Chapter 1
Medaka Management

1.1 Introduction

This chapter provides supplemental information about medaka breeding described in Chapter 2 Medaka Management of the first edition of *Medaka: Biology, Management, and Experimental Protocols* (2009). In this decade, the biological usefulness and convenience of medaka as a model animal have been recognized and the number of research fields and researchers using medaka has been expanding. In these situations, strict and sophisticated rearing methods are required to evaluate the effects of the gene of interest, chemicals, environmental conditions, and so on. Well-designed and regulated feeding is especially critical to evaluate growth and appetite. Additionally, ethical issues such as animal welfare have been emphasized including the "3Rs" concept: Replacement, Reduction, and Refinement. Therefore, it has become important to understand anesthesia and euthanasia methods.

Understanding the life-cycle of medaka in the wild is important to construct a research plan for successful breeding in the laboratory. First, in this chapter, the life-cycle of medaka in the wild is described. Then, the outline of the breeding process is mentioned before the detailed breeding procedure is explained. Finally, anesthesia methods are discussed.

1.2 Medaka Management for Scientific Research

In breeding model animal strains, the most critical point is to maintain the phenotypical characteristics of the various strains in order to obtain experimental reproducibility. Although the same model animal strain is subjected to a certain experiment, the results may differ due to different breeding and maintenance conditions. Therefore, it is very important to describe the proper breeding and maintenance conditions as well as the significant results in the research paper so that other researchers can reproduce these procedures as accurately as possible. In this section, the standard breeding procedures based on the life-cycle of medaka in the wild are described; they are helpful in setting up a medaka breeding system as a first step to begin a research project.

Medaka: Biology, Management, and Experimental Protocols, Volume 2, First Edition.
Edited by Kenji Murata, Masato Kinoshita, Kiyoshi Naruse, Minoru Tanaka, and Yasuhiro Kamei.

1.2.1 Outline of medaka life-cycle in the wild

Medaka (Japanese medaka, *Oryzias latipes*) has a widespread distribution in Japan, primarily in small ponds and rice paddies (See 1.2 Phylogeny in Chapter 1 in the first edition of *Medaka: Biology, Management, and Experimental Protocols* (2009) and Chapter 2 in this book). Medaka can live throughout the year in Japan (Shima and Mitani 2004), but has a limited spawning period in the wild. More than 12 hours of daylight and water temperature higher than 13 °C are required for oogenesis and spermatogenesis to take place. Then, the fish start to mate and the females spawn eggs. These climate conditions match those during spring and summer in Japan. Actually, the combination of a 14 hour light/10 hour dark (14L–10D) cycle and temperatures of 25–28 °C (which are consistent with those of early summer in Japan) and sufficient food provides the best conditions for spawning eggs in the wild. In spring, in the wild, when the water temperature is lower than 25 °C, larvae hatch in 10 or more days. The larvae reach maturity in the early summer. They mate and spawn eggs until the hours of daylight become shorter than the hours of darkness (late summer to autumn). A small number of the next generation survives the winter and a smaller number may survive two winters. The average lifespan in the wild is considered to be around one year because predation and/or seasonal environment change are the leading causes of death of medaka in the wild. Egami reported that the average lifespan is less than three years and the maximum lifespan is approximately five years under experimental conditions (Egami 1971).

1.2.2 Preparation of normal rearing conditions of medaka in the laboratory and procedures for breeding

The outline of the rearing schedule is shown in Figure 1-1. To obtain eggs, creating the conditions that are required for successful mating and spawning is important. Details of these conditions are described in 3.5 Necessary Conditions for Spawning in Chapter 3 in the first version of this book. Briefly, these conditions consist of feeding three times a day, a 14L–10D light cycle at 25–28 °C, and avoiding keeping the fish at a high density.

1.2.2.1 Breeding system set-up

Select the water system (flowing, recycled, or static water) according to the laboratory situation or the requirements of the experiment, and prepare the water to fill the fish tanks. Soft water (this means the water contains a concentration of calcium and magnesium ions lower than 120 mg/L) without chlorine is recommended. The light/dark cycle (14L–10D) and the room temperature (25–28 °C) of the breeding room should be controlled respectively using a timer and an air conditioner, before bringing medaka into the room. In case it is difficult to control the lighting conditions of the room where they have been set for the experiments and the regulation of the water temperatures in each breeding tank in the room as a single unit, the light/dark cycle and the temperatures of the water in the tanks can be independently regulated at each breeding tank or aquarium system. (See Chapter 2 Medaka Management in the first version of this book.)

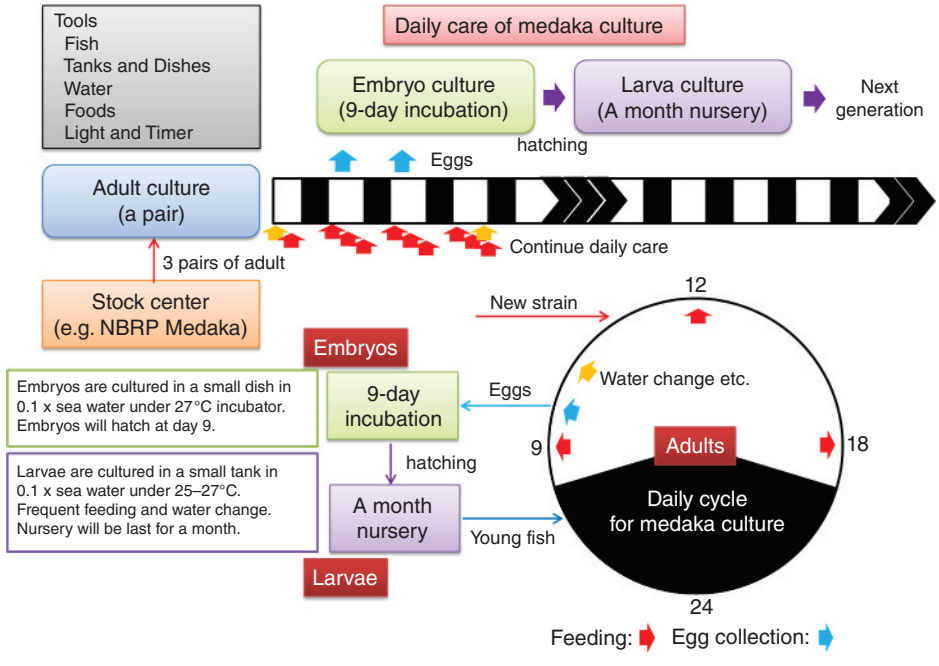

Figure 1-1. Outline of the rearing schedule.

1.2.2.2 Obtaining medaka

It is strongly recommended that medaka be obtained from researchers who are culturing medaka or from the National Bio-Resource Project (NBRP) Medaka (https://shigen.nig.ac.jp/medaka) to prevent the contamination and/or introduction of pathogens or parasites into the aquarium(s). The NBRP Medaka at the National Institute for Basic Biology in Japan distributes medaka strains, including wild, inbred, transgenic, and mutant strains, domestically and internationally.

In the wild, medaka habitats are located in areas that encompass tropical, temperate, and cold climate zones within various types of aquatic habitats, with water conditions ranging from still, freshwater lakes, flowing freshwater streams, and rice paddies to brackish and sea water; medaka have adapted to all these conditions. However, to avoid problems such as the contamination of pathogens and/or parasites from the environment as described above, the medaka used for research should be kept away from all wild aquatic animals, and water taken directly from the natural environment should never be used, since untreated water from natural habitats may harbor pathogens and/or parasites.

If it should be necessary to use medaka obtained from the wild or pet shops, these fish should be reared in an independent tank to collect more than 20 eggs from them. After pasteurizing (see section 1.3.2.2) these eggs, the larvae that hatch from these eggs can be introduced into the aquarium. Also see Chapter 2 Medaka Management in the first version of this book.

1.2.2.3 Collecting eggs in a laboratory setting

In order to obtain eggs from medaka to use for your experiment, introduce a pair of adult medaka into a half-full breeding tank. The volume of the tank should be at least 1 L

(10×10×10 cm). The fish will spawn eggs (10–30 eggs a day per a female) within two weeks with enough food and proper water temperature (25–28 °C).

1.2.2.4 Daily care and maintenance of eggs

The collected eggs are cultured with embryo culture medium* (ECM) or 0.3% (W/V) artificial sea water in a plastic dish (a, b, and c in Table 1-1) at 25–28 °C. Since a lack of oxygen causes delay of ontogeny and/or abnormal development of the embryos, an excessive number of embryos in a dish should be avoided. The proper number of embryos in a dish is shown in rows a, b, and c in Table 1-1. The dish should be examined every day for abnormal and/or dead embryos which should be promptly removed; otherwise fungi and bacteria will multiply sufficiently to kill the embryos. The details of sorting and cleaning methods of embryos are described in sections 1.3.2.2 and 1.3.2.3.

* 0.1% (W/V) NaCl, 0.003% (W/V) KCl, 0.004% (W/V) $CaCl_2$-$2H_2O$, 0.016%(W/V) $MgSO_4$-$7H_2O$.

1.2.2.5 Rearing medaka from the larval stage to adulthood

After bringing the newly acquired larvae to the breeding facility, transfer them to a culture tank (see d or e in Table 1-1) as soon as possible. Larvae can start eating in one or two days after hatching, therefore it is necessary to start feeding them just after the transfer of hatched larvae into the tanks. The amount of food and frequency of feeding depend on the type of food. The feeding methods using paramecia and commercial powdered food are described in section 1.3.2.4, and Table 1-2.

Leftover food on the surface and bottom causes deterioration of water quality. In breeding with static water, leftover food and debris should be removed every day and half of the breeding water should be changed every day. When breeding fish in a tank that has a water recycling system with filtration, it is recommended that leftover food and debris are removed every day. One method for removing them from the bottom of the tank is described in section 1.3.3.2. The amount of food should be increased as the larvae grow. The size of the food particles is another important matter and should be changed as the fish grow. Information about the particle size and the amount of commercial powdered food needed is described in Tables 1-2 and 1-4. Additionally, since the density of larvae in the tank/aquarium affects the growth rate, the size of the tank and the number of fish should be adjusted to the optimal conditions as shown in Table 1-3.

The fish grow to sexual maturity within three months in proper breeding conditions and they can continue spawning for at least for three months. Rearingmulti-generation will allow to obtain eggs throughout the year (Figure 1-2). It is helpful to keep in mind that a lower density of fish in a breeding tank results in a shorter time between generations.

1.2.2.6 Anesthesia and euthanasia

The ethics of animal welfare have become an important issue in daily life and even affect conditions in scientific fields. Therefore, the fish used in experiments should be treated properly after the experiments have finished. Principally, these fish should be treated according to the guidelines of experimental animals established by each institute or

Table 1-1. Tools for medaka embryonic/larval culture and treatments.

	Tools	Size, company, and remarks
a	Polystyrene dish (without coating)	35–40 × 10–13 [diameter × height mm] (similar products) To culture <15 embryos
b	Polystyrene dish (without coating)	57–60 × 15–16 [diameter × height mm] (similar products) To culture <40 embryos
c	Polystyrene dish (without coating)	90–100 × 15 [diameter × height mm] (similar products) To culture <80 embryos
d	Round plastic cup (deep type)	50–60 × 35–40 [diameter × height mm] (similar products) To culture <15 hatchlings (the primary larvae)
e	Round plastic cup (deep type)	90–100 × 50–60 [diameter × height mm] (similar products) To culture <40 hatchlings (the primary larvae)
f	Komagome pipette (glass pipette)	5–10 mL scale (similar products) To collect eggs from the abdomen of females
f'	Silicone nipple (for 10 mL)	For 1 Komagome pipettes Rubber types are similarly useful To collect eggs from the abdomen of females
g	Transfer pipette (polypropylene)	2–4 mL scale Disposable type is sufficient To transfer embryos and larvae and remove debris (cut the tip end to the appropriate size of the larvae)
h	Transfer pipette (polypropylene)	2–4 mL scale (another type)
i	Fine forceps	Straight 100–110 mm length with fine tip Dumont Inox No. 5 0203–54-PO (or similar products) For anatomical operation and handling of eggs/embryos
j	Steel needle with handle	1–3 mm diameter, 10–150 mm length (similar products) Tip should be ground into round shape To rotate and align the embryos and larvae
k	Medaka spoon	Custom made Made from *g* or *h* by cutting the head part To transfer medaka under anesthesia
l	Nonslip forceps (serrated, straight, fine tip)	Straight 110–120 mm length with fine tip and serrated Dumont INOX 08 0508-2-PO (or similar products) Stainless steel For anatomical operation
m	Small scissors	90–110 mm length, straight, 18–20 mm blades (or similar products) For anatomical operation
n	Micro scissors (spring type)	70–110 mm length, straight, 5–8 mm blades WPI Spring Scissors 501235 (or similar products) For anatomy

Table 1-2. Powdered food for medaka culture.

	Name/company	Particle size, suitable stages, and remarks
a	Ayu Super Gold/Marubeni Nisshin Feed Co.	Particle size: 0.074–0.125 mm For the primary larval stage to 3 wk stage Nutrition: protein >51.0%, fat >9%, fiber <2.0%, ash <19.0%, ca >2.00%, phosphate >1.60%
b	Otohime A/Marubeni Nisshin Feed Co.	Particle size: <0.25 mm For 3–6 wk stage Nutrition: protein >53.0%, fats >8.0%, fiber <3.0%, ash <16.0%, ca >2.30%, phosphate >1.50%
c	Otohime B1/Marubeni Nisshin Feed Co.	Particle size: 0.36 mm For 6–13 wk stage Nutrition: protein >50.0%, fat >10.0%, fiber <3.0%, ash <16.0%, ca >2.00%, phosphate >1.50%
d	Otohime B2/Marubeni Nisshin Feed Co.	Particle size: 0.36–0.65 mm For 13 wk stage to adulthood Nutrition: protein >50.0%, fat >10.0%, fiber <3.0%, ash <16.0%, ca >2.00%, phosphate >1.50%

Table 1-3. Optimal tank size and density (numbers of fish).

Stage[a]	Round container		Circulation system rack		
(body length)	d	e	2–3 L tank	5 L tank	9 L tank
First to second larval (<6 mm)	1~15	10~60			
Second larval to first juvenile (6–15 mm)			~20	~50	~100
First to second juvenile (15–20 mm)			~10	~20	~30
Second juvenile[b] (20–25 mm)			~5	~15	~20
Adult (>25 mm)			~5	~10	~15

[2] The photos of round containers *d* and *e* are in Figure 1-3.
[a] The definition of stages is described in Chapter 6 of the 1st edition.
[b] The fish display signs of secondary sexual characteristics but haven't produced eggs yet.

government. Although some governments have guidelines for welfare for mammals, birds, and reptiles, they generally lack guidelines for fish and amphibians. Nevertheless, the scientific community expects that experimental model fish are controlled by appropriate guidelines and that each research project using fish should be approved by local or regional ethics committees or related organizations involved in handling animal welfare before each project is started.

Therefore, considering this situation, knowledge of the effects of anesthesia on fish is required not only in regard to the welfare of the animals, but also in order to humanely

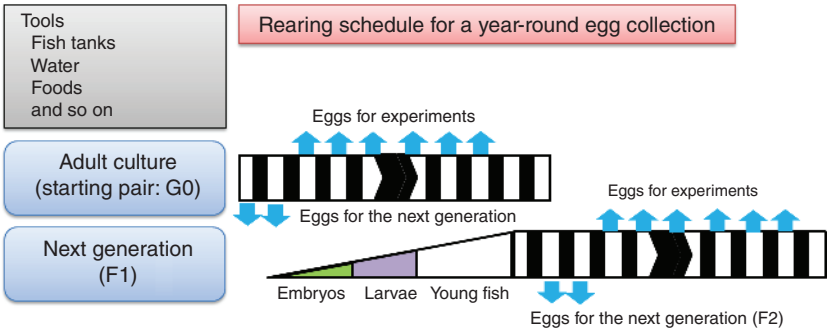

Figure 1-2. Outline of generating medaka eggs year round.

euthanize the fish at the end of experiments. The details of anesthesia methods are described in section 1.3.4.

1.3 Standardized Culture and Growth Curve

1.3.1 Characteristics and selection of strains

Medaka, as a model animal, has been used for more than 100 years. To date, many strains (such as the wild population in various regions in Japan and the Far East, color mutants, artificially induced mutants, and transgenic fishes) have been collected. Most of these strains have been kept in small-scale breeding facilities, for example keeping 10 females and 10 males of each generation of each strain. As a result of small-scale and long-term breeding, the genetic background has become unified in each strain. These strains are called "closed colonies" and each fish in a colony has homogeneous morphological characters, behaviors, and personalities. Moreover, the internal crossings of brother–sister (sib) mating for more than 20 generations has resulted in the establishment of "inbred strains." At present, about 10 inbred strains are kept in the NBRP Medaka stock center. These fish have no differences in their genetic background. Among experimental model animals, except for the mouse and rat, only medaka has multiple inbred strains. On the other hand, it is difficult to breed inbred strains. For example, the most famous inbred strain, Hd-rR, which was used for the Medaka Genome Project (https://shigen.nig.ac.jp/medaka/genome/top.jsp), has some disadvantages for experiments. The obvious disadvantages are the smaller numbers of spawned eggs and shorter spawning period compared with Cab which has been kept with mass mating and possesses polymorphism in its genome. This defect is often seen in many inbred strains, so at the beginning of research, it is important to select medaka strains that are suitable for the aim of the research. Although inbred strains retain some defects such as the smaller numbers of spawned eggs, their homogeneous genetic background is advantageous and attractive for specific studies, such as the assembly of genome sequence and fine quantitative trait locus (QTL) analysis. Refer to the first edition of this book and the NBRP Medaka HP (https://shigen.nig.ac.jp/medaka) for more information about inbred strains and other medaka lines, such as transgenic lines, natural populations, and mutants.

1.3.2 Management of medaka eggs and fish

The standard conditions of light and temperature are the 14L–10D cycle and 25–28 °C for medaka embryos, larvae, and adults.

1.3.2.1 Mating

A mature female prefers a familiar male to an unfamiliar male for mating and spawning (Okuyama et al. 2014). Therefore, it is better to breed a female and a male in a tank at least one day before the scheduled egg collection day. In the laboratory, under regulated lighting conditions, medaka spawn within one hour after the light is turned on. Occasionally, a female will not accept the male in the same tank. Therefore, if the pair does not spawn for a few days, replace the male fish with another one. A good pair spawns every day. Usually, females spawn 10–20 eggs a day, but this number is reduced in females of inbred strains (the number differs slightly according to the type of inbred strain). When many eggs are required, increase the number of breeding tanks where a male and a female are breeding or add one more female to the breeding tank to make a male to female ratio of 1:2. It is not recommended that more than one male fish is bred in a single tank for the following reasons: male fish fight each other for females, making certain males vulnerable; and the spawning performance of a male and a female is interrupted by another male. These behaviors reduce the number of eggs spawned.

It is also important to provide enough food to the breeding pairs to obtain many eggs. Details for feeding are given below.

1.3.2.2 Management of embryos

Keeping the ECM clean is one of the most critical matters in order to culture and maintain healthy embryos; otherwise bacteria and/or fungi will grow and cause harmful effects on embryonic development. Another critical matter is the number of embryos in a culture dish. Too many embryos in one dish disrupt normal embryonic development. The recommended number of embryos per culture dish is shown in Table 1-1. Under the appropriate culture conditions, the date of hatching can be controlled.

Tools

The tools used in embryo/larva culture are listed in Table 1-1 and Figure 1-3. Alternatives are listed below.

Reagents and Solutions

1. Sea water (SW): Dilute 3 g of artificial sea salt powder with 100 mL of reverse osmotic water (RO water) or distilled water (DW).
2. Embryonic water (EW): Dilute SW by 10% with RO water or DW (The final concentration of sea salt is 0.3%). This medium can be replaced by a balanced salt solution (BSS), Yamamoto's Ringer solution, or ECM (BSS: 0.65% NaCl, 0.04% KCl, 0.02% $MgSO_4$-$7H_2O$, 0.02% $CaCl_2$-$2H_2O$, 0.001% phenol red [sterilize and adjust to pH 8.3 with 5% $NaHCO_3$]. Yamamoto's Ringer solution: 0.75% NaCl, 0.02% KCl, 0.02% $CaCl_2$, 0.002% $NaHCO_3$ [adjust to pH 7.3 with 5% $NaHCO_3$].
   ECM: 0.1% NaCl, 0.003% KCl, 0.016% $MgSO_4$-$7H_2O$, 0.004% $CaCl_2$-$2H_2O$).

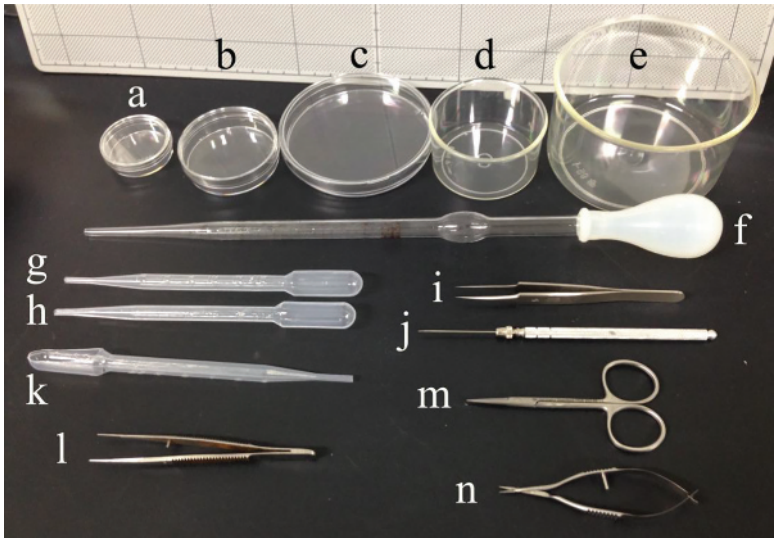

Figure 1-3. Tools for medaka embryo/larva culture and treatments. Standard use of each tool is explained in Table 1-1.

3. Methylene blue (MB): CAS number 61-73-4.
4. MB stock: 0.1% methylene blue stock solution in RO water or DW.
5. MB-EW*: Dilute MB stock with 1000 times volume of EW.
6. Sodium hypochlorite (SH) stock solution: Some chemical companies provide hypochlorite solution, for example Wako 194-02216. Be sure not to use certain hypochlorite products (for example, breach reagent for clothes) that contain NaOH and detergents.
7. SH-EW: Sodium hypochlorite working solution for the pasteurization of eggs. Dilute 150 µL SH stock with 1.5 L EW.

* For more information on methylene blue, refer to the last part of section 1.3.2.3.

Collecting and separating eggs

A female holds eggs on the outside of her cloaca region in her abdomen after spawning. To collect eggs, first scoop the female with a small net and then, using a pipette, suck the eggs off from the body (Refer to movies M3-2a and M3-2c in the first version of this book). The eggs form a cluster with their attaching filaments (see Figure 1-5). In order to more easily manipulate and/or move the eggs during experiments, the attaching filament on the egg envelope (chorion) should be removed and the clustered eggs should be separated individually. It is also important to remove unfertilized and abnormal eggs which may become feeding grounds for bacteria and/or fungus growth. Since the egg envelope is very soft just after spawning, it is better to collect the eggs after the envelope has hardened (wait for 1 hour) unless a very early stage of fertilized eggs is required; for example, one-cell stage eggs are required for microinjection. These eggs are firm enough to handle with fingers.

In this section, two methods for removing attaching filaments and separating the eggs from the cluster are described. One is rubbing the egg cluster with a finger onto a piece of paper or into a culture dish (Figure 1-4). The other is twisting each egg with forceps (Figure 1-5).

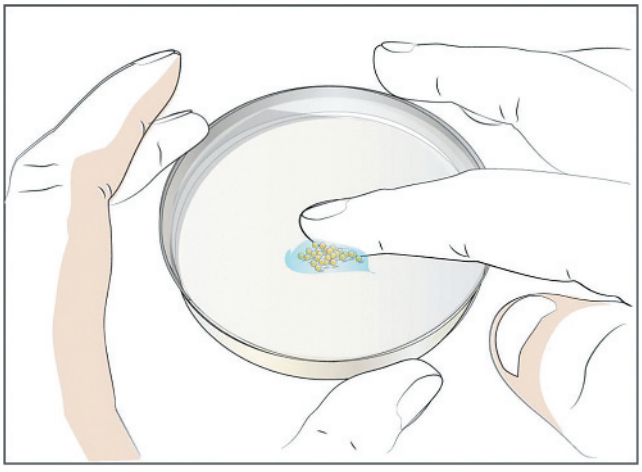

Figure 1-4. Egg separation with a finger (method 1).

Separation method 1 (rubbing the eggs with a finger) (Figure 1-4) This method is simple but be sure to use it only after the egg envelope has become hard enough (approximately one hour after fertilization). This method is demonstrated in Movie M3-3a in the first version of this book.

Materials

Plastic dishes (tool *c* in Table 1-1)
Two transfer pipettes (tool *g* or *h* in Table 1-1)
Fine forceps (tool *i* in Table 1-1)
EW (appropriate volume)
Disposable rubber gloves (if necessary)

Procedure

1. Transfer egg clusters into a dish filled with EW.
2. Remove debris using a pipette.
3. Gather the clusters in the center, and then push and rub eggs with a finger (with or without a rubber glove).
4. Keep rubbing gently by rotating the finger over the top of the egg cluster, just like drawing a small circle; the eggs will detach from the clutch one by one.
5. Stop rotating when each cluster contains a few eggs and then, clip a bundle body (a small clot of attaching filaments) directly with forceps and pull apart the remaining bundles to remove them from the eggs using the other forceps.
6. Clean the eggs by the method described in next page.

Modified Method

It is also possible to rub the eggs onto the surface of a newspaper or paper towel in order to separate the eggs, but some eggs may fly off the paper.

Separation method 2 (twisting the eggs with forceps) (Figure 1-5) This method requires two fine forceps and is suitable for eggs with soft egg envelopes (within 30 minutes after

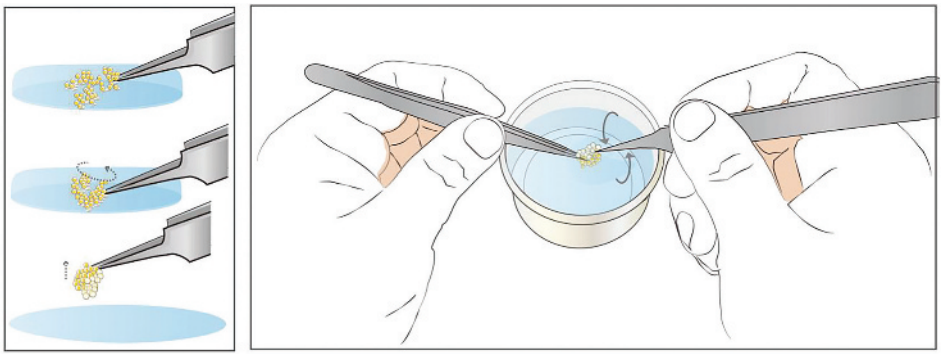

Figure 1-5. Egg separation with two forceps (method 2).

fertilization). Therefore, this method is useful to prepare fertilized eggs in the early stage required for microinjection. Refer to movies M3-3b to 3d and M7-1c in the first edition.

Materials

Fine forceps (× 2) (tool *i* in Table 1-1)
Plastic cup (tool *d* or *e* in Table 1-1)
Plastic dish (tool *a, b,* or *c* in Table 1-1)
EW (appropriate volume)

Method

1. Transfer the egg clusters onto a plastic dish or a plastic cup filled with EW. Use a stereo microscope to carry out the following procedures.
2. Gather the clusters into one big cluster as if tangling the attaching filaments of each cluster.
3. Pinch thick bundles of attaching filaments using one pair of forceps, and pinch other bundles with another pair of forceps.
4. Tangle or wind the attaching filaments around the bundles using the forceps.
5. The eggs will drop down onto the bottom of the dish or cup one by one (keep tangling the filaments).
6. Stop tangling the filaments when the cluster is reduced to a few eggs. Remove the remaining bundles from eggs with forceps.
7. Clean eggs using the method described in next section.

Egg cleaning
It is necessary to clean the eggs soon after their separation from the bundle in order to avoid the growth of microorganisms and fungi. Rinsing the eggs three to five times with EW is sufficient to clean the eggs. When the eggs are collected from the bottom of the tank (eggs that have already detached from the female's abdomen), it is likely that they will be contaminated with microorganisms and fungi, which will adversely affect incubation. In that situation, stricter cleaning methods using bleach (the pasteurization method) are recommended and will reliably maintain higher viability of the embryos.

This procedure can also be used to pasteurize eggs derived from the wild or another laboratory.

Simple rinsing method

Materials

Plastic dish, (tool *a, b* or *c* in Table 1-1)
Transfer pipette (tool *g* or *h* in Table 1-1)
EW (appropriate volume)

Method

1. Discard as much EW as possible from the dish containing eggs by decantation or use a pipette.
2. Fill the dish with new EW appropriately.
3. Repeat steps 1 and 2 for two to five times.

Pasteurization method (see movie M4-1 in the first version of this book) (Figure 1-6)

Materials

500 mL scale beaker (× 5)
Tea strainer (with smaller mesh than the eggs and that fits with the 500 mL beaker)

Figure 1-6. Egg cleaning (pasteurization method). (a) Set-up for pasteurization, (b) tea strainer, (c) eggs on the tea strainer, (d) eggs in the SH-EW, and (e) collection as the last step.

2.5 L EW
1.5 L Sodium hypochlorite solution (SH-EW) (1/10 000 diluted stock solution [8.5–13.5% SH] with EW)
Plastic dishes

Method

1. Put a series of solutions in five beakers as follows: #1 SH-EW, #2 EW, #3 SH-EW, #4 EW, #5 EW.
2. Transfer the eggs onto a strainer using a pipette. (minimize the volume of carry-in culture medium).
3. Move the strainer containing eggs into #1 and keep the eggs in the solution for five minutes.
4. Move the strainer from #1 to #2 beaker and keep the eggs in the solution for five minutes.
5. Move the strainer from #2 to #3 beaker and keep the eggs in the solution for five minutes.
6. Move the strainer from #3 to #4 beaker and keep the eggs in the solution for five minutes.
7. Move the strainer from #4 to #5 beaker and keep the eggs in the solution for five minutes.
8. Move the eggs from #5 beaker to a new dish with a small amount of solution.
9. Pour new EW into the dish and culture the eggs.
   For more information, see Chapter 3.8 Embryo Collection in the first version of this book.

1.3.2.3 Management of embryos before hatching

Separated and cleaned eggs are kept in a plastic dish at 28 °C in an incubator. The embryos should be checked every day; abnormal embryos must be removed. If there are moldy eggs in the dish, remove them and replace the contaminated dish and all the EW with a new dish and fresh EW. Although methylene blue is effective in inhibiting growth of microbes, the addition of methylene blue into the culture medium is not necessary to culture embryos (refer to the following additional information). The rate of embryo development depends on the temperature. At temperatures ranging from 25 to 30 °C, embryos progress normally throughout embryogenesis and hatch in about 10 days after fertilization. At temperatures higher than 32 °C, the risk of abnormal development increases and the expression of sex-related genes is also affected, resulting in production of reverse-sex individuals (Sato et al. 2005). At temperatures lower than around 10 °C, the developmental process will not be completed and the embryos will die. In order to ensure healthy development of the embryos, it is recommended that they are cultured at a constant temperature of 28 °C. Under this condition, the embryos will hatch within nine days after fertilization. Moderate increases or decreases in temperature can lead to faster or slower development, respectively.

Just before hatching, the embryo secretes a hatching enzyme from a gland located in its mouth cavity. This enzyme decomposes the egg envelope (refer to 6.1.3. Hatching Gland and 6.2. Medaka EGG Envelope and Hatching Enzyme in the first version of this book). It is better to incubate the embryos, as well as to rear fish, under a 14L–10D lighting cycle because the light stimulation triggers the secretion of the hatching enzyme. After hatching,

the hatching enzyme diffuses into the medium and acts on other embryos which are not ready to hatch because of their earlier developmental stage. Therefore, mixing embryos of totally different developmental stages should be avoided, otherwise the less mature embryos will come out of the egg envelope. Within a day after hatching, the larva should be transferred into a larval culture tank (refer to section 1.3.2.4).

Additional Information: Use of Methylene Blue in the Embryo Culture

Methylene blue is advantageous in the embryo culture for two reasons.

1. *The reduction of microorganisms (protoctista) and fungi*. The chemical structure of MB creates a clear blue color when dissolved in water and absorbs visible light to generate peroxides (reactive oxygen species). MB penetrates microbes through plasma membranes and works as a sterilization agent generating peroxides. For medaka embryo culture, 0.0001% MB solution diluted with EW (MB-EW) is often used instead of EW.
2. *Labeling of dead embryos*. Living embryos can actively excrete MB that flows into the chorions from the culture medium. On the other hand, in dead embryos MB is accumulated and the dead embryos look blue (arrows in Figure 1-7). This phenomenon makes it easy to distinguish dead embryos from living ones and is helpful when removing dead embryos from culture dishes.

However, since MB generates reactive oxygen species, its use must be avoided in some experiments, such as those using fluorescent observations and pharmacological evaluation of reagents in embryos.

1.3.2.4 Rearing from the larval stage to adulthood (to induce earlier maturation)

Scientific studies need to be reproduced even in aspects such as the growth curve, which is the record of body weight and body length congruent with the age of the animal. Controlling the breeding conditions (for example, frequency of feeding during a 24-hour period, the kind of food, the size of the tank, and so on) can achieve reproducibility with few errors, resulting in the creation of the required number of fish of the same age that are almost the same size.

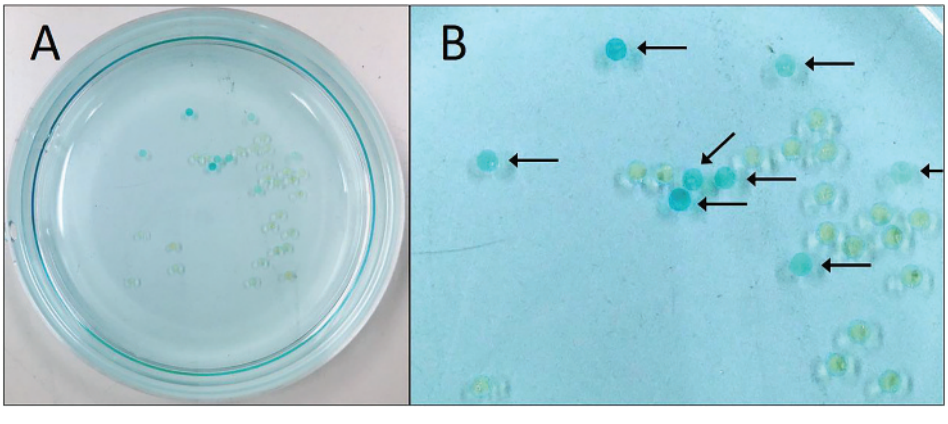

Figure 1-7. An easy method to identify dead eggs using MB.

Furthermore, rapid growth and maturation of medaka are required in studies dealing with multigenerations, for example, evaluating the effects of chemicals in subsequent generations and establishing homozygous transgenic strains which is usually completed in the F2 generation. In our experience, the history of nutrition (history of growth rate) in the larval stage and the history of breeding density strongly affect the growth rate in later stages and the rate of sexual maturation. Here we provide a description of the breeding method that will achieve rapid growth as the result of an ideal feeding program. The key to this method is to create an environment where the fish can eat constantly. For this purpose, there are two important points: one is feeding in a proper way and the other is maintaining good water quality. In this subsection, an ideal feeding program (how to feed at each growth stage of medaka) is described. The way to maintain water quality is described in section 1.3.3.

There is a good correlation between an ideal feeding program and growth in the length and weight of the fish. The feeding program is also important to achieve the maximum survival ratio and early maturation at two month post hatch.

In this subsection, feeding is separated into five stages, depending on the amount of growth.

1. Primary larval stage (<6 mm in body length).
2. Yong juvenile stage (6–15 mm).
3. Juvenile stage (15–20 mm).
4. Young adult stage (20–25 mm).
5. Adult stage (>25 mm).

Choice of food for larvae and adult medaka

It is important to obtain an appropriate diet that contains the nutrients necessary for optimal growth and also fits into the different sizes of the gape of the growing fish. Paramecia and brine shrimp provide lower nutrition than commercial powdered food (Goolish et al. 1999; Moren et al. 2006; Carvalho et al. 2006), but still lead to normal growth of medaka. The size of paramecia is smaller than the size of the gape at each stage. It is better to change the diet from paramecia to brine shrimp as soon as possible in order for the fish to grow faster because brine shrimp provide higher nutrition than paramecia (Goolish et al. 1999). Powdered food can be used instead of live food (for example, paramecia and brine shrimp) through all stages but leftover food should be removed using a pipette or the flow of the water recycling system. Furthermore, the different sizes of powdered food should be used according to each growth stage of the fish (Table 1-2). In the primary larval stage, it is better to mash the smallest commercial foods using a mortar and muddler and then feed the fish with the much smaller powders (<0.125 mm diameter). To avoid growth failure due to overcrowding in a narrow environment, the breeding tanks should also be changed according to the size of the growing fish (Table 1-3).

Powdered foods Powdered food is rich in nutrition for medaka. The particle size is very important because medaka cannot eat particles that are bigger than the size of their gapes. So, first, it is important to check the size of the particles carefully. The smallest commercial powdered food usually needs to be mashed into a finer powder using a mortar and muddler. Furthermore, it is recommended that the water in the container is changed before every

feeding to remove leftover food to maintain the water quality of the container during times when the circulating system is not operating.

Paramecium Medaka look at their food and prey on it (Beck et al. 2004). The paramecium is one of the optimum live foods for medaka at the primary larval stage. The authors usually use a small round container with static water for rearing larval fish. Even though many paramecia are added to the container, dissolved oxygen is not reduced as in the case of powdered food. Paramecia provide a lower nutrition level than commercial powdered food and brine shrimp. In order to determine when to change the food from paramecia to powdered food or brine shrimp, introduce a small amount of powdered food, such as "*a*" shown in Table 1-2, or brine shrimp and observe the results. If the growing larvae are able to ingest the powdered food or brine shrimp, each can be exchanged for the paramecia in their diet. The day to change the type of food will come in about one week.

Culturing of paramecia It is difficult to cryopreserve or store paramecia in the form of frozen stock. Paramecia should be continuously cultured and kept as a liquid medium. Generally, in order to avoid contamination, at least three beakers (beaker A, beaker B, and beaker C) are required to culture the paramecia. In cultivating of paramecia, it is necessary to prevent the entry of other animalcules, excluding bacteria which serve as food for the paramecia. Some animalcules inhibit the growth of paramecia. The cultivation of paramecia should be carried out without contamination.

Materials

1. Paramecium strain (obtained from research facility or stock on hand).
2. EBIOS tablets (a kind of supplement made from beer yeast; Asahi Food and Healthcare Co. Ltd., Japan) or equivalent.
3. Distilled water (deionized or RO water is also available).
4. 500 mL conical beaker (more than three).
5. Silicone cap or plug (for aerobic culture without contamination).
6. Sterilized pipette.

Method

The procedures below describe the preparation of one bottle containing paramecia in the EBIOS medium. During the time that the medaka larvae will be consuming paramecia, multiple bottles will be needed; these procedures should be applied to the preparation of subsequent bottles of the paramecia medium used to feed the medaka larvae as needed. After becoming fully grown, the medium contains approximately 200 paramecia per 1 mL medium.

1. Put 450 mL of DW into each 500 mL beaker (A, B, and C) and then add one EBIOS tablet into each beaker.
2. Cap the beakers with silicone caps.
3. Autoclave (120 °C for 20 minutes).
4. Allow the beakers to cool at room temperature (RT).
5. Add 10–15 mL of the paramecium culture medium to beakers A and B in a clean bench.
6. Culture at RT (approximately 23–26 °C).

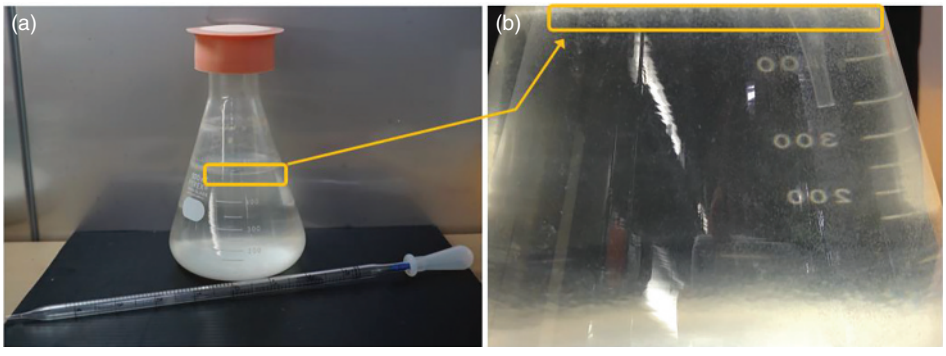

Figure 1-8. Paramecium culture. (a) Paramecia are cultured under aerobic conditions (top cap has air permeability) at RT. (b) After 4–5 days incubation, the paramecia will proliferate, reaching the stage where they can be fed to medaka larvae. The most concentrated portion (*yellow*) is suitable for feeding.

7. After becoming fully grown (generally 4–5 days: Figure 1-8), use the paramecia in beaker A to feed the medaka.
8. When the volume of the remaining medium in beaker A is reduced, use a pipette to suck 10 mL of the paramecia-rich medium (Figure 1-8b) from beaker B and add to beaker C.
9. Use the paramecia medium in beaker B to feed the medaka. Culture the paramecium in beaker C.
10. Rinse beaker A and return to steps 1–4.
11. When the volume of the remaining medium in beaker B is reduced, suck 10 mL of the paramecia-rich medium from beaker C and add it to beaker A.
12. Use beaker C for feeding. Culture paramecium in beaker A.
13. Repeat steps 8–12.

    Subculture the paramecia at least every two weeks. Paramecia can be stored in the beakers for two weeks; when feeding paramecia, it is necessary or desirable to take a brief pause in the culturing procedures.

Feeding paramecium Add 15 mL of the fully grown culture medium (beaker A in step 7 and beaker B in step 9 above) to the container of medaka larvae twice a day to provide sufficient food for 15 medaka larvae. In this case, many paramecia can be observed, even at eight hours after adding the medium (Figure 1-9). This situation allows the medaka larvae to eat paramecia continuously.

Brine shrimp Although the paramecium feeding program is one of the best methods to create an environment where the primary-stage larvae can eat food all the time and maintain a high quality of breeding water, it is important to change the food from paramecia to nutrient-rich foods such as brine shrimp or powdered food as soon as possible. When the larvae start to eat brine shrimp, they grow visibly day by day. The day that larvae become able to eat brine shrimp varies according to the medaka strain. Kyoto-Cab, HO5, d-rR, and Hd-rR strains can eat brine shrimp four days after hatching; the Kaga strain can start seven days after hatching; the HNI-II strain can start more than 10 days after hatching.

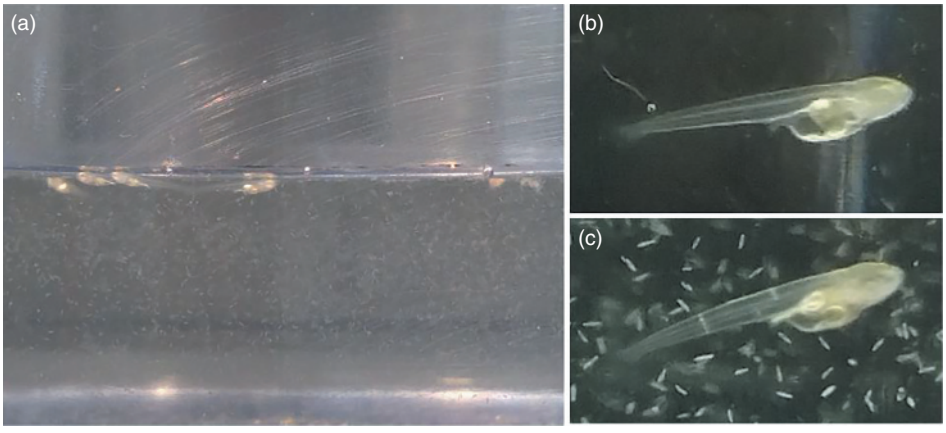

Figure 1-9. Paramecium medium and medaka larvae fed with paramecium medium. The high density of paramecium provides a suitable environment for medaka larvae at the primary stage. Under these conditions, the paramecia are continuously available for the larvae to eat. Feeding twice a day should provide sufficient food for the larvae. The abdomens of the larvae which ate paramecia appear to be expanded and white in color (c). In contrast, that of the larvae which did not eat paramecia looks thin and transparent (b).

In order to create uniform larval growth of medaka being raised in a fish tank, the feeding of paramecium should be continued until all larvae are mature enough to be able to eat brine shrimp. Otherwise, the individual differences in the size of the larvae become more pronounced since the larvae that eat brine shrimp grow faster than those that do not. On the other hand, in order to raise the next generation as quickly as possible, the introduction of brine shrimp to the rearing containers should be started as soon as only a few larvae become able to eat them. To determine whether larvae have grown enough to be able to eat brine shrimp, add a small amount of brine shrimp to the culture container and observe the behavior of the larvae. The larvae will target and chase the shrimp, and their abdomens appear orange after eating them (Figure 1-10). On the other hand, when the larvae do not eat the shrimp, paramecia should continue to be fed to the larvae and the test feeding of shrimp should be postponed.

Differences in individual growth of the larvae may occur when brine shrimp feeding starts. Bigger fish eat more food and this may increase the variation in growth among fish of the same age. In order to reduce the variation of body size among the same brood, it is effective to separate the larvae that can eat shrimp from those that cannot and put the larger

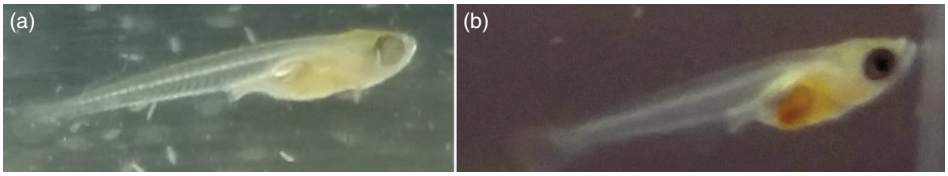

Figure 1-10. Discrimination of larval foods at seven days post hatching. (a) Paramecia-fed larva (whitish abdomen) and (b) brine shrimp-fed larva (orange abdomen).

larvae into a new container as a "second group" and continue feeding paramecia to the "first group." On the next day, larvae that are newly able to eat shrimp are put into the tank of the "first group." Continue this sorting and after all larvae can eat shrimp, the brine shrimp feeding should be started.

Feeding medaka from immediately after hatching to the larval stage

If it is possible to achieve breeding in a high growth manner from the posthatching period to two weeks post hatching, the fish will have a high survival rate and also spawn eggs at two months post hatching. Medaka larvae can start eating food within 1–2 days post hatching; however, until about seven days post hatching, the larvae appear to be not good at finding food. The larvae with delayed and insufficient feeding behavior display low growth and low survival rates which result in a slower onset of sexual development. So, it is necessary to ensure that the larvae are able to eat food constantly and that the water quality is kept high. Commercially available powdered foods and/or slipper animalcules can be given to the larvae as their first food. When using powdered food, it is especially important to carefully maintain the quality of the breeding water and not to overfeed. The feeding should be done three to four times a day, and the breeding water should be changed one hour after every feeding. However, when using paramecia, changing the breeding water twice a day is sufficient to maintain water quality. In this case, changing the water is performed as follows.

First, remove the water from the breeding container with a pipette or drain hose, and then add the new water containing a high concentration of living paramecia. After the larvae are able to eat brine shrimp or powdered food as shown in "*b*" in Figure 1-10, they should be transferred into a 2–3 L tank. The timing depends on the strain, for example four days after hatch for the Kyoto-Cab, d-rR, HO5, and Hd-rR strains, six days after hatch for the Quintet and STII stains, nine days after hatch for the Kaga and HNI-II strains. Then, the size of the tank and the number of larvae should be adjusted according to the optimal density shown in Table 1-3.

Feeding newly hatched larvae

Materials

Paramecia (culture method is described in Culturing paramecia or in section 2.3.3.1 in the first version of this book)
Breeding tanks (3 L)
Transfer pipette (*g* in Figure 1-3)
Round container (*d* and e in Figure 1-3)
EW (10% sea water: see section 1.3.2.2)

Method

This method starts just after hatching.

Day 1

1. Transfer the newly hatched larvae to a round container using a transfer pipette.
2. Add 15 mL of paramecia solution (see section "Feeding Paramecium") and EW into the container, and let it sit for a number of hours, until it is time to leave for the day.
3. Change the breeding water before leaving.

3-1. Remove the breeding water and as much of the debris on the surface and/or the bottom as possible using a pipette. But be sure to retain enough of the breeding water at a level where the larvae are still able to swim.
3-2. If there is still some remaining debris which needs to be removed, add a small amount of new breeding water into the container and then repeat step 3-1.
3-3. Add new paramecia solution and EW as described in step 2.

Day 2

Check whether the amount of paramecia solution is sufficient or not by observing two hours after adding the solution according to the following directions.
☐ Is the color of the abdomen (intestines) white?
→ If yes, it's a sign that the larva has eaten paramecia.
☐ Is the abdomen full?
→ If yes, it's a sign that larva has eaten enough paramecia.
☐ Is there any sign of feces?
→ If yes, it's a sign that larva has eaten paramecia.
If the answer is "no" for all the above questions, the amount of paramecia in the container was insufficient. In this case, add more high-density paramecia solution to the container (there are two areas of high- and low-density paramecia within the culture solution [Figure 1-8]). From this day on, check the abdomens and feces (three check questions above) at least two hours after feeding and keep checking until any sign that the larvae have eaten some of the paramecia is noticed.

4. At 9.00 a.m. (at the start of work, the sooner the better), change the breeding water according to step 3.
5. At 17.00 (time to leave for home), change the breeding water according to step 3.

Day 3

By day 3, almost all larvae usually have started to eat.

6. Feed and change the breeding water according to steps 2 and 3.
7. Check the color of the abdomens of the larvae and check for the presence or absence of feces in order to confirm that the larvae can eat paramecia and that the amount of paramecia is sufficient. If the amount of paramecia is not sufficient (in this case, the abdomens of larvae are not full), add more paramecia solution. Generally, almost all the larvae will be able to eat paramecia within one week after hatching.
8. Check the color of the abdomens of the larvae and for the presence of feces again.

Days 4–16

9. Repeat the cleaning of the container according to step 4.
10. Determine when to change the type of food. Add a small amount of brine shrimp or powdered food into the container, and then observe each larva within one hour after feeding according to the following checklist.
☐ What color are the intestines?
→ If the intestines are red (or the color of powdered food), it's a sign that the larva has eaten shrimp (or powdered food).
☐ Is the abdomen full?
→ If yes, it's a sign that the larva has eaten enough shrimp (or food used).

☐ Is there any sign of feces?
→ If yes, it's a sign that larva has eaten shrimp (or food used).
If uniformity of body size of the fish in a breeding tank is required, change the food from paramecia to brine shrimp (or powdered food) when almost all the larvae can eat brine shrimp (or powdered food). If a number of larvae cannot eat brine shrimp (or powdered food), remove the brine shrimp (or powdered food) and feed paramecia.
If a variety of body sizes is acceptable, move the larvae with red (or the color of powdered food) abdomens into a 3 L tank. The larvae that have been moved are fed as described in section Table 1-2 and 1-4.

11. Repeat step 10.

Feeding larvae that can eat brine shrimp and/or commercial powdered food This method is started when larvae have demonstrated the ability to eat brine shrimp (see Figure 1-10). However, sometimes, larvae that have successfully eaten brine shrimp in a narrow round container may not be able to catch the shrimp in a larger breeding tank. Therefore, feeding brine shrimp is started with a breeding tank (3 L) containing a small amount (1 L) of water. Then, after confirming that all larvae can eat brine shrimp in a small amount of water, increase the water volume to 3 L. Generally, this method will be started at four days post hatching (4 dph) for the Kyoto-Cab, d-rR, HO5, and Hd-rR strains, at 6 dph for the Quintet and STII strains, and at 9 dph for the Kaga and HNI-II strains.

Materials

Hatched brine shrimp (culture methods are described in section 2.3.3.1. in the first version of this book)
Breeding tanks (3 L)
Transfer pipette (*g* in Figure 1-3)
Glass pipette (Komagome 5–10 mL; *f* in Figure 1-3) and EW (1 L for each tank)

Method

1. Rear larvae in the 3 L tank containing 1 L water.
2. Add brine shrimp (or the smallest size of commercial powdered food).
3. Check the abdomen of each larva one hour after feeding. If all the larvae are able to eat brine shrimp, increase the amount of water to 3 L. If not all the larvae are able to eat the shrimp within one hour, keep the amount of water at 1 L and recheck them after the next feeding. Food intake is checked in the same way as described in Days 4–16 *Feeding newly hatched larvae*.

Management of fish after the second larval stage and the growth curve
After the second larval stage, the excess feeding program should be continued in order to achieve maximum growth and rapid maturation of the medaka. In order to maintain high-quality water conditions, it is important to use circulation system racks (see Chapter 2 in the first version of this book). However, a hobby aquarium system that contains an aeration and filtration system can also be used. In both cases, the number of fish in each tank should be about two adult fish per 1 L of breeding water (as shown in Table 1-3). As the fish grow in body length, the size of the tank should be increased according to Table 1-3 and simultaneously the type and amount of food should be changed according to Tables 1-2 and 1-4, respectively.

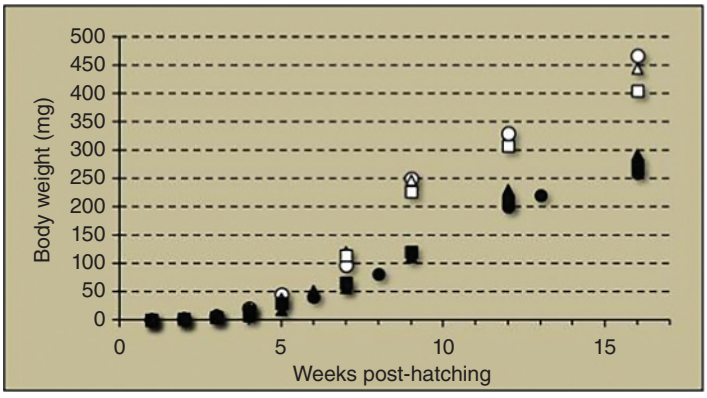

Figure 1-11. Growth curves of data resulting from different larval feeding programs. White and black marks on the graph represent the average body weight of 10 fish from the excess and half feeding programs, respectively. Each program was carried out in three independent trials.

Figure 1-11 shows standard growth (body weight) curves of fish fed under the excess feeding program (white) and the half feeding program (black). Fish reproducibly grew, as the white marks indicate, due to the excess feeding program for this test period. Both programs produced reproducible data about the growth curve. The measuring method for medaka body weight is described in Chisada et al. (2011, 2014). Excess feeding means that fish can eat food any time they want to. Nonfresh foods (leftover foods) are not suitable as fish food, so the standard feeding procedure is to feed 5–7 times a day; hence, it is important to know the relationship between the growth rate and the amount of feeding. The authors have tested and calculated the amount of feeding per day at each stage of larval development (weeks post hatching) as shown in Table 1-4. This table is useful to reduce

Table 1-4. Adequate amounts of powdered food consumption for the excess feeding program.

Stage (weeks)	Daily amount (mg/fish)	Standard body weight (mg) at starting period
2–3	2.1	2.5
3–4	2.8	9
4–5	2	20
5–6	4.7	40
6–7	5.4	65
7–8	6.3	100
8–9	7.5	150
9–10	10.7	220
10–11	12.9	260
11–12	13.7	290
12–13	15.9	330
13–14	17.8	340
14–15	18.2	350
15–16	18.7	360

leftover food which is the cause of reduced water quality. This feeding program produces the data shown as white marks in Figure 1-11.

Although the food listed in the table is the powdered food as described above (Table 1-2), brine shrimp are also suitable as food. In the case of shrimp, feeding three times a day is suitable from the second larval stage to the second juvenile stage; feeding five times a day is suitable from the second juvenile to the adult stage (these stages are defined in Chapter 6 in the first version of this book). The amount of food used for a single feeding is about 150–300 shrimp per fish; this shrimp feeding method results in the same growth curve as the use of powdered food. In addition, the half feeding program results in the growth curve shown in black in Figure 1-11.

Materials

Aquarium system (with water circulation system)
Breeding tanks (appropriate sizes for each stage: see Table 1-3)
Food (appropriate particle sizes)
Milliliter tubes and medicine spoon
Larvae

Method

1. Prepare the daily amount of food and insert it into a 1.5 mL tube according to Table 1-4.
2. In the daytime, feed 1/5–1/7 of the daily amount of food at each feeding.
3. Repeat feeding 5–7 times a day in order to feed the total daily amount of food. And then, change the amount of food according to Table 1-4 for the next week. If a group of fish at any developmental stage reaches the density limit for the breeding tank, move the fish to a new and larger tank so as to maintain the optimal density according to Table 1-3.

1.3.3 Maintenance of breeding tanks during breeding

The ideal feeding program can achieve a higher survival rate and rapid sexual maturation within two months post hatching. This is the shortest maturation interval for medaka to attain the ability to spawn. This program requires keeping the quality of the breeding water high.

1.3.3.1 Judgment of water quality

In order to determine the quality of the water in the breeding tank, check the following two points: the clarity of breeding water and the physical condition of the medaka in the breeding tank. Water clarity is a simple indicator of water quality. Generally, good-quality breeding water is clear, while cloudy water is poor in quality. The breeding water will become cloudy in the following situations: (i) the water has been motionless for a while, (ii) dead fish are left in the breeding tank for over a day, (iii) leftover food remains on the bottom of the tank for a few days. Even though the water may appear to be clear, sometimes the quality of the water can be poor. In such circumstances, observation of the physical condition of fish is another way to evaluate the water quality. In tanks with a high quality

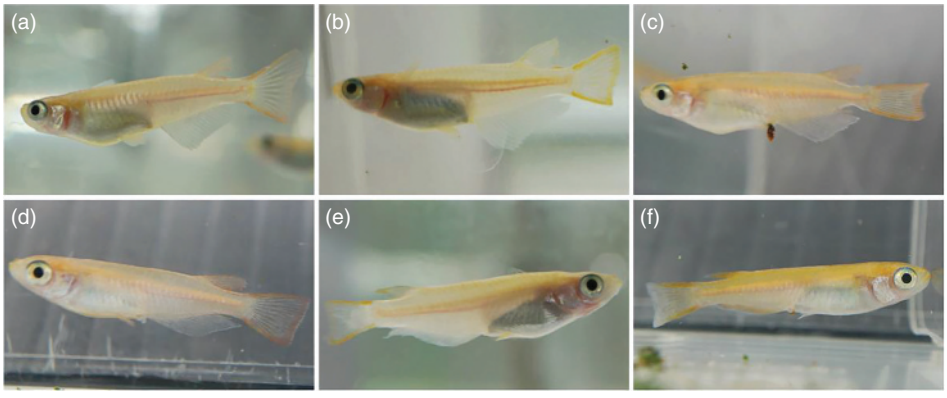

Figure 1-12. Observation of fin form. (a, b) Healthy fish. (c–f) Fish in poor physical condition, and fish in marginally poor to very poor physical conditions are in alphabetical order. (c) Fish with closed tail fin. (d) Fish with closed anal fin. (e) Fish with closed anal fin and cloudy white anal and tail fins. (f) Fish with closed and white cloudy anal and tail fins.

of breeding water, the fish have widened and transparent fins (Figure 1-12a,b). When the quality of the water is lowered, the fish feel stress and their fins become closed and/or cloudy white areas appear on their fins (Figure 1-12c–f).

1.3.3.2 Maintenance of breeding water

General procedures

Breeding healthy fish requires water with a sufficient amount of dissolved oxygen. Leftover food causes a proliferation of fungi, algae, and bacteria, following the decrease of dissolved oxygen. Therefore, carefully monitoring the amount of food given to the fish and thoroughly cleaning leftover food are the keys to maintaining a high quality of breeding water.

To maintain medaka strain. The appropriate amount of food for each feeding consists of the amount that healthy fish can completely consume within 5–10 minutes. If any food is left on the bottom one hour after feeding, all the leftover food should be removed as described below and the amount of food should be decreased at the next feeding.

To achieve the earliest possible maturity among the fish. The ideal feeding program can achieve the fastest maturation and the maximum viability. In this case, each feeding should contain the amount that healthy fish can completely consume within one hour. If any food is left on the bottom one hour after feeding, all the leftover food should be removed as described below and the amount of food should be decreased from the next feeding.

This subsection describes how to remove the leftover food with a siphon tube and pipette from the tanks.

Optionally, freshwater snails (red lamb's horn) may be useful for cleaning the walls of breeding tanks. Be aware that snails have the potential to bring disease factors into fish tanks. It is strongly recommended that the snails should be obtained from a reliable institution or laboratory where fish and snails are healthy.

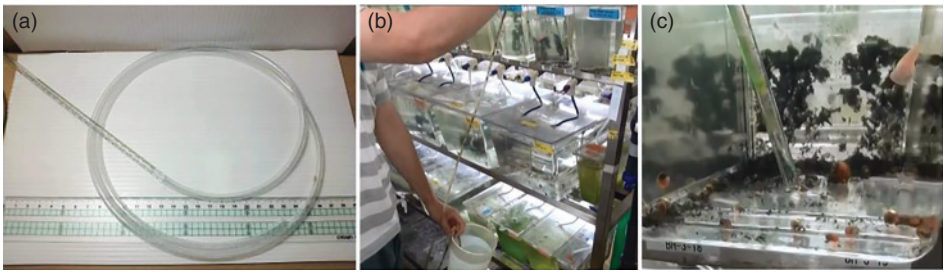

Figure 1-13. Cleaning of the tanks (bottom debris removal). A custom-made siphon tube (a) is useful for removing debris on the bottom of the tank (b,c).

Materials

Cleaning sponge (nonabrasive and nondetergent composition)
Siphon tube with a pipette (custom made as in Figure 1-13)
1–2 L bucket

Method

1. Wipe the front wall of breeding tank with the sponge (wipe the bottom if necessary).
2. Wait for about an hour until debris settles to the bottom of the tank.
3. Aspirate the debris with a siphon as shown in Figure 1-13b,c.

Management after finding fish with closed fins

When fish with closed fins are discovered, the following procedure is necessary to clean the breeding tank. When rearing with static water, it is better to change a tenth of the volume every 3–4 days to restore and maintain good water quality. Salt (a final concentration of 0.5–1.0%) is sometimes available to unhealthy fish for improving their health conditions. In cases where recycling and overflow systems are used, salt should be added to give a final concentration of 0.5–1.0% every morning until the fin gets wider. Methylene blue water (0.0001%) can also help unhealthy fin recovery.

Management of tanks after finding cloudy water

In each breeding situation (whether using a recycling system or static water), when cloudy water is discovered, first determine whether there are any dead fish in the tank and if so, remove them. After that, change the water. If the fish are in a recycle system, the same procedure is used for the fish, and the recycle system is filtered until the cloudiness disappears from the water. Next, observe the condition of the fins of the fish. If the fins are closed, it is necessary to improve the conditions of the breeding water according to the steps listed in above. If the fins appear normal and there are no dead fish in the tank, the low transparency of the water may possibly be due to water composition.

1.3.4 Anesthesia

An anesthetic procedure for medaka with the objective of providing for animal welfare is important. In general, fish are treated with careful consideration for their welfare; after they start to eat foods, the need to consider animal welfare becomes more significant. The major

role of anesthesia in fish husbandry is to minimize stress during observation, dissection, and fin clipping for genotyping. In previous articles regarding basic biology, MS-222 (tricaine methanesulfonate), the methane sulfonic acid salt of ethyl-m-aminobenzoate, has been used as an anesthetic agent for fishes. In the fishery sciences, eugenol (FA-100), 4-allyl-2-methoxyphenol, has been used as an anesthetic. McFarland and Jolly et al. defined the stages of anesthetic depth for fishes according to the behavior and physiology as shown in Table 1-5 (McFarland 1959; Jolly et al. 1972). Grush et al. reported the effects of MS-222 and eugenol as anesthesia for zebrafish (Grush et al. 2004). They indicated that anesthetic stages 4–5 should be used for dissection and recovery from anesthesia. MS-222 and eugenol are popular as anesthetic agents and are used for adult medaka at concentrations of 0.03% and 0.005%, respectively. For larvae, half of the adult dose is used. In this subsection, the time needed to achieve each anesthetic stage for medaka using MS-222 and eugenol is described. Furthermore, MS-222 has the potential to cause death for medaka at a specific growth stage.

1.3.4.1 Behavior under each anesthesia stage

The upper illustration in Figure 1-14 shows the progression of anesthesia stages in medaka. After dipping the fish into the solution containing the anesthetic reagent, the medaka starts swimming erratically (stage 2). Next, it loses a sense of equilibrium along with an increase of erratic behavior (stage 3), followed by a complete lack of balance (stage 4). Although it does not seem to move at stage 4, the fish can react to touch. At stage 5, the medaka does not move at all.

The lower image in Figure 1-14 is a comparison of time it takes to reach each stage between STII and Kyoto-Cab strains using 0.03% MS-222 and 0.005% eugenol. STII has a different sensitivity to anesthetic agents.

1.3.4.2 Difference in sensitivity to anesthesia among strains

Empirically, it is known that sensitivity to anesthetic reagents depends on the medaka strain. Here, the difference in sensitivity to MS-222 and eugenol between Kyoto-Cab and STII medaka is described as an example. We recorded the time periods for achieving each anesthesia stage (Table 1-5) and recovery from anesthesia with MS-222 and eugenol. The

Table 1-5. Stages of anesthesia in fish.

Stages	Description	Details
0	Normal	Reactive to external stimuli
1	Light sedation	Opercular rate slightly decreased; equilibrium normal
2	Deep sedation	Swimming slightly erratic; slight decrease in opercular rate; equilibrium normal
3	Partial loss of equilibrium	Swimming erratic; increased opercular rate; equilibrium erratic
4	Total loss of equilibrium	Total loss of equilibrium; slow but regular opercular rate; loss of some spinal reflexes
5	Loss of reflex reactivity	Total loss of reactivity; opercular movements slow and irregular; heart rate slow; loss of all reflexes
6	Medullary collapse	Opercular movements cease; cardiac arrest usually follows quickly

Source: Modified from Keene et al. (1998).

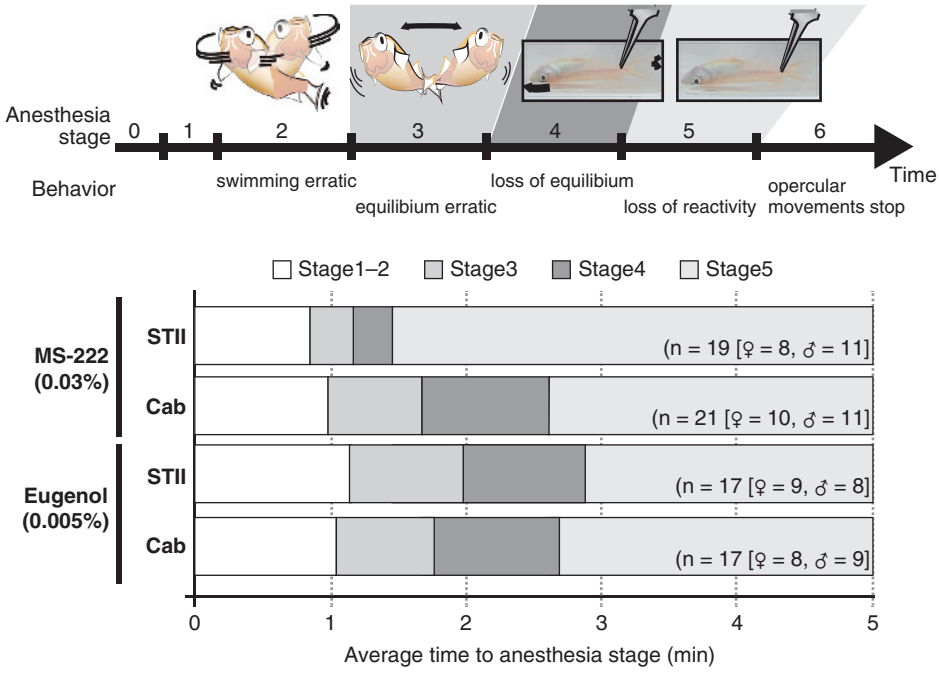

Figure 1-14. Duration of anesthesia stages and brief summary of anesthetic effects on two medaka strains.

results indicate that those time periods differ in the different strains. The lower illustration in Figure 1-14 shows the progression of anesthesia stages in adult Kyoto-Cab and STII strains. Between these strains, there are differences in the progression at the different anesthetic depths using MS-222. The STII anesthetized with 0.03% MS-222 reached stage 5 within 1.5 minutes, while Kyoto-Cab required over 2.5 minutes to achieve stage 5. Thus, the STII strain has a high sensitivity to MS-222. Furthermore, at a specific growth stage the STII strain is seriously damaged by MS-222, resulting in death (Figure 1-15). Using eugenol, there was no obvious difference between these strains. The time periods needed for the fish to reach each anesthesia stage become longer as the fish mature or increase in body weight.

As for strains other than Kyoto-Cab and STII, it will be necessary to investigate the sensitivity to anesthetic agents and determine the concentration of the reagents and exposure period to the reagents.

1.3.4.3 Growth stage specificity in sensitivity to MS-222

Recovery: For Kyoto-Cab and STII medaka at growth stage (G.St) 42 (four weeks post hatch) with a body length of 10–15 mm, it takes more than 15 minutes for the fish to recover from the effects of the 0.03% MS-222 anesthetic for treatment lasting for 10 minutes. On the other hand, fish at other G.St required only six minutes to recover from the effects of the anesthetic.

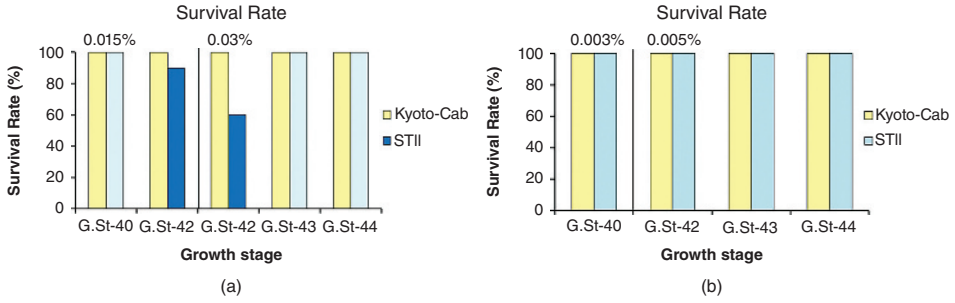

Figure 1-15. Survival rates from anesthesia using MS-222 (a) and eugenol (b). Fish were exposed to 10 mins of anesthetic reagents and then transferred to a container containing breeding water without anesthetic reagents and their survival rates were counted. G.Sts indicate the growth stages. The body length at G.St 40 ranges from 4 to 7 mm, at G.St 42 body lengths range from 10 to 15 mm, at G.St 43 the lengths range from 15 to 21 mm, and at G.St 44 lengths range from 21 to 28 mm. Each anesthetic concentration is shown in the panels.

Lethality: Figure 1-15a shows survival rates at each G.St from 10 minutes under anesthesia. In the STII strain at G.St 42, MS-222 treatments resulted in low viability (90% and 60% at 0.015% and 0.03% concentrations of MS-222). It is important to note that MS-222 has a serious effect upon the STII strain at specific larval stages (G.St 42).

1.3.4.4 Eugenol is recommended as an anesthetic reagent

As described above, MS-222 shows G.St-specific lethality for the STII strain. The same effects can be seen in other strains, d-rR and Kaga, while there is no lethal effect on Kyoto-Cab, Quintet, Hd-rR, and HO5.

As shown in Figure 1-15b, eugenol does not show a lethal effect at G.St 42 at a concentration of 0.005% which can achieve complete anesthesia (Figure 1-14). And no adverse effect has been found in other strains using eugenol.

We recommend using eugenol as an anesthetic, especially for the STII, d-rR, and Kaga strains at the larval stage.

1.3.4.5 Euthanasia

When it is necessary to euthanize the fish, they should be killed in a manner that causes minimal stress. Numerous articles have indicated that fish have various sensory organs that convey a sense of pain, but it is not yet clearly understood whether medaka feel pain or suffer from these procedures. Here, using preliminary data, the authors show the concentration (0.06%) at which medaka cannot recover from anesthesia with MS-222. We consider this to be the concentration at which medaka cannot feel any pain. The procedure for euthanizing medaka is to anesthetize the fish with MS-222 at a concentration of 0.06% and to sever its head or spinal cord. Figure 1-16 shows the dose–response effect of MS-222 on adult Kyoto-Cab medaka. The indices are the times needed to reach anesthetic stages 4 and 5, the recovery time, and the survival rates. Those are recorded after 15 minutes' exposure to anesthetic at each concentration. Adult Kyoto-Cab medaka anesthetized with 0.015% MS-222 were able to achieve anesthetic stage 4 within six minutes, although the

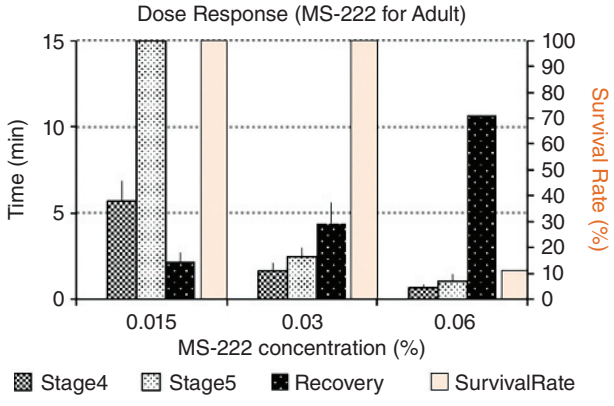

Figure 1-16. Dose–response effect of MS-222 on adult medaka. The white dotted and pale gray bars show the times needed to reach stages 4 and 5. The black bars indicate recovery times. The orange bars show the survival rates after 15 minutes' treatment with MS-222.

fish were unable to achieve stage 5 in 15 minutes. All the fish were able to recover from 0.015% MS-222 within two minutes. Adult Kyoto-Cab anesthetized with 0.03% MS-222 were able to achieve anesthetic stage 4 in about 1.5 minutes and stage 5 in about 2.5 minutes. Using 0.03% MS-222, all the fish were also able to recover from anesthesia, although the recovery time was longer than that of 0.015% MS-222. Using 0.06% MS-222 caused the fish to reach anesthesia stages 4 and 5 rapidly. All the fish showed much slower heart and respiratory rates after 15 minutes' exposure to anesthetic, but 90% of those were not able to recover within 15 minutes after being transferred to the recovery water. Therefore, a 15-minute treatment of 0.06% MS-222 was considered to be a very deep anesthetic state. The procedure to euthanize medaka requires anesthetizing the fish using 0.06% MS-222 for 15 minutes, followed by spinal transection.

1.3.4.6 Important reminders for euthanasia

There have been many reports about differences of effect between MS-222 and eugenol. MS-222 rapidly diffuses across the gill and passes the blood–brain barrier. It affects the central nervous system and can stop respiratory activity (Topic et al. 2012). Although eugenol decreases respiratory rates, it acts on the sensory system (Yang et al. 2003; Javahery et al. 2012). This might have caused the differences in the effects of these reagents on medaka. The authors recommend using 0.005% eugenol for anesthesia at least for the STII, d-rR, and Kaga strains at G.St 42 and using 0.06% MS-222 for euthanasia by inducing a deep state of anesthesia at all strains.

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