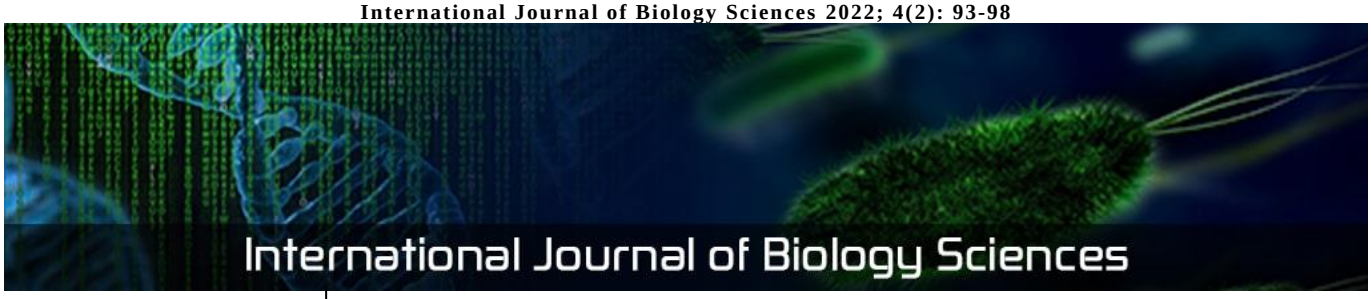

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Blood glucose level, blood profile and ectoparasite infestation of cantang grouper fish commercially fed with the addition of marine leeches *Zeylanicobdella*

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Abstract
The main problem that often occurs in grouper cultivation is the appearance of disease attacks caused by pathogens due to water quality that has not been optimal during maintenance. There are common problems in grouper cultivation, that is unstable environmental conditions that cause accumulation of organic matter which can cause fish to be stress, which is characterized by increased blood glucose levels and susceptible to disease. The types of ectoparasites that commonly attack groupers are *Trichodina, Cryptocaryon, Zeylanicobdella* and *Neobenedenia*. An alternative to control pathogen attacks on fish is by increasing the body's defense cells of fish using immunostimulants. *Zeylanicobdella* known to have molecular weight of proteins that are immunogenic, and is thought to increase the activity of cells defense of the fish body as an immunostimulant. This study aims to find out the blood glucose level, blood profile and ectoparasite infestation of cantang grouper fish given a marine leeches *Zeylanicobdella* on feed. The method in this research used an experimental method with a factorial pattern consisting of two factors. Factor P is the dose given and factor Q is the maintenance time. Statistical test results showed there was an influence (p<0.01) between the doses of marine leeches *Zeylanicobdella* on the number of erythrocytes and leukocytes, there was also interaction (p > 0.01) between the doses of marine leeches *Zeylanicobdella* with different maintenance times to the number of erythrocytes, leukocytes, differential leukocytes including lymparhocytes, neutrophils and monocytes. The results showed that there was a very significant difference between blood glucose levels and ectoparasite infestation of cantang grouper maintenance at different doses and times and there was an interaction effect (p < 0.01). The highest blood glucose level of 67.00 ± 5.148 mg/dL occurred in cantang grouper with a moderate degree of ectoparasite infestation of 36.20 ± 2.387 parasites on day 0 of maintenance with a dose of 0 ppm. The lowest blood glucose level of 28.80 ± 0.837 mg/dL occurred in cantang grouper with a mild degree of ectoparasite infestation, that is 12.60 ± 1.517 parasites on the 28th day of rearing with a dose of 9 ppm. The results showed that the highest of total erythrocytes was at a dose of 7 ppm with a maintenance time of the 21th day are 2,534 × 106 cell/ml. Total Leukocyte was at a dose of 9 ppm with a maintenance time of the 28th day are 1,4902 × 106 cell/ml. The percentage of lymphocytes is the highest with a range of 58-93%, neutrophils range from 5-38% and monocytes range from 1-9%.

Keywords: Blood glucose level, blood profile, ectoparasite infestation, cantang grouper, marine leeches *Zeylanicobdella* sp., dosages, maintenance times

Introduction
Cantang grouper is a type of reef fish that has high economic value and selling value in the world market (Chaniago, 2020) [3]. Common problems in grouper cultivation are slow fish growth, high FCR, and the main problem is the decline in water quality. This decrease in water quality can affect the condition of fish, for example fish become stressed and easily infected with pathogens (parasites, fungi, bacteria and viruses). The ectoparasites that commonly attack groupers are *Trichodina*, *Cryptocaryon* and *Zeylanicobdella*.
Based on the research of Mahasri *et al*. (2020) [6] found the average prevalence value of fish infested with *Zeylanicobdella* can reach 100% in soil-based ponds and 31.11% in concrete-bottom ponds. Efforts to overcome these problems need to take action, either treatment or prevention. Manoppo *et al*. (2016) [8] mentions that to improve growth performance, fish immunity, and disease control can be done by using immunostimulants. Immunostimulants can improve growth performance and control disease by increasing the body's defenses of fish, one of which is the addition of marine leeches *Zeylanicobdella* on feed.

The presence of marine leech protein *Zeylanicobdella* that enter the fish body can increase immunity, phagocytic ability, stimulation of cytokines from lymphocytes, coordination of humoral and cellular immunity (Wang *et al*., 2016) [15].

Analogous to the description above, this study will examine how the immune response is indicated by blood glucose level, blood profile and ectoparasite infestation in grouper treated with feeding with the addition of marine leeches *Zeylanicobdella* because there is no research on the effectiveness of the marine leeches *Zeylanicobdella* used as an immunostimulant.

Research method

Time and Place

This research was conducted from October 2020 to April 2021 at the Anatomy and Aquaculture Laboratory, Faculty of Fisheries and Marine Affairs, Airlangga University, Surabaya, East Java.

Research Material

The equipment used in this research includes plastic packing, seser, bucket, rubber band, styrofoam box, mortar, pestle, analytical scale, volume pipette, micro pipette, aquarium size (59 x 29 x 34) cm^3 as many as 20 pieces, aerator, stone aeration, aeration hose, filter, rockwool, 1 ml syringe, microtube, haemocytometer, stationery, object glass, cover glass, staining jar, hand counter, camera, stationery, binocular microscope, pH paper, refractometer, DO meter, ammonia kit, nitrite kits, and nitrate kits.

The materials used were grouper cantang measuring 7-9 cm, marine leeches *Zeylanicobdella* as many as 900 individuals, hayem marine leeches, turk marine leeches, EDTA (Ethylene Diaminetetra Acetic Acid), immersion oil, distilled water, hand gloves, masks, tissue, 100% methanol, 10% Giemsa, seawater with a salinity of 30-35 ppm, and feed fish that have been fed *Zeylanicobdella*.

Cantang grouper rearing was carried out for 30 days with a density of 12 fish/18 liters of water with a total density of 1 fish/1.5 liters. The feed used was test feed (P1, P2, P3) and commercial feed (P0: control). The frequency of feeding was 3 times a day in the morning and evening. Feeding as much as 3% of the average weight of fish.

Research Method

The method used in this study is an experimental method using a factorial completely randomized design (CRD) consisting of 5 treatments with 4 replications each as follows:

Factor P

P0 = Commercial feed without the addition of marine leeches *Zeylanicobdella* sp.

P1 = Commercial feed + 2,6455 µL marine leeches *Zeylanicobdella* sp.

P2 = Commercial feed + 3,7037 µL marine leeches *Zeylanicobdella* sp.

P3 = Commercial feed + 4,7619 µL marine leeches *Zeylanicobdella* sp.

Factor Q

Q0 = Maintenance time 0 day (before treatment).

Q1 = Maintenance time 7 day.

Q2 = Maintenance time 14 day.

Q3 = Maintenance time 21 day.

Q4 = Maintenance time 28 day.

Table 1: Combination Research Treatment

Treatment	Q0	Q1	Q2	Q3	Q4
P0	P0Q0	P0Q1	P0Q2	P0Q3	P0Q4
P1	P1Q0	P1Q1	P1Q2	P1Q3	P1Q4
P2	P2Q0	P2Q1	P2Q2	P2Q3	P2Q4
P3	P3Q0	P3Q1	P3Q2	P3Q3	P3Q4

Data Collection

The parameters tested in this study were blood glucose levels, erythrocyte and leukocyte counts, differential leukocytes, ectoparasite infestation, water quality and survival rate. Parameter value measurements were carried out on day 0 (before treatment), day 7, day 14 and day 28. The method of calculating the number of erythrocytes calculated based on Klontz (1994) [17] is:

Total erythrocytes = 5 areas x 10 x 5 x 200 (sel/mm^3)

The method of calculating the number of leukocytes calculated based on Klontz (1994) [17] is:

$$\text{Total leukocytes} = \frac{64 \text{ area x } 200 \text{ x } 10}{4} \text{ (sel/mm}^3\text{)}$$

The method for calculating the differential leukocytes count based on Santoso, *et al*. (2013) [18] are:

$$\text{Limfosit} = \frac{\text{Jumlah Limfosit}}{\text{Jumlah Total Leukosit}} \text{x100\%}$$

$$\text{Monosit} = \frac{\text{Jumlah Monosit}}{\text{Jumlah Total Leukosit}} \text{x100\%}$$

$$\text{Neutrofil} = \frac{\text{Jumlah Neutrofil}}{\text{Jumlah Total Leukosit}} \text{x100\%}$$

$$\text{Eosinofil} = \frac{\text{Jumlah Eosinofil}}{\text{Jumlah Total Leukosit}} \text{x100\%}$$

$$\text{Basofil} = \frac{\text{Jumlah Basofil}}{\text{Jumlah Total Leukosit}} \text{x100\%}$$

Determination of the degree of ectoparasite protozoan infestation based on Fegan *et al*. (1993) [4] can be seen in Table 2 and the determination of the degree of ectoparasite worm infestation based on Williams and Williams (1996) [16] can be seen in Table 3.

Tabel 2: Category of protozoa ectoparasite infestation degree

Jumlah Parasit	Kategori
5-25	Mild
26-50	Moderate
>50	Heavy

Table 3: Category of ectoparasite worm infestation degree

Jumlah Parasit	Kategori
0	Normal
1-5	Mild
6-50	Moderate
51-100	Heavy
>100	Very Heavy
>1000	Super Infection

While calculating the survival rate using the formula according to Manullang (2018) is:

$SR = \frac{(nt)}{n0} \times 100\%$ … (2)

Dimana: SR = Survival Rate (%); N_t = Number of fish at the end of the study; dan N_0 = Number of fish at the beginning of the study.

Data Analysis

The data obtained were analyzed using the ANOVA (Analysys of Variance) test, to determine the effect of the treatment given, if there are significant results, the calculation is continued with Duncan's multiple test with a significance level of 5%.

Result

Blood Glucose Level

The results of statistical tests for measuring blood glucose levels in cantang grouper can be seen in Table 4.

Table 4: Average blood glucose levels in grouper cantang

Maintenance Time	Blood Glucose Level (mg/dL)			
	Dose 0 ppm (P0)	Dose 5 ppm (P^1)	Dose 7 ppm (P2)	Dose 9 ppm (P^3)
Day 0 (QO)	$62,00^{a} \pm 5,148$	$57,80^{ab} \pm 1,304$	$52,60^{ab} \pm 2.302$	$56,00^{b} \pm 1,581$
Day 7 (Q1)	$54,00^{bc} \pm 4,848$	$53,20^{bc} \pm 3,768$	$48,40^{cd} \pm 3,362$	$38,60^{fgh} \pm 3,050$
Day 14 (Q2)	$52,20^{bc} \pm 3,114$	$44,60^{de} \pm 11,675$	$44,80^{de} \pm 5,404$	$37,40^{gh} \pm 4,159$
Day 21 (Q3)	$43,80^{def} \pm 2,387$	$36,40^{gh} \pm 3,782$	$40,604^{efg} \pm 3,362$	$36,00^{gh} \pm 6,892$
Day 28 (Q4)	$37,80^{gh} \pm 1,924$	$33,00^{hi} \pm 2,236$	$30,20^{i} \pm 1,304$	$28,80^{i} \pm 0,837$

Erythrocyte Count: Calculation of the number of erythrocytes of cantang grouper can be seen in Table 5.

Table 5: Average number of erythrocytes of cantang grouper

Waktu Pemeliharaan (Hari ke-)	Jumlah Eritrosit (10^6 sel/ml)			
	Dosis 0 ppm (P0)	Dosis 5 ppm (P1)	Dosis 7 ppm (P2)	Dosis 9 ppm (P3)
0 (Q0)	$1,61^{k} \pm 0.12$	$1,90^{ghi} \pm 0.17$	$2,17^{def} \pm 0.08$	$2,00^{fgh} \pm 0.12$
7 (Q1)	$1,67^{jk} \pm 0.12$	$2,37^{bcd} \pm 0.11$	$2,50^{ab} \pm 0.09$	$2,05^{efg} \pm 0.09$
14 (Q2)	$7,00^{fgh} \pm 0.20$	$2,10^{ef} \pm 0.10$	$2,24^{cde} \pm 0.17$	$1,74^{ijk} \pm 0.11$
21 (Q3)	$1,80^{ijk} \pm 0.11$	$2,23^{cde} \pm 0.09$	$2,46^{ab} \pm 0.13$	$2,32^{bcd} \pm 0.15$
28 (Q4)	$1,818^{hij} \pm 0.11$	$2,396^{abc} \pm 0.16$	$2,534^{a} \pm 0.11$	$2,344^{abcd} \pm 0.26$

Leukocyte Count: Calculation of the number of leukocytes of cantang grouper can be seen in Table 6.

Table 6: Average leukocyte count of cantang grouper

Waktu Pemeliharaan (Hari ke-)	Jnmlah Leukosit (10^3 sellml)			
	Dosis 0 ppm (PO)	Dons 5 ppm (21)	Dons 7 ppm (22)	Dosis 9ppm (P3)
0 (Q0)	$0,768^{i} \pm 0.12$	$0,7762^{hi} \pm 0.17$	0,7668k ± 0.08	$0,7998^{hi} \pm 0.12$
7 (Q1)	$0,7972^{hi} \pm 0.12$	$0,8212^{hi} \pm 0.11$	$0,824^{h} \pm 0.09$	$0,9136^{g} \pm 0.09$
14 (Q2)	$0,8182^{hi} \pm 0.20$	$1,011^{f} \pm 0.10$	$1,179^{d} \pm 10.17$	$1,217^{c} \pm 0.11$
21 (Q3)	$0,9538^{g} \pm 0.11$	$1,307^{c} \pm 0.09$	$1,3062^{c} \pm 0.13$	$1,4032^{b} \pm 0.15$
28 (Q4)	$1,1292^{e} \pm 0.11$	$1,3794^{b} \pm 0.16$	$1,3704^{b} \pm 10.11$	$1,4902^{a} \pm 0.26$

Differential Leukocytes

A. Lymphocyte Count: The lymphocyte count of cantang grouper can be seen in Table 7.

Table 7: The average lymphocyte of cantang grouper

Waktu Pemeliharaan (Hari ke-)	Jumlah Limfosit (%)			
	Dosis 0 ppm (P0)	Dosis 5 ppm (P1)	Dosis 7 ppm (P2)	Dosis 9 ppm (P3)
0 (Q0)	$61^{ij} \pm 3.46$	$61.8^{ij} \pm 5.9$	$58^{j} \pm 9.82$	$63.8^{i} \pm 3.11$
7 (Q1)	$72.8^{gh} \pm 3.56$	$72^{gh} \pm 1.87$	$69.2^{h} \pm 3.4$	$71.6^{gh} \pm 2.7$
14 (Q2)	$76^{fg} \pm 2.7$	$73^{gh} \pm 2$	$79^{ef} \pm 3.03$	$77^{fg} \pm 1.58$
21 (Q3)	$812^{def} \pm 5.54$	$76.2^{fg} \pm 2.58$	$82.2^{cde} \pm 3.8$	$84.2^{bcde} \pm 2.28$
28 (Q4)	$882^{b} \pm 4.32$	$87.4^{bc} \pm 2.7$	$85.2^{bcd} \pm 2.68$	$94.6^{a} \pm 2.88$

B. Neutrophil Count: Calculation of neutrophils of cantang grouper can be seen in Table 8.

Table 8: Average neutrophils of cantang grouper

Walctu. Pemeliharaan (Hari ke-)	Jumlah Neutmfil (%)			
	Dosis 0 ppm (PO)	Dons 5 ppm (P1)	Dom 7 ppm (P2)	Dom 9 ppm (P3)
0 (QO)	$31.6^{bc} \pm 0.12$	$33,2^{ab} \pm 0.17$	$35,4^{a} \pm 0.08$	$33,2^{th} \pm 0.12$
7 (Q1)	$25,2^{d} \pm 0.12$	$25,6^{d} \pm 0.11$	$28.2^{cd} \pm 0.09$	$26,4^{d} \pm 0.09$
14 (Q2)	$21,6^{ef} \pm 020$	$25^{de} \pm 0.10$	$19^{fg} \pm 0.17$	$18,4^{fg} \pm 0.11$
21 (Q3)	$16,4^{gh} \pm 0.11$	$18,6^{fg} \pm 0.09$	$14,8^{h} \pm 0.13$	$13^{hi} \pm 0.15$
28 (Q4)	$10,4^{i} \pm 0.11$	$10^{i} \pm 0.16$	$13^{hi} \pm 0.11$	$5,2^{j} \pm 026$

C. Monocyte Count: The calculation of cantang grouper monocytes can be seen in Table 9.

Table 9: The average monocytes of cantang grouper

Waktu Pemeliharaan (Hari ke-)	Jumlah Monosit (%)			
	Dosis 0 ppm (PO)	Dosis 5 ppm (P1)	Dosis 7 ppm (P2)	Dosis 9 ppm (P3)
0 (Q0)	$7,8^{a} \pm 0.12$	$5^{b} \pm 0.17$	$4,6^{bede} \pm 0.08$	$3^{cde} \pm 0.12$
7 (Q1)	$2^{cde} \pm 0.12$	$2,4^{bcde} \pm 0.11$	$2,6^{de} \pm 0.09$	$2^{bc} \pm 0.09$
14 (Q2)	$2^{cde} \pm 0.20$	$2^{cde} \pm 0.10$	$1.8^{bcde} \pm 0.17$	$1,6^{bcde} \pm 0.11$
21 (Q3)	$2,4^{bcde} \pm 0.11$	$2^{bcde} \pm 0.09$	$2.6^{de} \pm 0.13$	$2^{e} \pm 0.15$
28 (Q4)	$1,4^{de} \pm 0.11$	$1,6^{bc} \pm 0.16$	$1,8^{bcd} \pm 0.11$	1cde ± 0.26

Infestation Ectoparasite

The results of the calculation of the average number ectoparasites in cantang grouper in Table 10 show that during 28 days of rearing the values ranged from 12.60 to 36.20 parasites with the category of mild to moderate ectoparasite infestation according to Williams and Williams (1996) [16].

Table 10: Average number of ectoparasites in cantang grouper

Maintenance Time	Avenge Number of Ectoparasites Infestation Degree			
	Dose 0 ppm (PO)	Dose S ppm (P1)	Dose 7 ppm (P2)	Dose 9 ppm (P3)
Day 0 (Q0)	$36,20^{a} \pm 2,387$/Moderate	$31,60^{bcd} \pm 2,074$/Moderate	$29,40^{de} \pm 1,140$/Moderate	$30,40^{cd} \pm 2,074$Moderate
Day 7 (Q1)	$34,00^{ab} \pm 2,915$/Moderate	$29,60^{de} \pm 2,074$/Moderate	$26,40^{fg} \pm 1,140$/Moderate	$26,401^{fg} \pm 2,408$/Moderate
Day 14 (Q2)	$31,20^{bcd} \pm 2,950$/Moderate	$24,00^{gh}$ 3,162/Moderate	$20,40^{ijk} \pm 2,074$/Moderate	$18,40^{jk} \pm 2,302$/Moderate
Day 21 (Q3)	$32,80^{bc} \pm 2,490$/Moderate	$25,60^{fg} \pm 1,949$/Moderate	$21,00^{ij} \pm 1,581$/Moderate	$18,80^{ijk} \pm 1,304$/Moderate
Day 28 (Q4)	$27,20^{ef} \pm 2,280$/Moderate	$21,60^{hi} \pm 1,140$/Moderate	$17,60^{k} \pm 1,517$/Moderate	$12,60^{1} \pm 1,517$/Moderate

Water Quality: The results of measuring the water quality of cantang grouper can be seen in Table 11.

Table 11: Water quality of cantang grouper

Parameter	Perlakuan				Firdaus *et al.* (2016) [19]
	P0 (0 ppm)	P1 (5 ppm)	P2 (7 ppm)	P3 (9 ppm)	
Suhu (ºC)	27,3-28,5	28,8-29	27,8-28,4	27,8-29	27-32
Oksigen Terlarut (mg/1)	2,34-2,84	2,97-4	2,48-4,05	2,43-4,17	≥ 4
pH	7	8	8	7	7-8.5
Amonia (mg/1)	1	0,5-1	0,5-1	1	$\leq 0,01$
Nitrat (mg/1)	10-15	25-50	50-75	25-50	≤ 50
Nitrit (mg/1)	1	1	1	1	$\leq 0,06$

Survival Rate: The calculation of the average survival rate of cantang grouper during rearing can be seen in Table 12.

Table 12: Average survival rate of cantang grouper

Perlakuan	Jumlah Ikan Yang Dipelihara Awal (ekor)	Jumlah Ikan Yang Hidup Setelah 28 hari Pemeliharaan (ekor)	Tingkat Kelulushidupan (%)
P0	12	5	41,6%
P1	12	7	58,3%
P2	12	7	58,3%
P3	12	7	58,3%

Discussion

High blood glucose levels in cantang grouper at the beginning of rearing is an indicator of fish stress which can be caused by less than optimal environmental conditions and fish are still in the process of adapting to the rearing environment. This is supported by Nasichah *et al.* (2016) [9] which states that stress is a physiological response from the body that occurs when fish try to maintain a stable condition in the body, one of which is due to poor environmental conditions that affect the physiological conditions of the fish's body, one of which is increased blood glucose levels.

The results of statistical tests on the number of erythrocytes and leukocytes showed that there was an interaction between the dose of *Zeylanicobdella* with different maintenance times. The increase in erythrocytes after a decrease was due to the immunostimulant marine leech *Zeylanicobdella* that enters the body of the fish. The presence of protein content in *Zeylanicobdella* can increase the number of red blood cells. Furthermore, the increase in the number of leukocytes was due to the presence of immunostimulants that entered the fish's body. The increase in total leukocytes in fish given immunostimulants shows that immunostimulants that enter the body have a positive effect on increasing total leukocytes in the blood (Suhermanto, *et al.* 2013) [12].

The results of the observation of blood smear preparations, it is known that the types of leukocytes found were monocytes, lymphocytes and neutrophils. The average value of lymphocytes in cantang grouper before being fed a diet containing *Zeylanicobdella*, obtained a range of 58-69%. These results indicate that the average lymphocyte value of the tested fish is still in the below normal range (Svobodova *et al.*, 2006) [13]. The peak of the increase in the percentage of lymphocytes on the 28th day of treatment P1, P2 and P3

with P3 as the best dose, indicated that the marine leech *Zeylanicobdella* with its protein content is able to influence the percentage of lymphocytes in the blood of grouper cantang.

The results of statistical tests on the number of neutrophils showed that there was an interaction between doses of *Zeylanicobdella* with different maintenance times. Based on observations, the average neutrophil percentage value before being treated ranged from 28-38%, this percentage showed that the neutrophil value of cantang grouper was not optimal (Preanger *et al*., 2016) [10]. Giving marine leeches *Zeylanicobdella* in cantang grouper feed can cause a decrease in the number of neutrophils, although in some values it shows that it is not optimal, but the value is lower than the range before being given immunostimulants. Then, the results of statistical tests on the number of monocytes showed that there was an interaction between the dose of *Zeylanicobdella* with different maintenance times. The average value of the percentage of monocytes before being treated ranged from 2-10%, this percentage showed that the monocyte value of cantang grouper was not optimal. Monocytes which tend to decrease every week until the 14th week are related to monocytes that are not needed for phagocytosis, because there has been no infection that has entered the fish body (Syatma *et al*., 2015) [14].

In addition, the combined dose of *Zeylanicobdella* and different rearing times reduced the number of ectoparasite infestations in cantang grouper. This is because according to Mahasri *et al*. (2018) [7] stated that the entry of immunostimulants from the marine leeches *Zeylanicobdella*. Macrophage cells that are considered foreign bodies need time to respond in order to stimulate increased activity of phagocytic cells that function as cells that carry out phagocytosis of foreign objects that enter the host's body. Phagocytosis is a non-specific body defense system that is able to protect the body against disease attacks so that the fish's immune response increases. So that the combined dose of *Zeylanicobdella* and different rearing times can reduce the number of ectoparasite infestations in cantang grouper.

The results of the water quality examination showed that the dissolved oxygen level ranged from 2.75-4.17 mg/L which could be said to be not in accordance with the optimal value. The results showed that the content of nitrite during maintenance was in the range of 0.5-1 mg/L, nitrate during maintenance was 10-75 mg/L and ammonia in the range of 0.5-1 mg/L. The results of these three parameters indicate that the values are not optimal (Solichin *et al*., 2012) [11]. Furthermore, the high survival rate (58.3%) in the addition of *Zeylanicobdella*. This is because the protein *Zeylanicobdella* as an immunostimulant candidate, it can provide high protection or protection to cantang grouper, with an increase in the immune system.

Conclusion

The conclusion of this research is the provision of marine leeches *Zeylanicobdella* on feed with different doses and maintenance time had a very significant effect on blood glucose level, the number of erythrocytes, leukocytes, differential leukocytes and ectoparasite infestation of grouper cantang. The best interaction between doses of *Zeylanicobdella* on feed with different maintenance times is a dose of 9 ppm *Zeylanicobdella* sp. on feed with maintenance time of day 28.

Suggestion

Suggestions from this study are that it is necessary to rethink the effective method of feeding, and the injection must be done with the appropriate technique, because the wrong injection can cause stress to the fish.

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