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by Laksmi Sulmartiwi

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The Effect of Rubber Seed (*Havea brasiliensis*) as Anesthetic on Asian Sea Bass (*Lates calcarifer*, Centropomidae) during Transport

Megananda Paradewi[1], Dhita Maulida Saputri[1], Laksmi Sulmartiwi[2*],
Hari Suprapto[3] and Kriengkrai Satapornvanit[4]

ABSTRACT

The Asian sea bass is one of the most important marine aquaculture species in Indonesia. Transportation is often required for the distribution of sea bass fingerling, but this leads to stress on fish, as reflected by cortisol and blood glucose levels. Stress in fish during transportation can be suppressed using anesthetic substances. This study aims to determine the effectiveness of rubber seed extract as an anesthetic for Asian sea bass fingerling during transportation, with cortisol and blood glucose levels, and survival rate as indicators. The treatments consisted of four concentrations of rubber seed extract (70 ppm, 80 ppm, 90 ppm and 100 ppm), 5 ppm clove oil as a positive control, and water without any anesthetic as a negative control. Results show that rubber seed extract as an anesthetic material has a significant effect ($p<0.05$) in reducing cortisol and blood glucose levels at six hours of transport, with the optimal concentration of 90 $mg \cdot L^{-1}$.

Keywords: Asian sea bass, Cortisol, Glucose, Rubber seed, Survival rate, Transportation

INTRODUCTION

Sea bass is one of the most important aquaculture species in Indonesia. Transportation of sea bass fingerling is an important consideration as it can be costly. Closed transportation systems are reported to be more profitable than open systems due to their higher efficiency, by carrying more fingerlings and by covering longer distances (Junianto, 2003). According to Ross and Ross (2008), problems encountered in transportation include the occurrence of fish stress caused by fluctuating temperature and water chemical content. Fish stress can be evaluated by measuring hematological parameters such as serum cortisol and blood glucose levels (Kubilay and Ulukoy, 2002). Cortisol is a benchmark for determining stress levels and reflects a key mechanism of allostatic control of physiological stress. Plasma cortisol is an important marker used to assess mediators of allostatic loading (McEwen and Wingfield, 2010). Determining stress by cortisol level has been carried out in many fish species such as Atlantic cod, *Gadus morhua* (King and Berlinsky, 2006) and zebrafish, *Danio rerio* (Ramsay *et al*., 2006).

In addition to increases in plasma cortisol, stress typically causes increases in plasma glucose. Catecholamine triggers an increase of glycogenolysis, while cortisol triggers an increase of gluconeogenesis, which can cause glucose to increase (Begg and Pankhurst, 2004).

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[1]Aquaculture Study Program, Faculty of Fisheries and Marine, Airlangga University, Surabaya, Indonesia
[2]Department of Marine, Faculty of Fisheries and Marine, Airlangga University, Surabaya, Indonesia
[3]Department of Fish Health Managament and Aquaculture, Faculty of Fisheries and Marine, Airlangga University, Surabaya, Indonesia
[4]Department of Fishery Biology, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand
* Corresponding author. E-mail address: laksmi-s@fpk.unair.ac.id
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Mortality of fish seed caused by transportation and post-transportation handling is generally high, often reaching 30-40 % (Sulmartiwi, 2014). This obstacle can be managed by using fish anesthetical techniques. Currently, the chemicals MS-222 (Tricaine methosulfate) and Quinaldine (2-4 Methylquinoline) are used as commercial anesthetics for transporting fish (Ross and Ross, 2008). An alternative source of anesthetics is from natural substances, e.g, clove oil, which can be much cheaper than synthetic chemicals (Velisek *et al.*, 2005). Another natural substance which has potential for use as an anesthetic is from the seed of the rubber tree, *Havea brasiliensis*. The major component (17.14 %) of *H. brasiliensis* seeds is the alkaloid linamarin (Selmar *et al*., 1986). Alkaloid compounds in rubber seeds have pharmacological properties as anesthetics (Madziga *et al*., 2010). Alkaloids transmit nerve impulses (conduction blockade) by inhibiting the Na^+ ion delivery through the gates of the Na^+ ion-selective membranes of nerves. The failure of the permeability of the Na^+ ion gate to reduce the depolarization velocity can result in the potential threshold not being achieved. This prevents the action potential from being completed (Madziga *et al*., 2010). Research on the effects of rubber seed extract as an anesthetic during transportation of Asian sea bass fingerling has not previously been reported. Thus, this study was conducted to determine the effects of rubber seed extract on stress and survival of Asian sea bass fingerling and the optimal concentration to be used during transportation.

MATERIALS AND METHODS

Rubber seed extraction

Ten kg of rubber seeds obtained from the company CV. Alam Lestari were dried and ground with a blender, yielding 568 g of dry rubber seed powder. Then, 500 g of the powder was macerated with one liter of ethanol for 24 h; during maceration the mixture was stirred several times. The supernatant from the maceration process was evaporated with a rotary vacuum evaporator at 70 ºC with a speed of 50 rpm. This separated the rubber seed extract from the ethanol (Wildan *et al*., 2013).

Concentration determination

Preliminary experiments tested eight concentrations of the rubber seed extract tested (100 ppm, 150 ppm, 250 ppm, 350 ppm, 450 ppm, 550 ppm, 650 ppm and 750 ppm) on fingerlings similar in size to those used in this study; a concentration of 100 ppm gave the highest survival rate (100 %). Therefore, the concentrations used in this study were 70 ppm, 80 ppm, 90 ppm and 100 ppm. According to Hanggono (2006), a safe concentration of clove oil is 5 ppm or less.

Experimental procedure

Asian sea bass fingerlings (TL 10 cm, weight 18.46±2.16 g) were obtained from Balai Penelitian Budidaya Air Payau (BPBAP) or Brackishwater Aquaculture Research Institute located in Situbondo Regency, East Java Province. Fish were selected based on the desired size for transportation. Fish health status was determined by visually inspecting fish for external deformities and parasites or signs of fungal diseases. Fish were starved for one day before the experiments to reduce metabolic activity and excretion during transportation.

Rubber seed extract (RSE) and clove oil treatments were prepared based on the concentrations previously mentioned, with three replicates per treatment. Ten sea bass were packed in each plastic bag filled with 2 L of water with the designated concentration of anesthetic substance, and packed with oxygen following the procedures of World Wildlife Fund (2015).

In order to avoid error and stress of the fish during transport trials, sampling and repacking, two sets of experimental fish were prepared: one set (with 6 treatments and 3 replicates) for 6 h of transportation, and another set (with 6 treatments and 3 replicates) for 12 h of transportation.

Sedated fish in the plastic bags were observed before placing the bags in styrofoam boxes for transportation. The styrofoam boxes were filled with ice cubes to maintain a temperature between 21-22 °C (Lim *et al*., 2003). Transportation of fish was carried out in the afternoon to avoid a significant air temperature change at midday.

Blood sampling

Blood samples were taken to determine cortisol and glucose levels at four times: pre-transportation, at 6 h during transport, at 12 h during transport, and post-transportation (1 h after transportation). Blood samples were taken from the caudal fin (Hanley *et al*., 2010) using 1 mL syringe. Each blood sample was put in a plain 5 mL vacuum tube and left at room temperature for one hour for the separation of the serum and red blood cells. The blood serum was stored at -20 °C (Sink *et al*., 2008).

Cortisol levels

Cortisol levels in the blood samples were measured by non-competitive type enzyme-linked immunosorbent assay (ELISA) (Fish ELISA Kit; BT Laboratory, Shanghai Crystal Day Biotech Company, China) (Salavati *et al*., 2018).

Glucose levels

Glucose levels in the blood samples were measured using Blood Glucose Monitoring System, Lifescan Europe (One Touch Ultra, Johnson and Johnson) (Eames *et al*., 2010).

Survival rate

Sea bass survival rates for treatment and control groups were determined at 6 and 12 h of transportation, and after fish were exposed to clean water with aeration for one hour (post transport).

Data analysis

Data were analyzed with analysis of variance (ANOVA) to determine the effect of treatments on research variables. Duncan's multiple range test (DMRT) was further used to determine differences between treatments.

RESULTS

Cortisol levels

The mean cortisol level in fish before transportation was 359.35 µg·dL^{-1}. After 6 h of transportation, cortisol increased by 1.5 % (364.79 ±2.53 µg·dL^{-1}) in the negative control, while it decreased in fish transported with various anesthetic treatments (Table 1). The greatest decrease in cortisol was exhibited by fish transported in water treated with 90 ppm of RSE (42.3 %), followed by those in 5 ppm clove oil (38.3 %), 80 ppm of RSE (37.8 %), 70 ppm RSE (36.3 %), and 100 ppm RSE (33.7 %). The effect of rubber seed extract on fish was highly significant at 90 ppm. Treatments with 5 ppm clove oil and 70-80 ppm RSE had similar effects on cortisol levels of the experimental fish.

After another 6 h of transportation (12 h), the levels of cortisol in experimental fish increased by at least 50 %, while in the negative control cortisol decreased by 1.4 % (349.74± 10.00 µg·dL^{-1}). The cortisol levels at 12 h were slightly lower than the pre-transportation levels (from 2.7 to 9.8 % less), except for those in 100 ppm RSE, for which cortisol was 5 % higher.

One hour post transportation, the levels of cortisol in all treatments except the negative control were 0.9 to 2.8 % higher than the original level, i.e., prior to transportation.

Glucose levels

Table 2 shows the blood glucose levels of sea bass fingerlings in various anesthetic treatments at various stages of transport. The glucose level in fish before transportation was 116 mg·dL^{-1}, and increased by 14.9 % (133.33±6.11 mg·dL^{-1}) after 6 h in the negative control, while glucose decreased in all fish groups transported with anesthetic. The greatest decrease in glucose level was exhibited by fish in the 90 ppm RSE treatment (37.63 %), followed by those in 5 ppm clove oil (31.9 %), 80 ppm RSE (27.0 %), 70 ppm RSE (22.1 %), and 100 ppm RSE (15.5 %). The glucose levels for 90 ppm RSE and 5 % clove oil were not significantly different ($p>0.05$).

After another 6 h, i.e., at 12 h of transportation, the levels of glucose increased by 7.5 % (143.00±5.56 mg·dL^{-1}) in the negative control fish, by 53.6 % (121.33±13.57 mg·dL^{-1}) in 5 ppm clove oil, and by 46 % to 62 % in RSE treatments. There was no significant difference among treatments of clove oil and RSE from 70 ppm to 90 ppm. All of the glucose levels at 12 h of transportation were higher than the level before transportation.

One hour post transportation, the glucose levels in all treatments were higher than the pre-transport level, except for 5 ppm clove oil.

After 6 h of transportation, rubber seed extract and clove oil caused a decrease in cortisol and glucose levels in sea bass, at varying levels according to concentration. The effect of 5 ppm clove oil on cortisol levels was not significantly different from the effect of 70-80 ppm RSE, while for glucose, the effect of clove oil was not significantly different from that of 70-90 ppm RSE. The fish were still considered to be in good condition at 6 h, since no mortality was observed.

Survival rate

All fish survived after 6 h transportation in all treatments. After 12 h of transportation, the only mortality observed was at the highest concentraton of RSE (100 ppm), with a survival rate of 93±0.57 %. No further mortality was observed during the post-transportation period.

Table 1. Cortisol levels (µg·dL^{-1}) of Asian sea bass treated with rubber seed extract (RSE) at four concentrations and 5 ppm clove oil, measured before, during and after transport.

Treatment	**Cortisol (µg·dL^{-1}) (mean±SD)**			
	Pre-Transportation	**6 h**	**12 h**	**Post-Transportation**
K- (no treatment)	359.35	364.79±2.53^{d}	349.74±10.00^{b}	359.02±11.07^{a}
K+ (clove oil, 5 ppm)		221.64±1.92^{b}	331.80±8.92^{ab}	362.55±3.37^{a}
P1 (RSE, 70 ppm)		229.01±1.99^{bc}	343.33±11.46^{bc}	363.51±4.83^{a}
P2 (RSE, 80 ppm)		223.56±0.96^{b}	335.97±2.88^{bc}	365.75±2.53^{a}
P3 (RSE, 90 ppm)		207.23±2.88^{a}	324.12±3.37^{a}	368.63±4.40^{a}
P4 (RSE, 100 ppm)		238.29±11.85^{c}	377.28±2.53^{d}	369.59±4.19^{a}

Note: Means±SD in the same column with different superscripts are significantly different ($p<0.05$).

Table 2. Glucose levels (mg·dL^{-1}) of Asian sea bass treated with rubber seed extract (RSE) at four concentrations and clove oil (5 ppm), measured before, during and after transport.

Treatment	**Glucose levels (mg·dL^{-1}) (mean±SD)**			
	Pre-Transportation	**6 h**	**12 h**	**Post-Transportation**
K- (no treatment)	116.00	133.33±6.11^{d}	143.00±5.56^{bc}	114.33 ±9.07^{a}
K+ (clove oil 5 ppm)		79.00 ±6.24^{ab}	121.33±13.57^{a}	116.00 ±4.58^{a}
P1 (RSE, 70 ppm)		90.33±7.09^{bc}	132.00±4.00^{ab}	116.66 ±3.05^{a}
P2 (RSE, 80 ppm)		84.66±6.65^{b}	125.33±3.51^{a}	119.00 ±9.16^{a}
P3 (RSE, 90 ppm)		72.33±6.80^{a}	117.00a±5.56^{a}	121.33 ±3.21^{a}
P4 (RSE, 100 ppm)		98.00±3.00^{c}	158.00±14.73^{c}	123.33 ±4.04^{a}

Note: Means±SD in the same column with different superscripts are significantly different ($p<0.05$).

DISCUSSION

This research shows that rubber seed extract reduces stress levels of sea bass during transport, as evidenced by the decrease in cortisol and glucose levels after 6 h of transportation. According to Ortuño *et al*. (2001), the normal cortisol level in gilthead seabream is approximately 358 µg·dL^{-1} and normal blood glucose level is about 112 mg·dL^{-1} (Ortuño *et al*., 2001). In this study, the pre-transportation levels of cortisol and glucose in the Asian sea bass were 359.35 µg·dL^{-1} and 116 mg·dL^{-1}, respectively, quite similar to the normal levels cited above.

Rubber seed extract worked as an anesthetic during transportation of fish by inhibiting the release of catecholamine and corticosteroid hormones, so that there was a decrease in cortisol and glucose levels in fish. These pharmacological properties are due to alkaloids present in the rubber seed. Septiarusli *et al*. (2012) reported that the use of anesthesia aims to supress metabolic activity as well as reduce stress risk; the use of rubber seed extract provides these effects.

After 12 h of transportation, the cortisol and glucose levels increased in all treatments, although no mortalities were observed except in fish transported in 100 ppm RSE. Lisdiana (2012) stated that stress levels increase adrenocorticotropic hormone (ACTH) secretion, elevating the level of cortisol, which is a stress hormone regulated by the release of corticotropin releasing factor (CRF). CRF stimulates the pituitary gland to release a variety of adrenaline hormones. Strong stress signals are received by the hypothalamus, which increases CRF release, pituitary stimulation and secretion of cortisol by the adrenal gland.

Fish with 90 ppm RSE showed the lowest cortisol level among treatments and controls at 12 h of transportation, and the fish were still in a calm condition. This suggests that the anesthetic material in the media was still able to affect the nervous system.

Fish in 100 ppm RSE showed excessive signs of stress from the anesthesia at 12 h. In addition, the cortisol level in this group at 6 h was highest among the treatments; at 12 h, the level increased significantly further and exceeded the pre-transport level. Increased cortisol indicates that fish in 100 ppm RSE experienced stress, and this was also reflected by 7 % mortality after 12 h of transportation. Rubber seed extract has several compounds that can have negative physiological impacts to fish, and if their concentrations are too high, they can cause stress. Selye (1936) reported that chemical changes in blood occur between 6 and 48 h after fish are anesthesized, as was the case in the increase in cortisol we observed at 12 h of transport.

Post transportation, the negative control group (without rubber seed extract) showed the lowest cortisol levels at 359.02 µg·dL^{-1}, while for treatment groups, cortisol level increased with RSE concentration. Higher concentrations of the anesthetic may cause greater absorption of stress-causing compounds, and their effects were still apparent even after one hour in clean water following transportation. This is in accordance with the findings of Tampubolon (2012), wherein greater concentrations of anesthetic extract given to fish resulted in increased amounts of the active ingredients in the circulatory system.

It was also observed that cortisol and glucose levels increased in parallel across treatments. Glucose levels are affected by cortisol levels, as explained by Martinez-Porchaz *et al*. (2009), who stated that stress hormones associated with cortisol increase glucose production in fish through the process of glucogenesis and glycogenolysis to meet energy needs caused by the stressors. In this study, at 6 and 12 h of fish transportation, the treatment with 90 ppm RSE showed the lowest glucose level among all the treatments, whereas the highest glucose level was with 100 ppm RSE. In the post-transportation period, the lowest glucose level was found in fish transported in non-treated water (negative control), while the highest level was again found for 100 ppm RSE. Increased concentrations of circulating glucocorticoids are induced by stress (Li *et al*., 2009), wherein glucocorticoid increases with elevated stress levels, resulting in increased blood glucose levels to cope with high energy needs during stress.

Rubber seed extract used as an anesthethic during transportation of sea bass at all concentrations tested affected cortisol and blood glucose levels, whereas survival rate was only affected at 100 ppm. Based on this study, the optimum conditions during transport for Asian sea bass to effect the least stress, i.e., lowest cortisol and glucose levels, are to use 90 ppm of rubber seed extract in transport water and limit transportation time to six hours. However, if the cost of the rubber seed extract is high, a concentration of 70 ppm may be more appropriate. This concentration reduced cortisol and glucose levels by 36.3 % and 22.1 % after 6 h, respectively, and at post-transportation, both levels were lower than for 90 ppm RSE.

CONCLUSION

The use of rubber seed extract as an aenesthethic during fish transport affects cortisol levels, blood glucose levels, and survival rate of Asian sea bass. The greatest reduction in cortisol and glucose was observed at 90 ppm RSE. It is worth looking further at the economic implications of the use of rubber seed extract, and whether using 70 ppm is more cost-effective. Further research also needs to be done on the effects of rubber seed extract between 6 and 12 h of transportation of Asian sea bass.

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