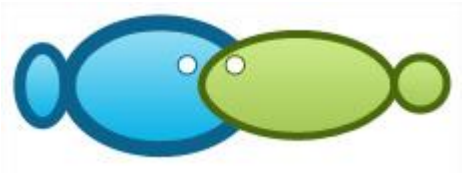

The secondary stres responses of transported glass eel (*Anguilla bicolor bicolor*) with various packing densities

[1,2]Ferdinand H. Taqwa, [2]Eddy Supriyono, [2]Tatag Budiardi, [2]Mia Setiawati, [3]Ridwan Affandi

[1] Department of Aquaculture, Faculty of Agriculture, Sriwijaya University, Indralaya, Indonesia; [2] Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University, Bogor, Indonesia; [3] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University, Bogor, Indonesia. Corresponding author: F. H. Taqwa, ferdinand@fp.unsri.ac.id

Abstract. The purpose of this study was to evaluate the secondary stress responses of transported glass eel (*Anguilla bicolor bicolor*) through a closed system with different densities, which could significantly improve the performance during the recovery period. The glass eel used originated from the fishing activities in the Cimandiri River, Pelabuhan Ratu, West Java (52.70±0.82 mm in length and 0.10±0.02 g in weight). The research was designed with a completely randomized design. The applied treatment was a closed transportation system with different densities of glass eel during 24 hours, i.e. 710, 770, 830, 890 glass eel L^{-1}, respectively. The results showed that glass eel transported with a density of 770 glass eel L^{-1} was able to suppress stress based on survival rate, blood glucose, glycogen, total protein and oxygen consumption rate, thus the gross energy content was still able to support physiological functions during the three days recovery period to maintain homeostatic condition. During the transport and recovery period, the physical and chemical water value were still within the range that supported the survival of glass eel.
Key Words: energy, homeostatic, physiological status, recovery, tropical eel.

Introduction. The demand for live eel products for consumption from East Asia, Europe and America markets is increasing every year, especially from aquaculture (Shiraishi & Crook 2015). This condition triggered the exploitation of eels from different stages and sizes, so the population of eels, including the species *Anguilla bicolor bicolor* has decreased (Kuroki et al 2014; Triyanto et al 2020). The high exploitation of glass eel in nature is a consequence of not yet well-established eel reproduction technology and low survival rate during seed rearing (Mulyani et al 2016). In accordance with this, the Ministry of Marine Affairs and Fisheries Republic of Indonesia has anticipated, by regulation Permen KP No.19/MEN/2012, the illegal export of eel (*Anguilla* spp.) seed by an export ban of less than 150 g per eel from Indonesia. The result of this regulation was a shift in the allocation of captured eel seed to the aquaculture sector in Indonesia.

On the other hand, the sustainability of the production of *Anguilla bicolor bicolor* farming in Indonesia is largely determined by fishing and distribution of glass eel from Pelabuhan Ratu, Sukabumi, West Java. The distribution process of *Anguilla bicolor bicolor* glass eel from the fishing site to various eel cultivation areas generally uses the road or air transport, which takes varying time depending on the distance travelled (Honda et al 2016). The current application of *Anguilla bicolor bicolor* glass eel transport systems in Indonesia has still varied between collectors and sellers, usually referring to the operational technique of transporting fish seeds or shrimp larvae in a closed system, such as in the Lagonoy Gulf region of the Philippines (Nieves & Nolial 2019). This has the potential to cause declining physiological conditions of post-transport glass eel until recovery periods. Unsuitable methods for preparing glass eel for transport can result in higher stress levels, a decrease in recovery capacity after transporting glass eel and a

mortality rate of up to 40% (EFSA 2008). Research results from Appelbaum et al (1998) have shown that *Anguilla rostrata* glass eel and *Anguilla anguilla* elver transported over 24 hours may cause more than 50% mortality until recovery, which is expected in aquaculture because it will have an impact on productivity and losses. Increasing fish density and decreasing water volume are common practical ways of increasing the efficiency of live fish transport, but this has the potential to increase fish stress and reduce water quality dramatically (Singh et al 2004; Kamalam et al 2017). Various studies of increased transport density have shown a higher response to stress that can lead to changes in behaviour, biochemical and physiological conditions, health status, and even higher mortality upon arrival (Chatterjee et al 2006; Hong et al 2019; Vanderzwalmen et al 2021).

Depending on the period of stressor effect, biochemical conditions and physiological status, stress responses in aquatic species can be divided into primary, secondary and tertiary stress status. The secondary stress response is a consequence over a period of physiological loads that can cause physiological disruptions in the form of increasing glucose levels, decreasing glycogen levels (Barton 2002), as well as osmoregulation and hydromineral balance (Taqwa et al 2018b; Jerez-Cepa et al 2021). In addition of being affected by genetic factors, fish quality can also be determined by the minimum stress level starting from pre-transport handling, transportation processes and post-transport recovery (Boerrigter et al 2015; Nieves & Nolial 2019; Vanderzwalmen et al 2021), so that the performance of production will be maximized. Research and scientific publications on density optimization in the transport of wild captured tropical glass eel with a duration of more than 12 hours without water change are currently still very limited (Taqwa et al 2018b; WWFI 2018; Nieves & Nolial 2019; Taufiq-Spj et al 2021). Thus, the innovation of live fish transport technology, which is more effective and efficient, needs to be studied more intensively (Purbosari et al 2019). The aim of this research was to evaluate the secondary stress reponses of *Anguilla bicolor bicolor* glass eel in a closed transportation system with different packaging densities, which continues to support the production performance of glass eel until the recovery period.

Material and Method

Design and research location. A completely randomized design method of various densities of glass eel during 24 hours of closed transportation system, i.e. 710, 770, 830, 890 glass eel L^{-1} was used in this research. The study was carried out in Pelabuhan Ratu, Sukabumi, Bogor and several cities in West Java Province, Indonesia from March to April 2018.

Acclimatization and transportation procedures. The test subjects used were captured glass eel by fishermen from the Cimandiri River, Pelabuhan Ratu, Sukabumi, West Java, with an average length and weight of 52.70±0.82 mm and 0.10±0.02 g, respectively. The captured glass eels were acclimatized for 24 hours at a salinity of 6 g L^{-1} and a medium temperature of 20°C before being transported (Taqwa et al 2018a). Physiological conditions of glass eel in starvation before transportation, i.e., glucose levels 88.67 mg dL^{-1}, glycogen 0.07 mg dL^{-1}, total protein 13.67 mg mL^{-1}, and gross energy 1777.10 cal g^{-1}.

The closed transportation system used a polyethylene (PE) plastic bag measuring 25 cm x 45 cm, double-lined, and the end of the plastic bag was tightly tied with a rubber band to prevent leakage. The range of physical and chemical water values used for the transport process is optimally pursued based on preliminary research by Taqwa et al (2018a,b), i.e., temperature 20.0-20.5°C, pH 6.8-7.0, dissolved oxygen 7.0-7.2 mg L^{-1}, carbon dioxide 6.0-8.0 mg L^{-1}, and ammonia 0.003-0.004 mg L^{-1}. Furthermore, the plastic bag was filled with 0.5 L of prepared water and added with a density of 355, 385, 415 and 445 glass eels that had been acclimatized for each test treatment. The ratio of water to oxygen in the plastic bag used was 1:3. Plastic bags were randomly put in a 75 cm x 42 cm x 32 cm Styrofoam box, in which we applied 650 g of ice cubes in a bottle of

mineral water. The Styrofoam box was then tightly closed and transported for 24 hours by road (Figure 1).

Figure 1. Placement of plastic bags containing glass eel in styrofoam (A) and road transport test (B).

After 24 hours of road transport, each plastic bag containing glass eel was acclimatized in an aquarium recovery unit (80 cm x 45 cm x 40 cm) containing 50 L water with a salinity of 6 g L^{-1} and temperature 29±1°C. All recovery containers were equipped with a recirculation system and a shelter, that added 40 mg L^{-1} of $CaCO_3$. The initial recovery process was done by placing glass eel near the air bubbles on the recovery medium. Dead glass eels were removed from the recovery aquarium and calculated for total mortality. Three days of glass eel recovery period was conditioned with a density of 2 g L^{-1} without feeding. During the recovery period, the management of water quality was carried out by siphoning the remaining feed and feces and exchanging water by 10% every day.

Data collection. For the calculation of the survival rate, the number of glass eels that died on arrival and at the end of the recovery period was recorded. Secondary stress responses of glass eel were observed before transport, at arrival and after three days of recovery period, including glucose, glycogen, total protein, oxygen consumption rate and gross energy. The stunning step of glass eel was performed at a medium temperature of 6°C for approximately one minute prior to the measurement of various secondary stress response parameters. The test sample used to measure these biochemical parameters was in the form of a whole body and partly in a supernatant. Glass eels were grinded, then transferred into an Eppendorf tube and centrifuged at a speed of 15000 g at 5°C for five minutes. Supernatant was separated and placed in a new Eppendorf tube and kept in the refrigerator at -20°C before further analysis.

The whole-body glucose measurements were carried out using a standard kit D (+)- Glucose (Merck, Germany) and a spectrophotometer reading. Determination of the glycogen content of the whole-body glass eel began with the drying of one g of the sample at 50ºC for 12 hours until it became flour. At the final stage, it was analyzed by a spectrophotometer reading. Measurement of the total gross energy of glass eel was performed using the Parr 6200 bomb calorimeter on the test sample, which had previously been dried in the oven at 60°C for 24 hours to determine the dry matter content. The total protein was determined from supernatant sample analysis with phenolic group detection reagents such as the folinic and ciocalteu reagents based on a Lowry's method with a spectrophotometer reading. The metabolic rate was based on the glass eel oxygen utilization rate measured in basal conditions immediately upon arrival and at the end of the three days recovery period.

Measurements of water physical and chemical parameters in plastic bags and recovery medium were carried out prior to transport and on arrival, as well as at the beginning and end of the three days recovery period. The tools used to measure the physical and chemical water value parameters, i.e., temperature, salinity, pH, dissolved oxygen, carbon dioxide and ammonia were digital thermometer, salinometer, pH meter, DO meter and spectrophotometer, consecutively.

Analysis procedure. The survival rate of glass eel refers to the formulation of Luo et al (2013), glucose, glycogen, and gross energy levels, based on the reference protocol of O'Connor et al (2011), Peungvicha et al (1998) and the manual book for the bomb calorimeter Parr 6200, respectively. The analysis of total protein was performed using the Lowry's method (Lowry et al 1951). The formulation for oxygen consumption rate refers to Lukas et al (2017). The measurement technique for water physical and chemical parameters was performed using the protocol from the American Public Health Association (APHA 2012).

Statistic analysis. The data were mostly presented as a mean value with standard deviation, which was processed using a one-way analysis of variance (Anova) using SPSS Ver. 22 software at 5% confidence level. If there was a significant difference between the mean value, a further analysis of the Least Significant Difference test (LSD) was performed (Steel & Torrie 1991). Water physical and chemical data were tabulated based on the lowest and highest range values, analyzed descriptively.

Results. The results showed that the densities of glass eel during 24 hours of the closed transportation system significantly affected the survival and secondary stress responses of *Anguilla bicolor bicolor* glass eel on arrival until the end of the recovery period, based on the content of blood glucose, glycogen, total protein, oxygen consumption and gross energy ($p<0.05$).

Survival rate on arrival and recovery period. The closed transportation system with various densities of glass eel showed that the lower density of glass eel led to a higher survival rate ($p<0.05$), but at a density of 770 L^{-1} was not significantly different with a density of 710 L^{-1}, either on arrival or at the end of the recovery period (Figure 2).

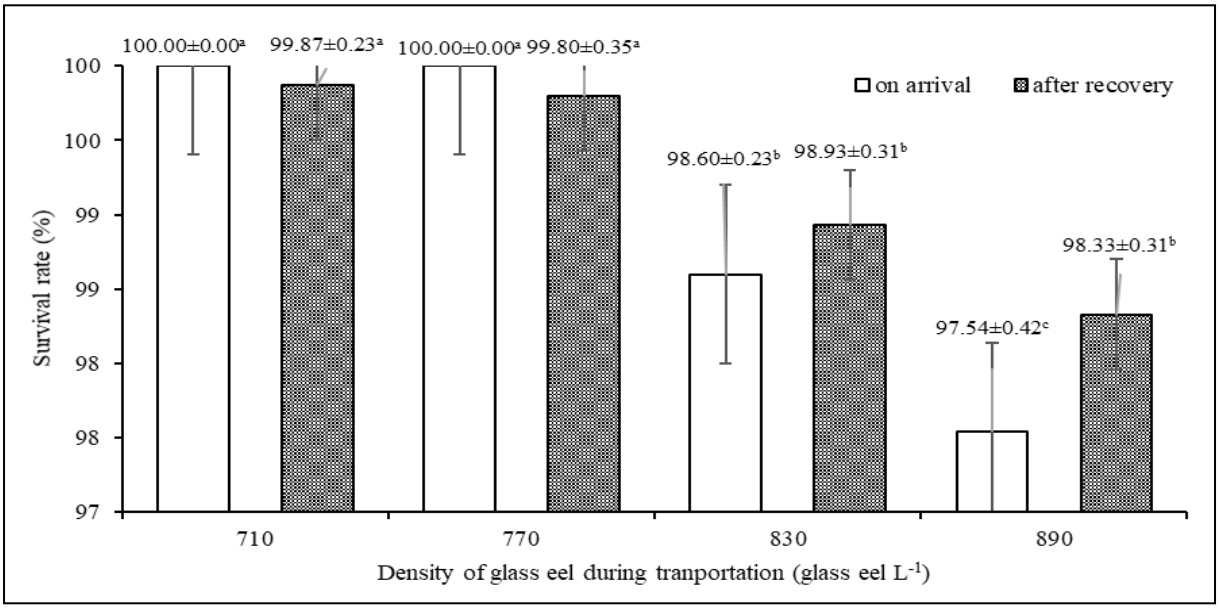

Figure 2. The survival rate of glass eel on arrival and the recovery period. Different superscript letters with the same bar pattern indicated significant differences between treatments ($p<0.05$).

Physical and chemical water values in plastic bag and recovery medium. The range of physical and chemical water value in plastic bags at the time of arrival and during the recovery period of glass eel presented in Table 1. Management of water physical and chemical in recovery medium through daily water exchange attempted in the proper range for maintaining glass eel, so that the range value was relatively homogeneous.

Table 1

The range of physical and chemical water value in plastic bag on arrivals and at the end of the recovery period

Parameter	*Transport densities treatment (glass eel L^{-1})*			
	710	*770*	*830*	*890*
On Arrival				
Temperature (°C)	24.00-24.1	24.1-24.2	24.3-24.4	24.5-24.6
pH (unit)	6.8-7.0	6.8-7.0	6.8-6.9	6.6-6.8
Dissolved oxygen (mg L^{-1})	11.3-11.9	9.3-9.6	6.6-7.7	4.8-5.3
Carbon dioxide (mg L^{-1})	11.10-11.80	11.99-13.98	12.99-15.98	15.98-17.98
Ammonia (mg L^{-1})	0.002-0.007	0.002-0.024	0.028-0.035	0.044-0.053
Recovery Period				
Temperature (°C)	27.8-28.7	28.7-29.0	28.4-28.7	28.3-28.7
pH (unit)	6.6-6.8	6.7-6.8	6.5-6.8	6.6-6.8
Dissolved oxygen (mg L^{-1})	6.9-7.2	6.8-7.2	7.0-7.3	6.8-7.0
Carbon dioxide (mg L^{-1})	7.99-15.98	7.99-13.98	7.99-13.98	7.99-13.99
Ammonia (mg L^{-1})	0.001-0.005	0.001-0.005	0.001-0.005	0.001-0.005

The secondary stress response of glass eel on arrival and recovery period. Although the survival rate of glass eel was generally high until the recovery period, the physiological conditions encountered varied based on the measurement of the test parameters performed. Results showed that glass eels values for 24 hours post-transportation was significantly lower along with the decreasing density of glass eel due to secondary stress responses (Table 2). The performance of glass eel at the end of the recovery period generally showed that levels of glucose, glycogen, total protein, oxygen consumption rate and gross energy were significantly different between treatments ($p<0.05$), except for glycogen levels ($p>0.05$).

Table 2

The secondary stress response of glass eel on arrival and at the end of the recovery period

*Parameter**	*Transport densities treatment (glass eel L^{-1})*			
	710	*770*	*830*	*890*
On Arrival				
Glucose (mg dL^{-1})	126.62 ± 6.07^{a}	130.86 ± 3.04^{ab}	134.52 ± 0.60^{b}	136.64 ± 0.68^{b}
Glycogen (mg dL^{-1})	0.06 ± 0.02^{a}	0.04 ± 0.01^{ab}	0.03 ± 0.01^{b}	0.03 ± 0.01^{b}
Total protein (mg mL^{-1})	11.81 ± 0.39^{a}	12.15 ± 0.10^{ab}	12.35 ± 0.32^{b}	12.58 ± 0.07^{b}
Oxygen consumption rate (mg O_2 g^{-1} h^{-1})	0.171 ± 0.010^{a}	0.258 ± 0.019^{b}	0.317 ± 0.001^{c}	0.364 ± 0.011^{d}
Gross energy (cal g^{-1})	1769.33 ± 15.3^{a}	1761.57 ± 6.62^{a}	1590.47 ± 9.03^{b}	1569.95 ± 6.94^{c}
Recovery Period				
Glucose (mg dL^{-1})	109.88 ± 0.41^{a}	113.36 ± 0.23^{b}	114.98 ± 0.30^{c}	119.68 ± 0.32^{d}
Glycogen (mg dL^{-1})	0.04 ± 0.01^{a}	0.03 ± 0.01^{a}	0.03 ± 0.00^{a}	0.03 ± 0.01^{a}
Total protein (mg mL^{1})	10.92 ± 0.17^{a}	11.28 ± 0.08^{ab}	11.26 ± 0.16^{ab}	11.40 ± 0.36^{b}
Oxygen consumption rate (mg O_2 g^{-1} h^{-1})	0.362 ± 0.011^{a}	0.368 ± 0.002^{a}	0.372 ± 0.001^{a}	0.391 ± 0.002^{b}
Gross energy (cal g^{-1})	107.74 ± 0.42^{a}	106.96 ± 0.20^{ab}	106.82 ± 0.34^{b}	105.52 ± 0.73^{c}

*Different superscript letter in the same row indicated significant difference between treatments ($p<0.05$).

Discussion. The high survival rate (97.54% to 100%) during the transportation test until the recovery period showed that, in addition to the suitability of the applied density, physical and chemical water parameters values were also supported, which were ideal for 24 hours of road transport up to the recovery period. The temperature of water in the plastic bags immediately after unloading were generally within the range of values that did not significantly different (24.0-24.6°C). Lower transport temperatures were generally intended to reduce the metabolic rate so that fish could be transported for a longer period (Islam & Hossain 2013). The results of CO2 and NH_3 measurements were in

a higher value range along with an increase in the density of glass eel in the plastic bag. Increased levels of CO_2 and NH_3 in plastic bags at higher transport densities can lead to higher levels of stress, leading to death (Golombieski et al 2003; Gomes et al 2003; Stieglitz et al 2012; Kamalam et al 2017). King (2009) reported that the tolerable levels of CO_2 during the live fish transport process ranged from 20-30 mg L^{-1}.

The range of dissolved oxygen values in plastic bags during transport increased in the application of a lower density of glass eel during transport. However, the overall dissolved oxygen value was within a reasonable range to support the viability of glass eel until the recovery period. The survival rate of glass eel at the end of the recovery period was significantly different ($p<0.05$), indicating the effect of the transport stress on the physiological condition that was still ongoing, which had an impact on the durability of glass eel for three days of the recovery process. Some points that need to be considered in determining the appropriate fish density during closed transportation system over a long period of time included species, fish weight, water volume, addition of pure oxygen, free space coefficient, temperature, water quality, time of transport, duration of fasting and use of fish sedatives (Balamurugan et al 2016; Kamalam et al 2017; Purbosari et al 2019; Vanderzwalmen et al 2021).

The highest survival rates on arrival until recovery were found in glass eel density treatment of 710 and 770 glass eel L^{-1}. Some factors affecting the survival rate of aquatic organisms with long transport durations without water and oxygen changes were largely dependent on the suitability of the transport system used, acclimatization techniques, density, use of sedatives, duration, temperature, size, stage, and fish resistance to water quality degradation (Lekang 2007; Harmon 2009; Hong et al 2019). In addition, high mortality for live fish transported could be caused by various stressors during fishing, handling, packing, transport, unloading and suboptimal conditions of water quality (Harmon 2009; Tacchi et al 2015; Sampaio & Freire 2016). Taufiq-Spj et al (2021) showed that even though the glass eel stadium can be transported for 23 hours, the ability to survive during recovery is limited to 12 days without feeding.

Biochemical parameters are indicators that can describe the physiological status of aquatic organisms (Ferry-Graham & Gibb 2001), including those related to primary, secondary, and tertiary stress responses (Chatterjee et al 2006). The secondary stress response of transported glass eel has previously been reported to cause changes in osmoregulation performance and body mineral composition in *Anguilla bicolor bicolor* glass eel during transport in a salinity medium of 6 mg L^{-1} (Taqwa et al 2018b). Glucose level of glass eel at a density treatment of 710 glass eel L^{-1} showed the lowest value (109.88-126.62 mg dL^{-1}) and was significantly different from other treatments up to the three days of recovery period. Singh et al (2004) stated that live fish transportation with a higher density could lead to higher levels of stress due to water quality degradation. Fish density in plastic bags is one of the factors that correlates with the fish stress response and varies between species (Costas et al 2008; Pakhira et al 2015; Honryo et al 2018; Kamalam et al 2017). Live fish transportation is a series of activities that cannot be separated in aquaculture, which can cause a variable stress response depending on density, handling, shocks, sound, and poor water conditions (Manuel et al 2014), resulting in more severe physiological disturbances (McEwen & Wingfield 2003; Koolhaas et al 2011) and behavioral changes (Vanderzwalmen et al 2020a,b).

The endocrine system will respond to the stress response of fish during transport until three days of recovery period by releasing cortisol and catecholamine hormones in the bloodstream as a primary response, followed by an increase in glucose levels as a secondary response (Pakhira et al 2015). In most fish, the cortisol hormone peaks at one hour after the stressor arises and returns to the basal point after six hours (Iwama et al 2006), making glucose level measurements (secondary response) more effective at longer observations. Glucose is a source of energy that comes from the glycogenolysis and gluconeogenesis processes that are needed to maintain homeostasis due to stressors (Iwama et al 1992; Baltzegar et al 2014) so that the stressed fish will increase this level (Hong et al 2019). In addition, glucose is the main source and essential substrate for cell metabolism, especially brain cells, so that it is continually needed (Stewart 1991; Hastuti

et al 2003). Thus, the energy requirements for glucose to maintain the homeostatic condition of glass eel due to transport stress until the recovery period must be met for the target cell to function well.

The glucose dynamics were in line with the phenomena that occurs in the oxygen consumption rate of glass eel in basal conditions, both upon arrival and at the end of the recovery period. The different density of glass eel during transport caused a high difference in the dissolved oxygen content in the plastic bag upon arrival (4.8-11.9 mg L^{-1}), which affected the oxygen consumption rate of glass eel during transport. The higher dissolved oxygen content in the plastic bag showed a contrast pattern with the measured oxygen consumption rate of glass eel at basal conditions. This showed that lower glass eel density during transport caused lower energy requirements for basal metabolism, so that the required oxygen consumption would be lower. Degani et al (1989) have shown that the lower basal metabolic rate plays a role in promoting the longer survival of European eel *Anguilla anguilla* in the biological cycle without feed.

The closed transportation system mainly used containers in polyethylene plastic bags, which were filled with water and added with pure oxygen at a ratio of 1:2 for a short time and 1:3 for a longer time (Da Silva et al 2017; Nieves & Nolial 2019; Taufiq-Spj et al 2021). Preparation time for fasting before transport generally ranges from 24 hours to 168 hours (Boerrigter et al 2015). Fish fasting prior to transport is one of the routine methods used to reduce metabolic rate and excretion but fasting over a long period of time can lead to a reduction in fish performance during transport and recovery in new environments (Shrivastava et al 2017). Several studies have shown that the respiratory rate of fish is an indicator that represents the physiological conditions and energy metabolism of fish during the live fish transport process (Xu et al 2017; Saputri et al 2019). Therefore, the varying levels of oxygen consumption in this study can define the energy requirements used to maintain the normal physiological status of *Anguilla bicolor bicolor* glass eel associated with higher stress on increased density during transport.

The highest levels of whole-body glycogen on arrival and at the end of the recovery period were found in glass eel density treatment of 710 eel L^{-1} (0.04 mg dL^{-1} and 0.06 mg dL^{-1}). This result showed that the lower density of glass eel during transport tended to increase the glycogen content of the whole body of glass eel. This condition was similar with Chatterjee et al (2006) that an increase in the density of fish in plastic bags during a closed transportation system of varying duration would result in a significant decrease in glycogen levels and would be very specific for each type of fish. Higher glycogen levels indicated lower stress levels of glass eel, as they reduced the use of glycogen as an energy source. Under conditions of increasing stress on glass eel, glycogen as a source of reserve energy will be converted back to glucose to achieve homeostatic conditions. The breakdown of glycogen into glucose is a continuous response of the catecholamines and cortisol hormone to the physiological functions of fish in maintaining homeostatic during transport and recovery (Barton 2011).

The total protein of glass eel at arrival was significantly lower in glass eel density treatment of 710 eel L^{-1} (11.81 mg mL^{-1}) compared to other treatment (12.15-12.58 mg mL^{-1}). This result has shown that higher stress levels will cause a significant increase in the protein content of aquatic organisms in body fluids. Similar findings have been documented from studies by Lorenzon et al (2007, 2008) and Arjona et al (2009) which have shown that suboptimal transport methods may lead to higher levels of total protein because protein was one of the energy sources used to maintain homeostatic conditions (McEwen & Wingfield 2003). In addition, based on various test results, the protein content of smaller eels was higher than that of larger eels, so that more protein synthesis than fat synthesis was observed in smaller eels (Degani et al 1989). Increased levels of total protein could be used as a biomarker for higher levels of stress in aquatic organisms because it was associated with higher energy requirements and a compensatory response (Lorenzon et al 2007).

The higher total protein of glass eels influenced the reduction of the energy content of post-transport glass eel until the end of the recovery period. The results

showed a significant decrease in the energy content of glass eel at arrival and the end of three days recovery period ($p<0.05$). The lowest energy content found in the highest density of transport treatment (890 glass eel L^{-1}). As shown in the glass eel transport treatment with density of 710 and 770 glass eel L^{-1}, the energy availability of glass eel for the homeostatic was significantly higher in this study. This result showed that the higher density of glass eel during transport would lead to an increase in the energy requirement for basal metabolism, thus reducing the energy available for other biological activities. Furthermore, the energy content of the captured glass eel is strongly influenced by the time or season of fishing, salinity, behaviour, or activity to reach varying distances of estuarine water (Du Colombier et al 2007). According to the results of the post-transport European eel (*Anguilla anguilla*), Boerrigter et al (2013) indicated that eel required an additional energy during the recovery process to adapt to new environmental conditions. Thus, the energy content can be used as an important indicator to determine the portion of energy used in the homeostatic process during the recovery period (Jerez-Cepa et al 2021).

Conclusions. The closed transportation system of *Anguilla bicolor bicolor* glass eel with a density up to 770 glass eel L^{-1} for 24 hours produces better performance, based on the survival rate and secondary stress responses of glass eel until the end of the recovery period. The level of glucose, total protein and oxygen consumption of glass eel increased along with an increase in the density of glass eel in the plastic bag. There are different conditions in higher glycogen and gross energy levels of glass eel with a decrease in the glass eel density during road transport. A similar phenomenon occurs during the recovery period, except that the content of glycogen is not significantly different.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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Authors:
Ferdinand Hukama Taqwa, Sriwijaya University, Agriculture Faculty, Aquaculture Program Study, Indralaya, South Sumatra, Indonesia, e-mail: ferdinand@fp.unsri.ac.id
Eddy Supriyono, Bogor Agricultural University, Fisheries and Marine Sciences Faculty, Aquaculture Department, Agatis Street, Lingkar Kampus IPB Darmaga, Bogor, West Java, Indonesia 16680, e-mail: eddysupriyonoipb@gmail.com
Tatag Budiardi, Bogor Agricultural University, Fisheries and Marine Sciences Faculty, Aquaculture Department, Agatis Street, Lingkar Kampus IPB Darmaga, Bogor, West Java, Indonesia 16680, e-mail: tatagbdp@yahoo.com
Mia Setiawati, Bogor Agricultural University, Fisheries and Marine Sciences Faculty, Aquaculture Department, Agatis Street, Lingkar Kampus IPB Darmaga, Bogor, West Java, Indonesia 16680, e-mail: miasetiawati25@yahoo.com
Ridwan Affandi, Bogor Agricultural University, Fisheries and Marine Sciences Faculty, Aquatic Resources Management Department, Agatis Street, Lingkar Kampus IPB Darmaga, Bogor, West Java, Indonesia 16680, e-mail: affandi_ridwan@yahoo.com

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