Mass-Marking of Ayu Eggs and Larvae by Tetracycline-Tagging of Otoliths

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The object of this study is to develop a method of mass-marking the egg and larval stages of fish in order to carry out field surveys of the early life history and population dynamics of the fish. In this method the eggs or newly hatched larvae are immersed in tetracycline hydrochloride (TC) solution and marked-fish or fish from treated eggs are detected by the presence of a fluorescent mark on otoliths under UV light.

To determine the optimum TC concentration and treatment time for marking the eggs and larvae of the ayu *Plecoglossus altivelis*, a diadromous fish, various combinations of TC concentration and treatment time (40-44 lots) were tested. As a result, 200-300 mg/l for 24-48 h was found to be appropriate for eggs and 200-300 mg/ml for 3-24 h for the larvae. Mark retention and the effect of TC treatment on survival and growth were examined by rearing marked larvae in 500 l plastic tanks for more than 5 months. The mark was easily discernible (100%) until 105 days after hatching without any special preparation for detection. Even 164 days after hatching the percentage of retention was 100% after grinding or etching in weak acid, compared with 83% before such treatment. The treatment had no effect on the survival and growth of the ayu. These results strongly suggest that this method can be applied to field surveys on the ayu during early life stage in the sea.

Mark-recapture methods are an effective technique for studies on migration, population size, growth rate and behavior of fish. Although various sophisticated techniques have been developed for field studies and aquaria experiments, none is foolproof. An investigator should select a method which complies as far as possible with the requirements of his experiment, in consideration of its objectives, duration, detection method, type of fish (*i.e.* species, size, stage of life cycle) and so on.¹⁾ The early life stages seem to hold the key to understanding fluctuations in fish population and fisheries biologists have long sought an effective means of marking fish eggs and recently hatched larval fish in a manner that would not alter the life functions or mortality of marked fish from those of unmarked fish. If a suitable marking method is available at this early life stage, it would be possible to determine the various population parameters such as density, mortality rate, exploitation rate, and recruitment rate, as well as age, growth and migration.

Vital staining, either by immersion or incorporation of a dye into food, is one of the few methods available for marking very small fish. ARNOLD²⁾ in a review of fish marking with dyes concludes

that immersion staining is of value only for short-term experiments. SAKURAI *et al.*³⁾ reported that the glass eel *Anguilla japonica* marked with a fluorescent red dye could be detected for 1-2 months after marking and TZENG⁴⁾ demonstrated the application of this method to a field survey of elvers for *c.a.* 1 month. BAGENAL⁵⁾ fed maturing female brown trout *Salmo trutta* with pelleted food mixed dry with powdered Sudan Black. The resulting eggs were darkly stained and the color remained in the fry for 6 weeks after they started to feed themselves. The use of fluorescent materials (*e.g.* tetracycline or oxytetracycline) as *in vivo* bone-tissue marker is now popular not only in marking experiments^{6,7)} but also as a time mark in estimates of growth of hard tissue.^{8,9)} TROJNAR¹⁰⁾ fed tetracycline hydrochloride to rainbow trout fry and found that although 100% success in initial marking was not achieved, successfully-marked fish were all recognizable one year later. CAMPANA and NEILSON⁹⁾ suggested that the otoliths of the starry flounder *Platichthys stellatus* can be marked by immersion in a tetracycline-saline solution. HETTLER¹¹⁾ in his recent work, demonstrated that otoliths of spot (SL, 13-16 mm) *Leiostomus xanthurus* and pinfish (SL, 12-15 mm) *Lagodon*

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rhomboides could be marked by immersion in oxytetracycline solution. In addition to the above techniques, trace element and nonradioactive isotopes appear to be effective for marking fish eggs and larvae.¹²⁻¹⁴⁾ By X-ray-excited optical luminescence spectroscopy, MUNCY and D'SILVA¹⁵⁾ detected Tb (terbium) in 3-week old walleye fry hatched from Tb-labeled eggs immersed in terbium dicitrate solution.

All of the above methods, however, have some disadvantages in regard to retention time, success of initial marking and/or involve trouble and cost in detection of marked fish and are not very suitable for mark-recapture studies in the wild. The object of this study is to develop a mass-marking method for fish eggs and newly hatched larvae. In this paper, I describe mass-marking of eggs and larvae of the ayu *Plecoglossus altivelis* by tetracycline-tagging of otoliths and discuss the possibility of application of this method to field surveys of this species.

Materials and Methods

The ayu used in this study is a commercially important species not only for fish culture but also for game fishing. Artificial fertilization and rearing of the species are carried out extensively in Japan. The ayu is categorized as a diadromous fish and is famous for its one-year life span and territorial behavior.¹⁶⁾ It feeds on adhesive algae on rocks. The fish matures in the river and lays adhesive eggs among gravel in the middle or lower reaches of the river in the fall. Eggs hatch out 10-14 days after fertilization at water temperature

of 15-20°C. Newly hatched larvae are soon carried out to sea and the fish spend *c.a.* 6 months in the sea before beginning upstream migration in the spring.

This study consists of three experiments. In Experiment 1, the optimum concentration of tetracycline (TC) and treatment time are examined. Experiment 2 deals with the retention time of the fluorescent mark on otoliths, while Experiment 3 is concerned with the effects of TC treatment on hatching, survival and growth of larvae. Materials and marking procedures in each experiment are summarized in Table 1. All materials were obtained through artificial fertilization following standard ayu hatchery procedures. Experiments were carried out at Gifu-ken Gyogyo Center (Gifu Pref. Ayu Hatchery), Mino City, Gifu Pref.

Experiment 1: Optimum TC Concentration and Treatment Time

This experiment is further divided into two tests (Table 1). In the egg test, 30 eggs at the eyed stage attached to a slide glass were used for each lot. Forty-four lots were subjected to various combinations of TC concentration (0-2000 mg/l) and treatment time (1.5-48 h) in fresh water in 100 ml covered beakers (see Fig. 2). In the larva test, 50 newly hatched larvae were used for each lot. In this case, forty lots were exposed to various combinations of TC concentration (0-1000 mg/l) and treatment time (1.5-24 h) in 200 ml covered beakers (see Fig. 3). All lots in both tests were incubated in a water bath at 20°C under light conditions of 0-400 lx. Fish were not fed

Table 1. Materials and processes used in the three experiments

Exp.	Initial materials		Marking treatment			No. of lot	Container	Test period (day)	Rearing temp. (°C)
	life stage	No./lot	TC conc. (mg/l)	time (h)	WT (°C)				
Exp. 1									
egg test	eggs 7-8 days after fertilization	30	0-2000	1.5-48	20	44	100 ml covered beaker	10	20
larva test	larvae 0-4 days after hatching	50	0-1000	1.5-24	20	40	200 ml covered beaker	3	20
Exp. 2	larvae 2 days after hatching	3000	100	7	20	1	500 l plastic tank (φ100 cm)	164	12-21
Exp. 3									
treated	larvae 2 days after hatching	6000*	100	7	20	1	500 l plastic tank (φ100 cm)	164	12-21
control			0	7	20				

* 3000 treated fish and 3000 control fish kept together in a tank.

Fig. 1. Comparison of sagitta otoliths between marked and unmarked fish 10 days after hatching. Photographs A and B are of the same samples without any changes in magnification, framing or focus, but with different lighting conditions, *i.e.* normal white light and UV light, respectively. The upper otolith in each photograph was extracted from TC treated fish (100 mg/l for 7 h, 2 days after hatching) and the lower, from control fish. **m**: fluorescent mark; **e**: edge of otolith. Open circle (○): the discontinuities corresponding to the time of the marking operation. Solid circle (●): the ring which seems to correspond to the day of hatching.

during the experiment. Ayu larvae survived at least one week after hatching without food¹⁷⁾,* and the results of this experiment do not seem to have affected by the feeding conditions.

Hatching success was observed 10 days after the start of treatment in the egg test. As for the larva test, survival rates were determined 3 days after the beginning of treatment. Five individuals were sampled out of the surviving fish in each lot. A pair of sagitta otoliths were extracted from each fish and mounted in euparal on a slide glass. The fluorescent mark on the otoliths was examined with a UV light microscope. Clearness of marking was estimated individually (not for each sagitta) and ranked "clear", "faint" or "no", each rank being given 2, 1 and 0 points, respectively. If the total number of points of 5 individuals in a lot was more than 6, the lot was considered to be successfully marked.

Experiment 2: Mark Retention Time

Larvae 2 days after hatching were immersed in

100 mg/l TC solution of fresh water for 7 h at 20°C. About 3000 marked larvae were kept in a 500 l plastic tank and reared with rotifer and commercial food for more than 5 months from October 1983 till March 1984. The water temperature ranged from 12 to 21°C. The rearing tank was placed indoors near a window. Since the ceiling and the wall of the rearing house were semitransparent, the fish were considered to be reared under natural photoperiod conditions.

Upon occasion, *c.a.* 15 times during the experiment, 50–100 fish were sampled from the rearing tank. A pair of otoliths was removed from each individual and mounted in the same manner as in the Experiment 1. The percentage of detectable fish, whose fluorescent mark on the otoliths was discernible, was determined for each sampling time. In the standard method of detection, no pre-treatment of the otolith was required. However, for otoliths the fluorescent mark of which was undetectable, the convex side of the otolith was ground a little on a whetstone or etched in weak

* TSUKAMOTO *et al.*, unpublished data.

formic acid.

Experiment 3: Effect of TC Treatment on Survival and Growth

About 3000 marked larvae from the same lot used in Experiment 2 and the same number of control fish, which were immersed in fresh water containing no TC for 7 h, were kept together in one rearing tank. They were reared in the same manner as in Experiment 2 and random sampling of 50–100 fish was similarly carried out on occasion. A pair of otoliths was extracted from each fish and examined by UV light microscopy to determine whether it was a marked or unmarked fish. The numbers of marked and unmarked fish were determined for each sampling time and the survival of both groups were compared based on the fluctuation of the percentage of marked fish in the rearing tank. At the same time, the changes in body length of both groups were monitored.

Results

An example of a marked otolith and an unmarked one is presented in Fig. 1. The marked otolith in the figure was sampled from the rearing tank of Experiment 2. Fig. 1-A is a photograph taken under normal lighting and Fig. 1-B under UV light. The upper otolith in Fig. 1-A which was marked in 100 mg/l TC solution for 7 h is clearly discernible in Fig. 1-B as a gleaming yellow ring standing out against a dark green background, while the lower one which is a control disappears in the UV light photo. Thus, marked fish can easily be distinguished from unmarked ones. If Fig. 1-B is observed closely, one can find a faint greenish yellow ring just at the edge of the control otolith (lower) and similarly outside the fluorescent mark of the treated one (upper). These faint rings seem to be caused by birefringence at the edge of otolith or autofluorescence of the margin.

We have found that the ayu exhibits exact daily growth increments and age determination in this species is possible throughout its life history.* This was determined by time marking through tetracycline injection and by comparing the actual age of reared fish from eggs with the number of increments. There were clear discontinuities 4–5 rings inside from the edges of both otoliths in Fig. 1-A. It was apparent that these discontinuities corresponded to the time of the marking operation since the diameter of the fluorescent mark in Fig. 1-B was exactly the same as that of

the discontinuity in the upper otolith. The time schedule of treatment, however, did not accord with the count of daily growth increments. Fish were treated 2 days after hatching and sampled a further 8 days after that (10 days after hatching). Outside of these discontinuities, 7–8 rings should be present, but in fact, only 4–5 rings were observed. The formation of daily growth increments might have ceased for 2–4 days and this period might corresponded to the discontinuities. Another point to note is that the discontinuities were not caused by the drug (TC) but probably by the other factors such as changes in salinity, nutritive conditions and the stress of handling *etc.*, since both TC marked and control fish showed similar discontinuities. If we could find the hatching marks, they should be located 2 rings inside of each discontinuity. However, neither TC marked nor control otolith had clear hatching marks. The stress following the marking operation might be more severe for the otolith formation than that at hatching.

Experiment 1: Optimum TC Concentration and Treatment Time

Figs. 2 and 3 show the range of TC concentration and treatment time that can be used for marking eggs and larvae, respectively. The upper line at the edge of the shaded area shows the lethal limit and the lower line the minimum treatment for successful marking. The effect of treatment time seems to be greater in eggs than in larvae, and the available range for marking is wider in larvae than in eggs. The time of day when treatment is carried out might affect marking success. TANAKA *et al.*¹⁸⁾ found a daily rhythm in otolith growth responding to laboratory photoperiod in *Tilapia nilotica*. Although a daily rhythm has not been established in ayu, yet, it seems preferable that the duration of treatment is kept sufficiently long to avoid the possible effects of such a rhythm. Water temperature also probably affects marking success. However, no data at any temperature other than 20°C was obtained. In conclusion, immersion in 200–300 mg/l for 24–48 h is recommended for marking eggs and in 200–300 mg/l for 3–24 h for larvae.

Experiment 2: Mark Retention Time

Figs. 4, 5 and 6 are pairs of photographs taken under normal and UV lighting at 61, 105 and 164 days after hatching, respectively. Fluorescent

* TSUKAMOTO and KAJIHARA, unpublished data.

Fig. 2. Optimum range of TC concentration and treatment time for marking ayu eggs. Circle ($\circ$): hatching rate $\geq 90\%$; triangle (Δ): 0–90%; cross ($\times$): =0%. Shaded symbol: marked clearly; open symbol: marked unsuccessfully; semi-shaded symbol: marked with intermediate success.

Fig. 3. Optimum range of TC concentration and treatment time for marking ayu larvae. Circle ($\circ$): survival rate $\geq 80\%$; triangle (Δ): 0–80%; cross ($\times$): =0%. Shaded symbol: marked clearly; open symbol: marked unsuccessfully; semi-shaded symbol: marked with intermediate success.

Fig. 4. Photographs of otoliths of 61-day old fish treated for 7 h in 100 mg/l TC solution 2 days after hatching. A: normal white lighting; B: UV lighting. m: fluorescent mark; e: edge of otolith.

Fig. 5. Photographs of otoliths of 105-day old fish sampled from the same lot as in Fig. 4. A: normal white lighting; B: UV lighting. m: fluorescent mark; e: edge of otolith.

Fig. 6. Photographs of otoliths of 164-day old fish sampled from the same lot as in Fig. 4. A: white lighting; B: UV lighting. m: fluorescent mark; c: cracks in otolith. The fluorescent mark at 164 days was not so clear as those at 61 and 105 days because of thickening and calcification of the otolith during growth.

Table 2. Retention time of the mark on otoliths of fish treated for 7 hours in 100 mg/l TC solution 2 days after hatching

Sampling date	Days after hatching	Fish with mark/ Examined fish	Percentage of fish with mark	SL* ¹ (mm)
10/24, '83	12	60/60	100	11±0.6
12/12, '83	61	30/30	100	15±1.5
1/25, '84	105	30/30	100	27±1.8
3/24, '84	164	25/30 (30/30)* ²	83 (100)* ²	49±4.5

*¹ mean±S.D.*² The otoliths without a detectable mark through the standard preparation were either ground or etched in weak acid.**Table 3.** Effect of TC marking on survival and growth in the ayu

Sampling date	Days after hatching	Marked fish/ Examined fish	Percentage of marked fish	SL* (mm)	
				marked	unmarked
10/24, '83	12	13/30	43	11±1.0	11±0.5
12/12, '83	61	18/30	60	18±1.0	18±0.8
1/25, '84	105	15/30	50	26±3.4	27±2.0
3/24, '84	164	12/30	40	52±3.9	53±4.4

* mean±S.D.

marks on otoliths are clearly discernible until 105 days after hatching (see Figs. 4 and 5). At 164 days after hatching, the mark became faint. The percentages of marked fish for each sampling time are shown in Table 2. Twelve days after hatching, all 60 fish examined had clear marks. This indicates 100% initial success in marking. Until 105 days, 100% detection was still possible. At 164 days, the percentage decreased to 83%, perhaps because of thickening and coloration following otolith growth. Since otoliths grow by accumulating calcium layer upon layer, the tetracycline labeled part of the otolith must be near the nuclei of the otolith. When undetectable otoliths were ground or etched in weak acid and observed again, the marks were clearly detected. Thus, the mark was retained for at least five and a half months. Moreover, the results of Experiment 1 indicate that the treatment of Experiment 2 was the absolute minimum required for marking success and, thus, more adequate treatment would undoubtedly raise the percentage of detection at 164 days.

Experiment 3: Effect of TC Treatment on Survival and Growth

The effects of marking treatment on survival and growth are shown in Table 3. At the beginning of this experiment, marked fish and unmarked control fish were present in roughly equal numbers in the tank. From the results in Experiment 2,

the fish without detectable mark could be regarded as untreated control fish at the beginning of the experiment and those with marks as marked fish. The percentages of marked fish show an almost constant value of about 50% for 164 days though a small fluctuation of less than 10% is observed. The fluctuation may merely result from the small number of fish examined. However, the following explanation may also be possible. The lots treated in 0 and 5 mg/l for 6 h in Fig. 3 showed lower survival rates than those treated at a higher TC concentration (10–300 mg/l). This suggests that TC may favor survival of the ayu larvae, and this may account for the increase in the percentage of marked fish from 43 to 60% between 12 and 61 days. Furthermore, since no special preparation of otolith, such as grinding or etching, was adopted for detection in this experiment, the decrease at 164 days may be explained by the small decrease in the percentage of detectable fish at 164 days in Experiment 2 when the standard procedure was followed. Whether or not this is correct, it can be concluded that TC treatment has no serious effect on the survival of the ayu.

Body lengths of marked and unmarked fish showed almost the same values throughout the experiment. It is thus apparent that growth is not affected by the treatment.

Discussion

The merits and demerits of the marking method

described here can be summarized as follows. Its merits are: (1) it can be used for eggs and newly hatched larval stages; (2) rapid mass-marking with 100% initial success can be achieved; (3) the retention time is comparatively long; (4) it has no effect on fish's growth or mortality; (5) in laboratory experiments, different groups of fish marked at the egg stage can be raised together under the same rearing conditions. On the other hand, the biggest disadvantage in this method is the troublesome process for detection, namely, the extraction of otoliths and detection by incident UV light microscopy. I am now trying to establish an easy and rapid method for mass-extraction of otoliths and fast preparation for detection. Another demerit is that only a few combinations of different colored marks seem to be possible in an experiment because few fluorescent materials are available at present that can be applied to otolith marking. Calcein and alizarin complexon have low toxicity and present clear fluorescence of palegreen and red, respectively.¹⁰⁾ These chemicals are now undergoing trial. Despite these demerits, this method of otolith marking still seems to have many advantages over previous marking methods. The principle of the marking method described here is essentially the same as that described by HETTLER who used 12-16 mm fish.¹¹⁾ He, however, tried to use the fluorescent mark on otolith as a time mark, not for mark-recapture studies in the field but for the validation of daily growth increment. He also suggested at the same time that the yellow fluorescence in heads and fins, especially caudal fins* might be used for mark-recapture studies if the longevity of the external fluorescence is known. ICHII and MUGIYA²⁰⁾ proved in ⁴⁵Ca-prelabeled goldfish *Carassius auratus* that the calcium turnover rate was highest in scales, next came bones and last otoliths and that, once deposited, calcium on otoliths was mobilized little if at all. From this point of view, therefore, otoliths are the ideal calcium-containing tissue for tetracycline marking. Although special preparation (*i.e.* grinding or etching) must be adopted for otoliths older than 5 months, the retention time of the tetracycline mark on otoliths is considered to be sufficient for field studies of the ayu whose life span is only one year. Tetracycline-tagging also seems to be suitable for other diadromous species such as *Salanx microdon* and *Leucopsarion petersi* which have thin flat sagittae and short life spans. Moreover, tag loss does not occur because otoliths are in effect

"biological internal tags". As LAIRD and STOTT¹⁾ pointed out, however, it is known that sunlight can deactivate tetracycline. However, once pigmentation around the fish head is completed at the juvenile stage, tetracycline combined with the calcium of otolith is probably protected from natural UV rays by the pigmentation. Mark retention is now being examined in eggs and larvae stocked in the wild and the outdoor rearing tanks.

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