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EFFECT OF FISH SIZE ON NITROGEN UTILIZATION IN JAPANESE FLOUNDER, *Paralichthys olivaceus* (TEMMINCK & SCHLEGEL)

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Abstract

Information on nitrogen utilization of varying size of Japanese flounder is essential in the formulation of cost-effective and low-pollutant diets. To do that, total ammonia nitrogen (TAN) excretion (mg-N/100 g fish) of different size of Japanese flounder (small size, $S_S = 13.5 \pm 0.40$ g; medium size, $S_M = 41.2 \pm 1.15$ g; large size, $S_L = 119.0 \pm 2.74$ g) was monitored every 2 h following 24 h after feeding with the diets containing 40, 46, 52, 58 or 64% protein. Results indicated that the TAN excretion (mg-N/100 g fish/d) of SS and SM were significantly ($P<0.05$) higher than that of the SL in each dietary protein level. The only peak on post-prandial TAN excretion occurred 2 - 4 h after feeding in S_S and S_M. However, the fish of S_L tended to reach to the peak with slightly longer time than those of S_S and S_L. TAN excretion significantly ($P<0.05$) increased with increasing nitrogen (protein) intake in all fish size. At the same protein level, TAN excretion decreased with increasing fish size. No interactive effect between dietary protein level and fish size was observed on TAN excretion. The relationship between nitrogen intake and TAN excretion was identified by linear regression in each size, and intercepts of the equations represented the ration of ingested nitrogen diverted to ammonia. S_L showed more effective nitrogen utilization compared to S_S and S_M. The results indicated that nitrogen utilization of Japanese flounder was size-dependent. In conclusion, to optimize the protein level in diets for Japanese flounder, size dependent absolute dietary protein requirement must be established.

Keywords: Nitrogen utilization, fish size, ammonia excretion, Japanese flounder, *Paralichthys olivaceus*

Introduction

The optimization of dietary nutrient levels in aquaculture feeds has received considerable attention. Their deficiencies and excesses reduce growth rates or lead to disease of fish as well as cause an unnecessary investment in a nutrient or add to the nutrient loading of the culture system (Allan Davis & Arnold, 1998). There is a lot of research focusing on the effect of dietary protein level on growth performance in Japanese flounder *Paralichthys olivaceus* because protein is a most important component in the diets of this fish (Kikuchi et al., 1992; Kikuchi et al., 2000; Kikuchi & Takeda, 2001; Alam et al., 2003; Uyan et al., 2022). According to these studies, recommended optimum dietary protein levels that vary from 40 to 64% seems to be primarily depends on the fish size ranging from 0.4 to 55 g (Kikuchi et al. 2000; Kikuchi & Furuta, 2000; Lee et al., 2000; Kim et al., 2002; Lee et al., 2002). It reveals that nitrogen utilization capability of Japanese flounder might vary depending on the fish size, which is a well-known phenomenon in fish nutrition. Although higher dietary protein requirement has been demonstrated for varying fish species during early growth period than during later phases of growth (Lovell, 1989; Garcia-Alcazar et al., 1994; Gristale-Helland, & Helland, 1997; Helland & Gristale-Helland, 1998; Sveier, 2000; Bai et al., 2001, Kim et al., 2002; Nordgarden et al., 2002; Wilson, 2002; Kim et al., 2003), limited information is available on Japanese flounder. The land-based farming system is very common on Japanese flounder culture in Japan, where water supply is provided by pumping from near seashores and/or by recirculating to the water using mechanic or biofilters (Kikuchi et al., 1991). In both cases, from the practical point of view, the amount of nitrogen loading should be known to realize effectiveness of the recirculation and/or to determine the amount of the water to be supplied under varying conditions. Serious fish health problems can occur if this toxic excretory product is allowed to accumulate in intensive culture unit with water reuse systems (biofilters for ammonia oxidation), which are particularly likely problem areas (Wedemeyer, 1996). If ambient ammonia reaches high levels, it may act as a limiting factor for not only fish growth but also survival (Person-Le Ruyet et al., 1995). Therefore, if ammonia peaks can be predicted, it might be possible to manage the culture pond in such a way as to reduce possibility of buildup of dangerous levels of ammonia in the system (Porter et al., 1987).

The present study was designed to investigate (1) the effect of fish size on nitrogen utilization of Japanese flounder as the references of TAN excretion and (2) to evaluate the amount of TAN loading under current experimental conditions.

Materials and Methods

Test fish

The Japanese flounder were transported from a commercial hatchery (Matsumoto Suisan Co., Miyazaki, Japan) to Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, and maintained on a commercial flounder diet (Higashimaru Foods, Kagoshima, Japan) and kept in 500 L round fish tank until used. The fish were sorted in three different body weights (BW small, BWS = 13.5 ± 0.40 g; BW medium, BWM = 41.2 ± 1.15 g; BW large, BWL = 119.0 ± 2.74 g), and stocked in five 50 L culture tanks of a recirculation unit with 30, 12 and 6 fish per tank for BWS, BWM and BWL, respectively. No significant difference was found among the mean body weights within each size group, but mean weights of different size were significantly ($P>0.05$) different from each other. Each group of fish was acclimatized to the respective experimental diets for one week prior to the experiment.

A series of experiments was conducted to evaluate the nitrogen utilization of different size of Japanese flounder. Each of the five test diets containing 40 to 64% dietary protein was fed to each different body weights of fish, and post-prandial ammonia nitrogen excretion was monitored following 24 h.

Test diets

The ingredients and proximate composition of the experimental diets are shown in Table 1. Five isocaloric (4.7 kcal/g energy) diets containing 40, 46, 52, 58 or 64% protein using milk casein and defatted brown fish meal as the protein sources, pollack liver oil as the lipid source, dextrin and α-starch as the carbohydrate or nitrogen free extract sources. Soybean lecithin was used as the phospholipids source. Lactoferrin was added to feeds to improve the resistance of the fish to infection with pathogenic bacteria and reinforce the fish against the environmental stress factors (Gallardo-Cigarro et al., 1999). Dietary protein level was increased by adjusting the proportion of the brown fishmeal at the expense of dextrin, α-starch and pollack liver oil.

Table 1. Formulation and proximate composition of the test diets

Ingredients	**Dietary protein levels (%)**				
	40	**46**	**52**	**58**	**64**
Brown fish meal (Defatted)[1]	25.6	33.0	40.0	47.6	55.0
Casein	20.0	20.0	20.0	20.0	20.0
Pollack liver oil[2]	12.0	9.2	6.2	3.3	0.7
Soybean lechitin[3]	5.0	5.0	5.0	5.0	5.0
HUFA[4]	1.0	1.0	1.0	1.0	1.0
Dextrin	4.6	3.8	3.1	2.4	1.7
α-starch	4.6	3.8	3.1	2.4	1.7
Cholesterol[5]	1.0	1.0	1.0	1.0	1.0
Lactoferrin[6]	0.1	0.1	0.1	0.1	0.1
Vitamin mixture[7]	2.5	2.5	2.5	2.5	2.5
Mineral mixture[8]	2.5	2.5	2.5	2.5	2.5
Attractant mixture[9]	1.0	1.0	1.0	1.0	1.0
Binder[10]	1.0	1.0	1.0	1.0	1.0
α-cellulose	19.1	16.1	13.5	10.2	6.8
	100	100	100	100	100
Proximate composition (% dry matter)					
Crude protein	40.5	46.4	53.9	59.1	65.1
Crude lipid	21.2	18.1	15.0	12.1	9.9
Crude ash	7.1	8.4	9.6	10.9	12.4
Crude fiber	21.3	18.6	14.6	12.5	8.8
N-free extract	9.9	8.5	6.9	5.4	3.8
Gross energy[11]	4.7	4.7	4.8	4.7	4.7

[1] Defatted with diethyl ether in the laboratory.
[2] Squid liver oil, Riken Vitamin, Tokyo, Japan.
[3] Lechithin from soybeans, Cica-Reagent, Kanto Chemical Co., Inc., Tokyo, Japan.
[4] Poweash A, Oriental Yeast Co. Ltd., Tokyo, Japan.
[5] Cholesterol (cholesterin), Nacalai Tesque.
[6] Morinaga Milk Industry Co. Ltd.
[7] Vitamin mixture (g kg^{-1} 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, ascorbyl-2-phosphate-Mg, 0.12; folic acid, 0.03 and choline chloride, 13.65

[8]Mineral mixture (g kg^{-1} diet); NaCl, 0.919; $MgSO_4$-$7H_2O$, 3.425; $NaHPO_4$-$2H_2O$, 2.18; KH_2PO_4, 5.995; $Ca(H_2PO_4)_2$-$2H_2O$, 3.395; Fe-citrate, 0.743; Ca-lactate, 8.175; $AlCl_3$-$6H_2O$, 0.004; $ZnSO_4$-$7H_2O$, 0.090; $CuCl_2$, 0.003; $MnSO_4$-$4H_2O$, 0.020; KI, 0.004 and $CaCl_2$, 0.025

[9]Attractant mixture (g kg^{-1} diet); DL-α-alanine, 3; Betaine monohydrate, 3; L(-)-proline, 2 and inosine 5-mono phosphate (IMP), 2.

[10]Berda, Rosch Co., Germany.

[11]Gross energy is calculated based on: protein, 5.65 kcal g^{-1}; lipid, 9.45 kcal g^{-1}; carbohydrates, 4.10 kcal g^{-1}.

To prepare test diets, the dry ingredients were mixed thoroughly for 30 min in a food mixer. Pollack liver oil, soybean lecithin and HUFA were previously mixed together in a sonicator and added to mixed dry ingredients. Then, the diets were remixed for 15 min. Finally, water (35% of the dry weight of ingredients) was added, and the diets mixed again for 15 min. The mixture was then passed through a meat grinder with an appropriate diameter disc size to produce the pellets (2.2 mm) and were dried in a dry air mechanical convection oven at 70 ºC by obtaining approximately 10% moisture level in each diet. The experimental diets were stored at -30 ºC until used. Crude protein and lipid of the diets were determined by the Kjeldahl method (N x 6.25) and by the Bligh and Dyer method (Blight & Dyer, 1959), respectively. Ash, fiber and moisture contents of diets were determined by Association of Official Analytical Chemistry (AOAC, 1995) method.

Rearing conditions and measurements of ammonia excretion

Water exchange rate of the recirculation unit was 30% a day. Water temperature, dissolved oxygen and salinity were maintained at 21 ± 1.0 ºC, 6.72 ± 0.12 mg O_2/l, 33.5 ± 0.18 ppt, respectively. Photoperiod was 12 h: 12 h light/dark.

Simplified schematic diagram of experimental unit for ammonia excretion was illustrated in Fig. 1. For the determinations of TAN excretion, the fish in the culture tank was fed the respective experimental diet at ratio of 1% (in dry matter) of their body weight following the starving period of 72 h. Then, the fish were transferred to six 5 L covered chamber which were completely filled with filtered sea water with a water inlet and outlet 30 min after feeding. Five, two and one fish were placed in a single chamber for S_S, S_M and S_L, respectively. Chambers were placed in a water bath connecting to the recirculation unit at 21 ºC constant water temperature. Filtered fresh seawater (21 ºC) was passed through the chambers during 24 h. All chambers were sampled after both inlet and outlet were interrupted for 100 min in every 2 h period. After sampling, the water mass of chambers was allowed to renew with filtered running sea water at same temperature during the 20 min of the rest of 2 h till further period started. The water samples were collected for ammonia measurement in each sampling period by pipetting from middle of a chamber every 2 h over a 24 h period (from 09:00 to 09:00). TAN excretion rates were determined based on the difference in ammonia concentration of filtered seawater and the water volume in the chambers. In starvation experiments, excretion rates were measured in the same manner as that described above without feeding after 72 h starving period.

TAN concentration in water samples was determined by phenol-hypochlorite method of Strickland and Parsons (1972) using a standard curve prepared from ammonium sulfate solution.

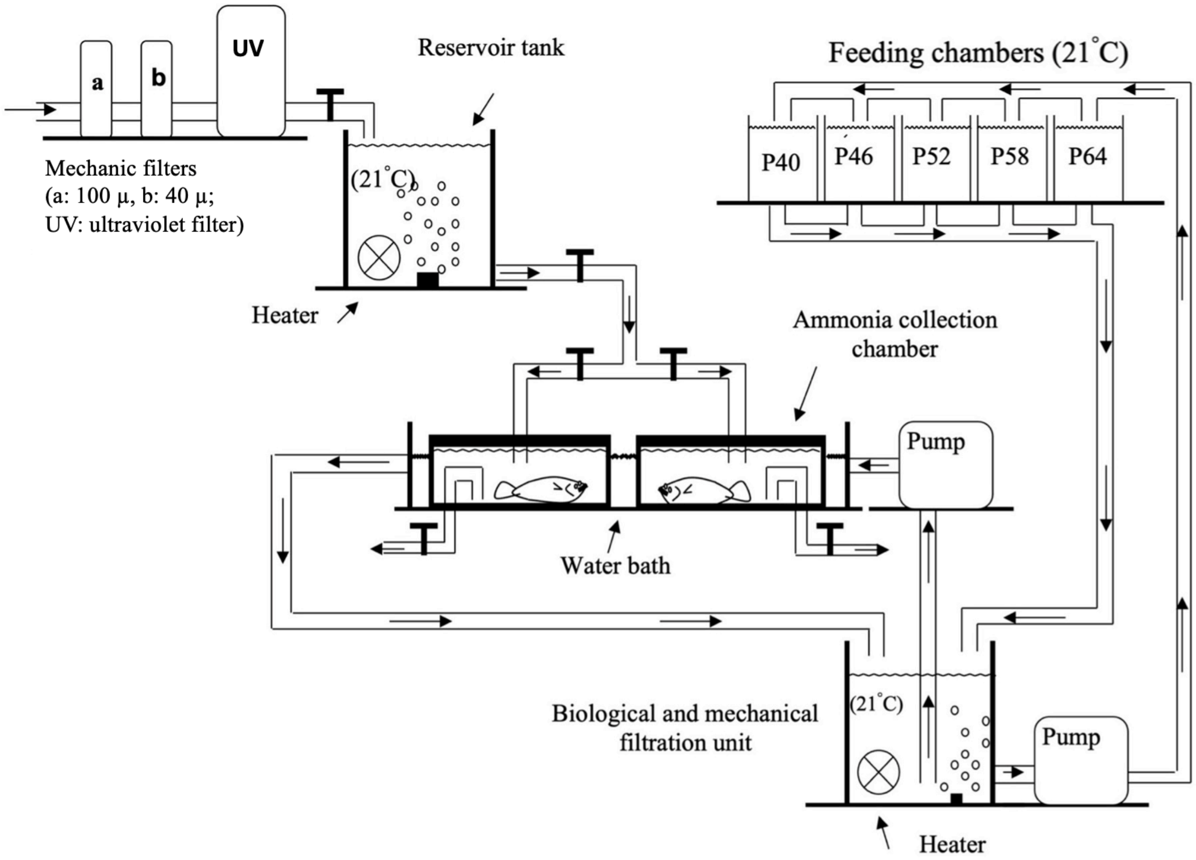

Figure 1. Schematic diagram of experimental unit for ammonia excretion.

Statistical analysis

Daily TAN excretion rates of experimental groups were compared using one or two-way ANOVA with significance level of 0.05 based on the size and dietary protein level (package super-ANOVA, Abacus Concepts, Berkeley, California, USA). Significant differences between treatments ($P<0.05$) were evaluated by Duncan`s new multiple range test (package super-ANOVA, Abacus Concepts, Berkeley, California, USA).

Results

Daily TAN excretion

Daily TAN excretion (mg-N/100 g fish/d) of different weights of fish fed the diets containing varying protein levels are presented in Table 2. Daily TAN excretions of BWS and BWM were significantly ($P<0.05$) higher than that of BWL. However, no significant difference was observed on daily TAN excretion between BWS and BWM. In starved fish, the daily TAN excretion of BWL was significantly ($P<0.05$) lower than BWS and BWM. No interactive effect between dietary protein level and fish weight was observed for daily TAN excretion. The relationships between nitrogen intake and daily TAN excretion showed highly linear correlation in each size of fish expressed in mg-N/100 g fish/d (Table 3).

Exogenous TAN excretion and the proportion of ingested nitrogen diverted to ammonia (INDA, %) were illustrated in Table 4. INDA represented that 20% of ingested nitrogen was excreted as ammonia in S_S and S_M, however, BWL showed more effective nitrogen utilization with 10% than those of BWS and BWM.

Table 2. Daily TAN excretion of different size of Japanese flounder fed varying dietary protein level.

Dietary Protein Level (%)	Daily TAN excretion (mg-N/100 g fish/d)[1]		
	S_S	S_M	S_L
Starved	13.88	13.67	8.30
40	24.88	25.90	14.74
46	31.64	30.07	15.58
52	33.14	31.41	17.11
58	36.11	33.81	18.79
64	39.23	36.64	19.27
Pooled SEM	2.22	1.75	1.11
Two-way ANOVA ($P<0.05$)			
Protein	0.0001		
Fish size	0.0001		
Interaction	0.6102		

[1] Means of six replicate groups.

Table 3. The relationships between nitrogen intake and daily TAN excretion.

Fish Size Group	Daily TAN production (y, mg-N/100 g fish/d)	
	Linear Equation (ax+b)	R^2
S_S	0.222x[1] + 12.83	0.99
S_M	$0.201x + 13.98$	0.98
S_L	$0.109x + 7.87$	0.97

[1] x = Nitrogen intake, (mg N/100 g fish)

S_S (small size): 13.5±0.40 g; S_M (medium size): 41.2±1.15 g; S_L (large size): 119.0±2.74 g)

Table 4. Nitrogen intake, exogenous TAN excretion and percentage of ingested N diverted to ammonia

Fish size		Dietary Protein Level (%)				
		40	46	52	58	64
S_S	N intake[1]	54.69±6.56	82.12±6.24	94.05±14.9	103.7±12.7	112.7±24.1
	E-TAN excretion[2]	11.00	17.76	19.26	22.23	25.35
	IN diverted to ammonia[3]	20.11	21.63	20.48	21.44	22.50
S_M	N intake	61.3±11.77	75.57±5.12	89.65±11.7	100.1±20.5	111.8±10.8
	E-TAN excretion	12.23	16.40	17.74	20.14	22.97
	IN diverted to ammonia	19.95	21.70	19.79	20.12	20.55
S_L	N intake	61.19±8.34	74.25±3.97	86.69±8.16	96.50±8.39	106.3±17.9
	E-TAN excretion	6.44	7.28	8.81	10.49	10.97
	IN diverted to ammonia	10.52	9.81	10.16	10.87	10.32

[1] (mg N/100 g fish)

[2] TAN excretion of fed fish (mg N/100 g fish/day) – TAN excretion of starved fish (endogenous excretion) (mg N/100 g fish/day)

[3] (Exogenous TAN / N intake) x 100

IN: Ingested N; E-TAN: Exogenous TAN

S_S (small size): 13.5±0.40 g; S_M (medium size): 41.2±1.15 g; S_L (large size): 119.0±2.74 g)

Starving period

During 24 h period, starved fish showed relatively constant excretion rate than the fed fish in corresponding fish weights (Fig. 2). Hourly TAN excretion (mg- N/100 g fish/h) of starved fish significantly decreased with fish weight. Under starved condition, the mean excretion of S_S and S_M were 0.58 ± 0.04 and 0.57 ± 0.04 mg-N/100 g fish/h, and were significantly ($P<0.05$) higher than that of the S_L (0.35±0.04 mg-N/100 g fish/h) ($P<0.05$) (Fig. 2)

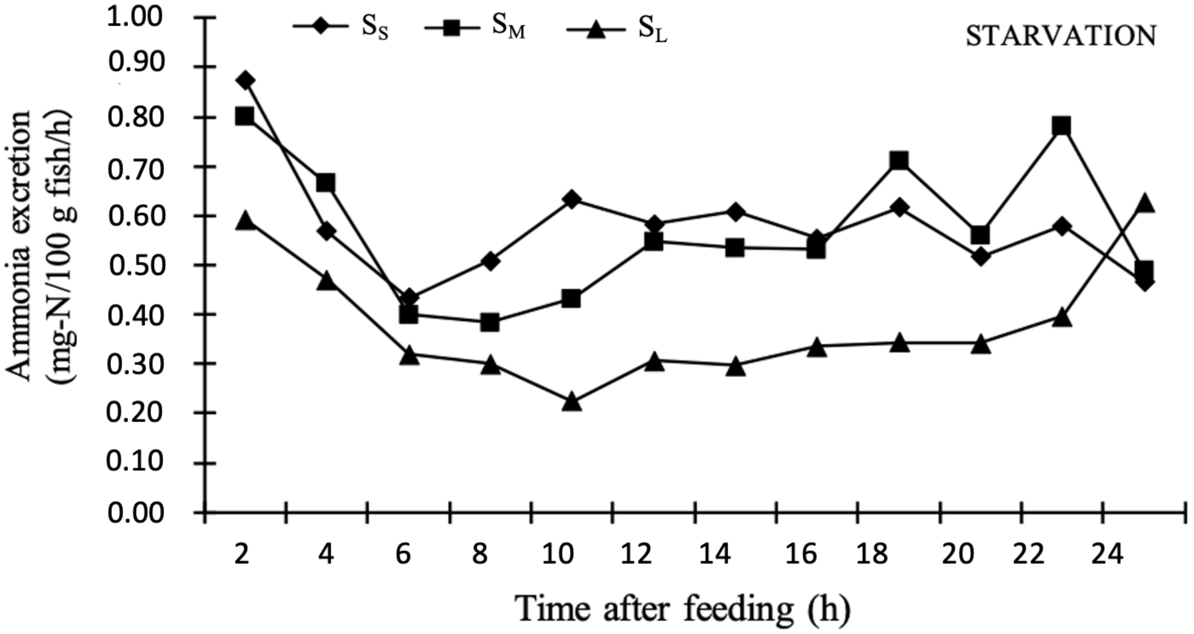

Figure 2. Starvation ammonia excretion pattern of each size of fish. S_S (small size): 13.5±0.40 g; S_M (medium size): 41.2±1.15 g; S_L (large size): 119.0±2.74 g)

Calculated endogenous TAN excretion (starvation period, no feed distribution) from the regression equations (intercepts) for S_S, S_M and S_L were 12.83, 13.98 and 7.87 mg-N/100 g fish/d, respectively. This was close to the mean values obtained for starved fish of S_S, S_M and S_L of 13.88 ± 0.91, 13.67 ± 0.85 and 8.30 ± 0.41 mg-N/100 g fish/d, respectively (Table 4).

Diurnal pattern of TAN excretion of fed and starved fish

Diurnal TAN (mg-N/100 g fish/h) excretion patterns of the fed and starved fish are represented in Fig. 2. TAN excretion (mg-N/100 g fish/h) increased shortly after feeding, and the only one peak of TAN excretion appeared between 2 to 4 hours for S_S and S_M for all dietary treatments whereas for S_L, it took slightly longer (between 4 to 8 hours) to reach the peak. Mean excretion rates of S_S, S_M and S_L fed the diets containing 52, 58 and 64%, respectively were still higher by the end of the 24 h period.

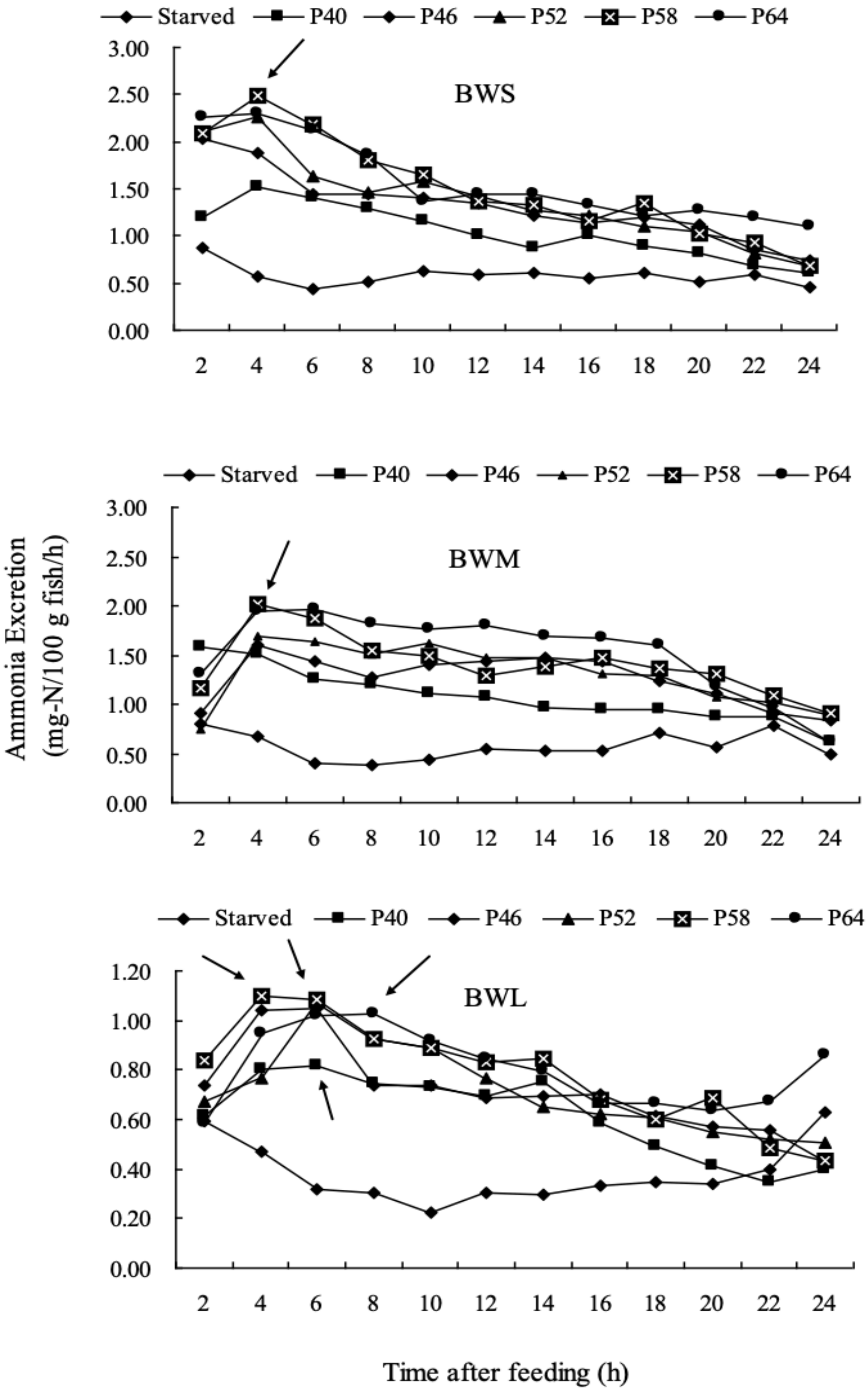

Figure 3. Diurnal TAN excretion rate of the Japanese flounder.

Discussion

The greatest part of the nitrogen excretion product of Japanese flounder is in the form of ammonia which amounts to more than 80% of total nitrogen excretion (Kikuchi et al., 1995) and increasing TAN excretion with increasing protein intake have been demonstrated in Japanese flounder (Kikuchi et al., 1991). TAN excretion (mg-N/100 g fish/d) of Japanese flounder significantly ($P<0.05$) increased in different weights of fish when they were fed the diets containing increasing dietary protein level in the present study. The increase in TAN excretion rate as nitrogen intake increases have also been confirmed for rainbow trout, (Rychly, 1980; Medale et al., 1995), Atlantic cod (Ramnarine et al., 1987), areolated grouper (Leung et al., 1999), European sea bass (Ballestrazzi et al., 1994) and Australian short-finned eel (Engin & Carter, 2001).

The largest source of ammonia is catabolism of dietary (exogenous excretion) or structural protein (endogenous excretion) in fish (Wood, 1993). Feed dependent nitrogen excretion (exogenous excretion) is considered to result from the direct deamination of amino acids ingested and absorbed from the food and is influenced by factors such as feeding rate and protein/amino acid composition of the diet (Jobling, 1994). In the present study, nitrogen intake of each weight of fish increased due to increasing dietary protein level, and it was resulted to increase in TAN excretion rate which was also explained by Beamish and Thomas who pointed out that the most important influence on ammonia excretion rate was the amount of dietary protein intake (Beamish & Thomas, 1984).

Ammonia excretion of fish consists of two fractions. Endogenous excretion represents the amount of ammonia excreted after a period of starvation while exogenous excretion is the resultant energy loss associated with the assimilation and deamination of protein after feeding. When the relationship between nitrogen intake and TAN excretion rate is described by simple regression, the intercept on the Y-axis of regression line of the equations approximately represents endogenous TAN excretion (considering nitrogen intake is zero, $x = 0$) while slope shows exogenous excretion (Jobling, 1981). Kikuchi et al. *(*1992) indicated that the slope of the equation must represent the indigestibility of dietary protein in feaces-N excretion. Dosdat *et.al.* (1996) demonstrated that decreasing regression coefficient of TAN against ingested nitrogen in accordance with the good protein utilization, the low exogenous losses and the low activity in turbot. In the present study, the daily TAN production, as the sum of endogenous and exogenous fraction, was described by linear regression for each weight, and calculated endogenous TAN excretions were in good agreement with the excretion rates obtained in the experiments. The intercept of TAN excretion in each weight (0.222, 0.201 and 0.109 for S_S, S_M and S_L, respectively) represented the percentage of INDA when nitrogen intake was plotted against the TAN excretion (Table 3). It decreased with increasing fish weight. This finding agrees with Kikuchi et al. (1992) who pointed out that proportions of TAN to consumed nitrogen were 23% and 21% for juvenile (1.5 - 6.5 g) and young (15 - 91 g) Japanese flounder, respectively. However, it is not possible to make a direct comparison between the present study and the Kikuchi et al. (1992) due to the differences of fish weight.

According to the intercepts of the regression lines and calculated INDA values, it might be concluded that per unit of weight between the mean weight of 13.5 to 41.2 g/fish, the amount of dietary protein used as energy was higher compared to that used in mean weight of 119 g/fish. Better nitrogen utilization with increasing body weight indicated that optimum dietary levels of protein of Japanese flounder might decrease with increasing body weight, possibly because nitrogen utilization for energy decreased as fish weight increasing in immature Japanese flounder. The growth of Japanese flounder highly depends on the dietary protein because the dietary lipid and carbohydrate could not be utilized as energy source (Kikuchi et al., 1992; Kikuchi et al., 2000; Kikuchi et al., 2001). Therefore, particularly in smaller weights, relatively higher amount of dietary protein must be provided to the fish to meet the energy requirement of fish as well as for growth. It is well known that energy requirement of fish decreased with increasing fish weight. Because of the fact that, in the present study, INDA was found to be lower in the biggest weight of fish (S_L) than those in smaller weights (S_S and S_M). As a result, the protein requirement of 119 g of Japanese flounder might be lower than that 13.5 to 41.2 g of fish. However, requirement level of dietary protein for 13.5 to 41.2 g of fish might be similar.

No interaction was found between fish size and nitrogen intake, revealing that nitrogen excretion was independently affected by nitrogen intake and fish weight in Japanese flounder. Generally, TAN excretion per unit weight tends to decrease with increasing fish size under starved and fed conditions. For instance, 3 g gilthead seabream produced 2.9 times as much TAN as do 40 or 90 g gilthead seabream which produced approximately the same amount (Wood, 1993). Daily TAN excretion in milk fish (*Chanos chanos*) decreased exponentially as fish weight increased but positively increased with feed ration (Sumagaysay-Chavoso, 2003). Dosdat et al. (1996) demonstrated that 10 g turbot excreted 3 times more ammonia than 100 g fish. Kikuchi et al. (1992) described that the TAN excretion of starved juvenile Japanese flounder (1.8 to 5.1 g) was 0.76 mg-N/100 g fish/h (equals 18.24 mg-N/100 g fish/d) and was about three times higher than that of young fish (15 to 49 g). Our findings are generally in agreement with Kikuchi et al. (1992) in terms of decreasing TAN excretion with increasing size, but average rates seem to be comparable. This may be caused that based on each body weight group, the variations of fish weights of the compared paper were higher than the present study.

At any fish weights examined, the results showed that a pattern of rapid rise or peak in TAN excretion (mg-N/100 g fish/h) occurred mainly between 2 to 4 h after feeding and/or occasionally stabilized slightly longer followed by a more prolonged and gradual decrease then slowly declined and reached to the almost mean excretion rate of starvation level after 24 h. Diurnal pattern of BWL seemed to be relatively stable than the other weights. However, some exceptions were also observed, particularly on higher dietary protein levels. The biggest fish (S_L) tended to reach to the peak with slightly longer time. This is almost in agreement within the results of the Kikuchi et al. (1992) who found that the rates of TAN excretion of juvenile and young flounder decreased from the period of 3 - 6 h to that of 12 - 24 h. These small varieties may stem from the methodology applied. We took the water sample in 2 h intervals instead of the 3 h as it was in mentioned experiment.

The present study showed that TAN excretion rate of Japanese flounder with different weights increased with increasing dietary protein but decreased with increasing fish size. In the practical point of view, using the equation described by simple regression, the assessment of the amount of protein to diverted the ammonia, and daily total ammonia nitrogen accumulation to the culture unit for varying size of Japanese flounder at 21±1.0 ^{o}C could be possible.

Conclusion

Data obtained here show that per unit of dietary protein utilized for energy decreased with increasing fish size on Japanese flounder. It reveals that nitrogen metabolism of Japanese flounder is size-dependent. Therefore, further studies are needed to clarify on size dependent absolute dietary protein requirement of Japanese flounder to optimize dietary protein level and decrease the amount of ammonia nitrogen excreted.

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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 graduate studies 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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