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SHORT COMMUNICATION

Artificial fertilization and generating families for a selective breeding programme of large yellow croaker (*Larimichthys crocea*)

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Abstract Large yellow croaker is an important marine aquaculture species in China. The aim was to determine an appropriate protocol of artificial fertilization for family construction in the breeding programme based on two trials. In trial 1, luteinizing hormone-releasing hormone A_3 ($LHRHA_3$) was injected once, with a dosage of 2 μg/kg for females and 1 μg/kg for males. The latency time was in the range of 30–34 h. The maturation stage was checked by extracting a few eggs with a Pasteur pipette. The fertilization rate and hatching rate were 27 and 52%, respectively. The percentage of females with spawning difficulties was 30%. In trial 2, the females were injected $LHRHA_3$ twice: with a first dose of 0.8 μg/kg and a second dose of 2 μg/kg, at an interval of 10 h, whereas the males were still injected once. The latency time was in the range of 29.5–35 h, determined by only observing courtship behaviour of males. The females with spawning difficulties decreased to 10%, and the fertilization rate and hatching rate also improved to 41 and 62%, respectively.

Keywords Large yellow croaker · Artificial fertilization · Hormone injections · Latency time

Introduction

Large yellow croaker (*Larimichthys crocea)* is a batch spawner that naturally occurs in temperate seawater regions of East Asia. The aquaculture production was near 1.5×10^5 tons in 2015, accounting for 10% of the cultured marine fish production in China (Tang 2016), and now it is the largest marine aquaculture fish species in this country. However, the production is still based on strains that have not been subjected to a modern breeding programme which is crucial for further development of the aquaculture industry. A basic requirement for implementing a family-based sustainable breeding programme for a new fish species is the knowledge of control of reproduction to establish enough full- and halfsib families. Natural spawning at first glance seems to be a good choice to generate families, since large yellow croaker is sensitive to stress caused by handling (Duan et al. 2001). By natural spawning, a certain number of females and males are kept in a tank, and the species reproduce naturally. The consequence of this approach is that full- and halfsib families are mixed in

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the tank, and DNA-typing is required to obtain pedigree information; this will introduce a certain cost when a relatively large number of fishes need to get parental assignment. Moreover, since sperm competition gives unequal contributions from males, some broodfish are bound to have much larger genetic contributions than others (Liu et al. 2012). Thus, a relatively large number of non-contributing broodfish will inevitably reduce the effective population size (N_e) and increase the inbreeding rate. Single pair mating by natural spawning was also tested in some initial trials, but it was not successful; most likely because positive social interaction signals were lacking for the initiation of sexual maturation.

Applying artificial fertilization is an alternative way to establish full- and halfsib families for the breeding programme, but high-quality gametes are critical for successful fertilization. Post-ovulation oocyte ageing in the ovary of fish is one of the limiting factors for successful artificial fertilization (Bahre Kazemi et al. 2010). Thus, it is especially important to know the latency time, the time interval between hormone injection and stripping, when conducting artificial fertilization. The sperm-quality is easier to control compared to eggs. For instance, it was reported that very good-quality sperm can be obtained during the reproductive period, even without hormone induction in pikeperch (Zakes and Demska-Zakes 2005). However, sea water should be avoided during milt stripping, as the sperm motility will be activated by water and the duration of motility will last for only a short period for most fish species (Cosson 2004). With sperm energy storage exhausted after mobilization, the ability to fertilize eggs will quickly be lost consequently.

For cultured large yellow croaker, it is essential to determine latency time to avoid egg over-ripening in artificial fertilization. Moreover, in the breeding programme, spawning time should be synchronized to make full- and halfsib families as uniform as possible, both in size and age, and to reduce seasonal or temporal environmental effects on families. It was previously reported that the latency time for large yellow croakers was about 48 h with a dose of 1–2 μg/kg $LHRHA_3$ at a water temperature of 22–24 °C (Zheng et al. 2006). This reported latency time was initially tested under similar conditions, but the over-ripening problem was found to be very severe.

Thus, the aim of this study was to find an appropriate way to determine the best time for egg stripping after ovulation, and to establish an artificial fertilization protocol for large yellow croaker, as a preparation for a future selective breeding programme.

Materials and methods

Trial 1

Broodstock managements

In March 2013, 150 two-year-old large yellow croakers (sex ratio♀:♂ = 2:1), from the Mindong strain, were selected as broodstock from a farm in Fuding, Fujian province. All the selected candidates were in good health condition, with no body wounds. They were reared in a tank (6 m × 8 m × 1.2 m), with 80% daily water exchange, and were fed on minced fresh mackerels mixed with complex vitamins and minerals additives, twice a day, at a ratio of 5% of body mass per day. One month before mating, the broodstock were sorted by sex and then reared in separate tanks; making each group consist of 20 females and 10 males. Males were identified by the appearance of milt when the abdomens were pressed gently.

Hormone stimulation

Before hormone injection, each group of fish (20 females and 10 males) were anaesthetized with MS-222 (30–40 ppm) to minimize stress. This treatment took place at 06:00 in the morning, and the dosage of $LHRHA_3$ injection was 2 μg/kg for females and 1 μg/kg for males. Treated fish were placed in a breeding tank with clean seawater (24 °C) and aeration. Then the tank was covered with black plastic sheets to obtain a dark environment. All the lamps were also turned off in the incubation room and noises were minimized. Females were checked every 2 h from 26 to 36 h post injection, i.e. from 08:00 to 18:00 the next day. Females with large bellies were gently caught for closer examination. If the abdomen was soft, a few eggs were checked from the genital pore by a Pasteur pipette with a long tip. When the eggs were spherical, translucent and slick,

rather than collapsed and flaccid, it was considered to be the right moment to strip eggs. A few females were selected randomly for dissections to visualize the ovaries status, both before injection and after injection.

Gametes collection and artificial fertilization

Ready to spawn females and males were again anesthetized with MS-222 (30–40 ppm), and washed with clean seawater, wrapped in wet towels and the genital openings were dried off. The females were then stripped by gently stroking of the abdomens, and eggs from each female were collected in a separate 1000 ml beaker. Milt was collected into a 1 ml syringe, with gentle pressure to the male's abdomen, and then kept on ice in a polystyrene box (4 °C) until fertilization. During stripping, it was ensured that no water or urine contaminated the gametes. To generate paternal halfsibs and fullsibs, the milt from one male was used to fertilize eggs from two females. After stripping, these fish were put into a well-aerated tank, containing 200 L clean sea water, for recovery. Forty grams of eggs (800 eggs/g) and 0.2 ml (1.45×10^{10} sperms/ml) milt were placed in the same beaker, without adding any water. They were mixed with a sterilized feather for 40 s, and then 1 L clean seawater (24 °C) was added gradually and stirred with the feather at the same time. After 5 min of incubation, two layers of eggs appeared. The buoyant ones were collected, rinsed and then transferred to 1 m^3 breeding cylinders with 24 °C seawater (Fig. 1a) for further incubation, whereas sinking and white ones were considered as non-viable and thrown away. The average hatching time was 25 h.

On the second day after hatching, rotifers were put into the breeding buckets as live weaning feed for the larvae, on day six artemia were added, and on day 12, live copepods were added. The hatching and start-feeding buckets had 30% water exchange every day. Fifty days after hatching, the fingerlings (5 ± 1.1 cm in total length, Fig. 1c) were transferred to the net cages in the sea and each family cultured separately until tagging.

Trial 2

In March 2014, two-year-old broodstocks from the Daiqu strain (90 fish from a farm in Xiangshan, Zhejiang province) and the Mindong strain (90 fish from a farm in Fuding, Fujian province) were selected with a sex ratio of 2:1(♀:♂). The selection criteria were the same as in trial 1. The two strains were crossed, and paternal half- and fullsib groups generated. These fish were grouped into two types of mating cohorts: one including 10 Daiqu females and 5 Mindong males (six cohorts) and the other including 10 Mindong females and 5 Daiqu males (six cohorts). Based on the evaluation of trial 1, some adjustments were made to the breeding protocol:

Fig. 1 **a** Buckets for incubation; **b** larvae at hatching; **c** fingerlings, 50 days after fertilization

Double injections

The females in this trial were injected twice, at an interval of 10 h. The first injection was given at 6:00 in the morning, with a dosage of 0.8 μg/kg, and the second treatment was given at 16:00 in the afternoon, with a dosage of 2 μg/kg. The males were still only injected once, at the same time as the second injection of the female, with the same dosage as in trial 1, i.e. 1 μg/kg.

Courtship behaviour observation

In order to stimulate social signals for sexual maturation, the treated fish were placed in cohorts, as described above. The behaviour of the broodfish was monitored every half an hour from 28 h post injection, but without invasive checking by a Pasteur pipette. The time was recorded as latency time when males' courtship behaviour appeared; i.e. males chased females and emitted repetitive throbbing or drumming sounds by vibration of the sonic muscles near the swimming bladder, thereby the name croaker. Then artificial fertilization was performed in the same manner as in trial 1.

Fertilization rate and hatching rate of the two trials

The fertilization rate and hatching rate were calculated from six randomly sampled fullsib families. The fertilization rate was first calculated by observation of 100 randomly fetched buoyant eggs 2 h after fertilization, examined under a microscope. At this time, the development of normal eggs had reached the stage of 32-cell-division, which was possible to identify in the fertilized eggs. Then the fertilization rate was converted to be based on 100 total stripped eggs by the ratio of buoyant and sinking eggs. The hatching rate was determined as the proportion of hatched larvae to 100 fertilized eggs. Fertilization and hatching rates of the two trials were compared using a *t* test.

$$\text{Fertilization rate} = \frac{\text{Number of fertilized eggs}}{\text{Total number of stripped eggs}} \times 100\%$$

$$\text{Hatching rate} = \frac{\text{Number of hatched larvae}}{\text{100 fertilized eggs}} \times 100\%$$

Results

In trial 1, the latency time for females was 30–34 h, using a single $LHRHA_3$ injection and by Pasteur pipette checking, in 23.6 °C seawater. In trial 2, the latency time was 29.5–35 h, using double injections and by courtship behaviour observations, in 23.7 °C seawater. The fertilization rates of the two trials were 27.5 ± 2.1 and 41.4 ± 2.3%, respectively; significantly different ($p < 0.05$). The hatching rates of the two trials were 52.3 ± 4.8 and 61.5 ± 8.7%, respectively; not significantly different ($p > 0.05$) (Table 1).

Before injections, the females ready to spawn had pink genital papilla (Fig. 2a). The white and tiny eggs in the ovary were connected with each other like one cohesive substance (Fig. 2b). After injections, most females had large bellies and soft abdomens. These females could be stripped easily and ovulated eggs were separable and transparent (Fig. 2c), but there were still some white immature eggs left in the ovaries.

Also, eggs in some females were found hard to strip even after full latency time, both in trial 1 and trial 2. Despite extremely large bellies, the abdomens did not soften. The eggs inside were white and still connected with each other, although some eggs' colour changed from white to semi-transparency (Fig. 2d). These females had spawning difficulties and were impossible to strip. In trial 1, the percentage of these poorly

Table 1 Fertilization rates and hatching rates

Trials	Water temperature ± SD (°C)	Latency time range (h)	Fertilization rate (%) ± SD	Hatching rate (%) ± SD
1	23.6 ± 0.16	30–34	27.5 ± 2.1	52.3 ± 4.8
2	23.7 ± 0.14	29.5–35	41.4 ± 2.3	61.5 ± 8.7

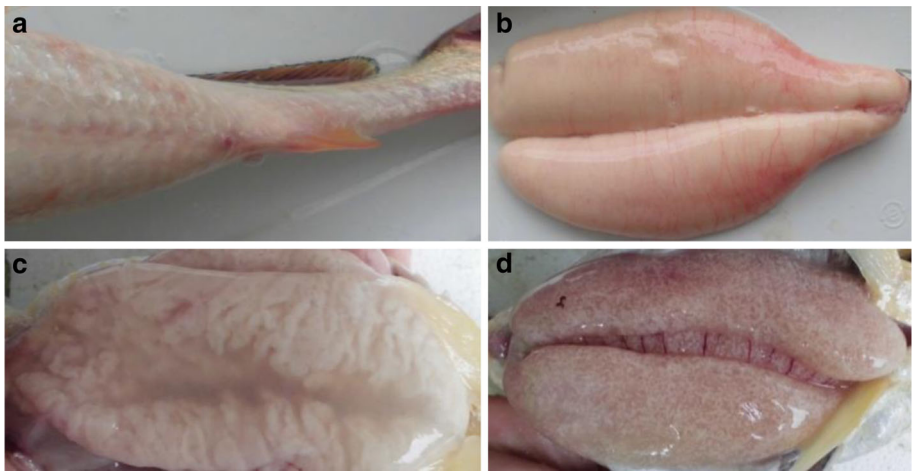

Fig. 2 **a** Pink genital papilla of females; **b** immature eggs in the ovaries, before hormone injection; **c** mature eggs, ready to be stripped; **d** non-mature eggs after injection that could not be stripped easily, even after expected latency time

matured females was about 30%, whereas in trial 2 it decreased to about 10%, both in the Mindong and the Daiqu strain. Eventually, 37 families were generated with 150 broodfish tested in trial 1, whereas 60 families were obtained with 180 broodfish tested in trial 2.

Discussion

For cultured large yellow croakers, hormone stimulation is necessary to induce final oocyte maturation and synchronize the ovulation. $LHRHA_3$ (a GnRH analogue) can have a stimulating effect on pituitary gonadotrophs and consequently increase the secretion of gonadotropins. In trial 1, $LHRHA_3$ was injected once, but there were still 30% poorly matured females whose eggs were hard to strip, and the fertilization rate was low. In trial 2, using double injections, the poorly matured females decreased to 10%, and the fertilization rate increased significantly. The hatching rate was also improved, although not significantly. The first injection was done to promote maturation of the eggs and the second one was to induce the release of eggs from the ovary (Shan Jian 1985). For Senegalese sole (*Solea senegalensis*), a repeated GnRHa injection protocol was also more effective than a single injection for stimulating egg production by stripping, without compromising egg quality (Rasines et al. 2012). Double injections with $LHRHA_3$ are thus recommended for large yellow croaker, especially for the less matured parent fish, where it can substantially accelerate the maturation of the eggs. The hatching rate could be affected partly by herterosis effect from crossing of two strains in trial 2.

Other spawning hormones may also be evaluated for more successful spawning, e.g. human chorionic gonadotropin (hCG). In pikeperch, the highest percentage of ovulated females was obtained in the group stimulated with hCG, giving up to 100% ovulation, and the development of the oocytes in this group was also more rapid than in the group stimulated with only GnRH analogue (Zakes and Demska-Zakes 2005).

The latency time is very important to determine the optimal egg-stripping time, and a significant determinant for the egg quality (Craik and Harvey 1984). If egg stripping is done much later than optimal latency time, ageing of ovulated eggs in the ovary or the coelomic cavity occurs, causing egg over-ripening, which is associated with a decrease in egg viability. In rainbow trout (*Oncorhynchus mykiss*), biochemical and histological changes occur inside the eggs and in the ovarian fluid during over-ripening (Lahnsteiner 2000), and the egg viability significantly decreased with the egg protein fragments being accumulated in the ovarian fluid (Hélène et al. 2004). The duration time of ovulated egg viability in the fish ovarian fluid seems to be temperature dependent. For example, cold water fish species like rainbow trout (*Salmo gairdneri* R.) can hold their eggs in the ovaries for 4–6 days post-ovulation, at about 10 °C, without decrease in viability (Springate et al. 1984), and Atlantic salmon eggs were still of good quality even for one week after ovulation at 9.1 °C (de Gaudemar and Beall 1998). In contrast, in warm-water fish species, eggs deteriorate rapidly after ovulation. For instance, the maximum period between ovulation and the deterioration for striped bass (*Morone*

saxatilis) was only 15–30 min (Rottmann and Chapman 1991). As large yellow croaker is a warm-water species (18–25 °C), the duration time of ovulated egg viability could be very short, and the latency time between hormone injection and stripping is thus critical to avoid egg over-ripening. The previous report only mentioned the latency time, but the measuring method was not given (Zheng et al. 2006). In this study, the latency time is much shorter and the over-ripening problem is improved by observing courtship behaviours.

Most females of large yellow croaker could be stripped easily, and good-quality eggs were obtained when the correct latency time was applied, but there were still some immature white eggs left in the ovaries. The reason could be that large yellow croaker is a batch spawner. Monitoring method may be another factor that affected eggs' maturation processes. In trial 1, monitoring by a long tip Pasteur pipette was a too invasive method, which caused the fish to become overly nervous and stressful.

The handling stress likely induces cortisol synthesis, which could affect reproductive characteristics of the fish by altering gonadal steroids levels through the hypothalamus–pituitary–gonadal axis. In jundia (*Rhamdia quelen*), handling stress of mature females resulted in higher cortisol level, but lower 17β-estradiol level, compared to the control group; fewer oocytes could be stripped from the stressed fish, and the quality of these eggs appeared reduced (Soso et al. 2008). Stress during reproductive development also delayed the ovulation and reduced the quality of gametes in rainbow trout (Campbell et al. 1992). Large yellow croaker is a stress-sensitive fish, and the maturation and ovulation of oocytes might be stopped as a reaction to the handling stress by Pasteur pipette checking applied in trial 1. Consequently, the higher occurrences of females' spawning difficulties could be due to the successive checking and handling before complete egg maturation. In trial 2, the latency time was determined by observing courtship behaviour and less spawning difficulties occurred. More non-invasive methods should thus be tested for measuring the stages of reproductive maturation in the stress-sensitive fish, such as measurement of sex steroid hormones in surface mucus and use of ultrasound imaging for monitoring gonadal development (Schulz et al. 2005; Novelo and Tiersch 2016).

Conclusion

From these two trials of artificial fertilization in large yellow croaker, we generated 37 and 60 families, respectively. Although the number of tested families in each generation was a bit low for an efficient breeding programme, the suggested protocol would still be helpful for family construction by artificial fertilization in a future selective breeding programme. The main points were to select broodfish at the same age and size, perform double hormone injections and keep handling of the fish at a minimum level. However, there are still additional improvements of the protocol before satisfactory artificial fertilization is achieved, such as routines to further minimize the handling stress.

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Compliance with ethical standards

Ethics statement This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Ocean University, Zhoushan, China, and all persons involved in fish handling had special training before conducting the relevant experiments.

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