A Manual for Breeding of Medaka in Large Scale Using Aquatic Habitats System

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Introduction

A genome-wide mutant screening was carried out using medaka fish (*Oryzias latipes*) by a JST Kyoto group. The general procedures used there and then to breed medaka fish in large scale will be described here, which used 6,000 individual fish tanks that can accommodate with more than 100,000 fish. It was an unprecedented scale of cultivation of medaka fish in the laboratories; we, of course, adopted the established know-how of medaka cultivation of fish farmers and research institutions using smaller scale systems in Japan and in abroad, and consulted with the experiences of research institutions abroad where large-scale zebrafish cultivation was already ongoing. Described here is based on several years of experience of the author and his colleagues involving many trials and errors, which may be improved by the readers. The conditions may be modified depending on the type of the fish cultivation facility, quality of available water source, purpose of fish breeding for experimentation, etc.

An important aspect of running a large-scale fish system is requirement of cooperation of all researchers and technicians to accomplish a good husbandry. It is necessary that all those involved have sufficient knowledge about breeding of medaka, the organization, and maintenance of the facility. For this purpose, we made rules in the fish rooms for them to strictly observe.

Outline of Breeding of Medaka Fish

Medaka fish were bred with the large-scale circulating water tank system placed indoor, where temperature and light cycle were automatically controlled. Three sizes of tank of 1 L, 2.75 L, and 9 L made of transparent polycarbonate were used. About 30–40 adult fish are maintained in a 9-L tank. System water was made by dissolving a small amount of natural seawater salt in the reverse osmosis (RO) water, as used in zebrafish cultivation, but in some cases filtered tap water (this was fine in Kyoto) was added to gain stability of the water system, especially during the period of starting a new tank system.

NB 1. Before start of large-scale breeding of fishes, an acclimation period must be prepared, by placing a small number of fish in the water system as pilots, during which time ceramic biofilters of the system is colonized enough with microbes to stabilize the water system. Alternatively, cultivating water and biofilters may be transferred from

other systems in operation for the quick startup of a new water tank system.

Water quality control included measurement and reinforcing stability of pH, conductivity, water temperature, ammonium concentration, nitrite concentration, nitrate concentration, and hardness of water. Water temperature was kept in the range of 25–28 °C, depending on the purpose of the experiments and fish lines by controlling a room temperature using air conditioners. Day and night periods were set for 14 and 10 hours, respectively, by timer control of light switch. Daytime brightness was maintained no lower than 100 lux. The fishes were fed with brine shrimp larvae and artificial powdered food in the morning and afternoon, namely twice a day.

NB 2. Two important points must be borne in mind to maintain fish health and get regular good spawning. First, constant good water quality; otherwise fish stop spawning of eggs and become sick. Second, disease-free culture condition. Any fish derived from outside of the fish facilities, most seriously those handled by pet shops, are potential source of infectious diseases. Therefore, a quarantine room was set, and those imported fish were isolated and bred in the quarantine.

Fish Facility

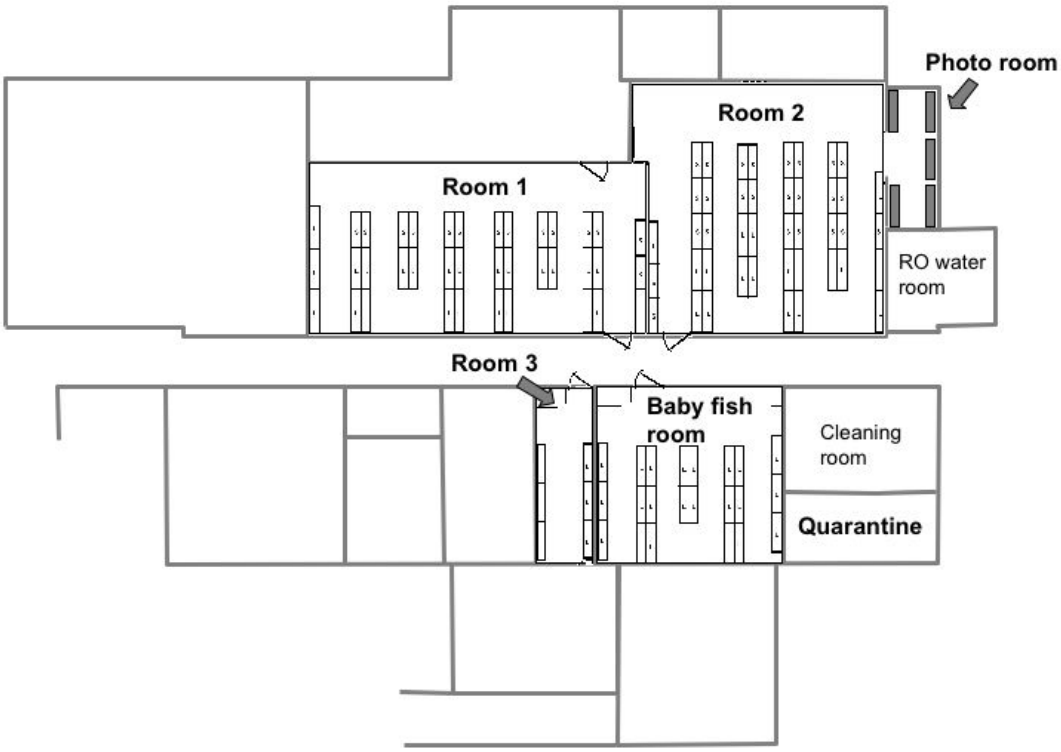
Outline of fish tank system

We operated a large fish facility made by Aquatic Habitats for Accelerated Bioresearch (AHAB: FL, USA; http://www.aquatichabitats.com/), including two kinds of rack system, multirack complex system and single-rack stand-alone system. A multirack system had many rack units (60"×89"×14" or 50.5"×89"×14") connected, and was operated by single water-circulation system. The single-rack system (60"×89"×14") also had one circulation system. Circulated water flowed through every tanks placed on the rack. In the JST Kyoto facility, we had six different rooms for the operation: Rooms 1 and 2 plus “photo room” (a room for variable light cycle) were used to maintain adult fish, to cross females with males and to collect spawned eggs. Room 3 was for low-temperature maintenance of adult fish at around 20 °C, suppressing activity of fish and increasing longevity. Baby fish room was used for raising fry to adult. Quarantine room was used to keep fishes brought in from the wild, other research institutions, or pet shops. The eggs collected in the quarantine may be brought into baby room after

sterilization by bleaching.

For information to readers: Room 1 housed two multirack units, each consisting of 19 racks, and Room 2 had two multirack units consisting of 21 or 22 racks. Photo room had five single-rack units, three of which had a light-tight cover, so that light cycles can be operated in individual rack. Room 3 had a short (60"×76"×14") six-rack unit. Baby fish room had single multirack unit of 22 racks. (60"×81"×14"). The quarantine room was equipped with four single-rack systems, three of which (1580mm×1900mm×340mm, MH type) were manufactured by Meito Suien (Nagoya). In the quarantine, it is important to have multiple isolated water circuit in order to prevent spread of infection hazard. The tank positions were registered and labeled on each rack in order to keep track of fish pedigrees.

Figure 1. Floor plan of the multirack tank systems used by the JST Kyoto group.



Water circuit with inlet and drainage

The flow of the water in a circuit in a multirack system is schematized as:

(1) Fish tanks → (2) Prefilter pads → (3) Sump/biofilter → (4) Main pump →

(5) Pressure regulator → (6) Column filters → (7) UV sterilizer → (1) Fish tanks

(1) Fish tanks

Water delivery lines brought filtered circulating water to each fish tank through individually assorted valves on manifolds. To breed adult fish, valves were adjusted for the water to flow through the tank at the rate of 300–320 mL/min. Then the water overflowed out of the tank and into drains.

(2) Prefilter pads

One prefilter pad with a pore size of 150 μm was installed in a rack. Drainage from all the water tanks was filtrated through the prefilter pads.

(3) Sump and biofilter

The water that passed through prefilter pads of all racks of a system was collected in a sump and passed through a series of sumps placed under a couple of racks. A biofilter was installed in a sump, which consists of porous ceramic "Reactballs (the diameter of 6 mm or 9 mm)." All sumps were strongly aerated by aerators and kept in a highly aerobic environment, which helped biological decomposition of ammonium and provides a high level of the dissolved oxygen in the breeding water.

(4) Main pump

The water collected into the last sump was given a pressure by the main pump and delivered into the series of column filters.

(5) Pressure regulator

A pressure regulator was installed in a tank system. This regulator was installed so that opening/closure of local valves to regulate flow rates into specific set of tanks would not affect the rate of water flows into other tanks.

(6) Column filters

All circulating water driven by the pump was filtered by serially passing through 50- or 100-μm pore-sized filters, then through charcoal column to remove organic materials.

(7) UV sterilizer

The circulating water was sterilized by passing through an UV sterilizer (powered by eight or ten 60-W UV lamps for a multirack system or by two 25-W UV lamps for a single-rack system). The sterilized water was distributed into individual racks of the tanks.

A large amount of RO water was produced by Milli-RO200 system

(Millipore, Tokyo, Japan), stored, and pumped out of the RO water room (Figure. 1). RO water was distributed to each multirack system through system-specific pipelines, and also to the cleaning room where the fish tanks were cleaned.

A multirack tank system received constant-rate inlet of RO water that would result in 10% exchange per day of the water held in the system. The water in a sump above a fixed level was drained through an overflow pipe. The drainage was treated with a chlorine chemical while passing through a sterilizer apparatus, without allowing escape of live fish or embryos.

Fish tanks

Fish tanks made of polycarbonate had three sizes, holding 9 L, 2.75 L, and 1 L of water. A rack can accommodate all these tank sizes, allowing various arrangements of tanks. Each tank was furnished with a lid and a baffle having a narrow opening slit at the bottom. Water stream going trough the baffle-bottom slit drained out the deposits at the tank bottom, allowing cleaning of the fish tank. 1-L tanks were mainly used for mating of a pair of adult fish. 2.75-L tanks were widely used to rear the fry derived from a mating pair, and to breed adult fish up to 20 individuals per tank. 9-L tanks were used to breed adult fish over about 30 individuals or to rear about 50 juveniles.

NB 3. A 60”-wide rack (indicated L in Figure 1) held six 9-L tanks, twelve 2.75-L tanks, or twenty 1-Ltanks on each shelf. A 50.5”-wide rack (indicated S in Figure 1) held five 9-L tanks, ten 2.75-L tanks, or twenty 1-Ltanks on each shelf. These racks had five racks. Total volume of water held were roughly 7,000 L for Rooms 1 and 2, 900 L for Room 3, 5,800 L for baby fish room, and 200 L for a single-rack (stand-alone) system.

Water Quality Control

Automatic operation

AquaNode (Aquadyne, CA, USA) system was installed in each multirack system. pH, conductivity, and water temperature were always measured by AquaNode, as well as the sump water level and water flow rate. Alarm was set to ring when a failure was detected by AquaNode. When the conductivity dropped below the threshold, the second automatic system was activated that delivered a high concentration salt solution kept in

salt tank into a sump. Conversely, when water level in a sump decreased, RO water was supplied automatically to the constant water level.

NB 4. All water exchange, salt addition, RO water supply, and checking water quality were carried out manually using a single-rack (stand-alone) system.

Water quality is important for good health, longevity, and fecundity. pH and conductivity were monitored automatically by AquaNode all the time. Ammonium concentration, nitrite concentration, nitrate concentration, and hardness were measured additionally twice a week. pH was kept between 6.8 and 7.5. Conductivity was kept between 200 and 450 μS/cm. Ammonium, nitrite, nitrate, and total hardness in system were kept less than 0.2 mg NH_4^+/L, 0.1 mg NO_2^-/L, 20 mg NO_3^-/L, 20-100 mg $CaCO_3$/L, respectively. These data were recorded using a checklist sheet.

The stability of the water quality of the system is maintained by interaction of three elements, fish themselves, microbes habitant in the system, and the ionic composition of the inlet water. To start up a large-scale tank system, it really took a while to attain a steady state that was maintained by the balance of three elements, until the fluctuation of the second element was settled. The difficulty of this start-up phase of a large cultivation system was already described in a chapter above. A particular care of the condition of the water must be taken during this start-up phase, much more frequently checking the parameters in particular the nitrogenous compounds.

Table 1. Water quality of fish breeding water in JST Kyoto systems.

Parameters	**Conditions to be satisfied**
pH	6.8–7.5
Conductivity	200–450 μS/cm
Water temperature	26–28 °C
Ammonia concentration	Less than 0.2 mg NH_4^+/L
Nitrite concentration	Less than 0.1 mg NO_2^-/L
Nitrate concentration	Less than 20 mg NO_3^-/L
Hardness	20–100 mg $CaCO_3$/L

pH

pH of the circulating water usually decreased as the period of fish breeding extended. Under this condition, sodium bicarbonate ($NaHCO_3$) was added to the circulating

system water (10–20 g at once at the site of aeration in a sump). The amount of sodium bicarbonate to be added to the system water varied depending on the total water volume of the system and the number of fish cultivated in the system. To provide some idea, the system of baby fish room was added with 20–30 g of $NaHCO_3$ per day. A caution is that the pH of the circulating water changed within a day, the highest in the early morning, and gradual decrease in the following hours, and a small rise in the evening. Too much addition of $NaHCO_3$ in the late afternoon should be avoided.

It is generally considered that neutral pH in the range of 6.5–7.0 is preferable for the medaka. It is to be noted, however, that in nature pH of the water environment of medaka can be as high as pH 8.0, probably reflecting the wide adaptability of medaka.

Conductivity

Conductivity reflects the amount of electrolytes dissolved in the water. It decreases by addition of RO water for 10% daily water replacement, and increases by addition of salts (Red Sea salts; Red Sea, Japan, Tokyo) or sodium bicarbonate. Otherwise, its increase indicates dirtiness of the water. Usually, conductivity was kept constant by the functions associated with AquaNode.

Ammonium, Nitrite, and Nitrate

High concentrations of ammonium or nitrite must be avoided, as these substances are toxic to fish. Increase of nitrate level of the water also causes trouble by promoting algal growth. Packtest kit (Kyoritsu Chemical-Check Lab., Corp., Tokyo) was used to measure the levels of these substances. In the kit, color development reacted by water samples was compared with color standards.

Nitrogeneous compounds are formed during fish breeding, deriving from discharges, food leftover, or dead fish. These are decomposed by the activity of deamination bacteria schematized as follows: (1) ammonium is oxidized to nitrite by *Nitrosomonas/Nitrosococcus,* and (2) nitrite oxidized further to nitrate by *Nitrobacter/Nitrospira.* These bacteria colonize in biofilter, and decompose nitrogenous compounds. As these oxidative reactions depend on aerobic environment, sump water containing biofilters were strongly aerated.

Nitrogenous compounds → Ammonia (1) → Nitrite (2) → Nitrate

(1) $NH_4^+ + ½O_2 \rightarrow NO_2^- + H_2O + 2H^+$

(2) $NO_2^- + ½O_2 \rightarrow NO_3^-$

Nitrate was removed from the circulating water by water exchange. Therefore, when the nitrate level became high, the water exchange rate was increased.

Ammonium in water exists in two forms, ionized (NH_4^+) and un-ionized (NH_3). NH_3 is strongly toxic for fish. The proportion of un-ionized NH_3 in water changes depending on the temperature and pH, higher with increase of temperature and pH.

Hardness of water

Total hardness (TH) is the sum of hardness derived from calcium and magnesium contents, which is shown as a value represented by the amount of calcium carbonate ($CaCO_3$). TH of the circulating water was adjusted by addition of calcium chloride ($CaCl_2 \cdot 2H_2O$) to increase, or by enhancing water exchange to decrease. As the majority of natural river water in Japan is soft (less than 120 mg $CaCO_3$/L), a soft water condition is considered favorable to medaka cultivation. However, this may depend on the particular medaka lines used in the experimentation.

Another important aspect of water hardness is the carbonate hardness, the sum of carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) ions, which to a large extent reflects the buffering capacity of the water.

In any event, the best conditions for the combination of parameters of water quality must be sought in each laboratory. It is our experience that when the chorion of eggs was too soft, increase of TH helped its hardening.

Water Temperature

Japanese medaka fish is adapted to the climate in Japan, and can tolerate a wide temperature range between 10 and 40 °C. Medaka fish lay their eggs every day when maintained under the conditions of 25–28 °C. In the JST Kyoto system, the temperature of water circulating through multirack systems was controlled by air-conditioning of the room temperature.

Lifespan of medaka is extended under low temperatures where the rate of energy metabolism is low. In the JST Kyoto system, the room temperature of Room 3

was maintained around 20 °C, and the fish lines to be maintained as livestocks were placed there.

Light Cycle

Light control is important for medaka fish breeding. Medaka spawns in the long day period, to which the light control of tank system rooms was adjusted. Constant light strength of 100–150 lux produced by ordinary fluorescent light was used for a day period of 14 hours; a night period was 10 hours. Medaka spawns around onset of morning light, the timing of spawning was adjusted for experimentation by changing the light cycle. (Using a single-rack system, it was possible to adjust light cycles individually.) To promote synchronous spawning in the morning to collect eggs for the study of early developmental stages, a strong (roughly 1,000 lux) light was shot to tanks using additional fluorescent lamps attached on top of the shelf of tanks for egg collection. (A caution is that a strong light tends to promote growth of green algae that make tanks dirty.)

Feeding of Fish

Medaka were fed with brine shrimp (*Artemia*) larvae and artificial food, Hikari Crest Guppy (Kyorin, Hyogo). Brine shrimp larvae just after hatching were given as live food. Hikari Crest Guppy is powdered fish food that stays on water surface. To feed fry, Hikari Crest Guppy was ground to finer powders using a mortar.

Dried brine shrimp eggs were cultured for 1 day in the salt water to get hatching of brine shrimp larvae. Brine shrimp larvae hatch under the conditions of 26–30 °C and vigorous aeration in the 2–3% salt water in 24 hours. Hatching is promoted when a photo-stimulus is given. Cultivation of brine shrimp eggs was done in the proportion of 2–3 g dry eggs in 1 L of RO water in which 20 g artificial sea salts were dissolved. The cultivation tanks were scaled up to hold 50 L of the medium. Electric heater was placed in a tank to keep the water temperatures in the range of 28–30°C, and an air compressor of constant operation was used for strong aeration of the culture.

The hatched larvae were collected just before they are given to fish. Therefore, brine shrimp hatching was set everyday for tomorrow's use. Brine shrimp egg culture was started at 8:30 every morning and in the following day brine shrimp larvae were collected and given to the fish both in the morning and in the afternoon.

Cultivation longer than 24 hours improved the hatching rate. Consumption of brine shrimp eggs was about 400 g per day, when the tank systems were in their maximum operation.

It is important to isolate hatched larvae out of unhatched eggs and hard shells left after hatching. The light eggshells floated on the surface of the water and heavy unhatched eggs sank to the bottom of the tank once the aeration was halted, while hatched larvae were left in the middle. Taking advantage of this separation, freshly hatched larvae were collected from the salt water using a mesh of 150 μm. Collected larvae were suspended in RO water to be given to fish. (It is important to avoid inclusion of shells as much as possible, as the shells are hardened in water and are not digested when taken by fry.)

Fish were fed twice a day in the morning and the afternoon. Depending on the stage of the development and the purpose of the use of fish, the feeding protocols were changed. In our practice, the feeding protocols were color coded on the labels put on the tank wall or a rack shelf as unambiguous notes to technicians, as exemplified in Table 2.

Baby fish were fed on powdered fish food. To help rapid growth of the babies, feeding them three times a day with a smaller amount, rather than regular protocol of twice a day, was effective. Young fish in the middle of growth were fed twice a day with brine shrimp and powdered food in this sequence. Brine shrimp larvae must be eaten up first, then the amount of powdered fish food to be eaten in 2–3 minutes was given to the tank.) Adults are fed twice a day only with brine shrimp larvae. Fish to be maintained for a long period were fed with brine shrimp larvae only once a day in the morning.

Fish were fed while water flow into a tank was halted. Fish food to be given to each tank must be sufficient for every fish to get access to the food, while the amount to be eaten up in 5 minutes was preferred. The amount of the food to be given to a tank of course varied depending on the fish size and fish numbers. Overfeeding should be avoided, which deteriorates the water quality.

Table 2. Five different feeding protocols marked by labels put on a tank wall.

Labels (in the order of fish	**Feeding in the morning**	**Feeding in the afternoon**

growth)

Blue dot encircled by red	Finely powdered food for baby fish	Finely powdered food for baby fish
Green dot encircled by red	Brine shrimp larvae Plus finely powdered food for baby fish	Brine shrimp larvae Plus finely powdered food for baby fish
Red	Brine shrimp larvae Plus powdered food	Brine shrimp larvae Plus powdered food
Yellow	Brine shrimp larvae	Brine shrimp larvae
Green	Brine shrimp larvae	None