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EFFECT OF DIETARY PHOSPHORUS LEVEL ON DEFENSE SYSTEM PARAMETERS AND LIVER HSP70 CONCENTRATION IN JUVENILE JAPANESE FLOUNDER, *Paralichthys olivaceus*

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Abstract

A feeding trail was performed to investigate the effect of dietary phosphorus (P) level on defense system in juvenile Japanese flounder. Calcium mono phosphate was used as dietary inorganic-P source. Three semi-purified diets with 3 levels of P (6, 10 and 18 g/kg diet, respectively) were prepared and fed to juveniles (average initial body weight, means ± S.E., 7.3 ± 0.3 g) for 30 days. After termination of the feeding trail, growth parameters were calculated, and mucus lysozyme activity, mucus bactericidal activity and the levels of heat shock protein 70 family (HSP70s) in liver were measured. Results indicated that final weight, weight gain and feed efficiency significantly improved with increasing dietary P level, and the highest growth parameters were obtained from the fish fed diet containing 18 g/kg P. Mucus lysozyme activity and mucus bactericidal activity were significantly improved with the increasing dietary P level. The highest level of HSP70s in liver (35.97 ng/mg protein) was observed on fish fed the diet containing 18g/kg P, and it was 2 and 2.6 folds higher (P<0.05) than those fish fed the diet with 10 and 6 g/kg P, respectively. In conclusion, P deficiency has significant effect on growth and measured defense system parameters, therefore, the dietary inclusion of P might affect the defense system and stress tolerance of juvenile Japanese flounder. Highest level of mucus lysozyme activity, HSP70s in liver, and bactericidal activities with reduced survival rate

of *E. coli* was observed in fish fed diets incorporated with 1.8% P, that also maintained best growth performance in Japanese flounder.

Keywords: Dietary phosphorus, mucus lysozyme activity, mucus bactericidal activity, HSP70s, growth, *Paralichthys olivaceus*

Introduction

Phosphorus (P) is one of the nutritionally important minerals for fish, but it is also a major nutrient for microbial activity in the aquatic environment, and can contribute to excessive algae and macrophyte growth in effluent waters (Pillay, 1992; Bureau & Cho, 1999; Storebakken et al., 2000). In recent years, therefore, there have been considerable efforts to reduce P concentration in commercial fish feeds based on species-specific minimum P requirement levels. One of the most common practises to decrease P level from the aquafeeds is to replace fishmeal by a single or combinations of lower P containing ingredients, mainly by plant proteins. However organic P (phytate) from dietary plant-based ingredients is not readily available for fish (Lall, 2002) therefore, this approach might occasionally cause P deficiency. If the P deficiency is minor, fish size is bigger or duration of the deficiency is not long enough to cause external malformations such as dark skin coloration or folded operculum, it might not be possible to detect the effect of deficiency to the fish, particularly in the early stages. However, this marginal inadequacy in dietary P level might still be harmful to the fish influencing the defense system and/or stress tolerance, since P is a required macromineral for various metabolic processes and functions in fish (Lovell, 1989; Tacon, 1990; NRC, 1993; Roy & Lall, 2003, Uyan et al., 2007).Therefore, the effect of dietary P deficiency on fish health must also be considered beside the external or visually detectible deficiency signs appear since the feed should be adequate not only for normal growth but also an intact immune defence (Jokinen et al., 2003).

The effects of P deficiency on immune functions or defense system of fish have not been studied well. Eya & Lovell (1998) found that, dietary P level influenced antibody production of channel catfish after challenging with a virulent strain of *Edwardsiella ictaluri*, and mortality of challenged fish increased when dietary P content decreased. Jokinen et al. (2003) concluded that P deficiency has only minor effects on the immune parameters in whitefish, and the practical aquafeed with P contents sufficient for normal growth does not compromise immune functions of this species. In the case of Japanese flounder, no study is available on the effect of P deficiency on defense system.

The synthesis of heat shock protein (HSP) is a general primary cellular event which occurs during both exogenous and endogenous stress situation, and has an ability of mediate misfolded or denatured functional proteins caused by various stressors in the cell (Iwama et al., 1999). Therefore, considering dietary P deficiency is a nutritional stress, tissue HSP level might be affected, and be an indicator demonstrating the potential stress tolerance of the fish under dietary P deficiency.

Therefore, present study was designed to demonstrate the effect of dietary P deficiency on growth, primary defense system parameters, namely mucus lysozyme activity and mucus bactericidal activity, and liver HSP level as a reference of potential stress tolerance of juvenile Japanese flounder.

Material and Method

Test Diets

Three semi-purified diets containing 6, 10 and 18 g/kg P was tested (Table 1) as deficient (diet 1), sub-requirement or marginal deficiency (diet 2) and sufficient (diet 3) groups in dietary P level. All diets contained 55% crude protein and 13% crude lipid. Defatted krill meal, defatted tuna muscle by-product powder and soybean protein isolate served as protein sources. Crystalline amino acid mixture (Methionine, tryptophan and taurine) was added to maintain the optimum amino acid level in test diet. The lipid sources were pollock liver oil. The P levels in test diets were obtained by adding sufficient amount of inorganic-P (mono calcium phosphate). Calcium carbonate was used as Ca source.

Table 1: Ingredient composition and proximate analysis of the experimental diets.

	Diets		
Ingredients (%)	1	2	3
Defatted krill meal	15.0	15.0	15.0
Defatted TMP[a]	15.0	15.0	15.0
Soybean protein isolate	27.0	27.0	27.0
Amino acid mixture[b]	2.5	2.5	2.5
Pollock liver oil[c]	11.0	11.0	11.0
HUFA[d]	1.0	1.0	1.0
Starch	4.0	4.0	4.0
Dextrin	4.0	4.0	4.0
Vitamin Mix[e]	4.0	4.0	4.0
Vitamin C ester[f]	0.1	0.1	0.1
Mineral Mix[g]	4.0	4.0	4.0
Calcium mono phosphate	0.0	2.0	4.0
$CaCO_3$	1.5	0.7	0.0
Activated gluten	5.0	5.0	5.0
Cellulose	5.9	4.7	3.4
Proximate composition (% in DM)			
Protein	55.3	55.6	55.4
Lipid	13.1	13.1	13.3
Total P	0.6	1.0	1.8
Total Ca	**0.8**	**0.8**	**0.8**
Crude ash	7.2	8.3	9.4

[a]Tuna muscle by-product powder; Marusho Inc., Shimizu, Shizuoka, Japan.
[b]Amino acid mixture (g/kg diet): Methionine 10; Tryptophan 5; Taurine 10. Ajinomoto Co. Inc., Japan.
[c]Riken Vitamin, Tokyo, Japan.
[d]Poweash A, (Tuna oil ethylester mixture; composition (%): DHA; 40, EPA; 20, lecithin; 5, 14:0, 1.5; 16:0, 3.1; 16:1, 2.8; 18:0, 1.0; 18:1, 3.0; 18:2, 2.6; 18:3, 0.2; 18:4ω3, 3.0; 20:1, 1.4; 20:4ω3, 0.9; 20:5ω3, 22.9; 20:4ω6, 4.8; 22:4, 2.2; 22:5ω3, 3.4; 22:6ω3, 43.9; 24:1, 1.2; others, 2.1. Oriental Yeast Co. Ltd., Tokyo, Japan.

[e]Vitamin mixture (g/kg diet): ρ-Aminobenzoic acid 0.67; Biotin 0.01; Inositol 6.68; Nicotinic acid 1.30; Ca-pantothenate 0.47; Pyridoxine-HCl 0.08; Riboflovin 0.33; Thiamin – HCl 0.10; Menadione 0.08; Vitamin A-palmitate 0.32; α-tocopherol 0.67; Cyanocobalamin 0.46; Calciferol 0.02; Folic acid 0.03 and Choline chloride 13.65.
[f]L-Ascorbyl-2-phosphate-Mg
[g]Mineral mixture (g/kg diet): KCl 1.8; MgSO4·7H2O 5.1; Fe-citrate 1.1; Ca-lactate 12.1; AlCl3·6H2O 0.006; ZnSO4·7H2O 0.132; CuCl2 0.005; MnSO4·4H2O 0.04; K(IO3)2 0.006; CoSO4·7H2O 0.04.

All dry ingredients were well mixed for 30 min in a food mixer. Then, previously blended lipid sources were added and mixed for 15 min. Finally, water (35% of the dry weight of ingredients) was added, and resulting mash was mixed for 15 min. The pH of the diets was adjusted to 7.0–7.5 with 4 N sodium hydroxide. The mixture was cold extruded to form 1.2 – 2.2 mm diameter pellets, and pellets were then dried in a mechanical convection oven (DK 400, Yamato Scientific, Japan) at 60 ºC for 30-65 min to lower moisture content to 10%. The experimental diets were stored at −30°C until used.

Fish and Feeding Protocol
Juvenile Japanese flounder *Paralichthys olivaceus* were obtained from the commercial hatchery, Matsumoto Suisan, Miyazaki, Japan, and reared in a 500-L tank at Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University where the trail was performed in May 2006. Prior to the feeding trial, all fish were acclimatized to the indoor rearing conditions for 2 weeks and fed commercial pellets (Higashimaru, Foods, Kagoshima, Japan). At the start of the feeding trial, the juveniles (initial body weight 7.3 ± 0.3 g) were stocked in 9 100-L polycarbonate tanks (filled with 80 l of water) with 15 fish per tank in triplicates per treatment. The juveniles were given their respective test diets at apparent satiation level at 8:00 and 16:00 h for 30 days. Uneaten diet was collected and dried to determine the feed intake. Fecal matter was removed 1 h after feeding by siphoning off the water. The water flow of the tank was 1.5 L/min and a photoperiod of 12 h light/12 h dark was maintained throughout the experiment. The water temperature was 26.8 ± 1.5 ºC (mean ± S.E.) throughout the trial. The pH and salinity of the water in the tanks during the study period was 8.2 ± 0.14 and 33.6 ± 0.19 ppt, respectively.

At the end of the feeding trial, fish were starved for 24 h. At the sampling, two fish were randomly chosen from each dietary replicate tank (six fish per treatment) and immediately dissected to collect their livers. After dissection, fish were bulk weighed considering the dissected liver weights. Livers were separately stored at -80 ºC until analysis was performed. Other fish were externally examined to identify the malformations.

Collection of Skin Mucus
Before dissection, skin mucus from each fish with a constant area on the body surface (200 mm^2) was collected by a small piece of sterilized cotton, and immediately suspended in 1 ml of phosphate buffer saline (PBS, pH=7.2) and centrifuged (2000 × *g*, 10 min, 4 ºC) (Kakuta et al., 1996). The mucus samples were stored at -80 ºC until analysis was performed.

Determination of mucus lysozyme activity
Lysozyme activity of mucus was determined with turbidimetric assays (Takahashi et al., 1986). Test mucus (0.5ml) was added to 2.5 ml of the suspension of *Micrococcus lysodeikticus* in a 5 mM sodium phosphate buffer (pH 6.2). The reactions were carried out at 25ºC, and absorbance

at 520 nm was measured after 1 and 20 min. The unit of enzyme activity was defined as the amount of enzyme that caused a decrease in absorbance of 0.001 per min.

Determination of mucus bactericidal activity

Escherichia coli strain IAM1239 was used for the assay of mucus bactericidal activity. The bacteria were incubated on Trypto-Soya agar (Nissui) at 25ºC for 24 h. The mucus bactericidal activity assay was conducted according to Yamamoto & Iida (1995) in the following manner. Mucus was diluted 4 times with a Tris buffer (pH 7.5, containing 0.5 mM Mg^{2+} and 0.15 mM Ca^{2+}). The diluted mucus was mixed with a bacterial suspension (0.001 mg/ml) and incubated at 25ºC for 4 h with shaking. Viable bacteria in the mixture were counted by the plate counting method and their survival rate was calculated.

Analysis of HSP70s in liver

In the present study, heat shock protein 70 family (HSP70s) was the targeted biochemical indicator of potential stress tolerance of juvenile Japanese flounder to dietary P deficiency. The levels of HSP70s, which cross-reacted with mouse HSP70 monoclonal antibody in the sampled organ, were analyzed by ELISA using a commercial quantitative kit (EKS-700; Stressgen Biotechnologies Corporation, Victoria, BC, Canada). This kit was designed for inducible HSP70 in humans, mice and rats. However, cDNA sequence of inducible HSP70 of Japanese flounder is highly conserved among various vertebrates even in bovine and humans (Yokoyama et al., 1998), Investigators have confirmed that monoclonal antibody originated from mammals cross-react with fish, shellfish crustacean and green alga HSP (Bierkens et al., 1998; Dubeau et al., 1998; Snyder et al., 2001; Cimino et al., 2002). Therefore, inducible HSP70s can be detected in these species. The HSP sample preparation was performed according to Lewis et al. (1999) with slight modification. Collected skin and liver were frozen immediately and then crushed in liquid nitrogen with a cold pestle and mortar to a powder. An extraction buffer (provided in kit) with protease inhibitor (Complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics Corporation, USA) was added to the powdered sample and homogenized with a glass potter homogenizer in an ice bath. The homogenate was centrifuged (17000 x g, 15 min, 4 ºC). Precipitate was discarded, and the supernatant was filtered with a cellulose acetate filter (DISMIC 3-CP filter unit; Advantec Toyo Kaisha, Tokyo, Japan) and subjected to analysis of HSP70s. Measurement of HSP70s level was performed a microplate reader. Total protein content of the supernatant was determined by the method of Lowry et al. (1951) using bovine serum albumin (Nakarai tesque, Tokyo, Japan) as standard. The levels of HSP70s were expressed as levels of HSP70s in total protein in the sample solution.

Biochemical analysis

Crude protein was determined by the Kjeldahl method with a Tecator Kjeltec System (1007 Digestion system, 1002 Distilling unit and Titration unit) using boric acid to trap ammonia. Lipids were extracted according to the Bligh & Dyer (1959). Ash and moisture contents were analyzed by standard methods (AOAC, 1990). P concentrations in the samples were determined photometrically by the method of Lowry & Lopez (1946).

Statistical analysis

Data were subjected to one-way analysis of variance (package super-ANOVA, Abacus Concepts, Berkeley, California, USA). Duncan's multiple range test was used as the mean separation procedure when significant ($P<0.05$) differences were found in the ANOVA. Survival was transformed by arcsine square root before statistical analysis.

Results

Growth performances of the fish fed diet containing graded levels of P are shown in Table 2. Final weight and weight gain of the fish fed the diet containing 6 and 10 g/kg P were significantly (P<0.05) lower than the fish fed the diet with 18g/kg P. Feed efficiency significantly improved with increasing dietary P level. The best feed efficiency (FE) was obtained from the fish fed diet containing 18g/kg P. All experimental fish grew well, and no mortality was recorded during the trial.

Table 2: Growth performances of and feed efficiencies in Japanese flounder fed the diets containing varying levels of dietary P for 30 days.

Dietary P level (g/kg diet)	Final weight (g)	Weigh gain (%)	Feed efficiency[1]	Survival
6	23.0 ± 0.3[a]	216.1 ± 5.9[a]	0.9 ± 0.1[a]	100.0 ± 0.0
10	23.5 ± 0.9[a]	229.6 ± 11.9[a]	1.0 ± 0.1[ab]	100.0 ± 0.0
18	26.2 ± 0.1[b]	257.9 ± 3.9[b]	1.1 ± 0.0[b]	100.0 ± 0.0

Values are means of triplicate groups ± S.E. Within a row, means with the same letters are not significantly different (P>0.05). Absence of letters indicates no significant difference between treatments. Average initial body weight, means ± S.E., 7.3 ± 0.3 g.
[1]weight gain (g)/dry feed intake (g).

Mucus lysozyme and bactericidal activities significantly improved with increasing dietary P level (Figure 1, 2). The highest (P>0.05) lysozyme activity (unit/ml) and the lowest (P>0.05) survival rate of *E. coli* were observed from fish fed the diet containing highest P level (diet 3, 18g/kg). The lysozyme activities of fish fed diet with 0.6 and 1.0% P did not significantly differ from each other (Figure 1).

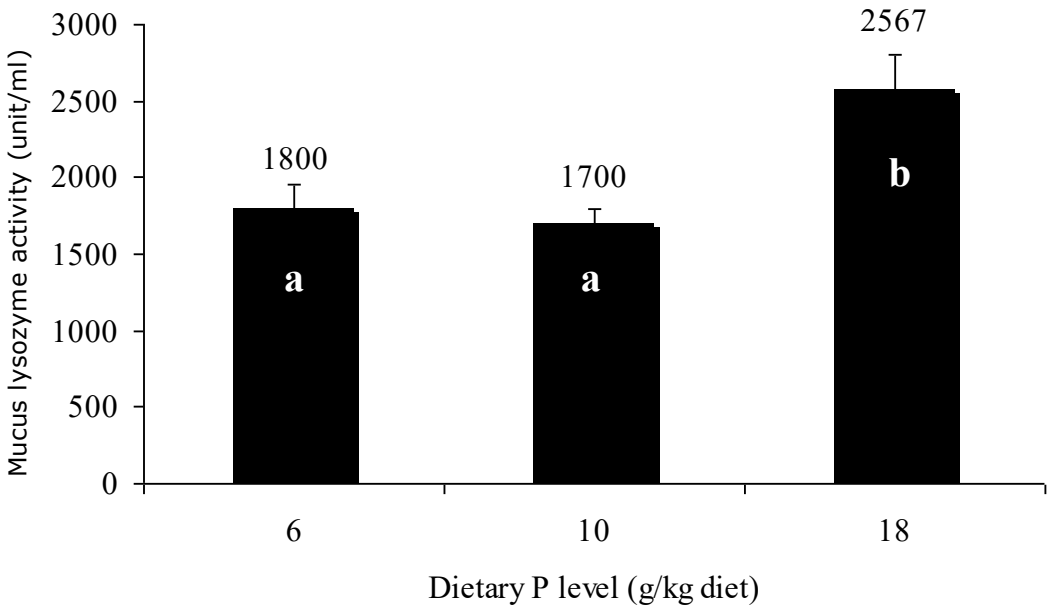

Figure 1: Mucus lysozyme activity of the fish diets containing varying levels of P. Values are expressed as mean ± S.E. (n=6). Letters indicate significant difference (*P*<0.05).

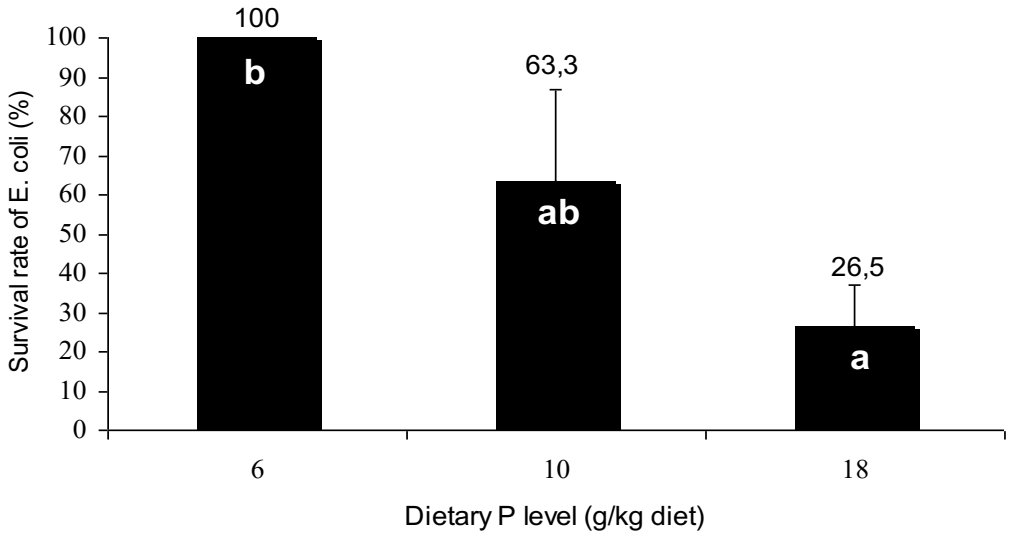

Figure 2. Mucus bactericidal activity as the survival rate of *E. coli* of fish fed the diets containing varying levels of P. Values are expressed as mean ± S.E. (n=6). Letters indicate significant difference (P<0.05).

Figure 3 is presenting liver tissue HSP70 concentrations of the experimental groups. A significantly higher level of HSP70 was detected in liver tissue of fish fed the diet supplemented with 18g/kg P compared to those of 6 and 10 g/kg P groups. The liver HSP70 concentration of the fish fed the diet containing 18g/kg P is almost triple than those the fish fed the diet containing 6 g/kg P. A similar tendency was also observed in the fish fed the diet containing 10g/kg P.

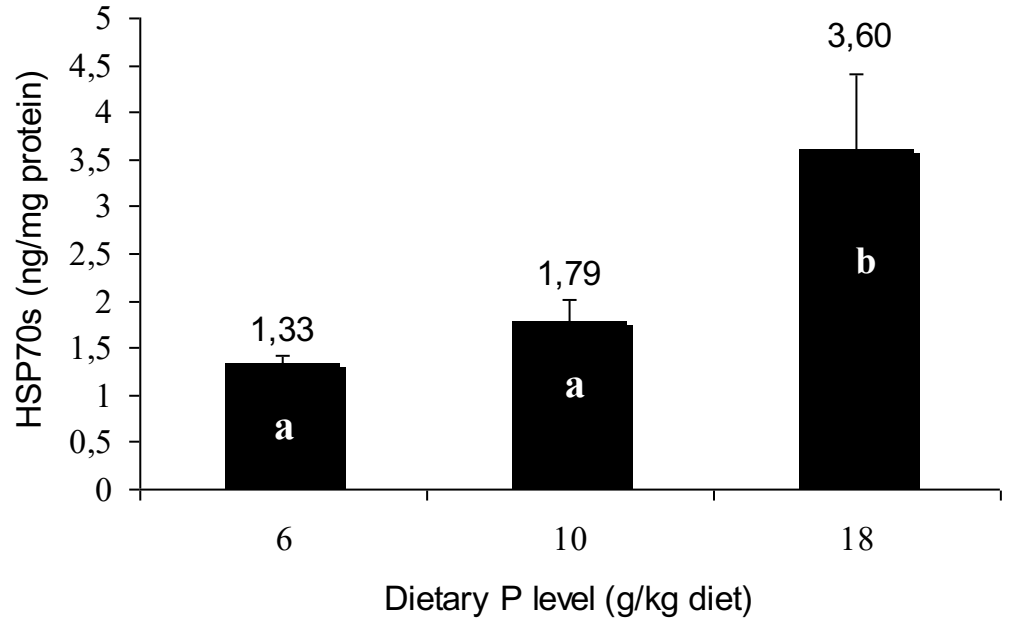

Figure 3. Levels of HSP70s detected from liver in juvenile Japanese flounder fed the diet containing varying levels of P over 30 days. Values are expressed as mean ± S.E. (n=6). Letters indicate significant difference (P<0.05).

Discussion

The rapid growth and efficient feed utilization in cultured organisms can only be achieved by optimizing dietary nutrient requirements. In contrast, dietary nutrient deficiencies, excesses or imbalances reduce growth rates or lead to disease of fish (NRC, 1993). As an essential mineral, P have a great importance for growth, bone mineralization, reproduction, synthesis of nucleic acids and structure of phospholipids as well as for energy metabolism in fish (Tacon, 1990; NRC, 1993; Roy & Lall, 2003). Lack of this mineral can cause a negative effect on fish health since nutrition is one of the most important factors interacting with the body and also has a profound influence on immune defense mechanisms (Jokinen et al., 2003).

In general, the P requirement of marine fish ranges from 0.5 to 1% of diet (Kim et al., 1998; Borlongan & Satoh, 2001; Roy & Lall, 2003; Oliva-Teles & Pimentel-Rodrigues, 2004; Pimentel-Rodrigues & Oliva-Teles, 2001). In the case of Japanese flounder, Wang et al. (2005) suggested the optimum dietary phosphorus level was between 0.45 and 0.51% for maximum weight gain of the juveniles. In the present study, the P levels of the diet 1 and 2 were 0.6 and 1.0%, respectively, and these diets caused reduced growth, revealing that the available dietary P was suboptimal for juvenile flounder. Although the dietary P level of 0.6% (diet 1) can be referred as the requirement level for Japanese flounder, major part of dietary P is originated from soybean protein that contains phytate as organic form of P. The intestinal mucosa of many fish does not secrete the enzyme phytase that is able to hydrolyze phytic acid to its moieties (Lall, 2002). Therefore, bioavailability of organic P from these diets might be fairly low, hence it caused growth retardation as a result of deficiency in the present study. In order to simulate the sub-requirement P deficiency condition (marginal P deficiency), diet 1 was supplemented with 4% calcium mono phosphate bringing about 0.4% increment in total P level. However, no further significant improvement on growth was observed. The highest growth was obtained from fish fed the diet containing 18g/kg P. In our previous study (Uyan et al., 2007), P deficiency caused growth retardation and operculum deformity in juvenile Japanese flounder with the initial weight of 1.02±0.0 g. However, no external deficiency sign was observed from the P deficient groups (diet 1 and 2) in the present study (initial weight was 7.3 ± 0.3 g). These results might reveal that fish size exposed to P deficiency is the primary factor to occurrence of malformation in juvenile Japanese flounder since the rest of the experimental conditions were similar in both trials.

Humoral immunity such as lysozyme activity or bactericidal activity functions as primary defence factors in the preference to cellular defence mechanism when attacked by invader (Ren et al., 2007). In the present study, mucus lysozyme activity was enhanced in fish fed high P supplemented diets. In other words, the low or non-P supplemented diets resulted in lowering mucus lysozyme activity. Lysozyme has an important role in non-specific immune defense system and has an antibiotic ability and can damage bacterial cell walls (Ellis, 1990; Grinde, 1989). Mucus bactericidal activity shown as the survival rate of *E. coli* was also affected by the dietary P level. It has been shown that fish skin mucus contains many antipathogenic substances such as immunoglobulin, lysozyme, complement, C-reactive protein, lectin and hemolysin that control the bactericidal activity (Ingram, 1980; Ellis, 1981; Fletcher, 1982), and they can be affected by stress (Takahashi et al., 1986; Thompson et al., 1993). In the present study, the mechanism by which dietary P affects the mucus lysozyme and bactericidal activity might be correlated with the nutritional stress because of P deficiency. It might also be possible if the P is not present, the energy required for the production of these antipathogenic substances may decrease since the synthesis of these antipathogenic substances requires ATP (energy) (Lehninger et al., 1993), which needs to continuous P supply to be able to synthesized. When the P deficiency is considered, mechanism affecting the defense system parameters could also

be speculatively attributed to possible failure in the protein phosphorylation, which is probably the most important regulatory event in eukaryotes.

HSP is a kind of stress protein which is synthesized when organisms are exposed to various stressors, e.g., heat shock, cold shock, viral infection, and heavy metal exposure (Iwama et al., 1998). Some of the vital roles that HSPs play in the cell include the maintenance of protein integrity, preventing premature folding and aggregation of proteins, protein translocation, and mediating steroid and receptor binding (Iwama et al., 1999). The synthesis of HSP is a general primary cellular event which occurs during both exogenous and endogenous stress situation, and has an ability of mediate misfolded or denatured functional proteins caused by various stressors in the cell (Iwama et al., 1999). In the present study, however, the fish were not exposed to experimental stressor such as air exposure, low salinity, low oxygen or high temperature, HSP70 was still detected in liver of fish. Besides being a mediator of denatured functional protein caused by various stressors (Iwama et al., 1999), HSP plays a role in novel protein synthesis as molecular chaperone (Ellis, 1999). Chaperones are proteins that mediate the translocation, folding, and assembly of other proteins but are not themselves components of the final structures (Ellis et al., 1989). Therefore, HSP70 would also be detected as chaperon when fish was under the non-stress conditions (Yokoyama et al., 2005). Hence, significant differences in liver HSP70s levels among the treatments could be attributed the dietary P level. Significantly higher liver HSP70 level of the fish fed diet containing 18g/kg P could demonstrate the higher stress resistance capability compared to those fed diet containing lower dietary P levels. Considering the growth parameters and immune response of the fish fed diet containing 18g/kg P, HSP70 level of 3.60 ng/mg protein of the fish fed diet containing 18g/kg P could be considered as the normal liver tissue HSP70 concentration of a healthy fish. Lower liver HSP70 concentration of fish fed diet containing 0.6 and 1.0% P might demonstrate impaired HSP70 synthesis because of inadequate dietary P consumption.

The rearing history of the fish including nutritional state can affect the stress response (Vijayan & Moon, 1992, 1994). However, up to date, the relationship between tissue HSP level and nutritional condition have not well documented in fish. Olsen et al. (2007) stated that one of the approaches to study suboptimal diets and their effect on the animal is to evaluate various stress-gene activations. They demonstrated that 100% fish meal protein replacement by the plant ingredients activate HSP70 stress-gene mRNA expression in the cod intestine. According to Cara et al. (2005), food-deprivation enhances HSP70 and HSP90 protein expression in larvae of sea bream and rainbow trout. In the latter study, they concluded that HSP70 and HSP90 protein expression in early life stages of fish have the potential to be used as markers of nutritional stress while elevation of the tissue HSPs content may be used as a means to increase stress tolerance during larval rearing. Based on these statements and findings, HSPs might be a sensitive indicator of nutritional stress in juvenile Japanese flounder. Further investigations are needed to clarify the influence of nutritional conditions such as deficiency and excess of certain nutrient on HSP70 expression in fish.

Conclusion

Dietary P deficiency significantly affected growth and defense system parameters in fish. Therefore, the dietary incorporation of P might improve stress tolerance of juvenile Japanese flounder. Under the conditions of the present study, dietary incorporation of 1.8% P was effective in improving the level of mucus lysozyme activity, HSP70s in liver, and bactericidal activities with reduced survival rate of *E. coli* of Japanese flounder while maintaining best growth performance.

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Ethical approval
The experiment was performed under the approval of the Committee on Animal Ethics, Kagoshima University, Japan, as a partial fulfilment of the PhD Thesis of first author.

Informed consent
Not available.

Data availability statement
The authors declare that data are available from authors upon reasonable request.

Conflicts of interest
There is no conflict of interests for publishing of this study.

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Contribution of authors
All authors in this study have equally contributed in terms of conceptualization, data curation, formal analysis, writing original draft, funding acquisition, investigation, methodology, resources, validation, and visualization, and finalizing paper.

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