Growth and morphological changes in relation to the maturation of male Japanese eel, *Anguilla japonica* injected with human chorionic gonadotrophin (HCG) in the different interval in the tropical region

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Abstract. In this study, human chorionic gonadotrophin (HCG) hormone of 1, 3, 5 and 7-days interval injection was done to investigate the morphological and growth changes in relation to maturation on male Japanese eel, *Anguilla japonica* in the tropical region within two months. No significant difference was found in all growth parameters. However, noticeable growth and morphological changes were only seen in the 5-day interval of hormone injection compared to other treatment. Hence, this study suggests that Japanese eel of 5-days injection interval has high possibility to achieve sexual maturation by taking growth and morphological changes into consideration.

1. Introduction

The Japanese eel *Anguilla japonica* which is native to East Asian, particularly in Japan of temperate climate and experiencing transition of the four distinct seasons, had been reported to have declined in population over the past four decades mainly due to uncontrolled fisheries [1]. From 1955 onwards, eel farming in Japan intensified and production has been increased with the introduction of paddle wheel aerators following by formulation of eel feed to replace trash fish in 1964 [2]. In 1992, the spawning ground of Japanese eel was discovered to be in the North Equatorial Current west of the Mariana Islands, at a salinity front near 15° N, 140° E [3].

In captivity, dopamine is a neurotransmitter that inhibits the sexual maturation of teleost fish unless by administration of exogenous gonadotropins to induce synthesis of reproductive hormones [4]. Japan is enthusiastic in research on the artificial seed production of Japanese eel and successfully produced the first preleptocephalus larvae in aquarium through hormonal treatment as reported in 1974 [5]. The breakthrough was in 2010 when the full-life cycle culture was attained from the hormone treated artificially hatched eels with shark roe feeding and proper water quality management [6].

Human chorionic gonadotrophin (HCG) is the most common hormone used for sexual maturation of various species of eel, the first attempt was experimented with male European eel, *Anguilla* sp. Meanwhile in 1936, European eel was reported with successfully induced spermatogenesis [7]. It was reported that weekly injection of HCG at 250 IU for 14 weeks on immature male Japanese eel reared
in sea water at 20°C induced spermiation and milt obtained was 1 to 4g at most for a single male eel [8].

There are many studies of HCG administration on the Japanese eel had been conducted under temperate climate and there is lack of information in the tropical climate. Since the spawning ground of Japanese eel in East Asian is temperate climate, this study was designed to investigate the morphological and growth changes in relation to maturation of male Japanese eel subjected to HCG injection under tropical region environments through the manipulation of the different interval of HCG injection that conducted within two months.

2. Material and methods
2.1. Japanese eels transportation and rearing
Japanese eels were transported by airline cargo from Nagasaka eel farm in Aichi Prefecture, Japan to Fish Hatchery of Borneo Marine Research Institute (BMRI) located in Universiti Malaysia Sabah (UMS), Malaysia. Upon arrival, the Japanese eels were in good condition which then acclimatised and reared under fixed temperature (29.44±0.33°C) in the 7500L of fibreglass tank prepared for a month. Tank was covered to prevent escape of Japanese eels. Water quality was taken twice a day (Table 1) using Hanna HI-9828 multiparameter during morning (8 am) and afternoon (4 pm) and trash fish feeding was given daily until satiation stage.

2.2. Japanese eels selection and injection
After a month of rearing under the hatchery condition, 16 Japanese eels were selected for the experiment. The Japanese eels were initially anaesthetised mildly with 4 ml of methylquinoline (Transmore, Nika brand, Japan) in 4 l of water, then insertion of tags to the dorsal body part of the Japanese eels were conducted for identification using Biomark 601 handheld reader. For 1-day interval, the ID of the tags were 2A74, 655E, 714C and 3023. For 3-days interval, the ID of the tags were 0F21, 686F, 4951 and 6454. For 5-days interval, the ID of the tags were 0C70, 2C3F, 1440 and 4901. For 7-days interval, the ID of the tags were 3D75, 4F2B, 1778 and 6751.

Then, the eels were slowly acclimatised to seawater in one tonne of fibre-glass tank for two days. Finally, the eels were kept separately according to treatment. Four different intervals of hormone injection were tested in this study: 1, 3, 5 and 7 days. Each treatment was kept in floating fibre-glass tank (length: 66.2 cm × width: 50.0 cm × height: 55.0 cm) installed inside the 150 tonnes of fibre-glass tank with water recirculation of about 20 cycles per day. Each floating fibre-glass tank containing four eels was covered with net to prevent eels escape and 40mm of polyvinyl chloride pipes were inserted as shelter to reduce stress of eels.

The hormone used in this experiment was HCG Pregnyl Injection 5000 IU Organon imported from Holland, one ampoule contains 5000 IU HCG was diluted with 5 ml of 1% saline water then kept in five syringes of 1 ml respectively to make 1000 IU per syringe. Before injection, the Japanese eels were anaesthetised to reduce movement and struggle. HCG hormone injection was carried out at the dosage of 1000 IUkg⁻¹ for all treatments.

In this experiment, the number of injections given for 1-day is 21 times in 21 days, 3-days is 21 times in 60 days, 5-days is 13 times in 60 days and 7-days is 9 times in 56 days. The final measurement was conducted at 70th day.

2.3. Data collection
The initial body weight of eels was weighed in gram with digital platform scale (model XK3190-A12, Accurate Measurement Malaysia Sdn Bhd) and initial body girth was measured with measuring tape, to the nearest 1 mm. Moreover, eel body, eye and anus were photographed using camera Canon EOS 50D and observed, then analysed photometrically using ImageJ software.

2.4. Data analysis
The morphological (eye index and condition factor) and growth (body weight and total length) changes were calculated according to following formula.

Mean body weight (BW) change was calculated according to the formula:

\[
\frac{\text{Mean (Final BW - Initial BW)}}{\text{Mean Initial BW}} \times 100
\]

Mean total length (TL) change was calculated according to the formula:

\[
\frac{\text{Mean (Final TL - Initial TL)}}{\text{Mean Initial TL}} \times 100
\]

Mean body girth (BG) change was calculated according to the formula:

\[
\frac{\text{Mean (Final BG - Initial BG)}}{\text{Mean Initial BG}} \times 100
\]

Condition factor (K) was calculated according to the formula:

\[
K = \frac{\text{BW (g)}}{\text{TL (cm)}^3} \times 1000
\]

The eye index (EI) was calculated as:

\[
\text{EI} = \left\{ \left( \frac{\text{A} + \text{B}}{4} \right)^2 \times \frac{\pi}{\text{TL}} \right\} \times 100 \quad [17]
\]

Where A is the horizontal eye diameter and B is the vertical eye diameter.

The statistical analysis was performed by means of one-way ANOVA (SPSS 21.0) with the data are presented as the mean ± standard deviation for continuous variables such as body weight, total length, body girth and condition factor and as percentages for eye index. One-way analysis of variance (ANOVA) was performed to compare 1, 3, 5 and 7-days treatments and significant effects from ANOVAs were further examined with Tukey’s post-hoc (HSD) test. The level of significance was set at \( P<0.05 \). All statistical procedures were performed using the Statistical Package for the Social Sciences 20.0 for Windows (SPSS 20, IBM, Armonk, NY, United States of America).

3. Results

3.1 Growth changes

There were no significant differences in mean body weight between treatments from 0\textsuperscript{th} to 40\textsuperscript{th} day (\( P>0.05 \), Figure 1). There were significant differences in mean body weight between treatments with the 3-days group (250.00±40.82g) having the highest mean body weight at 50\textsuperscript{th} day (\( P<0.05 \)). In contrast no significant differences in mean body weight were recorded between the 1-day (187.50±25.00g), 5-days (200.00±0.00g) and 7-days groups (187.50±25.00g)\( (P>0.05) \), whereas the 5-days group also having no significant differences with the 3-days group (\( P>0.05 \)). From 0\textsuperscript{th} day to 70\textsuperscript{th} day, the mean body weight of 1-day and 3-days groups decreased by 12.50g (-6.25\%, from 200.00±40.82g to 187.50±25.00g) and 25.00g (-10.00\%, from 250.00±40.82g to 225.00±50.00g) respectively, mean while the mean body weight of 5-days and 7-days groups remained the same which are 212.50±25.00g and 187.50±25.00g respectively but all showed no significant difference (\( P>0.05 \)).
Figure 1. Mean changes of body weight for 1-day, 3-days, 5-days and 7-days interval of HCG injection.

From 0th day to 70th day, each treatment showed decrease in mean total length as 1-day group is the highest which is 0.40cm (-0.68%, from 52.70±3.10cm to 52.30±3.20cm), followed by 7-days group is 0.30cm (-0.65%, from 52.80±2.20cm to 52.50±2.20cm), 5-days group is 0.20cm (-0.35%, from 55.00±2.30cm to 54.80±2.20cm) and lastly, 3-days group is 0.20cm (-0.29%, from 54.50±2.10cm to 54.30±1.80cm) but all showed no significant differences (P>0.05, Figure 2).

Figure 2. Mean changes of total length for 1-day, 3-days, 5-days and 7-days interval of HCG injection.
There were significant differences in mean body girth between treatments with the 3-days group having the highest mean body girth from 10th day onwards except at 30th, 40th and 70th (P<0.05, Figure3). In contrast no significant differences in mean body girth were recorded between the 1-day, 5-days and 7-days groups except at 50th day and 70th day (P>0.05), whereas 5-days group having no significant differences with 3-days group at 10th day (P>0.05). At 50th day, below 3-days group (8.50±0.54cm) is the 7-days group (7.53±0.53cm) having the higher mean body girth with significant differences than 1-day (6.65±0.13cm) and 5-days groups (7.33±0.24cm) (P<0.05). However, there were significant differences in mean body girth between treatments at 70th day with the 5-days group (8.25±0.26cm) having the highest mean body girth (P<0.05) whereas no significant differences in mean body girth were recorded between the 1-day (7.50±0.18cm), 3-days (7.13±0.57cm) and 7-days groups (7.60±0.67cm) (P>0.05).

![Mean changes of body girth for 1-day, 3-days, 5-days and 7-days interval of HCG injection.](image)

**Figure 3.** Mean changes of body girth for 1-day, 3-days, 5-days and 7-days interval of HCG injection.

From 0th day to 70th day, the mean condition factor of 5-days and 7-days groups increased by 0.01 (1.02%, from 1.28±0.12 to 1.29±0.12) and 0.01 (0.95%, from 1.28±0.24 to 1.29±0.11) respectively meanwhile 1-day and 3-days decreased by 0.05 (-3.43%, from 1.36±0.09 to 1.31±0.08) and 0.14 (-9.06%, from 1.54±0.11 to 1.40±0.24) respectively but all showed no significant difference (P>0.05, Figure 4).
3.2. Morphological change

There were significant differences in mean eye index between treatments with the 7-days group having the highest mean eye index from 50th day onwards to 60th day ($P<0.05$, Figure 5). In contrast no significant differences in mean eye index were recorded between the 1-day, 3-days and 5-days groups ($P>0.05$), whereas 3-days group showed a mean eye index below these groups from 10th day onwards to 60th day, significantly lower than 7-days group at 50th and 60th day ($P<0.05$). At 50th and 60th day, below 7-days group is 1-day and 5-days groups but having no significant differences ($P>0.05$). At 70th day, the mean eye index of Japanese eel injected with 7-days group is found with the highest mean eye index which is 8.09±2.52%, followed by 5-days is 7.10±1.10%, 3-days is 5.83±1.04% and 1-day is 5.64±1.15% respectively but no significant difference is found ($P>0.05$).
Japanese eel injected with 1-day interval in term of horizontal eye diameter, particularly specimen 655E showed lowest enlargement which is 1.89% from 0.53cm at 0th day to 0.54cm at 70th day(Figure 6). The highest enlargement showed by specimen 714C is32.14% from 0.56cm at 0th day to 0.74cm at 70th day, followed by specimen 3023 is 18.87% from 0.53cm to 0.63cm and lastly, specimen 2A74 is 25.45% from 0.55cm to 0.69cm. In term of vertical eye diameter, particularly specimen 655E showed lowest enlargement which is8.89% from 0.45cm at 0th day to 0.49cm at 70th day. The highest enlargement showed by specimen 2A74 is 25.00% from 0.48cm at 0th day to 0.60cm at 70th day, followed by specimen 714C is 22.45% from 0.49cm to 0.60cm and lastly, specimen 3023 is 18.00% from 0.50cm to 0.59cm.

![Image of eye enlargement in four Japanese eels of 1-day interval of HCG injection.](image)

Japanese eel injected with 3-days interval in term of horizontal eye diameter, particularly specimen 6454 showed lowest enlargement is 13.79% from 0.58cm to 0.66cm(Figure 7). The highest enlargement showed by specimen 4951 is 44.44% from 0.45cm at 0th day to 0.65cm at 70th day, followed by specimen 686F is 35.85% from 0.53cm to 0.72cm and lastly, specimen 0F21 is 35.42% from 0.48cm to 0.65cm. In term of vertical eye diameter, particularly specimen 6454 showed lowest enlargement which is 2.04% from 0.49cm at 0th day to 0.50cm at 70th day. The highest enlargement showed by specimen 4951 is 46.34% from 0.41 at 0th day to 0.60 at 70th day, followed by specimen 686F is 29.41% from 0.51cm to 0.66cm and lastly, specimen 6454 is 24.00% from 0.50cm to 0.62cm.
Figure 7. Eye enlargement in four eels of 3-days interval of HCG injection.

Japanese eel injected with 5-days interval in term of horizontal eye diameter, particularly specimen 4901 showed lowest enlargement is 23.44% from 0.64cm at 0th day to 0.79cm at 70th day (Figure 8). The highest enlargement showed by specimen 0C70 is 53.06% from 0.49cm at 0th day to 0.75cm at 70th day, followed by specimen 2C3F is 48.98% from 0.49cm to 0.73cm and lastly, specimen 1440 is 43.48% from 0.46cm to 0.66cm. In term of vertical eye diameter, particularly specimen 2C3F showed lowest enlargement which is 13.21% from 0.53cm at 0th day to 0.60cm at 70th day. The highest enlargement showed by specimen 1440 is 65.79% from 0.38cm at 0th day to 0.63cm at 70th day, followed by specimen 0C70 is 42.31% from 0.52cm to 0.74cm and lastly, specimen 4901 is 35.85% from 0.53cm to 0.72cm.
Japanese eel injected with 7-days interval in term of horizontal eye diameter, particularly specimen 6715 showed lowest enlargement is 39.13% from 0.46cm at 0th day to 0.64cm at 70th day (Figure 9). The highest enlargement showed by specimen 4F2B is 69.39% from 0.49cm at 0th day to 0.83cm at 70th day, followed by specimen 1778 is 54.17% from 0.48cm to 0.74cm and lastly, specimen 3D75 is 50.91% from 0.55cm to 0.83cm. In term of vertical eye diameter, particularly specimen 6715 showed lowest enlargement was 13.33% from 0.45cm at 0th day to 0.51cm at 70th day. The highest enlargement showed by specimen 4F2B is 128.57% from 0.35cm at 0th day to 0.8cm at 70th day, followed by specimen 3D75 is 97.56% from 0.41cm to 0.81cm and lastly, specimen 1778 is 43.48% from 0.46cm to 0.66cm.
Figure 9. Eye enlargement in four eels of 7-days interval of HCG injection.

3.3. Water quality parameters
Table 1 shows the mean average of temperature (°C), dissolved oxygen (DO, ppm), pH and salinity (ppt) throughout the experimental period. The readings of each water parameter were relatively constant in the morning and evening since these parameters were monitored strictly twice daily in order to provide ideal rearing condition to the experimental fish.

| Parameters     | Temperature (°C) | DO (ppm) | pH     | Salinity (ppt) |
|----------------|------------------|----------|--------|----------------|
| Morning (8am)  | 27.04±0.96       | 5.83±0.76| 7.66±0.33| 30.99±1.76    |
| Afternoon (4pm)| 27.29±0.88       | 5.89±0.73| 7.71±0.38| 31.18±0.99    |

4. Discussion
In the present study, Japanese eels in each treatment had ceased in consuming any feed given after they were acclimatized to seawater and kept inside the floating fibre-glass tank. It is presumable responsible for the reduction in overall body weight particularly when fish might had experienced from water loss and energy loss due to allocation to the adaptation to osmoregulation apart from starvation. Starvation alone insufficient to cause degeneration of the alimentary tract in gut morphology of hormone-treated eels, factors like osmoregulation in seawater and stress should be taken into consideration[9]. The seawater adaptation of Japanese eels such as drinking salt water has been similarly reported in European eels, revealed that salinity evoking enteric neuronal plasticity
caused a reduction of circular muscle layer in the eel gastrointestinal tract purposely for compensation of water loss during spawning migration [10]. It was reported that the intestine weight of silver eel reduced due to less developed circular muscle layer which will be more functional for osmoregulation rather than active nutrient absorption [11] and high efficiency of intestine epithelium for chloride (Cl\(^{-}\)) absorption as pre-adaptive strategy for long starvation time prior seaward spawning migration [12]. Moreover, the wild anguillids with degeneration of digestive tract had no stomach or intestine content was examined from captured by trawl near the spawning ground [13]. This statement in agreement with the Japanese eel observed throughout the experimental period.

Osmoregulation is energy demanding and energy can be supply from plasma levels of metabolites such as glucose, lactate, triglycerides or protein in the fish body [14]. Osmoregulation took place in gill that triggered by cortisol when fish under environmental stress tend to utilises lactate as an energy [14]. Hence, body weight of Japanese eels in the present study decreased by time as energy reserves was used for osmoregulation especially when eels refused to eat. Moreover, 1-day and 3-days groups experienced higher frequency of injections (21 times respectively) comparing to 5-days and 7-days groups (13 and 9 times respectively). Stress developed in 1-day and 3-days groups due to frequent handlings for injection. The growth reduced was demonstrated in European eels with degeneration of the gastric mucous membrane as a result of stress, accompanying by the disturbance of the endocrine control of mucous secretion, causing increased self-digestion and more vulnerable to invasion by pathogenic microorganisms [15].

In the present study, Japanese eels in each treatment probably experienced anorexia as total length decreased, resulted in shrinkage of size. Anorexia in fish is the decrease or cessation of feeding when fish is under unfavourable environmental condition [16]. Anorexia can be happened to any aquatic organisms including Japanese eels, as reported in feeding trial with formulated feed containing low concentration of ascorbic acid which caused poor growth [17]. Anorexia resulted in body shrinkage in term of body weight and total length in organisms, as this happened to hatchery-raised juvenile salmonids when released to natural environment in autumn due to lower preying ability compared to wild salmonids [18].

In the present study, Japanese eels in each treatment showed reduction in body girth except 5-days. The larger body girth of 5-days Japanese eels has high possibility of milt produced compared to other treatments with body girth reduced [19]. Body girth increases faster instead of total length during growth to sexual maturation as fat reserves in the body will be useful for spawning migration purpose whereas body girth decreased more rapidly than total length during starvation [20]. Body girth can be used to indicate maturation status of Japanese eel or other eel by taking the larger body girth producing more milt into consideration as cited by [19].

Japanese eels in each treatment showed reduction in condition factor except 5-days and 7-days groups. These findings reflect the positive outcome particularly in inducing gonad maturation of Japanese eel through 5-days and 7-days interval of hormone injection. Previous study reported on good condition factor of European eel, Anguilla anguilla is an ideal corpulence in relation to total length with condition factor value of 1.08 and values greater than that of 1.10-1.38 was considered in poor condition [20]. However, the condition factor value of European eels may not suitable as a guideline for ideal corpulence of Japanese eel since condition factor value in present study all were greater than 1.08. The higher value of condition factor was reported in 68 wild male Japanese eels captured in Yangtze River, China with mean condition factor value of 1.63±0.18 [21, [22]. showed that Japanese eel captured from Mikawa Bay, Japan with condition factor of late yellow eel (1.5±0.10), silver eel (1.4±0.20) and late silver eel (1.4±0.20) having no significant difference. The condition factor of present study is between 1.29±0.12 to 1.40±0.24 was smaller than the wild Japanese eels reported in previous study, possibly due to the farmed Japanese eel bought for this experiment was cultured in high stocking density in Japan. The difference in sex differentiation of A. Anguilla reared in stocking density of 3200gm\(^{-3}\) (96% males), 1600gm\(^{-3}\) (78% males) and 800g m\(^{-3}\) (69% males) was observed but male eels reared in low stocking density is significantly larger in size than male eels reared in high stocking density[23].
Japanese eels in each treatment showed increasing trend in eye index. Eye index (relative eye size) method of predicting the gonadal status of eel in particular has been widely used [24]. A correlation between eye index and sexual maturity for this species treated with an exogenous hormone and suggested its availability as a method of assessing sexual maturity during induced maturation. Previous study on the wild Japanese eels in silvery stage reported the eye index of range from 3.1-7.9% [25]. Another previous study on the wild Japanese eels in Kaoping River of Taiwan between November 1998 and November 2001 reported the mean eye index for silver eels was 5.10±0.32% [26]. The wild silver Japanese eels in Taiwan has smaller mean eye index (3.7-5.8%) [27]than the hormone-treated Japanese eels of present study conducted in Malaysia’s condition. In contrast, the previous study on the European eels reared in captivity mentioned that enlargement of eye can be triggered by other pituitary hormones of the eel itself instead by hormone injection [27]. The changes in eye index before and during silverying are in synchronisation with the maturation status as well-responsive HCG-treated Japanese eels showed larger eye enlargement indicating the eels achieving maturation [28].

5. Conclusion
This study suggests that the Japanese eel *A. japonica* of 5-days interval HCG injection has high possibility to reach sexual maturation in tropical climate by taking its positive growth and eye index with noticeable morphological changes into consideration.

6. References
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