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Induced Spawning and Larval Rearing of Japanese Eel *Anguilla japonica* in Taiwan

Abstract

In Taiwan, broodstock cultivation is a premise of the artificial propagation of Japanese eel *Anguilla japonica* due to the depletion of seaward migrating wild eel brooders. Aged Japanese eels reared in low salinities (5-10 ppt) and mud-based ponds were found with advanced gonads as well as high condition factor. The strategy of induced maturation of pond-reared female eel is commenced by injection of human chorionic gonadotropin (HCG) at the first month in order to increase the hormonal response of eel to the subsequent weekly injection of catfish pituitary extracts. At the final maturation stage, the full matured females were injected with catfish pituitary extracts and 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) to induce their ovulation and spawning in 100 *l* aquaria. By this way, fertilized eggs can be obtained year around. However, the problems of steadiness in fertilization and hatching rates as well as the survival rate of yolk-sac larvae are not yet solved. Eel eggs are buoyant and with multiple oil droplets. Potency of yolk-sac larvae to adapt wide range of water temperature and salinities were astonishingly strong, but they were typically sensitive to the disturbances such as aeration, oil film and water current. Darting behavior of yolk-sac larvae was often induced by mentioned disturbances and led to a crooked body shape and even death. The larvae started to exhibit a negative phototactic behavior since the forming of eye pigment. Taking together these observations, we speculate that the eel larvae may prefer a deeper, dim, serene and even low-temperature water layer at the onset of feeding. Despite evidences indicating that the eel larvae may depend on soluble organic matter in the water as their nutrient source, they could not survive beyond 25-day old in dissolved organic matter (DOM)-enriched rearing water or in green water. Based on the unique characteristics of eel larvae, finding appropriate foods and developing a suitable larval rearing system to meet their peculiar characteristics may be the key-point strategies for the successful larval rearing of the Japanese eel. The impediment of larval rearing of eel is due to the rudimentary and fragmented biological information of eel larvae. Undoubtedly, the mysterious life cycle of eels is considered as an attractive topic in the research field and remains to be overcome.

Key words: *Anguilla japonica*, Induced spawning, Early development, Larval rearing

The artificial propagation of eels has been performing for a long time since obtaining the sperms of European eel, *Anguilla anguilla* in 1937 and eggs in 1964[1]. After

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that, a lot of subsequent studies have been reported as summarized in Table 1. Recently, a breakthrough on eel larval rearing of Japanese eel has been achieved, extending the survival of eel larvae to around 250 days. In the same research, the longest body length recorded of the 209-day old larva was 31 mm[13].

Table 1. The advancement in obtaining gametes, embryos and larvae in artificial propagation of eel *Anguilla* spp.

Years	*Results*	*Sources or Remarks*
Anguilla anguilla		
1937 (1934)	Sperms	Boucher (1934), Cited from Bezdenezhnykh et al. (1984)[1]
1964	Eggs	Fontaine (1964), Cited from Bezdenezhnykh et al. (1984)[1]
1980	Gastrula embryos	Boëtius and Boëtius (1980)[2]
1983	3.5-days old larvae	Bezdenezhnykh et al. (1983)[3]
A. japonica		
Japan		
1966	Eggs	Hibiya (1966)[4]
1973	6-day old larvae	Yamamoto and Yamauchi (1974)[5]
1976	14-day old larvae	Yamauchi et al. (1976)[6]
1999	27-day old	Tanaka et al. (1999)[7]
2000	209-day old	Tanaka et al. (2000)[12], latest news reported the larvae surviving 253 day-old.
Taiwan		
1980	yolk-sac larvae	Ko and Tsay (1980)[8]
1993	25-day old larvae	Yu et al. (1993)[36]
1994	31-day old larvae	Yu and Tsai (1994)[9]
China		
1974	6-day old	Cited from Wang et al. (1980)[10]
1979	19-day old larvae	Wang et al. (1980)[10]
2000	32-day old larvae	Chen 2000[11]
A. rostrata		
1984	Gastrula embryos	Sorensen and winn (1984)[12]
1988	6-day old	Liao and Chang, unpublished data

Although the reproductive endocrinology of eels is well documented[14-18], the technique of induced breeding has not been completely established. The main reason is that the vitellogenesis of female eel caught in river or estuary is not completed. Among eels, the New Zealand longfinned eel *Anguilla dieffenbachii* was found as the only one that had reached a better gonadal development, with the GSI

of 5-10 % or secondary yolk globule stage before starting their seaward spawning migration[15]. Neither Japanese eel, European eeln or American eel is as mature as *A. dieffenbachii*, only with the oocyte development attained at the primary yolk globule stage. Therefore, the oogenesis, ovulation and spawning can only be achieved by long-term repeated and regular hormonal treatment. The hormones used for induced maturation include Synahorin (HCG + pituitary extracts of mammals), pituitary (carp, salmon, or catfish) and HCG. The success of induced spawning was impacted by many variables, such as asynchronization of oocyte development in the gonad and inconsistency in hormonal response among individuals. In addition, without additional treatment of DHP, most ovulations failed because of over-ripeness.

This report aim to discuss some problems encountered on eel broodstock cultivation, strategy of induced maturation, ovulation and spawning, egg quality, the unique characteristics of eel larvae, and larval rearing of the artificially propagated Japanese eel.

Broodstock cultivation

In Taiwan, there is practical difficulty in obtaining the catadromous silver Japanese eel as broodstock because of the environmental degradation. Therefore, most of the eel broodstock used for induced spawning must be obtained from aquaculture farms. The gonad development of pond-reared Japanese eel is effected by the age, seasonality, salinity, density and water temperature[19-21]. The best maturity of freshwater pond-reared aged Japanese eel (~5 years) was found with oocyte development of early primary yolk globule stage (GSI 2.23 %) in female and motility of sperms (GSI 3.5 %) in male[22].

Research results so far have shown that eel, which were reared in pond of low salinity (5-10 ppt), earth-based bottom and co-cultured with other less aggressive marine finfish species (e.g. *Acanthopagrus* spp. and *Pomadasys hasta*), having a gray pectoral fins, darken dorsal part, silver-colored abdomen, and with higher condition factor, are suitable as the broodstock. Some females also show a good maturity with the biggest oocyte of 0.27 mm in diameter even in July. In Taiwan, the growing phase of Japanese eel occurs in warm season from April to August, and the gonads start to develop from September[19]. Eels about 6 years of age which were cultured in earthen ponds with low saline water in the summer season can be found with light gray pectoral fins. The eye-pigmented larvae can still be obtained from those cultured eels with commencement of hormonal treatment in the summer season[23].

The guts of European eels were proved to degenerate during the maturing stage[24]. Feeding activity of eel brooders was normally interrupted during acclimating to saline environment in the laboratory before hormonal treatment. But the eel brooders were found to feed on the clam *Scapharca satowi* if they were stocked in fresh water environment before hormonal treatment[25]. It was considered that the eel brooders fed with the clams which is rich in highly unsaturated fatty acid (HUFA) 0.5-1 month before hormonal treatment will benefit the quality of eggs. Kagawa et al.[21] reported that the females were induced to vitellogenesis similar to the natural migrating silver eel with the primary yolk stage of oocytes prior to hormonal treatment after stocking in seawater for 3 months without feeding. Although the pond-reared eel brooders kept in seawater 3 months before hormonal treatment can be considered as the period needed for their migration to the spawning ground, the impacts of consuming and converting stored nutrition during the long-term acclimation period in seawater remain unclear. In addition, Lai et al.[19] found that Japanese eels cultured in seawater (22-28 ppt) were susceptible to parasites *Caligus* sp. and *Dactylogyrus* sp., which led to anorexia. Concerning appetite, condition factor and susceptibility to parasites, the aged eel reared in earth-bottom ponds with low salinity can be considered as alternative source of eel brooders.

Induced maturation, final maturation, ovulation and spawning

The seaward migrating spawning eel brooders are noticeable with the deep-black coloration of pectoral fins. It can be considered as an important indicator of nuptial coloration of eel. In order to facilitate the long-term hormonal treatment practically and efficiently, the maturity of pond-reared female is divided into 6 grades (0-5) based on the black coloration of pectoral fins[23]. The protocol for artificial maturation of female (800-1200 g) is summarized as follows: In the first month, the female are administered with 0.5-1 IU/g BW HCG depending on the black coloration of pectoral fins at the first and second week to increase the maturity to 3-4 grades at the end of first month. From the fourth week, weekly regular injections of 4 catfish pituitary (4.2 mg/pituitary) extracts per female are given, associated with reducing the dosage and shortening the injection period at the late stage [≧100 % Body weight index (BWI), BWI (%)=Body weight/Initial body weight x 100]. And then, the females are transferred from the large tank (2 x 1 x 1 m) to 100 *l* small aquaria (1 x 0.4 x 0.3 m) to facilitate the observation and handling of eel. Subsequently, the female administered with 2 pituitary extracts twice a week with the BWI between 100-105 %, and then 2 pituitary extracts 2 days interval with the BWI between 105-110 %. The injection protocol was modified to administer 1 pituitary extract per female daily while the BWI is over 110 % (Fig. 1). All brooders during hormonal treatment are stocked in a water temperature controlled re-circulating system (22-23 ℃).

HCG was found functional to enhance the black coloration of pectoral fins of eel brooders compared with the administration of catfish pituitary extracts only. According to the above mentioned protocol, 44.8 % of pond-reared females (210 females) could be induced to the final maturation stage[23]. It was higher than the result of Ijiri et al.[26] that only 29 % of pond-reared females reached the final maturation, but was lower than those of feminized females (64 %). In other words, there is about half of selected pond-reared in brooders that attained the final maturation stage. Low success rate of induced maturation may be due to the inconsistency in the hormonal response among individuals. Thus, the maturing rate of the female might be enhanced through improvement of technique in broodstock cultivation and increasing the culling rate of eel brooders before regular hormonal treatment. In addition, the effect of 17β-estrodiol on oogonia proliferation and vitellogenesis of Japanese eel are needed to be further studied.

In our previous studies, some of females ovulated at the end of 8th week, which were 5 days earlier than the result of Ohta et al.[27]. It means that the administration of HCG at the first month have the synergistic effect on the subsequent injection of catfish pituitary extracts. Although the function of HCG is similar to the attribute of GtH II, its effect on the early maturation of eel is obvious. The long-term efficacy of HCG on maturation of eel may be due to its long half life cycle. Half life cycle of HCG was 12 days in male European eel[28]. The synergistic effect of HCG co-injected with carp pituitaries was also proved effective in the induced maturation of European eel[2]. Since HCG has a function of stimulating the ovulation of teleost fishes, the timing of injection during final maturation in eels may become confusing if it is administered at the stage earlier before the final maturation.

The BWI of female slightly increased at 2 weeks after administration of HCG (Fig. 2). On the contrary, the BWI of those failing in success of induced maturation decreased continuously since the commencement of hormonal treatment. It means that the response of the female to HCG vary individually. Thus, the culling of female can be conducted at the second week based on the BWI after HCG administration. The response of female to HCG is positively correlated to the black coloration of pectoral fins. Therefore, the culling of female can also be conducted at the fourth week based on the coloration of pectoral fins.

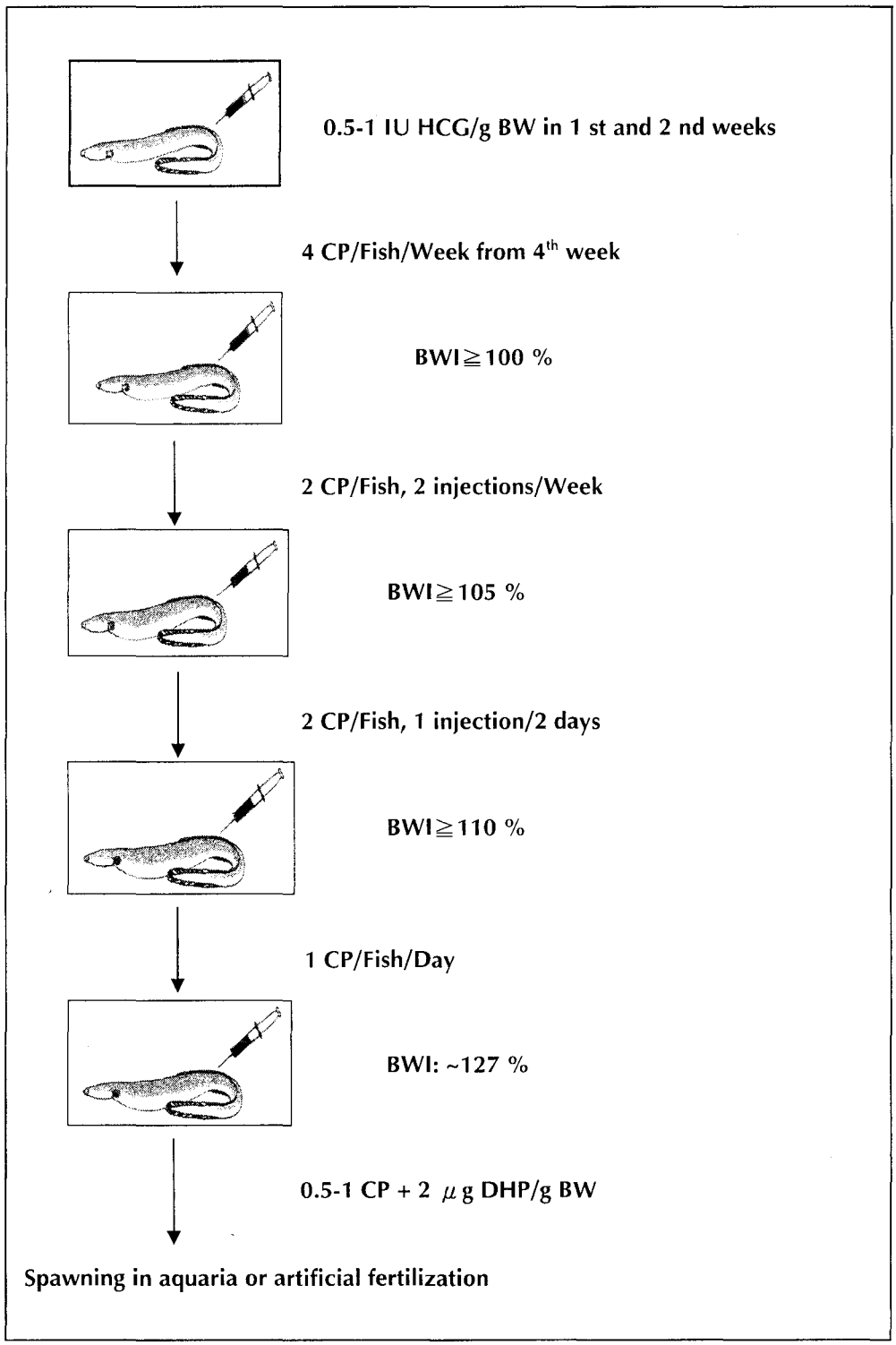

Fig. 1. The protocol of induced maturation and spawning in the artificial propagation of Japanese eel *Anguilla japonica*.

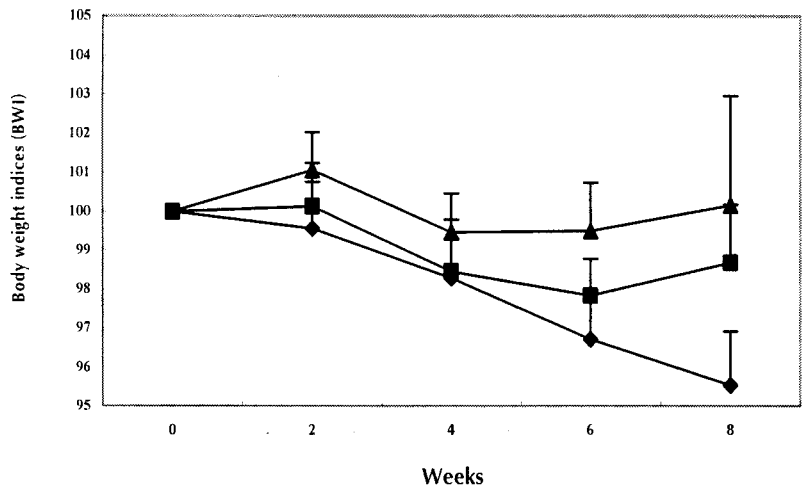

Fig. 2. The change of body weight index of female eel after hormonal treatment. ■Ovulated female (n=18) ▲Ovulated female with good egg quality (n=10) ◆Non-ovulated female (n= 9).

The ovulating female was identified by the BWI, the lapsed time from the abrupt body increase, the morphology of genital papillae, and the aggregation of oil globule of ova by intraovarian cannulation from genital pore. In the final treatment, a co-injection of 0.5-1 catfish pituitary extract and DHP are employed to induce eel ovulation and spawning. And then, three to five pituitary extracts-treated spermiating male are put together with the female. Most of ovulation or spawning occurred at 12-16 h after DHP administration, but mostly between 15-16 h In order to facilitate the further study of embryonic development, the timing for injection of DHP can be regulated to a certain period (Fig. 3).

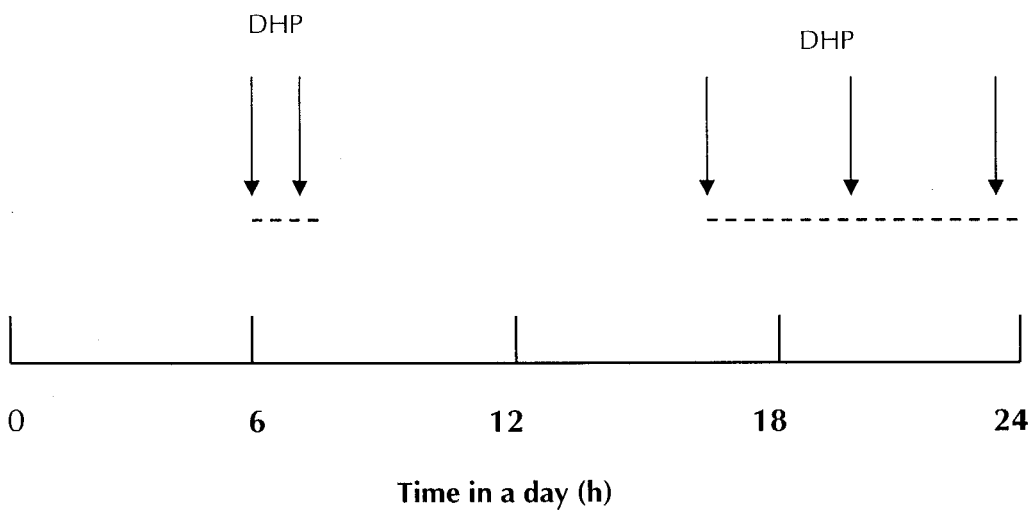

Fig. 3. The suitable timing for injection of DHP in order to induce the spawning occurred beyond the sleeping time.

Based on the hormonal administration mentioned above, seventy percent of females attained the final maturation stage within 2-4 months. Viable eye-pigmented larvae had been obtained from induction period of 61 to 175 days[23]. Since the feeding activity of eel brooders was interrupted in the stocking tanks for almost 6 months during the induced maturation, the effect of long-term starvation on egg quality and survival of larvae needs further study.

The success rate of ovulation after hormonal treatment in the brackish- (5-10 ppt) and freshwater-reared eels were 66.7 % and 38.5 %, respectively (Table 2). Higher percentage of female (58.8 %) reared in brackish water had ovulated within 2-3 months after hormonal treatment compared with those (28 %) reared in freshwater. It means that the hormonal response of female reared from brackish water is more sensitive than in freshwater. The result is comparable to the finding of Kagawa et al.[21].

The suitable timing for injection of DHP by judging with BWI might be affected by fecundity, induction period, condition factor, and synchronization in oocyte development. The response of pond-reared female brooders to hormonal treatment varied individually and seasonally, mostly resulting from asynchronous oocyte development which led to difference in induction period. Only few females performed a synchronous oocyte development. Ohta et al.[27] reported that the suitable timing for administration of DHP in feminized female was around BWI of 127 %. However, the inverse correlation was found between the induction period and BWI[23]. Thus, BWI of pond-reared eel brooders shoul not serve as the only indicator for the injection timing of DHP.

Table 2. Comparisons on the induced maturation of pond-reared female Japanese eel *Anguilla japonica* in freshwater or brackish water.

Rearing water	*Fresh water*	*Brackish water*
Number of female eel	65	51.0
Percentage of non-ovulated female (%)	61.5	33.3
Percentage of ovulated female (%)	38.5	66.7
Percentage of female eel ovulated between 2-3 months after hormonal treatment (%)	28	58.8
Percentage of female eel ovulated between 3-4 months after hormonal treatment (%)	36	17.7
Percentage of female eel ovulated beyond 4 months after hormonal treatment (%)	36	23.5

Induced maturation and spermiation of male

The maturity of male eel could be much advanced in captivity compared with the female. Aged male (~5 years) in captivity was reported to have motile sperms even when reared in freshwater[22]. Motility of sperms obtained from pituitary-treated pond-reared male eel lasted only 3-5 minutes[23], but those in the wild-caught male could last as long as 16-19 minutes [29]. In general, the protocol of induced maturation of male (150-450 g) could be conducted 1 month later than the female. The dosages of 1-2 IU HCG/g BW were administered weekly or 2 weeks interval depending on the maturity of male and female.

Spermiating males were moved into the spawning tanks (100 *l*) ready for mating with ovulating female.

The cost of hormonal treatment

In artificial propagation of eels, the ovulation and spermiation are induced through long-term hormonal treatment. It is not only laborious, but is also very expensive. About 100 USD on the average is needed for one mature female and three mature males during 3 months of induction period (Table 3). DHP is very expensive. *In vitro* experiment, oocytes at a migratory nucleus stage with diameter of 700-800 μm could respond to high dosage of 17 α-hydroxyprogesterone (17 α-OHP), a precursor of DHP[17]. The 17 α-OHP has a far lower price compared with DHP. Although there were several successful spawning induced by injection of 17 α-OHP, the suitable dosage of 17 α-OHP for induction of ovulation is needed to be established .

Egg collection and incubation of fertilized eggs

Egg collection is equipped with a cylindrical holding net (70 cm in diameter, 60 cm deep) connected with a tube to the spawning tanks. The holding net is suspending in a 2 m^3 tank (2 x 1 x 1 m). The eggs are incubated with light aeration and slow running water. The dead eggs are removed by siphon during incubation. The C-shaped embryos before hatching are transferred to the holding tanks fitted for the development of yolk-sac larvae and further larval rearing experiments.

Table 3. The cost of hormonal treatment for one matured female and three matured male.

Items	*Quantity needed*	*Sub-total (NT)*	*Remarks*
Female			Body weight of female is supposed to be 1 kg
HCG	1,500 IU	600	0.4 NT/IU
Pituitary of catfish	36 Pituitaries	540	15 NT/Pituitary
DHP	2,000 μg	1,000	0.5 NT/μg
Male			
HCG	3,600	1,440	0.4 NT/IU
Total		3,580	USD=103 (USD: NT=1:34.5)

Embryonic development

Spawned eggs of the eel are buoyant and have multiple oil droplets[23]. As the embryos developed, the small oil droplets converged and fused gradually. The pattern or the fusing degree of oil droplets can also be used to evaluate the quality of fertilized eggs. The oil droplets fuse into a single oil droplet at the blastula or gastrula stage in normal fertilized eggs.

Normally, the eel eggs form a big perivitelline space soon after fertilization. Most of fertilized eggs induced from the pond-reared eel have a smaller perivitelline space (1.1-1.2 mm) compared to the mean perivitelline space (~1.6 mm) in the eggs produced from female with a deep silvering abdomen. However, larvae hatched from the eggs with larger or smaller perivitelline space could both develop to the

eye-pigmented stage.

If the eggs were fertilized at 23 °C, the temperature range for embryonic development could be between 18 °C and 28 °C. The hatching time of eggs incubated at 20, 22, 24, 26 and 28 ℃ were 48, 40, 29, 26 and 24 hours, respectively[23]. The embryonic development was significantly retarded below 22 ℃. Taking floating attribute of eggs together, its ecological significance means that the fertilized eggs might float on warmer water layer soon after fertilization. The C-shaped embryos had already showed a strong tolerance to wide range of salinities (15-35 ppt). Sasai et al.[30] reported that a large number of the chloride cells on the yolk-sac membrane of C-shaped embryos of Japanese eel were found at 35 h after fertilization.

Peculiar characteristics of yolk-sac larvae

Sensitivity to disturbance and subsidence of yolk-sac larvae

After hatching, the larvae stretch its body very soon, and usually suspend vertically with its head up near the water surface[23]. In this period, eel larvae are almost motionless unless disturbed. After formation of neuromasts at about one-day old, the yolk-sac larvae often perform a darting behavior when disturbances are sensed. Accompanying with the yolk and oil droplet absorption, buoyancy is decreased and the larvae start to sink down gradually. These observations imply that, in the natural environment, eel larvae may suspend/locate in the middle- or upper-water layer initially, and then sink to the deeper water layer after forming the eye pigment. The route of subsidence must be quiet and serene. In this period, some factors like air bubbles, oil film and water flow may induce their acute body motion such as twitching that resulted in a crooked body shape and even death in the laboratory environment.

Subsiding attribute of yolk-sac larvae in rearing environment

In the artificial rearing environment, the developing larvae sink down on the bottom gradually and mostly lay there until 2-3-day old. Larvae sometimes perform a darting behavior and re-suspend in the water column temporarily, and then lay on the bottom. As mentioned above, the yolk-sac larvae are particularly sensitive to disturbances such as aeration, oil film and water flow. Survival of eye-pigmented larvae could still be maintained in the smaller and clean aquaria without any supply of aeration. In the bigger tanks or ponds, however, the survival of eye-pigmented larvae could not be maintained due to the difficulty in the separation of the dead eggs completely. As the result, water quality control becomes difficult. Furthermore, the subsidence of larvae is more rapid in lower salinity. In artificial rearing environment, the salinity is commonly lower than full seawater (32 ppt), which is due to the dilution from the rain or utilizing the source of lower salinity from underground seawater. The motionless larvae laid on the earth-bottom might be preyed by aquatic insects before developing to the eye-pigmented larvae. At present, the stocking technique of yolk-sac larvae needs to be improved.

Adaptation in wide range of water temperature

The fertilized eggs of Japanese eel are assumed to float on warmer upper or middle water layer after spawning based on floating attribute and temperature adaptation of embryo. After hatching, it takes about 4-5 days at 26-28 ℃ and 7-8 days at 22-23 ℃ for yolk-sac larvae developing to the eye-pigmented stage[23]. Survival of larvae had been found at 31-32 ℃ in the eye-pigmented stage. Surprisingly and doubtfully, the one-day old larvae already had a potency to tolerate the low water temperature of 3°C by decreasing the water temperature gradually from the 24℃. Besides, the eel larvae were reported having a higher feeding activity in water temperature of 26-28℃ in captivity[7]. The wild leptocephali of eel were found having a vertical migration behavior

between 50-100 m in daytime (27-28 ℃) and 200 m in nighttime (18-20℃)[31]. The adaptation to water temperature and the feeding habit of leptocephali might be different because of the obvious morphological changes in different stages.

Larval rearing

Concomitant with the eye pigmentation, the neuromasts disappear. Meanwhile, the darting behavior of larvae is taken place gradually by the snake-like swimming type with intermittently sinuate swimming behavior[23]. At this transitional stage, the larvae can withstand stress compared with the yolk-sac larvae. It can still survive after sucked by pipette. Transferring the larvae can be conducted at this stage. Apparently, the feeding larvae start to utilize their vision, in contrast to the yolk-sac larvae which depend on the neuromasts for sensing the motion of periphery. And then, the larvae exhibit a negative phototactic behavior after eye pigmentation. Concerning the subsidence of yolk-sac larvae and negative phototactic behavior of feeding larvae, it can be speculated that at the onset of feeding, the eel larvae prefer a deeper, serene, dim and may even inhabit low-temperature water layer. Feeding activity was found when the light intensity was regulated at the range of 40-100 luxes[7].

There are ample evidences to indicate that the leptocephali may subsist on dissolved organic matter (DOM) as their nutrient sources[32-35]. However, the larvae still neither survive beyond 25-day old in well DOM- (fermented from shrimp flake) enriched rearing water nor in green water in our experiment. No obvious active feeding behavior of larvae was found in our study and report of Wang et al.[10]. However, eel larvae were reported to prey rotifer at 13-day old[36]. Tanaka et al.[13] reported that the eel larvae survived over one month with feeding on the slurry-like compound feeds that were mainly made from shark egg powder and mixed with low molecular compounds, vitamin, and minerals. Feeding of larvae occurred on the bottom by controlling the suitable light intensity (40-100 luxes) to meet with their negative phototaxis. Yu et al.[37] reported that eel larvae survived 25 days by feeding rotifer and copepod. A lot of protozoa and labeled feeds were found in the gut of larvae[11]. Surprisingly, the growth of larvae in the latter two studies were significant faster with a total length of 19.2 mm at 25 days and 21.2 mm at 32-day old, respectively, than the former study of 11.9 mm at 30 days . Appelbaum and Riehl[38] speculated that the function of forward fang-like teeth of leptocephalous larva was considered as the filter for food while drifting. The feeding characteristics of eels need to be further studied.

It is quite difficult to control the water quality by supplying the slurry-like feeds for eel larvae[7]. Microorganisms were also considered blooming more quickly in higher temperature rearing water. In general, the biology of eel larvae is quite different to the larvae of typical marine fishes. According to peculiar characteristics of eel larvae, finding appropriate foods and designing or developing a suitable larval rearing system may be the key-point strategies for the successful larval rearing for the Japanese eel. It must be particularly different to the traditional larval rearing system for most of typical marine fishes.

Conclusion

At present, the technique of induced spawning of Japanese eel has been improved significantly. But it is still far compared with the successful techniques of producing fertilized eggs in other cultured marine fishes. Further studies of induced spawning need to be continued. The impediment of larval rearing is due to their complex life cycle. Undoubtedly, the mysterious biology of eel is regarded as a highlight topic in the research field and remains to be overcome.

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