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Effect of Vitamin B Complex and Phosphorus on Stress Resistance and Phosphorus Uptake in Barramundi

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Abstract

Barramundi, the Asian sea bass (*Lates calcarifer* Bloch 1790), is a valuable aquaculture commodity, but seed survival is generally low. This study used a completely randomized design (two factors; 16 treatments; 3 replicates) to examine the effect of adding vitamin B complex and phosphorus to the rearing media on the survival, stress resistance, and phosphorus content of barramundi larvae. Barramundi larvae aged two days were stocked at a density of 25 fish L^{-1} in 40 L green plastic basins (30 L water) and reared for 30 days with natural feed (Chlorella and rotifers). The factor I was vitamin B complex with 4 levels: 0, 75, 150, and 225 mg L^{-1}. Factor II was phosphorus with four levels: 0, 0.5, 1.0, and 1.5 mg L^{-1}. Commercially available B vitamin complex and phosphorus mineral supplements were dissolved in water and added to the larval-rearing media. Analysis of variance showed that the combination of vitamin B complex and phosphorus treatments had a significant effect ($P < 0.01$) on survival, stress resistance, and phosphorus uptake. Survival and phosphorus content in barramundi larvae were highest with a combination of 150 mg L^{-1} vitamin B complex and 1.0 mg L^{-1} phosphorus and lowest in control (0 mg L^{-1} of both additives).

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Introduction

Barramundi (*Lates calcarifer* Bloch 1790), also known as the Asian sea bass, is a high-value euryhaline fish with good potential as an aquaculture commodity because of its relatively fast growth and ease of adaptation to the culture environment, in particular concerning salinity (Wijayanto et al., 2020). One of the determining factors for the success of barramundi culture is the availability of seeds (Thépot et al., 2016). In Indonesia, most barramundi seeds are still wild-caught, and availability is seasonal. The development of seed production in hatcheries is needed to support the sustainable development of the barramundi culture (Wijayanto et al., 2020). Several hatcheries have succeeded in the captive breeding of barramundi, but the survival of larvae to fingerling size is often still very low (Thépot et al., 2016). The larvae and fingerlings up to around 7 cm in length the larvae/fingerlings are easily stressed, leading to high mortality rates, especially at high stocking densities, which can lead to reduced dissolved oxygen concentrations making the fish vulnerable to stress (Noval et al., 2019). Several studies have reported low survival rates from 0 % to around 30 % (Hassan et al., 2021; Noval et al., 2019; Srichanun et al., 2014; Thépot et al., 2016; Wijayanto et al., 2020). High larval mortality rates can be due to problems with environmental factors and poor quality or inappropriate feed (Srichanun et al., 2014; Thépot et al., 2016). Therefore, it is necessary to provide the larvae with suitable high quality feed and a rearing environment that meets their needs (Noval et al., 2019; Srichanun et al., 2014; Thépot et al., 2016; Wijayanto et al., 2020).

One way to increase barramundi larvae's survival and stress resistance is by improving nutrition. Two potential supplements are the vitamin B complex and the mineral phosphorus. The benefits of B vitamins in humans include reducing fatigue, supporting the normal functioning of the immune system. and nervous systems, and red blood cell formation (Shabbir et al., 2020). Similarly, in fish, the B vitamins, especially vitamin B6, play a role in forming red blood cells and synthesizing insulin and hemoglobin. In contrast, vitamin B12 regulates the brain and nervous system (Vilain and Baran, 2016). Research on the use of B vitamins in fish has shown positive effects. For example, supplementation with B vitamins resulted in 100 % survival of Atlantic salmon (*Salmo salar* Linnaeus 1758) (Hemre et al., 2016) with similar results reported in other taxa, including juvenile crabs and fishes (Hansen et al., 2015; Wei et al. 2014).

In addition to vitamin B complex, phosphorus supplementation could also be expected to reduce stress levels in barramundi larvae. Increasing the availability of phosphorus and, thereby, phosphorus uptake can significantly increase the growth performance (e.g., final body weight, specific growth rate, and feeding rate) of juvenile yellow catfish *Pelteobagrus fulvidraco* Richardson 1846 (Tang et al., 2012; Wang et al., 2016), rainbow trout *Oncorhynchus mykiss* Walbaum 1792 (Hernández and Roman, 2016; Morales et al., 2018), Prussian carp *Carassius auratus gibelio* var. Bloch 1782 (Jiang et al. 2012), and Nile tilapia *Oreochromis niloticus* Linnaeus 1758 (Dogan et al. 2015). Furthermore, the provision of phosphorus in a mineral form is expected to increase phosphorus uptake and, thus, the phosphorus content of larvae.

The combination of appropriate levels of supplementation with vitamin B complex and phosphorus should result in improved survival, stress resistance, and phosphorus uptake in barramundi larvae. However, there needs to be more data on phosphorus and B vitamin complex supplementation in barramundi larvae, including the appropriate dosage or quantity of each supplement. Therefore, research is needed to determine the appropriate levels of each supplement for the optimal combination of vitamin B complex and phosphorus when rearing barramundi larvae. This study examined the effect of combining different levels of vitamin B complex and phosphorus supplementation on the survival, stress resistance, and phosphorus content of barramundi (*L. calcarifer*) larvae.

Materials and Methods

Experimental material

The study was carried out at the Takalar Brackish Water Aquaculture Centre. The 48 rearing containers used were 40 L green plastic basins with a length of 30 cm, a width of 50 cm, and a height of 30 cm filled with 30 L of water and equipped with aeration equipment. Before use, the basins were sterilized by soaking in a 10 ppm chlorine solution for 24 hours; the chlorine was then neutralized with a 5 ppm solution of sodium thiosulfate ($Na_2S_2O_3$). The culture media was sea water obtained from the coastal waters in Galesong, Takalar Regency. The water was pumped using a submersible pump, and the pipe's end was covered with a filter bag (20x40 cm). The water was then filtered through a sand filter and stored in a water reservoir.

The experimental animals used were barramundi (*Lates calcarifer*) larvae aged two days after the egg yolk had been exhausted. The larvae were obtained from the spawning of barramundi broodstock at the Takalar Brackish Water Aquaculture Centre (BPBAP). Larvae were stocked at a density of 25 fish L^{-1} and reared for 30 days. Salinity was maintained within the 30-31 ppt range with a temperature range of 29-31°C. The experimental fish were fed with natural feed from Chlorella and rotifers. Both natural feeds were obtained from mass culture systems at the Takalar Brackish Water Aquaculture Centre (BPBAP).

Experimental Feed

The vitamin B complex was a readily available commercial formulation produced by PT. Indosco, Surabaya. The vitamin B complex composition was: Vitamin B1 (10,000 mg); B2 (20,000 mg); B6 (10,000 mg); B12 (10,000 mcg); Ca-d pantothenate (20,000 mg); nicotinic Acid (50,000 mg). The phosphorus mineral supplement used in this research was commercially available Na_2HPO_4. To get the desired phosphorus content in the compound Na_2HPO_4 (2:1:1:4), the mass ratio (46:1:31:64) was considered.

The vitamin B complex and phosphorus supplements were administered through the culture medium (water) on the first day after stocking in doses appropriate for each treatment. The same vitamin B complex and phosphorus doses were administered every 2 days after changing the water until the end of the study. The vitamin B complex and phosphorus, according to each treatment, were mixed with water in a 1 L dipper before being added to the rearing containers. This is according to the research of Nurfadilah (2021), that implemented the same study to evaluate the effect of vitamin B complex on the survival rate, growth, and stress resistance of Nile tilapia (*O. niloticus*).

The study used a completely randomized design (CRD) with two factors. The factor reference refers to Nurfadilah (2021), which uses the same treatment in the research. The factor I was phosphorus, with four levels: 0, 0.5, 1.0, and 1.5 mg L-1. Factor II was a soluble vitamin B complex with four levels: 0, 75, 150, and 225 mg L-1. With 16 treatment combinations and three replicates, there were 48 experimental units **(Table 1)**.

Table 1 Phosphorus (P) and vitamin B complex (B) treatment combinations

Treatment		P	B	Treatment		P	B
Letter	Code	(mg L^{-1})	(mg L^{-1})	Letter	Code	(mg)	(mg)
A	FIB1	0	0	I	F3B1	1.0	0
B	FIB2	0	75	J	F3B2	1.0	75
C	F1B3	0	150	K	F3B3	1.0	150
D	F1B4	0	225	L	F3B4	1.0	225
E	F2B1	0.5	0	M	F4B1	1.5	0
F	F2B2	0.5	75	N	F4B2	1.5	75
G	F2B3	0.5	150	O	F4B3	1.5	150
H	F2B4	0.5	225	P	F4B4	1.5	225

Procedures and variables

The variables observed in this study were larval survival, stress resistance, and the amount of phosphorus absorbed and deposited by the larvae. The survival rate was calculated as the percentage of live barramundi larvae at the end of the experiment. The stress resistance of the barramundi larvae was evaluated using the Cumulative Stress Index (CSI). A stress resistance test was carried out to evaluate the physiological condition of barramundi larvae under the various phosphorous and vitamin B complex treatments. The test followed the methods in Roy et al. (2002) to measure the resistance of barramundi larvae to osmotic shock. At the end of the study, ten barramundi larvae were randomly taken from each experimental unit and placed in a glass beaker filled with 1 L of low-salinity water (0 - 5 ppt). The number of stressed barramundi larvae was observed at 5-minute intervals for one hour. Stress assessment was based on behavioral responses such as abnormal movements of the barramundi larvae or death. Abnormal movements indicative of stress included unusual swimming up and down behavior and spinning around until death occurred. The Cumulative Stress Index (CSI) was calculated using the following formula adapted from Shabbir et al. (2020):

$$[\mathrm{CSI} = \mathrm{D5} + \mathrm{D10} + \mathrm{D15} + \ldots + \mathrm{D60}]$$

Where:

DX = number of larvae exhibiting stress after X minutes when exposed to the osmotic shock test.

The phosphorus content of the barramundi larvae was calculated using the procedures described in the Association of Analytical Chemists (2012) in adapting and summarizing from the original publication reports on two experiments conducted to characterize better the nutritional value of a high-quality fish meal against standard fish byproduct meal in barramundi and the potential for phosphorus supplementation (Schreck and Tort 2016). Supporting data on water quality were collected throughout the research period. Temperature and dissolved oxygen (DO) were measured using a DO meter (YSI 58, Yellow Springs Instrument Co. Inc., USA), and pH was measured using a pH meter (Meterlab PHM 201, Radiometer Analytical, S.A., France). Temperature, pH, and DO were measured twice a day, in the morning (08:00) and afternoon (16:00). Ammonia levels were measured three times at the experiment's start, middle, and end. Water samples were collected in bottles from each experimental unit and taken to the Water Quality Laboratory in the Fisheries Department of the Faculty of Marine Science and Fisheries at Hasanuddin University, where ammonia levels were measured using a spectrophotometer.

Statistical analysis

The data obtained were tabulated in Microsoft Excel 2010 and reported as mean values ± standard deviation (SD). All statistical analyses were carried out in SPSS version 23.0. Analysis of variance (ANOVA) was used to evaluate treatment differences at the 95 % confidence level ($\alpha = 0.05$). When significant differences were found, the W-Tukey posthoc test was applied. The water quality parameters were analyzed descriptively concerning the barramundi larvae survival rates.

Results

Survival rate, stress resistance, and phosphorus uptake

The survival rate, cumulative stress index (CSI), and phosphorus content of the barramundi larvae under the 16 vitamin B complex and phosphorus treatments are shown in **Table 2**. The ANOVA indicated that the vitamin B complex and phosphorus combination had a highly significant effect ($P < 0.01$) on barramundi larval survival rate, CSI, and phosphorus content. The data in Table 1 show that survival was highest under treatment K (150 mg L^{-1} vitamin B complex and 1.0 mg L^{-1} phosphorus), reaching values around 60 %, more than 10 times higher than the lowest survival rate of around 5 %

under the control treatment with no added vitamin B or phosphorus during the study, water quality was in fairly good condition, except for low dissolved oxygen in the morning. Temperatures ranged between 27 – 32°C, salinity 28 - 35 ppt, pH 6.9 – 8.9, dissolved oxygen 2.1 – 5.6 ppm, and ammonia 0.002 ppm.

Table 2 The survival rate, cumulative stress index (CSI), and phosphorus content of barramundi (*Lates calcarifer*) larvae under vitamin B complex and phosphorus treatments.

Vitamin B Complex and Phosphorus Treatment (mg L^{-1})		Survival Rate (%)	Cumulative Stress Index (CSI)	Phosphorus Content (%)
A	0 and 0	$5.06^{j} \pm 2.095$	$101.333^{a} \pm 0.577$	$0.365^{e} \pm 0.111$
B	75 and 0	$24.62^{ghi} \pm 1.776$	$93.333^{abcde} \pm 1.52$	$0.377^{e} \pm 0.034$
C	150 and 0	$28.93^{ij} \pm 1.638$	$87.333^{cdefgh} \pm 3.512$	$0.705^{b} \pm 0.034$
D	225 and 0	$15.06^{cde} \pm 0.833$	$91^{abcdef} \pm 6.245$	$0.361^{e} \pm 0.031$
E	0 and 0.5	$17.91^{efg} \pm 1.646$	$95.667^{abcd} \pm 2.082$	$0.548^{c} \pm 0.027$
F	75 and 0.5	$30^{de} \pm 2.800$	$83.333^{efgh} \pm 2.517$	$0.656^{b} \pm 0.010$
G	150 and 0.5	$32.35^{bc} \pm 0.758$	$78.333^{h} \pm 3.055$	$0.770^{a} \pm 0.029$
H	225 and 0.5	$19.95^{cde} \pm 2.694$	$89^{bcdefg} \pm 2.000$	$0.384^{e} \pm 0.039$
I	0 and 1.0	$10.93^{def} \pm 1.638$	$96.333^{abcd} \pm 3.055$	$0.213^{f} \pm 0.077$
J	75 and 1.0	$38.26^{cde} \pm 1.010$	$81.33^{fgh} \pm 5.100$	$0.382^{e} \pm 0.077$
K	150 and 1.0	$59.82^{a} \pm 3.041$	$78.667^{gh} \pm 3.512$	$0.781^{a} \pm 0.016$
L	225 and 1.0	$21.37^{b} \pm 0.658$	$97^{abc} \pm 2.646$	$0.513^{c} \pm 0.064$
M	0 and 1.5	$34.57^{hi} \pm 1.150$	$99^{ab} \pm 2.646$	$0.420^{d} \pm 0.077$
N	75 and 1.5	$30.8^{gh} \pm 0.611$	$88^{cdefgh} \pm 3.606$	$0.461^{d} \pm 0.214$
O	150 and 1.5	$45.06^{fgh} \pm 8.331$	$78^{h} \pm 5.196$	$0.724^{a} \pm 0.027$
P	225 and 1.5	$13.86^{hi} \pm 2.203$	$86.333^{defgh} \pm 3.786$	$0.444^{d} \pm 0.043$

Note: in each column different superscript letters indicate significantly different mean values at the 95 % confidence level ($P < 0.05$)

The cumulative stress index (CSI) data in **Table 2** show that the stress levels are lowest and therefore stress resistance highest under treatments G, K and O, which were not significantly different from each other. All three treatments had 150 mg L^{-1} B vitamin B complex with phosphorus levels of 0.5, 1.0 and 1.5 mg L^{-1}, respectively. The highest CSI and therefore the lowest stress resistance occurred under the control treatment A with no additives. The increased stress resistance associated with vitamin B complex was evident at 75 mg L^{-1}, highest at 150 mg L^{-1} and much lower at the highest rate of 225 mg L^{-1}, while all three doses of phosphorus had a similar positive effect.

The treatments had an effect on phosphorus intake, as evidenced by the significant differences ($P < 0.05$) in the phosphorus content of the barramundi larvae at the end of the study (**Table 2**). Barramundi larvae showed an increase in phosphorus content with increasing doses of phosphorus and vitamin B complex up to 1.5 mg and 150 mg L^{-1}, respectively. The highest phosphorus content in barramundi larvae was 0.78 %, obtained under treatment K with the combination of 150 mg L^{-1} vitamin B complex and 1.0 mg L^{-1} phosphorus. The lowest phosphorus content was 0.21 % under treatment A, with no additives.

Water quality

The physical and chemical water quality parameters measured during the experiment (**Table 3**) did not differ significantly between the 16 vitamin B complex and phosphorus enrichment treatments. Throughout the study, the parameters remained within the ranges considered suitable for barramundi larval rearing.

Ammonia

Ammonia causes stress and damages gills and other tissues, even in small amounts. Fish exposed to low levels of ammonia over time are more susceptible to bacterial infections, have poor growth, and will not tolerate routine handling as well as they otherwise would. Ammonia is a killer in higher concentrations, and many unexplained production losses have likely been caused by ammonia. But in this research, the

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average range of ammonia is in good condition to barramundi larvae between 0.002 – 0.005 ppm.

Table 3 Water quality parameters were measured in the barramundi larvae rearing units during the 30-day experimental period.

Parameter	Observed Range	Recommended Range*
Temperature (°C)	27 - 32	28.6 – 32.2
pH	6.9 – 8.9	7 – 9
Salinity (ppt)	28 - 35	
Dissolved oxygen (ppm)	2.1 – 5.6	> 4.0
Ammonia (ppm)	0.002 – 0.005	< 0.1

Discussion

The results indicate that providing barramundi larvae with a combination of phosphorus and vitamin B complex in appropriate dosages can improve the phosphorus content, and that a higher phosphorus content was associated with an improvement in the performance and quality of barramundi larvae, as evidenced by the results of the stress test. The very low survival rate of barramundi larvae in the control treatment A, with all supplementation treatments showing some increase in survival, most likely indicates a deficiency of these nutrients in the larvae.

Stressors can be external, such as environmental conditions, or internal such as injury, disease, or nutritional status. The ability to withstand and recover from stress and stress-induced health problems depends significantly on the condition of an organism, in particular the energy balance and metabolic processes (Rahmi and Ramses, 2017; Tang et al., 2012).

The B vitamins and phosphorus play essential roles in fish metabolic processes, while deficiencies will cause abnormal symptoms (Thépot et al., 2016; Tort, 2011; Vilain and Baran, 2016; Waagbø, 2010; Wang et al., 2021; Wang et al., 2016; Wei et al., 2014); these roles likely contributed to the observed the positive effect of supplementation. Vitamin B deficiency symptoms can include poor appetite, low feed efficiency, and severe digestive problems weight loss, while phosphorus deficiency can result in a loss of or low appetite; these effects can cause weakness and elevated mortality (Thépot et al., 2016; Tort, 2011; Vilain and Baran, 2016). Furthermore, the vitamin B complex also plays a role in antibody production, so the absorption of vitamin B complex by the larvae could improve immunity and resistance to stress as well as disease (Thépot et al., 2016; Tort, 2011).

The vitamin B complex functions as a coenzyme that helps the fish to obtain energy from food (Wijayanto et al., 2020). Thiamine (B1), in particular, aids in releasing energy from food and promotes appetite. Riboflavin (B2) also aids in releasing energy from food and is important for growth, cell development, and the proper functioning of cells within the body. Niacin (B3) plays a role in energy production and other crucial cell functions. Pyridoxine (B6) is crucial for protein metabolism, the production of red blood cells, and the transmission of nerve signals. The amino acid folate, often known as folic acid, aids in the digestion of proteins, encourages the production of red blood cells, and lowers the incidence of spinal abnormalities; Biotin aids in the release of energy from carbohydrates in food in the metabolism of lipids, proteins, carbohydrates; and Pantothenic acid (B5) in involved in energy and hormone production and the metabolism of lipids, proteins, and carbohydrate from food. Cyanocobalamin (B12) plays a role in developing genetic material, producing normal red blood cells, and maintaining the nervous system (Thépot et al., 2016; Vilain and Baran, 2016; Tort, 2011). In this study, the ability of the larvae to survive with vitamin B complex supplementation, alone and in combination with phosphorus, is thought to be due to positive effects on metabolic processes, in particular, an increased energy availability.

In particular, phosphorus plays essential roles in muscles and energy metabolism, carbohydrate metabolism, nervous tissue metabolism, regulating amino acids, normalizing blood chemistry, the transport of fatty acids and other lipids, the formation of protein, nucleic acids, and cell membranes; maintaining the balance of hormone production and functions; bone mineralization to form hard tissues, in particular the skeleton and other structural components in the fish body (Waagbø, 2010; Wang et al., 2021; Wang et al., 2016; Wei et al., 2014). However, fish cannot absorb phosphorus from water because the concentration of phosphorus dissolved in water is generally very low (Wang et al., 2016). Phosphorus deficiency affects not only hard tissue, and in humans, is responsible for malformations causing rickets but also affects various intermediate metabolism aspects, thereby affecting weight gain and feed conversion (Tort, 2011; Waagbø, 2010). Signs of phosphorus deficiency have been found in fish, especially bone abnormalities but also loss of appetite; the abnormalities can include curved spines, softened bones, reduced head/skull size, and other skeletal deformities, and in extreme cases, the deleterious effects on bone formation can lead to the death of the fish affected (Tort, 2011; Wei et al., 2014). In some fish species, phosphorus deficiency is characterized by slow growth, poor feed efficiency, and stunted bone mineralization (Waagbø, 2010; Wang et al., 2021; Wang et al., 2016; Wei et al., 2014). Conversely, excess phosphorus intake in the diet or through the gills is also deleterious to fish health (Vilain and Baran, 2016). In this study, phosphorus also appears to be a key factor in improving larval quality; furthermore, phosphorus assists in activating B vitamins through the attachment of phosphate groups (Thépot et al., 2016), the positive interaction between the two additives could be expected.

The high survival rate of barramundi larvae under treatment K indicates that the combination of 150 mg L^{-1} vitamin B complex and 1.0 mg L^{-1} phosphorus is optimal or very close to the optimum value of both additives for promoting barramundi larval survival. For each of the two additives, this combination represents the middle value in treatments tested, indicating that higher or lower values of each additive are less effective, alone or in combination. In these conditions, Singh (2016) in Nurfadilah (2021) stated that those treatments play a role in improving the function of the adrenal glands that manage stress. If the stress can be overcome, the bodywork system in fish larvae will return to normal, and larvae can exist to live and grow (Rehman et al., 2017).

In conclusion, adding a combination of vitamin B complex and phosphorus to the rearing medium (water) significantly affected the survival, stress resistance, and phosphorus uptake of barramundi (*Lates calcarifer*) larvae. The combination of 150 mg L^{-1} vitamin B complex and 1 mg L^{-1} phosphorus (treatment K) gave the best results overall, with the highest survival rate (around 60 %) and high-stress resistance and phosphorus uptake. The control treatment (no vitamin B complex or phosphorus intake) performed poorly on all three parameters, with a low survival rate (≈ 5 %), poor stress resistance, and low phosphorus content. All other treatments performed better than the control but less well than treatment K on one or more of the three variables measured, indicating that both higher and lower levels of the vitamin B complex (75 and 225 mg L^{-1}) and phosphorus (0.5 and 1.5 mg L^{-1}) enrichment were less effective. It can be concluded that the combination of 150 mg L^{-1} vitamin B complex and 1 mg L^{-1} phosphorus is effective and probably close to optimal for the rearing of barramundi larvae.

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