City University of Hong Kong

Department of Biology and Chemistry

Centre for Marine Environmental Research and Innovative

Technology (MERIT),

STANDARD OPERATING PROCEDURE

Cultivation of medaka in Hypoxia Studies

-Effects of Hypoxia on Marine Medaka (*Oryzias melastigma*) Juvenile

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1. General Test Conditions

The general test conditions of this hypoxia study have been described in table 1 (Appendix 1)

2. Fish Transfer and Acclimations

2.1. Tank Preparations

Prepare 2 tanks for the new fish at least 3 days before transferring them. The physical conditions for the new fish are as follows:

UV filter: ON
Temperature: 28 ± 1°C
pH: 7.5~8.0
Salinity: 30‰
Dissolved oxygen: 6.0-6.6 mg/L
Light-dark cycle: 14h light: 10h dark

2.2. Fish Transfer and Acclimations

Bring a bucket, a scoop and a clean plastic bag to medak stock (animal house of City University of Hong Kong). Put the bag into the bucket, add some water from the tank where fish are kept using the scoop. Collect some fish from the tank and put into the bag with a small fish net (10 fish each time, total 200 fish). Bring the bucket with the scoop on it (to prevent light, which can decrease the stress on fish caused by the transfer). On arrival the hypoxia chamber at AOE, place the bag of fish into the prepared tanks and let the temperature equilibrate for 15-20 min. Then open the bags and release the fish. Shut off the light to decrease the stress on fish for approx. 5h, then acclimate the fish according the standard conditions (refer to 2.1) for 3 days.

3. Hypoxic and normoxic systems

Two continuous flow systems, one with hypoxic water and the other with normoxic water will be set up to provide constant, desirable levels of ambient dissolved oxygen for the experiments. In the hypoxic system, water in test tank (238L =132 cm length × 60cm width × 30 height) will be pumped through a stripping column with plastic balls, where an appropriate amount of nitrogen/air and water are mixed to achieve the desirable oxygen level, which is controlled and maintained by a Dissolved Oxygen Controller (within ± 0.2 mg O2 L-1) throughout the entire experimental period. The water surface will be covered with a transparent plastic sheet to reduce atmospheric

oxygen diffusion and to maintain a stable oxygen concentration. A normoxic control system will be also set up in a similar fashion. Levels of dissolved oxygen in the hypoxic/normoxic system will be monitored daily, using a calibrated YSI Model 580 dissolved oxygen meter, and the Dissolved Oxygen Controller will be concurrent finely calibrated according to the readings of dissolved oxygen meter. These two systems are set-upped in the Controlled Environmental Chamber (Temperature: 24±1°C, light-dark cycle: 14h light: 10h dark).

4. Water Parameters Monitoring

Dissolved oxygen (DO), temperature and salinity are three most important water parameters, affecting the overall growth and reproduction of medaka, thus will be detected daily. In addition, weekly analysis of pH, phosphate and ammonia will offer the proper information needed to help monitoring the water quality.

4.1. Dissolved Oxygen (DO) and Temperature

DO and temperature will be detected using dissolved oxygen meter (YSI Model 52).

1) **Calibration:** Place a prepared probe in the plastic calibration bottle with a moistened sponge. Switch to **CALIBRATE**. The display will read: Select percent calibration by pressing **CONFIRM**. SKIP sets the value to 100.0%; Press CONFIRM. The instrument will display the legend “Please wait” for a few seconds. Then it will display: Calibrate to 100 %.
2) **Salinity:** Salt reduces the ability of water to hold oxygen in solution. Enter the salinity of the sample you are measuring, and the meter will automatically compensate for the effect of salinity on dissolved oxygen. Turn the function switch to **SALINITY**, enter the salinity of the sample (30.0 ppt). Press **CONFIRM**. You are now ready to measure dissolved oxygen.
3) **Operation:** Set the selector switch to **O_2-TEMP,** place the prepared probe in the sample to be measured. ***Allow 3 to 5 minutes*** for temperature equilibration. Begin **stirring at least 30 seconds before taking readings**. Observe readings for stability and record measurements (both DO and temperature).
4) To make sure the DO distribution in test tanks are homogeneous, DO levels at 6 positions (Fig.1) will be detected each time (twice: morning and night).

5) Dissolved Oxygen Controller, controlling and maintaining DO levels in test will be concurrent finely calibrated according to the readings of dissolved oxygen meter.

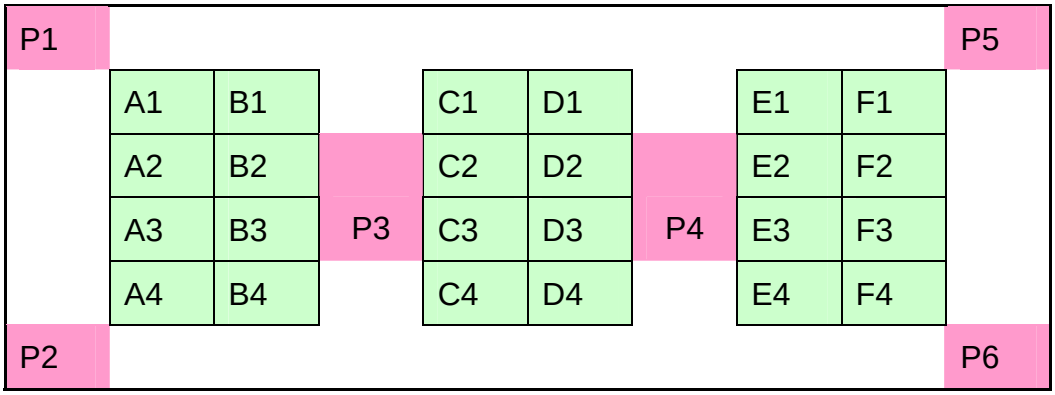

Fig. 1 Schematic digram of cages (green color) and DO detection position (red color) in test tanks.

4.2. Salinity

Salinity of seawater in test tanks will be measured by ATAGO 2412 S-10E Handheld

4.3. pH

pH will be monitored weekly using A-7812 Hagen Test Kit - pH High Range

4.4. Phosphate (PO4)

Phosphate (PO4) will be monitored weekly using A-7840 Hagen Test Kit – Phosphate.

4.5. Ammonia

Ammonia (NH3& NH4) will be monitored weekly using A-7855 Hagen Test Kit -Ammonia

5. Feeding & Brine Shrimp Hatching

5.1. Feeding

⇨ Fish are fed with 20-30 h hatched brine shrimp *Artemia cysts* (lucky Brand, Ocean Star International, Inc. Snowville UT USA) three times a day (every 3~4 hours: at 10:00-11:00 am, 2:00-3:00 pm and 7:00-8:00 pm). To avoid overload of the filter system, divide every meal into 3 turns with a dropper of brine shrimp (approx. 2ml) for each cage for each turn

5.2. Brine Shrimp Hatching:

1) Prepare approx. ***1.1 liters*** of hatching water (3/4 fresh water+ 1/4 30 ppt seawater) in the plastic hatching bottle (see attached photo).
2) Put ***1 grams*** of brine shrimp eggs
3) Bubble continuously and illuminate with a fluorescent tube and a table lamp for 20-30h.
4) Stop bubbling and stand for ***5-10min*** to separate hatched larvae (lower) from unhatched eggs and eggshells (upper). Shade the upper part of the bottle with aluminium foil will help more larvae swim to the lower part of the bottle.
5) Collect only the hatched larvae from the bottom of the hatching bottle using a 250ml conical flask.
6) Rinse hatched larvae with a mesh using filtered UV-treated fresh water several times. Diluted the "dried larvae" to ***300 ml*** (**2ml/dropper × 24 cages × 2 tanks × 3 times = 288 ml**). The brine shrimp eggs are now ready to be used.

6. Tank Cleaning and Water Changing

1) Switch off the thermostat, UV and filter.
2) Collect dirt on the tank floor with a fish net
3) Wipe the walls and floor of the tank with a black nylon spone (Only at week 3, week6 and week9).
4) Turn the valve at the bottom of tank to release water (1/10 for week 1, 2, 3 and 1/3 for week 4-12)
5) Add new seawater to the test tank (from seawater reservoir). Change water of the normoxia tank first, and then decrease the DO level in the seawater reservoir to 1.8 mg/L, and then add hypoxia water to hypoxia test tank.
6) Refill the filter with water using a water pump, switch on the UV filter, thermostat and filter to resume the circulation.

7. Filtering System and UV Sterilization system

1) **Filter:** The external filter comprises of a physical nylon barrier, activated charcoal filter, bio-rings. Wash physical nylon barrier and activated charcoal filter every two weeks (week 2, 4, 6,8, 10,12) and replace a new activated charcoal bag every month (week5, 9).

2) **UV lamps:**

UV lamp is of vial importance in sterizing the water. Switch off the white fluorescent tube and observe if the UV lamps glows blue in the dark, Check every week and replace a new on immediately once it is used.

8. Water Reservoirs & Embryo Rearing Tank

8.1. Water Reservoirs

Tap water can not be used immediately due to the fact that it usually contains chlorine and volatile chemicals. It is necessary to circulate the tap water overnight to remove all these hazards before use. One freshwater reservoir and one seawater reservoir are therefore prepared close to test tanks. The former are used for replenishing vapoured water while the later are used for changing water.

8.2. Embryo Raring Tank

An embryo rearing tank is also prepared for rearing embryos collected from test tanks when the fish begin spawning.

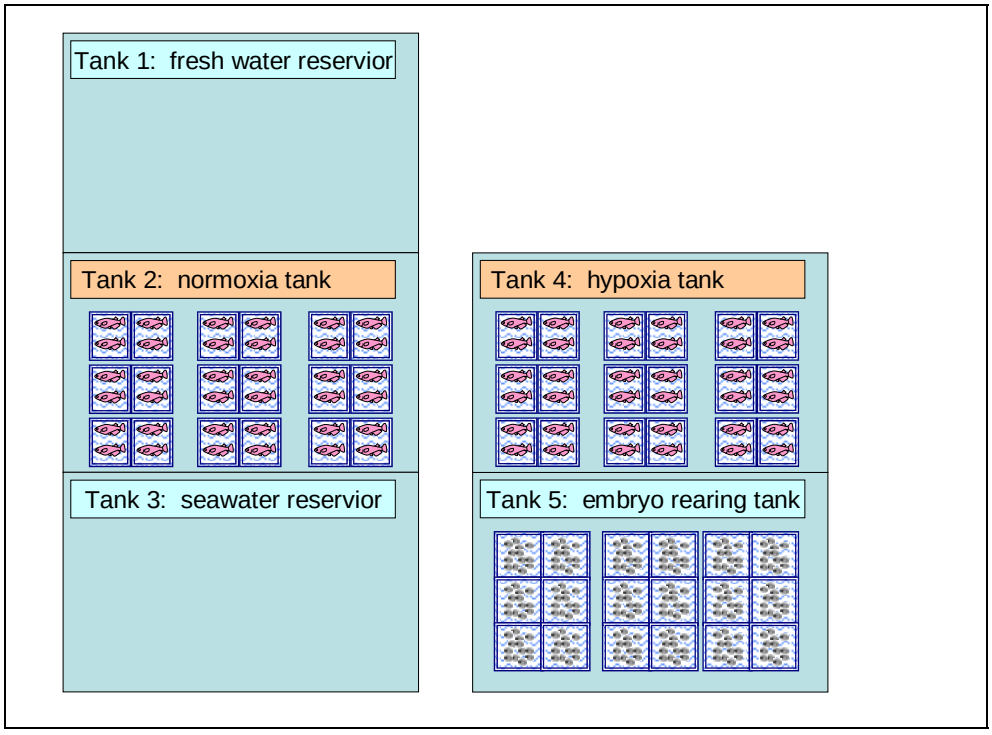

Fig.2 Schematic diagram of aquaria in AOE hypoxia chamber

9. Records & Documentation

Data of water parameter monitoring will be recorded on the appropriate data sheets (Appendix 2) and kept at AOE. A soft copy will also be concurrently kept.

Appendix 1

Table 1. Test Conditions		
1	Test species	Medaka (*Oryzias melastigma*)
2	Age of test organisms at initiation	3-4 weeks post hatching
3	Test type	Flow-through
4	Test tank size (hypoxia and control tanks)	238 L (132cm length × 60cm width × 30 height)
5	Test net cage size	2.68L (16.5cm length ×12.5cm width ×13cm height)
6	No. of net cages per test tank (hypoxia and control tanks)	24
7	No. of fish per test net cage	Approx. 20~30
9	No. of fish per test tank (hypoxia and control tanks)	~700
8	No. of treatments (test tanks)	2 (1 normoxic group, 1 hypoxic group)
8	DO of treatments	Normoxic group: 6.4 ±0.2 mg/L; Hypoxic group: 1.8 ±0.2 mg/L
10	Water temperature	28 ± 1°C
11	Salinity	30‰
12	pH	7.6~8.0
14	Photoperiod	14 h light,10 h dark
13	Feeding regime	3 times daily with ≤24 h hatched brine shrimp *Artemia salina*
17	Test acceptability	≥90% survival of fish in normoxic group; ≥ 70%~80% survival of fish in hypoxic group

Appendix 2 Data sheets for water parameter monitoring

Data sheet: DO, Temp (twice per day) & Salinity (once per day)
Date: ______________________________
Exp: ______________________________
Group: ______________________________

Week:		**DO (mg/L)**						**Temp (°C)**						**Salinity (‰)**
Date	**Time**	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	
	10-11am													
	6-7pm													
	10-11am													
	6-7pm													
	10-11am													
	6-7pm													
	10-11am													
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	6-7pm													
	10-11am													
	6-7pm													

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Data sheet: **pH, phosphage & ammonia (once per week)**

Date:

Exp:

		Normoxia			Hypoxia		
Week:	**Date**	**pH**	**PO4 (mg/L)**	**NH3/NH4 (mg/L)**	**pH**	**PO4 (mg/L)**	**NH3/NH4 (mg/L)**
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							