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RESEARCH PAPER

Effects of Temperature on Growth, Feed Intake and Antithrombin Activity of *Poecilobdella manillensis*

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Abstract

The effects of different water temperatures on the growth, feed intake and antithrombin activity of *P. manillensis* were studied in order to identify the optimum water temperature for growth. After *P. manillensis* were cultured at the water temperatures of 18, 22, 26, 30, 34, 38 °C and room temperature (RT), final body weight (FBW), weight gain rate (RWG), specific growth rate (SGR), feed conversion rate (FC), average feed intake per time (AFI), average feed intake rate per time (AFIR), survival rate (SR) and antithrombin activity (AT) were measured for 64 days. The result showed that FBW, RWG, SGR, AFI, and SR of *P. manillensis* presented different growth indices that were higher than other experimental treatments at 26-30 °C ($P<0.05$), while FC was lower than others. The optimal water temperature range for *P. manillensis* was 26-30 °C, and the results indicate that the maximum AT (295.52 U) occurs at 34 °C ($P<0.05$).

Keywords: The optimal water temperature, specific growth rate, feed conversion rate.

Introduction

Poecilobdella manillensis is subordinate to Arhynchobdellida, Hirudinidae, and *Poecilobdella* Blanchard (Yang, 1996) and is a type of blood-sucking leech used as a traditional medicine leech. As early as 1500 BC, a picture in an ancient Egyptian tomb shows treatment with medicinal leeches. An ancient Indian encyclopedia written from 500 to 200 BC noted the use of leeches to treat disease. Antithrombin activity is secreted by the salivary glands of *P. manillensis* and is one of the strongest natural antithrombin materials that can be used as a specific thrombin inhibitor. Modern research has shown that *P. manillensis* contains the properties of an anticoagulant (Guan *et al.*, 2012), anti-thrombus (Li, Yang and Li. 2006), a dissolving thrombus (Feng *et al.*, 2013), reducing blood lipid (Cao *et al.*, 2010) and so on. However, with the continued research on leech medicinal materials, the development and utilization of this kind of resource has been gradually diversified. The market demand for leeches is constantly expanding, which is leading to the rapid decline of wild leech populations as well as the constant rising price of leeches. To be able to reasonably development and utilize the resources of leeches, it is therefore necessary to study and optimize the artificial breeding of *P. manillensis*.

The water temperature, one of the most important factors effecting the growth of aquatic animals (Burel *et al.*, 1996), influences the metabolic reaction rate of the animals and thus affects their physiological and biochemical processes (Wang *et al.*, 2006; Liu *et al*., 2012) like food-intake (Benjamin *et al*., 2010), metabolic rate (Xiao *et al.*, 1992), and rate of protein synthesis (Wu *et al.*, 2002). Therefore, water temperature becomes an important environmental variable effecting the ingestion and growth of aquatic animals. Water temperature can also effect the normal functions of the circulatory and respiratory systems of aquatic animals, thereby influencing their survival (Han *et al.*, 2010; Enders *et al.*, 2006).

Thus, the artificial breeding of leeches has attracted considerable attention. At present, the main research on medicinal leeches has focused on their biological characteristics (Meng *et al.*, 2013; Liu *et al.*, 2014; Shi *et al.*, 2006; Ceylan *et al*,. 2015; Cheng *et al.*,2015; Liu *et al.*,2016a) and genetic structures (Liu *et al.*, 2013a; Liu *et al.*, 2013b; Liu *et al.*, 2011; Liu *et al.*, 2016b). Meanwhile, the main research of *P. manillensis* has focused on pharmacology and drug efficacy (Feng *et al.*, 2013). Previous research has seldom addressed the effect of temperature on the growth, feed intake and antithrombin activity of *P. manillensis*. By controlling the temperature gradient,

this study aims to identify the optimum water temperature range for the feed intake and growth of *P. manillensis* and explores the effect of breeding temperature on the antithrombin activity; accordingly, this study provides a scientific basis for the precise breeding of *P. manillensis*.

Materials and Methods

Ethics statement

All handling of *P. manillensis* was conducted in accordance with the guidelines for the care and use of animals for scientific purposes set up by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Agricultural University, China. The IACUC has specifically approved this study within the project "The artificial culture of medicinal leeches" (approval number NAU (F)-14-008).

Experimental Animals

P. manillensis was field-caught from Huangwutun in Guangxi Zhuang Autonomous Region, China and transferred to the Institute of Chinese Medicinal Materials using the car equipped with automatic aerator. They were acclimated in a rearing system (diameter 18 cm, jar volume 5 L, water volume 3 L) that the jars were sealed with gauze to prevent leeches to escape and that used water taken from the mountains that had been precipitated. During the acclimation period, *P. manillensis* that were selected for the experiments were not fed. A total of 1050 cultured *P. manillensis* with an average weight of 0.75 g were more active without surface scars in this study.

Examination of Antithrombin Activity

Measurement of antithrombin activity was conducted according to the Pharmacopoeia of the People's Republic of China (2015 edition) (Chinese Pharmacopoeia Editorial Committee, 2015a)

Content determination was performed by passing 1 g of the powder of *P. manillensis* through a number three sifter. The powder was precisely weighed, and 5 ml of a 0.9% sodium chloride solution was added. Then, 100 μl of the supermatant after leaching for 30 minutes was placed into a test tube (8mm×38mm), agitated and centrifuged a few times before the Tris-Hcl buffer (prepared when needed) containing (cattle) 200 μl of 0.5% fibrinogen (calculating as solid) was added, agitated equably and put into a test tube, which was then put into a water bath kettle (37 °C±5 °C). After 5 minutes, dropwising thrombin solution containing 40 units / 1 ml was added once per minute and 5 μl every time, gently agitating at the same time up to solidification. The volume of thrombin consumed was recorded and calculated using the following formula:

$$U = \frac{C_1 V_1}{C_2 V_2}$$

In this formula, U- the unit of thrombin activity for every g

C_1-the concentration of the thrombin solution

C_2-the concentration of the sample solution

V_1-the volume of the consumed thrombin solution

V_2-the amount of the sample solution that has been added

Examination of the Moisture Content

Moisture content was conducted according to the Pharmacopoeia of the People's Republic of China (2015 edition) (Chinese Pharmacopoeia Editorial Committee, 2015b)

To determine and examine the moisture content, samples that were approximately 2-5 g and had been dried to a constant weight were put into flat-shaped weighing bottles. The thickness of the samples could not be more than 5 mm. The thickness of the loose sample could not be more than 10 mm. Bottles containing the samples were dried at 100-105 °C without a bottle cap. After 5 hours, the cap was put back on and the bottles were moved into the dryer. Then, the bottles were cooled for 30 minutes and precisely weighed and dried at the above-mentioned temperature for one hour again. They were cooled and weighed until the consecutive difference was less than 5 mg, and the moisture content of the samples was calculated on the basis of reducing weight.

Experimental Procedures

Seven temperature gradients were set: 18, 22, 26, 30, 34, 38 °C and room temperature for control. This study was conducted indoors so the water temperature of control group was defined as room temperature. During the experiment, water temperature indoors (RT) ranged between 19.3 and 23.4 °C. Three replicates per group were used (50 leeches per replicate). The animals were fed with fresh animal blood in jars. At the beginning of the experiment, the water temperature of each jar was the same (19.5°C). However, subsequently the water was heated or cooled at a rate of 1 °C·(6 h)$^{-1}$ to reach the experimental temperature using constant temperature incubator with a 10 h light - 14 h dark (10L: 14D) photocycle under 500 lux. The official test started after the *P. manillensis* were acclimated to the water for 5 days. The following actions were taken during the experimental period of 64 days: thoroughly cleaning and changing all of the water in the jars every day; controlling the temperature difference at less than ±1 °C during the process of changing water; feeding them enough fresh animal blood every four days and noting the food ration. During the experiments, water that had been aerated for 24 h was supplied to the jars. The water quality was tested every three days, and the following parameters were maintained: pH between 6.50 and 7.50; H_2S and NH_3

concentrations at less than 0.01 mg/L and 0.04 mg/L, respectively; and dissolved oxygen concentrations at more than 5.5 mg/L.

Five days after the last feeding, a 200 g *P. manillensis* was randomly chosen from each experimental group and was dried in the sun before being tested for moisture content and antithrombin activity.

Parameters of growth and ingestion

Weight Gain Rate (RWG), Specific Growth Rate (SGR), Feed Conversion Rate (FC), Average Feed Intake per time (AFI), Average Feed Intake Rate per time (AFIR) and Survival Rate (SR) are calculated individually by the following formulas:

$$WG = W_2 - W_1$$
$$RWG = 100 \times WG / W_1 ;$$
$$SGR = 100 \times (\mathrm{Ln}W_2 - \mathrm{Ln}W_1) / (t_2 - t_1) ;$$
$$FC = F / [n (W_2 - W_1)] ;$$
$$AFI = F / n / t$$
$$AFIR = AFI / [(W_1 + W_2) / 2]$$
$$SR = 100 \times (N_t - N_0) / N_0$$

In these formulas: W_1 and W_2, respectively, represent the average weight of *P. manillensis* when t_1 and t_2 represent time, n represents the amounts of *P. manillensis*, F represents the total intake of *P. manillensis* during the period of t_2~t_1, N_t and N_0 represent the amount of *P. manillensis* at the beginning and at the end of experiment, respectively, and t represents the total amount of feeding times.

Statistical Analysis

All data are analyzed by one-way analysis of variance (ANOVA) and presented as the mean ± standard error (SE). Duncan's multiple range tests were analyzed among different group means. The significant level was set as P<0.05. All data analysis was performed using SPSS 19.0.

Results

The Effects of Temperature on the Growth of *P. manillensis*

After 64 days, the growth data of each temperature gradient are recorded, as presented in Table 1. It showed that FBW, WG, RWG, SGR, and SR of *P. manillensis* all presented a similar trend, which increased at first and then decreased with the rise of water temperature. When the feeding temperature was 26 °C, FBW, WG, and RWG of *P. manillensis* reached a maximum and exhibited significant differences with other groups (P<0.05). When the temperature was between 26 - 30 °C, the index of SGR and SR of *P. manillensis* were significantly higher than the others (P<0.05), and the index of SGR and SR of *P. manillensis* between 26-30 °C seldom had any significant difference (P<0.05).

Regression analysis shows that the relationship of quadratic functions exists between growth temperature and SGR. Regression equation: $SGR=-0.0456T^2+2.4313T-26.468$ (R_2=0.9876). Based on the regression equation, GSR of *P. manillensis* achieved the maximum (5.9400) at 26.66 °C (Figure 1).

The effects of different water temperatures on the feed intake

After 64 days, we demonstrated, as seen in Figure 2, that FC presents a trend that decreases at first and then increases as the temperature increases, and FC reaches the lowest value of 2.79 at 26 °C.

Contrary to FC, AFI presented a trend that increased at first and then decreased as the temperature increased (Figure 3.). Variance analysis shows that the AFI of *P. manillensis* is significantly higher than other experimental treatments at 26 °C (P<0.05). The value at 30 °C is slightly lower than the value at 26 °C and is significant higher than the other values (18 °C, 22 °C, 34 °C) (P<0.05).

From Figure 3, we can see that the value of AFIR is the highest at 34 °C, reaching 51.42%, and is the lowest at 18 °C, reaching 32.24%.

The effects of different water temperature on antithrombin activity

We can see from Table 2. that the moisture content of *P. manillensis* in every experimental treatment meets the standard of the Pharmacopoeia of the People's Republic of China (2015 edition) (no

Table 1. Growth indexes of *P. manillensis* cultured at different water temperatures

Index(°C)	IBW/g	FBW/g	WG/g	RWG/%	SGR/%/d	SR/%
18	0.74±0.01	4.30±1.73^{e}	3.56±1.73^{e}	479.00±230.55^{e}	2.75±0.81^{d}	52.00±4.90^{c}
22	0.74±0.00	11.32±0.73^{c}	10.57±0.73^{c}	1423.56±95.62^{c}	4.54±0.11^{b}	77.33±0.94^{b}
26	0.75±0.01	28.47±0.84^{a}	27.72±0.85^{a}	3716.78±124.33^{a}	6.07±0.05^{a}	92.00±4.90^{a}
30	0.74±0.00	20.36±1.56^{b}	19.61±1.56^{b}	2633.63±195.16^{b}	5.51±0.12^{a}	98.00±1.63^{a}
34	0.75±0.01	6.94±0.49^{d}	6.19±0.48^{d}	825.67±62.55^{d}	3.71±0.11^{c}	73.33±4.71^{b}
38	0.76±0.00	-	-	-	-	-
RT	0.74±0.00	18.55±0.10^{b}	17.81±0.10^{b}	2398.02±6.82^{b}	5.36±0.00^{a}	69.33±4.99^{b}

[1]At 38 °C, all *P. manillensis* died after 5 days of feeding. Different superscripts in each row indicate significant differences between treatments (P<0.05).

[2]RT: Room temperatures

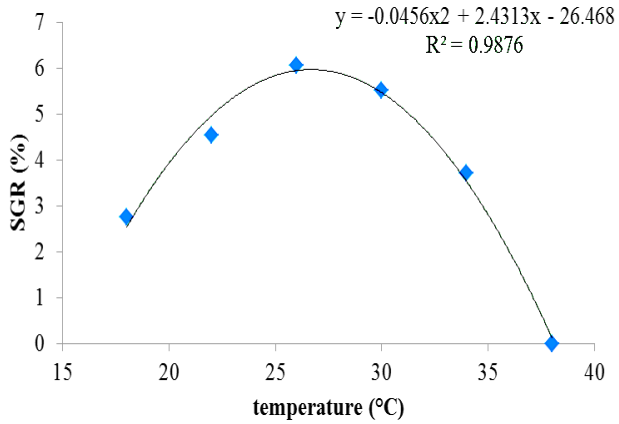

Figure 1. Regression curve between SGR and water temperature.

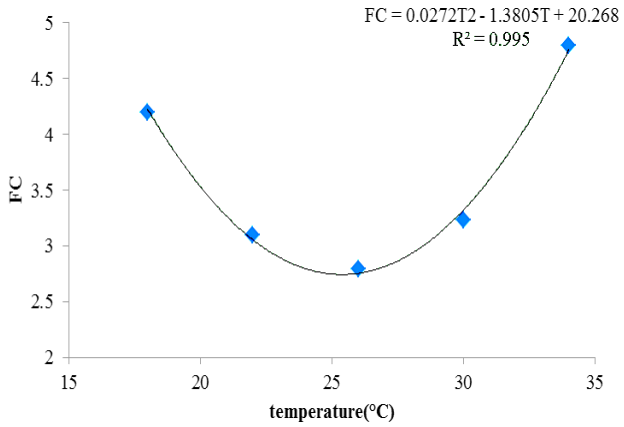

Figure 2. Regression curve between FC and water temperature.

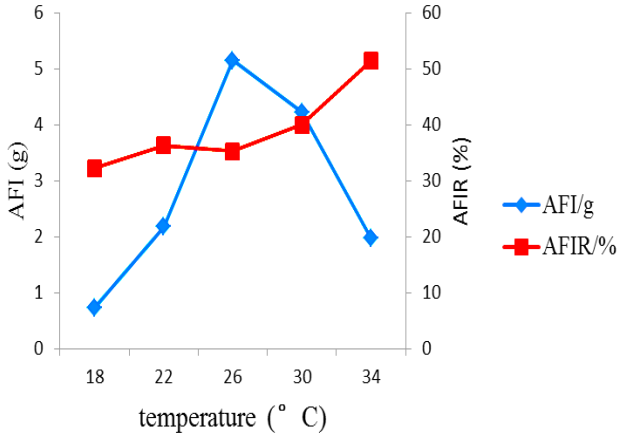

Figure 3. AFI and AFIR of *P. manillensis* cultured at different water temperatures

more than 18.0%) (Chinese Pharmacopoeia Editorial Committee, 2015a). The maximum antithrombin activity of *P. manillensis* is 295.52 U at 34 °C, which is significantly higher than other experiment groups ($P<0.05$).

Discussion

In a natural environment, the growth of aquatic animals is closely linked to water temperature, an environmental factor that changes with space and time (Wang *et al.*, 2006). As this 64 d cultivation experiment shows, different cultivation temperatures

Table 2 AT of *P. manillensis* cultured at different water temperatures

Index (°C)	moisture (%)	AT (U)
22	9.56±0.04	243.85±0.92[c]
26	10.32±0.01	178.06±0.31[d]
30	9.69±0.01	143.81±1.48[e]
34	8.97±0.02	295.52±3.09[a]
RT	9.23±0.02	259.49±1.91[b]

[1]At 38 °C, all *P.* manillensis died after 5 days of feeding. At 18 °C, their survival rate is only 4.00 %. The two experiments above did not make the tests due to the shortage of *P. manillensis*. Different superscripts in each row indicate significant differences between treatments (P<0.05).
[2]RT: Room temperatures

significantly influence growth indexes of *P. manillensis*, such as FBW, WG, RWG, and SGR (P<0.05). The research demonstrates a similar result with other research on aquatic animals like *Oreochromis niloticus* (Azaza *et al.*, 2008; Likongwe *et al.*, 1996), *Amphiprion clarkii* (Ye *et al.*, 2012) and *Oreochromis niloticus X O. aureus* (Hassanen *et al.*, 2014). Generally, the growth rate of aquatic animals rises with increasing temperature and decreases as the temperature rises past the suitable range (Russell, Fish and Wootton. 1996). The growth indexes of *P. manillensis*, such as FBW, WG, WGR, and SGR, decrease after a steady increase of temperature, which coincides with the results of the above research.

Aquatic animals can grow and breed within the appropriate range of temperature, however excessive temperature will slow the speed of growth, cease growth, or lead to the death of aquatic animals (Gao *et al.*, 2006). In the experiment, *P. manillensis*'s SRs reach 90% on average at 26 °C and 30 °C and have no significant difference (P<0.05), which is much higher than those of other experiment groups (P<0.05). However, all the *P. manillensis* died when cultivation temperature remained constant at 38 °C in the 5d experiment; the SR at 18 °C is just 52%, remarkably lower than the other experiment groups (P<0.05). Therefore, the temperature of 26-30 °C is most suitable for *P. manillensis* to grow.

Under normal conditions, aquatic animals grow as food consumption increases, and the growth of aquatic animals is impacted by illumination, density, and temperature when food is sufficient (Wang *et al.*, 2006). The growth rate of aquatic animals rises with increased temperature within the appropriate range. At the excessive temperature, the energy requirement that maintains growth increases with the rising temperature, however, there is a decline in growth rate, the proportion that accelerates growth in food, and food conversion efficiency decreases (Ruyet *et al.*, 2004).

The food coefficient reflects the trend of food conversion efficiency of aquatic animals. Research shows that the food coefficient decreases as temperature rises but increases when water temperature is higher than the optimum temperature (Xiao *et al.*,1992). The FC of *P. manillensis* falls and then increases with rising temperature during the experiment.

As the results indicate, the AFI of *P. manillensis* increases and then decreases as temperature rises, and the AFI at 26 °C and 30 °C are much higher than other experiment groups (P<0.05). Li (2011) conducted research on the impact of temperature on the AFI of turbot *Scophthatmus maximus* L.. From 14 °C to 18 °C, the AFI of turbot *Scophthatmus maximus* L. increases with rising temperature, but the AFI decreases sharply at 21 °C. The research shows a similar tendency with turbot *Scophthatmus maximus* L.. The AFIR of *P. manillensis* at 34 °C reaches the maximum as 51.42%, notably higher than other experiment groups (P<0.05). This research finds that *P. manillensis*'s activity is significantly higher at 34 °C and food conversion efficiency is lowered due to the consumed energy in *P. manillensis*'s activity. Similar to the study results of *Sepiella maindroni* conducted by Peng (2011), *P. manillensis*'s feeding efficiency increases and growth rate decreases if water temperature exceeds its optimum temperature.

In conclusion, the temperature range of 26-30 °C is most suitable for *P. manillensis*'s growth and feeding. Between these temperatures, the growth indices and feeding indices of *P. manillensis*, such as FBW, WG, RWG, SGR, SR, and AFI, are significantly higher than those of other experiment groups (P<0.05), while FC is relatively lower than those of other experiment groups and *P. manillensis* reaches the peak of growth and feeding.

Antithrombin activity is the main index of leech medication material in *The Pharmacopoeia of the People's Republic of China* because the natural hirudin extracted from the salivary glands is the most effective natural antithrombin substance currently known (Chinese Pharmacopoeia Editorial Committee. 2015a). It has good efficiency in treating cardiovascular disease, especially cerebral apoplexy (Zhou *et al.*, 2010; Wu and Yu. 2007).

The antithrombin activity, called heparin cofactor, is an important protease in the plasma coagulation system. It is a low molecular weight polypeptide consisting of 65 amino acids. Temperature is one of the key factors for protein accumulation because protein accumulation increases as temperature increases. However, when the temperature is above the optimum temperature, the protein accumulation decreases as the temperature increases (Zhou *et al.*, 2008). In our study, all the *P.*

manillensis held above 34 °C died after 5 days and for those held below 34 °C, the antithrombin activity is apparently lower than the group that is held at exactly 34 °C ($P<0.05$). A temperature of 34 °C is most likely the best temperature for the antithrombin activity inside of *P. manillensis*, however more studies are needed to confirm this assumption.

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