Protocols for Marine Medaka

Adapted from: "Chapter 10. Application of the Seawater Medaka *Oryzias melastigma* (McClelland) for Marine Ecotoxicology" in *Medaka: Biology, Management, and Experimental Protocols*, Volume 2, First Edition.

General features and basic biology of the marine medaka *Oryzias melastigma*	
Body length (adult)	3.5–4 cm
Time to sexual maturation *	Around three months
Secondary sex characteristics (phenotypic sex) *	Males with larger and longer caudal and anal fins, which are distinguishable as early as one month of age
Lifespan	Median lifespan 18 months; live up to three years
Spawning behavior	Daily
Daily egg production	5–15 eggs per female
Egg diameter	~1000 µm
Fertilization rate	>90%
Hatching rate	>90%
Embryonic development (from fertilization to hatching)	11 ± 2days
Density tolerance	0.3 L of seawater per adult fish
Seawater maintenance	Low; change seawater 1–3 times per week

* Rapid sexual maturation and phenotypic sex is observed under optimal rearing conditions (see below). In experimental conditions, egg spawning was observed as soon as 8-week post-hatching. Slower growth may delay significantly sexual and phenotypic sex development.

Rearing conditions for marine medaka	
Salinity	Optimal: 30‰ Minimum: ~ 5‰ Maximum: ~ 60‰
Artificial seawater	For 30‰ salinity: add approximately 450-500g of salt (~ 1000mL of dry salt) for 20L of reverse-osmosis water. The salt should dissolve completely and the seawater should age in aerated holding tanks overnight.. Salinity levels are checked using a calibrated refractometer and adjusted to 30‰ with artificial sea salt or reverse-osmosis water if necessary.
Aeration	Artificial aeration is optional. Avoid vigorous aeration, especially for larvae or juveniles. Air tubes and stones are cleaned monthly using ethanol or bleach and soaked overnight in dechlorinated tap water
Temperature	Temperature range: 24±3 °C Optimal temperature for egg production: 25 °C Optimal temperature for embryos: 26°C. (Development is slower at lower temperatures). In experimental conditions, fish could survive temperatures as low as 15 °C and as high as 30 °C for a few weeks without significant mortality.
Photoperiod	12:12 (light:dark) Full-spectrum overhead lighting (white- light fluorescent tubes, 100–500 lux) for rearing and breeding
Dissolved oxygen	6±1 mg/L O2
Nitrate	<20 mg/L
Nitrite	<0.1 mg/L
Ammonia	<0.01 mg/L
pH	8

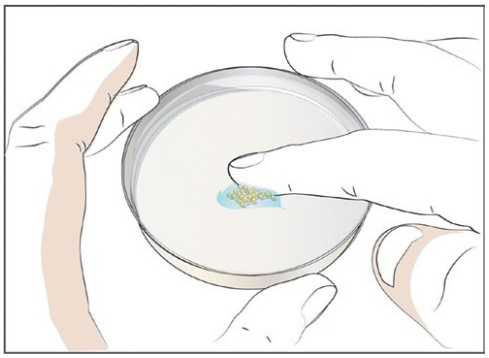
Feeding	
Feeding routine *	Morning: 9-10 am Afternoon (optional): 3-4 pm Evening: 6-7 pm Fish are fed to satiation, by feeding as much food as the fish can consume within five minutes.
Invertebrates (evening)	Newly hatched brine shrimp *Artemia nauplii*
Algae (for larvae) **	Light green or brown water (from algae present naturally in water serve as a food source.
Dry food	Ocean pearls (food flakes)

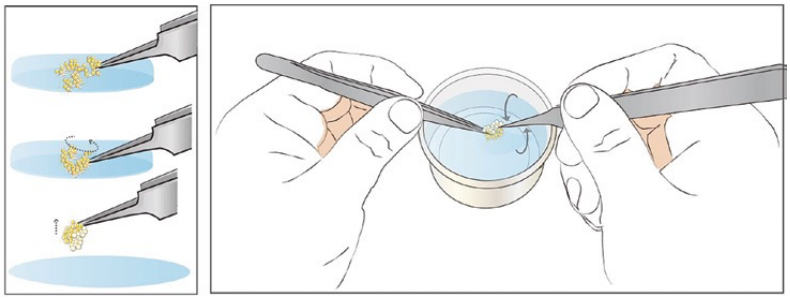
* For the first week, larvae should be fed only with dry larval feed, three times a day. After 1–2 weeks post hatching, larvae should be fed twice a day with dry larval food and once a day with brine shrimp to satiation.

** In general, algal growth usually does not have any negative impacts on egg production or fish mortality, but may obscure the visual observation of fish behavior. However, the bottom and sides of the tanks should be periodically scrubbed clean with a hard brush to prevent excess growth of fungi and algae.

Embryo collection and rearing (non-synchronized embryo development)

1. The eggs should be collected every morning (or they will be eaten, or may die more rapidly).
2. Wash your hands thoroughly.
3. Fill a Petri dish with artificial seawater.
4. Use an aquarium pipette (thoroughly washed with running dechlorinated tap water) to collect all the eggs, making sure to cover the entire bottom of the tank.
5. Place the eggs into a fish net (the mesh size should be smaller than the eggs but large enough to filter debris and fish droppings).
6. Flush tap water through the fish net to remove debris and fish droppings.
7. Transfer the eggs to the Petri dish.
8. Using your finger, gently press the eggs onto the bottom of the Petri dish and rotate your finger rapidly to detach filaments and reduce clumping of the eggs. (It is possible to use forceps. The chorion is relatively strong, and should not be damaged.)





9. Remove dead or unfertilized embryos with a plastic Pasteur pipette to reduce bacterial/fungal contamination (these eggs usually appear white and cloudy in color).
10. Let the eggs settle and carefully pour out the seawater supernatant.
11. Refill the Petri dish with clean seawater until the embryos are completely submerged.
12. Repeat steps 8 to 11 until you obtain a clean supernatant (3-5 times). The eggs should be completely separated.

13. Incubate the embryos in the Petri dishes at 26 °C, under 1000 lux illumination (with fluorescent tubes), with a 12L:12D light/dark cycle, and rotate in an orbital shaker at 36 rpm (optional).
    - Optimal temperatures and shaking result in faster hatching.
    - It is common for fish to reduce egg production in response to low temperature or external light during the dark cycle.
    - For a larger number of embryos (>50), place the eggs into separate Petri dishes or a 2L glass tank.
14. Artificial seawater should be completely changed daily. Dead embryos and chorions should be identified and discarded daily.
15. Use a pipette to collect newly hatched larvae into a separate tank containing seawater. (Newly hatched larvae can remain in a Petri dish for 1–2 days maximum).

Optimal population density for rearing marine medaka from larvae to adult *		
1–2 days **	60 in a Petri dish or 2 L glass tank	
1–4 weeks **	60 in a 2 L glass tank	75% water change should be done at least once a week, with tank cleaning performed at every second water change.
1–3 months	60 in a 40 L glass tank filled with 30 L of seawater(L×W×H:50×24×28cm)	
>3 months (for outdoor stock keeping)	200 in a 250 L fiberglass tank	75% of the seawater changed every other day
>3 months (for experimental use)	60 in a 30 L glass tank	75% of the seawater changed every other day
Male:female ratio (stock)	1:1	
Male:female ratio (breeding)	1:1 to 1:2	Male competition may reduce fertilization rate.

* Overcrowding of larvae and juveniles increases mortality. If a dead fish is found, remove it immediately and change the water. If a mass mortality event occurs (>5 fish), immediately remove all the surviving fish into a clean tank. The tank in which the mass mortality occurred should be thoroughly cleaned with soap and bleach.

** Larval growth and development are highly dependent on feeding conditions, the surface area of the water in the rearing tank, and the number of larvae in a single tank.

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