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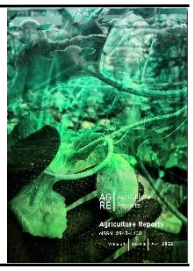

Processing and Characterization of Bioplastic Film Fabricated from a hybrid of Cocoa Pod Husk and Kenaf for the Application in Food Industries

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Abstract: The increase in synthetic plastic packaging usage has led to severe environmental problems due to their non-biodegradability. Replacement of synthetic with eco-friendly packaging films could reduce the ecological crisis and increase the potential of bio-material wastes to be utilized. Thus, this study focuses on developing a cost-effective film to be potentially exploited as a novel food packaging material using a combination of agricultural wastes, cocoa pod husks and kenaf fibre. Five different ratios of cocoa pod husk to kenaf fibre (100:0, 75:25, 50:50, 25:75, and 0:100) were used in the bioplastic preparation. The evaluated bioplastic reinforcement properties, including drying time to be bioplastic, water content, water absorption and water vapour permeability, were evaluated. This study found that the bioplastic with 50% cocoa pod husks integrated with 50% kenaf fibres exhibited the lowest water absorption and water vapour permeability. The moisture content of the 50% ratio of both materials presented an acceptable result (6.92%), while it took five days as the time taken for the bioplastic to be dried. The tested properties proved that the bioplastic from the ratio of 50% of cocoa pod husks and 50% of kenaf fibres is suitable as the novel of food packaging materials. The improvement of bioplastic made of cocoa pod husks incorporated with kenaf fibres could be exploited as a source for bio-plastic production in a food container and food wrapping.

INTRODUCTION

The plastic manufacturer reported that the usage of the total plastics over the year have been increasing. The enormous use of petroleum-based plastic compounds emphasized is an essential need for a sustainable alternative of renewable resources (Jain and Tiwari, 2015). There are various disadvantages present for petroleum-based plastic. This plastic type has been stated as a product that does not readily break down and is non-biodegradable as it is stable and hydrophobic. Apart from that,

the main disadvantage of this petroleum-derived plastic is it does not quickly decompose and biodegrade, which lead to the adverse effect of littering on the planet (Vaverková et al., 2012). Hence, an alternative method must be identified to save our environment, such as producing bioplastic to sustain greener earth (Shah et al., 2020).

Bioplastic is widely known as biodegradable plastics that can be decomposed within a shorter time (Shen et al., 2020). This bioplastic is generally made up of renewable resources such as starch, cellulose, and fibre. Besides, bioplastic has mainly used the crops as the vital ingredients rather than crude oil production like petroleum as its primary resources due to its degradability processes (Song et al., 2009). Kenaf fibre (automotive industries) and the cocoa pod husks (chocolate industries) are known as wastes that can be innovated and invented into a new material such as bioplastic that is "green" to nature.

Cocoa, Theobroma cacao L. is vitally crucial in the agricultural and economical crop that donating an enormous value to the economics of Malaysia (Adi-Dako et al., 2016). It is the primary source of cocoa beans in the chocolate production industry. Cocoa fruit consists of cocoa beans and cocoa pod husks (CPH), where this CPH becomes typically a waste of about 52- 76% of the cocoa fruit after extracted the cocoa bean from the fruit. After harvesting the cocoa beans, the CPH is generally abandoned and left as a waste to become rotten on a farm. It is reported that the massive amount of CPH waste is still underutilized and unexploited.

Kenaf originates from the Hibiscus cannabinus, a fast-growing plant of the hibiscus plant, and can be found in Malaysia as it is a tropical plant. This plant is mallow, a family of Malvaceae and composts of fibre from the bast fibre group. Kenaf fibres show excellent tensile strength and dense network fibre (Wambua et al., 2003). Kenaf is a polymer composite that is flexible and nature friendly. Besides, kenaf fibre is a secure and robust plant. The fibrous stalk possesses remarkable mechanical properties that replace glass fibres in polymer composites as reinforcing elements. The natural fibres source from kenaf can be initiated as glass replacement in fibre reinforced plastics. The mechanical properties of this natural fibre are lower compared to glass fibres (Wambua et al., 2003). Malaysian automotive industries use kenaf fibre to produce composite materials competitive with synthetic fibre (Mohanty et al., 2000).

Meanwhile, it is proven that kenaf natural fibre is lighter, about 50% of estimation than the glass fibres, and thus it is cheaper than the glass. Kenaf fibre has been added with the non-biodegradable polymers to produce bioplastic, which is biodegradability, lower density, higher strength and toughness, do not harmful, low cost and reduction of the use of the resources that cannot be easily renewed (Lee et al., 2009). This natural fibre is an innovative idea to replace and reinforce natural ingredients in bioplastics, especially for food product packaging, in prolonging their shelf life. The purpose of this kenaf fibre will be able to reduce the expenditure of plastic industries production. The kenaf addition will significantly impact and affect the mechanical properties of the type of initiative replacement of packaging.

Nowadays, plenty of research has been invented the idea of the potential of the combination of cellulose and fibre in bioplastic reinforcement. Specifically, the combination of natural cellulose and natural fibre in bioplastic production presents a lack of attention even though the product is beneficial and could be expended. Promising results from our previous study (Azmin et al., 2020) in formulating bioplastic films using a combination of cellulose (cocoa pod husks) and fibre (sugarcane bagasse) to be applied as food packaging contribute to this study. Thus, this research is conducted to analyze the physicochemical properties of the bioplastic reinforced with CPH and kenaf, the wastes from the chocolate and automotive industries, respectively. Cellulose from CPH and fibres from kenaf will be extracted and combined in five different ratios in bioplastic formulations before analyzing the physicochemical properties of these five bioplastics. This study found that the combination of CPH cellulose and kenaf fibre in bioplastic formation could be commercialized and safe for food packaging. Thus, the reduction of wastes from the chocolate and automotive industries can be made.

MATERIALS AND METHODS

Sample Collection and Pre-treatment

Ground and bleached kenaf sample was obtained from Kenaf and Tobacco State located at Kota Bharu Kelantan, Malaysia. In this study, the collected sample of kenaf fibre was ready to be used in bioplastic formulation. CPH sample was collected from the chocolate factory at Tanah Merah Kelantan, Malaysia. 2 kg of CPH sample was washed using running tap water to remove impurities before being cut into small sizes. CPH then was dried under sun drying until a constant weight was obtained. The dried CPH was milled into a fine powder form using a blender.

Cellulose Extraction from Cocoa Pod Husk

40 g of the CPH powder was weighed and diluted with 10% of the Sodium Hydroxide, NaOH solution in a beaker for 3 hours at 100°C. The CPH was washed in a muslin cloth several times with distilled water until pH 7 of the filtrate was obtained. These processes were conducted to separate the lignin components in the CPH, which mix with the cellulose in raw CPH. The CPH then was bleached with Hydrogen peroxide at a temperature of 60-70°C for 60 minutes to remove any impurities before rewashing it with the distilled water. Finally, the yellowish cellulose from the CPH was dried for 24 hours. Figure 1 presents the cellulose extraction procedure starting from a fine powder of CPH until yellowish cellulose was obtained.

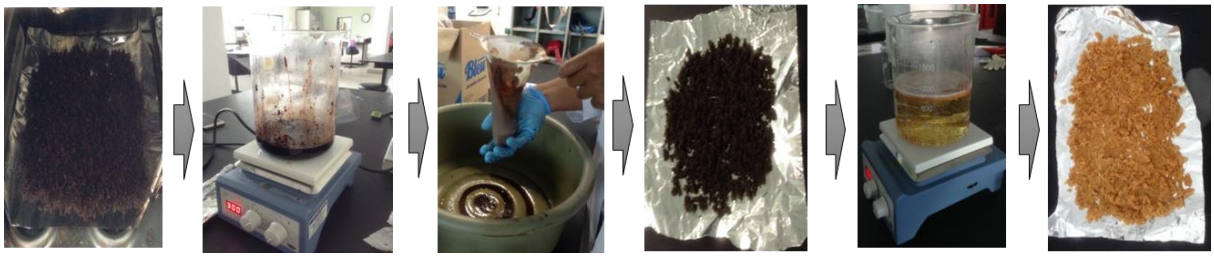


Figure 1. Cellulose extraction procedure from CPH

Bioplastic Development

In this study, five formulations (ratio of CPH to kenaf were 100:0, 75:25, 50:50 and 25:75) of bioplastics were developed where the bioplastic made from 100% CPH was set to be the control plastic. Control bioplastic was prepared using the following procedure. The mixture of 3 g of the starch, 1 g of bleached CPH powder, 1 ml of glycerine, 80 ml of distilled water and 0.5 g of sorbitol was stirred using the glass rod and heated on a hot plate at 90°C. The mixture was continuously stirred to ensure that there was no clump formed. The mixture was removed from the heat after the formation of yellowish liquid thickened occurred. The mixture was poured into a petri dish and left to dry in an oven at 50°C for 24 hours. Then, the undried bioplastic was leftover under sunshade for drying purposes. The other four bioplastics formulations were followed the controlled bioplastic procedure with 1 g of the total weight of the ratio of CPH to kenaf.

Bioplastic Characterisation and Measurement

The characterization and measurement of developed bioplastic were conducted for five parameters: sensory evaluation, drying time, water absorption, moisture content, and water vapour permeability (WVP). The sensory evaluation was tested for all five developed bioplastics where the bioplastic aroma, colour, and texture were observed.

After the bioplastic was developed, it was left under sunshade for drying purposes. Each plastic took a different time to dry. The time for bioplastics drying was recorded. The drying time measurement was done for triplicate to ensure the accuracy of the result.

The water absorption test of bioplastic was conducted by soaking the plastic into the water to measure the amount of water absorbed by the formed bioplastic. In this study, each developed plastic was soaked in 15 ml of water at room temperature. The bioplastic appearance was strictly observed where the time of bioplastic starting to break down was recorded. At this critical time, the bioplastic was taken out from the submerged water and immediately wiped off the access water on the plastic surface before weighing the bioplastic mass. The same procedure was triplicate done to ensure the accuracy of the measurement. The trial-and-error test was conducted before the actual experiment to estimate the duration for the bioplastic to be degraded. The percentage of weight increase was calculated for each bioplastic using equation 1.

$$A_w(\%) = \frac{W_f - W_i}{W_f}\ x\ 100\% \qquad \textit{Equation 1}$$

where A_w is water absorption, W_f is bioplastic final weight (wet weight) and W_i is the initial weight (dry weight).

The moisture content was tested by placing the developed plastic into an oven for 2 minutes at 105 °C. The initial and final weights of each bioplastic were recorded. The final weight was obtained by weighing the plastic using an electronic balance until the constant weight was gained. The test was repeated consecutively. The percentage of moisture content was calculated using equation 2.

$$M_c(\%) = \frac{m_f - m_i}{m_f}\ x\ 100\% \qquad \textit{Equation 2}$$

where M_c is moisture content, m_f is bioplastic final weight (constant weight after drying in an oven) and m_i is the initial weight (weight before drying in an oven).

An experimental study of water vapour permeability (WVP) for bioplastic was conducted following the gravimetric method at 25 °C as stated in ASTM E-96-00. The measurement started with sealing the plastic samples using elastic-plastic in Erlenmeyer flasks containing silica. The flasks were placed in a desiccator at 75% relative humidity (RH) at 25 °C, as Luchese et al. (2015) described. WVP was estimated by determining the sample weight gained after 48 h, according to Equation 3.

$$WVP = \frac{w}{t}.\frac{1}{A}.\frac{e}{P_S(RH_1 - RH_2)} \qquad \textit{Equation 3}$$

where $\frac{w}{t}$ is weight gained (g/d), A is biofilm area (m^2), e is the average thickness of biofilm (μm), P_S is saturation pressure of water vapour at tested temperature (mmHg), RH_1 is relative humidity inside the Erlenmeyer flask, and RH_2 is relative humidity inside the desiccator. This mathematical equation assumes that the film solubility and diffusivity are constant where Henry's law and Fick's first law are applied in computing plastic film WVP (Bertuzzi et al., 2007).

RESULTS AND DISCUSSSION

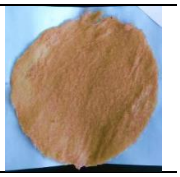
In this research, bioplastic with 100% CPH acted as a control. It was used to compare the bioplastic properties enhancement after incorporating CPH with kenaf fibre in bioplastic formulation.

Sensory Evaluation of Bioplastic

Table 1 shows the sensory evaluation of the developed bioplastics. Bioplastic made with 100% CPH showed a sweet smell due to the CPH contains 33.0 ± 0.6 g of sugar in 1 kg CPH as elaborated by Eghosa et al. (2010). This bioplastic also produced light brown and has a soft texture. Next, the two different ratios of bioplastic from 75% and 50% of CPH also shows the same result of the sensory evaluation, except the 50% CPH offers less sweet odour and more hard texture in the sensory evaluation.

Besides that, bioplastic with 25% of CPH shows no odour as the kenaf fibres help reduce the aroma of sweetness from the CPH component. Besides, the colour of this bioplastic is darker than the bioplastic made with 100%, 75% and 50% of CPH. Thus, it can be concluded that the higher the amount of kenaf, the darker the colour of bioplastic will be produced. This bioplastic texture is harder than the other bioplastic with a lower quantity of kenaf fibres. The bioplastic made from 100% kenaf fibres shows no odour, dark brown, and the hardest texture compared to the other four plastics.

Table 1. Sensory evaluation of bioplastic

Physical Appearance					
Ratio of CPH to Kenaf	100:0	75:25	50:50	25:75	0:100
Colour	Light yellowish brown	Yellowish brown	Dark yellowish brown	Reddish brown	Dark reddish brown
Texture	Soft, smooth, chewy	Soft, rough	Medium, rough	Slightly hard, rough	Hard, rough, brittle
Smell	Sweet Smell	Sweet Smell	Less Sweet smell	Odourless	Odourless

Drying Time of Bioplastic

The drying time of bioplastic is the time taken for the bioplastic to be completely dried. Plastic made from 100% CPH took seven days to dry, the longest drying time than the other bioplastics. The Time taken for the bioplastic made from 75% and 50% of CPH was the same (five days). The bioplastic produced from 25% of CPH took six days, while 100% of kenaf bioplastic only took two days to dry, as shown in Figure 2. The graph indicates that the amount of CPH to kenaf in bioplastic formation affects bioplastic drying time. For example, plastic from 100% CPH took the longest drying time while plastic from 0% CPH took the shortest drying time. However, plastic with 25% CPH presented a longer drying time than plastic with 75% and 50% CPH. This extended drying time might be due to an error which the weather and temperature surrounding the experimental workplace.

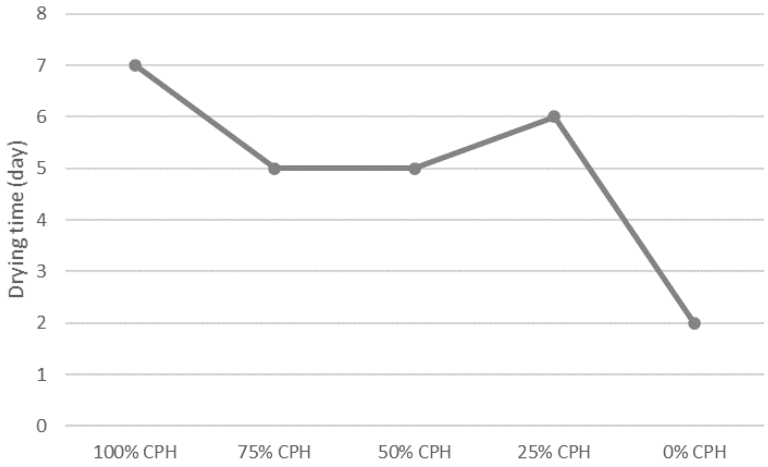


Figure 2. Drying time for the developed bioplastic films

Water Absorption

The percentage of water absorption for all developed bioplastics was measured and calculated using Equation 1. Water absorption for 100% CPH bioplastic shows 25.51%, the highest value exhibited in Figure 3. This result indicates that the CPH can easily absorb water around them. The addition of kenaf fibre in plastic formulation presents the reduction of water absorption in plastic. This phenomenon can be seen for 75% and 50% of CPH in bioplastic with only 15.02% and 14.71% of water absorption, respectively. There was a sharp decrease in the percentage of water absorption for these two ratios. These results indicate that the kenaf fibres help improve water absorption properties making the plastic more suitable for food packaging.

Water absorption for 25% of CPH gives a value of 24.57%, while 0% of CPH only gives a value of 6.47%, as presented in Figure 3. According to this figure, the bioplastic from 0% of CPH absorbs a

small amount of water. However, kenaf fibres are known as natural fibres, consist of hydroxyl groups. These groups can form the intermolecular hydrogen bonds (inside the macromolecule or intramolecular and between another cellulose macromolecule), which indicates that all-natural fibres are hydrophilic, loving water based on Aji et al. (2009). Even though this plastic absorbs a small amount of water, it is enough to destroy the hydrogen bonds to make the plastic melt. Thus, this plastic is not suitable to be applied as food packaging.

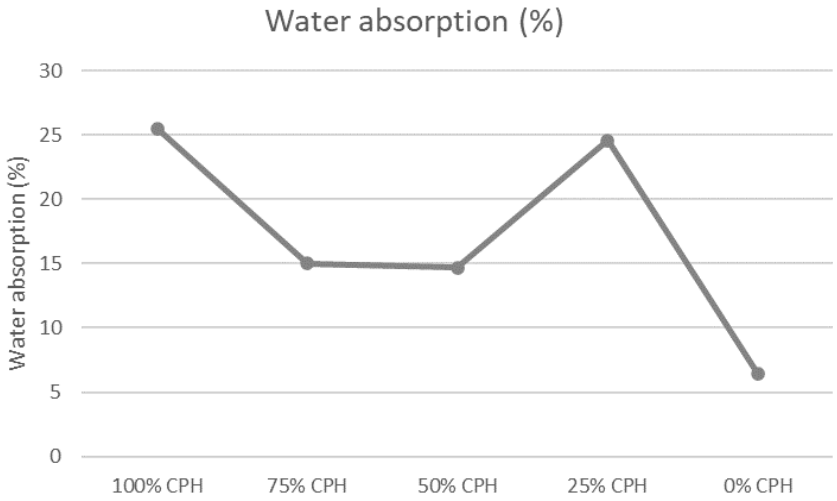


Figure 3. Percentage of water absorption for the developed bioplastic films

Moisture Content of Bioplastic

In this study, moisture content measures the amount of water inside the developed bioplastic films. Moisture content is vitally important because some polymers degrade when wet moulded, resulting in the property changing. The research shows that 100% of CPH moisture content is 20.89%, as presented in Figure 4. The result shows the highest reading as the CPH excellence in absorbing water and moisture.

Water content loss in the 25%, 50% and 75% of CPH bioplastic are 5.89%, 6.92% and 8.36%, respectively. The trend is increasing as the content of CPH increases. However, water content for 0% of CPH bioplastic shows the highest value, 12.87%. According to Mohanty et al. (2000), moisture content for the natural fibres obtained for jute, flax, and hemp was 12.6%, 10.0% and 10.8%, respectively. The moisture content for 100% of kenaf bioplastic is 12.87%, slightly higher than the previous research. The natural fibres show the value of moisture content is more than 10%, and it is parallel with the results from this study.

Furthermore, the moisture content reading decreases as increasing the percentage of CPH extract in bioplastic development. The moisture content reading drops from 12.87% to 5.89% and slightly increases in 50% of bioplastic from cocoa pod husks which are 6.92%. For the result of the bioplastic ratio of 75% cocoa pod husks, the reading shows 8.36% of moisture content and an increase sharply for 100% cocoa pod husks bioplastic. Daud et al. (2013) tested the chemical composition and morphology in CPH and found that the water content for the pure CPH is 14.1±0.05 (%). The moisture content obtained from this research is within 8.38% and 20.89%, which is parallel with the previous study. However, the inconsistent result may be occurred due to the types of additives used or incompatibility of the procedure in bioplastic production.

Sorbitol used in the bioplastic formulation comprises several hydroxyl groups in the molecules, which increase the ability to interact with water by hydrogen bonding (Al-Hassan and Norziah, 2012) as it is immersed in the water. The presence of sorbitol increases the intermolecular forces and lower the capability to interact with water (García-Ayuso et al., 2000). Consequently, the chances of sorbitol interacting with the cellulose chain in CPH are low. This makes the cellulose chain act as a water absorber and creates the high moisture content of a higher concentration of CPH.

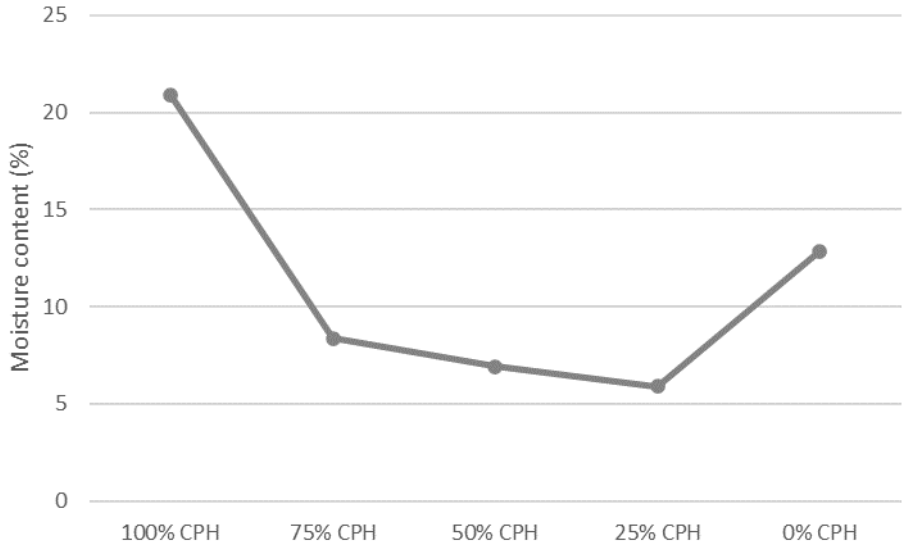


Figure 4. Moisture content for the developed bioplastic films

Water Vapor Permeability (WVP)

Figure 5 presents the results for water vapour permeability for all prepared bioplastic films. Bioplastic with a ratio of 0% CPH shows the highest value of WVP, while bioplastic with a ratio of 50% CPH displays the lowest value of WVP. These WVP values determine the ability of bioplastic to permit the moisture transfer between food and environment, where bioplastic with a low value of WVP exhibits the highest possibility to prevent the moisture transfer between food and environment (Azmin et al., 2020; Luchese et al., 2015). Hence, bioplastic film with a 50% CPH could be applied as food packaging because it showed the lowest value of WVP. Other research found that the edible films and coatings associated with cellulose derivatives could be used for food packaging and preservation (Bertuzzi et al., 2007).

The sorbitol applied in bioplastic formulation modifies the cellulose network's molecular organization. It increases the free volume resulting in a less dense network that results in more permeable films to water (Bourtoom et al., 2006). This phenomenon has been proven (displayed in Figure 5) as increase the ratio of CPH also increase the value of WVP. However, the interaction of the same ratios of CPH and kenaf does not permit the water space in the bioplastic. Adding more kenaf fibre in the bioplastic increases moisture's potential to be absorbed by the plastic. The results in this study are parallel with the research done by McHugh et al. (1993).

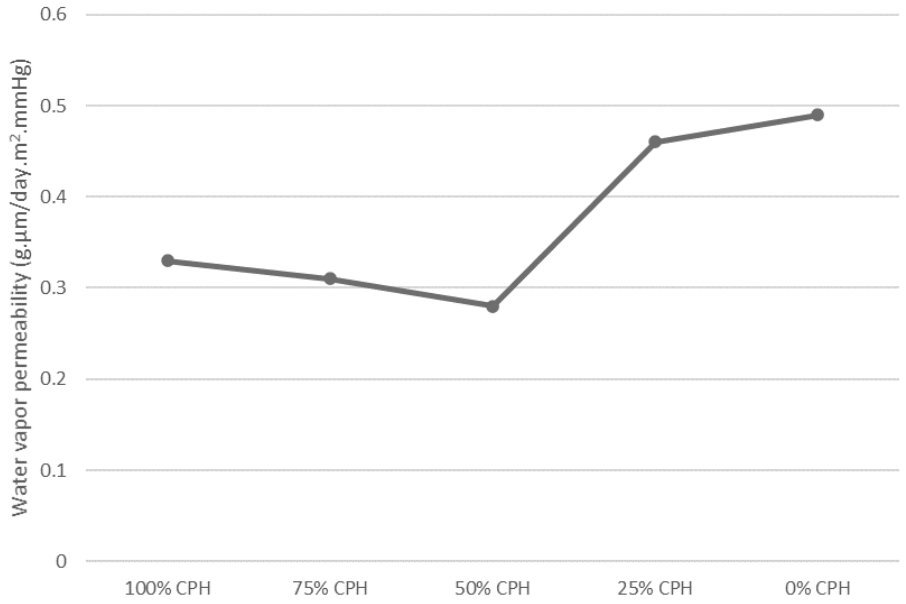


Figure 5. Water vapour permeability for the developed bioplastic films

CONCLUSION

Sensory evaluation and physicochemical properties tests of five bioplastic films containing different ratios of CPH to kenaf fibre were successfully conducted. 50% of CPH bioplastic was found the best film after accessing sensory, drying time, water absorption and moisture content. This plastic showed the best in sensory, moderate in drying time (five days), water absorption (and moisture content (6.92%). 100% of kenaf bioplastic ranked the lowest in sensory but shortest drying time (2%), lowest in the percentage of water absorption (6.47%) but highest in the percentage of moisture content (12.87%). However, this plastic melts in a short time (less than 3 minutes) in a water absorption test. Thus 100% of kenaf bioplastic is not suitable to be utilized as a food packaging container.

Patents: Not applicable.

Author Contribution

For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, Siti Nuurul Huda Mohammad Azmin and Nur Syazwin Eleena Mohamad Sharif; methodology, Siti Nuurul Huda Mohammad Azmin and Mohd Shukri Mat Nor; software; validation, Siti Nuurul Huda Mohammad Azmin, Nur Syazwin Eleena Mohamad Sharif and Palsan Sannasi Abdullah.; formal analysis, Nur Syazwin Eleena Mohamad Sharif; investigation, Siti Nuurul Huda Mohammad Azmin, Nur Syazwin Eleena Mohamad Sharif, Mohd Shukri Mat Nor and Palsan Sannasi Abdullah; resources, Nur Syazwin Eleena Mohamad Sharif; writing—original draft preparation, Siti Nuurul Huda Mohammad Azmin and Nur Syazwin Eleena Mohamad Sharif; writing—review and editing, Siti Nuurul Huda Mohammad Azmin; visualization, Siti Nuurul Huda Mohammad Azmin and Nur Syazwin Eleena Mohamad Sharif; supervision, Siti Nuurul Huda Mohammad Azmin; project administration, X.X.; funding acquisition, Siti Nuurul Huda Mohammad Azmin. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: No conflicts of interest

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Antibiogram and heavy metal resistance pattern of *Aeromonas hydrophila* isolated from Guppy, *Poecilla reticulata*, ornamental fish from aquarium shop

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Abstract: This study was carried out to investigate the mass mortality in ornamental fish Guppy, *Poecilia reticulata*, infected Motile Aeromonad Septicemia (MAS) due to *Aeromonas hydrophila* from ornamental fish aquarium shop in Tanah Merah, Kelantan, Malaysia with emphasis the bacterial isolates antibiogram and heavy metal resistance pattern. MAS due to *A. hydrophila* is an important disease to both human and aquatic animals. Mass mortality of MAS infection among ornamental fish lead to significant economic lose. Hence, there is a must to find out solution to overcome the arise problem. In the present study, a total of 100 bacterial isolates was successfully isolated and identified from diseased Guppy fish by using Glutamate Starch Pseudomonad (GSP) agar media. The bacterial isolates were then subjected to antimicrobial agent sensitivity test and heavy metal resistance assay by using disc diffusion and two fold dilution method, respectively. The findings of the present study showed Oxolinic acid was found can be effectively control the present bacterial isolates. High heavy metal resistance pattern and MAR index revealed that the sampled fish were highly exposed to the tested antimicrobial agent and heavy metals.

INTRODUCTION

World ornamental fish industry is recorded billions USD value where more than 125 countries involved in ornamental fish trading annually (Karthick et al., 2019). Guppy and zebra danio alone contributed about 14% of the total world ornamental fish industry value (Karthick et al., 2019). United States is the largest ornamental fish importer whereas European is the largest ornamental fish market in the world (Casey, 2016). In Asia, Singapore is remain largest ornamental fish exporter mainly Koi, Guppy and Goldfish where ornamental fish products of Singapore is mainly derivate from Malaysia. In Malaysia there are 259 companies involved in exporting ornamental fish throughout the world and has significant economic contribution (Casey, 2016). Total value of Malaysia ornamental fish industry was recorded about RM 340 million with the main ornamental fish families were Cyprinids and Poecilids (Department of Fisheries Malaysia, 2015). One of the famous ornamental fish under Poecilids family in Malaysia is Guppy fish. Guppy fish is not Malaysia native species and it was imported to this country to control mosquito population in natural and artificial waterbodies. The Guppy was found can control mosquito population by eating mosquito larvae subsequently can reduce transmission of diseases such as dengue

fever and malaria (Misha et al., 2017). Beside Guppy was used as fish to control mosquito population, it was also accepted as ornamental fish in worldwide and through selective breeding program in ornamental fish generating new varieties of ornamental fish including Guppy (Asem et al., 2010). However, Guppy is reported prone to be infected by bacterial diseases and one of the diseases is Motile Aeromonas Septicemia (MAS) due to *Aeromonas hydrophila*.

Motile *Aeromonas Septicemia* (MAS) due to *Aeromonas hydrophila* was reported as a disease that may lead to significant economically lose (Austin and Austin, 2016). This bacterium was also reported is a zoonotic pathogen to human (Li and Saghaian, 2011). Hence, *A. hydrophila* is an important pathogen to human and aquatic animals. *A. hydrophila* was reported infected various of aquatic animals. For example, *A. hydrophila* was successfully isolated from diseased Guppy (Cigdem, 2020), Malaysian Giant Freshwater Prawn (*Macrobrachium rosenbergii*) (Lee et al., 2009), red hybrid tilapia (*Oreochromis* spp.) (Lee and Wendy, 2017), silver catfish (*Pangasius sutchi*) (Lee et al., 2010) and freshwater Asian sea bass (*Lates calcarifer*) (Lee et al., 2010). In the present study, antibiogram and heavy metal resistance pattern of *A. hydrophila* were characterised in order to reveal the effectiveness of tested antibiotics in controlling the bacteria at the mean time to determine the exposure level of tested heavy metals against the isolated bacteria.

MATERIALS AND METHODS

Fish sampling

A total of 50 diseased Guppy, *Poecilla reticulata*, was sampled from ornamental fish shop located in Tanah Merah, Kelantan, Malaysia. The sampled fish appeared skin lesions, swollen abdomen, swollen eye and eroded fin. The sampled fish were brought back to laboratory in Universiti Malaysia Kelantan Jeli Campus for microbiology analysis.

Bacterial isolation and identification

A total of 100 bacterial isolates were successfully isolated from diseased Guppy. Sterile cotton bud was swab on swollen eye, skin lesion area, kidney and liver and streaked directly onto Glutamate Starch Pseudomonad (GSP) agar media (Merck, Germany). The media plates were then incubated for 24 to 48 h at room temperature. Bacterial colonies appeared yellow in color on medium plate with surrounded with yellow color were selected for bacterial identification. Selected bacterial isolates were screened with conventional biochemical test such as Gram staining, oxidase and catalase test. Only Gram-negative bacterial isolates performed positive in both oxidase and catalase tests were further identified by using commercial bacterial identification kit (BBL Crystal, Singapore).

Antimicrobial sensitivity test

Antimicrobial sensitivity test was carried out by using Kirby Bauer disk diffusion method as described in the study of Lee and Wendy (2017). The isolated bacteria were cultured in Tryptic Soy Broth (TSB) (Merck, Germany) with supplemented with 0.85% of NaCl (R&M Chemical, Malaysia) and incubated for 24 to 48 h at room temperature. The bacterial cells was collected by using MiniSpin (Eppendorf, Germany) at 14,500 rpm for 10 min. The bacterial cells suspension was prepared by using physiological saline and the concentration of the bacterial cells was adjusted to 10^9 colony forming unit (CFU) by monitored using Biophotometer (Eppendorf, Germany). The bacterial suspension was then swabbed entirely on the surface area of Tryptic Soy Agar (TSA) (Merck, Germany) by using sterile cotton bud. After 10 mins swabbing bacterial cells on the agar surface, 16 antimicrobial agents were placed on the agar media. The inoculated agar media were then incubated at room temperature for 24 h. The results of inhibition zone were measured by using ruler and the size of the inhibition zone was analysed by referred to CLSI to determine the sensitivity of the antibiotic against the bacterial isolates (CLSI, 2015). The antimicrobial agents (n = 16) were used in the present study were Compound sulphonamides (300 µg/disk), Nalidixic acid (30 µg/disk), Ampicillin (10 µg/disk), Doxycycline (30 µg/disk), Novobiocin (30 µg/disk), Oxytetracycline (30 µg/disk), Chloramphenicol (30 µg/disk), Erythromycin (15 µg/disk), Sulphamethoxazole (25 µg/disk), Flumequine (30 µg/disk), Kanamycin (30 µg/disk), Oxolinic acid (2 µg/disk), Spiramycin (100 µg/disk), Fosfomycin (50 µg/disk), Amoxycillin (25 µg/disk) and Tetracycline (30 µg/disk).

Multiple Antibiotic Resistance (MAR) index determination

Multiple Antibiotic Resistance (MAR) index determination of the present study was calculated as described in the study of Lee et al. (2013) as follow:

Multiple antibiotic resistance (MAR) index = X/(Y × Z)

Where,

X = Total number of antibiotic resistance cases;

Y = Total number of antibiotics applied;

Z = Total number of bacteria isolates.

MAR index equal to or <0.2 indicated that the sampled Guppy fish were seldom or never exposed to the tested antibiotics; on the other hand, the sampled Guppy fish may have a high risk of exposure to the tested antibiotics if the MAR index is more than 0.2 (Lee et al., 2013).

Characterization of bacterial isolates heavy metal resistance pattern

Characterization of bacterial isolates heavy metal resistance pattern was carried out by using two-fold dilution method as described in the study of Lee et al. (2009) and Lee and Wendy (2017). Heavy metals (mercury Hg^{2+}, chromium Cr^{6+}, zinc Zn^{2+}, and copper Cu^{2+}) were supplemented in Tryptic Soy Agar (TSA) (Merck, Germany) at five different concentrations each by using mercury II chloride, potassium dichromate, zinc sulphate, and copper II sulphate (Merck, Germany). 5 concentrations of Cr^{6+} and Zn^{2+}were same from 25 to 400 µg/mL whereas Hg^{2+} and Cu^{2+} were ranging from 2.5 to 40 µg/mL and 150 to 2400 µg/mL, respectively. Bacterial suspension was prepared as mentioned in the antimicrobial sensitivity test. The bacterial suspension was swabbed onto prepared heavy metal media agar surface followed by 24 h incubation period. After incubation period, the growth of the bacterial isolates on TSA supplemented with heavy metals was recorded. Any bacterial isolate was able to grow on TSA supplemented with 10 µg/mL of Hg^{2+}, 100 µg/mL of Zn^{2+} and Cr^{6+}, and 600 µg/mL of Cu^{2+} was categorised as resistant to the tested heavy metals (Allen et al., 1977; Lee et al., 2013).

RESULTS

A total of 100 bacterial isolates, *Aeromonas hydrophila* was successfully isolated and identified from disease Guppy fish sampled from an aquarium shop located in Tanah Merah, Kelantan. Antibiogram of the bacterial isolates showed in Table 1 whereas their heavy metal resistance pattern showed in Table 2. Overall of antimicrobial sensitive case and intermediary sensitive case were 64.3% and 4.6 %, respectively. On the other hand, antimicrobial resistance case was recorded as 31.3 %. All bacterial isolates were found resistant to ampicillin and sulphamethoxazole on the other hand oxolinic acid was able to inhibit the growth of all the present bacterial isolates. High antimicrobial sensitive case (more than 90%) was observed among antimicrobial agents such as spiramycin, fosfomycin, kanamycin, flumequine, doxycycline and nalidixic acid. Moderate antimicrobial resistance case (more than 60%) was recorded for amoxycillin, novobiocin and compound sulphonamides. Besides that, tetracycline, erythromycin, chloramphenicol and oxytetracycline were found can control present bacterial isolates moderately. MAR index calculation recorded as 0.311. Heavy metal resistance test revealed the present bacterial isolates showed highly resistant to Cu^{2+}(90%). This was followed by Cr^{6+} (72 %), Zn^{2+} (36 %) and Hg^{2+}(11 %), respectively.

Table 1. Antibiogram of *Aeromonas hydrophila* isolated from Guppy, *Poecilla reticulata*, ornamental fish from aquarium shop

Antibiotic (µg/disk)	Resistance case (%)	Intermediary sensitive case (%)	Sensitive case (%)
Compound sulphonamides (300)	66	5	29
Nalidixic acid (30)	0	7	93
Ampicillin (10)	100	0	0
Doxycycline (30)	0	2	98
Novobiocin (30)	75	5	20
Oxytetracycline (30)	24	2	74
Chloramphenicol (30)	31	11	58
Erythromycin (15)	12	10	78
Sulphamethoxazole (25)	100	0	0
Flumequine (30)	0	2	98
Kanamycin (30)	3	2	95
Oxolinic acid (2)	0	0	100
Spiramycin (100)	0	3	97
Fosfomycin (50)	0	4	96
Amoxycillin (25)	73	16	11
Tetracycline (30)	14	4	82
Overall	31.1 %	4.6 %	64.3 %
Total Incidence cases	498	73	1029

Table 2. Susceptibility of bacterial isolates to four types of heavy metal

Heavy metals	Sensitive (%) (Total cases)	Resistance (%) (Total cases)
Hg^{2+}	89 (89)	11 (11)
Zn^{2+}	64 (64)	36 (36)
Cr^{6+}	28 (28)	72 (72)
Cu^{2+}	10 (10)	90 (90)

DISCUSSION

The findings of the present study revealed that the mass mortality of sampled Guppy fish from aquarium shop was infected by MAS due to *A. hydrophila*. *A. hydrophila* was responsible to many cases of ornamental fish in world wide. For example, Raja et al. (2018) claimed that *A. hydrophila* was identified as causative agent of mass mortality of gold fish, *Carassuis auratus*, from four ornamental fish farms in Kerala, India. Park et al. (2009) was reported that *A. hydrophila* was responsible to mass mortality of adult koi carp, *Cyprinus carpio* in the early winter in Samchunpo city, Korea. Other than ornamental fish, *A. hydrophila* was also reported responsible to mass mortality to other freshwater fish. For instance, Abadi et al. (2020) was reported that *A. hydrophila* infected in Nile tilapia (*Oreochromis niloticus*) and led to mass mortality in Sorong District, West Papua, Indonesia. Hence we may conclude that *A. hydrophila* is a bacterium can lead to mass mortality of fish and there is a must to find suitable antimicrobial agent to overcome MAS due to *A. hydrophila*.

Based on the result in the present study showed oxolinic acid is the most effective antimicrobial agent in controlling *A. hydrophila*. Many studies revealed the potential of oxolinic acid in fish disease treatment. For instance, Katharios et al. (2015) mentioned oxolinic acid was found effective in the treatment of edwardsiellosis infection in sharpsnout sea bream, *Diplodus puntazzo,* in Mediterranean. Beside oxolinic acid, flumequine is also can be used as antimicrobial agent to against MAS due to *A. hydrophila* in the present study. Both antimicrobial agents are under quinolone antimicrobial agent group. These quinolone antimicrobial agents were reported no lead to antimicrobial agent resistant case among bacteria in the environment of aquaculture system although high dose of the antimicrobial agent was used (Giraud et al., 2006). Furthermore, oxolinic acid and flumequine were found can be degraded faster

when exposed to sun light (Lai and Lin, 2009). Therefore, we may suggest that these antimicrobial agents can be used in the treatment of MAS due to *A. hydrophila* infected in Guppy fish. However, in the recent year, quinolone antimicrobial agents (oxolinic acid dan flumequine) were not allowed to use in Chilean salmon industry (Claudio et al., 2018). Therefore, Chilean salmon industry player mainly using florfenicol and oxytetracycline in managing salmon health. In spite Chilean government restrict salmon farmer from using quinolone antimicrobial agent but this group of antimicrobial agent was reported widely used in worldwide (Done et al., 2015; Quesada et al., 2013) and in Mediterranean aquaculture (Rigos and Troisi, 2005).

Beside of using oxolinic acid and flumequine, fish farmer can consider antimicrobial agents such as nalidixic acid, doxycycline, kanamycin, spiramycin, fosfomycin and tetracycline in the treatment of MAS due to *A. hydrophila* in Guppy. However, nalidixic acid and tetracycline were banned to applied in Malaysia aquaculture (Thiang et al., 2021). Application of doxycycline was also reported used in disease treatment of striped catfish, *Pangasianodon hypohthalmus* in Vietnam (Phu et al., 2015). Kanamycin and spiramycin was seldom or none reported use in aquaculture however fosfomycin was found effectively used in controlling streptococcosis in Nile tilapia (*Oreochromis niloticus*) (Hussien and Hassan, 2011). Instead of using commercial antimicrobial agent in controlling MAS due to *A. hydrophila*, farmer may have other options like using plant-based treatment. Many studies was conducted showed plant based treatment was promising against MAS. For instance, Riauwaty et al. (2020) revealed that turmeric can be used in the treatment of MAS infection due to *A. hydrophila* in *Pangasius hypothalmus*. High MAR index was observed in the present study indicating the sampled Guppy fish were highly exposed to the tested antimicrobial agent. Therefore scientist may explored new plant based antimicrobial agent to alternate the current commercial antimicrobial agent for environment betterment.

High resistant to chromium and copper was observed among the present bacterial isolates. These trace elements are important in animal immune system but will tend to persist in environment hence contribute to heavy metal resistance case among bacteria (Resende et al., 2012). Furthermore, bacterial may adapt with the presence of heavy metals because it have genetic to resistant the heavy metals (Yu et al., 2017). Although the finding in the present study showed low resistance case of zinc and mercury case but if bacteria were continuously expose to these heavy metals slowly the bacteria will resistant to the heavy metals. Low resistance case was found among the present bacterial isolates against mercury. This finding was agreed with the study of Hassen et al. (1998) where claimed that mercury is the most toxic against bacteria if compared to chromium, copper and zinc. The main source of heavy metals contamination into environment is from agricultural activities where fertilizers contain heavy metal elements were widely used allowed these heavy metals seeping into aquaculture system through soil and water. However, further study need to be carry out before we can come to a conclusion.

CONCLUSION

Based on the present study findings showed that oxolinic acid can be used in controlling MAS due to *A. hydrophila* in Guppy fish. However, further study can be used before it can be used. High MAR index and heavy metal resistance pattern copper and chromium indicating the sampled Guppy fish were highly exposed to the tested antimicrobial agent and heavy metals.

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The effects of partial replacement of fishmeal with hermetia meal on the growth and fatty acid profile of African catfish fry

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Abstract: Fishmeal is becoming an increasingly expensive resource. Therefore, it is important to find other sources of protein to reduce feed costs. The larvae of the black soldier fly, *Hermetia illucens*, was evaluated as an alternative protein source for the African catfish, *Clarias gariepinus*, in six-week feeding trials. Three experimental diets were iso-nitrogenous (35% crude protein) and iso-lipidic (18 % crude fat). The following ratios of fish meal were used in the study: 1 fish meal (FM, control); 1:2 hermetia meal to fish meal (1HM:2FM); and 1:1 hermetia meal to fish meal (1HM: 1 FM). Two hundred and seventy catfish fingerlings (mean initial weight: 2.45 g) were distributed to nine tanks corresponding to three treatments with three replicates. The experiment examined weight gain, specific growth rate (SGR), feed conversion ratio (FCR) and muscular body composition as growth parameters. The study found that substitution of one-third of fish meal protein with hermetia meal had no negative effect on the growth performance of African catfish fry fed 1HM:2FM, which showed greater weight gain (17.74 ± 0.722g). The muscle composition of fish fed 1H:1FM showed a significant proportion of protein between diets. The fatty acid profile of fish muscle reflected the content of the diet. Based on the results, it was concluded that the protein meal of the black soldier fly can serve as a substitute for the diet of the African catfish.

INTRODUCTION

Aquaculture is one of the fastest growing food production industries in the world. Production increased from 61.8 million tonnes in 2010 to about 80 million tonnes in 2016 and is projected to increase by another 36.7 per cent to about 109 million tonnes in 2030 (FAO, 2018). That the aquaculture industry can grow so rapidly is due to the availability of external supplies of nutrients in the form of feed, and if growth is to be sustained, the supply of feed must also grow more or less rapidly (Tacon et al., 2011, Turchini et al., 2019). Due to its high content of easily digestible proteins, amino acid composition, highly digestible lipids and long-chain polyunsaturated fatty acids, fishmeal is one of the most important ingredients for feed (Turchini et al., 2019, Trushenski and Rombenso, 2020). Increasing demand with a limited supply of fishmeal has caused the price of fishmeal to skyrocket over the years (Imtiaz, 2022). This has led to many studies being conducted to find ways to reduce the reliance on fishmeal in animal

feed (Shekarabi et al., 2022; Kari et al., 2021; Kari et al., 2022; Kari et al., 2020; Zulhisyam et al., 2020; Kumar et al., 2020; Liu et al., 2020).

Insect protein has been identified as a possible ingredient to partially or completely replace fishmeal as a protein source in aquafeed for omnivorous species (Riddick, 2014). Insects grow and reproduce easily, have a high feed conversion ratio as they are cold-blooded and can be raised on organic waste (Palma et al., 2019, Spranghers et al., 2017). This enables the commercial production of insect meal. A favourable insect species as an alternative protein source to fishmeal is the pre-pupae of the black soldier fly (*Hermetia illucens*), as the pre-pupae have a high protein content of about 40 to 44 per cent crude protein and several experiments conducted show that black soldier fly larvae can partially or completely replace fishmeal in fish feed (Abdel-Tawwab et al., 2020, Belghit et al., 2019, Bolton et al., 2021).

Catfish is one of the most commonly farmed finfish in the world and one of the farmed catfish species is the African catfish (*Clarias gariepinus*) (FAO, 2014). Although the production of African catfish is not very large globally, it is the most farmed fish species in Africa, Indonesia and Malaysia. In Malaysia, the African catfish having been introduced through aquaculture from Thailand between 1986 and 1989, and since 2008, it has overtaken the production of red tilapia and plays an important role in the aquaculture industry (Dauda et al., 2018). The African catfish is an opportunistic omnivore that feeds on a variety of foods, with insects being one of the most important foods, especially when it is still small (Tesfahun, 2018). There is therefore a possibility that insect meal can be included in the diet of African catfish in aquaculture, as insects are part of their natural diet. The aim of this study is to determine the effects of replacing fish meal with different ratio of hermetia meal larvae on growth performance and fatty acid profile of the fish.

MATERIALS AND METHODS

Experimental fish and husbandry conditions

The African catfish fry used in this experiment were purchased from a commercial supplier Chia Bee Aquaculture, Sungai Petani, Kedah. The fish were quarantined in a fibreglass tank for one week before being used in the experiment. They were properly treated, such as with methylene blue and salt to rule out possible diseases and transported to the Kompleks Penyelidikan Akuatik (L24) at Universiti Sains Malaysia. Two weeks prior to the experiment, the fish were acclimatised to the environmental conditions in the laboratory upon arrival. During the acclimatisation period, the fish were fed twice daily with a commercial tilapia pellet containing 35% protein and 8% lipid.

Healthy fish with an average weight of 2.45g were randomly placed in the round polyester polytank (37-inch x 24-inch) with the water level in all tanks 1/3 the height of the tank. Each feeding treatment had three replicate tanks and each tank contained 30 fish. All tanks were continuously aerated and fish were reared in carbon filtered water. The water in the rearing tank was changed daily. The light cycle of the indoor system used for this experiment was set to 12 hours. The feeding experiment lasted for 12 weeks, during which the fish were fed the formulated diets twice daily at 09:00 and 17:00 until they were full. Each diet was replicated in three tanks. Every fortnight, growth progress was monitored by weighing the fish in large quantities, depending on the tank. The fish were not fed for 24 hours before being touched to avoid stress. Food intake was measured daily. The fish were weighed individually at the last sampling to determine the final weight. During the study, temperature, pH and DO of the culture conditions were maintained at 25.84 ± 0.07 °C, 6.70 ± 0.06 and 7.2 ± 0.09 mg/L, respectively.

Experimental Design and Feed Preparation

A feeding trial was performed to assess the potential inclusion of hermetia meal in aquafeed for African catfish. Three practical diets comprising the same protein level (35%), lipid (8%), and energy were prepared. Table 1 shows the formulation and chemical analysis of the experimental diets.

Table 1: Ingredients and proximate composition of experimental diet (g/100g dry matter).

Ingredients (g/100g)	FM (Control)	1HM:2FM	1HM:1FM
Fish meal[1]	40.00	30.00	20.00
Hermetia meal[2]	0.00	20.20	33.00
Soybean meal[3]	18.00	18.00	18.00
Palm oil[4]	13.50	10.34	6.95
Corn starch[5]	20.2	18.60	12.50
Cellulose[6]	1.50	1.36	0.95
Vitamin premix[7]	0.50	0.50	0.50
Mineral premix[8]	0.50	0.50	0.50
Chromium oxide[9]	0.50	0.50	0.50
Proximate composition (g/100g)			
Moisture	7.05	6.33	6.63
Protein	34.68	34.92	34.75
Lipid	17.92	17.35	17.88
Ash	11.27	11.21	11.06
Fibre	5.87	6.37	6.63
NFE[10]	20.26	17.17	19.68

[1]Danish fishmeal
[2]Hermetia meal
[3]Soybean meal
[4]Palm oil
[5]Corn starch
[6]Cellulose
[7]Vitamin premix (Rovimix 6288; F.Hoffman La-Roche Ltd, Basel, Switzerland), containing (per kg, dry weight): Vitamin A, 50 million IU; Vitamin D3, 10 million IU; Vitamin E, 130 g; Vitamin B1, 10 g; Vitamin B2, 25 g; Vitamin B6, 16 g; Vitamin B12, 100 mg; biotin,500 mg; panthothenic acid, 56 g; folic acid, 8 g; niacin, 200 g; anti-cake20 g; antioxidant, 200 mg; Vitamin K3, 10 g; and Vitamin c, 35 g
[8]Mineral premix (g/kg)-cobalt carbonate, 100mg; copper sulphate, 780 mg; magnesium sulphate, 137 g;mmanganese oxide, 800 mg; potassium chloride, 50 g; potassium iodide, 150 mg; sodium chloride, 60 g; sodium selenite, 200 mg and zinc oxide, 1.5 g; calcium lactate, 327 g; ferrous sulphate, 25 g; calcium phosphate (monobasic), 397.5 g.
[9]chromium oxide
[10]Nitrogen free extract: 100 - (moisture + protein+ lipid +ash+ fibre)

Growth performance and body indices

The collected samples were analyzed to estimate the growth performances using the following formulae:

Weight gain (WG)= final weight – initial weight
Feed conversion ratio= total feed intake. weight gain^{-1}
Specific Growth Rate = (ln Wtfinal – lnWtinitial). duration^{-1}
Protein efficiency ratio (PER) = weight gain.total protein intake^{-1}
Survival = final number fish/ initial number fish x 100
Hepatosomatic index (HSI %) = (liver weight.body weight^{-1}) x 100
Viscerosomatic Index (VSI %) = (viscera weight.body weight^{-1}) x 100

Proximate Analysis

AOAC methods were used to determine the moisture, protein, lipid, fibre, and ash content of raw ingredients, experimental diets, and fish (AOAC, 1997).

Tissue Collection

Fish were euthanised at the last sampling by exposing them to an overdose of clove oil in an ice-cold water bath (20 ml L^{-1}). Three fish from each tank were randomly selected to measure HSI and VSI levels on the whole body, viscera and liver.

Fatty Acid Methyl Ester Extraction and Analysis by Gas Chromatography

Fish muscle tissues were subjected to total lipid extraction and fatty acid methyl esters (FAME) were prepared by methylation and transesterification with boron trifluoride in methanol (Cuniff 1997). Tissues (0.5 g-1.0 g) were mechanically homogenised in chloroform/methanol (2:1, v/v) to obtain total lipid (Folch et al. 1957). A gas chromatograph (GC-2010, Shimadzu) equipped with a flame ionisation detector and a highly polar fused silica cyanosiloxane column (SP -2380 (30 m length, 0.25 mm inner diameter, 0.20 µm film thickness; Supelco, USA) was used to separate the FAME. The temperature was programmed to rise from 100°C to 230°C at a split ratio of 1:50 at a rate of 1.5°C/min, using nitrogen as the carrier gas. The injector and detector temperatures were set at 250°C and 260°C respectively. The individual FAME were identified by comparing the retention times with commercially available standards: 37 Component FAME Mix (Supelco) and PUFA No. 3 from Menhaden Oil (Supelco).

Statistical analysis

Statistical analysis of data on proximate composition, body indices, growth performance, digestive enzyme activities, and haematology was performed using SPSS 26 software. One-way analysis ANOVA was used as statistical analysis and a post hoc test was performed when necessary.

RESULTS

Growth Performance

An average of 2.45 ± 0.02 g of hybrid red tilapia was used for this study ($p > 0.05$), as shown in Table 2. At week 6, fish fed the 1HM: 2FM treatment (20.09 ± 0.731 g) showed no significant difference from fish fed the control diet (FM) (18.85 ± 0.70 g; $p > 0.05$). However, the fish fed 1HM:1FM had a significantly lower final weight than the other two treatments.

The weight gain of fish fed 1HM:2FM (17.74 ± 0.722 g) was not different from that of fish fed FM (16.42 ± 0.159 g; $p > 0.05$). Fish fed 1HM:1FM recorded the lowest weight gain, which was 14.25 ± 0.572 g ($p < 0.05$). A similar trend was also observed in the SGR value, where fish fed 1HM:2FM (5.02 ± 0.085) showed no significant difference compared to fish fed FM (4.86 ± 0.018; $p > 0.05$). The lowest FCR value was found in fish fed FM (1.29 ± 0.006) and this was not a significant difference from fish fed the diet 1HM:2FM. The 1HM:1FM diet had the highest FCR of 1.55 ± 0.021 ($p < 0.05$). The HSI, VSI and survival rate of the fish were not affected by the treatments.

Table 2 : Growth parameters and body indices of African catfish fed with formulated diets for 6 weeks. Data are presented in mean ± SEM. Different superscripts in each row indicate a significant difference ($p < 0.05$).

Parameters	FM (Control)	1HM:2FM	1HM:1FM
Initial weight (g)	2.45 ± 0.024	2.45 ± 0.025	2.44 ± 0.025
Final weight (g)	18.85 ± 0.701^{B}	20.09 ± 0.731^{B}	16.73 ± 0.623^{A}
Weight gain (g)	16.42 ± 0.159^{B}	17.74 ± 0.722^{B}	14.25 ± 0.572^{A}
Average Daily Weight Gain (g)	0.39 ± 0.006^{B}	0.42 ± 0.017^{B}	0.34 ± 0.015^{A}

Specific Growth Rate (SGR)	4.86 ± 0.018^{B}	5.02 ± 0.085^{B}	4.57 ± 0.081^{A}
Food Conversion Ratio (FCR)	1.29 ± 0.006^{A}	1.39 ± 0.047^{A}	1.55 ± 0.021^{B}
Feed intake (g)	21.18 ± 0.296^{A}	24.68 ± 1.213^{B}	22.10 ± 0.584^{A}
Viscerasomatic Index (VSI)	0.04 ± 0.006	0.04 ± 0.004	0.05 ± 0.007
Hepatosomatic Index (HIS)	0.01 ± 0.001	0.01 ± 0.002	0.01 ± 0.002
Survival Rates (%)	85.56 ± 5.557	74.44 ± 14.184	93.33 ± 3.848

Proximate composition

Proximate composition of fish muscle was affected by diet treatment, as shown in Table 3. Fish fed 1HM:1FM (8.64 ± 0.05%) contained the highest moisture content than fish fed FM and 1HM:2FM, 8.21 ± 0.10% and 8.00 ± 0.04%, respectively ($p < 0.05$). Muscle protein composition increased with the increase in Hermetia meal. Fish fed the diet FM contained the lowest amount of muscle protein (77.87 ± 0.05%, $p < 0.05$) and fish fed the diet 1HM:1FM contained the highest protein in muscle (86.87 ± 0.03, $p < 0.05$).

Table 3: Proximate composition of muscle of African catfish fingerlings fed with experimental diets.

Composition (%)	FM (Control)	1HM:2FM	1HM:1FM
Moisture (%)	8.21 ± 0.10^{A}	8.00 ± 0.04^{A}	8.64 ± 0.05^{B}
Protein (%)	77.87 ± 0.05^{A}	84.50 ± 0.09^{B}	86.87 ± 0.03^{C}
Lipid (%)	20.84 ± 0.43^{A}	25.07 ± 0.52^{B}	19.55 ± 0.19^{A}

Value presented are means ± SEM of three replicates group. Means value having different superscript are significantly difference ($p<0.05$)

Fatty acid profile

Table 4 shows the fatty acid composition of the experimental feeds. The total saturated fatty acid (SFA) content increased with the increasing level of hermetia meal. FM feed contained the lowest SFA (18.62 $\pm$ 0.08), followed by 1HM:2FM (27.15 $\pm$ 0.23) ($p < 0.05$). Diet 1HM1: FM had the highest SFA content, 35.78 $\pm$ 0.12 ($p < 0.05$). In general, the profile of unsaturated fatty acids decreased with increasing intake of hermetia meal. FM diet contained the highest levels of monounsaturated fatty acids (MUFA), n-3 polyunsaturated fatty acids (n-3 PUFA) and n-6 polyunsaturated fatty acids (n-6 PUFA), namely 28.76 $\pm$ 0.23, 7.30 $\pm$ 0.07 and 45.32 $\pm$ 0.05, respectively ($p < 0.05$). The lowest MUFA, n-3 PUFA and n-6 PUFA values were in the 1HM:1FM diet with 23.42 $\pm$ 0.16, 4.92 $\pm$ 0.04 and 35.78 $\pm$ 0.07, respectively ($p < 0.05$).

Table 4: Fatty acids composition in the experimental diets. Value presented are means ± SEM of three replicates group. Means value having different superscript are significantly difference ($p<0.05$)

Component	Diet FM (Control)	1HM:2FM	1HM:1FM
Saturated fatty acids			
C10:0			0.28 ± 0.00
C12:0	0.00 ± 0.00^{a}	5.75 ± 0.04^{b}	11.66 ± 0.02^{c}
C14:0	0.63 ± 0.00^{a}	2.38 ± 0.01^{b}	4.03 ± 0.03^{c}
C15:0	0.00 ± 0.00^{a}	0.14 ± 0.00^{b}	0.18 ± 0.01^{c}
C16:0	13.05 ± 0.02^{a}	14.09 ± 0.10^{b}	15.07 ± 0.03^{c}
C17:0	0.22 ± 0.01^{ab}	0.21 ± 0.01^{a}	0.25 ± 0.01^{b}
C18:0	4.28 ± 0.04^{b}	4.18 ± 0.05^{b}	3.99 ± 0.01^{a}
C20:0	0.32 ± 0.00	0.29 ± 0.02	0.24 ± 0.00
C22:0			
C24:0	0.12 ± 0.00	0.13 ± 0.01	0.09 ± 0.01
Total SFA	18.62 ± 0.08^{a}	27.15 ± 0.23^{b}	35.78 ± 0.12^{c}
Monounsaturated fatty acids			
C14:1	0.00 ± 0.00^{a}	0.17 ± 0.01^{b}	0.31 ± 0.00^{c}
C16:1	0.86 ± 0.01^{a}	1.16 ± 0.02^{b}	1.46 ± 0.05^{c}
C17:1	0.13 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
C18:1n9	23.80 ± 0.10^{c}	20.77 ± 0.24^{b}	19.50 ± 0.02^{a}
C18:1n7	0.92 ± 0.04^{b}	0.58 ± 0.13^{a}	0.69 ± 0.03^{ab}
C20:1n9	1.33 ± 0.05^{c}	0.95 ± 0.02^{b}	0.68 ± 0.03^{a}
C22:1n9	1.48 ± 0.03^{a}	1.09 ± 0.02^{c}	0.68 ± 0.02^{b}
C24:1n9	0.24 ± 0.01^{c}	0.15 ± 0.01^{b}	0.09 ± 0.01^{a}
Total MUFA	28.76 ± 0.23^{c}	24.97 ± 0.46^{b}	23.42 ± 0.16^{a}
n-3 Polyunsaturated fatty acids			
C18:3n3	4.83 ± 0.01^{a}	3.89 ± 0.01^{c}	3.38 ± 0.01^{b}
C20:3n3	0.00 ± 0.00^{a}	0.28 ± 0.02^{b}	0.00 ± 0.00^{a}
C20:4n3			
C20:5n3	0.87 ± 0.02^{c}	0.73 ± 0.01^{b}	0.54 ± 0.02^{a}
C22:5n3	0.09 ± 0.01	0.08 ± 0.01	0.06 ± 0.01
C22:6n3	1.51 ± 0.04^{c}	1.28 ± 0.03^{b}	0.93 ± 0.01^{a}
Total n-3 PUFA	7.30 ± 0.07^{c}	5.98 ± 0.06^{b}	4.92 ± 0.04^{a}
n-6 Polyunsaturated fatty acids			
C18:2n6	45.32 ± 0.05^{c}	41.51 ± 0.14^{b}	35.73 ± 0.07^{a}
C20:3n6			
C20:4n6	0.00 ± 0.00	0.07 ± 0.01	0.05 ± 0.00
Total n-6 PUFA	45.32 ± 0.05^{c}	41.58 ± 0.15^{b}	35.78 ± 0.07^{a}
Total PUFA	52.62 ± 0.12^{c}	47.56 ± 0.20^{b}	40.69 ± 0.12^{a}

The fatty acid composition in the muscle of the experimental fish reflect the profile of diets and presented in Table 5. Overall, the level of SFA in the muscle was increased with the inclusion level of dietary hermetia meal. The lowest level of muscular SFA was in the fish fed with FM diet that was 20.82 ± 0.35 and the highest level was detected in fish fed with 1HM:1FM diet that was 34.11 ± 0.27 ($p<0.05$). Similar to dietary fatty acid profile, unsaturated fatty acid in the muscle also reduced with an increase of dietary hermetia meal. The highest level of MUFA, n-3 PUFA and n-6 PUFA were in fish fed fish FM diet that are 29.60 ± 1.64, 8.60 ± 0.16 and 40.60 ± 0.67, respectively ($p<0.05$)

Table 5: Fatty acids composition in the muscle of fish fed with the experimental diets. Value presented are means ± SEM of three replicates group. Means value having different superscript are significantly difference ($p<0.05$)

Component	Muscle FM (Control)	1HM:2FM	1HM:1FM
Saturated fatty acids			
C10:0	-	-	-
C12:0	0.00 ± 0.00^{a}	3.90 ± 0.04^{b}	7.61 ± 0.04^{c}
C14:0	0.59 ± 0.01^{a}	2.28 ± 0.03^{b}	3.93 ± 0.02^{c}
C15:0	0.00 ± 0.00^{a}	0.17 ± 0.01^{b}	0.24 ± 0.04^{b}
C16:0	14.21 ± 0.22^{a}	16.19 ± 0.12^{b}	16.20 ± 0.02^{b}
C17:0	0.24 ± 0.02	0.26 ± 0.01	0.29 ± 0.01
C18:0	4.86 ± 0.07^{b}	5.08 ± 0.04^{b}	4.51 ± 0.11^{a}
C20:0	0.22 ± 0.00	0.19 ± 0.02	0.17 ± 0.02
C22:0	0.71 ± 0.03	0.83 ± 0.03	1.17 ± 0.02
C24:0	-	-	-
Total SFA	20.82 ± 0.35^{a}	28.91 ± 0.30^{b}	34.11 ± 0.27^{c}
Monounsaturated fatty acids			
C14:1	0.11 ± 0.01^{a}	0.16 ± 0.01^{b}	0.27 ± 0.01^{c}
C16:1	0.94 ± 0.03^{a}	1.39 ± 0.01^{b}	1.67 ± 0.04^{c}
C17:1	0.13 ± 0.08	0.00 ± 0.00	0.00 ± 0.00
C18:1n9	24.71 ± 1.27^{b}	21.19 ± 0.17^{a}	20.27 ± 0.12^{a}
C18:1n7	1.39 ± 0.16	0.96 ± 0.08	1.11 ± 0.08
C20:1n9	1.19 ± 0.05^{c}	0.94 ± 0.05^{b}	0.73 ± 0.01^{a}
C22:1n9	0.96 ± 0.03^{b}	0.07 ± 0.02^{a}	0.00 ± 0.00^{a}
C24:1n9	0.18 ± 0.01^{b}	0.17 ± 0.02^{b}	0.11 ± 0.00^{a}
Total MUFA	29.60 ± 1.64^{b}	24.88 ± 0.36^{a}	24.16 ± 0.27^{a}
n-3 Polyunsaturated fatty acids			
C18:3n3	3.96 ± 0.03^{c}	3.22 ± 0.01^{b}	2.64 ± 0.04^{a}
C20:3n3	-	-	-
C20:4n3	0.18 ± 0.01^{a}	0.16 ± 0.01^{b}	0.13 ± 0.01^{b}
C20:5n3	0.70 ± 0.03^{c}	0.50 ± 0.01^{b}	0.38 ± 0.00^{a}
C22:5n3	0.32 ± 0.01	0.27 ± 0.01	0.29 ± 0.01
C22:6n3	3.43 ± 0.07^{a}	2.68 ± 0.04^{b}	2.83 ± 0.03^{b}
Total n-3 PUFA	8.60 ± 0.16^{c}	6.84 ± 0.08^{b}	6.28 ± 0.09^{a}
n-6 Polyunsaturated fatty acids			
C18:2n6	39.88 ± 0.57^{c}	37.99 ± 0.17^{b}	33.67 ± 0.18^{a}
C18:3n6	0.39 ± 0.02^{a}	0.51 ± 0.03^{b}	0.88 ± 0.01^{c}
C20:3n6	0.33 ± 0.08^{a}	0.41 ± 0.05^{a}	0.43 ± 0.05^{b}
Total n-6 PUFA	40.60 ± 0.67^{c}	38.90 ± 0.25^{b}	34.98 ± 0.24^{a}
Total PUFA	49.20 ± 0.83^{c}	45.75 ± 0.34^{b}	41.26 ± 0.33^{a}

DISCUSSION

The partially replacement of fishmeal using hermetia meal was aimed to assess the potential of direct use of hermetia meal and to reduce the use of fishmeal dependent in aquafeed. Indeed many studies have been done to reduce fishmeal inclusion in aquafeed. In this study, African catfish fingerling can tolerate up to 25% of hermetia meal in the diet (1HM:2FM) to reduce the usage of fishmeal protein without compromising the growth performance of the fish. In this study, hermetia meal used was not undergo lipid removal and the chitin residue may exist naturally in the feed. Shiau & Yu, (1999) reported that hermetia meal contains 20 to 100.0 g kg^{-1}, dry matter amount of chitin in which could interfere with

digestibility and nutrient absorption. Study conducted by Kroeckel et al., (2012) supports this reporting that the presence of chitin may have decreased feed intake and thus decreased growth efficiency.

Li et al., (2017) stated that up to 100% fishmeal can be substituted for defatted hermetia meal in diets for Jian carp with no adverse effect on performance growth. However, in grass carp, 50% dietary inclusion is the most highest level can be tolerate by the fish without adverse effect (Lu et al., 2020). Another study also found that dietary replacement fishmeal with hermetia meal did not result in the growth performance difference in Atlantic salmon. Defatting hermetia larvae would lead to meals with higher protein values, than those commonly found in soybean meals (Veldkamp and Bosch, 2015). According to Tschimer & Simon (2015), these varied findings may be attributed to variation in insect species, insect substrates and insect processing, fish size and fish species.

Based on this experiment, at the end of the feeding trial, weight gain and specific growth rate (SGR) was higher in fish feed with 25% hermetia meal (1HM:2FM) replacement compared to other diets. Supplying more than 25% of hermetia meal in the diet reduced the growth of African catfish and in this study the lowest weight gain displayed by fish fed with 50% replacement. Although the growth of African catfish fed with 50% hermetia meal (1HM:1FM) was the lowest compared to other diets, there were no signs of nutritional shortage or higher mortality. A similar observation was also reported in rainbow trout where 50% hermetia meal was considered as an inclusion but feeding the fish with 75% of hermetia meal did not cause negative impact on the fish weight gain (Stamer et al., 2014). In addition to that, protein content in muscle increased in fish fed hermetia meal compared to controls. Although it is assumed that an increase in muscle protein is associated with an increase in dietary protein content (Ng et al., 2001), there has been no report of muscle protein content being altered by different protein sources. In contrast to protein, lipid content was higher in fish fed 25% BSFL, which could be because the amount of food ingested was higher than in fish fed other diets. The amount of food ingested was associated with the muscle lipid content of the fish (Noeske-Hallin et al., 1985).

The fatty acid content of the fish appears to reflect the fatty acid profile of the diet, with fish fed fishmeal having a higher content of LC-PUFA, which decreased with increasing intake of hermetia meal. A similar observation was also note in Atlantic salmon when dietary hermetia meal has been increased (Bruni et al., 2020). Hermetia meal contains a high proportion of saturated fatty acids (Ewald et al., 2020), possibly due to the larval food source, palm kernel cake (PKC). The hermetia meal used in this study was commercially produced and the pupae were fed PKC. This is a common practise because hermetia larvae are also a bioconverter that converts waste into higher value products. Malaysia is one of the world's palm oil producers and using PKC to make hermetia meal is one of the ways to contribute to sustainability. In the case of African catfish, the change in fatty acid profile due to the hermetia meal is not critical to the market value as the fish is consumed as an affordable source of protein and not for its fatty acid content like marine fish.

CONCLUSION

African catfish can tolerate a higher admixture of fishmeal in the diet. This reduces the use of fishmeal as the main protein source. The change in the fatty acid profile in the fish with increasing admixture of hermetia meal reflects the feed offered and should not cause negative affect the quality of the fish and its value.

Patents

Not applicable.

Author Contribution

For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, N.K.A.H, Z.A.K; methodology, N.A.M.A., L.K.C., A.J.R; software, N.A.M.A.; validation, N.K.A.H., A.J.R. and.; formal analysis, N.K.A.H., A.J.R., N.A.M.A., L.K.C; investigation, N.A.M.A., L.K.C.; resources, N.K.A.H., H.A.H; data curation, N.A.M.A., A.J.R., N.K.A.H ; writing—original draft preparation, NKAH.; writing—review and editing,

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The larval development of the Asian clam, *Corbicula fluminea* in the hatchery

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Abstract: The larval stage is given high priority in seed production, which is an essential aspect of any farming practice. Induced breeding was carried out by integrating temperature, salinity, and gametes suspension. Individuals from spawning broodstocks were sacrificed to observe larval development at an early stage. The released larvae (veliger) development was monitored until they settled on the substrate. As a result, three developmental stages have been identified: marsupial, planktotrophic, and benthic. The marsupial stage lasts until the pediveliger (D-shaped with velum and developed ciliated foot) is brooded in the innerdemibranches. The planktotrophic stage begins when the broodstocks release the first straight-hinged veliger (D-shape). Finally, the juveniles reach the benthic stage when they start to settle at the bottom of the culture chamber. Before metamorphosing into planktonic juveniles, the veligers were nurtured for about a month. Internal organs such as double-looped gills, digestive, mantle, foot, and prominent shell ridges developed and matured in planktonic juveniles. Therefore, the documentation of *C.fluminea* larval revealed significant development stages for seed production in the hatchery conditions.

INTRODUCTION

The *Corbicula* was captured and consumed as food in Asia, and it was considered one of the most economically important aquatic species (Korniushin & Glaubrecht, 2003; Wang, Zhang, Zhang, Li, & Xiao, 2014). Instead of *C.sandai* and *C.leana*, the *Corbicula fluminea* is the most prominent species of the genus *Corbicula* that lives in freshwater and is found throughout this region (Ramli, Ayyapan, Yusoff, Eh Rak, & Lee, 2020). Currently, the clam has been overfished, particularly in several countries such as China (Zhu *et al.,* 2018), Japan (Okawa, Kurita, Kanno, Koyama, & Onikura, 2016), and Malaysia (Yusof, Sow, Ramli, Rak, & Wei, 2020). This species was solely harvested from the natural habitat to cater to the market demands. For instance, the total annual catch was 12 MT in East Asian countries (Chen *et al.,* 2013), whereas the earlier record was 20 MT per annum. However, relying solely on wild stocks has led to drastic depreciation instead of water pollution and habitat destruction. There is no specific record of *C.fluminea* being captured in Malaysia, although their current presence on market shelves was gained speculatively from Thailand and Cambodia. The market price of clams has escalated dramatically due to

this status quo. This continuous occurrence has driven scientists to develop seeds to fulfil market demand through aquaculture.

Given these circumstances, the clam industry needs new sources in addition to the hatchery-produced seeds that look to be a feasible alternative. However, a lack of knowledge on larval production, growth, and development hindered the hatchery's capacity to produce this clam. The *Corbicula fluminea* is a well-known monoecious species that fertilises internally and broods its larvae in innerdemibranches, with the ability to produce over 10 million embryos. (Glaubrecht, Fehér, & Köhler, 2007; Okawa *et al.,* 2016). Previously, two techniques for producing clam seed were advocated: collecting from gravid broodstocks in natural habitats or using a hatchery system (Aji, 2011). Furthermore, the previous study emphasised the laboratory-bred, in which shelled larvae were acquired by dissecting wild clams in the laboratory and kept in laboratory conditions. (Nichols & Black, 1994). On the other hand, field-caught and continually grown in the hatchery is a typical method of obtaining shelled larvae.

Consequently, producing seeds could be a viable option for discovering a different source of *C. fluminea*. Attempts to artificially produce *C.fluminea* seeds in laboratory conditions have been made to date (King *et al.,* 1986). On the other hand, this study was performed to explore the early development of larvae in laboratory culture. Other freshwater bivalves, such as *Margaritifera spp*. have been extensively cultivated and reproduced in the laboratory and hatchery, but no *Corbicula* species has. (Hastie & Young, 2000; Preston *et al.,* 2007; Kovitvadhi *et al.,* 2008). In brief, external influences induced the fertilisation and spawning of hatchery-produced bivalves. As a result of the early study, three parameters were discovered to be significant: temperature, salinity, and feed supply. Therefore, this research discusses broodstock husbandry, induced breeding procedures, spawning, larval development of *C.fluminea* juveniles in a hatchery.

MATERIALS AND METHODS

Collection of the Corbicula fluminea.

The *C.fluminea* were obtained from the Pergau Lake, Jeli, Kelantan, Malaysia (5°37'13.4" N, 101°42'11.5" E). This lake is the largest source of *C.fluminea* for the local market, and a dredger (1.1m x 0.9m x 0.3m) was dragged from the boat to the permitted area (Rak *et al.*, 2021). Then, the clams with more than 20 mm were separated from the clutch and placed in a cold box to prevent stress or spontaneous spawning. Finally, they were transported to the Aquaculture Laboratory, Universiti Malaysia Kelantan, Jeli Campus. The clam's unwanted sizes (<10 mm) were released into the lake.

Broodstocks conditioning

The freshwater was gradually introduced into the box until achieving the equilibrium temperature. Then, 1000 broodstocks of *C.fluminea* were cleaned and placed in the recirculating aquaculture system (RAS) tank (700L) with supplemented sediment (fine sand), continuous, and aeration for eight weeks. The size is varied due to the sexual maturity of the clams being independent of the size. The broodstocks were fed by the diluted microalgae *Nannochloropsis sp*. (Reed Mariculture Inc, USA) and were continuously supplied at a density of 15 x 10^6 cellsmL^{-1} through a modified feeder (5 L). The microalgae volume was adjusted by observing the consumption through daily counts of the algal residual in the Neubauer chamber (Lima *et al.,* 2011). The dead clams were removed, the condition index (CI) was monitored, and the breeding induction was carried out when the CI value was at a range of 3.0-4.0 or above (Rahim, Idris, Kamal, Wong, & Arshad, 2012).

Breeding inductions

The *C.fluminea* (n=10) were randomly selected and sacrificed to obtain the gametes. Extracted gametes comprised of spermatozoa and ova. The gonads area was dissected under a microscope and ground using plastic micro mortar in the centrifuge tube (15 ml) with distilled water. The pulverised gonads are used for inseminating other broodstocks. Then, the gametes were diluted in the distilled water at 1 x 10^3 to 10^5 spermatozoa/ml. The concentration of the gametes was determined using a haematocytometer and observed under a light microscope (Leica, USA). Five hundred broodstocks were maintained in breeding tanks (10 L) with eight L adjusted water and without substrate (duplicate). Salinity adjustment to eight parts per million (ppm) using dechlorinated water or sodium chloride (NaCl), and the salinity was measured with a portable refractometer. The temperature was gradually increased from 27.0°C to 32.0°C, controlled by a water heater (DoPhin Heater 100W, China). The diluted microalgae

Nannochloropsis sp. was pipetted onto the broodstocks in the breeding tanks, stimulating them to extend their siphons. Then, the pulverised gonads were sieved using a 60 µM sieve, and 10 mL pipetted thoroughly to their extended siphons. The spawning induction was repeated every 48 h since the changes in turbidity of the water could not be the primary indicator of success (Baba *et al.,* 1999). The broodstocks spawning activities and responses were observed. However, the sperms or veliger releasing could not be seen through the naked-eyed. Every seven days after the induction process, the tank water was sequentially filtered using 150 µm, 120 µm,100 µm, and 60 µm plankton net to catch the released veliger and juveniles. They were harvested five times after induction was carried out.

Spawning

The spawning activity of the *C.fluminea,* such as releasing sperms and eggs were not viable since the fertilisation occurred inside the parent body. Furthermore, this freshwater clam incubated its embryos inside the brood chambers in the gills for two weeks up to a month (Rajagopal *et al.,* 2000). The spawning of *C.fluminea* was defined when the broodstocks released the pediveliger (usually in marsupial) or early juvenile (Rajagopal *et al.,* 2000). A recent study scarcely found pediveliger while early juveniles were abundant in the breeding tank.

Larval rearing and development

Great care during dissection was taken to preserve the integrity of the mantle and visceral mass. The dissection was conducted under the dissecting microscope (Leica, USA). The live clam was gently forced apart by scalpel and holding while iridectomy scissors were used to cut the adductor muscle that holds the mantle lobes. Then, the left shell valve was removed, and the detached mantle lobe was lowered onto the visceral mass. The left mantle lobe was pulled back to examine the clam gills, exposing the gills and visceral mass. Embryo or larvae were observed *in situ* before both sides' gills were removed by incised using iridectomy scissors. After the gills were removed, a small incision was carried out on the exposed visceral mass. The covering epithelium layer was gently pulled back, which exposed the superficially located oogenic and spermatogenic follicles. Usually, the follicles were whitish and finely granular masses. The follicles were carefully removed from several locations on the visceral mass to avoid contamination and smeared on the glass slide. Then, smeared tissues were examined under light microscopes (Leica, USA). Further examination was carried out on the four removal gills. Typically, the embryos are incubated in the inner demibranch (second layer gills). Therefore, it was placed on a glass slide with a few drops of distilled water. The incubated embryos were gently teased and freed from the marsupial gill. The stages development of embryos was determined as follows: (a) no embryo present; (b) blastula; (c) gastrula; (d) trochophore; (e) veliger; (f) pediveliger; (g) early straight-hinged juvenile and (h) straight-hinged juvenile (Kraemer & Galloway, 1986). The removal of brooded veliger could not be survived in the conditioned water (culture water). As a result, the larval development examination was initiated after the early straight-hinged juvenile was discharged from the broodstocks. The released larvae were reared in the closed recirculating aquaculture system (RAS) supplemented with the substrate (125-250µm) and fed with the commercial microalgae, *Nanochloropsis sp.* at density 1-2 x 10^6 cells/ml. Images determined the development of larvae, captured using a compound camera microscope (Leica, USA). This study determined three stages of life: marsupial, planktotrophic, and benthic stages.

RESULTS

Spawning and Larval Development of Corbicula fluminea

Spawning (release gametes) events were intricated to determine since the occurrence is unnoticeable. However, the extracted gametes (sperms and eggs) from the mature broodstocks (Fig.1) were the sperms found in biflagellate. The oogenesis and spermatogenesis were found in all evaluated broodstocks. The sperms (S) were approximately in range 12-16 µm linear distance from end to end and biflagellate (Fig. 1). These male gametes can be found in all dissected broodstocks. Meanwhile, the blastulae abundantly found were removed from the innerdemibranches of the sacrificed broodstocks in the form of a gelatinous envelope (GE) and suspended in water (Fig. 2). The presence of blastulae reduced the sperm's existence in the sample. However, this observation was found not in all dissected broodstocks.

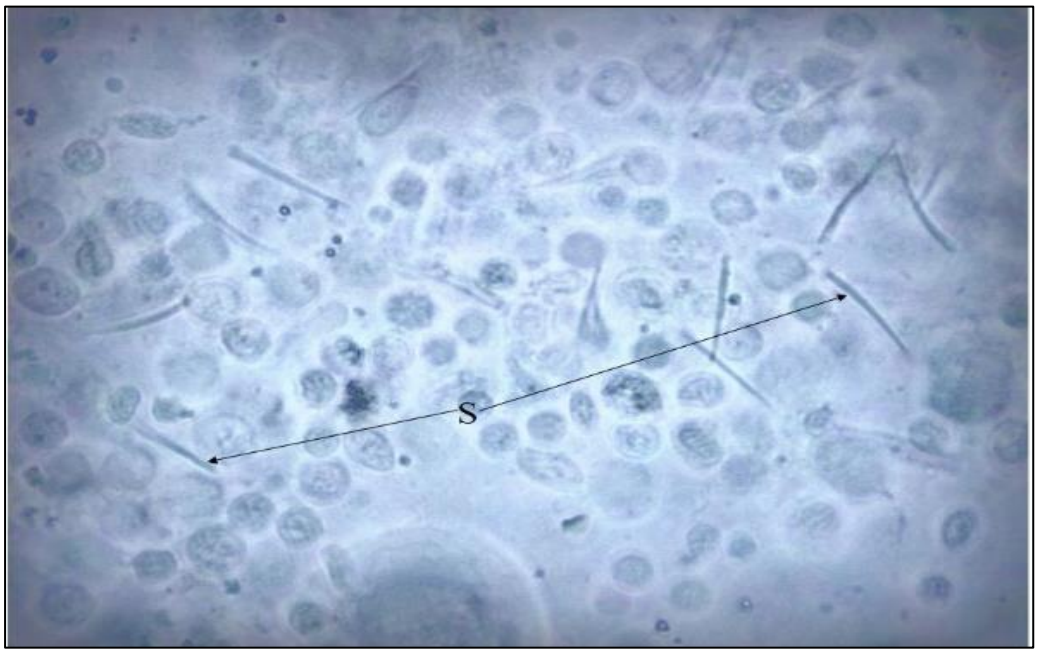

Figure 1. *Corbicula fluminea* sperms extracted from the gonad (400x magnification). S=Sperm

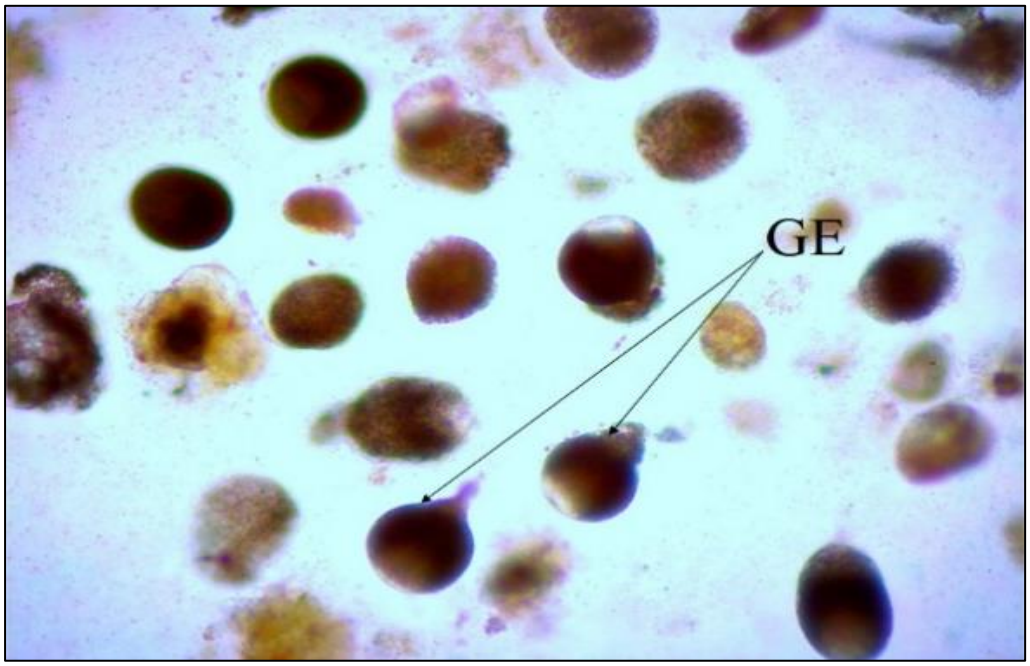

Figure 2. Gonad smear showing abundant blastulae (400x magnification). GE= Gelatinous envelope

The sperms (S) were approximately in range 12-16 µm linear distance from end to end and biflagellate (Fig. 1). These male gametes can be found in all dissected broodstocks. Meanwhile, the blastulae abundantly found were removed from the innerdemibranches of the sacrificed broodstocks in the form of a gelatinous envelope (GE) and suspended in water (Fig. 2). The presence of blastulae reduced the sperm's existence in the sample. However, this observation was found not in all dissected broodstocks.

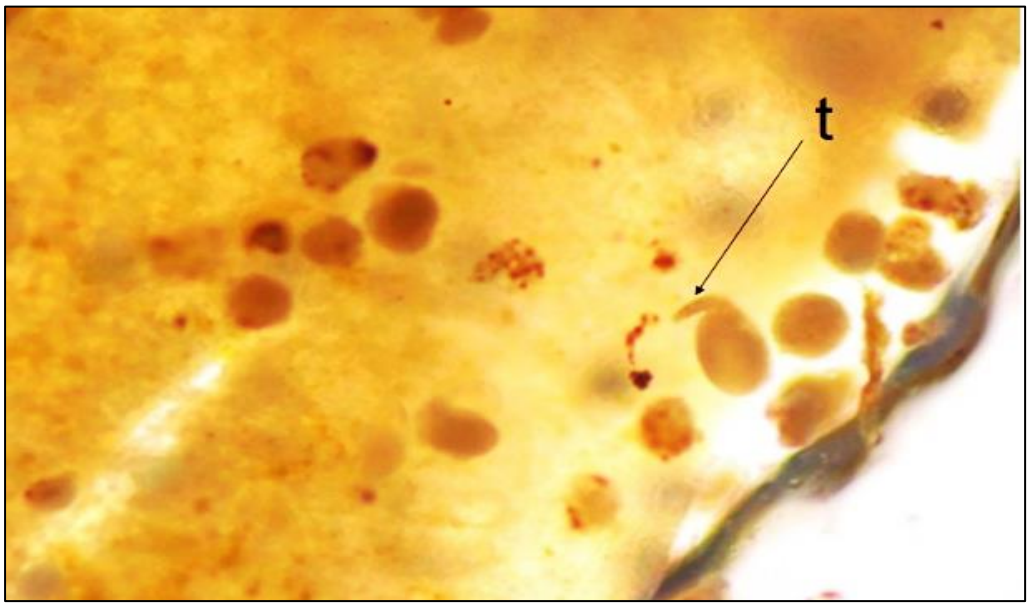

Figure3. Trochophore larva of *Corbicula fluminea* developed in innerdemibranch and apical tut (t) (400x magnification).

Early trochophore larvae developed after 14 h (Fig. 3). Cilia were not visible at 14 h on trochophores removed from parental gills, liberated from the gelatinous envelope, and suspended in water. However, particles moving in currents around the larvae were observed. After 17 h, tiny cilia covering the apical surface were visible, and by 18 h, the larvae's whole surface was covered with cilia. After that, trochophores were motionless while retained on the innerdemibranch. After 20 h, trochophores formed an apical ciliary tuft (t), which looked like a spike (Fig. 3).

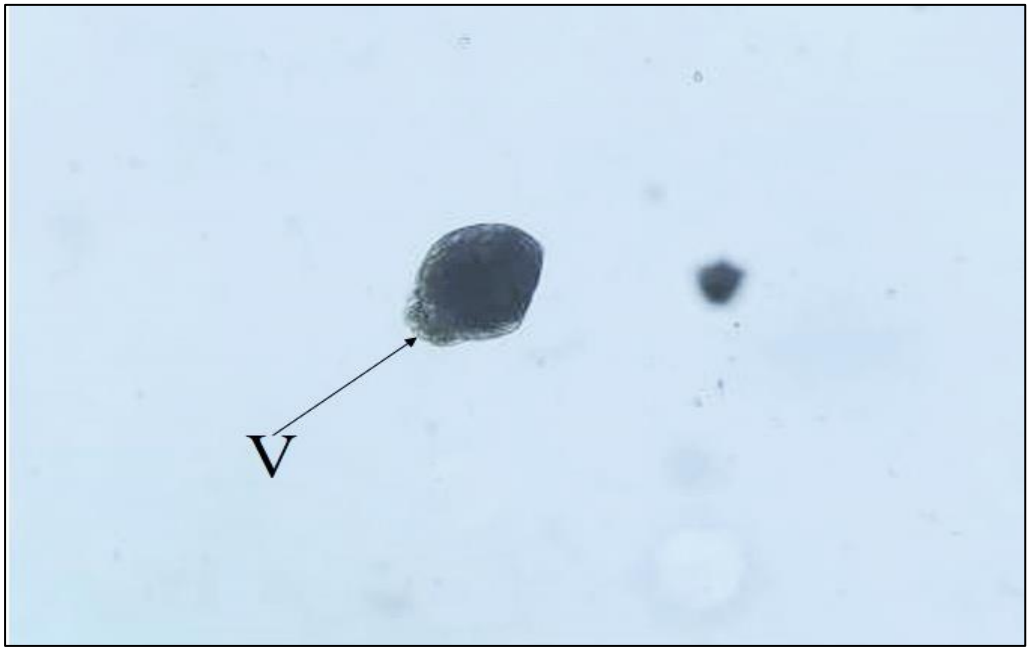

Figure 4. The early straight-hinged (veliger) larva of *Corbicula fluminea* 36 h after spawning bearing a velum (V) (400x magnification).

Early straight-hinged larvae (veliger) were first observed at 42 h after spawning (Fig. 4). At this stage, the larvae are immotile, retained in the gills. In this study, the early straight-hinged larvae were flushed out from the gills of sacrificed broodstocks and suspended in the water. The velum of the D-shaped larvae was seen to be extended (Fig. 4). There was little movement detected, and the veliger could not move against the current. This veliger uses the cilia on the velum to trap microalgae cells. Inner organs were not visible at this planktotrophic stage, and the velum was expected to shed. However, all

veliger were not survived, suggesting that the planktotrophic is the immature larvae that withstand outside conditions.

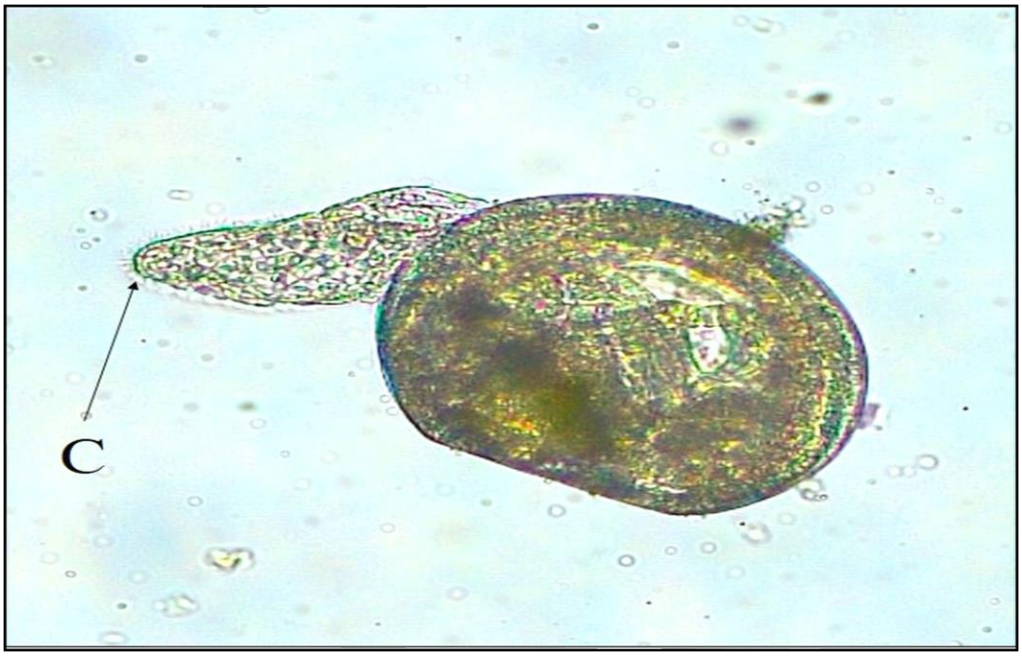

Figure 5. Late straight-hinged juveniles (100 x magnification). C= cilia

Late straight-hinged juveniles were found as first released by the broodstocks after spawning (Fig. 5). At this stage, the late straight-hinged juveniles bearing a large ciliated foot (C) were found crawling at the bottom of the observation disc. The vela, which sheds during metamorphosis, is missing in late straight-hinged juveniles. Gills and other internal organs were observable at this stage. The broad ciliated foot is utilised to crawl towards the feed particles while anchoring against the water current. During the observation, the late straight-hinged juveniles are actively crawling.

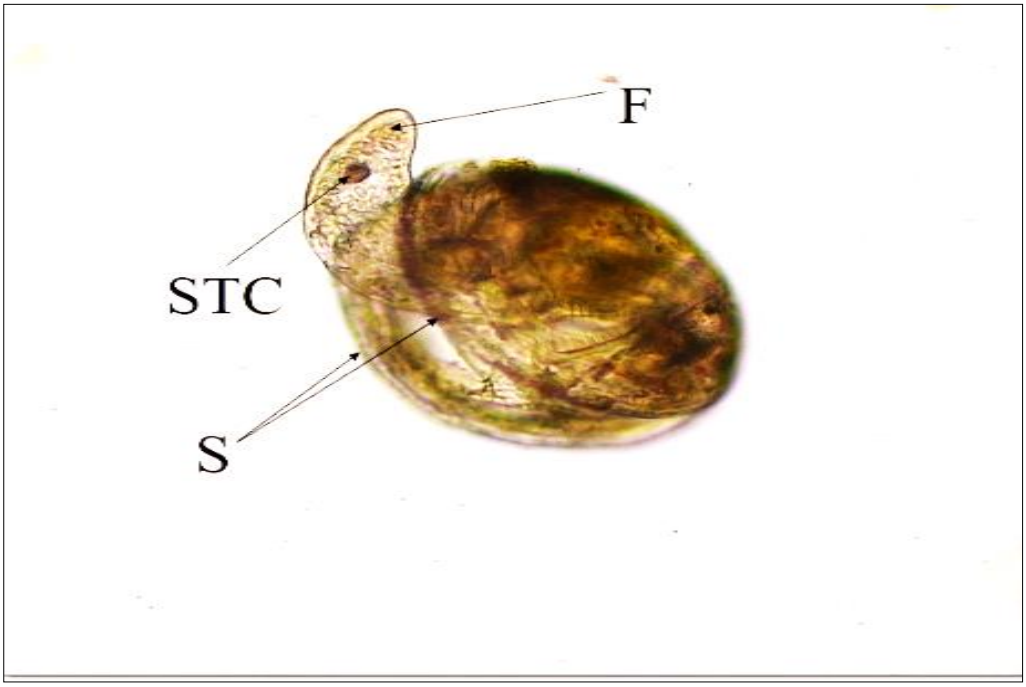

Figure 6. Late straight-hinged juveniles showing conspicuous statocyst (100x magnification). F=foot, STC=Statocyst, S=shells.

The juvenile of *C.fluminea* grown in the rearing chamber is shown in Fig. 6. The juveniles' movement is characterised by the opening of their feet (F) and shells (S). The statocyst (STC) is visible on the juvenile's foot. The STC aids the juveniles to stay oriented and balanced during swimming. The

STC acts as a sensory receptor, allowing the foot to approach the feed particles. At this point, microscopic villi can still be seen, and the ingestion feed mechanism is similar to that of late straight-hinged juveniles. The juvenile is sensitive to the stimulus. Due to the high-water current-like suction, the juveniles' shells closed for a while, and they drifted in the suction direction. This mechanism explains the easy dispersion in the natural water bodies.

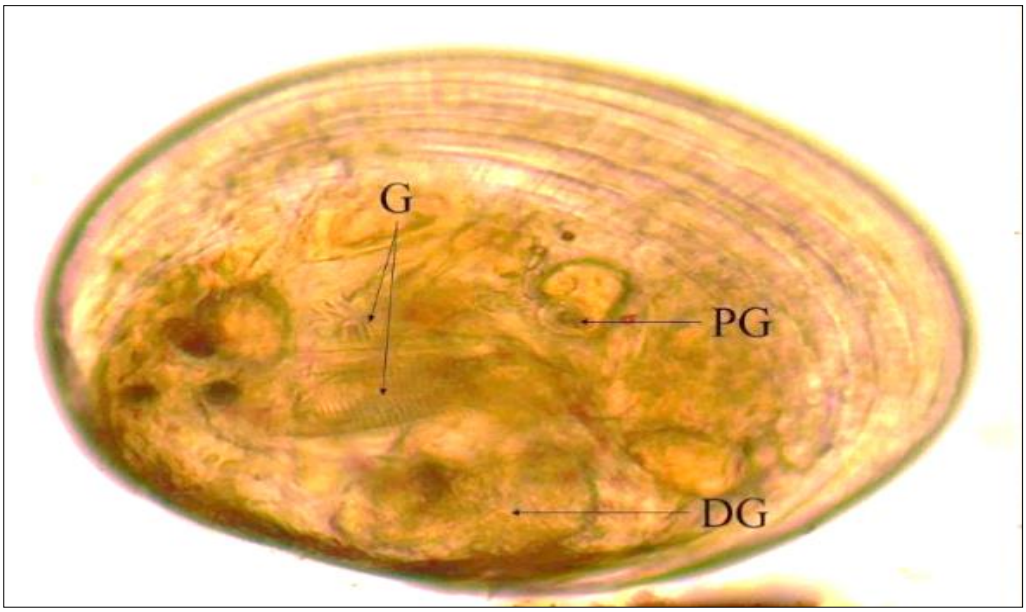

Figure 7. Late juvenile showing conspicuous double-looped gills, pedal ganglion, and digestive organs (40x magnification). G=Gills, PG=Pedal ganglion, DG=Digestive.

The late juvenile of *C.fluminea* grown approximately two months showed the shell ridges. This juvenile has well-developed a digestive system (DG) and respiration organ (G=gills). In Fig. 7, pedal ganglion (PG) could be seen under transparent shells responsible for the foot movement. Internal organs such as gills and digestive organs, and shell ridges become visible at this phase. Late juveniles are less active than earlier stages and rarely extend their foot to move. Late juveniles allow water to enter the mantle by opening the shell valves. The labial palps use this mechanism to catch and filter the feed particles for ingestion. The aperture frequently excretes pseudofaeces.

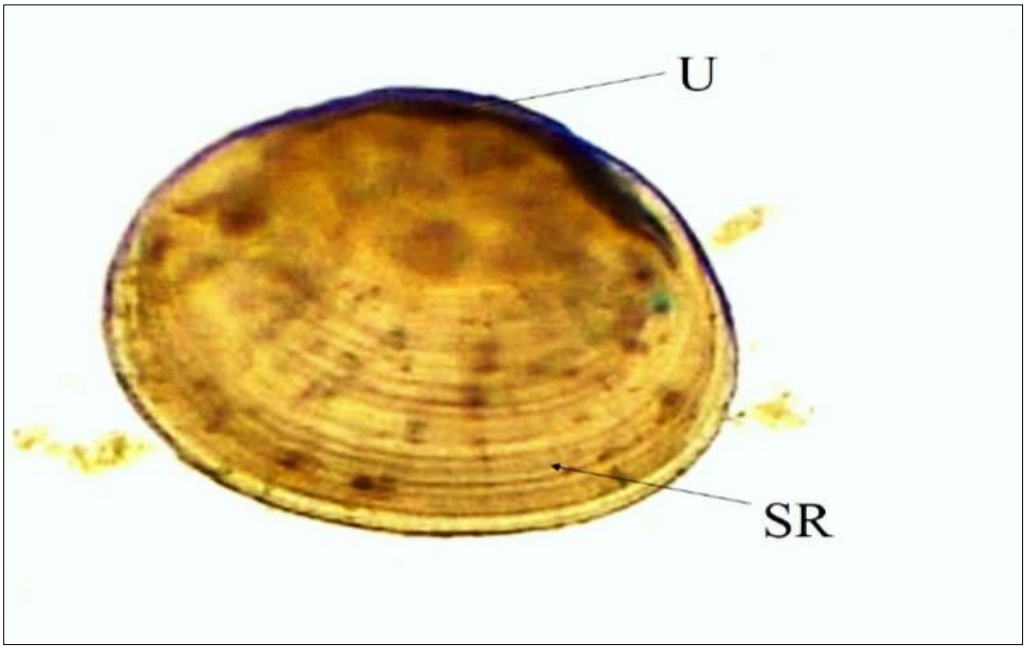

Figure 8. Umbonal juveniles of *Corbicula fluminea* (40x magnification).

The calcified shell of the juvenile is impervious to observe the organs inside. This umbonal juvenile embedded themselves in the sediment. Fig. 8 clearly shows the shell ridges (SR) and umbon (U) of the shell. At this stage, the juveniles rarely extend their foot to move. During the observation, the juvenile

remained still and closed the valves. The movement activity of the juveniles gradually decreased as their size approached one mm. The juveniles easily flow with the water current when there is no substrate. The juveniles died as a result of extensive valve closure.

DISCUSSION

Hatchery-produced seeds were successfully produced by inducing the rearing broodstocks by integrating temperature, salinity, and gamete suspension. This methodology was adopted from the natural conditions where the temperature and salinity cued the fertilisation and spawning of the *Corbicula* (Kimura, Soutome, & Sekiguchi, 2004; Rajagopal *et al.,* 2000). In the development history of *ex-situ*-produced seeds, King *et al.* (1986) employed temperature and salinity as the breeding parameters for *C.fluminea* reproduced in laboratory conditions. These two natural physical parameters critically affect the aquatic animal. These parameters influence the ingestion rates, feeding, respiration, metabolic activity, growth, gametogenesis, and reproduction (Xiao *et al.,* 2014). A present study employing salinity in range 0-8 ppt which the *C.fluminea* is tolerated to that range. Other studies advocate the salinity tolerance for this freshwater species as high as 24 ppt (Aji, 2011.). Recent observation shows the *C.fluminea* remains cessation and lack activities as the increasing of salinities over the range. This indicates the salinity limiting the distribution of the *C.fluminea* in brackish areas. Similarly, the salinity was found to affect the physiological process of the estuarine species, such as survival, osmolarity of the hemolymph, tissue water content, and have other sublethal effects (Xiao *et al.,* 2014; Matsuda *et al.,* 2008). On the other hand, additional gamete suspension into the breeding tank escalated the fertilisation time in a recent study. The reproductive biology of *C.fluminea* classified them as hermaphrodites, with mature sperms found in any individual, as shown in Fig.1. Though, in a more recent finding, *C.fluminea* was identified as hermaphrodites where the androgenesis dominated the reproductive biology (Komaru, Yamada, & Houki, 2013). Therefore, results from these studies were varied due to different periods of examination like in a recent study, and all the sacrificed individuals contained matured sperms and eggs.

The previous study had integrated temperature, mechanical, and salinity shocks to stimulate the spawning of the *C.fluminea* in laboratory conditions (King *et al.,* 1986). Sperms and eggs of the *Corbicula* in different degrees of detail. For instance, the sperms of the *C.fluminea* were found comprised of the conical-headed with biflagellate (Gomes *et al.,* 2016; Lee, Siripattrawan, Foighil, & Ituarte, 2005). Meanwhile, in the earlier study, King *et al.* (1986) reported that the American *Corbicula* produced sperms with a spherical head diameter of two µm and bear a single flagellum around 15 µm in length. In a recent study, the sperms were biflagellate and conical-headed, where the length is estimated in the range of 15-18 µm. On the other hand, several reports on the size of *Corbicula* eggs vary from 20 µm to 280 µm (Kimura *et al.,* 2004; King *et al.,* 1986). Unfortunately, all the sacrificed clams (n=10) contained small numbers of rounded eggs during the experiment, estimated in the range of 95-120 µm. Kimura *et al.* (2004) described the eggs released by *C.japonica* was found in spherical with a diameter of 103 µm. During the examination, the gamete sizes and shape variation could be due to species, environmental factors, or different gametogenesis stages. On the other hand, energy supply during gametogenesis is also responsible for determining egg size. In this case, food availability significantly contributes to carbohydrates and lipid as energy sources during gametogenic development.

The fertilisation inside the broodstocks was intricating the observation in early cell division. In an attempt to observe the cell division, a recent finding found the early embryo encased with the gelatinous envelope (GE). It is abundantly extracted from the innerdemibranches of the broodstocks (Fig.2). According to previous work, the brooded embryos were estimated seven h after fertilisation (King *et al.,* 1986). The time sequence of development stages varied, overlapping consecutive stages among the evaluated individuals. Gonads' maturity and brooding period vary, possibly affecting the development stages of the embryo observed recently. Several studies described the embryo of *C.fluminea* developed into trochophore larvae which were estimated around 14 h after fertilisation occurred (King *et al.,* 1986; Kraemer & Galloway, 1986). In a recent study, the trochophore larvae were found 24 to 48 h after fertilisation, bearing a flagellum-like, namely apical tuft (t) projected from the gelatinous envelope (GE) (Fig. 3) which coinciding with the photomicrograph taken by King *et al.* (1986). This apical tuft (t) facilitates the larvae to swim and acts as a sensory function directing the larvae to feed. Further details, the apical tuft is composed of cilia that joint together and remain until reached the pediveliger stage (Britton & Morton, 1982). The apical tuft is significant to the species that released the planktonic larvae without brooding period, usually found in estuarine bivalves (Morton, 1982). Thus, for brooded species such as *C.fluminea,* the trochophore larvae grew inside as marsupial larvae.

During the examination of the broodstock innerdemibranches, the marsupial larvae were teased out from the brooding chambers, and various stages of development were found. For instance, the veliger (Fig. 4) was observed bearing a velum. At this stage, the veliger could not use the velum for swimming, and they were scarcely being found in the water. The premature larvae were observed recently, which indicates the aborted brood due to environmental stress. The newly released juveniles are considered in the planktotrophic stage. The juveniles bearing a ciliated foot and anatomically rounded shells allow them to be well adapted on substrate and water current (Fig. 5). The juvenile was actively moving at this stage, foraging the food using its foot. By locomotion, the juvenile elongates the foot while the cilia around the foot sense and attracts the food particles. Then, the foot is contracted and pulled the shells in the direction. During this stage, the juveniles grew significantly but had low survival during the first 20 d after the juveniles were detected. In a recent study, the poor rearing conditions such as pseudofeces particles attached to the juvenile were not easy to clean due to indistinguishable sizes.

As the juvenile grows, the dark spot on the body and foot, such as statocyst (STC), a bead-like structure, emerges (Fig.6). The statocyst consists of a small sac with sensory cilia that detects the movement of mineral mass (Mackie & Claudi, 2009). In this study, the juveniles move toward and capture the microalgae at their ciliated foot. Besides, the statocyst is significant for maintaining balance, correcting the orientation and sense. It is positioned in the distal semi or the proximal third of the foot. Statocysts are positioned differently as the foot lengthens and differentiates (King *et al.,* 1986). Meanwhile, during the late juvenile stage, internal organs such as gills (G), digestive organs (DG), and pedal ganglion (PG) are observed under the light microscope (Fig.7). The pedal ganglion is a chemoreceptive sense organ (osphradium) connected to the cerebropleural ganglia via nerve fibres that control the foot movement (Mackie & Claudi, 2009).

Furthermore, juveniles at this size could be seen by naked eyes, and they are moving in the water like scattered "tiny whitish dots". In a recent study, these juveniles were transferred into the sand substrates chamber for further growing. The juveniles grew better in substrate conditions compared with none. However, monitoring the growth is complicated due to indistinct sizes between the juvenile and sands grains. The internal organ of the juveniles could not be seen due to the calcified shells impeding the observation while distinct umbonal could be observed (Fig. 8). On the other hand, clear ridges on the shells were observed, and they were inactive once they were settled in the benthic area. Hence, the growth of the juveniles was found at slow rates for growing and settled calcified juveniles (Fig. 8). The time estimation of the sequence of the developmental stages is based on the first observation of each developmental stage. However, there were consecutive stages that overlapped. Developmental time may vary due to water temperature, feed availability, and time-released from the brooder.

CONCLUSION

In conclusion, the present finding discovered the marsupial, planktotrophic and benthic stages of *C.fluminea* seeds produced in captive rearing. The broodstocks were not synchronised spawned, although they were induced. Hence, the recent study would be a reference for propagating this freshwater bivalve

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First record of biological invasion stages of the Asian clam *Corbicula fluminea* (Müller, 1774) in the Lake Pergau, Kelantan, Malaysia

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Abstract: The present study presents the first record of Asian clam, *Corbicula fluminea* (Müller, 1774), in Lake Pergau, Kelantan, Malaysia. Currently, this freshwater bivalve was harvested at a density between 2.21-113.63 individuals per m^2. Population structure and the dates of the first records suggest that the introductions occurred in the year 2000.The *C.fluminea* existence in this lake indicates that human activity was introduced into the novel location and aided in dispersal rather than life strategy characteristics. Thus, the biological invasion stages and the importance of monitoring this species' invasion to forecast their dispersion into the upstream tributary in Kelantan.

INTRODUCTION

The Asian clam, *Corbicula fluminea* is freshwater bivalve species that natively inhabited freshwater of the South East Asia region, including Malaysia and became a daily snack in Kelantan (Yusof et al., 2020). It has a high ability to disperse in freshwater and has become an invasive species in certain continents, such as Europe and America (Ramli et al., 2020). The invasion success and subsequent dispersal of the *C.fluminea* due to its high fecundity and short life-span (r-strategy) highly associated with human activities (Schmidlin, 2011; Sousa et al., 2008).

Lake Pergau (4.6 km^2) is a man-made dam with a depth of 3-10 m, constructed in 1994 and completed in 2000, making it Kelantan's largest lake. It is located within a mountainous terrain with upstream flowing water as an input source. Previously, it was the primary source for *C.fluminea* in Kelantan (Rak et al., 2021). *Corbicula fluminea* was believed to have been accidentally introduced into the lake through sand deposited during dam construction. As a result, *C.fluminea* was introduced and established in the lake and upstream rivers. The biological invasion phases of *C.fluminea* in Pergau Lake

were determined in this paper, and significant further monitoring for invasion and saturation stages in this lake anticipate the dispersion into the upstream tributary. .

MATERIALS AND METHODS

A quantitative density survey was carried out in Lake Pergau, Jeli, Kelantan (Figure 1). The coordinates (n=10) in the lake were determined (Table 1) according to the Department of Fisheries (DOF) Kelantan and marked by using the Global Positioning System (GPS). However, due to safety concerns and benthic topography, only five locations could harvest the *C.fluminea*. The tow dredger (1.1m x 0.9m x0.3m) with the mesh at size five mm was used to obtain the *C.fluminea* from the lake benthic. For each point, a boat and rope pulled the dredger 100 m forward and backwards two times (Syed Omar et al., 2020). The clams were flushed in the lake water to retrieve. Then, the clams were kept in the zipper bags, stored in the icebox and counted in the aquaculture laboratory, Universiti Malaysia Kelantan. Mean population density (the number of live individuals per m^2) was estimated from the data. In addition, the shell length (SL) was measured to the nearest 0.1mm using a vernier calliper, and shell length-frequency distributions were demonstrated in a bar graph. The water parameters such as temperature (°C), dissolved oxygen (DO), and pH were recorded using a multiparameter (YSI, USA) (Table 2).

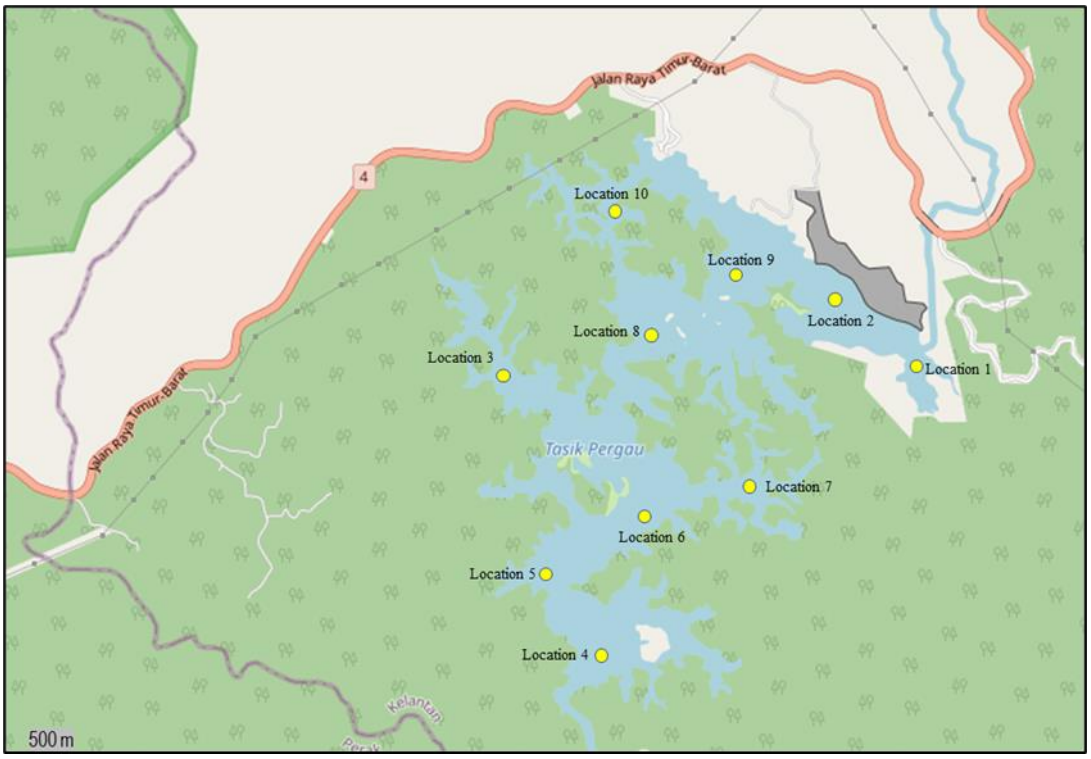

Figure 1. Sampling locations (n=10) in the Lake Pergau, Kelantan.

Table 1. Sampling locations and coordinates.

Sampling Locations	Coordinates
Location 1	5° 37' 8.4504" N, 101° 42' 18.9324" E
Location 2	5° 37' 23.5416" N, 101° 42' 2.9916" E
Location 3	5° 37' 5.3328" N, 101° 40' 52.9284" E
Location 4	5° 36' 9.9108" N, 101° 41' 12.7968" E
Location 5	5° 36' 29.1636" N, 101° 41' 3.9048" E

Location 6	5° 36' 41.9184" N, 101° 41' 23.2512" E
Location 7	5° 36' 44.2584" N, 101° 41' 40.4988" E
Location 8	5° 37' 15.2004" N, 101° 41' 24.2988" E
Location 9	5° 37' 12.6192" N, 101° 41' 43.6488" E
Location 10	5° 37' 44.868" N, 101° 41' 6.0036" E

Table 2. The water quality parameters of ten sampling locations in Lake Pergau

	Temperature (°C)	Dissolved Oxygen (ppm)	pH
Location 1	27.0	8.33	8.79
Location 2	27.9	8.65	8.58
Location 3	27.6	9.53	8.74
Location 4	22.9	8.52	8.51
Location 5	27.3	7.86	9.66
Location 6	27.5	8.59	10.2
Location 7	27.6	7.73	7.49
Location 8	27.6	8.51	8.61
Location 9	27.6	8.65	9.02
Location 10	27.4	8.58	9.01

RESULTS

A total of 1162 samples of *Corbicula fluminea* were caught in four sampling locations, absent in Location 3 (Figure 2). The collected clams were divided into five classes according to the shell length (SL): 5-7 mm; 8-10 mm; 11-13 mm; 14-16 mm; 17-20 mm (Figure 2). The highest density (individuals per m^2) of *C.fluminea* was recorded in Location 1 (113.63 individuals per m^2). The approximate age distribution of the clams in the lake is more than one year (66.61%), whereas the rest comprises one-year-old individuals (33.39%).

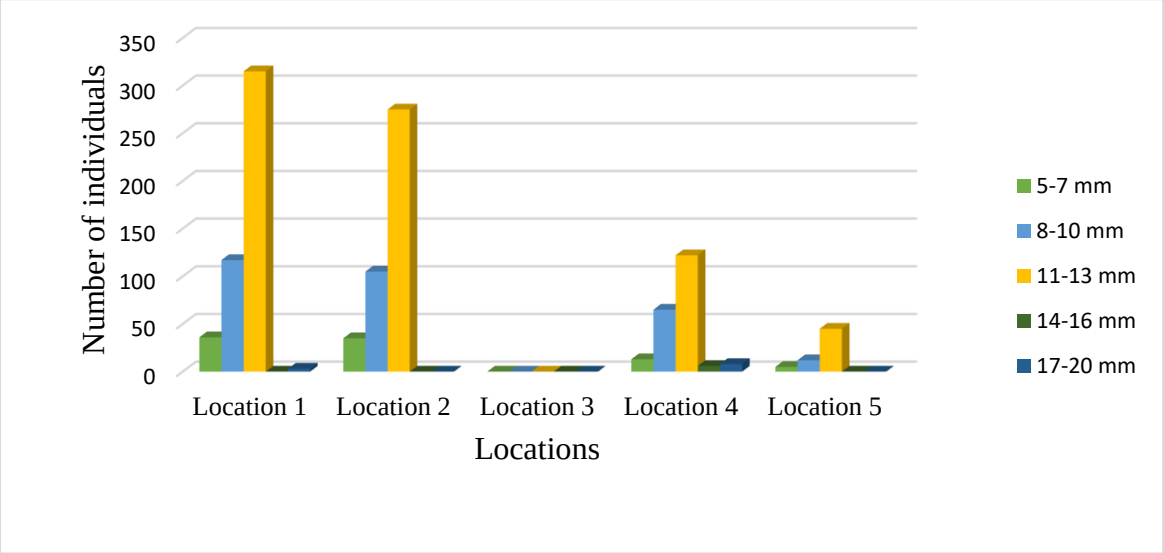

Figure 2. Size class distribution of *Corbicula fluminea* obtained from five sampling locations in Lake Pergau, Kelantan.

DISCUSSION

The *C.fluminea* reached maturity at 10 mm (Zeswita et al., 2016; Sousa et al., 2008), where 90.87% (1056 individuals) of the evaluated samples in the Lake Pergau were categorised as a mature clam. Based on these age estimates, density, and the date of first records, the biological invasion stages can be divided into four stages: introduction, establishment and adaptation, invasion, and saturation (Schmidlin, 2011). In history, the introduction of *C.fluminea* in Lake Pergau was circa 2000. A recent study found that *C.fluminea* is dispersed in all lake areas. However, during sampling work, the live *C.fluminea* was absent in Location 3 due to high siltation loads in that area. The highest population density was found in Locations 1 (Figure 2). This location was located adjacent to the turbine and wall of the dam. This finding elucidates that sand dumping activity in the lake happened in that area. The sands were obtained from the Kelantan River, which deposited *C.fluminea* seeds in the lake. In addition, the dam watergate was located in this area, and flowing water through the gate also carries drifted *C.fluminea* into this area. Since there are no scientific records on the *C.fluminea* in this lake, hence current study may become the first record where biological invasion stages were determined after almost 20 years of this freshwater bivalve introduction.

The density of the *C.fluminea* in Lake Pergau was recorded at 2.21-113.63 individuals per m^2. Compared to a previous study, the *C.fluminea* was more significant at 87-1249 individuals per m^2 in Lake Maggiore, Italy (Kamburska et al., 2013). The current report may be a reference to monitor the escalation density of *C.fluminea* in Lake Pergau in the future. For instance, the *C.fluminea* was found at low density (2-20 individuals per m^2) in 2002, but in 2008, the population density surged to 6000 individuals per m^2 within six years in Lake Tahoe (Wittmann et al., 2011). Meanwhile, 55 individuals per m^2 in Lake Seminole, USA, where the total population was estimated at 4.3 billion adults *C.fluminea* (Patrick et al., 2017). This rapid establishment and invasion in the lake altered organic matter cycling, decreased the phytoplankton, increased substrate for other species, altered biodiversity, and changed the water chemistry to toxic levels for other species (Wittman et al., 2011). Schmidlin (2011) emphasised that the establishment may take several decades, low impact on native biodiversity, and depend on life-history traits. With the current density in the lake and all considerations above-mentioned, it is appropriate to conclude that the *C.fluminea* is at the establishment and adaptation stages. Further study is needed to investigate the effect towards the native benthic fauna associated with *C.fluminea* in the lake.

On the other hand, the Pergau River (5 km) is the only river connected with this lake. No density of population structure data is available yet for the latter location. However, it existed at this location. The *C.fluminea* in this river has become an attraction for locals to collect this clam for food and ornamental purposes. Since the *C.fluminea* occurs in this river (upstream), it can be anticipated that this bivalve's existence in all its downstream tributaries in Kelantan. For example, Shannon River is an upstream connection with 105 km^2 Lough Ree Lake in Ireland. The spreading *C.fluminea* was introduced in 2006, and this species was found at a density of 400-750 individuals per m^2 in 2010 (Minchin & Boelens, 2018). Hence, the current study provides an insight view on the introduction of *C.fluminea* in the Pergau River and its tributaries.

In this study, only freshwater mussel, *Pilsbryoconcha exilis* was found together with the *C.fluminea* instead of the other gastropods. However, the population of this mussel was little compared to *C.fluminea.* Both species were endemic to Lake Pergau. The *C.fluminea* present in the lake does not foul native bivalves in the same way that the zebra mussel (*Dreissena polymorpha*) does, but still, it is competing for space and food (Pérez-Bote & Fernández, 2008). Therefore, the competition for basic needs probably endangered other bivalves or aquatic species in the vicinity.

CONCLUSION

Therefore, recent work disclosed the *C.fluminea* establishment in the Lake Pergau which potentially further invasion into the upstream tributaries. The need for extend monitor the dispersion in upstream tributaries in Kelantan should be noted.

Patents

Not applicable.

Author Contribution

Conceptualization, Zharif Ramli and Aweng Eh Rak.; methodology, Aweng Eh Rak.; software, Dee Koh Han.; validation, Zulhisyam Abdul Kari and Lee Seong Wei; formal analysis, Dee Koh Han; investigation, Zharif Ramli; resources, Aweng Eh Rak.; data curation, Faizuan Abdullah; writing—original draft preparation, Zharif Ramli.; writing—review and editing, Zulhisyam Abdul Kari.; supervision, Aweng Eh Rak, Faizuan Abdullah; project administration, Aweng Eh Rak.; funding acquisition, Lee Seong Wei. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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