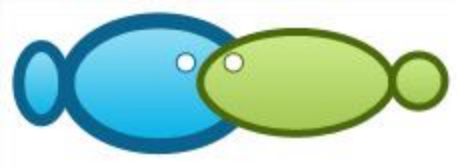

The physiological responses of two-sized juvenile barramundi (*Lates calcarifer*) when duckweed (*Lemna minor*) as a biofilter medium is incorporated into the rearing system

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Abstract. This study evaluated the physiological stress response of juvenile barramundi (*Lates calcarifer*) of different weights; the average weight of 13.34±0.12 g and 26.67±0.14 g reared at a high stocking density of 21.63 kg m^{-3} in two different rearing systems, namely recirculating aquaculture systems (RAS) and integrated recirculating aquaculture systems (IRAS) for 48 days. At the start and at the end of the experiment, the fish were anesthetized, and the blood was drawn to measure the physiological stress parameters such as hemoglobine (Hb), plasma cortisol, glucose, lactate, total thyroxine (T_4), total triiodothyronine (T_3), and plasma ions (Na sodium, K potassium, Cl chlorine). Growth performance and water quality parameters were also monitored throughout the experimental period. Levels of glucose, lactate, T_4, Hb, and Na were significantly higher in large fish, while cortisol and T_3 were significantly lower in large fish. No effects of fish size and different rearing systems were observed on plasma Cl and K. There were no significant differences in water quality parameters except for total nitrogen (TN) and nitrate levels in which TN and nitrate levels were higher in the tank of small-size fish. Weight gain (WG), specific growth rate (SGR), and feed conversion rate (FCR) were significantly influenced by size but not by rearing systems. The results indicated that a high stocking density of 21.63 kg m^{-3} affected the physiological stress responses in a differential manner according to fish size. However, no differences were observed in the experimental fish reared in RAS or IRAS.

Key Words: barramundi culture, growth performance, integrated recirculating aquaculture systems, physiological stress response, stocking density.

Introduction. The intensification of aquaculture development is raising concerns about the environmental consequences and sustainability. All existing aquaculture systems produce waste nutrients, solid wastes, and organic matter. These wastes are either dumped into the environment or treated on-site (Tucker et al 2008). This eventually leads to eutrophication and nitrification of effluent-receiving ecosystems and the pollution of water intended for other uses (Martinez-Porchas & Martinez-Cordova 2012).

A reduction in the volume of wastewater is essential to enhance the sustainability of aquaculture. Hence, recirculation aquaculture systems (RAS) have been proposed for this purpose (Ramirez-Godinez et al 2013; Dauda et al 2019). In these systems, water is mostly reused in the process after undergoing proper treatment, thereby reducing water usage and improving effluent quality. RAS reduces water dependence by 93% compared to the flow-through system (Ramirez-Godinez et al 2013). Moreover, RAS eutrophication potentially results in being 26-38% lower than that of flow-through systems (Nguyen et al 2019). However, some dissolved materials such as C, N, and P cannot be effectively removed in RAS as these dissolved substances are dependent on the intensity of production. Higher C, N, and P concentrations are obtained in more intensive systems with higher feeding rates and fewer water exchanges (Ramli et al 2020).

Although integrated multi-trophic aquaculture (IMTA) plays a significant role in coastal wastewater filtration and bioaccumulation (Klinger & Naylor 2012; Arumagam et al 2018), IMTA requires relevant research as it is highly site and species-specific; one must evaluate factors that affect macroalgae growth and nutrient uptake capacity for extrapolation on a commercial scale. Most of the studies using macroalgae to treat fish effluents used macroalgae stocked at discharge channels or outflow ponds, with no recirculation back to the fish cultivation system (Nelson et al 2010; Bambaranda et al 2019). Therefore, IMTA principles should be applied to the recirculation of fish effluent through macroalgae bio-filters in land-based intensive aquaculture farms. It can also be a more effective tool to increase recirculation practices and establish integrated recirculating aquaculture systems (IRAS) with all the known associated benefits (Robledoe et al 2014). In IRAS, water treatment can be adequately controlled as nutrient availability to the macroalgae can be increased by adjustment of water flow rates and mixing (Schuenhoff et al 2003; Ramli et al 2020). IRAS allows for the directing of the nutrients to an integrated recycling system by plants. Thus, IRAS offers a promising future because of its potential environmental and economic benefits.

As economic and resource conditions change in the short term, together with environmental pressures and the market for the cultivation of high-value species, the utilization of RAS and IRAS is predicted to increase shortly. However, various strategies and procedures adopted can be a source of stress for the fish. Commercial-scale production characterized by high stocking density is directly related to animal comfort and the productivity of fish culture. This could be the determining factor of the economic return on production (Braun et al 2010), due to stocking densities' profound effects on metabolism, growth, and stress associated with fish rearing conditions (Oyarzún et al 2020).

It is well known that physiological processes and metabolic rates are affected by, and depend on the body size. The same is also valid for fish species (Glazier 2008; Urbina & Glover 2013). However, the effect of body size with a duration of noxious stimuli on the cortisol stress response had not been studied extensively in fish species. Studies on Atlantic salmon (*Salmo salar*) have shown a higher plasma cortisol secretion in smolts than in smaller-sized parrs exposed to the same acute stressor (Madaro et al 2016). In a previous preliminary study of European sea bass, *Dicentrarchus labrax* of two different sizes exposed to repeated stressors, smaller fish showed peak plasma cortisol concentration at 4 h, while larger fish at 8 h post-stress (Fatira et al 2014).

As the fish size is highly variable and, in general, not necessarily correlated with age, we posed the following question: what crucial role do the fish size and rearing systems play in physiological responses? Therefore, the study aimed to investigate the effects of the higher stocking density of the two sizes of juvenile barramundi (*Lates calcarifer*) on the growth performance and physiological responses, which were then used to compare the physiological status of the juveniles under two different rearing systems in order to validate the efficacy of IRAS.

Material and Method

Fish and stress factors. The experiment was carried out from July 25th to September 10th 2017 and approved by the Animal Ethics Committee of Curtin University following the Australian Code for the Care and Use of Animals for Scientific Purposes (Approval number: AEC_2013_16). The study was conducted at Curtin Aquatic Research Laboratory, and the juveniles of barramundi used were provided by Challenger Marine Hatchery, Fremantle, Western Australia. Upon the first arrival, the juvenile barramundi used in this experiment were transferred to large storage tanks and reared for 48 days under the temperature of 26^{o}C until reaching an average weight of 26.67 g. Three weeks after the first arrival, the other juveniles were transferred to the large storage tanks and reared for 24 days until reaching an average weight of 13.34 g. The stress responses tested were a higher fish density and two rearing systems. In our previous study, the juveniles were stocked up to 18.75 kg m^{-3} in an IRAS (Ardiansyah & Fotedar 2016). In the present study, the stocking density was increased to 21.63 kg m^{-3}. The effects of the

higher fish density of two sizes reared in different rearing systems were evaluated for the 48-day experimental period.

Experimental design. The experimental fish was divided into two different weight groups; the fish with an average body weight of 13.34±0.12 g, and 26.67±0.14 g. Each weight group was randomly stocked at a density of 21.63 kg m^{-3} in four experimental units of RAS and four experimental units of IRAS. Each unit of the experimental RAS consisted of a fish-rearing tank (400 L circular tank), a waste collection tank (100 L circular plastic drum), and a filtration system that used two external filters (Fluval 406, Hagen, Rome, Italy), whereas the experimental IRAS consisted of a fish-rearing tank (400 L circular tank), a waste collection tank (100 L circular plastic drum) and a bio-filter tank (100 L circular tank) that housed duckweed (*Lemna minor*) as the bio-filter media to replace the external filter.

Both groups of fish were hand-fed twice daily (at 09.00 a.m. and 4.00 p.m.) at the same fixed rate of 2.5% of total body weight per day with a formulated diet. The amount of feed distributed to each rearing tank was recorded after each meal. The proximate composition of the formulated diet was crude protein 45%, crude fat 10%, crude ash 9.3%, and crude fiber 5.8%. The proximate composition of the feed ingredients was estimated using standard methods of AOAC (2000). Moisture was determined by oven drying at 105^{o}C for 22 h. Crude protein was analyzed by the Kjeldahl method while crude lipid was determined using chloroform-methanol extraction. The total ash content was determined by oven incineration at 550°C for 24 h in a muffle furnace. In both rearing systems, the replacement water flow rate was adjusted weekly to the ingested food quantity and water quality conditions.

Water quality parameters were periodically monitored and maintained at a temperature of 26-28^{o}C and a pH of 7.2-8.0, water flow rates of 4.1 L min^{-1}, a TAN of 0.98-1.03 mg L^{-1}, a nitrate nitrogen of 9.93-10.07 mg L^{-1}, a nitrite nitrogen of 0.23-0.25 mg L^{-1}, total nitrogen of 12.41-12.49 mg L^{-1}, orthophosphate of 1.37-1.41 mg L^{-1}, and total phosphorus of 1.85-1.88 mg L^{-1}. Aeration kept dissolved oxygen (DO) levels above 6.0 mg L^{-1} with % saturation of > 90%. At the end of the experiment, and after 24 h of fasting, the fish were weighed in groups and counted. The final mean body weight was calculated.

Growth performance. The total weight of the fish per unit was measured at the beginning and the end of the experimental period. The morphological growth parameters were calculated as follows: weight gain (WG) (g) = final weight (g) - initial weight (g); weight gain (WG) % = 100 x [final weight (g) - initial weight (g)]/initial weight (g); specific growth rate (SGR; % day^{-1}) = 100 x [Ln final weight (g) - Ln initial weight (g)]/time (days); feed conversion ratio (FCR) = feed intake/weight gain (g); survival rate (SR) (%) = 100 x [final number of fish/initial number of fish].

Physiological response analyses. Blood sampling was conducted at the beginning (basal condition) and at the end of the 48-day experimental period. Ten juvenile barramundi of each unit were anesthetized with Aqui-S (60 mg L^{-1}) and heparinized blood was drawn from the caudal vessel to determine hemoglobin (Hb) concentration. Plasma was separated by centrifugation and analyzed immediately for cortisol, glucose, lactate, and total T_4 and T_3 concentrations. The remaining plasma was then stored frozen for determination of plasma ions.

Plasma cortisol was determined by indirect enzyme immunoassay (ELISA) validated for rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) (Tintos et al 2006) and expressed as nmol L^{-1}. Plasma total T_4 and T_3 were measured following the manufacturer's instructions (Pointe Scientific, No T1007-96 and T1005-96, respectively, Lincoln Park, USA). Plasma glucose and lactate levels were measured by the enzymatic colorimetric method using the commercial kit (Pointe Scientific, No. G7521 and L7596 respectively, Lincoln Park, USA) and expressed as mmol L^{-1}. Hemoglobin (Hb) concentration was determined spectrophotometrically at 540 nm (UV-Visible Spectrophotometer, 1201, Shimadzu Co. Ltd., Japan), using the standard

cyanmethemoglobin method recommended by Suhartono et al (2018) and expressed as g dl^{-1}, whereas the concentrations of plasma Cl, K, and Na were determined by Bayer Rapidlab 865 Blood Gas Analyser - direct ion-specific electrode (ISE) (Bayer Diagnostics, Australia).

Statistical analyses. A two-way analysis of variance (ANOVA) was used to examine the significance of any differences between mean concentrations of data obtained, followed by the Tukey HSD test. All of the data obtained were expressed as means±standard error of the mean (SEM). All computations were performed with IBM SPSS Statistics 22.0. Statistical significance was measured at $p \leq 0.05$ in all cases.

Results

Physiological responses. High stocking density induced a significant ($p < 0.05$) increase in plasma cortisol, glucose, and lactate levels over the 48-day experimental period in both rearing systems (Figures 1, 2, and 3).

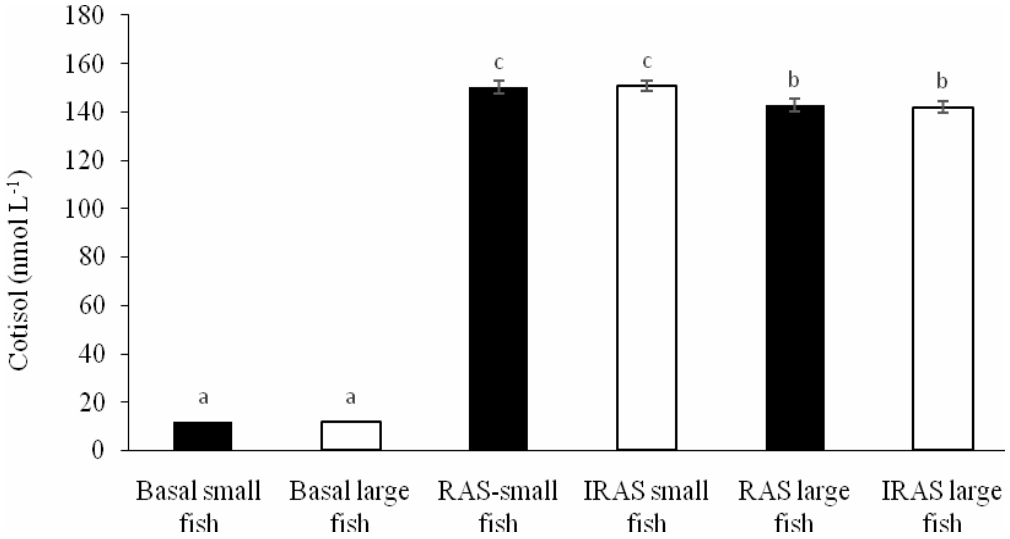

Figure 1. Cortisol concentrations of plasma blood in small and large fish after the 48-day experimental period. Data are shown as the mean±SEM.

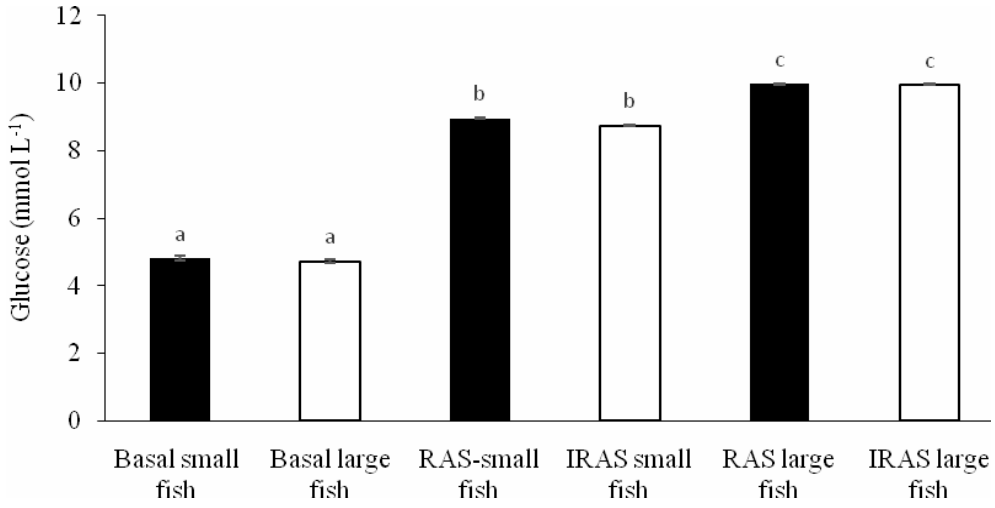

Figure 2. Glucose concentrations of plasma blood in small and large fish after the 48-day experimental period. Data are shown as the mean±SEM.

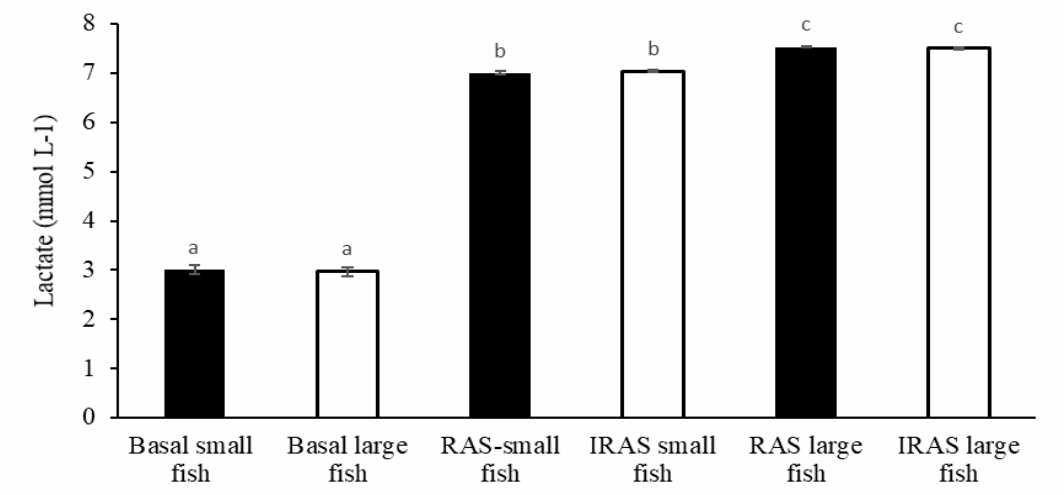

Figure 3. Lactate concentrations of plasma blood in small and large fish after the 48-day experimental period. Data are shown as the mean±SEM.

Differences in the fish size influenced the physiological responses to the stressful conditions, in which large-size fish had higher ($p < 0.05$) glucose and lactate levels but lower ($p < 0.05$) cortisol levels. However, no significant differences were observed as a result of different rearing systems. High stocking density also increased ($p < 0.05$) thyroxine (T_4) levels and decreased triiodothyronine (T_3), Hb, and plasma sodium levels during the 48-day experimental period (Table 1), in which plasma T_4 levels were higher in large-size fish compared to small-size fish, whereas T_3 levels were significantly higher ($p < 0.05$) in small-size fish compared to large-size fish, but no significant differences were observed due to the rearing system ($p > 0.05$).

Table 1

Alterations in thyroid hormones and plasma ions in small and large juvenile barramundi (*L. calcarifer*) when reared at two different rearing systems

Blood parameters	*Basal*		*RAS*		*IRAS*	
	Small fish	*Large fish*	*Small fish*	*Large fish*	*Small fish*	*Large fish*
T_4 (nmol L^{-1})	9.86±0.09^a	10.00±0.07^a	24.26±0.22^b	26.09±0.20^c	24.60±0.21^b	26.21±0.21^b
T_3 (nmol L^{-1})	5.13±0.05^c	5.10±0.06^c	4.86±0.08^b	4.70±0.07^a	4.86±0.07^b	4.71±0.08^a
Na (mmol L^{-1})	164.18±0.78^c	165.96±0.52^c	158.20±1.40^a	161.73±1.01^b	157.71±1.34^a	161.47±0.99^b
K (mmol L^{-1})	4.67±0.07^a	4.66±0.07^a	4.41±0.07^b	4.40±0.07^b	4.41±0.08^b	4.38±0.06^b
Cl (mmol L^{-1})	131.62±0.34^a	132.60±0.42^a	128.04±0.66^b	127.89±0.66^b	127.89±0.64^b	127.91±0.70^b
Hb (g dL^{-1})	9.51±0.07^c	9.66±0.05^c	8.22±0.08^a	8.44±0.07^b	8.22±0.08^a	8.45±0.06^b

Values are expressed as mean±SEM. Different letters in the same row indicate a significant difference at $p < 0.05$.

High stocking density also decreased mean hemoglobin (Hb) and plasma Na levels, in which the small-size fish had lower levels ($p < 0.05$) of Hb and Na compared to the large-size fish (Table 1). Similarly, plasma Cl and K levels significantly decreased ($p < 0.05$) following the 48-day rearing period in both RAS and IRAS, but no significant differences were observed due to either fish size or the rearing systems (Table 1).

Water quality and growth performance. Data showed that there were no significant differences ($p > 0.05$) in water temperature, DO, or pH among treatments of different densities (Table 2). Similarly, the mean TAN, nitrite-N levels, TP, and orthophosphate were similar for all rearing systems, whereas total nitrogen and nitrate-N levels were

significantly higher ($p < 0.05$) in the water tank of the small-size fish reared in RAS and IRAS (Table 2).

Table 2

Summary of water quality assessment in *L. calcarifer* RAS and IRAS over the 48-day experimental period

Water quality parameters	*RAS*		*IRAS*	
	Smaller fish	*Larger fish*	*Smaller fish*	*Larger fish*
Dissolved oxygen (mg L^{-1})	7.73±0.03	7.77±0.04	7.71±0.04	7.75±0.04
pH	7.51±0.01	7.53±0.03	7.49±0.02	7.52±0.04
Temperature (^oC)	26.77±0.22	26.74±0.32	26.78±0.34	26.75±0.26
TAN (mg L^{-1})	1.02±0.07	0.98±0.05	1.03±0.05	0.99±0.07
Nitrate nitrogen (mg L^{-1})	$10.07±0.73^b$	$9.98±0.68^{ab}$	$10.02±0.69^{ab}$	9.93±0.70
Nitrite nitrogen (mg L^{-1})	0.25±0.04	0.23±0.04	0.25±0.03	0.24±0.03
Total nitrogen (mg L^{-1})	12.49±0.80	12.44±0.80	12.46±0.92	12.41±0.92
Orthophosphate (mg L^{-1})	1.40±0.11	1.37±0.12	1.41±0.14	1.37±0.14
Total phosphorus (mg L^{-1})	1.88±0.13	1.85±0.13	1.87±0.11	1.85±0.12

Values are expressed as mean±SEM. Different letters in the same row indicate a significant difference at $p< 0.05$.

In the present study, high stocking density also influences the WG, SGR, and FCR of the experimental fish, in which the small-size fish had higher WG and SGR, and lower FCR ($p > 0.05$) than the large-size fish (Table 3). However, no significant differences ($p > 0.05$) in the mean WG, SGR, and FCR were observed due to the differences in the rearing systems. Similarly, no significant difference ($p > 0.05$) was observed in the SR.

Table 3

Growth of juvenile *L. calcarifer* reared at two different rearing systems for 48 days

Parameters	*RAS*		*IRAS*	
	Smaller fish	*Larger fish*	*Smaller fish*	*Larger fish*
WG	$59.96±0.52^a$	$58.96±0.47^b$	$60.21±0.62^a$	$59.21±1.27^b$
SGR	$0.98±0.01^b$	$0.94±0.01^a$	$0.97±0.02^b$	$0.94±0.01^a$
FCR	$2.19±0.02^a$	$2.31±0.01^b$	$2.23±0.04^a$	$2.31±0.01^b$
SR	100±0.00	100±0.00	99±0.01	99±0.01

Values are expressed as mean±SEM. Different letters in the same row indicate a significant difference at $p < 0.05$.

Discussion. When barramundi juveniles of different sizes and ages were reared at a higher stocking density of 21.63 kg m^{-3} in RAS and IRAS, differences in physiological stress responses and growth performances were observed. Rearing the juveniles at a higher stocking density of 21.63 kg m^{-3} over the 48-day rearing period induced significant changes in endocrine (basal plasma cortisol) and metabolic indicators (basal plasma glucose and lactate), impaired hydromineral balances (basal plasma Na^+, K^+, and Cl^-), and affected growth performances (WG and SGR) of juvenile barramundi. However, differences in the rearing systems did not cause significant differences in endocrine, metabolic indicators, hydromineral balances, and growth performances of the smaller and larger juveniles.

Although baseline cortisol values for barramundi have not been established, our results showed that basal cortisol concentrations of the small and large-size juveniles were in agreement with that previously described by Vo et al (2020) and Ellis et al (2012) in unstressed salmonid fish and most wild teleosts, respectively. After the 48-day experimental period, the elevation of plasma cortisol concentrations was observed in both IRAS and RAS. However, the elevated cortisol concentrations found in this study are considered a mild stress level, as reported by Metz et al (2005) that plasma cortisol concentrations between 138 and 160 nmol L^{-1} can be regarded as a mild stress level.

Furthermore, the elevated concentrations of plasma cortisol observed in this study were near values previously reported by Van der Salm et al (2006) when reared red porgy (*Pagrus pagrus*) at 20 kg m^{-3} in RAS. Findings from European sea bass showed similar responses in the fish reared at 40 kg m^{-3} in RAS (d'Orbcastel et al 2010) and 100 kg m^{-3} in RAS (Sammouth et al 2009). The value was very much higher than the cortisol concentrations reported on European sea bass (72.56 nmol L^{-1}, Tintos et al 2006) but the values were very much lower than the concentrations documented on *D. labrax* after exposure to the combination of trimethoprim and sulfamethoxazole (353 nmol L^{-1}, Yildiz & Altunay 2011). Cortisol increase is typical after stress (Hosseini & Hoseini 2012).

The current results showed that the high stocking density is a stress factor that activates the hypothalamic-pituitary-interrenal (HPI)-axis in the smaller and larger barramundi juvenile. Culturing of juveniles of different sizes and ages under high stocking density induced significant differences in the cortisol stress responses. A similar trend was seen by Koakoski et al (2012), in which the stress response as reflected by plasma cortisol lasts longer in 12- month-old jundia (*Rhamdia quelen*) than the 6-month juveniles. In another experiment on jundia, Barcellos et al (2012) concluded that fish age, instead of weight and size, is the decisive factor for time course differences observed in cortisol stress responses. Rearing fish of different sizes and ages at higher stocking density may lead to a sustained increase in plasma cortisol concentrations (Fatira et al 2014), to which fish may adapt through a down-regulation of the HPI-axis that is induced by the negative feedback mechanism of cortisol (Faught & Vijayan 2018).

The primary response comprises a neuroendocrine response, which involves the release of catecholamine and the activation of the HPI-axis. The corticotrophin-releasing factor (CRF) from the hypothalamus acts on the pituitary to synthesize and release adrenocorticotropic hormones, which subsequently stimulate the synthesis and mobilization of glucocorticoid hormones (cortisol) from the interrenal tissue located in the head kidney (Huntingford et al 2006; Ashley 2007; Iwama 2007). However, the effects of a lack of interaction on rearing systems and fish sizes and cortisol concentrations in this study might be caused by the acclamation of internal tissue to chronic stress induced by crowding over time (Delfosse et al 2021). Another explanation may be that a significant source of energy derived from the fish feed ameliorates the crowding effects and enables the fish to cope with the stressor (Ramsay et al 2006).

Both smaller and larger-size juveniles exhibited elevated glucose and lactate concentrations over the 48-day experimental period. Increased glucose levels of small and large juveniles barramundi in both rearing systems agreed with the previous study in juvenile tilapia reared at densities of up to 33 kg m^{-3} (8.62-10.77 mmol L^{-1}, Kpundeh et al 2013). Changes in metabolic indicators, measured as plasma glucose and lactate, can also be used as general stress indicators in fish (Sopinka et al 2016). The plasma glucose concentration in circulation is a function of its production versus absorption by tissues. Under stressful situations, glucose is generated to provide energy substrates to tissues, in order to deal with the increased energy demand (Teles et al 2007). Lactate has been widely used as a measure of anaerobic metabolism and rapid response to the depletion of tissue energy stores. Our results show that the size of the fish significantly affected the physiological stress response of the fish to high stocking density as indicated by the high levels of plasma glucose and lactate. This result confirms the previous findings in European sea bass, where fish size had significant effects on plasma glucose concentrations (Fatira et al 2014), and in rainbow trout, where the larger fish showed higher lactate concentrations compared to the small-size fish (Goolish 1989). However, in previous studies, the effect of treatment was only performed in a relatively short period (acute stressor). According to Yue et al (2006), the effect of familiarity with the fish is stronger rather than size. Mariette et al (2010) showed the preference for guppy (*Poecilia reticulata*) developed in familiar individuals after they had been placed together over 13 days.

Decreased concentrations of Hb after the 48-days rearing period may be related to the release of immature cells from the hemopoietic tissue into the blood as well as by disturbance of iron metabolism that leads to a decrease in Hb synthesis (Wiciński et al 2020). Additionally, the reduction in Hb levels may be due to the alteration in the

properties of Hb by lowering their affinity for oxygen, increasing the fragility of erythrocytes and reducing deformability. Listiyani et al (2018) reported that a decrease in the Hb concentration of tilapia indicates that the fish's ability to provide sufficient oxygen to the tissues is restricted considerably; this resulted in the reduction of physical activity. In our study, the reduction in Hb was accompanied by hyperglycemia (Tavares-Dias et al 2001) due to cortisol release, which led to increased hepatic glycogenesis (Panase et al 2018) that stimulated the production of glucose (Al-Asgah et al 2015). A decreasing trend in Hb levels was also reported in *Oncorhynchus clarki* stocked at 2998 fish m^{-3} (Bektas & Ayik 2009). When subjected to the stress of capture and handling, decreased Hb levels, increased plasma cortisol, and glucose levels were observed for tambaqui (*Colossoma macropomum*) (Tavares-Dias et al 2001).

Stress associated rises in plasma cortisol concentrations depressed thyroid activity (Walpita et al 2007), increased basal metabolic rates, and reallocated energy away from immunity, growth, and reproduction (Pettersen et al 2016). Previous studies have revealed the effects of cortisol on thyroid hormone metabolism. Cortisol stimulated the conversion of T_4 to T_3 in brook trout (*Salvelinus fontinalis*) liver *in vitro* by ORD (the outer ring deiodination, or 5-deiodination) (Arjona et al 2011). However, in our experiment, significantly elevated cortisol concentrations resulted in elevated plasma T_4 concentrations and decreased plasma T_3. T_4 is a tyrosine compound with 4 atoms of iodine, whereas T_3 is a tyrosine compound with 3 atoms of iodine. Most of the thyroid hormones made are T_4, but T_3 is a more active form of thyroid hormone. Decreased plasma T_3 has been reported in Nile tilapia (*Oreochromis niloticus*) (Van der Geyten et al 2005). Similarly, the hepatic conversion of T_4 to T_3 was also inhibited in *Oncorhynchus mykiss* (Deal & Volkoff 2020), which could well represent a mechanism for adaptation by down-regulating energy expenditure away from growth and reproduction toward physiological functions required for coping with the stressor and to restore homeostasis (Schreck 2000; Van de Pol et al 2017). Our results suggest that plasma T_4 and cortisol concentrations might follow similar patterns during exposure to the highest stocking density. However, the negative correlations between plasma cortisol and T_3 concentrations could be due to the interaction of the hypothalamic-pituitary-adrenal (HPA)-axis and hypothalamic-pituitary-thyroid (HPT)-axis in the brain of the fish is impaired under stressful situations (Geven et al 2006). Elevated cortisol concentrations cause the inhibition of thyroid hormone deiodination. The deiodination involves the enzymatic removal of an iodine atom from the outer (phenolic) ring and the inner (tyrosyl) ring of the iodothyronine molecule. The outer ring deiodination of T_4 is required to yield the most potently bioactive hormone T_3 (Moreno et al 2008).

In teleost fish, glucocorticoids have mineralocorticoid properties, which are involved in the regulation of the water-salt metabolism (Evans 2002). Thyroid hormones play an essential function in the making of ionic mechanisms and osmotic homeostasis in fish (Walpita et al 2007; Arjona et al 2011). The previous finding by Babitha & Peter (2010) revealed that elevated cortisol concentrations reduced plasma T_3 concentrations and altered the parameters of water-salt, carbohydrate, and nitrogen balance in *Clarias gariepinus*. This is also evident in our study; high cortisol concentrations decreased plasma T_3, plasma Na^+, K^+, and Cl^- after the 48-day experimental period. High stocking density induces osmotic disturbances that activate the HPI-axis and elevate plasma cortisol. McCormick et al (2020) reported that cortisol is an osmoregulatory hormone released from the interrenal tissue in response to the stress condition.

Responses to stress conditions are controlled by a complex neuroendocrine system that releases catecholamines (epinephrine, noradrenaline) and cortisol (Conde-Sieira et al 2018), both of which are related to the control of ion regulation (McCormick 2001) and the transport of ions in freshwater fish (Evans 2002). Stress causes a significant rise in cortisol, characteristic of the primary stress response, changed secondary and tertiary responses such as elevated plasma glucose levels, and suppressed thyroid hormones, growth hormones, and plasma ions (Sadoul & Vijayan 2016). The decreasing concentrations of the plasma ions may be due to the damaged epidermis and loss of the integument integrity, and hence there is an increase in the permeability with the loss of the ions or being diffused from water into the fish body,

potentially leading to the failure of osmoregulatory and disturbance of acid-base and electrolyte homeostasis (Tripathi et al 2005). In the present study, plasma concentrations of Na^+ of the smaller juveniles were lower than the larger fish, a condition that indicates higher stress in the larger fish. Stress increases the blood flow in gills and the permeability of the epithelium, resulting in ionic losses in freshwater fish (McDonald 2011).

Juvenile barramundi of different sizes showed different responses to higher stocking density. Growth rates and FCR of the smaller juvenile were significantly better than the larger juveniles. The results agreed with the previous studies of Tran-Duy et al (2008) and Abdel-Tawwab et al (2010) who found that the growth performance of the smaller tilapia (*O. niloticus*) was significantly higher than the larger tilapia. Significant alteration in fish growth and FCR due to differences in fish size or fish weight has been concluded in a number of studies. Booth et al (2008) stated that the growth of the Australian snapper (*Pagrus auratus*) was significantly influenced by fish size. Handeland et al (2008) reported that fish size significantly affected the growth rate and FCRs of Atlantic salmon. Policar et al (2013) found that small-sized pikeperch (*Sander lucioperca*) demonstrated higher SGR compared to the larger ones. Sun & Chen (2014) confirmed growth rate and feed consumption of cobia (*Rachycentron canadum*) were significantly influenced by fish size. It is observed in the present study, that the survival of the juvenile barramundi was not affected by higher stocking density. In comparison with literature (North et al 2006), the mortality rate was very low in both systems. Good water quality can partly explain those results. Throughout the experimental period, water quality parameters in IRAS and RAS were maintained within the acceptable limits for the indoor production of barramundi in RAS (Al-Tawaha et al 2021). Therefore, even though the juvenile of different sizes had different growth performances, the high survival observed in both systems confirmed the efficacy of duckweed incorporated into barramundi IRAS.

Conclusions. The high stocking density of 21.63 kg m^{-3} is a stress factor for the small and large-sized barramundi juveniles reared in both RAS and IRAS. Differences in the juvenile size also impacted on growth and feed conversion ratio. However, there are no differences in stress responses and the growth performance between the juvenile reared in RAS or IRAS. Further research on the role of IRAS in maintaining the carrying capacity of the aquaculture environment to ensure sustainable fish production needs to be carried out.

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