being elicited by PDGF and VEGF receptors and its ligands (Duchek et al., 2011; Cho et al., 2000, Munier et al., 2002). Also, Pvf1-3 is involved in cell proliferation and migration [Bruckner et al 2004, Ishimaru et al., 2004, Olofsson and Page 2005). Pvf2 on the other hand, has a key role in hemocyte proliferation (Jung et al., 2005). Quantitative Real Time-PCR (qRT-PCR) results confirmed the *in vivo* gene knockdown of *PvRaf* using RNA interference. Expression of *PvRaf* in *PvRaf*-dsRNA treated shrimps were (0.62, 0.34, 0.35, 0.39); in GFP-dsRNA treated shrimps (0.86, 0.52, 0.62, 0.80); in PBS-treated shrimps (1.00, 0.52, 0.91, 0.50) in each of its post-infection days, respectively. Expression of *PvRaf* exhibited its peak down regulation from Day 0 to Day 1 for all setups (Figure 6). *PvRaf* knockdown showed significant therapeutic effect in shrimps as it may play a role in the antitumor activity and disease stabilization functions of Raf. Moreover, there are Raf scaffold proteins namely KSR and CNK which are evolutionary conserved even during Raf knockdown that may also be factors in the increased survival of shrimps treated with *PvRaf*-dsRNA.

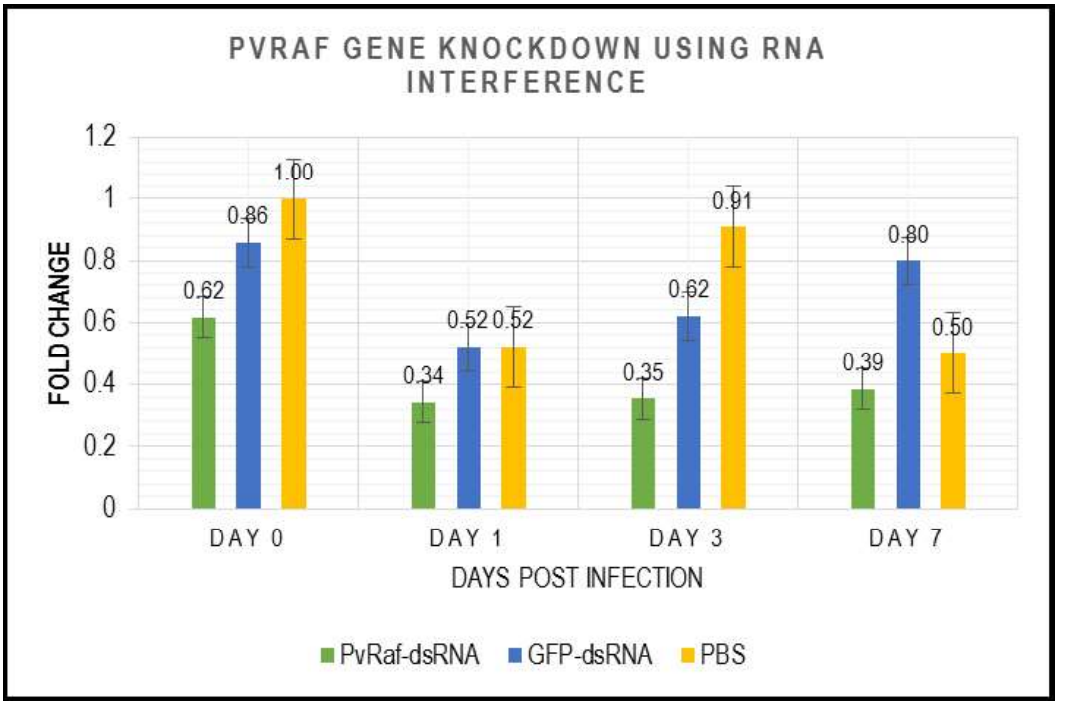

Figure 6: Quantitative Real-Time PCR (qRT-PCR) results of PvRaf gene knockdown using RNA interference. X-axis corresponds to Days post-infection (0, 1, 3, and 7). Y-axis corresponds to fold change

Challenge test and survival analysis

Survival rates of shrimp with (1) *PvRaf*-dsRNA treatment, (2) GFP-dsRNA treatment, and (3) PBS treatment were plotted after the samples were challenged with WSSV. Survival rates of shrimps in each treatment (x-axis) were recorded against days post-infection (y-axis) (Figure 7). WSSV challenge test was done to investigate the role of *PvRaf* in *P. vannamei* (Maningas et al., 2008). Interestingly, the result revealed that there were significant differences between the survival rate of *PvRaf*-dsRNA treatment against PBS treatment and GFP-dsRNA treatment.

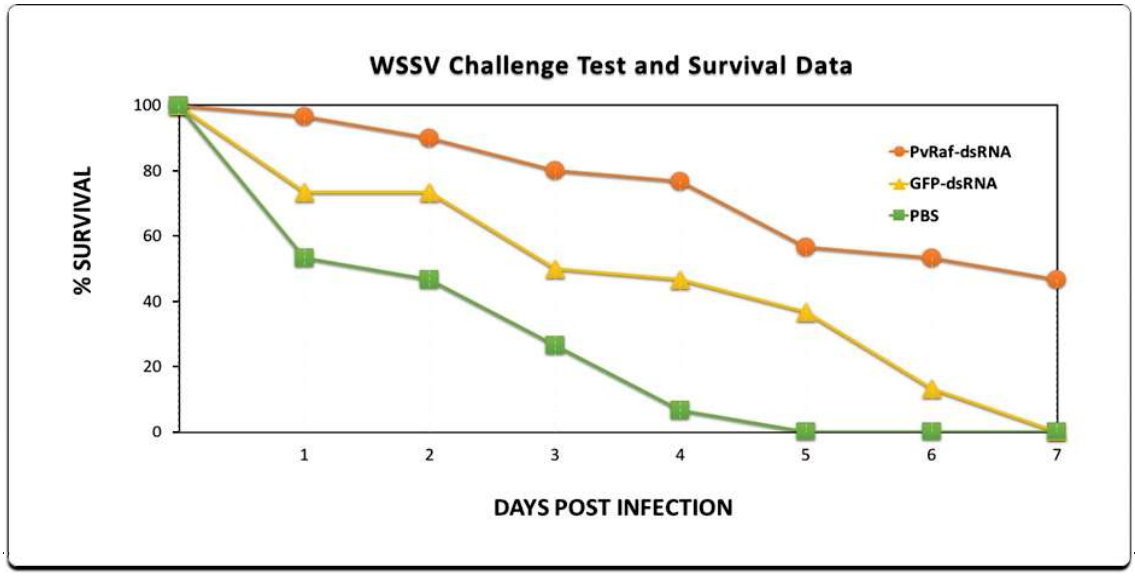

Figure 7: WSSV Challenge Test and Survival Data of shrimps with (1) *PvRaf*-dsRNA treatment, (2) GFP-dsRNA treatment, and (3) PBS treatment. X-axis shows Days post-infection while Y-axis shows percentage survival

The *Drosophila* Ras/Raf/MAPK pathway regulates the innate responses in its immune system. *In vivo* experiments in *Drosophila* sp. Demonstrated that Ras/Raf/MAPK pathway is a requirement in restricting innate immune responses in hemocytes. This pathway promotes cell proliferation and has simultaneously been utilized to limit the innate immune response. Therapeutics studies targeting Raf inhibition using RNA interference found that ISIS 5232, a 20-base phosphorothioate oligonucleotide antisense RNA for C-Raf showed efficient tumor inhibition and disease stabilization [Cunningham et al., 2000, Oza et al., 2003, Cripps et al 2002, Couderf et al 2001, Tolcher et al 2002). It can be deduced from these findings that *PvRaf* may contribute to the prolonged disease stabilization of shrimps thereby delaying mortality. Consequently, the survival data of shrimps treated with *PvRaf*-dsRNA shows significant difference against GFP-dsRNA and PBS treatments. It can be inferred that *PvRaf*-dsRNA has a significant therapeutic effect in a shrimp's system, similar to that of *Drosophila* sp.. D-Raf knockdown in *Drosophila* sp. showed fly embryos failed to differentiate into structured embryos and displayed lethal phenotype (Ragab et al., 2011). However, more recent studies discovered that this pathway has Raf scaffold proteins, Kinase Suppressor of Ras (KSR) and a Connector Enhancer of KSR (CNK) which are evolutionary conserved during Raf knockdown. KSR and CNK interact with multiple components of the Ras/Raf/MAPK pathway where they play the key role in the assembly and the localization of the activated ERK into the plasma membrane [Muller et al., 2001, Therrien et al., 1998, Chong et al 2001).

Conclusion

Raf promotes cell proliferation and survival in a mechanism that is independent of MEK-ERK pathway. Raf also has an antitumor activity and exhibits disease stabilization. Raf may directly act as a critical component of the shrimp's cellular apoptotic pathway through protein-to-protein interactions. One of the key reasons why Raf has been chosen for this study aside from being a key effector in the Ras/Raf/MAPK pathway is that Raf has a kinase independent function which is necessary to

ensure cell survival and proliferation. Raf can replace the wild-type kinase to inhibit ASK1. Moreover, Raf may have dual functions: (1) activating MEK-ERK pathway through enzymatic activity and (2) inhibiting ASK1 through protein-to-protein interactions. The process on how Raf inhibits ASK1 and the mechanism behind the process are yet to be established and need further studies. Raf in shrimp suggests that it is an effective target in RNA therapeutics as *PvRaf*-dsRNA treated shrimps showed increased survival. Moreover, *PvRaf* may have a function in the antitumor activity and the disease stabilization in shrimps. Further investigations and studies must be done involving Ras/Raf/MAPK pathway in shrimp involving other bacterial pathogens, fungi, parasites, DNA and RNA based viruses. Lastly, further biological assays must also be established for other key components of the Ras/Raf/MAPK pathway in a shrimp's system.

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Declarations

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Short Notes

Description of a Fish Epidemiology and Health Economics (FEHE) Survey Tool forPerformance and Risk Factor Assessment

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Abstrak: Usaha kawalan penyakit oleh petani dan kerajaan biasanya terhalang oleh kekurangan data mengenai status kesihatan ikan, faktor risiko, dan kerugian ekonomi akibat penyakit. Untuk memenuhi keperluan untuk penilaian bersepadu epidemiologi ikan dan ekonomi kesihatan di Asia dan Afrika, alat tinjauan Epidemiologi Ikan dan Ekonomi Kesihatan (FEHE) telah diwujudkan dengan objektif untuk mengumpul data asas mengenai amalan sedia ada ladang, pengeluaran, input, epidemiologi, kerugian dan kesan ekonomi akibat penyakit dan kematian. Alat FEHE melibatkan penggunaan aplikasi mudah alih Open Data Kit (ODK) Collect untuk mengakses soal selidik tinjauan di lokasi ladang terpencil dengan sambungan internet yang terhad. Soal selidik disimpan di KoboToolbox, platform dalam talian sumber terbuka percuma untuk mengurus tinjauan dan mengumpul data lapangan. Menggunakan alat tinjauan, penemuan epidemiologi diperoleh daripada 550 ladang di Bangladesh dan 110 ladang di Mesir. Tindak balas tinjauan dianalisis untuk mengenal pasti faktor risiko dan untuk memahami prestasi sistem makanan akuatik tempatan termasuk kesan ekonomi penyakit berjangkit. Penemuan akan digunakan selanjutnya untuk membangunkan campur tangan pengurangan risiko yang disasarkan dan strategi kesihatan haiwan akuatik kebangsaan untuk akuakultur mampan di kawasan yang disasarkan. Versi alat kontekstual telah digunakan baru-baru ini di Nigeria sebagai sebahagian daripada projek Agensi Pembangunan Antarabangsa Amerika Syarikat Feed the Future Innovation Lab for Fish (USAID FIL) dan juga akan digunakan di Ghana dan Kenya di bawah projek yang disokong Norad. Untuk tujuan pembinaan kapasiti, kursus dalam talian mesra mudah alih (‘Tinjauan Akuakultur dengan ODK’) telah dibuat pada platform Learn.ink untuk melatih pengguna alat tinjauan. Alat dan garis panduan yang disertakan telah diterangkan oleh WorldFish dalam Pakej Amalan Kesihatan Haiwan Akuatik (POPs): Epidemiologi Ikan dan Ekonomi Kesihatan.

Abstract: Disease control efforts by farmers and national governments are normally hindered by lack of data on the status of fish health, risk factors, and the economics of losses due to disease. To fulfil the need for an integrated assessment of fish epidemiology and health economics in Asia and Africa, the Fish Epidemiology and Health Economics (FEHE) survey tool was created with the objective of collecting baseline data on existing farm practices, production, inputs, epidemiology, losses and economic impacts due to disease and mortalities. The FEHE tool involves the use of the Open Data Kit (ODK) Collect mobile application to access the survey questionnaire in remote farm locations with limited internet connectivity. The questionnaire is stored on KoboToolbox, a free, open source online platform for managing surveys and collecting field data. Using the survey tool, epidemiological findings were obtained from 550 farms in Bangladesh and 110 farms in Egypt.

Survey responses were analyzed to identify risk factors and to understand the performance of local aquatic food systems including economic impact of infectious diseases. Findings will be further used to develop targeted risk mitigation interventions and national aquatic animal health strategies for sustainable aquaculture in the targeted regions. A contextualized version of the tool has been recently used in Nigeria as part of the United States Agency for International Development Feed the Future Innovation Lab for Fish (USAID FIL) project and will also be applied in Ghana and Kenya under the Norad supported projects. For the purpose of capacity building, a mobile friendly online course ('Aquaculture survey with ODK') has been created on Learn.ink platform to train survey tool users. The tool and its accompanying guidelines have been described by WorldFish in an Aquatic Animal Health Package of Practices (POPs): Fish Epidemiology and Health Economics.

Keywords: Farm survey tool, Aquaculture baseline survey, Aquaculture risk factor, Fish epidemiology study, Aquaculture performance

Introduction

Fish farmers and national governments face barriers in disease control efforts due to uncertainties on the status of the fish health, the risk factors and the economics of mortalities. Since local farm data are often communicated by field officers using traditional paper forms or phone calls, the process is often lengthy and fails to trigger a timely response from aquatic animal health advisors. Thus, to fill the gap in information, the Fish Epidemiology and Health Economics (FEHE) survey tool was created to efficiently collect baseline data on existing farm practices, losses and economic impacts due to diseases and mortalities. The survey tool is aimed at standardizing data collection and providing an integrated assessment of fish epidemiology and health economics in Asia and Africa.

Materials and Methods

The tool is accessed through ODK Collect, a free Android mobile application which allows users to enter survey data into mobile devices. Survey forms can be downloaded and submitted using the mobile application at internet connection points. However, data can also be saved to the mobile device when no internet is available. The application leverages the capabilities of the mobile device to record GPS location and to submit photos from the survey site. The survey questionnaire is stored online on KoboToolbox, which is a free, open source software developed by the Harvard Humanitarian Initiative (HHI). KoboToolbox allows registered users to upload and deploy surveys on the platform using an uploaded template survey code in XLS format. Through the platform, research teams are able to manage the survey questionnaire, to validate responses, to view descriptive statistic reports, to map survey information, and to download the survey data. Using one account on the platform, multiple surveys can be deployed and shared with other account holders, allowing standardized survey data to be collated from different field teams and across geographic locations.

Results and Discussion

The survey tool was previously used in epidemiological studies on 550 farms in Bangladesh and 110 farms in Egypt (https://doi.org/10.1016/j.aquaculture.2020.735438). WorldFish and partners have applied the tool in several bilateral projects, including the United States Agency for International Development the Feed the Future Innovation Lab for Fish (USAID FIL) project in Nigeria, Agence Française de Développement (AFD) projects in Malawi and Zambia, the Centre for Environment, Fisheries and Aquaculture Science (Cefas) antimicrobial resistance and CGIAR COVID-19 country

projects in Bangladesh, and the Norwegian Agency for Development Cooperation (Norad) projects in Egypt, Ghana and Kenya. The survey tool provides findings that could be used to develop risk mitigation interventions and to inform national aquatic animal health strategies. The survey can be adapted to include local species, farming systems and administrative levels for scaling in countries within targeted regions. The contextualized version of the tool has been used in the Nigeria USAID FIL project and will also be applied in Ghana and Kenya under the Norad supported projects. In order to provide training on the survey tool, an online mobile phone course ('Aquaculture survey with ODK') has been made available at https://bit.ly/3AYXUGQ on Learn.ink. Through this training, users will be able to start and to stop the course at their own pace, or to refer back to the previous completed course modules on demand. The tool and its accompanying guidelines have been described by WorldFish in an Aquatic Animal Health Package of Practices (POPs): Fish Epidemiology and Health Economics. The POPs lists the available resources for conducting digital surveys for aquaculture performance assessment for first time users.-

INSTRUCTION TO AUTHORS

MALAYSIAN FISHERIES JOURNAL

Aim and Scope

The journal seeks to provide a forum for dissemination of research findings in all aspects of fisheries science. Manuscripts describing research work relevant to local communities are most welcome to aid in the advancement of sustainable fisheries. The standardized format set below is an adaption from some international journal.

Submission of Papers

A paper is considered for publication on the understanding that:

- It reports original unpublished work
- It is approved by all named authors
- It does not contain tables and figures that have been published elsewhere
- All acceptable manuscripts will be reviewed by the Publication Committee
- Acknowledgement and action on each point raised by the reviewer will be requested from the author if the manuscripts to be accepted

Different type of Submissions

1. Full length paper

These should describe new and carefully confirmed findings and experimental procedures that should be given in sufficient detail for others to verify work. The length of a full paper should be the minimum required to describe and interpret the work clearly. The paper should comprise the following sections: (a) Abstract; (b) Introduction; (c) Materials and Methods; (d) Results; (e) Discussion; (f) Acknowledgement; (g) References; (h) Tables; (i) Legends to figures; (j) Figures. The results and discussion section may be combined.

2. Short Communication

A Short Communication is suitable for recording the results of complete small investigation or giving details of new methods, techniques or apparatus, not more than 3000 words. The style of main sections need not conform to that of full-length papers. Progress reports are not acceptable.

3. Short Notes

Short Notes are one to two printed pages in length. They are suitable for reports of simple findings such as properties of an already well-described enzyme or of observations not requiring elaboration. They should be written with a short summary, with no main sub-division, may contain one table or figure, or two if the text is brief and no more than three references.

4. Technical Communication

These are reports of processes or procedures which may be published as an annex to a full length of paper or on their own provided that the work is of sufficient interests to other workers in the field.

5. Review Papers

These should be centered on current issues that are of interests to all. The length of the paper is between 6000 – 10000 words. The references must be more than 30.

Preparation of Manuscripts

Manuscript should be prepared in Microsoft Word. The paper must be typed with a double spacing throughout, including references, tables, footnotes, figures legends, etc on A4 size paper leaving margins of 25 mm minimum. Line numbers should be insert for review purposes. Headings should be centered, upper case in bold, size 14. Sub-headings should be lower case, centered and in **bold**. Sub-sub-headings should be in *italics* at left margin. The font used throughout your document should be in Times New Roman, 12-point font size.

Title Page:

a) A concise and informative title unobscured by taxonomic detail.
b) Name of author(s) should be in full, capital letters, font size 12.
c) Address of institution(s) where the work was done should be written in full, italic, font size 11.
d) Corresponding author email - mark with *, *italic.*

Abstract and Keywords

Abstract: Hanging indent, *italic,* **bold**, follow by text
Two abstracts required: English and Bahasa Malaysia version
Provide: i. No more than 200 words summarizing the main points of the paper
ii. Up to six keywords or phrases

Introduction

It should include key references to appropriate works and up-to-date primary literature. The rationale of the research undertaken should be explained. The introduction should clearly state the aims and objectives of the paper.

Materials and Methods

Materials and Methods should be described in sufficient detail to allow the work to be repeated. Specify and describe the study site and test animals where appropriate. Sub headings are used to itemize the main parts. Materials and methods should be written in the past tense either in active of passive voice. In this section, study dates, number of subjects, groups, evaluation criteria, exclusion criteria and statistical methods should be described sequentially. The origin of materials and/or suppliers of equipment should be named if necessary.

Results and Discussion

The sections may be separated, though authors may find it's easier to combine them. Use tables or graphs as appropriate but do not repeat information in the text. The reproducibility of the findings must be clearly stated, the number of times the experiment was conducted, the number of replicate samples, etc., should be stated. Statistical analysis of results must specify the procedure being used with a reference given. If results are given as a percentage of a control value, the 100% should be given.
Discussion should provide the explanation and interpretation of results or findings by comparing with the prior studies. It should bring out those essential points of the work, the implications and practical significance of the findings, their limitation and relevance to previous studies. It should not be a recapitulation of the results.

References

The references follow APA style. In the text, references should be cited as: Smith (1993) or (Smith, 1993). Two authors as: Smith and Brown (1993) or (Smith and Brown, 1993). Three or more authors as:

Smith et al. (1993) or (Smith et al., 1993). A series of references should be appearing in chronological order, e.g., White and Black 1991; Black and White 1992. References to papers by the same authors in the same year are distinguished by letter a, b, etc. (e.g., 1989a, or 1991a, b). Publications having no obvious authors are cited as Anon. (1990) in the text and bibliography. References with 4 and more authors should be written as: Abbasi, A. A., Paparidis, Z., Malik, S., Goode, D. K., et al. (2007). References to grey literature such as in-house reports, contract reports and non-referred papers are not appropriate and should be avoided. At the end of papers, References are listed in alphabetical order by the first word in the reference (usually the author's last name). References with three or more authors should be placed in chronological order after considering of the names of the first and second authors. The author must ensure that references cited in the text agree with those listed in the bibliography. Some sample reference styles follow:

a) Citation for Journals

i. In print

Sequence of citation: author's name, initials (for each author) (year of publication). Full title of paper. *Name of journal* (abbreviated in accordance with the Bibliographic Guide), **volume** (issue nu), first page **-** last page.

Example: Saha, B. C. and Zeikus, J. G. (1989). Improve method for preparing high maltose conversion syrup. *Biotechnology and Bioengineering*, **34**, 229-303.

Example: Debnath, P. P., Delamare-Deboutteville, J., Jansen, M. D., Phiwsaiya, K., et.al (2020). Two-year surveillance of tilapia lake virus (TiLV) reveals its wide circulation in tilapia farms and hatcheries from multiple districts of Bangladesh. *J Fish Dis.* **43**(11),1381-1389.

ii. Online

These references are formatted the same way as the print versions, except the DOI or URL is included at the end. If the article has a corresponding DOI number, use it instead of the URL. No URL? Use the homepage of the journal's website for the URL.

Author's Last name, F. M. (Year published). Title of article. *Title of Journal*, volume number (issue number), page range. https://doi.org/10.xxxx/xxxxxx OR URL

Example:

Spreer, P., and Rauschnabel, P. A. (2016). Selling with technology: Understanding the resistance to mobile sales assistant use in retailing. *Journal of Personal Selling and Sales Management*, **36**(3), 240**-**263. https://doi.org/10.1080/08853134.2016.1208100

b) Citation for Books

i. Sequence of citation for print books: author's or editor's name, initial, (year of publication), *book title.* Publisher, and place of publication.

Capitalize the first letter of the first word of the title and any subtitles, as well as the first letter of any proper nouns. The full title of the book, including any subtitles, should be stated and italicized.

Example: Primrose, G. B. (1987). *Modern Biotechnology*. Blackwell Scientific, Oxford.

ii. Citations for Edited Books

Most edited books state on the cover or title page that they are edited by an author or multiple authors. The format is the same as a print book, except the editor's name is in the author's position. Include a parenthesis afterwards with the abbreviation (Ed.) for an edited book by one author or (Eds.) for an edited book with two or more authors.

Editor, F. M. (Ed.). (Year published). *Title of edited book*. Publisher.

Example:

a) Primrose, G.B. (Ed.). (1987). *Modern Biotechnology*. Blackwell Scientific, Oxford.

b) Gudding, R., Lillehaug, A. and Evensen, O. (Eds.). (2014). *Fish Vaccination*, John Wiley & Sons Ltd., UK.

iii. Citations for Chapters in Edited Books

Some edited books contain chapters written by various authors. Use the format below to cite an author's individual chapter in an edited book.

Chapter author's Last name, F. M. (Year published). Title of chapter. In F. M. Last name of Editor (Ed.), *Title of book* (p. x or pp. x-x). Publisher.

The title of the chapter is not italicized, while the title of the book is. The chapter author's name is reversed at the beginning of the reference, but the editor's name is written in standard order.

Example:

a) Longacre, W. A., and Ayres, J. E. (1968). Archeological lessons from an Apache wickiup. In S. R. Binford and L. R. Binford (Eds.), *Archeology in cultural systems* (pp. 151-160). Blackwell, Oxford, UK.

In the above example, Longacre and Ayers are the authors of the individual chapter and Binford and Binford are the editors of the entire book.

b) Gudding, R. (2014). Vaccination as a preventive measure. In R. Gudding, , A. Lillehaug, and O. Evensen, (Eds.), *Fish Vaccination* (pp. 12-21). John Wiley & Sons Ltd, West Sussex, UK.

c) Citations for conference/proceedings

Conference proceedings published as a whole book follow the same reference format as whole: i) journal, ii) edited books or iii) book chapter

Example:

i. Duckworth, A. L., Quirk, A., Gallop, R., Hoyle, R. H., Kelly, D. R., and Matthews, M. D. (2019). Cognitive and noncognitive predictors of success. *Proceedings of the National Academy of Sciences*, *USA*, *116*(47), 23499–23504. https://doi.org/10.1073/pnas.1910510116

ii. Kushilevitz, E., and Malkin, T. (Eds.). (2016). *Lecture notes in computer science: Vol. 9562. Theory of cryptography*. Springer. https://doi.org/10.1007/978-3-662-49096-9

iii. Benedel, A. L., Jourdan, L. and Biernacki, C. (2019). Probability estimation by an adapted genetic algorithm in web insurance. In R. Battiti, M. Brunato, I. Kotsireas & P. Pardalos (Eds.), *Lecture notes in computer science*: Vol. 11353. Learning and intelligent optimization (pp. 225-240), Springer. https://doi.org/10.1007/978-3-030-05348-2 21

a) Citations for Newspapers found Online
Use this structure when referencing a newspaper article found on a website or database:

Author's Last name, F. M. (Year, Month Day of Publication). Title of article. *Title of Newspaper.* URL of newspaper's homepage

Example:
Rosenberg, G. (1997, March 31). Electronic discovery proves an effective legal weapon. *The New York Times.* http://www.nytimes.com

b) Composite works of serials:
Sequence of citation: author's name, initials, year of publication, publisher, place of publication, first and last page no.

Example:
Guilbot, A. and Marcier, C. 1985. Starch. *In:* Aspinall, G.O. (ed). The polysaccharides, Academic Press, New York, pp. 209-283.

c) Full publication details must be given for any citation that does not fit into any of the above categories such as unpublished in-house reports, contract reports, etc.

Acknowledgement
Brief of appreciation to whom it is due.

Table
Plain Tables should be used for data which cannot be described in the text. Type each table double spaced and position in the manuscript. Table number and caption should be positioned at the top. Explanatory footnotes in lower case letters should be concise to enable them to stand independent of the main text. Tables are numbered with Arabic numerals.

Figures
Figures should be selected to illustrate points which cannot be easily made in the text. They are numbered with Arabic numerals. Graphs, photos and diagrams with caption should be positioned in the manuscript. Diagrams must be drawn and lettered in black ink on good quality white paper for camera-ready use. Lettering should be parallel to the axes. Photocopies, hand-drawn diagrams and typewritten labels are not acceptable. Scale marks on graphs should be within the axes. Graphs should avoid as far as possible large areas of unused space.

Photographs should be will-contrasted black and white prints. For photomicrographs, the magnification should be given a scale (or marker) bar on each photograph and the length of this represents given in

the legend. Photographs of the original material should be submitted for the reviewer's scrutiny and the purpose of printing.

Units, Abbreviations and Nomenclature

Use only recommended SI Units. Use superscripts presentation (mg mL^{-1}). Below are few examples of abbreviations of the most commonly used SI units:

Base quantity	**Name**	**Abbreviation**
Length	Meter	m
Mass	Kilogram	kg
Time	Second	s
Time	Minute	min
Electric current	Ampere	A
Area	square meter	m^2
Volume	cubic meter	m^3
Frequency	Hertz	Hz

The correct Latin names of organisms must be used on first mention in the text. A widely recognized and designated common name should be used for subsequent mention.

References

American Psychological Association. (2020). *Publication manual of the American Psychological Association* (7th ed.). https://doi.org/101037/0000165-000

Paper Title: A concise and informative title unobscured by taxonomic detail

FULL NAME ALL AUTHORS: AUTHOR[1,2], AUTHOR[3], AUTHOR[3], AUTHOR[1], AUTHOR[4,*]

[1]Institution with complete current address, including post code

[2] Institution with complete current address, including post code

[3] Institution with complete current address, including post code

[4] Institution with complete current address, including post code

Corresponding author: author@institution.domain

***Abstract**:* A concise and factual abstract is required and must be written in English (maximum length of 250 words). The abstract should state briefly the purpose of the research, the methods used, the principal results and major conclusions. Please try to keep each sentence as specific as possible and avoid such general statements as "The fisheries status are discussed'. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, they must be cited in full, without reference to the reference list. Any abbreviations should be avoided, but if needed they must be defined at their mentioned in the abstract.

Keywords: Provide a maximum of 6 keywords, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abstrak: Abstrak di dalam Bahasa Malaysia mestilah mempunyai makna dan terjemahan maksud yang sama seperti dalam abstrak Bahasa Inggeris. Penggunaan 'google translate' dibenarkan dengan syarat penulis memeriksa kembali setiap patah perkataan dan membuat pembetulan mengikut tatabahasa yang betul.

Introduction

This is template for MFJ modified from Journal of Aquaculture format. Author(s) should use this template to format their manuscript before submission. These guidelines include complete descriptions of the fonts, spacing, and related information for producing your manuscripts. All manuscripts preferably in English but Bahasa Malaysia is also accepted. If you have any questions, please email the editor. Thanks.

This template provides authors with most of the formatting specifications needed for preparing electronic versions of their papers. All standard paper components have been specified using A4 size paper, with margins of 2.5 cm. (top, bottom, left, and right). Margins, column widths, line spacing, and type styles need to be followed as in this example. PLEASE DO NOT RE-ADJUST THESE FONTS SIZES AND MARGINS. The softcopy of the template can be requested from editormfj@gmail.com.

Materials and methods

Materials

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described. The correct Latin names of organisms must be used on first mention in the text. A widely recognized and designated common name should be used for subsequent mention.

Methods

Sub headings are used to itemize the main parts. Materials and methods should be written in the past tense either in active of passive voice. In this section, study dates, number of subjects, groups, evaluation criteria, exclusion criteria and statistical methods should be described sequentially. The origin of materials and/or suppliers of equipment should be named if necessary.

Sub-section

Third level section should be italic. We do not encourage additional sub-levels after the third level. Please try to make your paper concise and clear.

Units, Abbreviations and Nomenclature

Use only recommended SI Units. Use superscripts presentation (e.g: mg mL^{-1}) and common abbreviations such as ‘m’ for meter, ‘kg’ for kilogram, ‘min’ for minute and so on.

Results and Discussion

Results should be clear and concise. The discussion should explore the significance of the results of the work. Avoid extensive citations and discussion of published literature. If appropriate, Results can be written in a separate section from Discussion. This especially if the Discussion is extensive and includes all the Results of the study**.**

Table

Please submit tables as **editable text** and not as images. Tables use double space and 12pt Times New Roman fonts.

Table 1. Use Times New Roman 12 font

Component	Content (%, w/w)
Protein	44.9 ± 0.37
Carbohydrate	22.3 ± 0.94
Water content	13.7 ± 0.02
Ash	6.1 ± 0.19

Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

Figures

Please embed the figures in the text with minimum resolution of 300 dpi. Separate **figure files in JPEG or PNG formats can be supplied if it feels necessary**. Ensure that each figure has a caption. A caption should comprise a brief title (not on the figure itself) and a description of the figure. Keep text in the figure themselves to a minimum but explain all symbols and abbreviations used.

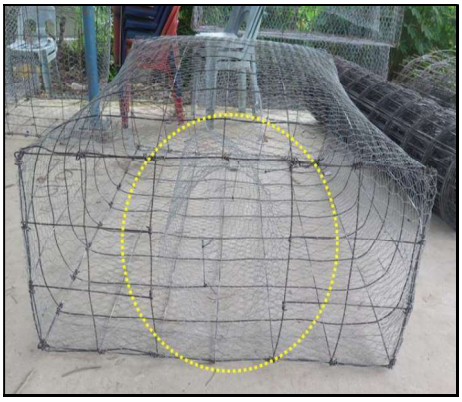

Figure 1. Left: Trap one funnel (1F)

Graphs

Graphs must be supplied in figure formats. The fonts of the graph must be clear and readable. Black and white graphs are preferred.

Citation

Please ensure that **every reference cited in the text is also present** in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list, they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication. All manuscripts should be formatted using the American Association style (APA). You can download the APA style for reference manager (Mendeley, Zotero, etc.) from the trusted website.

Text

All citations in the text should refer to:

- Single author: the author's name (without initials, unless there is ambiguity) and the year of publication (Ratledge, 2002); OR Ratledge (2002).
- Two authors: both authors' names and the year of publication (Triyaswati & Ilmi, 2020);
- Three or more authors: first author's name followed by 'et al,' and the year of publication (Papanikolaou et al., 2011).

Reference list

References should be arranged first alphabetically and then further sorted chronologically. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of Results and Discussion section.

Author contribution (Optional)

Please list the contribution of each author here, e.g.: M.I. designed the research and supervised all the process, L.A. collected and analyzed the data and wrote the manuscript.

Acknowledgments

List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proofreading the article, etc.).

Conflict of Interest (Optional)

Please state any conflict of interest regarding the research or the research funding.

References

Debnath, P.P., Delamare-Deboutteville, J., Jansen, M.D., Phiwsaiya, K. et al. (2020). Two-year surveillance of tilapia lake virus (TiLV) reveals its wide circulation in tilapia farms and hatcheries from multiple districts of Bangladesh. *Journal Fish Disease,* **43**(11),1381-1389.

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Kushilevitz, E., & Malkin, T. (Eds.). (2016). *Lecture notes in computer science: Vol. 9562. Theory of cryptography*. Springer. Retrieved from https://doi.org/10.1007/978-3-662-49096-9

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Rosenberg, G. (1997, March 31). Electronic discovery proves an effective legal weapon. *The New York Times*, Section D, pp.5. Retrieved from http://www.nytimes.com

Saha, B.C. and Zeikus, J.G. (1989). Improve method for preparing high maltos conversion syrup. *Biotechnology and Bioengineering,* **34**, 229-303.

PAGES	TITLES
1-9	Fish Wear Their Immune System on the Outside – What This Means for Aquaculture and Ecology PITTMAN KARIN, MERKIN GRIGORY, OKUBAMICHAEL MEARGE, POWELL MARK, ANDERSEN LINDA, CARLO C. LAZADO
10-17	Gamma Ray Irradiation: A Valuable Tool for Fresh Feed Disinfection PIK NENG TEOH, CHE ZULKIFLI CHE ISMAIL, NOR AIDA SUZANA ABDUL RAHMAN AND NAZARIA MOHD NAZRI
18-29	Ectoparasites Recovered from Whole Cage Freshwater Treatment on Cultured Marine Fish Disease Outbreak in Floating Cages LEONG TAK SENG
30-39	An Intelligent Protozoan White Spot Fish Disease Detection SITI NAQUIAH MD PAUZI; AMIERA SYAZLIN MD AZHAR, NOR HAZLYNA BINTI HARUN, MOHAMAD GHOZALI HASSAN, NORAINI YUSOFF AND KUA BENG CHU
40-49	Advances in Technologies towards Enhancing Shellfish Wellbeing for Optimum Aquaculture Production BENEDICT TERKULA IBER, HIDAYAH MANAN, MUHAMMAD SYAFIQ ABD RAZAK, HASSIMI ABU HASSAN AND NOR AZMAN KASAN
50-59	Mass Mortalities of Golden Pomfret (*Trichinotus blochii*) at Floating Cages Pulau Aman, Penang Associated with Oxygen Crisis, Multiple Infections of Parasites and *Vibriosis* PADILAH BAKAR, RIMATULHANA RAMLY, FAHMI SUDIRWAN AND KUA BENG CHU
60-74	*PvRaf* knockdown using RNA interference increased survival of *Litopenaeus vannamei* infected with white spot syndrome virus JOSEPH CARLO V. VERGEL AND MARY BETH B. MANINGAS
75-77	Description of a Fish Epidemiology and Health Economics (FEHE) Survey Tool forPerformance and Risk Factor Assessment LAURA KHOR, PARTHO PRATIM DEBNATH, SHIMAA F. ALI, MONA DVERDAL JANSEN, CHADAG VISHNUMURTHY MOHANAND JEROME DELAMARE-DEBOUTTEVILLEA

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