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# Optimum levels of crystalline amino acids in diets for larval red sea bream (*Pagrus major*)

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To study the nutritional requirements of essential amino acids in fish larvae, a purified diet is needed, and to assess the optimum level of crystalline amino acids (CAAs) in larval diets, red sea bream larvae (*Pagrus major* Temminck and Schlegel) were fed five microbound diets with different levels of CAAs. The larvae were cultured for 30 d. Assessment of growth, survival, and body amino acid composition after the culture period indicates that replacing casein with low levels of CAAs is beneficial. Growth and survival were enhanced when 5 g casein per 100 g of diet was replaced with CAAs. The effect of replacing 10 g casein per 100 g of diet with CAAs was that the larval performance was similar to that of the control diet. However, replacing 15 g or more of casein produced a detrimental effect on larval performance. The amino acid leakage from the diet particles is high and microencapsulation of CAAs is therefore recommended.

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## Introduction

The dietary requirements of amino acids in aquatic organisms have been studied by many workers. In fish, requirements of essential amino acids have been studied in adults and in juveniles, but, to date, not much work has been done on amino acid requirements in fish larvae. Nutrition of the larval stages is crucial in aquaculture. Larvae have very limited body reserves, and the absence or deficit of a nutrient in the diet can cause disease, poor growth, and high mortality. To define the nutritional quality of a larval feed, it is necessary to know the nutritional requirements of the larvae, and to study the amino acid requirements of fish larvae it is necessary to produce a defined microdiet with graded levels of each amino acid. Since all the essential amino acids are present in most protein source, it is necessary to prepare a diet with part of the amino acids given by crystalline amino acids (CAA), so that the amount of each amino acid in the diet can be adjusted to the desired levels.

CAAs have previously been used as ingredients in fish diets, ranging feeds from 0.69% to 50% (Thébault *et al.*, 1985; Hidalgo *et al.*, 1987; Ravi and Devaraj, 1991; Tibaldi and Lanari, 1991). In a preliminary study (López-Alvarado and Kanazawa, 1993), larval diets with 30–50 g CAAs per 100 g of diet produced total mortality after 16–17 days of rearing. But diets with 10% CAAs gave a similar growth and survival to that of a control group fed diets without CAAs. Free amino acids were shown to be an important energy substrate in marine fish embryos and larvae (Fyhn, 1989; Rønnestad and Fyhn, 1993), and low levels of CAAs in the diet may have contributed to a better larval performance. In this study it is intended to establish the optimum level of CAAs in experimental diets for nutritional studies, as well as the maximum level of CAA in these diets, without serious effect on survival. Five microbound diets were prepared, with levels of CAA of 0, 5, 10, 15, and 20% of the diets on a dry weight basis. All diets also had casein, which decreased as the level of CAAs increased in the diets, and fish meal and krill meal to increase palatability.

## Materials and methods

### Diets

Zein microbound diets were prepared following a method modified from Teshima *et al.* (1982). The lipids were blended with 300 ml of 60% ethanol. Zein was

Table 1. Ingredients of experimental diets for red sea bream larvae (g/100 g of dry diet).

| Ingredients | Diet 1 | Diet 2 | Diet 3 | Diet 4 | Diet 5 |
|---|---|---|---|---|---|
| Casein | 30.00 | 25.00 | 20.00 | 15.00 | 10.00 |
| White fish meal | 20.00 | 20.00 | 20.00 | 20.00 | 20.00 |
| Krill meal | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 |
| Essential amino acids: | | | | | |
| MET | 0.00 | 0.13 | 0.27 | 0.40 | 0.53 |
| THR | 0.00 | 0.16 | 0.32 | 0.49 | 0.65 |
| VAL | 0.00 | 0.26 | 0.53 | 0.79 | 1.06 |
| ILE | 0.00 | 0.20 | 0.40 | 0.60 | 0.80 |
| LEU | 0.00 | 0.39 | 0.77 | 1.16 | 1.55 |
| PHE | 0.00 | 0.23 | 0.46 | 0.69 | 0.92 |
| HIS | 0.00 | 0.13 | 0.25 | 0.38 | 0.50 |
| LYS | 1.70 | 1.99 | 2.28 | 2.57 | 2.86 |
| TRP | 0.00 | 0.05 | 0.10 | 0.15 | 0.21 |
| ARG | 1.30 | 1.47 | 1.70 | 1.80 | 1.97 |
| Non-essential amino acids: | | | | | |
| TAU | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| ASP | 0.00 | 0.34 | 0.70 | 1.06 | 1.42 |
| SER | 0.20 | 0.49 | 0.78 | 1.07 | 1.35 |
| GLU | 0.00 | 1.21 | 2.44 | 3.71 | 4.97 |
| PRO | 0.10 | 0.65 | 1.21 | 1.78 | 2.35 |
| GLY | 0.20 | 0.31 | 0.38 | 0.47 | 0.56 |
| ALA | 0.30 | 0.49 | 0.63 | 0.78 | 0.91 |
| CYS | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| TYR | 0.00 | 0.30 | 0.58 | 0.90 | 1.19 |
| Betaine | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| I.M.P.[a] | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| Vitamins | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 |
| Minerals | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |
| Squid liver oil | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Soybean lecithin | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 |
| n-3HUFA | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Dextrin | 6.70 | 6.70 | 6.70 | 6.70 | 6.70 |
| Zein | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 |
| Total | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |

[a] Inosine-5′-monophosphoric acid disodium salt.

added and blended and then all the powdered ingredients added and blended until the ethanol had evaporated. The mixture was freeze-dried for 48 h, and ground and sieved to produce particles ranging from less than 125 μm to 250 μm. Five experimental diets were tried, all with approximately the same amount of each essential amino acid, but with different levels of casein and CAAs. All diets had 60% protein. Five levels of casein substitution were used: 0, 5, 10, 15, and 20 g per 100 g of diet. These levels of casein were replaced by CAAs. In addition, all the diets had supplement levels of arginine and lysine to cover the requirements of the amino acids, and some non-essential amino acids were used as attractants. The ingredient composition of the diets is presented in Table 1. The elaboration of a diet with 30% CAAs was tried, but the mixture of ingredients did not bind and remained in a liquid state.

## Fish and culture conditions

Eggs of red sea bream (*Pagrus major*) were received from Kagoshima Prefectural Fisheries Research Center, and transferred to the Laboratory of Fish Culture, Kagoshima University. The eggs were placed in a 500 l tank with sea water at a temperature of 17°C, with gentle aeration and moderate flow of water. Two days after hatching, larvae were fed on rotifers (*Brachionus plicatilis*) at a concentration of 1 rotifer $ml^{-1}$. The feeding rate increased daily until a concentration of 5 rotifers $ml^{-1}$ was reached. Ten days after hatching, larvae were separated and stocked in 100 l tanks at a density of 1000 larvae per tank (10 larvae $l^{-1}$). The tanks were gently aerated, and the water exchanged between three and six times per day. Feeding of the microbound diets was carried out every half hour during the morning and every hour during the afternoon. In the evening, rotifers were fed at a density of 5 rotifers $ml^{-1}$ to provide food during the night. The particle size of the diets ranged from less than

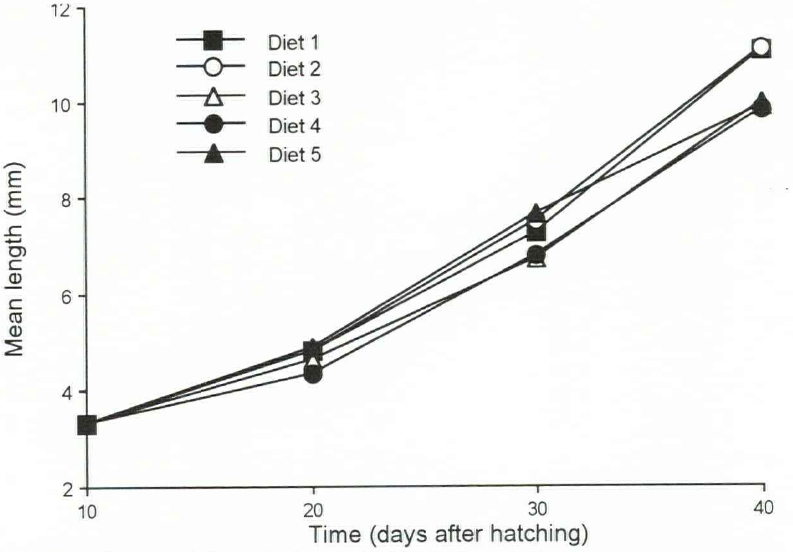


Figure 1. Growth of red sea bream larvae fed diets with increasing levels of crystalline amino acids (CAAs). Diet 1: no CAAs; Diet 2: 5% CAAs; Diet 3: 10% CAAs; Diet 4: 15% CAAs; Diet 5: 20% CAAs, as % of dry diet.

125 μm to 250 μm. Growth was estimated every 10 days by measuring a sample of the larvae. Survival was estimated every second day, counting the dead larvae siphoned from the bottom of the tanks.

### Analytical methods

At the end of the experiment, samples from all the treatments were taken and tissue and dietary amino acids were determined using a Shimadzu HPLC after hydrolysis of the protein at 110°C for 22 h with methane sulphonic acid. N-leucine was used as internal standard. The crude protein of the diets was determined using the Kjeldal method. The leaching of CAAs to the water was estimated by analysing the water in which a known amount of diet had been placed for 1 and 5 min. All data were analysed using Statview in a Macintosh PC.

## Results and discussion

### Larval performance

Figures 1 and 2 show the growth and survival of the larvae, respectively. Larvae fed a diet with 5% of the casein replaced with CAAs had a slightly better final length than the control group. There were no major differences between these two groups. The larvae fed diets with 10, 15, and 20% of the diet like CAAs had a lower final length than the control group in all three groups.

Larvae survival was best for the group fed a diet with 5% amino acid substitute, supporting the idea proposed by Fyhn (1989) that free amino acids are an important energy source during the embryonic development of marine fish.

The larvae fed a diet with 10% amino acid substitute had a similar survival rate to the control group, while those fed diets with 15 and 20% substitute had a much lower survival rate than the control group.

Even though all experimental groups were fed rotifers during the night, the feeding rate was very low and great differences were observed in both growth and survival among the different treatments. This indicates that the results were only slightly affected by the rotifers and that they were mainly determined by differences in composition of the microdiets.

Since the survival of the larvae ranged from 11.9% to 43.3%, an analysis of growth response is not enough on its own to evaluate these diets. The final size of larvae fed diets with high levels of CAAs was quite high. However, since survival of these experimental groups was low, mean size was probably overestimated, assuming that smaller larvae are more likely to die than bigger larvae in the same tank. Therefore, a new index was defined:

Larval performance index (LPI) = SGR × survival (at end of the experiment)

SGR: specific growth rate =

$$\frac{\text{L(final length)} - \text{L(initial length)}}{\text{time(days)}} \times 100$$

$$\text{Survival} = \frac{\text{Initial number} - \text{final number}}{\text{Initial number}} \times 100$$

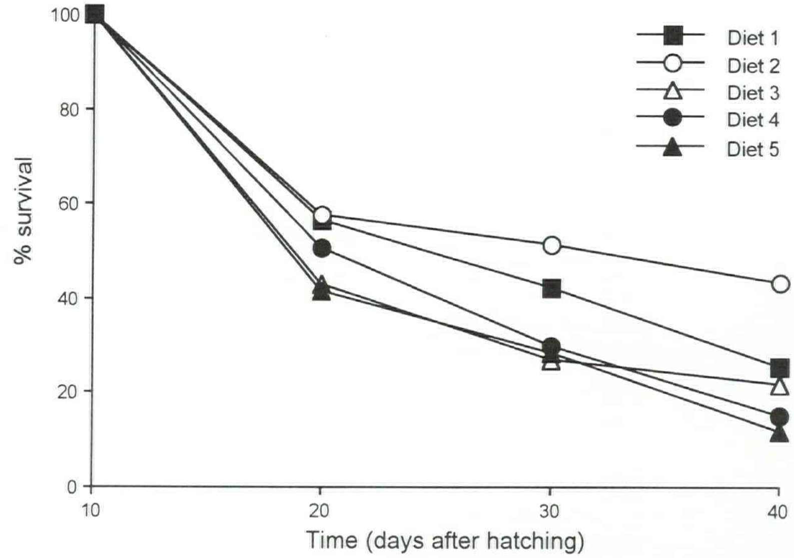


Figure 2. Survival of red sea bream larvae fed diets with increasing levels of crystalline amino acids.

Table 2. Specific growth rate and larval performance index of red sea bream larvae fed diets 1–5.

| Diet | Specific growth rate | Larval performance index |
|---|---|---|
| 1 | 4.01 ± 0.39 | 101.95 ± 9.95 |
| 2 | 4.04 ± 0.37 | 174.83 ± 15.96 |
| 3 | 3.66 ± 0.49 | 79.04 ± 10.49 |
| 4 | 3.62 ± 0.49 | 54.99 ± 7.47 |
| 5 | 3.64 ± 0.45 | 43.31 ± 5.35 |

$$SGR = \frac{L(\text{final body length}) - L(\text{initial body length})}{\text{time (d)}} \times 100$$

LPI = SGR Survival.

This index is a better indicator of larval performance than either larval growth or survival on their own. The control group had a LPI of 101.95 (Table 2), a value very close to 100. Therefore, an index greater than 100 will be considered a good LPI and an index lower than 100 a bad LPI. According to this index, diet 2 (LPI = 174.83) has a strong positive effect on the larval performance, and its LPI is significantly higher than that of the control. The LPIs of larvae fed diets 3, 4, and 5 are all lower than the control group. Groups 4 and 5 have fairly low LPIs, indicating that the diets fed had a negative impact on larval performance.

## Biochemical composition

The amino acid composition in the diets and in the larvae after 30 days of culture are given in Table 3A and B, respectively. The amino acid profiles of the diets used are all very similar, so the main difference among the diets is the source of the protein and amino acids, and not the amount of each amino acid in the diets. The amino acid compositions of the larvae are also very similar in all the experimental groups. This indicates that fish larvae have specific nutritional requirements for amino acids, and if some of the essential amino acids are not readily available fish show reduced protein synthesis and growth, even if the rest of the nutrients are present in adequate quantities in the diet. Some of the groups receiving diets with high levels of CAAs were in fact, owing to high leaching of CAAs, ingesting lower levels of amino acids than the groups fed low levels of CAAs. Nevertheless, the amino acid composition of the larvae was similar in all the experimental groups. The lower amino acid intake resulted in lower specific growth rates due to nutritional deficiency. This probably happened in fish from groups 4 and 5: even though the growth of these groups was lower than the growth of groups 1–3, and survival was much lower, the amino acid profiles of all these groups of fish were very similar.

## Leaching of CAAs

The loss of CAAs to the water amounted to 66.67% in the diet after 1 min in water, and up to 95.81% in the diet after 5 min in water. These leaching rates are very high, considering that fish larvae are slow feeders, and that it may take 1 to 5 min from the time the food is offered until the larvae find and ingest it.

# Conclusion

Red sea bream larvae are able to utilize free amino acids in the diet up to a certain level. Levels of 5% of the diet

Table 3A. Total amino acids in the diets (g/100 g of dry diet).

| Amino acid | Diet 1 | Diet 2 | Diet 3 | Diet 4 | Diet 5 |
|---|---|---|---|---|---|
| TAU | 0.22 | 0.22 | 0.27 | 0.23 | 0.23 |
| HYPRO | 0.06 | 0.07 | n.d. | 0.04 | 0.04 |
| ASP | 2.92 | 3.11 | 3.35 | 2.93 | 3.02 |
| THR | 1.50 | 1.36 | 1.36 | 1.09 | 0.99 |
| SER | 1.97 | 1.91 | 2.21 | 1.84 | 1.87 |
| GLU | 8.92 | 9.43 | 10.14 | 8.75 | 8.87 |
| PRO | 3.60 | 3.60 | 4.16 | 3.69 | 3.82 |
| GLY | 1.31 | 1.32 | 1.43 | 1.28 | 1.32 |
| ALA | 2.11 | 2.19 | 2.36 | 2.15 | 2.21 |
| CYS | 0.04 | 0.05 | n.d. | 0.07 | 0.04 |
| VAL | 1.75 | 1.85 | 2.19 | 1.90 | 1.88 |
| MET | 1.02 | 1.05 | 0.56 | 0.98 | 0.39 |
| ILE | 1.64 | 1.74 | 1.98 | 1.79 | 1.74 |
| LEU | 4.96 | 5.02 | 5.64 | 4.89 | 4.81 |
| TYR | 1.89 | 1.90 | 2.85 | 1.76 | 2.07 |
| PHE | 1.97 | 1.96 | 2.20 | 1.98 | 1.94 |
| HIS | 0.87 | 0.82 | 1.02 | 0.79 | 0.77 |
| LYS | 3.40 | 3.33 | 3.36 | 3.20 | 3.37 |
| TRP | 0.40 | 0.45 | 0.12 | 0.64 | 0.07 |
| ARG | 2.54 | 2.55 | 2.72 | 2.52 | 2.48 |
| Total | 43.08 | 43.93 | 47.92 | 42.52 | 41.93 |

Table 3B. Total amino acids in the larvae after 30 d feeding different diets (g/100 g dry weight).

| Amino acid | Diet 1 | Diet 2 | Diet 3 | Diet 4 | Diet 5 |
|---|---|---|---|---|---|
| TAU | 0.02 | 0.01 | 0.04 | 0.04 | 0.02 |
| HYPRO | 0.19 | 0.03 | 0.22 | 0.22 | 0.22 |
| ASP | 4.11 | 3.46 | 3.67 | 3.90 | 3.73 |
| THR | 1.84 | 1.55 | 1.67 | 1.83 | 1.70 |
| SER | 1.80 | 1.64 | 1.64 | 1.86 | 1.71 |
| GLU | 7.44 | 6.27 | 6.77 | 6.96 | 6.69 |
| PRO | 2.31 | 2.09 | 2.13 | 2.01 | 1.93 |
| GLY | 2.50 | 2.35 | 2.36 | 2.32 | 2.36 |
| ALA | 2.50 | 2.13 | 2.29 | 2.48 | 2.33 |
| CYS | 0.25 | 0.02 | 0.21 | 0.15 | 0.18 |
| VAL | 2.10 | 1.67 | 2.03 | 1.93 | 1.99 |
| MET | 0.99 | 1.36 | 1.33 | 1.32 | 1.30 |
| ILE | 1.88 | 1.51 | 1.82 | 1.68 | 1.74 |
| LEU | 4.09 | 3.41 | 3.85 | 4.01 | 3.88 |
| TYR | 1.85 | 1.63 | 1.55 | 1.53 | 1.53 |
| PHE | 1.97 | 1.76 | 1.78 | 1.82 | 1.78 |
| HIS | 1.19 | 1.05 | 1.18 | 1.14 | 1.15 |
| LYS | 3.58 | 2.75 | 3.09 | 3.22 | 3.01 |
| TRP | n.d. | 0.49 | 0.48 | 0.56 | 0.51 |
| ARG | 3.14 | 2.89 | 2.97 | 2.94 | 2.96 |
| Total | 43.75 | 38.07 | 41.03 | 41.92 | 40.72 |

as CAAs had a beneficial effect in larval performance, compared with a control diet without CAAs. This is in accordance with a recent review on the importance of free amino acids in fish larval nutrition (Rønnestad and Fyhn, 1993). A level of 10% of the diet as CAAs produced a larval performance slightly lower than a control diet. This level of (10%) CAAs in the diet is considered the maximum inclusion level without seriously affecting larval survival. This result is in agreement with a previous report on the utilization of free amino acids by Japanese flounder larvae (López-Alvarado and Kanazawa, 1993). Larvae fed diets with 15 and 20% CAA had a much lower larval performance than the control group. Leaching of CAAs from these diets was very high, and these larvae were in fact ingesting diets with quite low protein levels, resulting in lower growth and survival.

It is recommended that experimental diets for fish larvae contain levels of CAAs of up to 10% of the diet.

Higher levels proved to be detrimental. Due to the high leaching rates of CAAs in water, microencapsulation of these amino acids is recommended.

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