# LYSINE REQUIREMENT IN THE DIET OF GLASS EELS, *ANGUILLA BICOLOR*, AGAINST GENE EXPRESSION ASSOCIATED WITH GROWTH HORMONE

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**ABSTRACT**

**Traditional eel farmers face the problem of poor quality and high price of commercial glass eel diet, this feed has produced poor fish performance, as well. The lack of essential amino acids may be the cause. Molecular methods are used to determine the rapid response of glass eels to a formulated diet. The purpose of this study was to determine how the effect of adding lysine to commercial feed affects the expression of genes related to growth hormone GH. Pasta feed was given to glass eels (1600 heads) which were cultured for 60 days. The cylindrical plastic ponds were framed with steel and covered with HDPE plastic (300µm) in size {(3.14cm) x (85cmx85cm) x (60cm)} filled with water 907,460 L ≈ 0.91 m3. Real time RT-PCR results in GH suggest that the level of lysine (2%) added to the diet has strong effect on GH gene expression during eight weeks cultivation.**

**Key words: formulation diet, molecular technology, nutrition, optimum growth.**



## متطلبات الليسين في حمية ثعبان البحر الزجاجي ، أنغيلا ثنائي اللون ، ضد التعبير الجيني المرتبط بالنمو(GH)

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**المستخلص**

**يواجه مزارعو ثعبان السمك التقليديون مشكلة الجودة الرديئة والسعر المرتفع للنظام الغذائي التجاري لثعبان السمك الزجاجي ، وقد أدى هذا العلف إلى ضعف أداء الأسماك أيضًا .قد يكون السبب هو نقص الأحماض الأمينية الأساسية .تم تطبيق التقنية الجزيئية لمعرفة الاستجابة السريعة لثعبان السمك الزجاجي للنظام الغذائي المركب .كان الهدف من هذه الدراسة هو توضيح كيفية تأثير مكملات اللايسين الغذائية في نظام غذائي تجاري للثعابين على التعبير الجيني المرتبط بالنمو .(GH) تمت تربية الثعابين الزجاجية 1600) رأس (لمدة 60 يومًا وتغذيتها على علف المعكرونة .تم تأطير الأحواض البلاستيكية الأسطوانية بالفولاذ ومغطاة ببلاستيك 300) HDPE ميكرون (بحجم 3.14)} سم 85) × (سم 85 × سم 60) × (سم {(مملوءة بالماء 907460لتر 0.91 م .3 تشير نتائج RT–PCR في الوقت الحقيقي في GH إلى أن مستوى اللايسين2) ٪ (المضاف إلى النظام الغذائي له تأثير قوي على التعبير الجيني لـ GH خلال ثمانية أسابيع من الزراعة**

**الكلمات المفتاحية: هرمونات النظام الغذائي، هرمون النمو (GH)، ثعابين زجاجية، ليسين**

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## INTRODUCTION

Eels have high commercial value in Asian countries, especially Japan, China, Korea and Taiwan. This is an opportunity for Indonesia to increase eel yields and exports due to greatly reduced production in these countries. (14, 20 23). The addition of important amino acids such as lysine to feed increases the growth rate and survival of gas eels reared at the nursery stage (6,16). Essential amino acids that are not manufactured by animals such as lysine will be admit as proteins containing these amino acids (3, 5,17). Lysine content that does not meet the needs in the fish's body will cause decreased appetite, stunted growth, damaged fins and a large number of deaths (18, 24, 25) as shown in Japanese sea bass *Lateolabrax japanicus*, milk fish *Chanos chanos*. Many findings have been conducted to see changes in growth performance with the addition of lysine such as Penaeus monodon, Marsupenaeus japonicus (2). Lysine added to feed as much as 5.1% for striped bass (Morone saxatilis) will increase weight growth by 200% and feed conversion ratio value by 85%. In Pasific White Shrimp, *Litopenaeus vannamei*, the addition of lysine of 2.49% showed that a significant increase in the value of weight gain and specific growth ratio. In *Anguilla anguilla* reared for 2 months, feed containing 2.8% lysine showed a better average growth performance (12). The anterior pituitary gland of the vertebrae secretes growth hormone in the form of a single-chain pleiotropic polypeptide (10). All vertebrates are driven by a strong endogenous anabolic hormone which is growth hormone (GH) secreted by the pituitary gland (4,13). The stimulation of growth hormone (GH) encourages the liver and other tissues to secrete insulin like growth factor. Growth hormone controls growth, somatic development and metabolism, reproduction, appetite, osmoregulation, social behavior, and immunity which are physiological functions, as well. Transcription in target genes is the result of high stimulation of growth hormone (GH) and growth hormone receptors (GHR) in target tissues (27). In the early larval and embryonic stages of several types of fish, expression of growth hormone (GH) can be found. The presence of GH at the early stage of development indicates that it plays an important role in the growth and survival of larvae. This has been further supported by the stimulating effect of exogenous GH growth, transfer and overexpression of the GH gene that is shown in teleost larvae (22). This study aimed to explore lysine supplementation in glass eels' diet against gene expression associated with growth hormone (GH).

## MATERIALS AND METHODS

**Experimental design and sample preparation:** Glass eels (0.128 ± 0.002 g; mean ± SD) were obtained from glass eel fishermen and brought to the Cultivation Laboratory, Faculty of Fisheries and Maritime Sciences. During adaptation in the experimental pond, they were trained to eat artificial feed for 7 days. They were selected, and then transferred to plastic ponds. The cylindrical plastic ponds were framed with steel and covered with HDPE plastic (300μm) in size {(3.14cm) x (85cmx85cm) x (60cm)} filled with water 907,460 L ≈ 0.91 $m^3$ and each pond stocked 400 fish, with three replicate ponds. Glass eels were reared for 60 days and fed artificial feed in the form of pasta. The content of crude protein, crude fiber, ash and levels can be seen in Table 1. Feed was given as much as 3% of body weight every day until the end of the experiment. Pasta feed was given four times a day at 0.600, 11.00, 16.00 and 20.00, respectively.

**Table 1. Proximate content of feed (mean ± SD) based on the results of laboratory analysis (n=2)**

| Nutrients | Control (%) | Lysine (1%) | Lysine (2%) | Lysine (3%) |
|---|---|---|---|---|
| Organic matter[a] | 79.87±0.02 | 79.84±0.01 | 79.81±0.03 | 79.81±0.02 |
| Crude protein | 47.10 ±0.02 | 49.04 ± 0.17 | 49.13 ± 0.36 | 49.12 ± 0.73 |
| Crude lipid | 6.48 ± 0.04 | 6,52± 0.04 | 6.57± 0.05 | 6.45 ± 1.6 |
| Crude fiber | 7.70± 0.04 | 7.72 ± 0.04 | 7.80 ± 0.01 | 7.72 ± 0.04 |
| Moisture | 7.35 ± 0.03 | 7.37 ± 0.03 | 7.61 ± 0.28 | 7.41 ± 0.02 |
| Ash | 20.14±0.02 | 20.16±0.01 | 20.19±0.03 | 20.20±0.02 |
| NFE[b] | 11.25±0.13 | 9.19±0.28 | 8.71±0.15 | 9.11±0.48 |
| Gross energy (kcal/100g)[c] | 367.68±0.40 | 379.50±4,80 | 377.81±2.30 | 376.68±1.90 |

**a. organic matter = 100-Ash (%); b. NFE = 100-(CP + EE + CF + ash + moisture); c. Gross energy (GE)= (CP×5.6) + (EE×9.44) + (CF×4.1) + (NFE×4.1) kcal/100 g (NRC, 1993)**

**Diet preparation:** The glass eel diet is practically prepared as practiced by local aquaculturist, however the difference is the addition of vitamins, fish oil and lysine. Tapioca was used as a binder and used to adjust the percentage ratio, as well. Fish oil was bought from fishermen who make it traditionally. The lysine used was food grade lysine. The mixture of feed ingredients (Table 1) was made into a paste by adding enough water. The compositions of the experimental diets were prepared as in Table 2 and proximate compositions showed in Table 1. The fish oil added as feed stimulants to improve the appetite of larva. Three diets (containing 49% protein) were at similar protein levels by adjusting the content of lysine. The feed formulation made to find out how much lysine supplementation was suitable for glass eels.

**Specific Growth Rate:** Specific growth rate was calculated according to the following equation to

$$\mathbf{SGR= (\frac{Ln\ W2-Lnw1}{T}) \times 100 \ldots (\%\ g/day).}$$

**Survival Rate**

Survival rate was calculated according to the following equation to (21).

$$\mathbf{SR= \frac{Final\ number\ of\ fish\ survivor}{initial\ number\ of\ stocked\ fish}\ x100\ (\%).}$$

**Table 2. The compositions of the experimental diet**

| Ingredients | Control | Lysine-1% | Lysine-2% | Lysine-3% |
|---|---|---|---|---|
| commercial feed g(%/W) | 74 | 72 | 72 | 72 |
| Rice bran g(%/W) | 10 | 10 | 10 | 10 |
| Tapioca g(%/W) | 14 | 15 | 14 | 13 |
| Vitamin mix g(%/W) | 1 | 1 | 1 | 1 |
| Fish oil g(%/W) | 1 | 1 | 1 | 1 |
| Lysine g(%/W) | | 1 | 2 | 3 |

**RNA Isolation and RT-PCR:** The whole body of the sample glass eels was extracted using the Sepasol R-RNA super 1 blue reagent (nacalai tesque) with the Ethanol-phenol-chloroform extraction method to obtain the total mRNA. DNAse free RNAse (Takara) was used for all samples. Agarose gel electrophoresis was used to test the quality and concentration of total RNA and was read at 260 and 280 nm. RNA was loaded in batches and frozen at -70 C.

**RT-PCR :** cDNA synthezis kit (PrimeScript™ Reverse Transcriptase) from Takara was used for reverse transcription of a total of 1µl of mRNA samples. Primer Random 6 mers (50µM) and Prime script R-tase were used according to the manufacturer's instructions.

**Quantitative Real Time Analysis:** Primer 3.0 software was used to design primers based on GH Anguilla anguilla (AY148493.1), Anguilla japonica (M24066.1), and Anguilla australis (HQ436341.1). Anguila actin used as endogenous control, was amplified by the following primers —actin forward 5-GAGCTATGAGCTCCCTGACGG-3, and actin reverse 5-AAACGCTCATTGCCAATGGT-3 were used to normalize variations in RNA (Table 3). After optimization was achieved, the PCR reaction used a 10 µL volume containing 2 µL cDNA, 5 µL SYBR mixture (Applied Biosystem), 0.3 µL forward primer, 0.3 µL reverse primer, and 2.4 µL DDW. The standard curve is used to analyze the results obtained according to the recommendations of Applied Biosystems.

**Table 3. The primer used to amplify the GH and the PCR product**

| No | Name/Primer Code | DNA sequence (primer) | Tm | PCR product |
|---|---|---|---|---|
| 1 | Forward GH real time (F5) | CGTGGATCHYTGMARTACGAGTT | 60,23 | 360 bp |
| 2 | Reverse GH real time (R5) | ATGGTGGCRTCATTGAT | 69,67 | |
| 3 | Forward Actin (FA) | GAG CTA TGA GCT CCC TGA CGG | 58,3 | 53 bp |
| 4 | Reverse Actin (RA) | AAA CGC TCA TTG CCA ATG GT | 55,6 | |

**Data Analysis:** The SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA) was used to analyze the study results. Analysis of variance (ANOVA) was used to test for

differences between treatments followed by Tukey's Post Hoc Test. Mean values are expressed as mean ± SD (P<0 ,05)

## RESULTS AND DISCUSSION

All experimental pasta diet was well accepted and the glass eels approached the feed in 22-30 seconds. The addition of 2% lysine showed that the weight gain was significantly higher when compared to the addition of 1%, 3% lysine and without the addition of lysine (control) (Table 4), while the survival of glass eels was not affected by the addition of lysine. In this study, the maximum lysine requirement was estimated at 2% of the dry weight (Table 4), the higher lysine content in the feed (3%) resulted in reduced weight gain. In the Pacific white shrimp *Litopenaeus vannamei*, the addition of 2.49% lysine significantly increased body weight gain, whereas in juvenile Chinese Sucker (*Myxocyprinus asiaticus*), lysine required 1.04% of the feed given. Pacu *Piaractus mesopotamicus* (Holmberg, 1887) requires the addition of 1.44% lysine to its feed, while the striped bass, *Morone chrysops X M.Saxatilis* requires the addition of 5.1% lysine to grow properly. The addition of 2.49% lysine increased the growth performance of Penaeus monodon.

**Table 4. The Growth of glass eels during 8 weeks**

| Treatments | Control | Lysine (1%) | Lysine (2%) | Lysine (3%) |
|---|---|---|---|---|
| **Initial weight (g)** | **0.127± 0.003$^{a}$** | **0.128±0.003$^{a}$** | **0.128±0.002$^{a}$** | **0.128±0.002$^{a}$** |
| **Final weight (g)** | **1.31±0.02$^{a}$** | **1.33± 0.02$^{a}$** | **1.35± 0.02$^{b}$** | **1,31±0.02$^{a}$** |
| **Weight gain (g)** | **1.18±0.02$^{a}$** | **1.20±0.02$^{a}$** | **1.30 ± 0.08$^{b}$** | **1.18±0.02$^{a}$** |
| **SGR (%/day)** | **3.70±0.03$^{a}$** | **3.71±0.03$^{a}$** | **3.78±0.06$^{a}$** | **3.69±0.03$^{a}$** |
| **Survival (%)** | **29.92±2.67$^{a}$** | **31.92±2.75$^{a}$** | **31.42±0.76$^{a}$** | **31.83±1.81$^{a}$** |

**Means within a row followed by different superscript letters were significantly different (P < 0.05)**

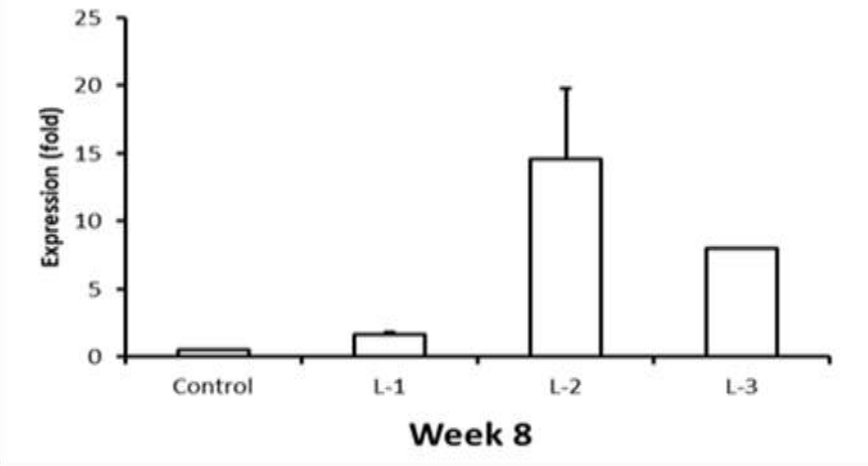


**Figure 1. Expression of GH in the week 8**

The results of Real-Time PCR from glass eel showed that feed with lysine 2% supplementation had a strong effect on GH gene expression during eighth weeks cultivation. These findings were in agreement with many studies showed that there was an overall increasing lysine supplementation would increase GH gene expression. In dietary studies, exogenous supplements, such as the addition of amino acids, carbohydrates, enzymes and hormones, show that the addition of these supplements affects the expression of GH and IGF. In Nile tilapia, *Oreochromis niloticus*, modulation of protein sources and exogenous protease affect significantly to GH expression in brain and liver (15). Feed with a high vegetable protein content (75%) also showed high GH gene expression in the liver as shown in rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (*Sparus aurata*) (1). In this study, addition of lysine in the diet showed that the relative level of GH gene expression was increased greatly in glass eels with increasing of lysine level in the diet. These findings were in agreement with many studies showed that in the early development of larvae when there was an overall increasing specific diet needed supplementation would increasing GH gene expression. The growth hormone (GH) gene increased rapidly in Siberian sturgeon (*Acipenser baerii*) at an early developmental stage after being treated with a high-carbohydrate diet and above normal temperature. *Megalobrama*

*amblycephala* increased growth hormone (GH) gene expression levels after being treated with high carbohydrate feed (26). GH mRNA was detected well in the early developmental stages of the larvae of Chanos chanos (11), grouper Epinephelus coioides (19) and Chinese sturgeon Acipenser sinensis Gray (9).

## CONCLUSION

In conclusion, this research molecularly reveals that the success of feed intake will occur simultaneously with the emergence of hormones. This is important information that the addition of lysine intake will improve nutritional quality and value in practical fish diets. Intake of lysine from outside shows that it can be used as a functional feed to improve the growth performance of glass eels amino acids as a feed supplement is feasible commercially. It is clear that research and development in the field of nutrition to assess essential for fish, especially glass eels.

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