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Studies on hatching and early larval survival on marble goby *Oxyeleotris marmoratus* for improvement of production techniques

マーブルゴビー*Oxyeleotris marmoratus*種苗生産における初期生残率向上に関する研究

Nguang Siew Ing

Contents

CHAPTER 1

General Introduction

Marble goby *Oxyeleotris marmoratus* (Bleeker 1852) (Fig. 1.1), is the largest goby fish and a freshwater Eleotridae species that has a wide distribution in Southeast Asia (Mohsin and Ambak 1983; Robert 1989; Cheah et al. 1994; Rainboth 1996; Inger and Chin 2002; Amornsakun et al. 2002; Luong et al. 2005). This fish is also known as "marble sleeper" in English, "Soon Hock" in Chinese, "Pla bu" in Thai, "ikan ketutu" and "ikan malas" in Malay (Ukkatawewat 1984; Robert 1993; Sadovy and Cornish 2000; Senoo et al. 2001a). It grows to 50 cm in total length (TL) and 2 kg in body weight (BW) (Mohsin and Ambak 1983; Roberts 1989; Inger and Chin 2002). There is different population of marble goby due to geographical distribution, genetic population and environment history (Ha et al. 2011).

Marble goby is a freshwater fish with commercial importance in Southeast Asia owning to its taste, firm and white flesh and high protein value, it being considered a first grade fish (Cheah et al. 1994; Inger and Chin 2002; Amornsakun et al. 2002; Luong et al. 2005). Marble goby is typically retailing at US$50-60/kg and it is the highest priced of any edible freshwater fish in Southeast Asian countries (Senoo 2003a; Lam et al. 2008). It is considered to be one of the most promising finfish for aquaculture, especially in Malaysia and Thailand (Amornsakun et al. 2002; Luong et al. 2005; Suwanjarat et al. 2005), as great international export to countries of Asian origin are made, especially ethnic Chinese, for instance China, Hong Kong, Taiwan, Singapore and Malaysia (Suraniranat 1998; Rakbankerd 2005; Phoomthai 2007).

Marble goby is also a good candidate species for research as they have several biological advantages for culture (Leatherland et al. 1990; Jow et al. 1999; Sayer 2005; Masaya et al. 2006). For instance, they remain motionless and require only to be kept a little moist during live transportation, thus only minimal use of water and oxygen is required (Rakbankerd 2005). Besides, marble goby also appear to be able to detoxify endogenous ammonia to glutamine in the muscle that reducing endogenous ammonia production and excretion (Jow et al. 1999). Other studies on marble goby were about fish culture (Tan and Lam 1973; Cheah et al. 1994), rearing conditions (Abol et al. 2005), growth and feeding performance cultured in re-circulating aquaculture systems and also effects of different diets on growth and survival rate of larval stages (Liem et al. 2001).

Fig.1.1: Marble goby *Oxyeleotris marmoratus*, a mature female (453 g in body weight, 30 cm in total length).

Since 1970s, governments of Malaysia, Thailand, Vietnam, and Indonesia have encouraged marble goby culture (Senoo et al. 1994a; 2008). Marble goby culture generally relies heavily on seed collection from the wild (Brohmanonda and Thanakumcheep 1983). Over the last 30 years, wild resource of marble goby has decreased drastically due to overfishing (Ikenoue 1991; Senoo et al. 1992; Senoo 2006) and this has also affected fish farmers who require a steady fish seed supply. Therefore, the insufficient and unreliable supplies of marble goby seeds became the main constraint to the marble goby culture, so culture as a limited scale (Tan and Lam 1973; Ang 1990; Senoo et al. 1993a, 1993b; Senoo et al. 1994a, 1994b; Senoo et al. 1997).

To meet this demand and to protect wild resources, artificial techniques for seed production have been developed. However, there is limited information available, especially for early life stage of this species. In 1990's, mass seed production techniques had yet to be established due to high mortality during early larval stage (Tavarutmaneegul and Lin 1988; Ang 1990; Senoo et al. 1994a, 1994b). In West Malaysia (Peninsular Malaysia), Senoo et al. (1994a, 1994b) described egg development, hatching, and larval development of marble goby in freshwater (FW), and have reported behavioural changes of the larvae. However, in East Malaysia (the State of Sabah), high larval mortality presents a significant obstacle for the successful larval rearing of this species. In 2008, marble goby seeds were reported to be successfully produced by increasing the salinity from FW to 10 psu diluted seawater (SW) after the larvae hatched (Senoo et al. 2008). They concluded that 10 psu SW was necessary for effective larval rearing during the first 10 days. In their experiment, eggs were incubated in FW and hatched larvae were transferred and reared in 10 psu.

However, whether incubation of the eggs in elevated salinities would lead to higher hatching success remains unknown for marble goby. Generally, development and growth of

fish take place following characteristic steps for each species and more or less directly under control of environmental factors. Fish are dependent on internal and external (ecological) factors, which control and synchronize many activities and functions, including development and growth capacity (Boeuf and Payan 2001). The ecological factors classify into limiting factors and determining factors, which this study highlighted for marble goby seed production. The determining factors include temperature and salinity, which act directly to growth and survival (Boeuf and Payan 2001).

Among the ecological factors, salinity is specific to the aquatic environment. Several studies have demonstrated the influence of external salinity on egg development, larval growth and survival in teleost fish (Blaxter 1969; Suresh and Lin 1992; Likongwe et al. 1996). Unfavorably high salinity increased embryo mortality and decreased body length at hatching in other freshwater fish such as tilapias and catfishes (Vetemaa and Saat 1996). Interestingly, previous studies (Senoo et al. 2008) showed that marble goby larvae could survive in saline water even though it is a freshwater species. Many juveniles stay intermediary salinities, which affected their survival and growth including marble goby (Blaxter 1969; Darwis et al. 2008).

Beside salinity, temperature is one of the most decisive factor of all the environmental conditions affecting fish eggs and larvae (Kamler 2002). Eggs and larvae are the most vulnerable stages in the development, especially susceptible to temperature changes (Brett 1970; Luczynski and Kolman 1987). Previous studies showed that lethal temperature to the eggs, larvae and adult of freshwater fishes in tropical region can be found even in only 2°C range (Allanson and Noble 1964; Subasinghe and Sommerville 1992).

The effects of salinity and temperature must be considered when developing fish culture (Boeuf 2001), which essentially aims to produce fish larvae of the best quality at the most economical cost. This requires judicious and careful use of ecological factors to provide products of consistent quality. In conclusion, for successful fish eggs incubation and larval rearing, proper management of water quality parameters is required, as they are crucial for egg and larval survival.

Yet, there is limited information concerning the effect of salinity and temperature on egg development, hatching and larval survival in marble goby. Thus, in an attempt to improve seed production techniques, a series of experiments were carried out to determine the optimum salinity and temperature for egg and larval survival of marble goby.

There were three main experiments in the study. First parameter study, the salinity effects on marble goby eggs were determined, comparing among FW, 5, 10, 15, 20 and 30

psu SW. Salinity effects were performed to quantify total hatching and larval deformation (chapter 2, section 1). Then, the hatching period and larval survival of marble goby eggs in FW and 10 psu SW were compared to determine the optimal salinity (chapter 2, section 2) and egg development in FW and 10 psu SW were observed (chapter 2, section 3). In the chapter 3, differences of larval survival in FW and 10 psu were determined. Second parameter study, temperature effects on egg development, hatching and larval survival were compared at 24, 26, 28, 30 and 32°C (chapter 4).

CHAPTER 2

Egg development, hatching and larval deformation in different salinities of marble goby *Oxyeleotris marmoratus*

2.1 Introduction

In West Malaysia (Peninsular Malaysia), Senoo et al. (1994a, 1994b) have described egg development, hatching and larval development of marble goby in FW and have reported behavioural changes of the larvae. However, in East Malaysia (the State of Sabah), high larval mortality presents a significant obstacle for the successful larval rearing of this species. Senoo et al. (2008) successfully produced marble goby seeds by increasing the salinity from FW to 10 psu SW after the larvae were hatched. They concluded that 10 psu SW was necessary for effective larval rearing during the first 10 days. However, it remained unknown whether incubation of the eggs in elevated salinities would leads to higher hatching success.

Developed embryos and newly hatched larvae are the most fragile and delicate of the stages in the life history of a fish. Therefore, great care must be taken to provide them with the proper incubating and hatching environment. There is limited information concerning the effect of salinity on egg development and hatching in this species. Thus, in an attempt to improve seed production techniques, a series of experiments was carried out to determine the optimum salinity for incubation of marble goby eggs.

2.2 Materials and methods

2.2.1 Brood fish management

The experiments were conducted at the Centre of Collaborative Research in Aquaculture (Universiti Malaysia Sabah-Kinki University), Sabah, Malaysia during September to October, 2010. Twenty female and 30 male of brood fish with body weight (BW) 259.9 ± 52.7 g and 307.5 ± 77.9 g (mean ± SD) were collected from a river in the Penampang area (Fig. 2.1). Female and male fish were separately reared for five months in FW in 1,000 L of high density polyethylene tanks (1.5 m diameter x 0.6 m deep). They were fed with fresh fish (*Sardinella* sp.) until satiation at two days intervals. Water temperature, dissolved oxygen (DO) and pH ranges of the brood fish

tanks were 26.2-30.8°C (28.4 ± 2.5)°C, 6.8-8.2 (7.7 ± 0.5) mg/L and 7.3-8.5 (8.0 ± 0.5), respectively (mean ± SD).

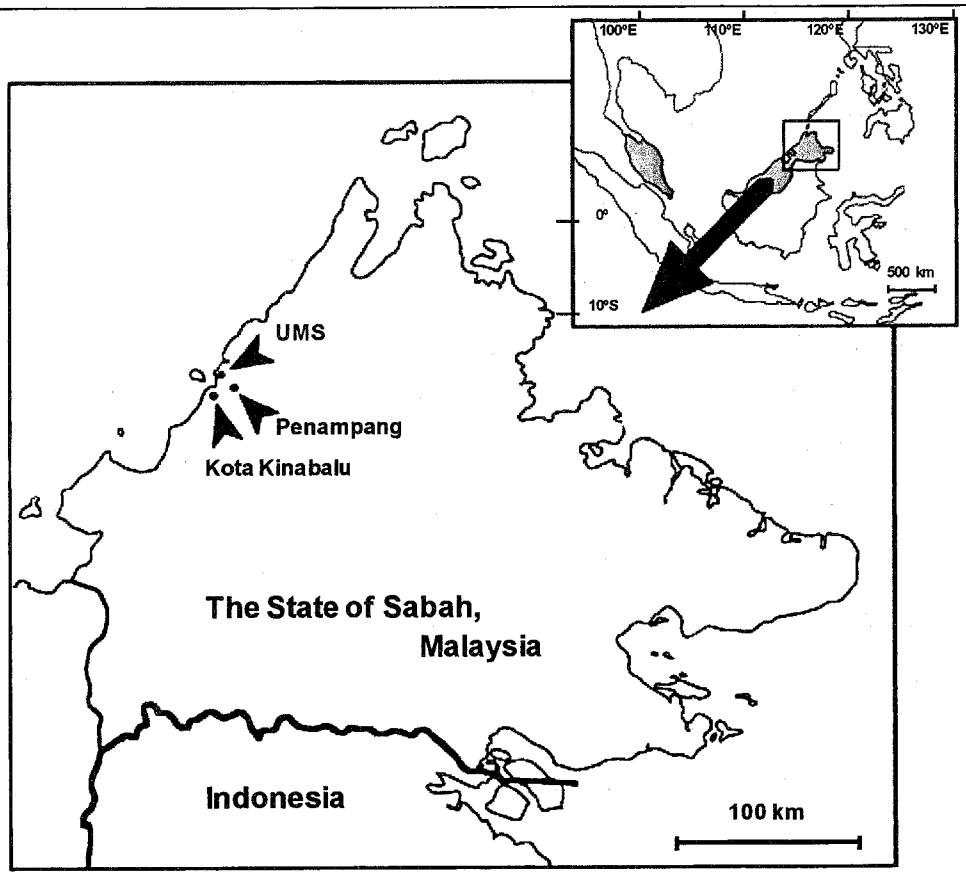

Fig. 2.1: Map showing the location of Penampang in the State of Sabah, Malaysia, where the brood fish of marble goby were caught. The experiment was conducted at the Fish Hatchery, Universiti Malaysia Sabah (UMS). Kota Kinabalu is the capital city in the State of Sabah.

2.2.2 Experiment 1.1 Determination of the optimal salinity for egg incubation of marble goby in FW, 5, 10, 15, 20 and 30 psu SW

2.2.2.1 Egg collection

The aim of this experiment was to determine the optimum salinity for hatching of marble goby eggs. Fertilized eggs were obtained from a pair of sexually mature brood fish (female, 265 g; male, 280 g) using the method described by Senoo et al. (1993b). The female was injected intramuscularly with human chorionic gonadotropin (Profasi, Laboratories Serono, Switzerland) with the dosage of 1,000 IU/kg BW (Senoo et al. 1993b; Senoo 2001a, 2003a, 2006). At three days post-injection, approximately 20,000

eggs were collected from a natural spawning with a fertilization rate of 95%. The fertilized eggs measured 1.84 ± 0.03 and 0.64 ± 0.02 mm (mean ± SD, n = 20) in the long and short axes, respectively, and weighed 1,668 eggs/g (n=3).

2.2.2.2 Incubation and observation

Eighteen 7 L transparent tanks (length, width, height; 18 x 26 x 17 cm) were prepared for incubation. They were divided into three groups of six tanks filled with either FW or SW diluted to a range of salinity 5, 10, 15, 20 or 30 psu. Salinity was adjusted by mixing filtered seawater with aged dechlorinated tap water and measured using a hand refractometer (H-50, ATAGO, Japan), precalibrated with distilled water. The prepared water was filtered through a 40-μm mesh plankton net.

One hundred eggs contained in a 9-cm diameter Petri dish were placed into each of the incubation tanks. Hatched larvae were removed and counted each day for six days. The removed larvae were observed with an optical microscope and the number of deformed individuals was recorded. Total hatching rate was defined as the percentage of stocked embryos that hatched. The deformation rate was the percentage of deformed larvae with a bent notochord among the hatched larvae.

2.2.3 Experiment 1.2 Determination of the optimal salinity for egg incubation of marble goby between FW and 10 psu SW

2.2.3.1 Egg collection

Based on the results of experiment 1.1, hatching time, hatching rate and larval deformation rate of marble goby were compared between FW and 10 psu SW. To obtain eggs fertilized at a known time, eggs were collected by stripping and were fertilized artificially. Fertilized eggs were obtained from six pairs of brood fish (females, 287.5 ± 7.6 g BW, 24.2 ± 2.9 cm TL; males, 307.5 ± 14.1 g BW, 28.8 ± 1.0 cm TL, mean ± SD) following hormone injection, as described above. At three days post-injection, ovulated eggs (approximately 15 g) were stripped from the female. Stripped eggs measured 0.89 ± 0.04 mm and 0.64 ± 0.03 mm (mean ± SD, n=6) in the long and short axes, respectively, and weighed 1,675 eggs/g (n=6). Milt was then stripped from the male and mixed with the eggs in FW in a 250 mL bottle previously coated with Vaseline™ to prevent the eggs from clumping (Senoo 2001a, 2003a, 2006).

2.2.3.2 Incubation and observation

In each trial, approximately 25,000 fertilized eggs were randomly deposited on two rectangular wooden-framed nets (30 x 38 cm) fitted with plankton gauze (250-μm mesh) and were incubated in FW or 10 psu SW. For the incubation, two aquarium tanks (60 x 39 x 36 cm, 70 L; Fig. 2.2) were prepared with a filtering system. One of the wooden-framed nets was floated on the water surface of each tank and aerated at a rate of 500 *ml*/min. An electric heater was installed in each tank to maintain the temperature at 28-29 (28.5 ± 0.05)° C (mean ± SD). Eggs were incubated under natural light conditions.

For determination of the hatching time and hatching rate, all hatched larvae were removed and counted every 12 hours from 24 to 144 hours after fertilization (hAF). Hatching rates and deformation rates at each observation time were determined as previously described. To compare larval survival in the two salinities, all larvae were transferred to 7 L transparent tanks (18 x 26 x 17 cm) once they were hatched (24 to 144 hAF) and reared into FW and 10 psu SW until 10 days after fertilization (dAF) (Senoo et al. 2008), recording mortality daily.

The experiments were repeated six times under the same experimental conditions using different egg batches. During the observation period, water temperature, DO and pH were recorded at 06:00 and 18:00 h. In FW the ranges were 28.2-29.0 (28.3 ± 0.3)°C, 7.2-7.8 (7.5 ± 0.2) mg/L and 7.4-8.2 (7.8 ± 0.3); and in 10 psu SW they were 28.1-29.0 (28.3 ± 0.2)°C, 7.0-7.6 (7.2 ± 0.2) mg/L and 7.2-8.3 (7.8 ± 0.3), respectively (mean ± SD). For 10 days larval rearing, water quality recorded; 27.8-29.0°C (28.2 ± 0.5)°C, 6.6-7.8 (7.2 ± 0.4) mg/L and 6.6-8.0 (7.6 ± 0.4) of pH in FW; 28.0-29.0°C (28.2 ± 0.2)°C, 6.8-7.9 (7.4 ± 0.5) mg/L and 6.6-8.2 (7.8 ± 0.3) in 10 psu SW, respectively (mean ± SD).The egg development and hatching were observed repeatedly; however, the results were similar to the observation made during incubation so this study mainly shows the incubation results.

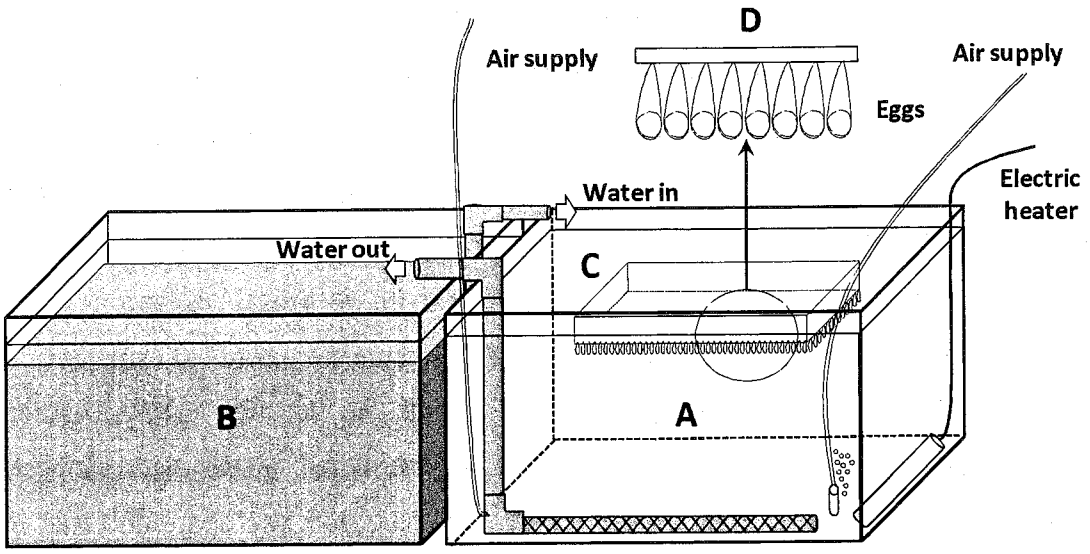

Fig. 2.2: Illustration showing the set of incubation tanks and the filtering system. Two sets of incubation tanks were prepared for egg incubation in freshwater (FW) and 10 psu diluted seawater (SW). A, incubation tank; B, filter tank with stone filter; C, wooden-frame net, D is magnification of C; D, magnification of C. Each tank was filled with 70 L water, aerated at a rate of 500 mL/min and maintained the temperature at 28.5 ± 0.05°C (mean ± SD) with electric heater (100 W). Eggs were dangling from the net (D).

2.2.4 Experiment 1.3 Observation of egg development of marble goby in FW and 10 psu SW

2.2.4.1 Morphological and sensory organ observation on egg development

Eggs from experiment 1.2 were used for observations on development. Approximately 20 eggs each from the FW and the 10 psu SW tanks, were transferred to a Petri dish with some of their water and observed under an optical microscope (Eclipse E600, Nikon, Japan) at every minute to observe the division of cells up to morula stage, at 15 minutes intervals for 24 hours (morula stage to embryo ready to hatch) and then every 12 hours until 120 hAF. Each developmental stage was timed and photographed with a digital camera (Digital 600, Olympus). Development of the embryonic sensory organs was assessed using a scanning electron microscope (SEM) (JSM-5610, JEOL, Japan). For the SEM observations, 20 eggs each from both waters were sampled at 12 hours intervals from 24 to 144 hAF and preserved in 10% buffered formalin for one month. The egg membranes were peeled off using micro-tweezers and the embryonic parts

were processed by dehydration and gold sputtering for SEM observation (Senoo et al. 1994a; Kawamura et al. 2003).

2.2.4.2 Counting of hatching glands cells (HGC) numbers

For observation of HGC, ten eggs from both waters were sampled randomly and deposited on a Petri dish with some water. A profile projector (Mitutoyo, PJ-3000) and an optical microscope (Nikon, Eclipse E600) with a digital camera (Olympus, Digital600) were used for the observation. HGC numbers were counted using an analog hand tally counter (model 7655, Canada) at 24, 48, 72, 96, and 120 hAF under an optical microscope. The hatching gland cells are distributed on the outer surface and visualized as dense giant cells on the embryonic head, yolk sac and embryonic body under an optical microscope. HGC also identified before hatching by means of a light-microscopic immunocytochemical method (Hiroi et al 1999).

2.2.5 Statistical analysis

Total hatching rates and deformation rates were compared between salinities by one-way analysis of variance (ANOVA) followed by a Tukey's honest significance different (HSD) test (experiment 1.1) or *t* test (experiment 1.2). To examine differences in the hatching period between two groups, repeated-measures ANOVA was used. Kaplan-Meier survival probabilities were computed for experiment 1.2 and the differences in larval survival between FW and 10 psu SW were tested using the log-rank test. All statistical analyses were carried out using JMP version 8 (SAS Institute, USA) and SPSS Statistics 17.0 software (IBM Corp., New York, USA).

2.3 Result

2.3.1 Experiment 1.1 Determination of the optimal salinity for egg incubation of marble goby in FW, 5, 10, 15, 20 and 30 psu SW

2.3.1.1 Rates of hatching and deformation

Salinity significantly affected the hatching rate of marble goby (ANOVA, $F_{4, 10} = 66.2$, Tukey's HSD test, $P<0.05$). The highest hatching rate was observed in 10 psu SW (60.0 ± 2.0%), followed by FW (44.7 ± 5.0%), 5 psu SW (44.3 ± 5.5%) and 15 psu SW (37.0 ± 6.2%) with the lowest rate at 20 psu SW (3.0 ± 1.7%, mean ± SD). No hatching

occurred in 30 psu SW (Fig. 2.3). Salinity also affected the occurrence of larval deformation (ANOVA, $F_{4,10} = 27.4$, Tukey's HSD test, $P<0.05$). All larvae that hatched in 20 psu SW were deformed and died within three days. The deformation rate was lower in 15 psu SW (49.6 ± 1.9%), followed by FW (33.1 ± 3.9%) and 5 psu SW (29.1 ± 2.8%, mean ± SD). The deformation rate in 10 psu SW (11.1 ± 2.4%) was significantly lower than at all other salinities (Fig. 2.4).

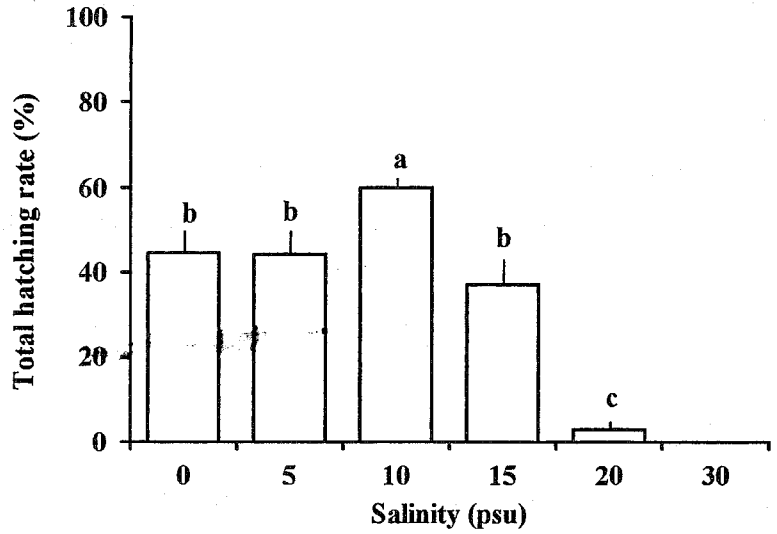

Fig. 2.3: Total hatching rates of marble goby eggs incubated in FW and in 5, 10, 15, 20 and 30 psu SW (means ± SD, n=3). Different letters above each bar indicate significant differences between treatments (ANOVA, $F_{4,10} = 66.2$, Tukey's HSD test, $P<0.05$).

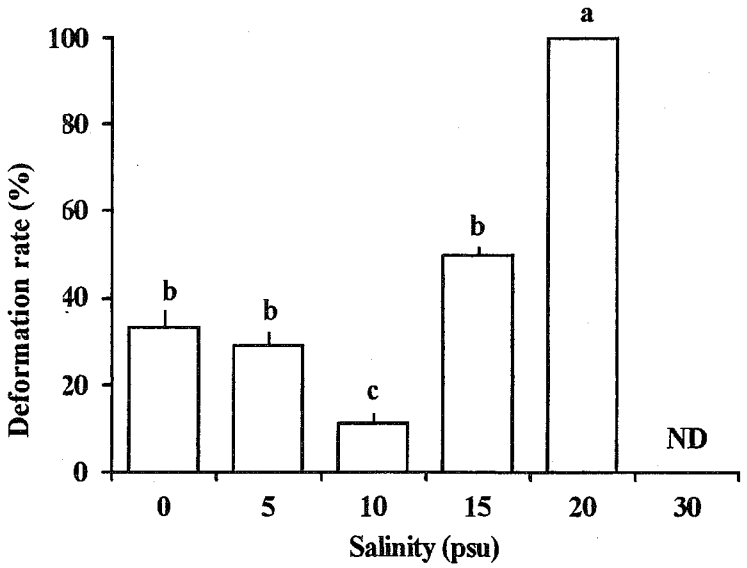

Fig. 2.4: Deformation rates of marble goby larvae hatched in FW and in 5, 10, 15, 20 and 30 psu SW (means ± SD, n=3). Different letters above each bar indicate significant difference between treatments (ANOVA, $F_{4,10} = 27.4$, Tukey's HSD test, $P<0.05$) and ND shows no data.

2.3.2 Experiment 1.2 Determination of the optimal salinity for egg incubation of marble goby between FW and 10 psu SW

2.3.2.1 Rates of hatching, deformation and larval survival

As shown in experiment 1, egg hatching and larval deformation rates were improved in 10 psu SW relative to FW. The mean hatching rate of eggs incubated in 10 psu SW (70.1 ± 13.2%) was significantly higher than in FW (52.3 ± 12.5%; t = - 2.40, P = 0.0376) and the overall larval deformation rate was significantly lower in 10 psu SW (4.8 ± 4.4%) than in FW (26.8 ± 11.6%, mean ± SD; t = 4.37, P = 0.0014) (Fig. 2.5).

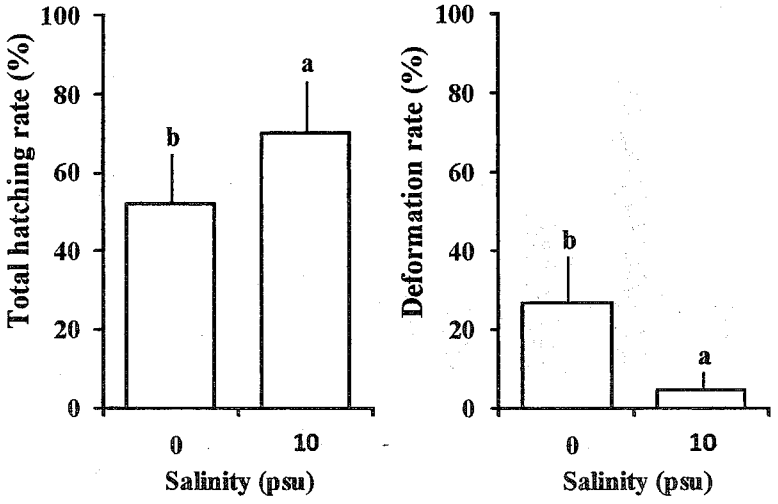

Fig. 2.5: Total hatching rates (independent-samples t-test, t = - 2.40, P = 0.0376) and deformation rates (t = 4.37, P = 0.0014) of marble goby eggs incubated in FW and in 10 psu SW (means ± SD, n=6). Different letters above each bar indicate a significant difference between treatments.

The repeated-measures ANOVA showed a significant effect of the interaction between time and salinity on hatching time ($F_{1,118}$ = 0.568, P = 0.037), indicating a difference in the hatching period between the groups (Fig. 2.6). Hatching was commenced between 24-36 hAF in both groups and the eggs hatched in 10 psu SW were more rapidly than those in FW. In FW, hatching was occurred from 24 to 132 hAF (ANOVA, $F_{9,50}$ = 19.5, Tukey's HSD test, P<0.05). The total hatching rates were significant higher during 60-72 and 72-84 hAF (9.7 ± 3.8% and 10.6 ± 3.4%), followed by a relatively constant rate of hatching during 36-48, 48-60, 84-96 and 96-108 hAF (4.8 ± 2.4%, 8.1 ± 2.6%, 8.8 ± 2.0% and 7.0 ± 2.6%, respectively), and then significantly lower during 24-36, 108-120, 120-132 hAF (0.7 ± 0.4%, 2.0 ± 0.9% and

0.7 ± 0.8%, mean ± SD, respectively). Of the eggs that hatched in 10 psu SW, significantly higher hatched in the period during 48-60 and 60-72 hAF (ANOVA, $F_{9,50}$ = 98.5, Tukey's HSD test, P<0.05, 33.1 ± 5.6% and 18.2 ± 4.5%), followed by 36-48, 72-84 and 84-96 hAF (7.6 ± 2.4%, 7.3 ± 3.3% and 3.0 ± 1.8%, mean ± SD, respectively). The hatching rate gradually declined, significantly lower during 24-36 hAF (0.8 ± 0.3%) and no further hatching was observed beyond 96 hAF in 10 psu SW.

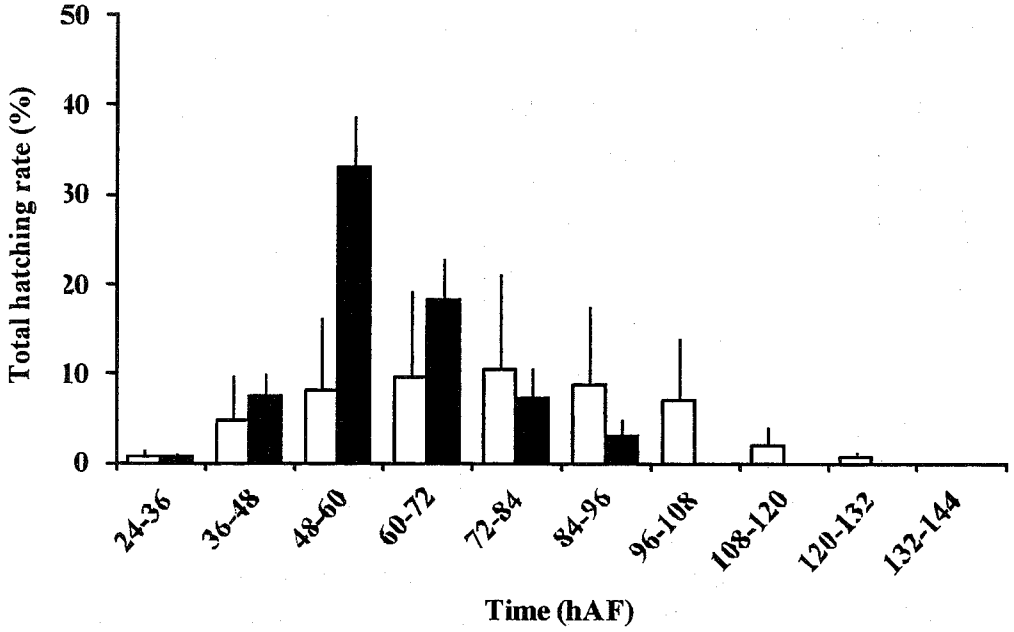

Fig. 2.6: Total hatching rates of marble goby eggs incubated in FW (white bar; ANOVA, $F_{9,50}$ = 19.5, Tukey's HSD test, P<0.05) and in 10 psu SW (black bar; ANOVA, $F_{9,50}$ = 98.5, Tukey's HSD test, P<0.05,) in different time intervals in hours after fertilization (hAF). Results are expressed as the means ± SD (n=6). The repeated-measures ANOVA also showed a significant effect of the interaction between time and salinity on hatching time ($F_{1,118}$ = 0.568, P = 0.037).

Deformed larvae (Fig. 2.7) were clearly observed after 72 hAF in both waters. In FW, all of the larvae that hatched during 108-132 hAF were deformed and eventually died on the tank bottom (ANOVA, $F_{8,45}$ = 46.5, Tukey's HSD test, P<0.05), followed by 84-96 and 96-108 hAF (44.7 ± 33.2% and 72.8 ± 20.7% of total hatching rates at each period; Fig. 2.8), then 72-84 hAF (21.8 ± 6.9% of total hatching rates), significantly lower at 60-72 hAF (2.2 ± 5.4% of total hatching rates, mean ± SD) and no deformation were observed from 24 to 60 hAF.

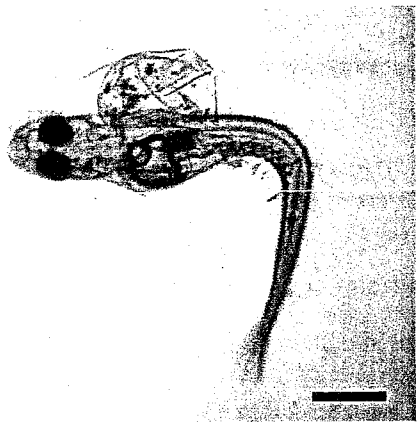

Fig. 2.7: Deformed larva of marble goby immediately after hatching at 96 hAF in FW. Scale bar, 0.5 mm.

The highest deformation rate for the larvae in 10 psu SW was 48.4 ± 32.2% of total hatching rates, which was observed in the interval 84-96 hAF (ANOVA, $F_{5,30}$= 4.0, Tukey's HSD test, P<0.05), followed by 72-84 hAF (23.8 ± 38.2% of total hatching rates), significantly lower during 60-72 hAF (1.8 ± 4.5% of total hatching rates, mean ± SD), and no deformation were observed from 24 to 60 hAF (Fig. 2.8).

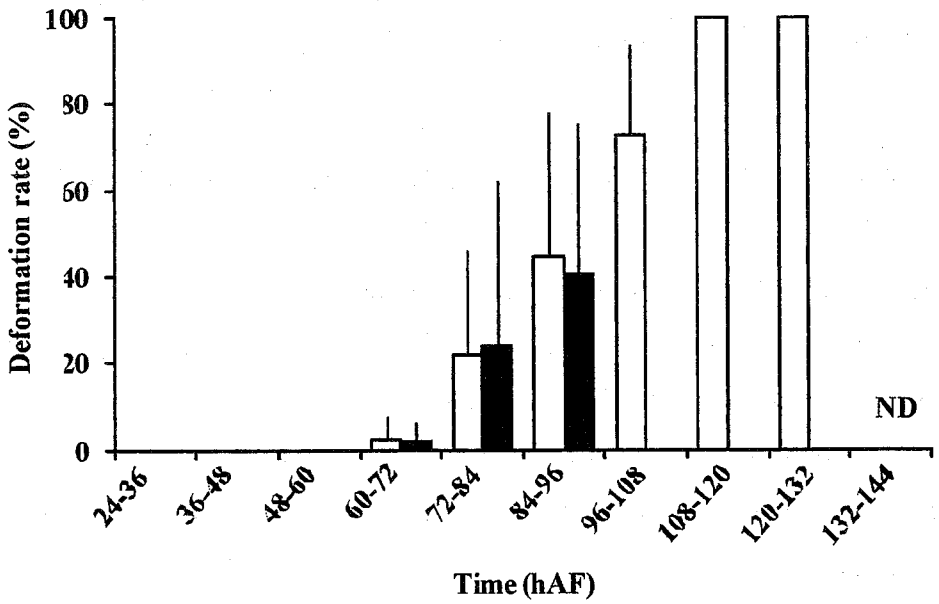

Fig. 2.8: Deformation rates of marble goby larvae hatched in FW (white bar; ANOVA, $F_{8,45}$= 46.5, Tukey's HSD test, P < 0.05) and in 10 psu SW (black bar; ANOVA, $F_{5,30}$= 4.0, Tukey's HSD test, P < 0.05) in different time intervals in hAF. Results are expressed as the means ± SD (n=6). ND shows no data.

In both groups, the larvae that hatched in later periods tended to have higher deformation rates. At 10 dAF, the larval survival rate in 10 psu SW was 52.7 ± 20.1%

(mean ± SD), while almost all larvae were dead by 9 dAF in FW (Fig. 2.9). Kaplan-Meier survival analysis indicated that there was a significant difference in survival between the two groups (P<0.0001).

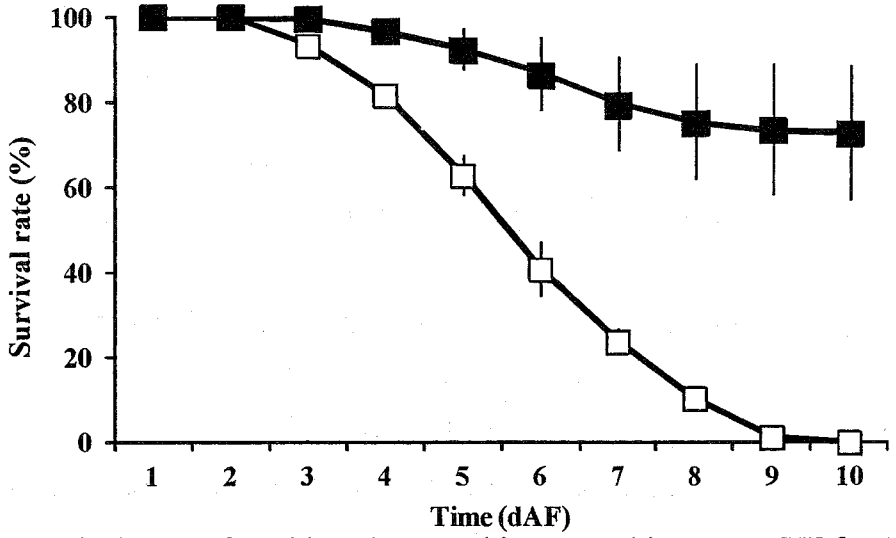

Fig. 2.9: Survival rates of marble goby reared in FW and in 10 psu SW for 10 dAF. Results are expressed as the means ± SD (n=6). Kaplan-Meier survival analysis showed significant different of survival rates in 10 dAF (P<0.0001).

2.3.3 Experiment 1.3 Observation of egg development of marble goby in FW and 10 psu SW

2.3.3.1 Morphological observation on egg development

Egg morphology was developed similarly in FW and 10 psu SW, as shown in Table 2.1. This result showed there is no salinity effects towards the egg development observed under the condition of the present study in both waters. Soon after fertilization, the eggs elongated along their vertical axis and were suspended below the plankton nets. At 1 hAF, the mean dimensions of the eggs in FW were 1.85 ± 0.04 and 0.65 ± 0.03 mm in long and short axes, respectively and, in 10 psu SW the dimensions were 1.87 ± 0.06 by 0.66 ± 0.03 mm (mean ± SD). Fertilization rates at the 2- to 4-cell stages were 99.0% in both FW and 10 psu SW.

Two to 16-celled stages were observed in 1 hAF, followed by the morula, blastula, and gastrula stages. At approximately 9 hAF, embryos had formed in eggs in FW and 10 psu SW. In both waters, embryos began to form at around 9 hAF with the agrippa condition (Fig. 2.10). Hatching was commenced at 24 hAF. Hatched larvae

were developed coincide as the embryo developed in both waters. At 36 hAF, hatching was occurred similarly, as shown in Fig. 2.11; however, afterward, eggs in 10 psu SW hatched more rapidly than those in FW. Over the whole embryonic stage, embryos in 10 psu SW were observed to move more actively, to vibrate and twist within the egg, compared to those in FW.

Table 2.1: The time course of egg development of marble goby incubated in freshwater (FW) and in seawater diluted to 10 psu diluted seawater (SW).

Egg Developmental Stages	FW	10 psu SW
Two-celled stage	00:24	00:22
Four-celled stage	00:42	00:40
Sixteen-celled stage	00:51	00:49
Morula stage	02:30	02:30
Blastula stage	04:15	04:15
Gastrula stage	05:00	05:00
Blastopore nearly closed	08:00	08:00
Embryo formed	09:00	09:00
Five-myomeres formed	10:15	10:15
Kupffer's vesicle appeared	11:30	11:30
Optic vesicle appeared	12:30	12:30
Tail separated from the yolk sac	13:00	13:00
Otocyst vesicle appeared	14:30	14:30
Head formed	15:00	15:00
Lens and heart formed	17:15	17:15
Embryo commenced moving	20:15	20:15
First hatching commenced	24:00	24:00
Embryonic tail elongated	36:00	36:00
Embryonic mouth formed	48:00	48:00
Eyes commenced pigmentation, vesicle of air bladder formed, olfactory pit opened, free neuromast with cupulae observed	60:00	60:00
Eyes deeply pigmented	72:00	72:00
Embryonic pectoral fin formed, tail elongated to the head in FW	96:00	96:00 (End of hatching)
Egg membrane transformed by developed embryonic head in FW	120:00 (End of hatching)	

Observation was done at every minute to observe the division of cells up to morula stage, at 15 minutes intervals for 24 hours (morula stage to embryo ready to hatch), and then every 12 hours until 120 hours after fertilization (hAF). The time showed at each egg developmental stage was first observed.

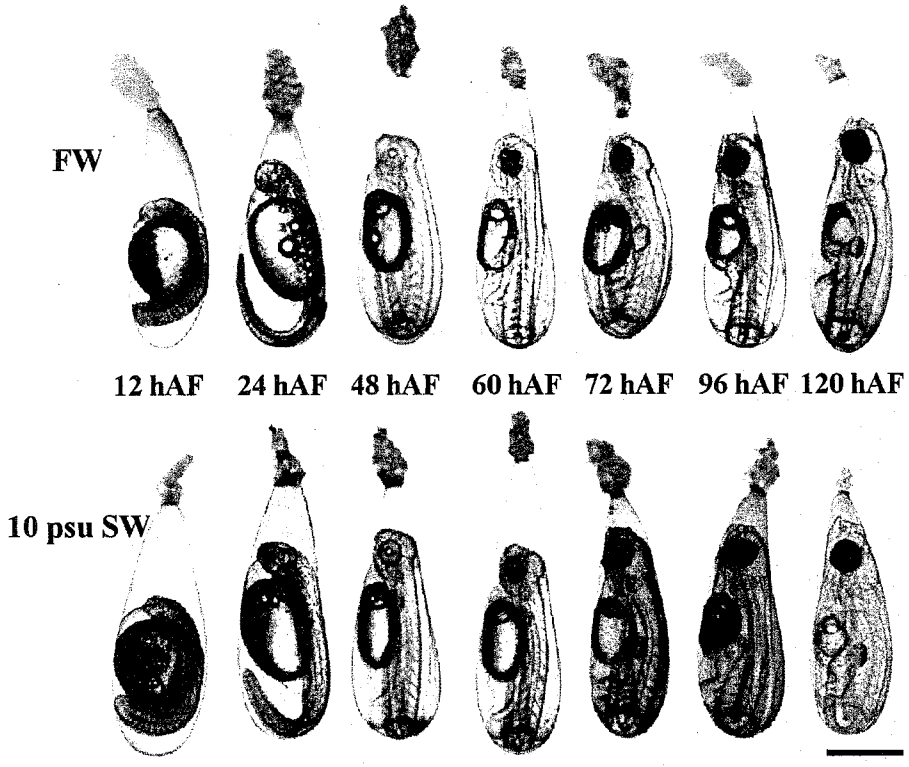

Fig. 2.10: Microscopic photographs showing changes of morphological egg development in FW (above) and 10 psu SW (below). Embryos developed similarly as agrippa condition. Scale bar, 0.5 mm.

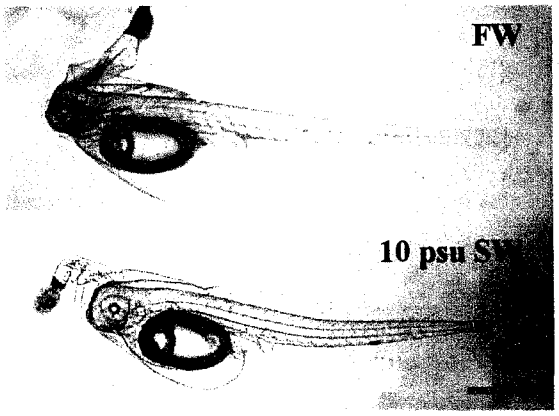

Fig. 2.11: Larvae of marble goby immediately after hatching at 36 hAF in both FW (above) and 10 psu SW (below), hatching characteristic were similarly. Scale bar, 0.5 mm.

2.3.3.2 Hatching gland cells (HGC)

The appearance of HGC was first observed on embryonic heads at 24 hAF (Fig. 2.12), and hatching commenced. Thereafter, HGC numbers increased and peaked from 48 to 72 hAF, and then decreased up to the end of hatching in both waters. Change of HGC numbers are shown in Fig. 2.13. Significant differences were not observed between FW and 10 psu SW. At 36 hAF, HGC were clearly observed under the optical microscope (Fig. 2.14). HGC were observed only in the upper parts of embryos, especially on heads (Figs. 2.12 and 2.14).

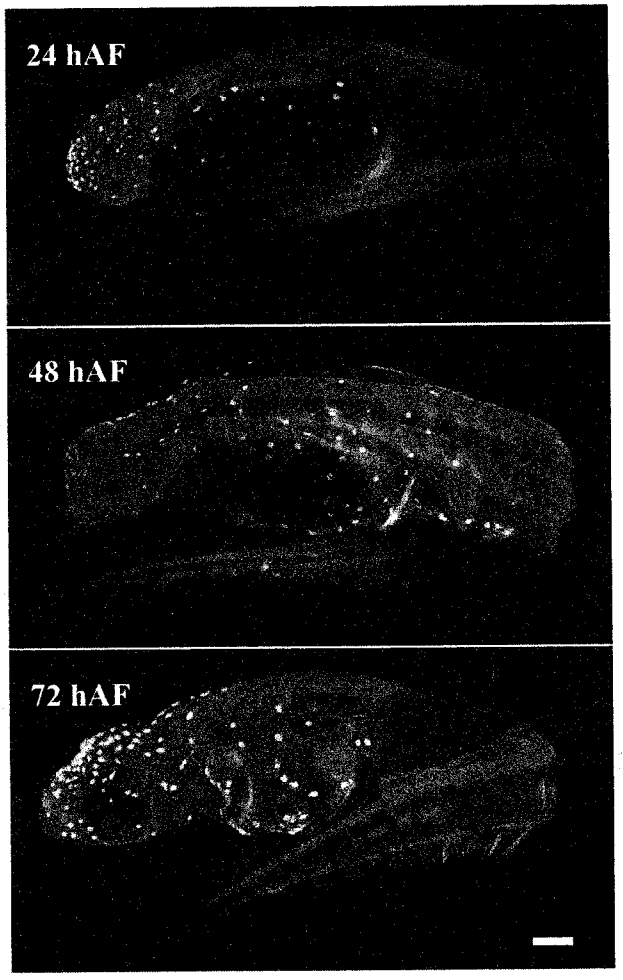

Fig. 2.12: The immunofluorescence image showing appearances of hatching gland cells (HGC) (green dots) were first observed on embryonic heads at 24, 48 and 72 hAF. Scale, 0.1 mm.

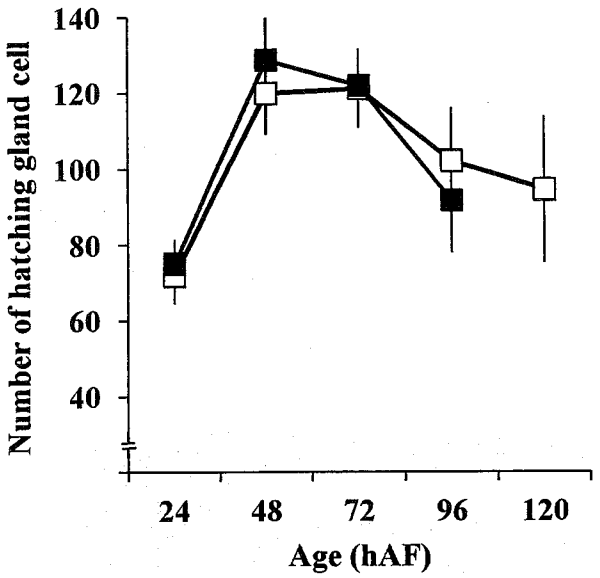

Fig. 2.13: Changes of mean numbers of hatching gland cells (HGC) of marble goby incubated in FW and 10 psu SW (mean ± S.D., *n*=10).

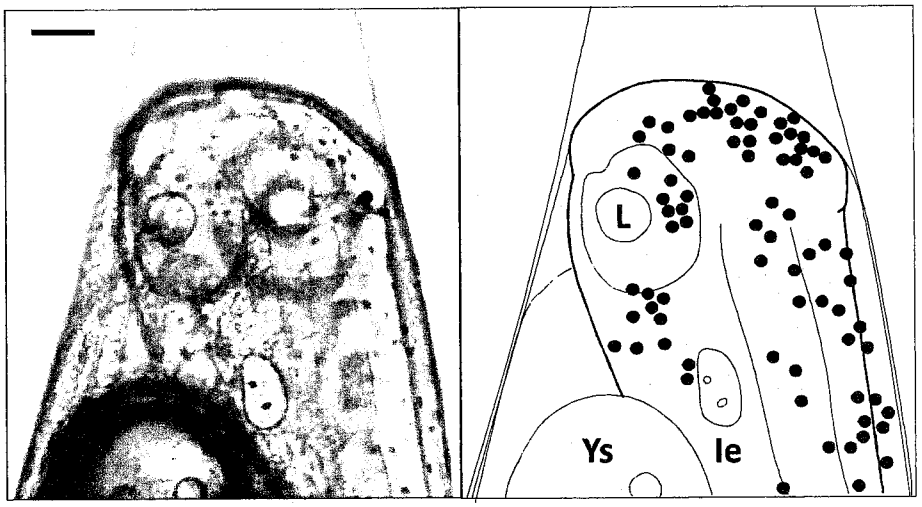

Fig. 2.14: Microscopic photograph (left) showing the upper part of embryo of marble goby at 36 hAF in FW. Illustration (right) showing position of HGC with black dots (Ie, Inner ear; L, Lens; Ys, Yolk sac). Scale bar, 0.1 mm.

2.3.3.3 Embryonic sensory organs

Sensory organs developed similarly in both FW and 10 psu SW eggs (Table 2.1). When hatching commenced at 27 hAF, embryonic eyes were unpigmented but lens and otic vesicles were already apparent (Fig. 2.15), while both larvae have not open mouth and anus, and floated in water column. In both FW and 10 psu SW eggs, free neuromasts (FNM) with cupulae were observed on the embryonic head at 60 hAF (Fig. 2.16). Eye pigmentation commenced at 60 hAF and eyes were deeply pigmented at 72 hAF in both FW and 10 psu SW eggs. In both waters, embryonic developed inner ears and olfactory pits opened at 60 hAF. At 78 hAF, mouths opened and pectoral fins were observed. At 96 hAF, opened mouth had occurred and some hatched larvae developed an air bladder filled with air and commenced active swimming in both waters (Fig. 2.17). Embryonic free neuromast and cupula, and olfactory epithelium with ciliated receptor cells in the olfactory pits at 120 hAF in FW were recorded by SEM (Fig. 2.18).

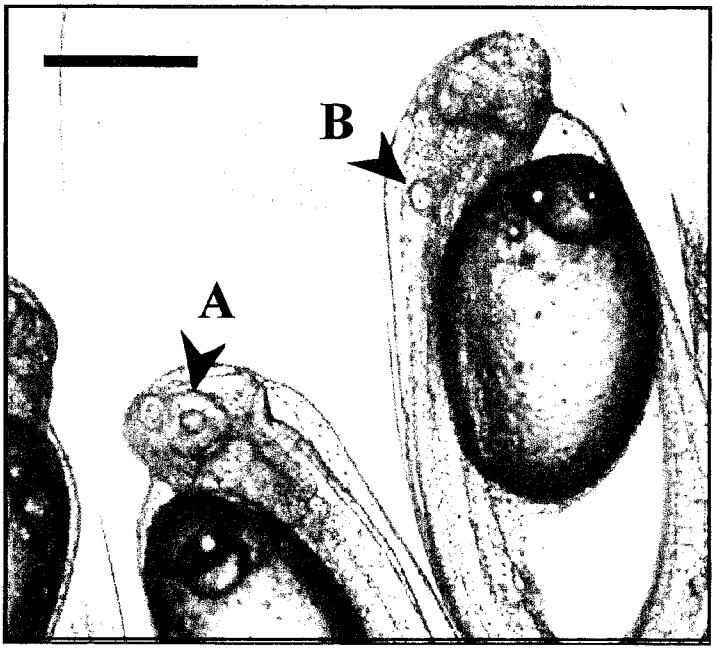

Fig. 2.15: Microscopic photograph showing the embryonic eyes of marble goby (A) were un-pigmented and otic vesicles (B) were apparent at 27 hAF in FW. Scale bar, 0.5 mm

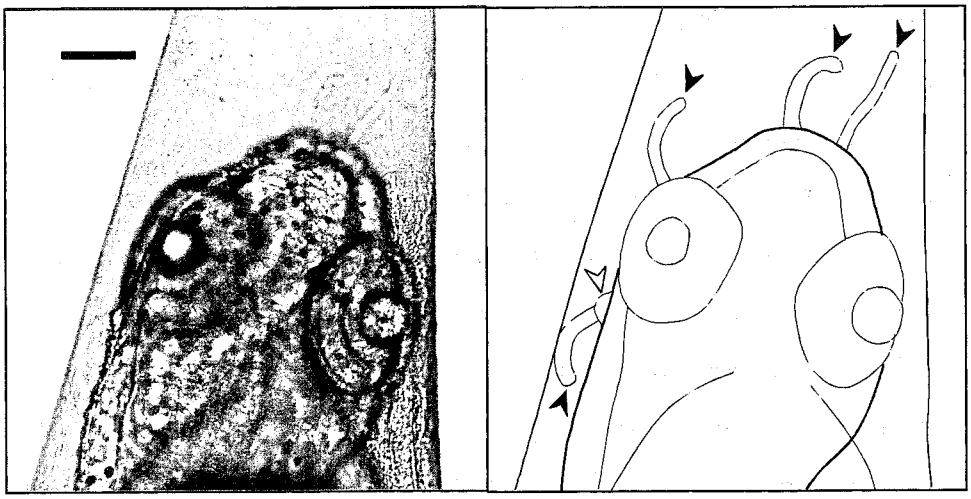

Fig. 2.16: Microscopic photograph (left) showing the appearance of free neuromasts (FNM) with cupulae on an embryonic head of marble goby at 60 hAF in FW. Illustration (right) showing the positions of the FNM (black arrows) and cupulae (white arrow). Scale bar, 0.1 mm.

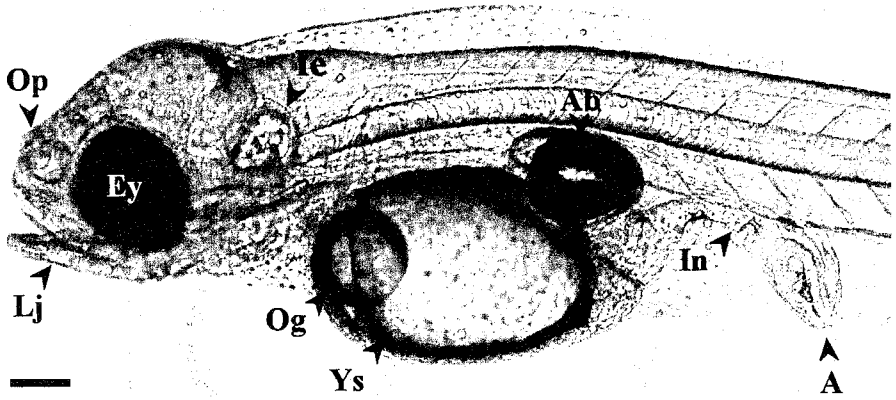

Fig. 2.17: Microscopic photograph showing marble goby larva developed an air bladder filled with air and commenced active swimming at 78 hAF in FW (A, Anus; Ab, Air bladder; Ey, Eye; Ie, Inner ear; In, Intestine; Lj, Lower jaw; Og, Oil globule; Op, Olfactory pits; Ys, Yolk sac). Scale bar, 0.1 mm.

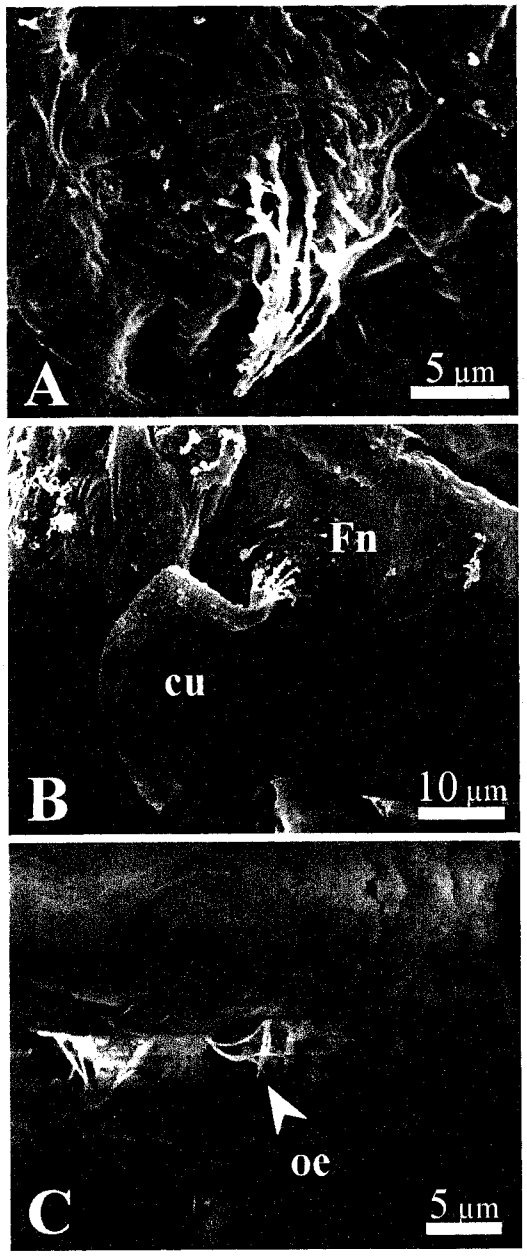

Fig. 2.18: Scanning electron micrographs showing the FNM with sensory hair cells on the head (A) and FNM with cupula (cu) on the embryonic head (B), olfactory epithelium (oe) with ciliated receptor cells in the olfactory pits of marble goby at 120 hAF in FW.

All through the observation, no differences were seen in the development of sense organs between un-hatched embryos and hatched larvae of the same age in both waters (Fig. 2.19). The development of embryonic taste buds was not observed in the present study.

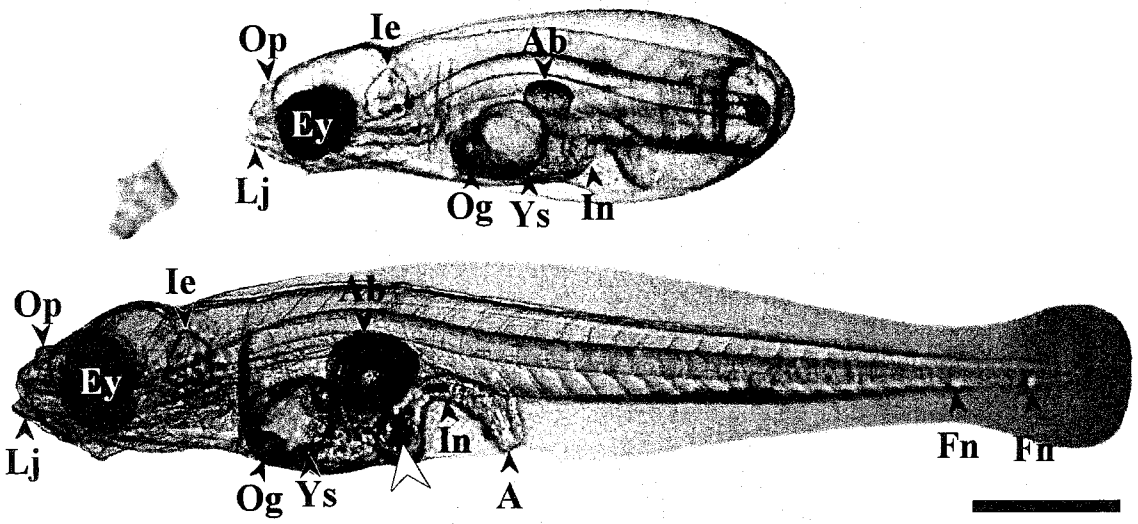

Fig. 2.19. Micrographs of an un-hatched egg (upper) in FW and a larva (lower) in 10 psu SW of marble goby at 120 hAF. The larva commenced feeding at this time and a rotifer provided as food is visible in the intestine (white arrow). A, anus; Ab, air bladder; Ey, eye; Fn, free neuromast; Ie, inner ear; In, intestine; Lj, lower jaw; Og, oil globule; Op, olfactory pits; Ys, yolk sac. Scale bar = 0.5 mm.

2.4 Discussion

The present study showed clearly that salinity did not effect to egg development observed under the condition of the present study but had significant effects on egg hatching and larval deformation of marble goby. Egg hatching occurred within a wide salinity range, from FW to 20 psu SW. However, the eggs died in 30 psu SW and all larvae that hatched in 20 psu SW were deformed and died within three days. Previously, high salinity has been shown to delay hatching or in some cases, to cause precocious hatching, and also to cause malformations and death of fish eggs (Vetemaa and Saat 1996; Albert et al. 2006). For marble goby, eggs hatched into viable larvae at salinities of 15 psu SW or lower. Generally, their natural habitat ranges from freshwater to brackish environments including canals, rivers, reservoirs and swamps (Robert 1993; Rainboth 1996). However, there is lack information of distributional environments of spawning and larval stages in natural water body. Interestingly, despite the inhabited in FW and brackish environment, incubation of the eggs in 10 psu SW provided the best outcome, i.e., significantly higher hatching rate (60.0 ± 2.0% and 70.1 ± 13.2%) and

lower deformation rate (11.1 ± 2.4% and 4.8 ± 4.4%, mean ± SD) than those in FW in experiment 1.1 and 1.2.

On closer comparison of the FW and 10 psu SW incubation it appeared that the hatching period is a key factor affecting embryo deformation. The deformation rate increased when egg hatching was delayed beyond 72 hAF, which coincides with the time of eye pigmentation. The majority of the eggs in 10 psu SW hatched before 72 hAF and the deformation rate was relatively low. On the other hand, approximately 66.5% of larvae in FW that hatched between 72-84 hAF and 84-96 hAF (44.7 ± 33.2% and 21.8 ± 6.9% of total hatching rates) were deformed, while almost all that hatched more than 96 hAF were deformed. This is consistent with previous studies that showed "late hatching" results in high larval mortality (Senoo et al. 1994a; Senoo 2001b, 2003b). Few studies have examined the problems of delayed hatching. For marble goby, hatching later than 72 hAF, was considered to be delayed. This information could be of practical value for checking the incubation conditions during seed production of marble goby. Observation of eye pigmentation could also be used to indicate whether hatching was delayed, with an expected low larval survival in eggs hatched after the appearance of deep eye pigmentation.

At the commencement of exogenous feeding, fish larvae face death from starvation as the first feeding is delayed past the point of no return (PNR) (Blaxter and Hempel 1963). First feeding of fish larvae is crucial for their subsequent growth and survival. Most fish larvae develop with an inability to swim and feed adequately result in inferior growth if they fail to successfully initiate first feeding (Houde 1974; Dou et al. 2002). Many researchers have reported low larval survival during and after the first feeding period (Mookerji and Rao, 1999; Gisbert et al. 2004; Dou et al. 2005; Pena and Dumas 2005; Kailasam et al. 2007) and this is attributed to factors including light intensity, food supply, egg size, yolk quantity, feeding behaviour and time of first feeding (Blaxter, 1974; Dou et al., 2000). In this study, marble goby fish larvae were morphologically prepared to be feed during 84-96 hAF, as their eyes pigmented, intestine peristaltic, anus opened, lower jaw functional and horizontal swim for foraging. The embryos were developed to coincide as the hatched larvae.

Besides causing the deformation and mortality of eggs and larvae, suboptimal salinity also influences the timing and the duration of the hatching period. Yang and Chen (2006) showed that hatching time increased with increasing in water salinities for eggs of the obscure puffer *Takifugu obscures.* At low salinities (0-8 psu), hatching occurred from 155-166 hours, whereas at high salinities (12-32 psu) hatching occurred from 179-209 hours. A few embryos hatched at 12-28 psu but all larvae were deformed and died within 24 hours, no embryo hatched and all embryo died on the day 4 at 32 psu. Mai et al. (2005) showed time to hatch tends to decrease with increasing salinity for most marine fishes. In eggs of the black bream *Acanthopargus butcheri*, there was high incidence (up to 93%) of deformities at salinities below 15 psu, characterised mainly by curvature of spine and bent tail (Haddy and Pankhurst 2000).

Fish egg membrane consists of external and internal layers, and hatching occur when the external layer was mechanically break by embryonic muscular motion and internal layer was degraded by the hatching enzyme (Yamagami 1988; Ishida 1994a; 1994b). Hatching enzyme secreted from the specially differentiated HGC before at the time of hatching and disappeared completely in the larvae after hatching (Yamagami 1988), as reported in chum salmon *Oncorhynchus keta* (Ishida 1948) and medaka fish *Oryzias latipes* (Ishida 1994a; 1994b). In the case of marble goby, the development of HGC was recorded for the first time in FW and 10 psu SW. Their HGC are visible as opalescent giant cells on the surface of embryonic head and membrane of yolk sac. These cells appear just before first hatching occur at 24 hAF and also clearly seen along the hatching occurred from 24 to 144 hAF. HGC numbers peaked at 48 hAF and decreased thereafter to the end of hatching. However, there is no significant difference in the number of HGC in FW and 10 psu SW. Yet, there is possibility to be difference in enzyme activities in both waters. The hatching enzyme is believed to be stimulated for solubilization of the egg chorion during hatching as reported in many studies (Yamagami 1981), but enzyme activities and hatching mechanism of marble goby were not investigated in this study.

The behaviour of fish larvae is closely related to the development of their sensory organs (Iwai 1972; Kawamura et. al. 2003). Larvae hatched with developed sensory organs have higher chances to survive than those hatched with undeveloped sensory organs (Blaxter 1969; Kawamura et al. 2003). FNM with cupula is an important

sensory organ in fish larvae (Kawamura and Ishida 1985; Blaxter and Fuiman 1989; Kawamura and Washiyama 1989; Jones and Janssen 1992; Mukai et al. 1994; Kawamura et al. 2003; Mukai 2006; Mukai et al. 2007; Tuzan et al. 2006). FNM play an important role in detecting prey, for instance, larvae of willow shiner *Gnathopogon elongatus caerulescens* (Mukai 2006) are able to detect rotifer in water and larvae of mottled sculpin *Cottus bairdi* (Jones and Janssen 1992) could feed on *Artemia* in the dark by using FNM. In the case of marble goby, at 60 hAF, both FW and 10 psu SW of embryos developed an inner ear, olfactory pit, and FNM with cupula. Larvae at this stage are believed able to develop shortly and able to swim and detect prey for foraging. The results indicate that the correct hatching stage of marble goby is after the peak appearance HGC and before eye pigmentation commences, i.e. 48-60 hAF. This considered as one of the reason of high larval survival in 10 psu SW.

In the present study, the salinities tolerated by marble goby eggs ranged from FW to 15 psu SW. In this and a previous study, larval survival was better in 10 psu SW than in FW (Senoo et al. 2008). This suggests that marble goby may be capable of reproducing in low salinity water and perhaps indicates that is should be classified as a euryhaline fish. The salinity tolerance pattern displayed by marble goby is similar to that of other euryhaline fish. For example, eggs of killifish *Fundulus heteroclitus* hatched from FW to 35 psu (Grosell et al. 2007). Eggs of the obscure puffer hatched at salinities from FW to 28 psu (Yang and Chen 2006) and hatching rates of the tawny puffer *T. flavidus* were above 70% from 5 to 40 psu (Zhang et al. 2010). Similarly, eggs of the Iceland capelin *Mallotus villosus* are able to hatch between 1.5 and 34.0 psu (Davenport 1989). The reproductive biology of marble goby in its natural environment is still unknown. Because marble goby has a wide distribution and diverse habitats, it is believed that there are several genetically distinct populations of this fish. Ha et al. (2011) revealed highly significant differences in the mitochondrial DNA control region between fish sampled in East Malaysia and West Malaysia. The marble goby brood fish used in the present study was captured from a "river" population and may possess different reproductive characteristics from "landlocked" fish inhabiting enclosed ponds and lakes, as used in other studies (Tavarutmaneegul and Lin 1988; Senoo et al. 1994a; Luong et al. 2005). Understanding the differences in biological and reproductive characteristics of river and landlocked marble goby needs further investigation.

The eggs used in the present study can be incubated in both FW and 10 psu SW. However, the eggs incubated at 10 psu SW had a shorter hatching period, higher hatching rate, and better larval survival than eggs incubated in FW. The present study showed that egg incubation at 10 psu is recommended for marble goby, at least for the brood fish used in the study. The most favourable incubation conditions for egg survival and hatching are considered to be those that result in the greatest numbers of normal larvae. Incubation in 10 psu SW, which resulted in a high hatching rate and a low deformation rate may produce better success in hatcheries. Hatched larvae should be reared for a further 10 days in 10 psu SW as this period is considered to be the most important period for larval survival. Larvae then can be reared in FW (Senoo et al. 2008). This study presents the first observations on the optimal salinity for egg incubation and hatching of marble goby, the information will be useful for its aquaculture and seed production. However, further studies are required to understand the mechanisms underlying the observed phenomena and to develop practical rearing techniques for this species.

CHAPTER 3

Early larval development of marble goby, *Oxyeleotris marmoratus* in freshwater and 10 psu seawater

3.1 Introduction

The studies on larviculture of marble goby have been doing since 1970s in Thailand, West Malaysia, Singapore and Indonesia. However, those studies were conducted in FW, Senoo et al in 2008 reported that the rearing with 10 psu SW is indispensable for the larval survival for the first 10 days on marble goby in the State of Sabah, Malaysia. It is considered therefore there are some different strains in this species depend on different habitats as well as Ayu *Plecoglossus altivelis altivelis* which has amphidromous type and land-locked type (Iguchi and Yamaguchi 1994; Takeshima et al. 2009) and its rearing method in hatchery is different depend on the type. Hatchery technique must be kept up with biological features of the fish. In this chapter newly hatched larvae of marble goby in the State of Sabah, Malaysia were obtained, reared in FW and 10 psu SW, and observed their developments, then cleared the cause of larval mortality reared in FW. Moreover, the correlation among developments of morphology, sensory organs, and behavior was recorded to discuss the optimum rearing technique for the early larval stage on this species.

3.2 Materials and methods

3.2.1 Broodfish management

The experiment was conducted at the Center of Collaborative Research in Aquaculture (Universiti Malaysia Sabah-Kinki University) in the State of Sabah, Malaysia from August to November, 2010. Brood fish of the marble goby were captured in a river in Penampang area in the State of Sabah and held in the hatchery for five months on a diet of trash fish. A mature male (20.0 cm TL, 310 g) and a mature female (24.0 cm TL, 280 g) were stocked in a glass aquarium (108 × 76 × 80 cm) (Fig. 3.1A) with a filter system (Fig. 3.1B). Water temperature was maintained with a 150 W electric heater at 28-29°C (recorded at 8:00 h and 17:00 h; Fig. 3.1C) and bubble aeration (Fig. 3.1D) was done

with 500 mL/min. A concrete substrate was installed on the tank bottom (Fig. 3.1E). DO (6.8-8.2 mg/L) and pH (7.3-8.5) were within the suitable range.

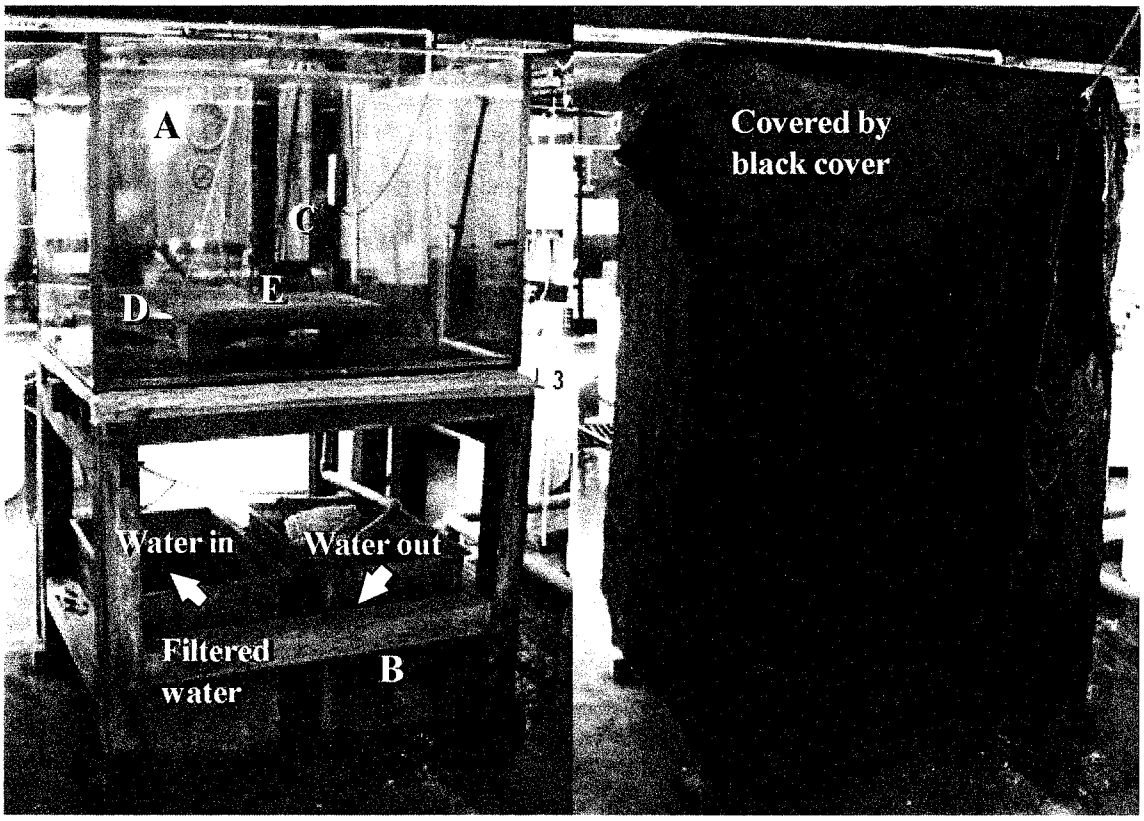

Fig. 3.1: Natural spawning tank of marble goby in UMS hatchery. A, spawning tank; B, filter tank with clean water storage tank; C, heater; D, bubble aeration; E, and spawning substrate installed on the tank bottom .

3.2.2 Larval rearing for observation

Newly hatched larvae were obtained following procedure. The female marble goby was injected with human chorionic gonadotropin at a dose of 1,000 IU/kg and the male at 500 IU/kg (Senoo et al. 1992; 1993b; 1994b). Three days after the injection, eggs were natural spawned and deposited under a concrete block at the aquarium bottom. After spawning, the female was taken out from the tank. The male cared for the eggs by brushing and fanning them until they all hatched. Hatching occurred starting at 24 hours after spawning and lasted until 96 hours, with a peak at 60 hours.

During the peak spawning, about 20,000 hatched larvae were harvested and divided into two groups and reared in FW or 10 psu SW in cylindrical plastic tanks (150 cm diameter, 60 cm high). The FW for larval rearing was tap water neutralized with sodium thiosulfate and aerated for one night. This fresh water was mixed with filtered sea water and used as 10 psu SW (following Senoo et al. 2008). During larval rearing through 9 days after hatching (dAH), water temperature was maintained with a 100 W electric heater at 28-29°C and bubble aeration was provided at 500 mL/min. The larvae in both FW and 10 psu SW were given live rotifers *Brachionus* sp. at a density of 20/mL. The water temperature, DO and pH during the rearing period were recorded at 6:00 and 18:00 hours. They were similarly maintained in both tanks and ranged from 28.0-29.0°C, 7.0-7.8 mg/L, and 7.4-8.2 in FW, and from 28.0-29.0°C, 6.8-7.6 mg/L, and 7.4-8.4 in 10 psu SW, respectively.

3.2.3 Measurements and analysis

In this paper, comparison was focus on the larval survival in FW and 10 psu SW, therefore, time was reckoned from hatching, i.e., 1 day old larvae were sampled next day from hatching and so on. Larval age was based on the first hatching time and shown in hours or days. It is therefore, when the larval rearing was commenced in FW and 10 psu SW, the larval age was 36 hours after hatching (hAH) or 1.5 days after hatching (dAH). The larvae were sampled randomly from both rearing tanks every day (*n=10*), and anesthetized with 0.1% of Transmore (NIKA, α-methylquinoline).

The larvae were observed for morphological development under a profile projector (Mitutoyo, PJ-3000) and a light microscope (Nikon, Eclipse E600) with a digital camera (Olympus, Digital 600). The larvae (*n=10*) were measured for TL and body depth (BD) to the nearest 0.1 mm. Yolk and oil absorption was determined from the change in the volume of the yolk sac and the oil globule with time. Yolk sac volume was approximated by the formula for a prolate spheroid $V = \pi/6\ L\ h^2$, where L is yolk sac length and h is yolk sac height; oil globule volume was computed from $V = \pi/6\ d^3$, where d is oil globule diameter (Bagarinao 1986). Larval behaviour was observed with the naked eye or under a light microscope. Larval feeding intake was determined by calculating the average number of ingested rotifer in the gut daily at

09:00 and 15:00 hours. The rotifer ingestion rate was determined as the number of rotifers in the digestive organ of larvae.

For histological examination of sense organs, the larvae were sampled at hatching, at 4 and 9 dAH (n=10) when all larvae in FW died. The specimens were preserved in Bouin's solution, then dehydrated in an ethanol series, embedded in paraffin, cut into 6 μm thick sagittal and cross sections, stained with haematoxylin-eosin, and examined under the light microscope. Following Kawamura et al. (1990), growth is defined here as quantitative changes in body size, and development as the appearance and qualitative changes in structures such as the sense organs, the internal organs, the muscles, and the fins.

3.2.4 Statistical analysis

Body sizes, gut epithelium, rotifer ingestion and larval survival were compared between salinities by analysis of variance (ANOVA) followed by a Tukey's honest significance different (HSD) test. All statistical analyses were carried out using JMP version 8 (SAS Institute, USA) and SPSS Statistics 17.0 software (IBM Corp., New York, USA). Differences and effects were considered significant at $P<0.05$.

3.2 Results

3.3.1 Morphological Development and Behaviour Changes

Changes of morphological development of marble goby larvae reared in FW and 10 psu SW were shown in Fig. 3.2. The correlation between the behavioral changes and morphological development was shown in Table 3.1.

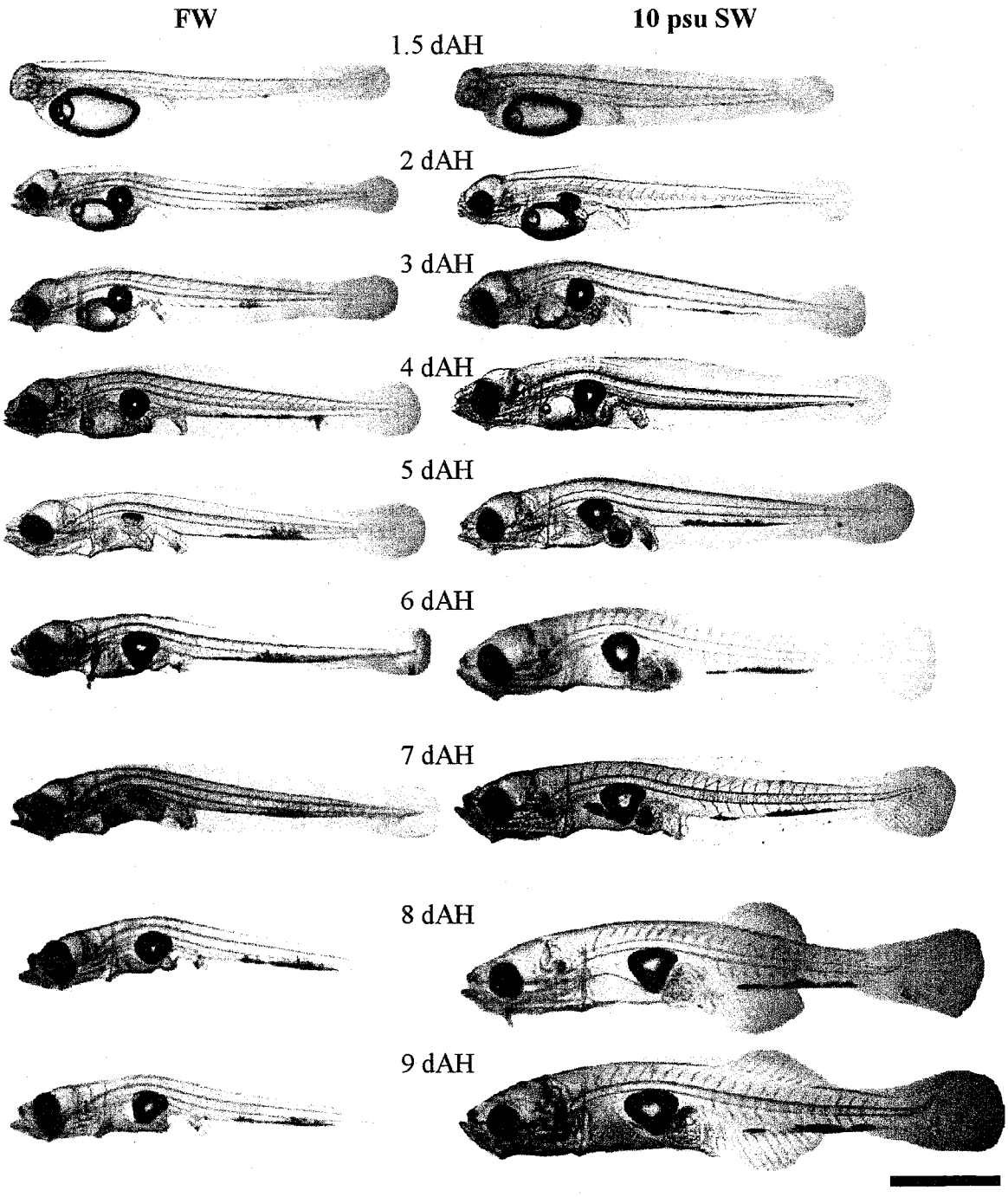

Fig. 3.2: Changes of morphological development of marble goby larvae reared in FW and 10 psu SW. Scale bar, 1 mm.

Table 3.1. Stages with times observed early larval development in FW and 10 psu SW.

Age (days after hatching, dAH)	Reared in FW	Reared in 10 psu SW
0	• Hatching commenced • Eyes unpigmented, olfactory pits not opened, otic vesicle with two otolith, no free neuromast • Mouth not formed, anus not opened • Larvae stay at the bottom • Newly hatched larvae measured 3.06 ± 0.06 mm TL in both water	
1	• Mouth formed, body elongated, yolk sac volume decreasing • Vertically swimming	
2	• Eye deeply pigmented, otic vesicle epithelium ciliated, free neuromast observed, olfactory pits opened, olfactory epithelium ciliated • Air bladder inflated, pectoral fin opened, horizontal swimming	
3	• Eye movement • Active jaw and gut movement	
4	• Active jaw movement • no consumed food (3.66 ± 0.15 mm TL)	• Active jaw movement • first feeding on rotifer (3.69 ± 0.09 mm TL)
5	• Body elongated and thin • Yolk sac absorption finished (3.71 ± 0.10 mm TL)	• Body elongated and muscle increase • Yolk sac absorption finished (3.85 ± 0.05 mm TL)
6	• Body become bended (3.80 ± 0.11 mm TL)	• Body mass increase (4.08 ± 0.27 mm TL)
7	• No ray formed (3.72 ± 0.13 mm TL)	• Caudal fin formed • Ray formed at the fin (4.27 ± 0.27 mm TL)
8	• No flexion occurred • fin no developed (3.71 ± 0.19 mm TL)	• Flexion occurred, second dorsal fin, and anal fin formed (4.49 ± 0.08 mm TL)
9	• Larvae dying (3.70 ± 0.16 mm TL)	• Larvae growing and developing (4.60 ± 0.20 mm TL)

Larvae were similar in size in both salinities at 1 dAH, 3.0 ± 0.1 mm in TL with yolk sac volume 0.052 ± 0.005 mm^3. The larvae reared in 10 psu SW and in FW showed equal growth rates during the first 4 days (Fig. 3.3).

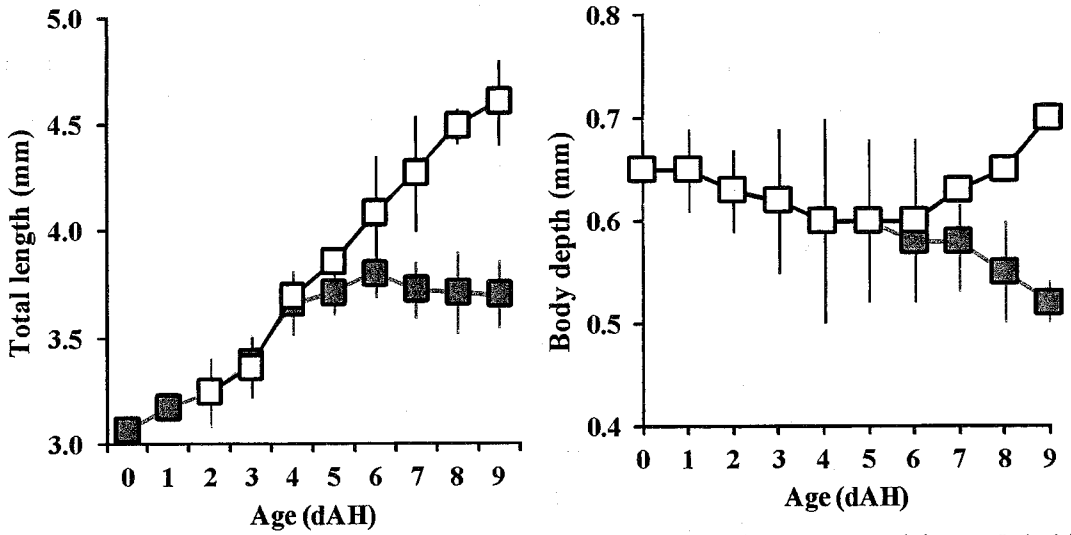

Fig. 3.3: Total length and body depth of marble goby larvae reared in FW (white square) and in 10 psu SW (red square) until 9 dAH. Results are expressed as the means ± SD (n=10). ANOVA analysis showed significant different of body in 9 dAH (P<0.05).

The yolk sac and the oil globule decreased markedly after 2 dAH and were completely absorbed by 5 dAH in both waters (Fig. 3.4). There was no difference in the volume of the yolk sac and the oil globule between two groups ($P>0.05$).

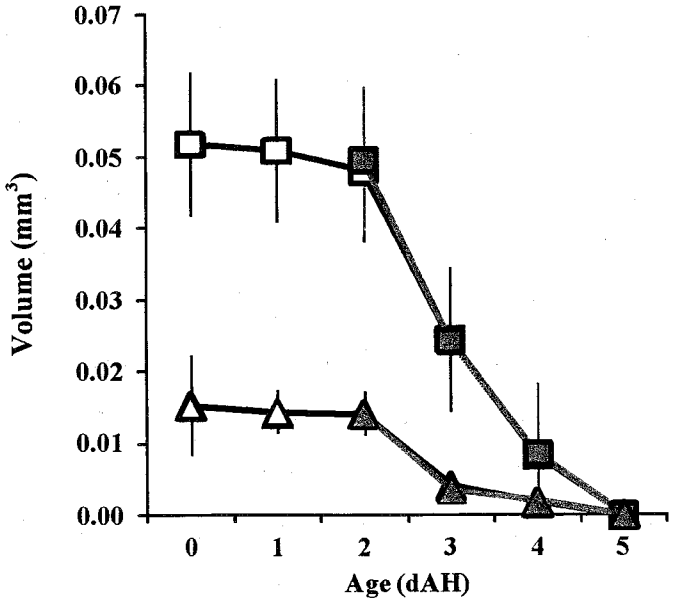

Fig. 3.4: Volume of yolk sac and oil globule of marble goby larvae reared in FW and in 10 psu SW for 5 dAH. Results are expressed as the means ± SD (n=10, P>0.05).

There was no apparent difference in larval morphology and behaviour in both waters during the first three days. Notable changes were noticed after first feeding was commenced. The larvae reared in 10 psu SW commenced feeding on rotifers at 4 dAH and continuously grew to 4.60 ± 0.20 mm TL at 9 dAH. On the other hand, the larvae reared in FW did not ingest rotifers at all. The ANR of larvae in 10 psu SW was 5.7 ± 2.3 at 4 dAH and gradually increased throughout the experiment period (19.0 ± 3.3 at 9 dAH) (Fig. 3.5).

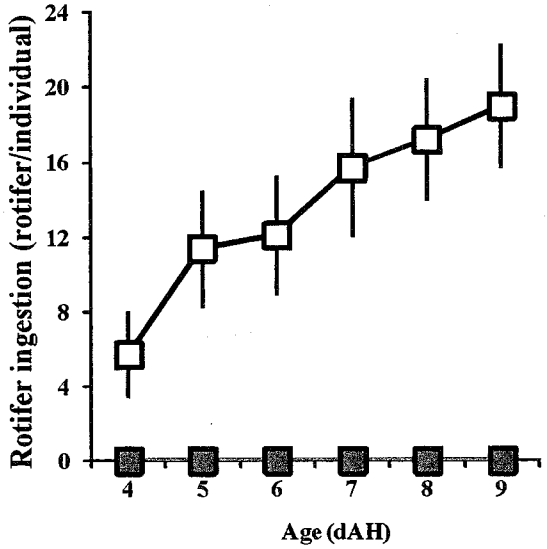

Fig. 3.5: Rotifer ingestion of marble goby larvae reared in FW (red square) and in 10 psu SW (white square) until 9 dAH. Results are expressed as the means ± SD (n=10, P<0.05).

Larval growth in FW was retarded at 5 dAH at 3.71 ± 0.10 mm in TL and 0.60 ± 0.04 mm BD at the completion of yolk sac absorption stage, compared to the larvae in 10 psu SW (3.85 ± 0.05 mm TL, 0.60 ± 0.07 mm BD). They grew slowly to 3.71 ± 0.10 mm TL to 3.80 ± 0.11 mm TL at 5 to 6 dAH, and all died at 9 dAH in FW (3.70 ± 0.10 mm TL, 0.45 ± 0.03 mm BD). While the larvae in 10 psu SW were survived with 69.0 ± 2.0% at 9 dAH (4.62 ± 0.15 mm TL, 0.72 ± 0.05 mm BD).

The larvae reared in 10 psu SW are hereafter also referred to as feeding larvae, and those reared in FW as non-feeding larvae. Jaw and gut movements were evident at 2 dAH in both groups of larvae. Among the feeding larvae (larvae in 10 psu), notochord flexion occurred at 8 dAH and the dorsal and anal fin rays were formed at the same time. Neither notochord flexion nor fin formation occurred in non-feeding larvae (larvae in FW). These growth and morphological development in the fed larvae reared were

similar to those reported by Senoo et al. (1994a, 1994b). After 6 dAH (0.55 ± 0.03 mm BD) the body depth of the non-feeding larvae decreased, indicating starvation and loss of body mass.

Histological analyses of gut epithelium of larvae at 9 dAH revealed remarkable difference in two salinities. The gut of larvae reared in FW was significantly thinner, which were ranged from 0.8 ± 0.3 µm at 4 dAH (ANOVA, $F_{1,58}$ = 37.9, P<0.0001) to 9.1 ± 2.6 µm at 9 d AH (ANOVA, $F_{1,58}$ = 432.6, P<0.0001) compared to those in 10 psu SW that ranged from 1.8 ± 0.6 µm to 23.7 ± 3.3 µm (mean ± SD, *n = 10*), respectively (Fig. 3.6). Histological sections of non-feeding larvae at 9 dAH showed reduced muscle mass and extremely thin epithelium and degenerate cells in the gut (Fig. 3.7).

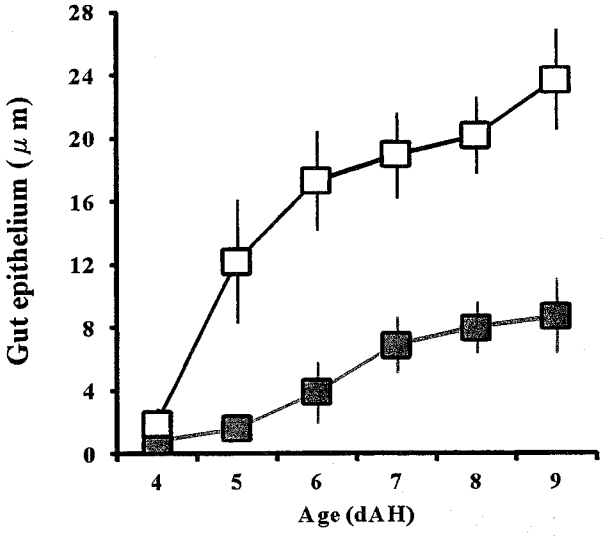

Fig. 3.6: Gut epithelium of marble goby larvae reared in FW (red square) and in 10 psu SW (white square) until 9 dAH. Results are expressed as the means ± SD (n=10, P<0.05).

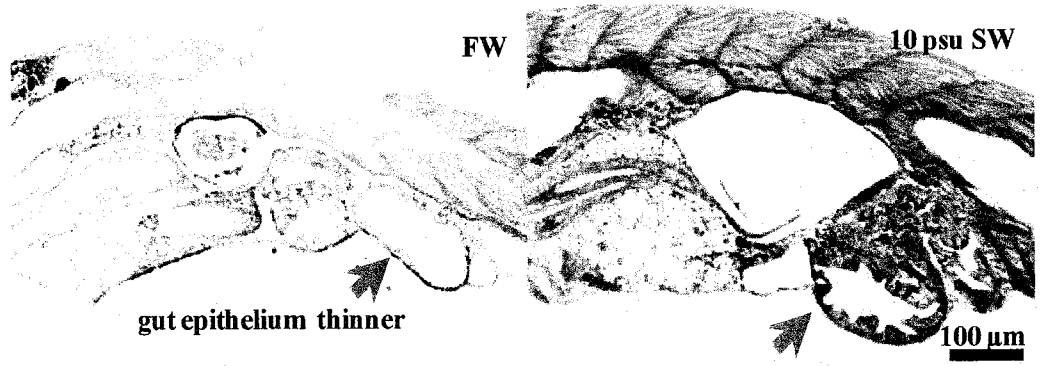

Fig. 3.7: Histological section showed the gut epithelium of marble goby larvae reared in FW and in 10 psu SW.

3.3.2 Development of Sensory Organs

The larvae showed similar development in sensory organs in FW and 10 psu SW (Table 3.1) by 4 dAH. In the newly hatched larvae, the eyes were not pigmented and the cornea was not developed (Fig. 3.8A). The eyes were deeply pigmented as they were growing, which clearly showed in larvae at their first feeding (Fig. 3.8C).

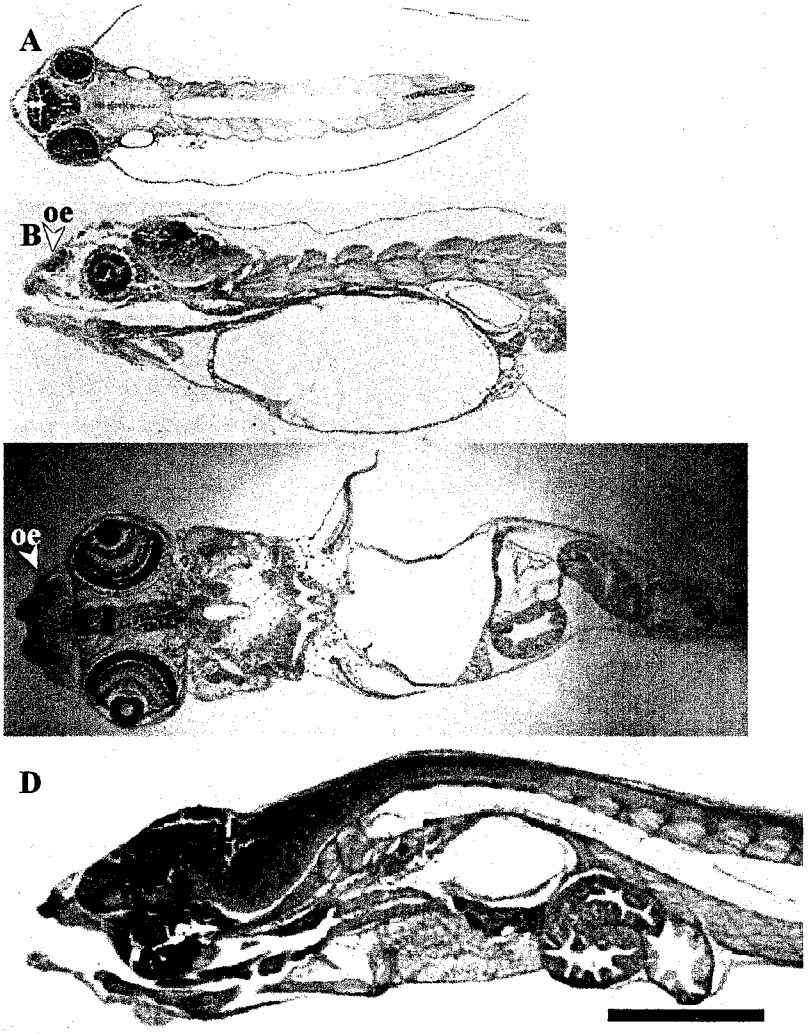

Fig. 3.8: Micrograph showed histological section of marble goby larvae in 10 psu SW. A, early hatching stage at 1 dAH, B, mouth opened stage, C, first feeding stage and, D, end of yolk sac stage. White arrows indicated well developed olfactory epithelium in each stage (oe, olfactory epithelium). Scale bar, 1 mm.

The eyes showed well layered retina at 9 dAH marble goby larvae in both water (Fig. 3.9). The inner ear of the newly hatched larvae was an oval-shaped otic vesicle containing two otoliths inside (Fig. 3.8A). The ossification progressed further with the growth of the larvae, but was not identified in this study. The inner ear well ossified and developed at 9 dAH marble goby larvae in both water (Fig. 3.10).

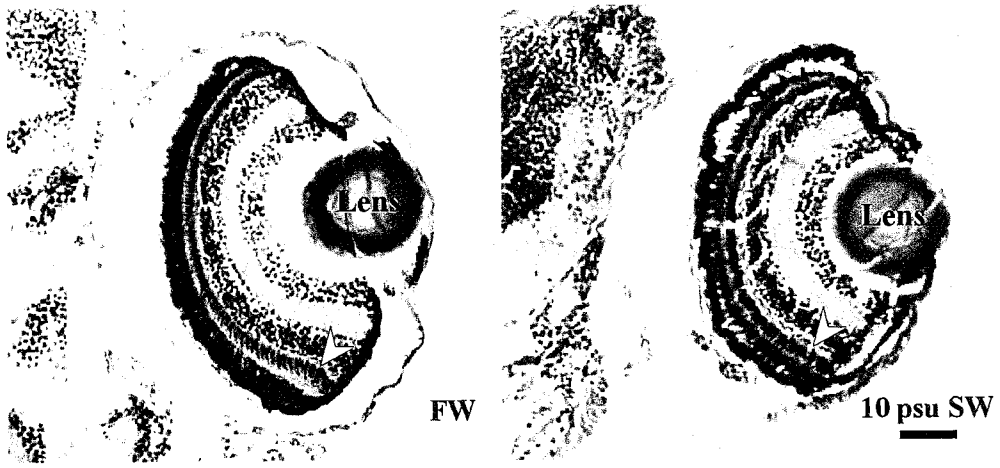

Fig. 3.9. Photomicrographs showing the well layered retina with an area (white arrow) at 9 dAH marble goby larvae in FW and 10 psu SW. Scale bar, 200 µm.

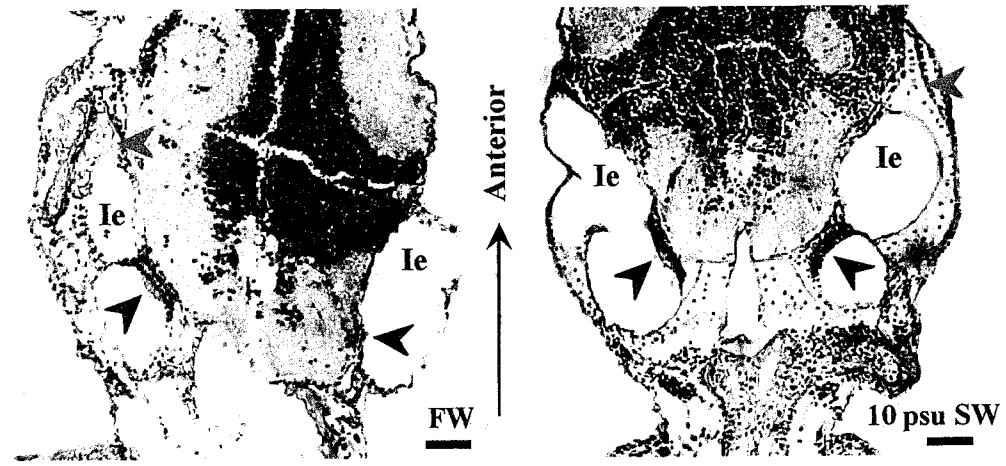

Fig. 3.10. Photos showing the well ossified (red arrows) and cilliated epithelium of inner ear (black arrows) at 9 dAH marble goby larvae in FW and 10 psu SW (Ie, inner ear). Scale bar, 200 µm

The olfactory pits developed with ciliated epithelium at mouth open stage. The ciliated olfactory epithelium cells were arranged radially to form a bud without taste bud (Fig. 3.8A). The olfactory epithelium with ciliated receptor cells in the olfactory pits clearly detected in 9 dAH larvae in both waters (Figs. 3.11 and 3.14). The taste buds were observed in oral cavity at 9 dAH marble goby larvae in FW and 10 psu SW (Fig. 3.12). The FNM was found on the head of the larvae at 2 dAH (showed in Fig. 2.16). The FNM were clearly observed on upper jaw at first feed stage (Fig. 3.8C) and larvae at 9 dAH in both waters (Fig. 3.13). The FNM attached with a well-developed cupula under observation of SEM (Fig. 3.15).

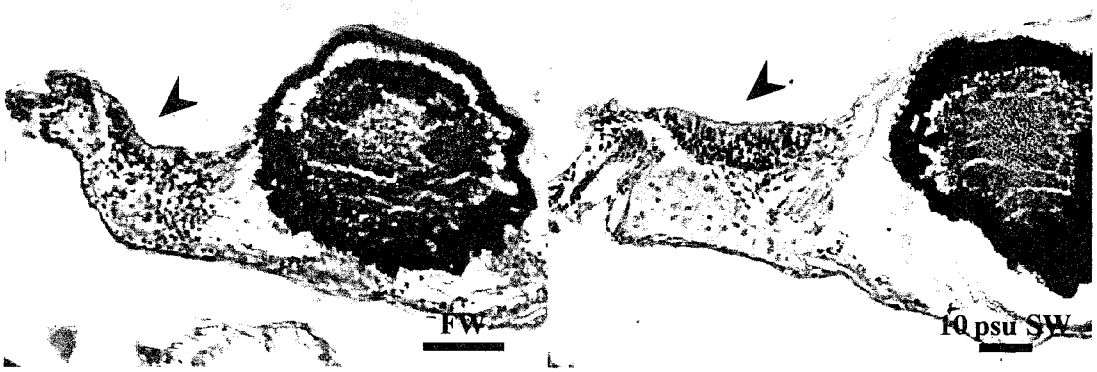

Fig. 3.11. Photos showing the olfactory epithelium ciliated (black arrows) in 9 dAH marble goby larvae in FW and 10 psu SW. Scale bar, 200 μm.

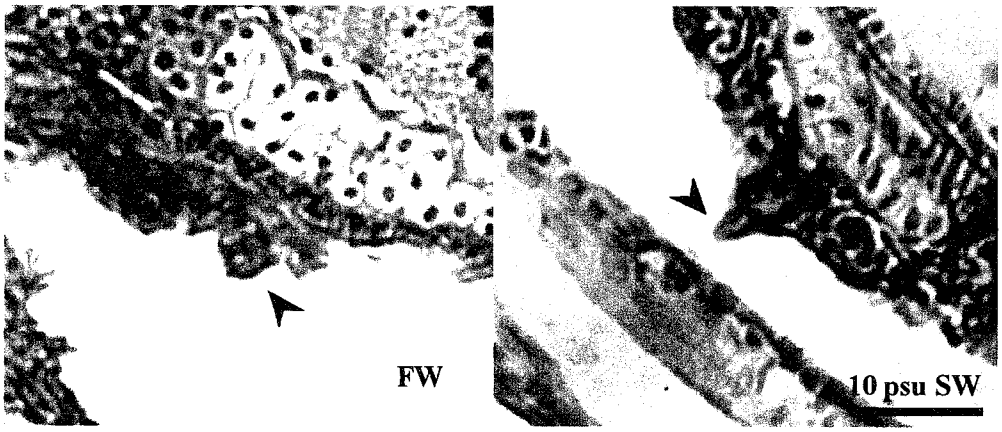

Fig. 3.12: Photos showing the taste buds observed in oral cavity at 9 dAH marble goby larvae in FW and 10 psu SW. Scale bar, 100 μm.

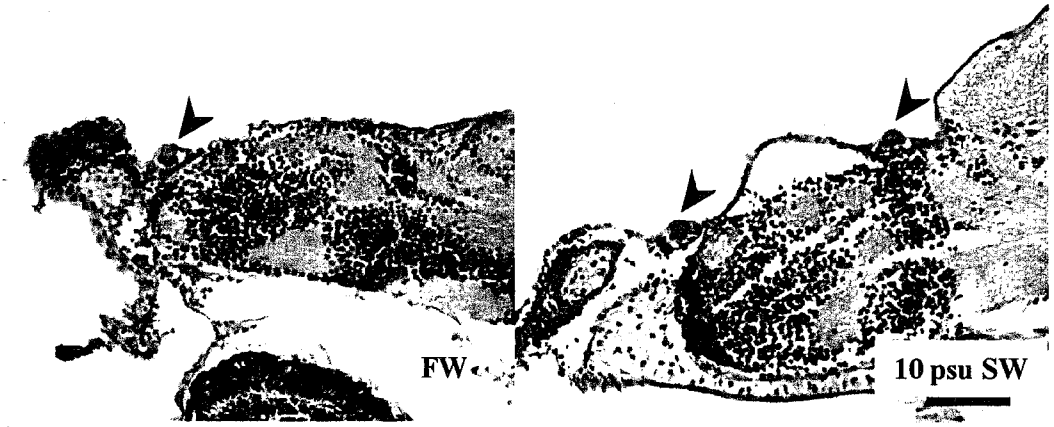

Fig. 3.13: Photomicrograps showing the free neuromasts (FNM) on upper jaw at 9 dAH marble goby larvae in FW and 10 psu SW. Scale bar, 200 μm.

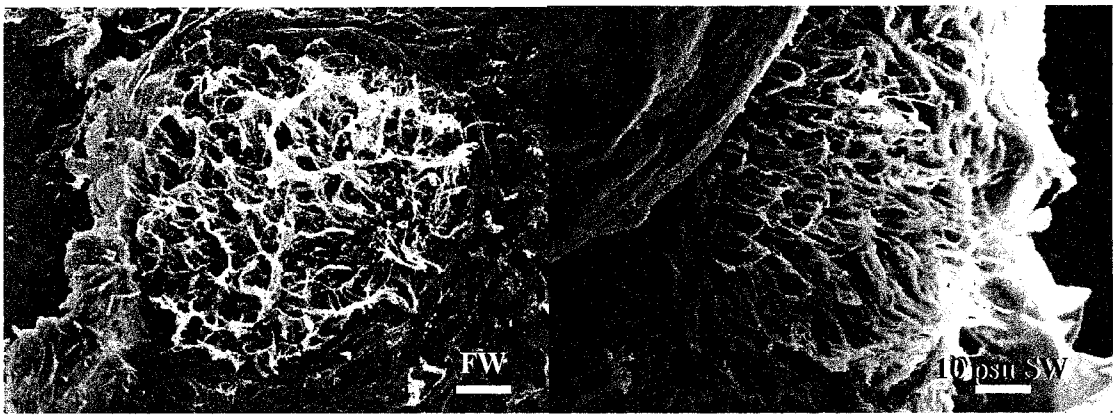

Fig. 3.14: Scanning electron micrographs showing the olfactory epithelium with ciliated receptor cells in the olfactory pits of 9 dAH marble goby larvae reared in FW and 10 psu SW. Scale bar, 10 μm.

Fig. 3.15: Scanning electron micrographs showing the FNM with cupula (white arrows) of 9 dAH marble goby larvae reared in FW and 10 psu SW. Scale bar, 20 μm.

3.4 Discussion

Larvae possess their strategy to survive in the wild (Thresher 1984; Fuiman 1994; Irisson et al. 2004). They will choose favorable condition to have higher hatching and survival (Fuiman and Magurran 1994; Irisson et al. 2004; Fuiman et al. 2006). In this study, larvae survive in 10 psu SW rather than in FW. The larvae in both waters showed similar yolk absorption rates and growth in length to 4 dAH. Remarkable differences were clearly seen between the two larvae after first feeding was commenced. Larvae in FW failed to start feeding and died at 9 dAH. Surprisingly, both feeding and non-feeding larvae developed the qualitatively similar sensory organs at 4 dAH and 9 dAH, respectively. Light microscopy detected no qualitative difference in histological

characteristics of the sensory organs of the two groups of larvae, but revealed a marked degeneration in the digestive tract epithelium and the muscle mass in non-feeding larvae. The intestine were thinner as other species of starve fish, for instance gilthead seabream *Spams aurata* L., California halibut *Paralichthys californicus*, sea bass *Dicentrarchus labrax* (Yufera et al. 1993; Gisbert et al. 2004; Rekecki et al. 2009).

Within the tolerance limits, salinity did not affect the yolk absorption rates in marble goby larvae in this study, nor the greenback flounder *Rhombosolea tapirina* (Hart et al. 1996), and the Atlantic halibut *Hippoglossus hippoglossus* (Lein et al. 1997). But a slight salinity effect was seen in the croaker *Bairdiella icistia* (May 1974), and significant effects were observed in the rabbitfish *Siganus guttatus* (Young and Dueňas 1993), milkfish *Chanos chanos* (Swanson 1996), and the striped bass *Morone saxatilis* (Peterson et al. 1996).

Generally, fish regulate their internal osmotic concentration of their body fluids at equivalent to approximately 8-10 ‰ salinity (Giese 1973). Gametes of teleosts are reported that either iso-osmotic or hypo-osmotic to the body fluid of the parent fish (Holliday 1969). Yet, the information spawning and distributional of larvae of marble goby still remained unknown. Nevertheless, the results in Experiment 1 showed that hatching of marble goby eggs occurred within the salinity FW and 15 psu SW and the optimal salinity was 10 psu SW. The brood fish is presumably a euryhaline fish. Besides, the marble goby was found to be in brackish water region during early life (unpublished data). Energetic costs were reported to be lower in an iso-osmotic environment compared to hyper-and hypoosmotic environment and that the energy savings are sufficient to permit to grow and survive (Morgan and Iwama 1991). This is consistent with the results of this study, where the growth and survival of marble goby larvae have successfully produced in 10 psu SW rather than in FW. Thus, it is considered that larvae in 10 psu SW had more energy to grow and survive than those in FW.

Following yolk and oil absorption, the fast growth of marble goby larvae in 10 psu SW and slow growth and death of those in FW was probably due to complications of osmo-regulatory energy requirements on the ability to feed. Osmo-regulation uses a high proportion of the available energy, from 20 to 50% of the total energy expenditure depending on the environmental salinity (Boeuf and Payan 2001). Energetic costs are

lower in an iso-osmotic environment compared to hyper- and hypoosmotic environment and that the energy savings are sufficient to permit to grow (Morgan and Iwama 1991). In this study, the larvae in 10 psu SW were in their preferred osmo-regulatory environment and presumably saved the energy otherwise used to maintain osmotic balance in the less favourable FW. The larvae in 10 psu SW presumably had more energy available to swim and start feeding. The larvae in FW had less energy to start feeding, and because they had difficulty or delay in feeding, they could not gain the energy to start growing. In fact, in preliminary experiment, the larvae were reared without food supply and the larvae in FW died within 5 days but larvae in 10 psu SW survive until 15 days. The finding that both feeding and non-feeding larvae at 4 dAH and 9 dAH developed the qualitatively similar sensory organs might indicate that sensory development is a "mandatory" energy expenditure and a conserved genetic character not easily altered by changes in environmental factors.

Non-feeding will resulted starvation and affected to the larval body and their feeding ability for instances sight and balance ability (Yin and Blaxter 1987; Shan et al. 2009; Bustos and Silva 2011). However, the sensory organ of larvae reared in both FW and 10 psu SW were developed. Preliminary experiment of frozen rotifer feeding was carried out to examine the feeding ability of larvae in both waters. The results showed than the larvae in FW did not feed and died eventually, and larvae in 10 psu SW fed well and survived. Starved larvae will be consumed endogenous feed and catabolized their body to survive until feed were found (Blaxter and Hempel 1963; Yin and Blaxter 1987). This is consistent to the marble goby larvae in FW. The larval growth in FW was retarded on 5 dAH, where the stage of yolk sac volume was fully consumed. They were unable to undergo the morphology and physiology development, and these resulted degradation body size of larvae in FW.

CHAPTER 4

Effects of water temperature on egg development, hatching success and early larval survival of marble goby *Oxyeleotris marmoratus* in 10 psu diluted seawater

4.1 Introduction

An essential step in the successful culture of all the species is to understand the optimal environmental conditions for egg incubation and larval rearing. However, little is known about the biological and ecological requirements of early life of this species as a basis for commercial cultivation. This leads to inconsistent and low larval survival that limits the seedling production of this species (Tavarutmaneegul and Lin 1988; Cheah et al. 1994). Previous studies showed that salinity (Watanabe et al. 1998) and temperature (Iglesias et al. 1995) are the key environmental factors affecting eggs hatching and larval survival.

Chapter 2 demonstrated that the egg incubated in 10 psu SW (diluted seawater) have shorter hatching period, higher hatching rate and better larval survival than those in freshwater (FW). Chapter 3 presented the larvae could not survive in FW but only 10 psu SW. Besides, Senoo et al. (2008) successfully produced marble goby seeds by increasing the salinity from FW to 10 psu SW after the larvae were hatched for the first 10 days. We have found that one of the factors contributing to high larval mortality is the delayed hatching which causes larval deformation (Nguang et al. 2012). The duration of hatching period can be shortened by controlling temperature is one of the most decisive environmental variables affecting fish egg and larval survival (Kamler 2002). However, there is no empirical study comparing the early development of marble goby under different water temperature. In this study, we aim to determine the optimal temperature for egg incubation and larval rearing to improve seedling production techniques of marble goby.

4.2 Materials and Methods

4.2.1 Brood fish management and egg collection

The experiment was carried out at the Centre for Collaborative Research in Aquaculture, Universiti Malaysia Sabah-Kinki University in October to December 2010. Marble

goby were collected from Penampang river, Sabah, Malaysia. Male and female brood fish reared in separate stock tanks at the hatchery for a year. Water temperature, DO, and pH for stock tanks ranged from 25.7-31.0°C, 5.8-8.0 mg/L, and 5.9-7.7 respectively. Brood fish were fed with the formulated moist pellet and *Sardinella* sp. until satiation every two days. For egg collection, a male of 300 g in BW and a female of 285 g were chosen. The female were injected intra-peritoneally with 1,000 IU/kg HCG (Senoo et al. 1993). After three days from the injection, approximately 20,000 eggs were collected with 98% fertility. Ages of the eggs and larvae were measured in hours and days starting from spawning time.

4.2.2 Incubation condition

At 15 minutes after spawning, 100 fertilized eggs were placed in a 9-cm diameter glass Petri dish and placed in each of fifteen 7 L transparent tanks (length x width x height; 18 x 26 x 17 cm) filled with 10 psu SW. Incubation water was prepared by mixing filtered seawater with aged dechlorinated tap water and filtered through a 40-μm mesh plankton net. Three tanks were placed together in a water bath with temperature of 24, 26, 28, 30 and 32°C and kept until hatching was completed. The hatched larvae were reared in each treatment until 10 dAS and given live rotifers *Brachionus* sp. at a density of 20/mL. Each tank provided with continuous aeration at 500 mL/min and kept under natural light conditions. Approximately 10% of larval rearing water was changed daily.

4.2.3 Data collection

Developmental stages of all eggs in the petri dish were monitored every 12 hours from 24 to 144 hAS under an optical-microscope (Nikon, Eclipse E600, Japan). Embryo developmental stage was differentiated into following four embryonic periods; cell cleavage, epiboly, organogenesis and organogenesis-growth. Figure 4.1 showed 20 stages of embryo development of marble goby during four embryonic periods. The time taken for 50% of the embryo to reach blastula stage, embryo formation and tails separated from embryo (stage G, J and N in Fig. 4.1, respectively) was compared between the treatment groups. Opaque eggs were considered dead and removed at the stage of G, J and N. Hatched larvae were removed, measured in total length and yolk sac sizes under a profile projector (Mitutoyo, PJ-3000) and photographed.

Effects of temperature on egg and larval survival were evaluated by comparing following criteria between the treatment groups: Egg mortality calculated as total number of dead eggs in stocked 100 eggs; total hatching rate calculated as the proportion of hatched eggs in stocked 100 eggs; deformation rate calculated as the total deformed larvae with a bent notochord among the hatched larvae; cumulative hatching rate calculated as the accumulated hatching rate at interval 12 hours from 24 to 144 hAS; and larval survival rate calculated as the total survived larvae in stocked 100 eggs at 10 dAS. The larval feeding intake was measured at two hours after feeding by observing gut content of 10 randomly selected larvae from each tank under an optical-microscope. Feed intake was determined by calculating the average number of ingested rotifer in the gut of larvae.

4.2.4 Statistical analyses

Effects of temperature on egg mortality, total hatching rates, deformation rates and larval survival rates was analyzed by analysis of variance (ANOVA) followed by Tukey's HSD test. Quadratic equation was used to describe the relationship between the hatching time and incubation temperature using the equation $y=a+bT+cT^2$ (where y = time in days; T = incubation temperature in degrees Celsius; and a, b, and c are constants) (Kamler 2002). All statistical analyses were preformed on SPSS Statistics 15.0 software (IBM Corp., New York, USA) and StatView Statistics 5.0 (Abacus Corp., Canada).

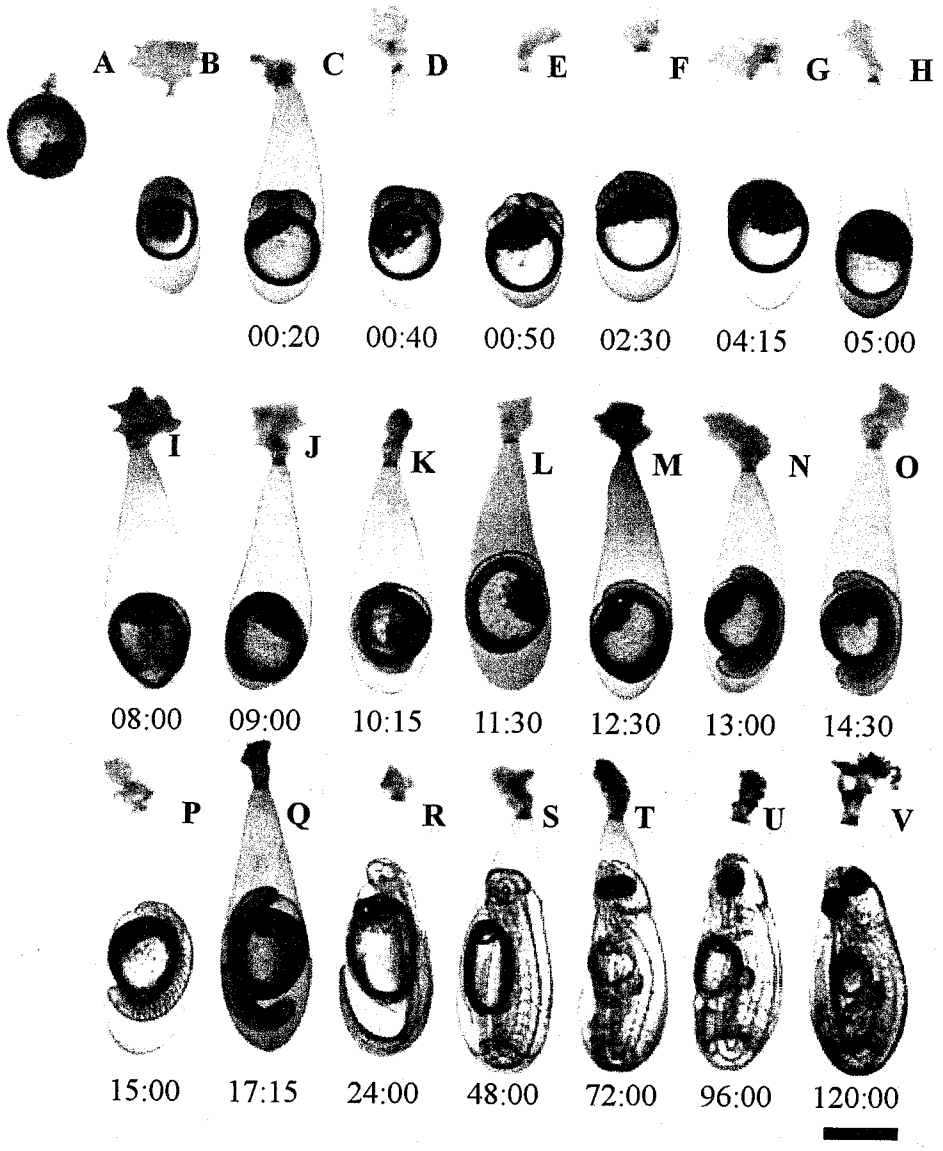

Fig. 4.1: Egg development of marble goby at 28°C in 10 psu SW: Four egg development periods: I, cell cleavage, formation of the blastodisc (B-F); II, epiboly, blastodisc spreading over yolk (G-I); III, organogenesis, formation internal organs (J-Q) and IV, organogenesis growth, frequent twitching observed and hatching occurred (R-V). (A) unfertilized egg, (B) fertilized egg, (C) 2-celled stage, (D) 4-celled stage, (E) 16-celled stage, (F) morula stage, (G) blastula stage, (H) gastula stage, (I) blastopore nearly closed, (J) embryo formation, (K) 5-myomere formed, (L) Kupffer's vesicle appeared, (M) optic vesicle appeared, (N) tail separated from the yolk sac, (O) otocyst vesicle appered, (P) head formed, (Q) lens and heart formed and embryo commenced moving, (R) hatching started, (S) body elongated, (T) vesicle of air bladder formed, (U) tail elongated to the head, (V) egg membrane transformed by developed embryonic head. The time showed at each egg developmental stage was first observed. Scale bar, 500 μm.

4.3 Results

4.3.1. Egg development and egg mortality

The embryonic development was accelerated by the increase of incubation temperature (Fig. 4.2). The quadratic equations fitted for each stage was summarised in Table 4.1.

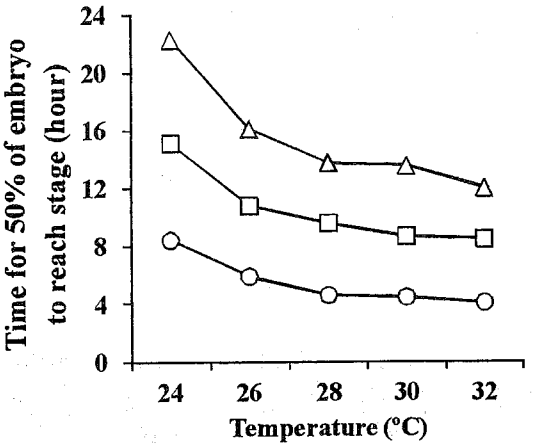

Fig. 4.2: Effect of temperature on the incubation time taken for 50% of marble goby eggs to reach three stages of egg development in 10 psu SW after spawning. Circle indicated blastula stage, square indicated the embryo formation and triangle indicated the stage of the tail separation from yolk sac.

Table 4.1: Quadratic equation $y = a + bT + cT^2$ for each development stage where y is the development time, T is the incubation temperature in degree Celsius (°C) and a, b and c are constants. The correlation coefficient (r^2) and level of significance (P) of the models are also indicated.

Stage	Temperature (°C)	a	b	c	r^2	P
Blastula stage	24-32	0.0971	-5.9488	95.134	0.9809	<0.05
Embryo formation	24-32	0.1523	-9.2957	150.21	0.9809	<0.05
Tail separation from yolk sac	24-32	0.2053	-12.651	207.19	0.9586	<0.05

For the total egg mortality, highest rate was noted at 24°C (87.7 ± 3.8%, mean ± SD, $n=3$) than those in 28°C (13.3 ± 3.2%), 30°C (17.0 ± 4.6%) and 32°C (11.7 ± 2.9 %) (ANOVA, $F_{4,10} = 533.8$, Tukey's HSD test, $P<0.001$) (Fig. 4.3).

Temperature had a significant effect on egg mortality in periods of cell cleavage, epiboly, organogenesis and organogenesis growth (ANOVA, Tukey's HSD test, $P<0.05$) (Fig. 4.4). Egg mortality in epiboly was significantly higher than other periods, with significantly higher at 24°C (11.3 ± 1.5%) than 32°C (2.3 ± 0.6%, mean ± SD, $n=3$).

Low egg mortality was observed in higher temperature. While there was no egg mortality observed at 30 and 32°C in cleavage period and in organogenesis growth period from 26 to 32°C. Meanwhile, significantly higher egg mortality was observed at 24°C: cell cleavage (3.7 ± 2.3%), epiboly (11.3 ± 1.5%), organogenesis (4.7 ± 1.9%) and organogenesis growth (3.7 ± 1.2%).

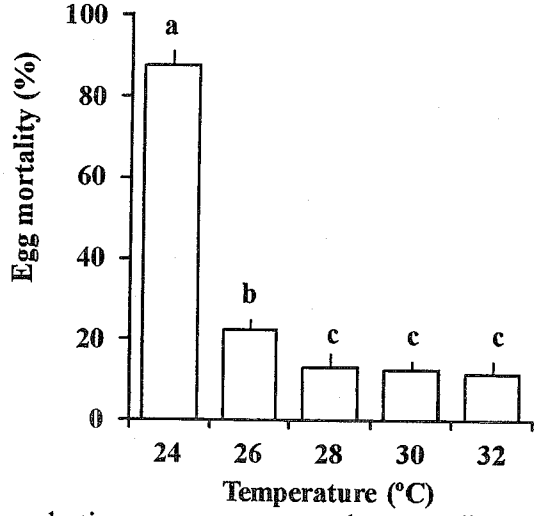

Fig. 4.3: Effect of incubation temperature on the mortality of marble goby eggs in 10 psu SW (means ± SD, n=3). Different letters above each bar indicate significant difference between treatments (ANOVA, $F_{4,10}$= 533.8, Tukey's HSD test, P<0.001).

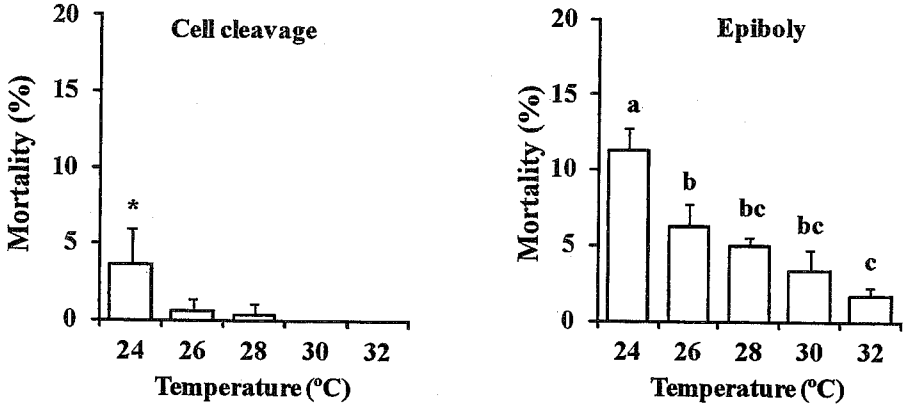

Fig. 4.4: Effect of incubation temperature on the mortality of marble goby eggs during periods of cell cleavage and epiboly. Different letters and asterisk above each bar indicate significant difference between treatments (ANOVA, $F_{2,8}$= 9.8 for cell cleavage period, $F_{4,10}$= 50.8 for epiboly period, Tukey's HSD test, P<0.05).

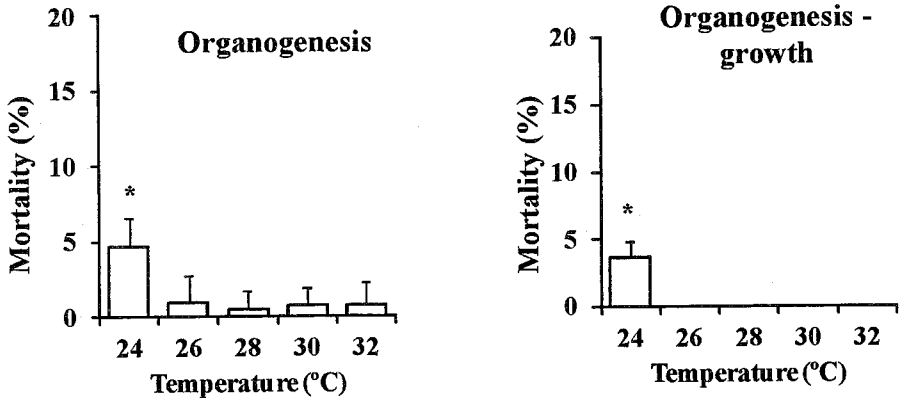

Fig. 4.5: Effect of incubation temperature on the mortality of marble goby eggs during periods of organogenesis and organogenesis growth. Different letters and asterisk above each bar indicate significant difference between treatments (ANOVA, $F_{4,35}$ = 5.4 for organogenesis period, Tukey's HSD test, P<0.05).

4.3.2 Total hatching, deformation rate and hatching period

Significant effects of incubation temperatures was also noted on total hatching rates (ANOVA, $F_{4,10}$ = 120.3, Tukey's HSD test, P<0.001) (Fig. 4.6). The hatching rates at the temperature between 26 and 32°C were significantly higher (77.0 ± 2.6% to 86.7 ± 3.2%) than at 24°C (12.3 ± 3.8%, n=3).

Deformation rate calculated as proportion of deformed larvae in the total eggs was also different between the incubation temperatures. All larvae hatched at 24°C were deformed with abnormally bended spines and eventually died on the tank bottom. The deformation rate at 26°C was 27.4±5.8% and it was significantly greater (ANOVA, $F_{4,10}$ = 16.7, Tukey's HSD test, P<0.05) than higher temperatures. Those at 28, 30 and 32°C were 9.2 ± 2.1%, 8.0 ± 2.9% and 3.5 ± 1.1%, respectively and were not different from each other (Fig. 4.6).

The first hatching commenced as early as 18 hAS at 32°C and the onset of hatching was temperature dependent. Hatching period of marble goby was relatively long and persisted until 48 hAS at 32°C (30 hours). This was even longer at lower temperature and lasted until 120 hAS at 24 and 26°C (Fig. 4.7).

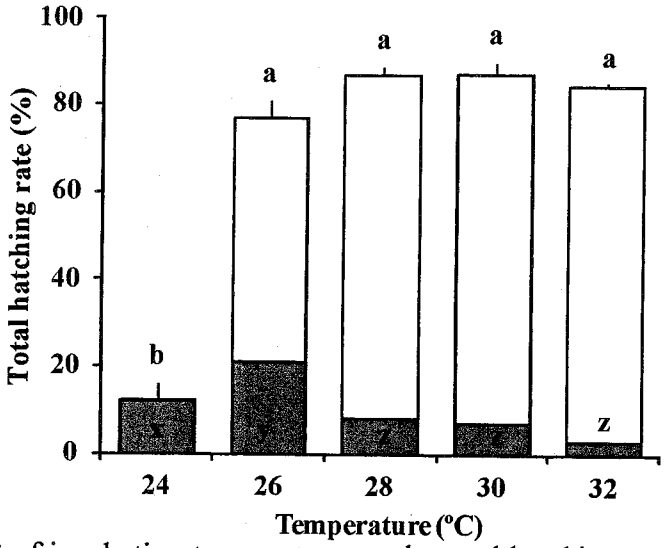

Fig. 4.6: Effect of incubation temperature on the total hatching rates of marble goby in 10 psu SW (means ± SD, n=3). White bar indicated normal larvae and gray bar indicated the deformed larvae. Different letters above each bar indicate significant difference between treatments (ANOVA, $F_{4,10}$= 120.3, Tukey's HSD test, P<0.001).

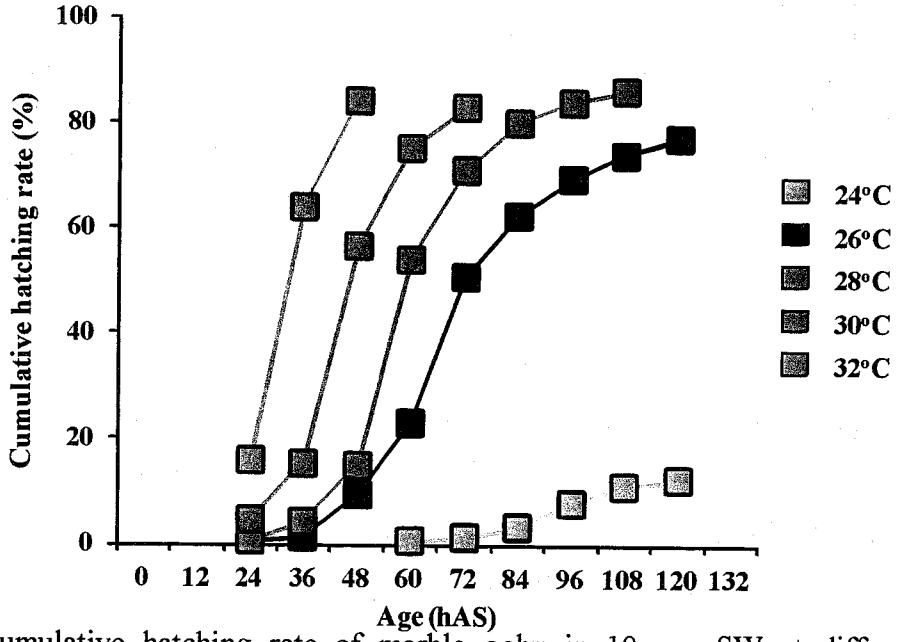

Fig. 4.7: Cumulative hatching rate of marble goby in 10 psu SW at different temperatures (n = 3).

4.3.3 Larval morphology

Average total length of larvae at first hatch at 30°C (2.88 ± 0.10 mm at 19 hAS) and 32°C (2.87 ± 0.12 mm at 18 hAS) were smaller compared to those hatched at 24°C (3.08 ± 0.11 mm at 60 hAS), 26°C (3.01 ± 0.10 mm at 24 hAS) and 28°C (3.01 ± 0.07 mm at

24 hAS) (ANOVA, $F_{4,10}$ = 4.8, Tukey's HSD test, P<0.05). All larvae hatched at 24°C were died within 5 dAS. Larvae showed rapid growth after 4 dAS and the growth rate at 26°C was lower than the higher temperatures (Fig. 4.8). Larval at 10 dAS at 26°C was significantly smaller (3.85 ± 0.13 mm) than those in the higher temperature (4.21 ± 0.09 mm to 4.26 ± 0.07 mm) (ANOVA, $F_{3,8}$ = 34.7, Tukey's HSD test, P<0.05).

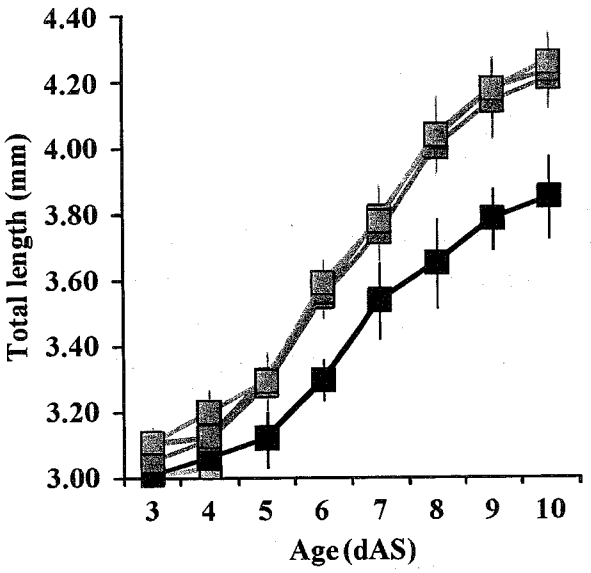

Fig. 4.8 Effect of temperature on total length of marble goby larvae until 10 dAS in 10 psu SW (means ± SD, *n*=3).

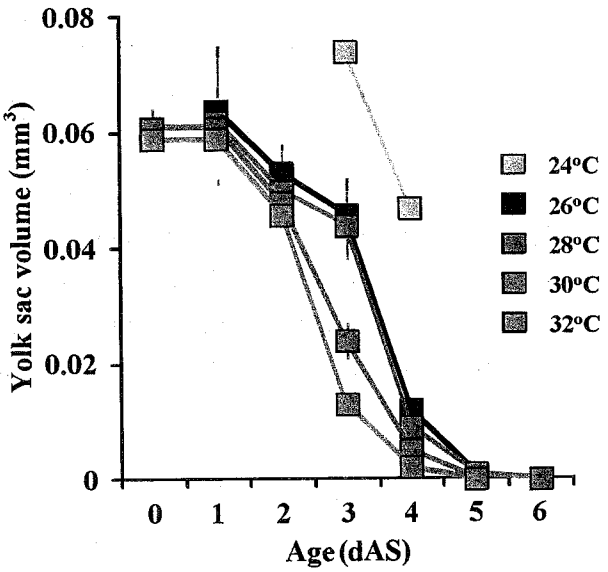

Fig. 4.9: Effect of temperature on yolk sac volume of marble goby larvae until 6 dAS in 10 psu SW (means ± SD, *n*=3).

Yolk sac volume at the first hatching was significantly larger at 24°C (0.074 ± 0.047 mm^3) bigger than other temperatures (ANOVA, $F_{4,10}$ = 16.1, Tukey's HSD test, P<0.05). The yolk sac absorption was completed by 5 dAS at 30 and 32°C and by 6 dAS at 28°C. No data was collected for the eggs kept at 24°C because all larvae died before yolk sac was completely absorbed (Fig. 4.9).

4.3.4 Rotifer ingestion and Larval Survival

The first feeding of rotifer was confirmed at 120 hAS at 26°C, 96 hAS at 28°C, 84 hAS at 30°C and 78 hAS at 32°C. Rotifer ingestion of larvae was significantly increase in higher temperature at first day feeding (ANOVA, $F_{3,36}$ = 11.3, Tukey's HSD test, P<0.05), with 5.8 ± 1.2 at 30°C and 7.0 ± 1.6 in 32°C, compared than those at 26°C (4.0

± 1.3) and 28°C (4.1 ± 1.2). The number of ingested rotifer gradually increased throughout the experiment period and reached 22.3 ± 2.5 and 22.7 ± 2.2 per fish at 10 dAS for those at 30°C and 32°C, respectively. These values were significantly greater compared to the larvae kept in 26°C (18.1 ± 2.3) and 28°C (19.0 ± 2.3) (ANOVA, $F_{3,36}$ = 7.8, Tukey's HSD test, P<0.05) (Fig. 4.10).

Larval survival was significantly affected temperature (ANOVA, $F_{3,8}$ = 63.5, Tukey's HSD test, P<0.05). Total survival rate at 10 dAS 20.0 ± 2.0%, 56.0 ± 4.0%, 46.0 ± 2.0% and 26.0 ± 3.6% for 26, 28, 30 and 32°C (Fig. 4.11).

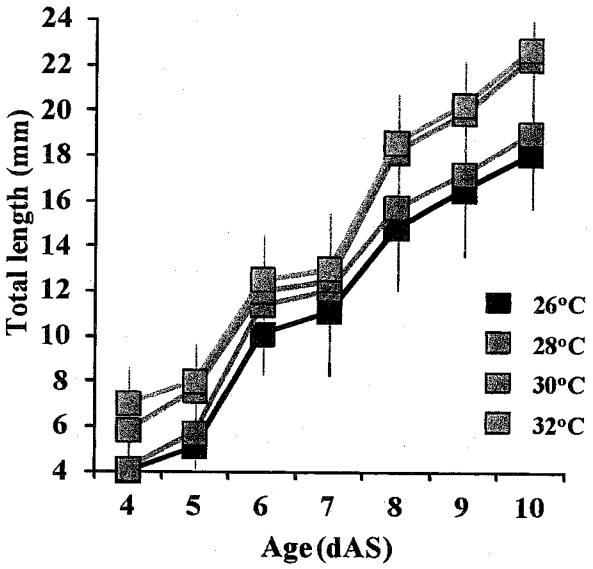

Fig. 4.10: Rotifer ingestion of larvae in different temperature until 10 dAS in 10 psu SW (means ± SD, n=3).

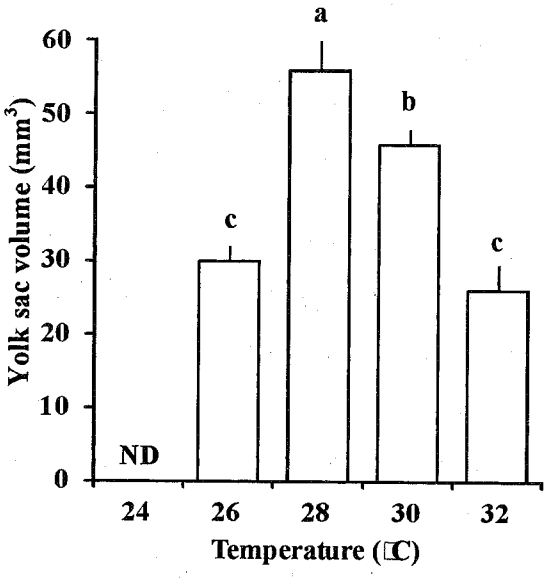

Fig. 4.11: Effect of temperature on larval survival of marble goby larvae until 10 dAS in 10 SW (means ± SD, n=3). Different letters above each bar indicate significant difference between treatments (ANOVA, $F_{3,8}$ = 63.5, Tukey's HSD test, P<0.05).

4.4 Discussion

This study clearly showed that temperature affects to egg development, hatching, larval deformation and survival of marble goby. Egg hatching occurred at temperatures within 24-32°C but 87.7 ± 3.8% of the eggs died at 24°C. Higher normal hatching and viable larvae was observed in temperature ranged from 26 to 32°C. Larval survival at 10 dAS was notably higher at 28°C than other temperatures. This is the first empirical data on hatching and larval survival under different temperature effects. Accordingly, natural

habitat of marble goby was recorded ranged from 22 to 28°C (tropical coordinator, 23°N to 18°S) (Riehl and Baensch 1996; Inger and Chin 2002). Yet, there is no information available on spawning and larval distribution in natural water body. Several studies showed the ambient temperature of brood fish, for instance leopard grouper *Mycteroperca rosacea* (Lopez et al. 2004), Senegalese sole *Solea senegalensis* (Anguis et al. 2005), Atlantic mackerel *Scomber scombrus* (Mendiola et al. 2006) and tiger puffer *Takifugu obscures* (Yang and Chen 2006), to be optimal for their egg incubation temperature. Some studies also found that the thermal tolerance of eggs was influenced by the temperature experienced of brood fish and spawning site (Anguis and Canavate 2004; Suquet et al. 2005; Nissling et al. 2006). In this study, marble goby spawning tank was maintained at 28°C. Base on the results, spawning temperature of the brood fish could be considered to be above 24°C, which is ranged from 26 to 32°C.

Tolerance limits of eggs to incubation temperature are commonly determined in relation to the egg mortality, which is tended to increase markedly at lower and upper extremes of temperature tolerance (Kinne 1963; Brooke 1975; Camus and Koustikopoulos 1984). Low temperature retarding resulted delaying in egg development, deformation and death of egg, for instance, greenback flounder *Rhombosolea tapirina* incubated at below 2°C (Blood 2001) and Atlantic mackerel *Scomber scombrus* at below 9°C (Mendiola et al. 2006). Some studies showed that fish egg incubated at low temperature had not developed eyes and misshapen tails (Nakatani and Maeda 1984; Blood 2001). Another studies showed that eggs developed in areas at low temperatures would experience higher mortality rates due to growth abnormalities (Jordaan and Kling 2003; Blood 2001). This is consistent to the results of marble goby in the present study, which the incubation temperature at 24°C was showed extremes under tolerance with most of the eggs died and all of the hatched larvae were deformed. While, the egg morphological development of marble goby at temperature from 26 to 32°C was normal. The morphological patterns of development that observed during this study were consistent with those reported by Senoo et al. (1994).

In egg development, high mortality particularly occurs during epibly stage, as the blastodisc spreading over the yolk, due to unsuitable incubation temperature (Camus and Koustikopoulos 1984), low oxygen level (Kaur and Toor 1978) and physical shock

(Holmefjord and Bolla 1988). This blastula stage is a sensitive morphogenetic period due to the forces involved in the cell migration process (Blaxter 1969). Low temperature at the early gastrula stage could cause delay in epiboly of periblast (Kazuyuki et al 1988). This is consistent to the result in this study, as the egg mortality of marble goby was particularly high during epiboly stage in all temperature treatments. Embryos died at low temperature as the embryo was torn physically in the cell migration process and at the margin between the germ ring or at the embryo shield (Ballard 1973; Wood and Timmermans 1988; Kimmel et al. 1995). This observation has provided important information on the probability of collection or transportation of marble goby egg for production.

Generally, lower temperature retards the rate of egg development and higher temperature accelerates it in the normal temperature range for hatching (Hart et al. 1996; Mihelakakis and Yoshimatsu 1998; Kamler 2002). In this particular species that possess long hatching period, hatching period was shorter at 32°C (18-48 hAS) and was consistent with the widely observed phenomena in many other fishes (Hart et al. 1996; Hansen and Falk-Petersen 2001; Kamler 2002). Previous study showed that deformation rate increased as egg hatching delayed beyond 72 hAF, which coincides with the time of eye pigmentation. The majority of the eggs in 10 psu SW hatched before 72 hAF and the deformation rate was relatively low (Nguang et al 2012). In this study, larvae hatched at 30 and 32°C were earlier and hatching period shorter lead to significantly lower deformation in hatching larvae (8.4±2.7% and 3.5±1.1% of hatched larvae, respectively) compared to others. Thus, higher temperature could be manipulated to hasten the egg hatching and decreased deformation of hatched larvae in marble goby seed production.

Growth, larval development and survival in temperate fish species have distinctive responses to temperature change. The relative importance of temperature change in the tropical region has been implied (Rombough 1997; Hunt von Herbing 2002) but rarely examined. Generally, tropical latitudes have little temperature fluctuation relative to temperate environments, due to the large ocean surfaces and absence of a cold season (McGregor and Nieuwolt 1998). Thus, small changes in temperature could have a greater impact on development of tropical fish larvae than larvae in temperate systems with naturally large temperature variation. In this study,

larval survival was significantly effects even in two degrees differences temperature from 24 to 32°C for marble goby

Growth is the most commonly measured response in temperature to fish (McMullen and Middaugh 1985; Zhang and Runham 1992). Fish larvae in higher temperature, which have an elevated metabolism, will consume nearly three times as much food to achieve average growth at ambient temperatures (Houde 1989), for instances, in striped trumpeter *Latris lineate* (Bermudes and Ritar 1999), Atlantic halibut *Hippoglossus hippoglossus* (Pittman et al. 1990a; 1990b) and sea bass *Lates calcarifer* (Marino et al. 1991). This indicates increased energy expenditure on maintenance metabolism at higher temperatures. Furthermore, the assimilation efficiency for fish larvae declined as temperature increased at high ingestion rates (Boehlert and Yoklavich 1984; Theilacker 1987; Houde 1989). This is consistent to the results of the present study, there is no significant different in total length among 28 to 32°C at 10 dAS, but the rotifer ingestion at 28°C was significantly lower than at 30 and 32°C at 10 dAS.

High egg mortality of marble goby at 24°C suggested that egg incubation at or below this temperature is not suitable for this species. There was no significant difference in total hatching rates among the higher temperatures (26 to 32°C), but deformation rate significant higher in 26°C and trend towards higher early larval survival existed at 28°C. The most favourable incubation conditions for egg survival and hatching are considered to be those that result in the greatest numbers of normal larvae. Thus, the suitable temperatures for incubating marble goby eggs suggested to be ranged from 28 to 32°C and the optimal temperature for early larval rearing is recommended at 28°C under the conditions of the present study.

CHAPTER 5

Recommendations and conclusion

The study results of salinity effects on egg incubation demonstrated that marble goby eggs hatched into viable larvae within FW to 15 psu SW. However, eggs incubated in 10 psu SW have significantly higher hatching rates, lower larval deformation and better larval survival than those in FW, even though, marble goby is a freshwater species. The most favourable incubation conditions for egg survival and hatching are considered to be those that result in the greatest numbers of normal larvae. Incubation in 10 psu SW, which resulted in a high hatching rate and a low deformation rate may produce better success in hatcheries. Thus, 10 psu SW was recommended for egg incubation of the marble goby. Microscopy observation on embryonic morphology and sensory organs showed similarity development in both FW and 10 psu SW. However, larvae that hatched at later period tended to be deformed, particularly hatched beyond 72 hAF, which is coincide with the deeply eye pigmentation. Hatching after 72 hAF was indicated delay hatching in marble goby. This information could be of practical value for checking the incubation conditions during seed production of marble goby.

The study of salinity effects on larval survival showed no apparent difference in larval morphology and behaviour in both FW and 10 psu SW during the first four days. Remarkable changes were noticed after first feeding was commenced. Larvae in FW become inactive and were unable to feed while larvae in 10 psu actively foraged and fed. Histological analyses of gut epithelium of larvae at 9 dAH showed significantly thinner of the gut of larvae in FW compared to those in 10 psu SW. All larvae in FW died at 9 dAH while the survival rate in 10 psu SW was 69.0 ± 2.0%. Therefore, the larvae were considered required brackish water physiologically. The results suggested that suitable salinity for early larval rearing of the marble goby is also 10 psu.

In the study of temperature effects on hatchability and early larval survival of marble goby, normal development, significantly lower egg mortality and higher hatching was observed in temperature from 28 to 32°C. Hatching period negatively

correlated with incubation temperature with the fastest hatching period of at 32°C (18 to 48 hAS). Higher temperatures hastened the egg development, hatching time and period. Significantly higher larval survival was observed at 28°C (56.0 ± 4.0%). These results showed the suitable temperature for incubation ranged from 28 to 32°C and for early larval rearing was at 28°C for the marble goby.

In the studies (Experiment 1), eggs were obtained via stripping to observe the development of fertilized eggs at the same time. However, for the egg collection and incubation techniques, Stripping is not recommended for fish farmers under hatchery conditions as it requires skill (Senoo et al. 1992; Senoo 2001b) and assessing final ovulation is difficult. Senoo (2003a; 2006) recommended natural spawning using a pair of marble goby brood fish, because the male parental eggs care of brushing and fanning are useful during hatching. All in all, from the experience of marble goby culture and the results that obtained from the studies, with natural spawning of marble goby is recommended under the following conditions:

1) eggs are spawned using a pair of brood fish in a tank with FW,
2) after spawning, the female is removed from the tank,
3) seawater is added the tank to give a final salinity of 10 psu SW,
4) incubation temperature settle ranged from 28 to 32°C,
5) the male cares for the eggs by brushing and fanning to the end of hatching,
6) the larvae are reared continuously in 10 psu SW at 28°C.

The most important matter to carry out stable production of marble goby under rearing condition is to keep and maintain optimum salinity and temperature condition during vulnerable and critical period. These methods are applicable on egg incubation and larval rearing and contributed to enhance the seed production of marble goby. However, the result is based on the eggs and larvae obtained from the brood stock captured in the river. The landlocked population may require different salinity and/or temperature for embryonic and larval development. Determination of population is likely important for application of the present data in egg incubation and larviculture of marble goby.

SUMMARY

Marble goby *Oxyeleotris marmoratus*, widely distributed in Southeast Asia and is one of most expensive freshwater fishes in the region. Owning to its taste, firm and white flesh and high protein value, marble goby being considered a first grade fish especially in Chinese restaurant and big cities such as Singapore, Kuala Lumpur and Hong Kong. Government in Thailand, Malaysia, Singapore and Indonesia encourage the seed production of marble goby since 1970's. However, wild resource has decreased due to overfishing for the last 40 years and fish farmer require a steady fish seed supply. To protect the wild resource and fulfill the demands, artificial seed production technique has been developed. Several studies on larviculture in this species had been conducted in FW, but Senoo et al (2008) reported that the rearing with 10 psu SW is indispensable for the larval survival for the first 10 days on marble goby in the State of Sabah, Malaysia. Yet, there is limited information concerning the effect of salinity on egg development, hatching and larval survival in marble goby. Little is known about the biological and ecological requirements for early life stage of this species. An essential step in the successful larviculture is to understand the optimal environmental conditions for egg incubation and larval rearing. Therefore, a series of experiments were carried out to determine the optimum salinity and temperature for eggs and larval survival of marble goby.

Salinity effects on marble goby eggs were investigated. In Experiment 1.1, hatching rate and larval deformation rate were first compared in eggs collected from a natural spawning and incubated in FW, 5, 10, 15, 20 and 30 psu SW. (1) eggs able to hatched into viable larvae in FW to 15 psu SW; (2) significantly higher total hatching rate and lower larval deformation rate found in 10 psu SW even though marble goby is regarded as freshwater species. The tolerance of egg in FW and 15 psu SW showed there is possibility of marble goby to spawn in low salinity and perhaps indicates that is should be classified as a euryhaline fish. However, there is lack information of distributional environments of spawning and larval stages in natural water body. In Experiment 1.2, the stripped eggs of marble goby were fertilized and incubated in FW and 10 psu SW for the closer comparison on hatching time and period. (1) egg in 10 psu SW hatching

more rapidly, had a shorter hatching period, higher hatching rate, with low deformation rate and better larval survival than those in FW; (2) Peak hatching in 10 psu SW (33.1 ± 5.6%) and FW (10.6 ± 3.4%) were observed in 48-60 and 72-84 hAF, respectively. Larvae hatched at later period tended to have higher deformation rates. The deformation rate increased when egg hatching was delayed beyond 72 hAF, which coincides with the time of eye pigmentation. The majority of the eggs in 10 psu SW hatched before 72 hAF and the deformation rate was relatively low. Hatching later than 72 hAF, was considered to be delayed in marble goby. This study presented that the first observations on the optimal salinity for egg incubation and hatching of marble goby, and the information which will be useful for its aquaculture and seed production.

Differences of larval survival of marble goby larvae in FW and 10 psu SW were determined in Experiment 2. Larvae obtained by natural spawning were reared in FW and 10 psu SW. No apparent difference in larval morphology and behaviour in both FW and 10 psu SW during the first four days. When larvae were morphologically prepared to be fed, larvae in FW become inactive and were unable to feed, while larvae in 10 psu were actively foraged and fed. The average number of ingested rotifer in the gut of larvae recorded only in 10 psu SW was 5.7 ± 2.3 at 4 dAH and gradually increased throughout the experiment period (19.0 ± 3.3 at 9 dAH). Histological analyses showed the gut epithelium of larvae in FW (9.0 ± 2.8 μm) at 9 dAH was significantly thinner those in 10 psu SW (23.7 ± 3.3 μm). All larvae in FW died at 9 dAH while the survival rate in 10 psu SW was 69.0 ± 2.0%. These larvae showed qualitatively similar development in sensory organs even though larvae not feeding in FW and the results suggest that sensory development is a mandatory energy expenditure and a conserved genetic character not easily altered by changes in environmental factors. The larvae were considered required brackish water physiologically for the survival and the results of Experiment 2 revealed that 10 psu SW is suitable for larval rearing of the marble goby.

Temperature effects on marble goby eggs and early larvae in 10 psu SW were investigated in Experiment 3. (1) significantly higher egg mortality observed in 24°C (11.3 ± 1.5%, mean ± SD, $n=3$) than others. Significant higher mortality occurred during the epiboly stage, in which the blastodisc spreading over the yolk, in all

temperature treatments; (2) no significantly different of total hatching rates in 26 to 32°C, all larvae hatched at 24°C were deformed. Hatching period negatively correlated with incubation temperature with the shortest hatching period of 48 hours at 32°C (18 to 48 hAS); (3) total length of larvae were no significant difference among 28, 30 and 32°C. The yolk sac absorption was completed in 30 and 32°C at 5 dAS and in 26 and 28°C at 6 dAS. No data collected at 24°C due to all died before yolk sac completely absorbed; (4) significantly higher larval survival was observed at 28°C (56.0 ± 4.0%). Higher temperatures hastened the egg development, hatching time and period. These results showed the suitable temperature for incubation ranged from 28°C to 32°C and for early larval rearing was at 28°C for marble goby.

These experiment results indicated that egg incubation and larviculture of marble goby should be conducted using 10 psu SW with temperature between 28 to 32°C, and preferably 28°C for early larval rearing. Both methods are applicable on egg and larval survival and contributed to enhance the seed production of marble goby. However, the result is based on the eggs and larvae obtained from the brood stock captured in the river. Other population such as landlocked population of marble goby may require different salinity and temperature for their egg and larval development.

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