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Optimization of Growth Conditions of *Hirudinea* sp.

Zulhisyam A.K,, A. Anwar Ismail and Ibrahim Che Omar

Department of Agro Industry, Faculty of Agro Industry and Natural Resources,
Universiti Malaysia Kelantan, Pengkalan Chepa, 16100, Kota Bharu, Kelantan.

Abstract: Leeches (Phylum: Annelida, Class: Hirudinea) are widely distributed all over the world in various habitats, such as freshwater, seas, desert, and oases (Gouda, 2006). In this study, the effect of light intensity, temperature and diet on the reproductive efficiency of *Hirudinea* sp. was examined with eight different conditions. After 3 months of culture, the number of cocoons produced was very significantly different among the different conditions (*p*=0.00). The average number of hatchlings per cocoon was significantly different ($p \leq 0.05$) where condition 1 gave the highest number (6.23±0.25), but hatching rate was not (*p*=0.354). The condition 5 produced the highest mortality of parent leeches (52±13.86%). The sizes of the cocoons were not significantly different among the treatments, with the condition 1 having the largest cocoon of 22.19±0.92mm and 13.26±0.07mm according to their length and diameter, respectively. The wet weight of cocoons was significantly different ($p \leq 0.05$) with the condition 1 producing the heaviest cocoons of 1.26±0.11g compared to condition 5 producing the lightest cocoons of 0.22±0.38g. The effect of diet (FT1: fresh eel blood and FT2: booster) on the growth and survivorship of the juvenile leeches was also studied. After 2 months of culture, the final body weight was significantly different among the treatments, with juveniles in the FT1 (fresh eel blood) had the highest final body weight (0.8893±0.012g). Percentage weight gain (WG) and specific growth rate (SGR) of the juveniles in the treatment FT2 (booster) were lowest with mean and standard deviation of 769.41±11.54% and 3.6±0.02%, respectively. Juveniles in the FT2 (fresh eel blood) treatment had the highest survival rate (93.33±5.77%).

Key words: *Hirudinea* sp., temperature, light intensity, survivorship, reproduction, growth

INTRODUCTION

Leeches are distributed all over the world in a variety of habitats; in freshwaters, seas, deserts, and oases. They are important components in food chains; as predators, vectors of parasites, preys of aquatic animals (Sawyer, 1986). They occur in habitats that range from terrestrial to aquatic (both marine and freshwater) environments and are found on all continents. Leech was used by toxicologists and pharmacologists as a convenient tool for various investigations (Mann, 1962; Herter, 1968; Sawyer, 1986) in the past when its natural resources were boundless. In recent years, some leech populations have declined dramatically due to over-exploitation for fishing bait and medicinal purposes (particularly in Europe and Asia), and due to pollution (Sawyer, 1981; Elliot and Tullett, 1984; Wells and Coombes, 1987; Petrauskiene, 2003; Trontelj and Utevsky, 2005).

Hirudinea sp. is a sanguivorous, freshwater leech, with a wide distribution in Southeast Asia, such as in southern China, the Philippines, Thailand, Vietnam and Malaysia. In Malaysia, these leeches are known as 'Lintah Kerbau' (406th Medical Lab. Special Report., 1968). Traditionally, leeches are widely used as a model animal in toxicological, physiological, neurobiological, biochemical, histological and many other studies (Mann, 1962; Flerov and Lapkina, 1976; Lapkina and Flerov, 1979; Sawyer, 1986; Lapkina, 1992; Huguet and Molinas, 1992, 1996; Blackshaw and Nicholls, 1995; Petrauskienė, 2001). There has been an increasing harvest of this species for medical purposes in the 20th century (Steiner *et al*., 1990; Electricwala *et al*., 1993; Singhal and Davies, 1996) and so is in Malaysia. In this country, it is not known or proven conclusively that the locally named Buffalo Leech is not of *H. manillensis*. Local taxonomists have not been able to identify the species used those for medical purposes and would rather refer to its genus only as *Hirudinea* sp.

During the reproductive process, parent leeches secrete cocoons that protect and often nurture the developing eggs during the critical stages of early development (Sawyer *et al*., 1981; Yang, 1996). Components of the cocoons are released from specialized glands situated within the clitellar sex segments, forming a sheath

Corresponding Author: Zulhisyam A.K, Department of Agro Industry, Faculty of Agro Industry and Natural Resources, Universiti Malaysia Kelantan, Pengkalan Chepa, 16100, Kota Bharu, Kelantan.
Email: mrzulhisyam@yahoo.com Tel: +6013-9115161

around the clitellum into which fertilized eggs are deposited. The cocoon membrane is then passed over the head and sealed at both ends forming "plugs" at either end (Mason *et.al*, 2004). Embryos are dependent upon cocoon fluid contained in hard-shelled cocoons, while embryos from membranous cocoons can develop independently of the cocoon (Marotta and Shain, 2007).

The breeding of leeches for medical purposes has bright commercial potential and of late many entrepreneurs have embarked on the farming of leeches. To meet the demand from clinical use, Chinese traditional medicine and other scientific research, there has been growing interest in culturing and breeding leeches in many countries (Yang, 1996; Trontelj and Utevsky, 2005). Several factors determine leech distribution in freshwater environments such as availability of food organisms; nature of the substrate; depth of water; presence of water currents; size and nature of the body of water; hardness and pH; temperature of the water; dissolved oxygen; siltation and turbidity; and salinity (Sawyer 1986). However, no study has investigated the factors affecting growth and production of leeches in the country. Particularly lacking is the effect of water, temperature, dissolved oxygen, pH, and light intensity on growth conditions of these leeches bred in a farm as well as the feeding requirements. The aim of the present study was to test the interactive effects of different temperatures, light intensities and diet on the reproductive performance of this leech species. In addition, the effect of different diets on juvenile growth and survivor rates were also studied.

MATERIAL AND METHODS

Origin of Broodstock:

Hirudinea sp. (Buffalo Leech) used in the study was provided by PT Dynamic Consultant Co., Kota Bharu, Kelantan. The leeches were cultured in concrete tanks (20 ×10 × 20 m) filled with non-chlorine water source which were from river, well and rain to a depth of 25 cm. The concrete tanks were divided to four compartments. Approximately 1000 leeches were cultured in every compartment. The water in the concrete tanks was not aerated and exposed to direct sun light. Water hyacinth was placed in the concrete tanks and the leeches were fed once on live eel blood every week and once with an artificial booster every month. Sand was placed in the concrete tank to a height of 12 cm. Before the start of the experiment, leeches were cultured for 1 week in an indoor aquarium filled with non-chlorine freshwater (30cm depth, 600L),aerated and 50% of the water changed once every 3 days. The temperature, pH and light intensity were maintained at 27.92 ± 6.62 °C, 6.8 ± 0.3 and 1000-1500 lx, respectively. Leeches were fed once on live eel blood and once with an artificial booster in the preceding week before the proper study was initiated.

Reproductive Performance or Efficiency:

The effect of temperature, light intensity and diet on reproductive efficiency of leeches was examined in a fully orthogonal experiment with three factors, each with two levels as presented in table 1.

Table 1: Different conditions tested on reproductive efficiency of leeches.

Treatments	Feeding		Light Intensity (lx)		Temperature (°C)	
Condition/Level	FT 1	FT 2	Low	High	Low	High
C_1	Eel blood	-	0	-	25-28	-
C_2	Eel blood	-	0	-	-	30-32
C_3	Eel blood	-	-	100-150	-	30-32
C_4	Eel blood	-	-	100-150	25-28	-
C_5	-	Booster	-	100-150	-	30-32
C_6	-	Booster	-	100-150	25-28	-
C_7	-	Booster	0	-	25-28	-
C_8	-	Booster	0	-	-	30-32

Each treatment consisted of three replicates with a total of 24 aquarium tanks (30 x 19 x 26 cm) with 25 leeches in each replicate tanks. Approximately 600 leeches were collected from the holding tank, and randomly placed into the assigned experimental aquariums. Soil to about 25 cm depth was provided as substrate in each aquarium. Over the three months period (January 1, 2010 to March 31, 2010), daily observations were made.

Experimental Methods:

Cocoon Deposition Number:

At the end of the experiment, all the cocoons deposited by the broodstock were collected and counted and the average deposition number of cocoons for each growth condition was calculated.

Hatching Number and Hatching Rate:

After the juveniles were released from the cocoons, they were collected and counted. The ratio of the numbers of juveniles to deposited cocoons was calculated for each growth condition to obtain the average hatching number.

During the experiment, the abortion rate was high and the number of dead cocoons (juveniles failed to hatch) were counted and recorded. The ratio of dead to the deposited cocoons was then calculated as the average hatching rate for each treatment.

Broodstock Mortality:

The number of dead broodstock leeches was recorded during the daily observation.

Cocoon Size and Wet Weight:

All cocoons from each replicate were collected and their length and diameter measured. The wet weight of each cocoon was taken before hatching occurred.

Data Analysis:

Statistical analyses were conducted using the software SPSS 17.0 (Statistical Program for Social Sciences 17.0) to test the difference among the growth conditions and any differences obtained were considered significant at $p \leq 0.05$. The cocoon deposition number of broodstock leeches, hatching number and hatching rate of cocoon, survivorship of parent leeches, cocoon size and wet weight were analyzed by one-way ANOVA and where this effect was significant; Duncan test was performed to compare the treatments.

Effect of Diet on Juvenile Growth:

Experimental Design:

Newly released juvenile leeches used in this experiment were cultured in the hatchery tanks for 3 days prior to the experiment. Two feeding treatments (FT1: fresh eel blood and FT2: booster) in three replicates per treatments with 20 juveniles per replicate were tested. The experiment lasted for 60 days. The indoor tanks used were the same size as that of the broodstock experiment (20 ×10 × 20m) and each was filled with non-chlorine water. The temperature, dissolved oxygen, pH, ammonia and light intensity were 27.34 ± 6.53 °C, 7.6 ±0.2 mg L^{-1}, 7.4 ± 0.3 and 0.05 mg L^{-1}, 1000-1500lx respectively. During the experiment, the juveniles were fed once every 10 days and the uneaten food was removed after each feeding. Any dead juveniles were removed and recorded daily. The ingredients of experimental diet FT2 (Booster) are shown in Table 2. The booster diet was mixed with soil with proportion of 1:4 (kg) before being fed to the juveniles. The FT1 (fresh eel blood) diet was fresh and placed directly into the tanks during feeding.

Table 2: Ingredients of the booster experimental diet.

Feeding treatment	Ingredients
Booster (FT2)	Compost
	Molina
	Ziolite
	Allobohpora rosea
	Phosphorus
	Calcium

Methodology:

Juvenile Growth:

At the beginning of the experiment, 20 juveniles of each replicated were sampled to obtain the original wet body weight for each treatment. At the end of the treatment, the final body weight of juveniles in each treatment was determined again. The percentage weight gain (% WG) and specific growth rate (SGR) of the juveniles in each treatment was calculated using the following formulae:

% WG = 100 x (final body weight - initial body weight) / initial body weight (g)

SGR = 100 x (ln final body weight – ln initial body weight) / 60 days

Juvenile Survivorship:

At the end of the experiment, the number of live juveniles in each treatment was counted and recorded, and then the survival rate was calculated.

Data Analysis:

Statistical analyses were conducted using the software SPSS 17.0 (Statistical Program for Social Sciences 17.0) to compare the means of two independent samples and any differences were considered significant at $p \leq 0.05$. The final weight gain, percentage growth rate, specific growth rate and survival rate were analyzed by independent sample t-test.

RESULTS AND DISCUSSION

Cocoon Deposition Number:

The number of cocoon deposited by the broodstock leeches was significantly different among the different of culture conditions (p=0.00). The average number of cocoons was highest in the C_1 with mean and standard deviation of 6±1, followed by C_2 with mean and standard deviation of 4±1. Both treatments differ significantly from the rest of the growth conditions of C_3, C_4, C_5, C_6, C_7 and C_8 which among themselves were not significantly different from one another (Table 3).

Hatching Number:

Hatching number was significantly different under different growth conditions ($p \leq 0.05$). The condition under C_1 had the highest hatching number with mean and standard deviation of 6.23±0.25 while under the C_3 conditions the lowest hatching number was obtained with mean and standard deviation of 0.5±0.87. Hatching numbers of the cocoons in the C_2, C_4, C_5, C_6, C_7 and C_8 did not differ significantly (Table 3).

Hatching rate:

Hatching rates of cocoons under different conditions were not significantly different ($p \geq 0.05$). Although the C_1 treatment had the highest hatching rate with value of mean and standard deviation of 95.23±8.26% but this was not significantly different among the eight conditions tested (Table 3).

Mortality of Broodstock Leeches:

Mortalities of broodstock leeches differed significantly under different growth conditions (p=0). The C_1 had the lowest mortality rate with mean and standard deviation of 2.67±2.31% compared with C_5 which gave the highest mortality rate of 52±13.86%. Mortality under other condition also showed a significant difference (Table 3).

Cocoon size:

The different temperature, light intensity and diet did not significantly influence the standard length and diameter of the cocoons produced ($p \geq 0.05$, p=0.153). Cocoon standard length and diameter in the C_1 were the largest with mean and standard deviation of 22.19±0.92 mm and 13.26±0.07 mm, respectively, whereas the cocoon standard length and diameter in the C_5 treatment were the smallest with mean and standard deviation of 4.74 ± 8.22 mm and 3.34 ± 5.78 mm, respectively. There was no difference in cocoon standard length and diameter obtained those cultures in C_2, C_3, C_4, C_6, C_7, and C_8 growing conditions (Table 3).

Cocoon Wet Weight:

The temperature, light intensity and diet imposed significantly influenced cocoon wet weight (p=0). Broodstock in the C_1 treatment produced the heavier cocoons with mean and standard deviation of 1.26 ± 0.11 g, whereas the C_5 treatment had the smallest cocoon wet weight with mean and standard deviation of 0.22 ± 0.38 g. Cocoon wet weight under the C_3, C_4, C_6, C_7 and C_8 regimes did not differ significantly (Table 3).

Table 3: Comparison of reproductive features of the *Hirudinea* sp. under different of culture conditions: cocoon number, hatching number, hatching rate (%), mortality rate of parent leeches (%), cocoon standard length (mm), diameter (mm) and cocoon wet weight (g).

	Culture conditions							
Reproductive parameters	C1	C2	C3	C4	C5	C6	C7	C8
Cocoon number (p=0.00)	6±1a#	4±1b	0.67±1.16c	1.33±0.58c	0.67±1.16c	0.67±0.58c	2±1c	1.67±1.16c
Hatching number ($p \leq 0.05$)	6.23±0.25a	3.14±1.27b	0.5±0.87b	2.83±2.47b	2.17±0.29b	2.33±2.08b	1.67±1.53b	2±2b
Hatching rate (%) ($p \geq 0.05$)	95.23±8.26a	62.77±25.64ab	16.67±28.87b	70±51.96ab	80±26.46ab	66.67±57.74ab	72.33±25.42ab	74.33±36.14ab
Cocoon length (mm) ($p \geq 0.05$)	22.19±0.92a	19.27±0.19ab	5.58±9.67bc	16.6±0.39c	4.74±8.22c	9.48±8.21c	12.24±11.4c	9.41±8.15c
Cocoon diameter (mm) (p =0.153)	13.26±0.07a	11.49±0.13ab	3.39±5.87b	11.17±0.3ab	3.34±5.78b	6.7±5.8ab	7.68±6.79ab	6.75±5.85ab
Cocoon wet weight (g) ($p \leq 0.05$)	1.26±0.11a	1.05±0.01a	0.23±0.4b	1.13±0.07a	0.22±0.38b	0.51±0.44ab	0.76±0.67ab	0.67±0.58ab
Mortality rate (%) *(p=0.00)*	2.67±2.31a	13.33±2.31a	44±4d	33.3±2.3bc	52±13.86e	29.33±8.33b	37.33±2.31c	46.67±6.11de

#Data in the table were mean and standard deviation (mean ± S.D). Means with the same letter within the same column are not different at the 5% of significant level as determined by Duncan test.

Growth Parameters of Juvenile Leeches as Influenced by Diet:

Final body weight, weight gain, and specific growth rate of the juveniles were significantly different among the treatments (*p*=0). Final body weight of the juveniles when fed with fresh eel blood (FT1) was highest with mean and standard deviation of 0.8893±0.012 g and that fed with booster (FT2) was the lowest with mean and standard deviation of 0.665±0.004 g. Weight gain and specific growth rate under FT1 treatment was high, and in the FT2 treatment was the lowest with mean and standard deviation of 769.41±11.54 g and 3.6±0.02 g, respectively (Table 4).

Table 4: Effect of diet on the survivorship and growth performance of juveniles of *Hirudinea* sp.

	Diet	
Growth parameters	Fresh eel blood (FT1)	Booster (FT2)
Initial body weight (g) (*p*=0.02)	0.08±0.00	0.07±0.00
Final body weight (g) (*p*=0.00)	0.8893 ± 0.012	0.665 ± 0.004
Weight gain (%) (*p*=0.00)	1016.29 ± 7.93	769.41 ±11.54
Specific growth rate (*p*=0.00)	4.04 ± 0.03	3.6 ± 0.02
Survivorship (%) (*p*=0.04)	93.33 ± 5.77	71.67 ± 2.89

Data in the table were means and standard deviations (mean±SD.).

Influence of Diet on Juvenile Survivorship:

Survival rates of the juveniles under different treatments differed significantly (*p*=0.04). Juveniles when fed with fresh eel blood had a high survival rate of 93.33 ± 5.77 % when compared to those fed with booster (Tab

Discussion:

Determining optimum condition is a key factor for successful leech culture and reproduction. For example, mortality of the leech *Hellobdella stagnalis* is influenced by broodstock density and the density of their offspring (Mann, 1957). In this study, increasing temperature and light intensity had a negative effect on the number of cocoons that the broodstock produced. In general, under the condition where the temperature was 25-28°C with a light intensity of 0 lx and fed with a fresh eel blood cocoon deposition number was optimal depositing an average of 6 ± 1 cocoons per replicate. In the present study, it was found that each cocoon that was produced was laid on top of the soil (Fig.1). This number was lower within the range of that obtained with *Haemadispa hainana* (ranging from 4 to 8.15 cocoons deposited) (Tan *et al*., 1992), and that of *N. obscura*, (average of 8.33 ± 0.68 cocoons deposited) (Collins and Holmstrand, 1984). According to B. Zhang *et al*. (2008), increasing broodstock density had a negative effect on the number of cocoons that the broodstock produced. In his study, a density of 5 leeches per tank was optimal for cocoon deposition, with each leech depositing an average of 3.84±0.12 cocoons where the temperature and light intensity were 25.92±6.61 and 1000-1500 lx, respectively. The differences in the cocoon deposition number obtained in that study from the present one is that from the former it was derived from each leech in each treatment whilst in the latter study the number was based on 25 leeches per replicate treatment. B. Zhang *et al*., (2008) stated that the low cocoon numbers of broodstock leeches under high density appeared to be related to competition for food and space among the leeches, creating a stressful condition which directly affects the natural reproductive behavior. However, in the present study the effect of the broodstock density was eliminated by the use of the same density (25 leeches) in all cases.

The duration of leech growth, development and reproduction can often be different due to different culture temperatures (Tan *et al*., 1992). In the present study, the duration of reproduction was 102 days with a temperature range of 25-28 °C, which is within the normal range for growth and reproduction (19-32°C) for *Hirudinaria manillensis* (Yang, 1996; Tan *et al*., 2002). Hatching number for cocoons was significantly influenced by temperature, light intensity and diet, with the highest obtained (6.23±0.25) under the temperature regime of 27-28°C with light intensity 0 lx and diet fed with fresh eel blood. However, hatching rate was not significantly influenced as is shown in Table 4. In the study conducted by Tan *et al*. (1992) with *H.hainana* each cocoon produce 6-17 juveniles with a hatching rate of 77.4 %. In contrast, the present study showed that the growing condition with low temperature and low light intensity and fed with fresh eel blood gave the highest hatching rate with mean and standard deviation of 95.23 ± 8.26 %. Other than the inherent inter-specific differences between *Hirudinea* sp. and *H. hainana*, the differences in the culture preparation between Tan *et al*.'s study and this study may explain the higher number in *Hirudinea* sp.

Fig. 1: Individual cocoon produced by an adult *Hirudinea* sp. laid on top of the soil

In this study, the different treatments significantly influenced the size and wet weight of the cocoons that were produced, with the largest cocoons produced under the treatment which had low temperature, no light intensity and fed with fresh eel blood. Cocoon sizes in this study were approximately equal to the size (about 22 mm in mean length and 13 mm in mean diameter) reported by Tan *et al*. (2002). Generally, the wet weight of leech cocoons can be markedly different between leech species, e.g. 1.6-2.0 g in *Whitmania pigra* (Shi *et al*., 2006a) and 0.15-0.18 g in *H.hainana* (Tan *et al*., 1992). In this study, the wet weight of the cocoons ranged from 1.26±0.11 g under the low temperature, zero light intensity and fed with fresh eel blood to 0.22±0.38 g under the condition fed with booster at high temperature and high light intensity which gave the lowest wet weight of cocoon.

Water quality, temperature and parasitism are known to significantly influence the survivorship of the leech species (Sawyer, 1970; Tan, 2005; Shi *et al*., 2006b). Life-span of leech was also one of the key impediments for leech culture (Mann, 1957). In the present study, it was found that the different conditions of temperature, light intensity and diet could also markedly influence the survival rate of the parent leeches. A higher temperature and light intensity led to greater mortality as the growth condition was probably too extreme a result which was different with the study conducted by B. Zhang *et al*. (2008) where broodstock density had a significant influence on the survival rate of leeches. In this study, many leeches were found to be infected by parasitic protozoans and flatworms at the higher temperature and light intensity during the course of the experiment, which greatly influenced the survival and growth of the leeches (Fig.2).

Fig. 2: Dead leeches infected by parasitic protozoans and flatworms

Sawyer *et al*. (1981) and Peterson (1983) reported that newly hatched juveniles of the leech are independent of their parent once they emerge from the cocoons, and may show differential growth and survival rate in the wild due to diet (Tan *et al*., 1992). The juveniles of *Hirudo orientalis*, *H. verbena* and *H. medicinalis* were reportedly consuming their first blood meal from amphibians whereas successive meals could be obtained from amphibians, fish or mammals (Sawyer, 1986; Keim, 1993; Graf *et al*., 2006). In this study, the final body weight of juvenile *Hirudinea* sp. fed with different diets were significantly different. Those fed with fresh eel blood (FT1) had the highest final juvenile body weight compared with booster (FT2) with means and standard deviations of 0.8893±0.012g and 0.665±0.004g, respectively (Table 4). During the course of the experiment, the juveniles in all the treatments were fed 6 times during the 60 days study in comparison to Tan *et al*. (2002) who fed the new-born juvenile of *H.manillensis* once during the 60 day culture. In their study, the final body weight of juveniles was only 0.27 g much lower that was obtained in this study. However, B. Zhang *et al*. (2008) reported a higher final body weight of juveniles of 0.98 g and 0.92 g fed with live bullfrog and fresh cattle blood, respectively, when compared with the present study although a different diet was used.

Diet composition can be important for growth and survival rates (Zapkuvene, 1972). In this study, it was determined that weight gain and specific growth rate of juvenile *Hirudinea* sp. fed with different diets were significantly different. The juveniles fed with fresh eel blood diet had the highest weight gain and specific growth rate with means and standard deviations of 1016.29 ± 7.93 % and 4.04 ±0.03, respectively. From the study conducted by B. Zhang *et al*. (2008) juveniles fed with live bullfrog had a highest weight gain and specific growth rate of 948.89 ± 35.76 % and 3.92 ± 0.06, respectively a result which was much lower when compared with the present study. The juvenile fed with fresh eel blood diet had the highest survival rate compared with those fed with booster during 60 days of culturing. B. Zhang *et al*. (2008) attributed that most of the juvenile leeches died mainly from hematemesis, probably due to their weak digestive system.

Conclusion and Recommendation:

This investigation has demonstrated that temperature, light intensity and diet significantly affect the reproductive efficiency of the leech, *Hirudinea* sp. A growth condition at a temperature of 25-28°C and zero light intensity fed with fresh eel blood is recommended for the commercial breeding of this species. The choice of diet is crucial on the growth and survival of the juvenile *Hirudinea* sp. where fresh eel blood is recommended as the diet in the first two months after cocoon release.

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