Effectiveness of giving clove oil as an anaesthetic for survival rate and number of leucocytes in cantang grouper (Epinephelus sp.) in the closed transportation

S Nurkomaria ¹, H Suprapto²*, Sudarno²

¹Program Study of Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Kampus C Jalan Mulyorejo, Surabaya 60115 East Java, Indonesia
²Department of Fish Health Management and Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Kampus C Jalan Mulyorejo, Surabaya 60115 East Java, Indonesia

*Corresponding author: hari-s@fpk.unair.ac.id

Abstract. Cantang groupers is one of fish commodities that have high economic value. Live fish transport has several problems for fish and one of the problem is stress in fish. Many stressors can cause mortality of fish if the fish body can not tolerate it. One way that can be used to reduce stress on fish which can increase the value of survival and decrease leucocytes during the transport is giving clove oil as an anaesthetic. This study aim to determine the effect of clove oil as an anesthetic substance on the survival rate (SR) and number of leucocytes in cantang grouper (Epinephelus sp.) in the closed transport. The method is an experimental method with a completely randomized design (CRD), with five treatments and three replications. The main parameters observed were survival rate and leucocytes of cantang groupers. The data obtained will be analyzed using ANOVA followed by Duncan's Multiple Range Test. The results showed that administration of clove oil as an anaesthetic may improve the survival rate and lower the number of leucocytes of cantang grouper, after the closed transport for 10 hours. The highest survival rate is 86.67% and the Leucocytes is 2.35x10⁴ cell/mm³ with 10ppm of clove oil dosage.

1. Introduction

Indonesia is one of the grouper producing countries, one of the commodity of grouper which is now in great demand is the abstraction grouper fish (Epinephelus sp.). Market demand for the grouper fish is also increasing, especially for the domestic market. The distance between aquaculture centers and consumers locations are far apart, resulting in the need for a transportation that can maintain the quality and quantity of fish during the transportation process [1].

Transportation of alive fish with long shipping distances has several obstacles, one of them is stress in fish. Fish are very easily stressed due to handling and transport [2]. One of the hematological parameters that can be used as an indicator of stress in fish is the number of leukocytes. Leukocytes are blood cells that play a role in the immune system. Leukocytes help cleanse the body of foreign objects, including invading pathogens through the immune response system and other responses [3].

Death during the transportation process can be caused by exposure to one or several stressors at once. Fish mortality mainly due to exposure to stressors during the transportation process depends on the length of time the fish are exposed to stress, the severity of the stressor (acute or chronic), and also
the condition of the fish that being transported [4]. One way that can be used to reduce stress on fish and increase survival value during transportation is by administering the anesthetic ingredients [5]. Anesthetics which are often used are Tricaine methanesulfonate (MS-222) and clove oil. Clove oil is the result of distillation from clove plants from leaves, flowers and stems. Clove oil has a large amount of eugenol components which have properties as stimulants, local anesthetics, carminative, antinetic, antiseptic and antipasmedic [6]. According to Velisek [7], clove oil can be used as fish anesthesia before handling or treating spawning, blood sampling or for some anesthetic needs for animals.

2. Materials and methods

2.1 Place and time
The research was conducted in Feed Laboratory of Brackish Water Aquaculture Institute (BPBAP) Situbondo, in August, 2016.

2.2 Tools
The tools needed in this research are aquarium, tub plastic, packing plastic, micropipette, hose and aeration stone, airblow, pH meter, DO meter, refractometer, thermometer, stopwatch, handcounter, rubber band, falcon tube, sterofoam, analytical balance microscopes, microtubes, haemocytometers and micropipette tips.

2.3 Materials
Materials needed are 7-9 cm sized of grouper fish, MS 222 (Tricaine methanesulfonate), clove oil, sea water, oxygen, turk solution, Ethylenediaminetetraacetic acid (EDTA), aquades, and ice cubes.

2.4 Research design
This study used an experimental method with five treatments and three replications, that is: P1 (negative control (without the addition of anesthesia)), P2 (positive control (administration of MS 222 at a dose of 70 ppm)), P3 (administration of clove oil at a dose of 5 ppm), P4 (administration of clove oil at a dose of 10 ppm) and P5 (administration of clove oil at a dose of 15 ppm).

2.5 Research preparation
Preparation that must be done before conducting research is to prepare the tools that will needed. The next preparation is to select groups of 7-9 cm abstraction grouper with a total of 150 fish. The selected fish is a healthy fish, meaning that it is free from disease and not deformed. The fish that have been selected are moved to the aquarium to be acclimatized and fed (satisfied) for 24 hours.

2.6 Research implementation
Fish transportation begins by packing the grouper abstraction into a plastic bag. Each bag was filled with as much as 2000 ml of sea water and then given clove oil and MS 222 in accordance with the treatment. Each plastic bag contains 10 fish which will be used to observe the survival value and the number of leukocytes. The grouper fish that have been packaged are placed in Styrofoam with temperatures between 21-23°C. Transportation is carried out at night for 10 hours. Observation of water quality is carried out twice, that is before transportation and after transportation. Water quality observed included temperature, dissolved oxygen (DO), carbon dioxide, salinity and pH.

2.7 Survival rate calculation
Calculation of the survival rate of the grouper fish is carried out after the fish transportation process is complete. After transportation, grouper is recovered or restored. The survival value of fish can be calculated using the following formula:
SR = \frac{N_t}{N_0} \times 100\%

Information:
SR = Survival rate of grouper seed (%).
N_t = Number of fish seed at the beginning of the study.
N_0 = Number of fish seed at the end of the study.

2.8 Leukocytes count calculation
Leukocyte count is done by sucking blood with a pipette containing white stirrers to a scale of 0.5. Then, add the Turk's solution to scale 11, the pipette is swung into an 8 for 3-5 minutes so that the blood is mixed evenly. Drop the solution on the haemocytometer, then cover with a cover glass. The liquid will fill the capillary space. The number of white blood cells or total leukocytes is calculated with the help of a microscope with a magnification of 400x. The total number of leukocytes is calculated by counting the cells contained in 4 large boxes. The number of leukocytes can be calculated using the following formula:
White blood cell count = counted white blood cells x 50 cells / mm³

2.9 Research parameters
The main parameters in this study are the survival rate (SR) and the number of leukocytes. Supporting parameters in this study are measurements of water quality including temperature, salinity, pH, dissolved oxygen (DO), and carbon dioxide.

2.10 Data analysis
This research uses ANAVA data analysis (Variation Analysis) to determine the differences between treatments. Followed by Duncan's distance test using the SPSS (Statistical Program for Social Science) program.

3. Result and discussion
3.1 Result
3.1.1 The level of survival
Table 1. Survival rate percentage of cantang groupers

| Treatment | Level of survival (%) ± SD |
|-----------|---------------------------|
| P1        | 60.00 ± 0.58c             |
| P2        | 90.00 ± 0.00a             |
| P3        | 76.67 ± 0.00bc            |
| P4        | 86.67 ± 0.58ab            |
| P5        | 80.00 ± 0.00bc            |

Description: different superscript in the same column indicate significant differences (p <0.05) P1 negative control (without the addition of anesthesia) P2 positive control (giving MS 222 at a dose of 70 ppm). P3 (clove oil anesthesia administration of a dose of 5 ppm) P4 (clove oil anesthesia administration of a dose of 10 ppm) P5 (clove oil anesthesia administration of a dose of 15 ppm).

3.1.2 Total of leukocytes
Table 2. Results of leukocytes total measurement

| Treatment | Level of survival (%) ± SD |
|-----------|---------------------------|
| P1        | 2.56 ± 0.0125a            |
| P2        | 2.28 ± 0.009b             |
| P3        | 2.38 ± 0.0230ab           |
| P4        | 2.35 ± 0.0b               |
Description: different superscript in the same column indicate significant differences (p < 0.05) P1 negative control (without the addition of anesthesia) P2 positive control (giving MS 222 at a dose of 70 ppm). P3 (clove oil anesthesia administration of a dose of 5 ppm) P4 (clove oil anesthesia administration of a dose of 10 ppm) P5 (clove oil anesthesia administration of a dose of 15 ppm)

### 3.1.3 Water quality

Table 3. Results of Water Quality Measurement

| Water Quality Parameters | Observation Time |
|--------------------------|------------------|
|                          | Before           | After            |
| Salinity (ppt)           | 31-32            | 30-31            |
| pH                       | 7.9 to 8.1       | 7.4 to 7.5       |
| Temperature (°C)          | 24-25            | 27-28            |
| Dissolved Oxygen (Ppm)    | 5.08             | 3.7 to 3.8       |
| Carbon dioxide (ppm)      | 0.22             | 7.2 to 7.4       |

#### 3.2 Discussion

Based on data on the survival rate of grouper fish in Table 1. After transportation for 10 hours, death occurred in all treatments. In P1 treatment (negative control) without anesthesia, the survival rate of the grouper fish is 60%. Whereas in P2 (positive control) that is by giving MS 222 70 ppm the survival rate reached 90%. Whereas in the provision of clove oil 5, 10, 15 ppm the survival rate was 76.67%, 86.67%, and 80%. The difference in survival between treatments can be caused by several factors, one of the factor is stressors in fish. Stress in fish that lasts continuously and in a long time can cause death [4].

Stress in fish can be caused by several factors including poor mechanical handling, stocking density that is too high, netting, decreased water quality, adaptation of fish to new environments, and others [4, 1]. Exposure to stressors in fish during transportation results in changes in physiological conditions in the body of the fish in response to the fish's body to stress. The response of fish to stress can be seen by observing several parameters including hematological parameters and changes in fish behavior. The response is done as a form of fish adaptation to stress conditions [8].

Clove oil is one of the natural anesthetics that has been developed and used as a substitute for chemical anesthetics such as MS 222. Clove oil is obtained from the extracts of leaves, stems and flowers of clove plants. Clove oil is known to contain eugenol compounds that are analgesic or reduce pain [9]. Besides eugenol clove oil also contains cartiophylline compounds which are also useful as antiseptics. Clove oil is used as a local anesthetic in animals and humans and has a sedating effect on aquatic animals [10]. However, these compounds also have insecticidal activity so that if they are used in excess they can cause death in fish [1].

Giving clove oil as an anesthetic affects the survival rate of the grouper abstraction on closed transportation. The survival rate of the grouper abstraction with clove oil treatment showed a higher average value than the negative control (without anesthesia). But it is still lower when compared to positive control (MS 222). MS 222 is one of the anesthetics that has been registered safely used in several countries.

The use of MS 222 requires a withdrawal period that is the time of maintenance before the fish are consumed [11]. The time used varies from 5 to 21 days it aims to make the residue that remains can be decomposed. MS 222 contains aminobenzenzoate compounds which have anesthetic properties, release vapor and can give a sharp odor in water that is pungent so as to cause sedation effects for fish [12].

Clove oil is included in the group of terpenes which are soluble in fat and works on the central nervous system [13]. These compounds can increase the concentration of GABA in the central nervous system [1]. Increasing the concentration of GABA in the nervous system will trigger the effects of
sedation in animals and mammals [14]. The effect of sedation on fish makes the fish more relaxed and passive so that the metabolic process of fish is reduced.

The administration of clove oil anesthesia also affected the total leukocytes in the grouper fish after transportation for 10 hours. In this study, total of leukocytes experienced significant differences between negative treatments and controls. In P2, P3, P4, and P5 treatments were not significantly different, but the treatment was significantly different from P1 treatment which was a negative control that is without administration of anesthesia. Leukocytes are one part of the blood that functions as non-specific antibodies. When fish experience stress conditions, the number of leukocytes can increase as a form of defense measures due to stressors [15].

Total of leukocytes in normal fish generally range between 2x10⁴ - 1.5x10⁵, while total leukocytes in tiger grouper fish are 2.72 x 10⁴ cells / mm³ [16]. In the treatment of P1, P2, P3, P4, and P5, the total leukocytes of the grouper fish after transportation for 10 hours are still included in the normal range of around 2.2x10⁴ - 2.5x10⁴. In the P2, P3, P4 and P5 treatments were significantly different from the P1 treatment where the total P1 leukocytes were higher than other treatments but remained within the normal range. The difference in total leukocytes between treatments can be influenced by added anesthetic ingredients. The increase in total leukocytes can also be affected by the poor quality of the delivery media. Giving anesthetic ingredients makes the fish more relaxed so that stress conditions can be minimized. Because the condition of water quality is more stable, the total leukocytes also do not increase rapidly.

Water quality is one of the limiting factors in fish transportation [1]. Declining water quality during transportation can cause stress and death in fish [4, 1]. After transporting the grouper abstraction for 10 hours the dissolved oxygen content of the fish decreased to 3.7-3.8 ppm. According to Langkosono [17] the normal oxygen level for groupers in general is > 3.5 ppm. Decreased levels of dissolved oxygen can cause fish to breathe not properly, if it lasts a long time it can result death in fish. This has an effect on the declining yield of the grouper bush life. Optimal dissolved oxygen levels must be maintained during the transportation process by adding pure oxygen in a plastic bag [1]. According to Harmon [4] dissolved oxygen levels are the main limiting levels in the transportation of fish.

Temperature is one of the environmental factors that can cause stress on fish. Large and sudden changes in temperature can cause stress on fish [18]. Fish sensitivity to temperature changes is caused by fish that are poikilothermic that is following the ambient temperature. Rising temperatures can cause metabolic activity in the body of the fish to increase and secretions also increase. The results of these secretions can make water quality decline so that fish can become stressed and lead to death.

The pH of the water in the plastic bag decreased after the grouper fish was transported for 10 hours. The decrease in water pH is caused by increased levels of carbon dioxide (CO₂) in the water due to the process of respiration [1]. Increased levels of CO₂ in water cause the formation of carbonic acid which is a weak acid so that the pH becomes decreased [19]. In all treatments CO₂ levels in the water increased in the range of 7.2-7.4 ppm. Besides CO₂, the decrease in pH can also be caused by the rest of fish metabolism in the form of feces. Stool that is in water can become ammonia so that the water becomes more acidic and pH decreases. The pH range for all treatments was 7.4-7.5. The range is below normal, the normal pH for groupers is 7.8-8.0 [18].

One of the parameters of water quality that was also observed was salinity. There was a decrease in salinity in all treatments, but still within normal limits, ranging between 30-31 ppt. The range is normal, because the general salinity range for grouper is 30-33 ppt [18]. The decrease in salinity in water can be caused by several factors including the administration of anesthetics or the decline in other water quality parameters.

4. Conclusion
The addition of clove oil as an anesthetic affects the number of leucocytes and survival rate of cantang grouper on closed transportation for 10 hours. The highest survival rate of 86.7% is found in P4 treatment with 10 ppm of clove oil. The lowest leucocytes of cantang grouper was found in treatment
P4 which 2.35x10 4 cells / mm 3 with 10 ppm of clove oil. There needs to be further research on the influence and eugenol activity in clove oil so that in the future it can be developed and applied in the world of fisheries, especially in aquaculture.

5. References

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