Establishment of Hatchery-Reared Broodstock of Spotted Scat (*Scatophagus argus* Linnaeus, 1766) and Its Reproductive Performance

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I hereby certify that this work has not been accepted in substance for any other degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การเตรียมพ่อแม่พันธุ์ปลาตะกรับ (*Scatophagus argus* Linnaeus, 1766) จากการเลี้ยงและศักยภาพในการเพาะขยายพันธุ์
ผู้เขียน นายจิระยุทธ รื่นศิริกุล
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บทคัดย่อ

ศึกษาการเตรียมพ่อแม่พันธุ์ปลาตะกรับ (*Scatophagus argus* Linnaeus, 1766) จากการเลี้ยงในโรงเพาะฟักและศักยภาพการเพาะขยายพันธุ์เพื่อรองรับการผลิตลูกปลาเชิงปริมาณในอนาคต โดยมีการศึกษา 4 เรื่อง ได้แก่ ผลของความเค็มของน้ำต่อการผสมเทียมปลาตะกรับและอัตรารอดของลูกปลาวัยอ่อน ผลของความเค็มของน้ำต่อประสิทธิภาพการสืบพันธุ์ของแม่ปลาตะกรับในโรงเพาะฟัก ระยะเวลาการสร้างไข่ชุดใหม่และประสิทธิภาพการสืบพันธุ์ของแม่ปลาตะกรับหลังการผสมเทียม และความสมบูรณ์เพศและความเป็นไปได้ในการเปลี่ยนเพศของปลาตะกรับ

การศึกษาที่ 1 ศึกษาผลของความเค็มของน้ำ 8 ระดับ (0, 5, 10, 15, 20, 25, 30 และ 35 พีพีที) ต่อการผสมเทียมปลาตะกรับที่จับจากทะเลสาบสงขลาและผลการอนุบาลลูกปลาวัยอ่อน โดยใช้แม่พันธุ์ผสมเทียมกับพ่อพันธุ์จำนวน 5 ครั้ง ผลการผสมเทียมพบว่า เปอร์เซ็นต์การตกไข่ของแม่ปลาที่ความเค็ม 0 พีพีที (20 เปอร์เซ็นต์) มีค่าต่ำกว่าความเค็มอื่นๆ (80 - 100 เปอร์เซ็นต์) อัตราการปฏิสนธิและอัตราการฟักมีค่าสูงที่ความเค็ม 25-35 พีพีที (62.8 - 66.9 เปอร์เซ็นต์ และ 53.6 - 54.9 เปอร์เซ็นต์ ตามลำดับ) อัตรารอดของลูกปลาอายุ 15 วัน มีค่าสูงที่ระดับความเค็มของน้ำ 10 และ 15 พีพีที (40.5 - 41.0 เปอร์เซ็นต์) การศึกษาที่ 2 ศึกษาผลของความเค็มของน้ำต่อประสิทธิภาพการสืบพันธุ์ของแม่ปลาตะกรับที่ได้จากการเพาะพันธุ์และเลี้ยงในโรงเพาะฟักจนถึงวัยเจริญพันธุ์ โดยคัดเลือกแม่ปลาที่มีรังไข่ระยะพักและพ่อปลาสมบูรณ์เพศเลี้ยงในถังพลาสติกความจุ 1 ลูกบาศก์เมตรที่ระดับความเค็มของน้ำ 5, 15, 25 และ 33 พีพีที ถังละ 10 ตัว (เพศผู้ 4 ตัวและเพศเมีย 6 ตัว) เป็นระยะเวลา 4 เดือน พบว่าศักยภาพการเพาะขยายพันธุ์ ฮอร์โมนเพศ (เอส-ตราไดออล) และค่าออสโมลาลิตี้ของซีรัมของปลาที่เลี้ยงแต่ละความเค็มไม่มีความแตกต่างกัน (p>0.05) ยกเว้นอัตราการตกไข่ของแม่ปลาที่เลี้ยงที่ระดับความเค็มน้ำ 15 และ 25 พีพีที มีค่าสูงกว่าระดับความเค็มอื่นๆ

(p<0.05) การศึกษาที่ 3 ศึกษาระยะเวลาการสร้างไข่ชุดใหม่และประสิทธิภาพการสืบพันธุ์ของแม่ปลาตะกรับที่เลี้ยงในโรงเพาะฟักหลังการผสมเทียม โดยผสมเทียมปลา 5 ครั้งและศึกษาการพัฒนาการของเซลล์สืบพันธุ์ทางเนื้อเยื่อวิทยา พบว่าแม่ปลาใช้ระยะเวลาพัฒนาไข่ชุดใหม่หลังการรีดไข่เฉลี่ย 41.6 ± 6.8 วัน ศักยภาพการเพาะพันธุ์ของการผสมเทียมครั้งแรกและครั้งที่สองภายหลังการพัฒนาไข่ชุดใหม่ส่วนใหญ่ไม่แตกต่างกันยกเว้นขนาดไข่ก่อนฉีดฮอร์โมน เปอร์เซ็นต์การตกไข่และเปอร์เซ็นต์ไข่ดีของแม่ปลาที่สร้างไข่ชุดใหม่ (381.3 ± 16.0 ไมครอน, 37.5 ± 26.2เปอร์เซ็นต์ และ 54.8 ± 21.2 เปอร์เซ็นต์ ตามลำดับ) ที่เล็กกว่า/น้อยกว่าแม่ปลาที่มีไข่ในครั้งแรก (422.8 ± 12.1 ไมครอน, 72.9 ± 17.0 เปอร์เซ็นต์ และ 81.3 ± 7.2 เปอร์เซ็นต์ ตามลำดับ) (p<0.05) ผลทางเนื้อเยื่อวิทยาพบเซลล์ไข่ระยะ hydrated oocytes, pre-vitellogenic oocytes, vitellogenic oocytes และ post-vitellogenic oocytes ซึ่งพบเป็นส่วนใหญ่ภายในรังไข่ของปลาอายุ 1 15 30 และ 45 วัน หลังรีดไข่ตามลำดับ การศึกษาที่ 4 ศึกษาความสมบูรณ์เพศและความเป็นไปได้ของการเปลี่ยนเพศของปลาตะกรับเพศผู้ที่เลี้ยงในโรงเพาะฟักแบบ protandrous คือเป็นเพศผู้ก่อนแล้วจึงเปลี่ยนเป็นเพศเมียในภายหลัง โดยการเลี้ยงสองแบบคือเลี้ยงพ่อปลาเพียงเพศเดียว 50 ตัว เป็นระยะเวลา 18 เดือน และเลี้ยงพ่อปลาร่วมกับแม่ปลารวม 70 ตัว (สัดส่วน 1 : 2.5) เป็นเวลา 12 เดือน ในบ่อซีเมนต์ความจุ 28 ลูกบาศก์เมตร ตรวจสอบความสมบูรณ์เพศและการเปลี่ยนเพศทุก 3 เดือน พบว่าความสมบูรณ์เพศของพ่อปลาที่เลี้ยงทั้งสองแบบไม่แตกต่างกันยกเว้นปริมาณน้ำเชื้อของปลาที่เลี้ยงเพศเดี่ยวมีแนวโน้มลดลง และไม่พบการเปลี่ยนเพศของปลาเพศผู้ในการเลี้ยงทั้งสองแบบ

การศึกษาครั้งนี้ชี้ให้เห็นว่าปลาตะกรับเป็นปลาชนิดที่ทนความเค็มน้ำในช่วงกว้างอย่างดีเยี่ยม อย่างไรก็ตามการผสมเทียมปลาตะกรับต้องการความเค็มสูงแต่การอนุบาลลูกปลาควรใช้ความเค็มลดลงตามพัฒนาการของลูกปลา การเลี้ยงแม่พันธุ์สามารถใช้ระดับความเค็มของน้ำได้ในช่วงกว้างโดยไม่ส่งผลลบต่อการสร้างไข่และคุณภาพไข่ แม่ปลาตะกรับสร้างไข่ชุดใหม่หลังจากการรีดผสมเทียมโดยที่ศักยภาพการเพาะพันธุ์เมื่อเปรียบเทียบกับไข่ชุดแรกส่วนใหญ่ไม่แตกต่างกัน รวมทั้งไม่พบการเปลี่ยนเพศของพ่อปลาในการศึกษาครั้งนี้ ข้อมูลที่ได้เป็นประโยชน์ต่อการเตรียมพ่อแม่พันธุ์ปลาตะกรับจากการเลี้ยงในโรงเพาะฟักเพื่อการวางแผนการผลิตลูกปลาเชิงปริมาณเพื่อการเพาะเลี้ยงเชิงพาณิชย์ในอนาคต

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ABSTRACT

Establishment of cultured spotted scat (*Scatophagus argus* Linnaeus, 1766) in hatchery and its reproductive performance for mass seed production in the future were determined. Four studies were investigated: 1) effects of water salinity on the artificial insemination of spotted scat and the survival rate of the larvae, 2) effects of water salinity on reproductive performance of female spotted scat, 3) timing for oocyte recruitment and reproductive performance of female hatchery-reared spotted scat after artificial insemination and 4) the maturation and possible protandrous sex change of hatchery-reared spotted scat.

The first study, effects of eight water salinity levels (0, 5, 10, 15, 20, 25, 30 and 35 ppt) on five artificial insemination of spotted scat caught in Songkhla Lake and larval rearing were investigated. The results showed that female broodfish held at 0 ppt produced the lowest ovulation rate (20%) while significantly (p<0.05) greater ovulation rates (80 - 100%) were observed at other water salinity levels. The fertilization and hatching rates were also significantly higher (p<0.05) at 25-35 ppt than at other water salinity levels (62.8 - 66.9 % and 53.6 - 54.9 % respectively). A high survival rate of 15-day-old larvae was observed at 10 and 15 ppt (40.50 - 41.00%). The second study, effect of water salinity on the reproductive performance, serum 17β-estradiol (E2) levels and osmolality of female hatchery-reared spotted scat were investigated. Female broodfish in resting stage and mature male were selected and maintained in 1-m^3 tanks with holding water salinities of 5, 15, 25 and 33 ppt at density of 10 fish/tank (4 males and 6 females) for 4 months. The results showed that none of the reproductive parameters including the E2 profile and blood osmolality differed

significantly between treatments except that the ovulation rate of the fish held in water salinities of 15 and 25 ppt were significantly higher than those of other salinities (p<0.05). The third study, timing for oocyte recruitment and reproductive performance of spotted scat from 5 consecutive inseminations were determined. The results showed that the timing for oocyte recruitment was 41.6 ± 6.8 days. Most reproductive performance parameters of the first and the second artificial insemination were not significantly different (p>0.05), except for oocyte diameter before hormone injection, ovulation rate and viable egg of recruited females (381.3 ± 16.0 µm, 37.5 ± 26.2 % and 54.8 ± 21.2 %, respectively) that were smaller/lower than those of the former matured female broodfish (422.8 ± 12.1 µm, 72.9 ± 17.0 % and 81.3±7.2%, respectively). Histological investigation of the ovarian found hydrated oocytes, pre-vitellogenic oocytes, vitellogenic oocytes and post-vitellogenic oocytes were predominantly located in the mature ovaries of 1, 15, 30 and 45 days after stripping, respectively. The final study, maturation and the possible protandrous sex change (male in early age, and female when elder) of hatchery-reared spotted scat was undertaken. Mature male fish were selected for 2 rearing regimes in different 28 m^3 tanks: in tank 1, only 50 males were reared for 18 months, and in tank 2, both males and females were reared together at a ratio of 1:2.5 (70 fish in total) for 12 months. Maturation and sex change were investigated every 3 months. The reproductive performance of two rearing regimes was not significantly different (p>0.05) except milt volume of the male fish in tank 1 seemed to have decreased gradually. There was no finding of protandrous sex change in both culture regimes.

These studies indicate that spotted scat is an excellent euryhaline species. However, artificial insemination of spotted scat should be conducted at high salinities while lower water salinity levels should be applied for larval rearing following development of the larvae. Broodstock rearing can be applied wide range of water salinity which was unaffected oocyte development and egg quality. Mature female scat recruited new oocyte clutch after stripping which was indifference in most of reproductive performance compare with the former clutch. The evidence of sex change was not found in this study. Obtained data are useful for spotted scat broodstock establishment in hatchery for mass seed production planning which support commercial aquaculture in the future.

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CHAPTER 1

INTRODUCTION

The spotted scat (*Scatophagus argus* Linnaeus, 1766) (Figure 1.1) is classified in the family, Scatophagidae. It is a euryhaline teleost which is commonly distributed in the Indo-Pacific region (Nelson, 1976) and inhabits a range of water salinity, from fresh or brackish water to pure marine conditions. It is found in estuaries, mangroves and river mouths, environments which are constantly changing. As a result, the spotted scat is well adapted to living in fluctuating conditions. Moreover, it has also shown good feeding adaptation and flexibility and is an omnivorous and opportunistic feeder (Wongchinawit and Paphavasit, 2009). Thus, this fish has a high potential for coastal aquaculture (Barry and Fast, 1988).

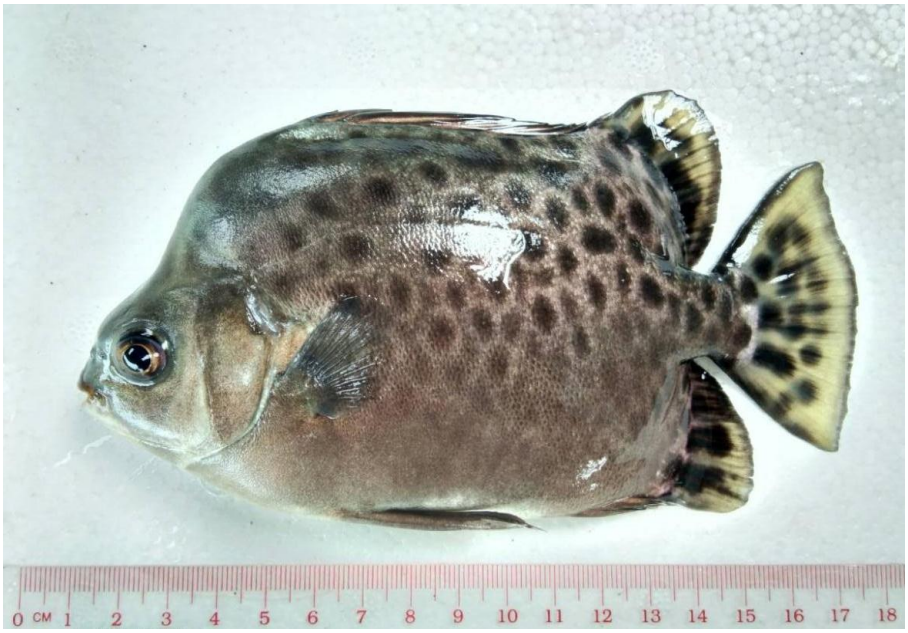

Figure 1.1 Spotted scat (*Scatophagus argus* Linnaeus, 1766)

The spotted scat is an edible fish and in some countries such as the Philippines, it is one of the highest-priced fish (Barry and Fast, 1992) as well as being famous as an ornamental fish which is traded in both domestic and international markets (Jayalal and Ramachandran, 2012) because of their attractive shape and color, and scat fry collected in the wild are in market demand (Barry and Fast, 1992). Because of the many advantages of this species, many countries have tried to study spotted scat reproduction and breeding, including the Philippines (Barry *et al*., 1988), Taiwan (Chang and Hsieh, 1997), China (Cai *et al*., 2010), India (Gandhi *et al*., 2014), Vietnam (Khanh *et al*., 2012) and Thailand (Ruensirikul *et al.*, 2008).

In Thailand, the spotted scat was successfully bred by the Department of Fisheries in 2008 using artificial fertilization (Ruensirikul *et al.*, 2008), and since then larvae have been reared (Ruensirikul *et al.*, 2009) and juvenile fish have been produced for fish farmers to rear to marketable size. However, most of the broodfish were collected from the wild and the main problem encountered has been uncertainty about the quantity and quality of the wild broodstock. Moreover, wild broodfish are available only in some seasons (Mair, 2002). Thus, plans for the long term production of spotted scat fry on a commercial scale should include domesticated hatchery-reared broodstock (Duarte *et al*., 2007) in a similar manner to other economic fish that have been successfully farmed, such as sea bass, *Lates calcarifer* (Fuchs and Nedelec, 1989), milk fish, *Chanos chanos* (Emata *et al*., 1992), grouper, *Epinephelus striatus* (Ranjan *et al*., 2014) and salmon, *Oncorhynchus nerka* (Maynard *et al*., 2012).

A previous study of the culture of captive spotted scat (Ruensirikul *et al*., 2012) found that the egg size of hatchery-reared broodstock was smaller than that of wild broodfish and the quality of the eggs was also lower. Moreover, it has been

commonly found that the oocyte of captive broodfish were unable to reach the final oocyte maturation stage (Mylonas and Zohar, 1998). One of the most important causes may be an inappropriate culture environment, which does not meet the requirements of the fish. Stress can occur in this condition and negatively affect the fish's immunology (Davis *et al*., 2002) and its reproductive ability (Campbell *et al*., 1994; Castranova *et al*., 2005). Thus, it is important to understand the ecology of the target fish in natural conditions when establishing fish broodstock in a hatchery. Suitable culture environmental conditions will help the fish to adapt themselves to captive conditions and to develop their reproductive system normally (Mylonas *et al*., 2010). Therefore, in spotted scat, an understanding of the natural environment where the broodfish were caught is essential for successful gonad development and spawning (Barry and Fast, 1988). In particular, the water salinity is very important in euryhaline species or brackish water fish. Several studies have been conducted either in natural waters, such as in three species of flatfish; *Limanda limanda*, *Pleuronectes platessa* and *P. flesus* (Nissling *et al*., 2002) and turbot, *Scophthalmus maximus* (Nissling *et al*., 2006) or in laboratory/hatchery conditions of, for instance, black bream, *Acanthopagrus butcheri* (Haddy and Pankhurst, 2000) and Brazilian flounder, *Paralichthys orbignyanus* (Sampaio *et al.*, 2007). In addition, the effect of water salinity on the artificial fertilization and survival of spotted scat larvae was investigated by Khanh *et al.* (2012) who found that a level of water salinity of 30 ppt produced the highest hatching rate of 43.3% in spotted scat. However, the optimum water salinity for artificial fertilization and early larval rearing were not reported.

Apart from the manipulation of the environment to minimize stress in broodstock and to ensure the satisfactory quality of reproductive cells, the effective

utilization of hatchery-reared broodstock is one of the most important factors in successful fish breeding. Maintaining excessive broodfish may increase the production costs while having only a small number may result in there being insufficient broodfish for efficient mass seed production. Thus, understanding the reproductive cycle of broodfish in terms of timing and the quality of reproductive cells in each development period needs to be intensively considered. Additionally, sex reversal in broodfish can affect the sex ratio (Allsop and West, 2004) and the number of available male/female broodfish can affect broodstock management planning. Changes in the sex ratio of fish due to sex reversal have been found in several marine fish species. However, in spotted scat, the role of sex reversal is still unclear. Arrunyakasemsuke (1975) suggested that the spotted scat might be a protandric hermaphrodite, being male in the early stage of its life cycle and then changing into a female later, since in the wild, females are commonly found in greater numbers than males. This agrees with the findings of Shao *et al.* (2004), who observed ovotestis in two captive male spotted scat.

The aims of this study were to establish hatchery-reared spotted scat broodstock and to utilize those broodfish for mass seed production, which will benefit commercial spotted scat aquaculture in the future.

Objectives

The specific objectives were as follows:

1. To study the effect of water salinity on the artificial fertilization and survival rate of the larvae of spotted scat.
2. To study the effect of water salinity on the development and quality of the reproductive cells of hatchery-reared spotted scat.

3. To study the development and quality of the reproductive cells of hatchery-reared spotted scat after artificial fertilization.
4. To study sex reversal in hatchery-reared spotted scat.

This thesis is organized in a sequence reflecting the above objectives. **Chapter 1** includes the introduction, overview and objectives of this study while **Chapter 2** consists of a literature review relating to basic knowledge and previous findings related to the study. **Chapters 3** and **4** focus on the effects of water salinity on the artificial insemination, larval rearing and reproductive performance of hatchery-reared spotted scat. **Chapters 5** and **6** focus on the oocyte development, reproductive performance after artificial breeding and the possibility of protandric sex change in hatchery-reared spotted scat. Finally in **Chapter 7,** the overall data from all the chapters is summarized. The results obtained by this study and reported in this thesis represent key knowledge applicable to the establishment of hatchery-reared spotted scat for mass seed production and the promotion of the commercial scale coastal aquaculture of this species.

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CHAPTER 2

REVIEW OF LITERATURE

2.1 Spotted scat and their reproductive biology

The spotted scat (*Scatophagus argus* Linnaeus, 1766) is classified in the family Scatophagidae. It is a euryhaline teleost which is commonly distributed in the Indo-Pacific region (Nelson, 1976).The spotted scat has a deep, strongly compressed body form with a small mouth and head. Its food preference is mainly plant material such as filamentous algae. It demonstrates allometric growth (Sawusdee, 2010). The largest scat found was 33.4 cm in total length and 1.2 kg in weight. The length-weight relationship has been estimated as $w = 0.07058 \times SL^{2.881}$ and $w = 0.18976 \times SL^{2.526}$ in males and females, respectively, where w = weight in grams and SL = standard length in centimeters (Barry and Fast, 1992). In the wild, the number of females is higher than that of males at a ratio of male: female of 1: 2.2 (Gandhi *et al*., 2014). Sexual differentiation can be determined by head shape.

The earliest females mature at 7-9 months of age, when they are around 14 cm in length and weigh approximately 150 g, and the smallest male found with running milt was 11.5 cm in length and weighed 83.5 g. In the Philippines, the spawning season of the spotted scat is June-July, which is the period of the SW monsoon (Barry and Fast, 1992). Gandhi *et al*. (2014) stated that the smallest mature scat were12.0-12.4 cm (male) and 14.0-14.5 cm (female) and the spawning season in India was observed to be between June and November and between April and August in China (Cai *et al*., 2010). Khanh *et al.* (2012) found that the highest number of gravid female spotted scat of 26.9% occurred in August.

This species performs multiple-spawnings during the spawning season (Cai *et al*. 2010). Fecundity depends on female weight according to the equation: n = 983w - 66,904, where n = number of eggs and w = wet weight of female broodfish in grams (Barry and Fast, 1992).The fecundity of 265 g females was 115, 038 and that of 350 g females was 153, 661 (Gandhi *et al*., 2014), while the reports of Khanh *et al*. (2012) and Cai *et al*. (2010) was were 2,469 egg/g and 720-963 egg/g respectively. Gonadal development was classified into seven stages by Gandhi *et al*. (2014): immature, developing immature/recovering spent, maturing, mature, advanced mature, ripe and spent. The oocyte size ranged from 0.10-0.75 mm (Gandhi *et al*., 2014).

A number of investigations of sex steroid hormone levels in spotted scat have been reported. Zhang *et al*. (2013) studied the sex hormone level of 2-year old mature females fed with different feed-stuffs for 8 weeks and found that the estradiol-17β hormone was increased following the culture period, in the range of 15-40 pg/ml, while testosterone varied between10 and 20 ng/ml.

2.2 Spotted scat breeding

Spotted scat breeding was firstly investigated by Barry and Fast (1992). They observed the pairing and mating behavior of captive spotted scat in 200-l rearing tanks after an LHRH hormone-induced female and two males were placed together. However, at that time, spawning behavior was not observed. In 1997, artificial breeding of spotted scat was successfully accomplished in Taiwan (Chang and Hsieh, 1997) following which, research in several different countries was carried out on breeding this species, such as Thailand (Ruensirikul *et al*., 2008; 2009), China (Cai *et al*., 2010), and Vietnam (Khanh *et al*.,2012). Almost all the breeding studies have been done by the artificial propagation method. Ovulation inducement is applied by the injection of

hormones such as LHRHa or HCG. Khanh *et al*. (2012) found that a dosage the hormone, Ovaprim of1 ml/kg obtained the highest ovulation rate ins cat of 93.3%, with a fertilization rate of 76.5% and a hatching rate of 69.5% as well as the lowest rate of abnormal larvae at 2.16%. Cai *et al*. (2010) applied LHRHa at a dosage of 15 µg/kg and DOM (Domperidone) at 3.0-4.0 mg/kg or LHRHa at 15 µg/kg and HCG at 1000-1500 IU/kg to induce natural spawning in captivity while Ruensirikul *et al*. (2008) induced gravid females solely with LHRHa at 10-20µg/kg and obtained a 60-80% ovulation rate,70-92% fertilization anda 43-88% hatching rate. Zhang *et al*. (2018) found that are combinant of LH and FSH can activate gonadal maturation in spotted scat. To date, the success of mass seed production of this species has not been reported.

2.3 Relationship of water salinity and biology of spotted scat

Changes in water salinity especially in an estuarine environment require fish to adjust their internal osmotic balance in order to survive. In freshwater conditions, teleost suffer from salt loss and water gain while the opposite phenomena occurs in marine conditions. However, the osmotic tolerance of euryhaline fish has not been well investigated (Gui *et al.*, 2016). Almost all studies on the effect of water salinity on spotted scat have been focused on water salinity tolerance and culture performance in different water salinity conditions, and better growth and survival rates have been found in brackish water than in fresh or salt water. Socorro *et al.* (1988) stated that spotted scat larvae can tolerate a wide range of water salinity of 0-40 ppt. Chang *et al*. (2005) found that 24-day-old spotted scat larvae were more tolerant of abruptly changing water salinity than were 12-day-old larvae. Khanh *et al.* (2012) studied the effect of water salinity on the hatching ability of spotted scat and found that water salinity of 30 ppt provided the best hatching rate (43.3 %) while 5 ppt produced the best growth (0.026

g/day) and survival rate (92.8%) of juvenile scat (30-60 days old). This finding was similar to that of Ruensirikul *et al*. (2012) that gradually decreasing water salinity at the age of 10-20 days old produced a significantly higher survival rate (90.0-93.8%) as compared to a stable water salinity level throughout the larval rearing period (51.7-52.7%).

Studies of the effect of water salinity on the reproductive performance of spotted scat have been rare and almost all the investigations which have been conducted have involved the relationship between water salinity and sperm quality. Chang *et al*. (2005) found that a longer period of scat sperm availability was observed when the sperm was activated in water with a water salinity of 15-20 ppt. This was similar to the study of Ruensirikul *et al*. (2009), which reported a high sperm motility of 97.2-100% after activation in water with a water salinity of 15-25 ppt. The spawning behavior of this speciesis, however, still unclear. Barry and Fast (1988) suggested that the spawning ground of spotted scat was brackish water and that scat will not spawn where the water salinity is lower than 10 ppt. Moreover, Cai *et al*. (2010) affirmed that scat need high water salinity water for spawning.

Because water salinity requires osmoregulation in the fish body, the study of the effect of water salinity on the biology or ecology of fish, especially euryhaline fish usually focuses on the relation of blood osmolality and the osmolality of the surrounding water, with the aim of finding the isosmotic point and the water salinity level at that point. At the isosmotic point, the energy demand is minimized, which benefits growth and reproductive development. Typically, blood or water osmolality will increase following the water salinity level. In flounder (*Paralichthys orbignyanus*), the isosmotic point was found to be 328.6 mOsm/kg at a water salinity

of 10.9 ppt (Sampaio and Bianchini, 2002) while in sole (*Dicologoglossa cuneata*), the isosmotic point was 284 mOsm/kg at a water salinity of 10.4 ppt (Herrera *et al.*, 2009). In a study of spotted scat Chang *et al*. (2005) determined that spotted scat fry reared in fresh and marine water presented close blood osmolality levels of 420 mOsm/kg and 452 mOsm/kg, respectively.

2.4 Sex reversal in spotted scat

Because the ratio of female to male spotted scat in natural conditions is high, some researchers have suggested that spotted scat may be sequentially hermaphroditic fish (Arrunyakasemsuke, 1975) and that it is probably a protandric species which is male in the early stages of development but will change into female later. However, scientific confirmation of this has not yet been adduced. Only the study of Shao *et al*. (2004) seems to provide strong evidence in support of this hypothesis. They found two inter-sex spotted scats with sizes of 15 and 17 cm total length, which had ovotestis. A transverse section of the ovotestis showed pre-vitellogenic oocytes scattered throughout the testicular tissue (Figure 2.1)

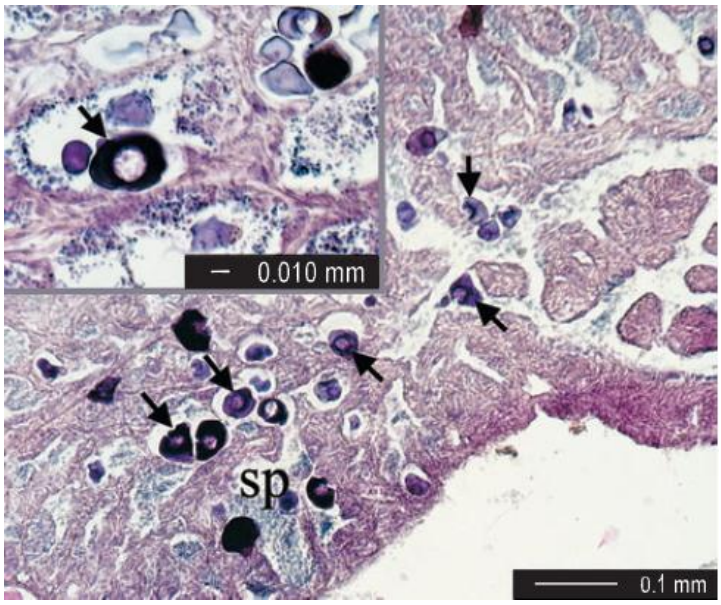

Figure 2.1 Histological micrographs of ovotestis of the spotted scat showing pre vitellogenic oocytes (arrows) in the testis (sp= spermatozoa) (Shao *et al*., 2004).

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CHAPTER 3

EFFECTS OF WATER SALINITY ON THE ARTIFICIAL INSEMINATION OF SPOTTED SCAT (*Scatophagus argus* Linnaeus, 1766) AND THE SURVIVAL RATE OF THE LARVAE

Abstract

The effects of eight water salinity levels (0, 5, 10, 15, 20, 25, 30 and 35 ppt) on the artificial insemination of spotted scat caught in Songkhla Lake were investigated. Female spawners, average size 115.79 ± 13.71 g (92.50 - 127.67 g) were taken for artificial fertilization with mature male individuals, size 72.30 ± 22.88 g (51.26 - 110.23 g). Five artificial inseminations were conducted with eight female spawners and 30 male individuals, each. The female spawners were separately maintained in tanks with different water salinity levels after injection with the LHRHa hormone. After ovulation, ovulation rate, latency time, viable eggs, egg size and oil globule diameter at different water salinities were determined. After artificial fertilization, the fertilized eggs were placed in tanks with different water salinity levels. The fertilization and hatching rate were monitored. The abnormality rate total length and survival rate of the larvae at each different water salinity level, from hatching out until day 15 were determined on five occasions. The results showed that broodfish held at 0 ppt produced the lowest ovulation rate (20%) while significantly (p<0.05) greater (80-100%) ovulation rates were observed at other water salinity levels. The fertilization and hatching rates were also significantly higher (p<0.05) at 25-35 ppt than at other water salinity levels (62.76 - 66.88% and 53.59 - 54.85% respectively) but the fertilization rate was not significantly different (p>0.05) at 20 ppt (55.20%). The

abnormality rate of larvae was significantly lower (p<0.05) between 25 and 30 ppt (3.87 and 4.93%, respectively) but not significantly different between 20 ppt (7.30%) and 35 ppt (5.53%). A high survival rate of 15-day-old larvae was observed at 10 and 15 ppt (40.50-41.00%) but the rates were not significantly different (p>0.05) from those obtained at 5, 20 and 25 ppt. These results indicate that the artificial insemination of spotted scat should be conducted at high salinities while lower water salinity levels should be applied for the first 15 days of larval rearing to increase breeding performance.

Introduction

The spotted scat (*S. argus*) is classified in the Scatophagidae family. It is a euryhaline species with a high ability to adjust to and inhabit any water salinity gradient around the Indo-Pacific region (Nelson, 1976). The environmental conditions of its habitat fluctuate widely. Consequently, the spotted scat has to adapt itself to survive at all times in different conditions. These characteristics suggest that the spotted scat is a promising species for coastal aquaculture.

Due to demand both as a food fish and an aquarium fish, the spotted scat fry mostly collected from the wild are insufficient to meet market demand (Gandhi *et al*., 2014). Researchers in several countries have tried to breed this species (Barry *et al*., 1988; Chang and Hsieh, 1997; Cai *et al*., 2010; Khanh *et al*., 2012) including in Thailand (Ruensirikul *et al*., 2008; 2009a). In Thailand, spotted scat juveniles have been produced both to increase the natural stock as well as to provide fish to fish farmer to culture to a marketable size. However, breeding performance has been mixed and the number of fish fry produced is still inadequate to meet the quantities required by fish

farmers to undertake commercial spotted scat culture. The development and improvement of efficient breeding and larval rearing techniques will benefit fish fry production both through increased quantity and quality which are important factors for expanding spotted scat culture in the future.

Water salinity is one of the most important factors affecting fish growth, spawning and larval rearing. Understanding what level of water salinity is appropriate for the natural reproductive mode of fish is very useful in hatchery operations. Several brackish water fish have been studied (Haddy and Pankhurst, 2000; Nissling *et al*., 2002; 2006; Sampaio *et al.*, 2007) but spotted scat have not, to date, been well investigated. In Songkhla lake, the natural water habitat includes a water salinity gradient from salt, through brackish to freshwater depending on the area and season. In the past, spotted scat were abundant in Songkhla Lake (Sirimontaporn, 1984). Gravid female spotted scat were collected in various water salinity levels although the most suitable water salinity for spawning and the conditions most favorable for newly hatched larvae were still unknown. The data from this study will be useful for the development of spotted scat breeding and scat hatchery-reared broodstock in the future.

Khan *et al*. (2012) studied the effect of water salinity on the hatching success of scat and found that a water salinity level of 30 ppt produced the highest fertilization rate at 43.3 %. However, there has been no report of the optimal water salinity level for artificial fertilization or that for newly hatched larvae during the critical period for marine finfish larval rearing during in the first 10 days after hatching. Almost all the studies of the effect of water salinity on scat larval rearing have focused on either juvenile scat or the older stage. Those studies have found that the best results were obtained in brackish water at a salinity level of 5 ppt (Khanh *et al*., 2012).

The objective of this study was to examine the effect of water salinity on the success of the artificial insemination of spotted scat caught in Songkhla Lake and its effect on the early stage of scat larval rearing. The results will provide baseline information for mass seed production and subsequent commercial aquaculture of this species in the future.

Materials and methods

Experimental design

The experimental design of this study was Randomized Complete Block Design, (RCBD). The study was divided into two experiments: experiment 1 was a study on the effect of water salinity on the artificial insemination of spotted scat,which comprised eight treatments (eight water salinity levels of 0, 5, 10, 15, 20, 25, 30 and 35 ppt) with five replications. Experiment 2 was the study of the effect of water salinity on the survival of spotted scat larvae, which comprised eight treatments which were similar to those in the first experiment.

Water preparation

Eight water salinity levels were prepared by diluting cleaned sea water with a water salinity of 30-33 ppt with freshwater, or mixing it with salt until it reached the target water salinity level, checked by a refracto-salinometer (ATAGO). Each batch of tested water was kept in 1 m^3 tank for further use in artificial propagation and larval rearing.

Broodstock preparation

Spotted scat broodstock were captured from the mouth of the outer part of Songkhla Lake (water salinity of 27-30 ppt, water temperature of 28-29 ^oC) using a

net trap, ten times (the first five times for experiment 1 and the last five times for experiment 2). Mature captured broodstock were selected according to the method described in Ruensirikul *et al*. (2008). For each time, eight mature females (115.8 ± 13.7 g weight, 14.5 ± 0.6 cm total length) and 30 mature males (72.3 ± 22.9 g weight, 12.9 ± 0.9 cm total length) were selected and transferred for acclimation into the hatchery of the Coastal Aquaculture Research Institute, Songkhla, Thailand. The broodstock were kept in a 1 m^3tank with gentle aeration until the time that hormone injection was performed to induce ovulation.

Experimental protocols

Experiment 1: study on the effect of water salinity on the artificial insemination of spotted scat.

Artificial insemination of the spotted scat was conducted five times according to the method of Ruensirikul *et al*. (2008). Each mature female was injected with 20 µg/kg LHRHa hormone to induce ovulation. Then, the injected fish were transferred and kept separately in 200l tanks, each of which contained one of the eight water salinity levels. When the broodfish ovulated, the latency time and percentage of viable eggs were recorded (Rasines *et al*., 2012). The female broodfish were stripped of their ovulated eggs by applying gentle pressure to the belly then 1 ml of ovulated eggs was placed into each of eight beakers containing 50 ml of the designated water salinity levels. Then, 20 µl pooled milt stripped from three mature male broodfish were mixed with the ovulated eggs in each beaker. The fertilization rate in each water salinity level was monitored at the cleavage stage (2-4 cell division). The fertilized eggs in the beaker which obtained the highest fertilization rate (at least 50 %) of each insemination was equally divided and transferred to hatch in eight 2-liter plastic jars containing the

eight water salinity levels being tested. Each hatching jar was aerated mildly until the larvae hatched out. The hatching rate and abnormality rate of the larvae (the number of crooked-body larvae in100 sampled larvae) were checked. The total length of day 0larvae (6-12 h post hatching) was checked for 30 larvae in each jar by using an ocular micrometer with a light microscope. At the end of the experiment, the percentage ovulation rate at each water salinity level was calculated by comparing the number of injected broodfish that ovulated in 32-44 h after hormone injection with the total number of injected females.

Experiment 2: study on the effect of water salinity on the survival of spotted scat larvae.

The artificial breeding of spotted scat was conducted according to the method of Ruensirikul *et al*. (2008) using three to five mature females each time at a water salinity of 25-30 ppt, to produce at least 200,000 larvae/time. These larvae were used for larval rearing at the same eight water salinity levels employed in Experiment 1. Spotted scat larval rearing was performed in 1 m^3 plastic tanks at a density of 10 larvae/liter (Ruensirikul *et al*., 2009a) until the larvae grew to the flexion stage at about 15 days old. There was no feeding for 0-2-day-old larvae. But thereafter, rotifers (*Brachionus rotundiformis*) at a density of 5-10 ind/ml were fed to the 3-15 days old larvae after the yolk sac was completely absorbed. Scat larval rearing was conducted five times in the same way as in Experiment 1. The water quality was controlled to the optimal level for marine fish nursing, i.e., a temperature of 27-29 ^oC, a pH of 7.4-8.1, dissolved oxygen of 5.8-6.1 mg/l, total ammonia of 0.62-0.84 mg/l and nitrite of 0.016-0.080 mg/l (Philminaq, 2008). The larvae obtained from each water salinity level were sampled for total length measurement at the ages of 3, 7 and 15 days old (50 ind/water

salinity level). At those ages, the survival rate of each treatment was estimated using the volumetric method. The water in each tank was drained until 200 liter remained then the sample of larvae (100 ml) were counted in triplicates for subsequent calculation of the total number of larvae in each tank.

Statistical analysis

All data were presented as means ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare the parameters (latency time, viable egg, egg size, oil globule diameter, fertilization rate, hatching rate, abnormal larvae survival rate and total length of larvae) of each treatment, with a significance level of $p<0.05$. Duncan's multiple range test was applied to determine significant differences among means of the water salinity treatments. The Chi-Square test was used to analyze the difference in the ratio of the ovulation rate.

Results

Effect of water salinity on artificial insemination

The ovulation rate of the broodfish in water salinity levels of 5, 10, 15, 20, 25, 30 and 35 ppt were not significantly different ($p>0.05$) and were within a range of 80-100%, while there were significant differences at all positive levels of water salinity with a level of 0 ppt which produced only a 20% ovulation rate since under that condition, almost all the broodfish delayed their ovulation. The latency time of the broodfish held in salinities of 5, 15, 25 and 35 ppt (36.1-38.4 h) was significantly lower than that of 0 ppt (42.1 h; $p<0.05$) but not significantly different from that of 10, 20 and 30 ppt (39.1-39.6 h). The percentage of viable eggs (61.0-81.7%), egg size (587.5-609.4

μm) and oil globule diameter (220.9-232.7 μm) of each treatment were not significantly different (p>0.05; Table 3.1).

Table 3.1 Ovulation rate, latency time, viable eggs, egg size and oil globule diameter of spotted scat artificial insemination at different water salinities

	Water salinity (ppt)							
	0	5	10	15	20	25	30	35
Ovulation rate[1] (%, n=5)	20	100	100	100	100	80	80	80
Latency time[2] (h)	42.1	36.1±2.1^{b}	39.6±1.9^{ab}	38.4±2.2^{b}	39.5±1.3^{ab}	38.1±2.2^{b}	39.1±3.5^{ab}	36.7±4.1^{b}
Viable egg[3] (%)	67.41	78.9±7.0^{a}	70.1±13.8^{a}	76.4±15.1^{a}	74.3±23.4^{a}	81.7±21.9^{a}	61.0±15.1^{a}	61.4±34.1^{a}
Egg size[4] (μm)	609.4	591.7±39.3^{a}	595.0±38.1^{a}	599.4±34.2^{a}	602.9±43.7^{a}	599.0±32.7^{a}	587.5±56.0^{a}	598.6±32.6^{a}
Oil globule diameter[5] (μm)	232.7	231.0±7.7^{a}	228.8±12.0^{a}	228.2±8.9^{a}	224.1±6.2^{a}	220.9±3.5^{a}	225.0±6.9^{a}	227.1±11.0^{a}

[1] Ovulation rate: number of ovulated females/ number of injected females (1/5=20%, 4/5=80%, 5/5=100%)

Ovulation rates of 80% and 100% were similar (p>0.05) but higher (p<0.05) than 20% (the chi-square test)

[2-5]Values are mean±SD. Value within the same row with different superscript letters are significantly different (p<0.05)

[3-5]Values of 0 ppt treatment were not included for ANOVA analysis because of no replicated data

The fertilization rate and hatching rate were positively correlated with water salinity. The fertilization rate at salinities of 25, 30 and 35 ppt (62.8-66.9%) were higher than those at 0, 5, 10 and 15 ppt (10.6-37.4%) (p<0.05) but did not differ significantly from the 20 ppt treatment (55.2%). The hatching rate at the salinities of 25, 30 and 35 ppt (53.6-54.9%) were higher than other salinities (0-39.5%) (p<0.05). No fertilized eggs at all hatched at salinities of 0 and 5 ppt (Figure 3.1).

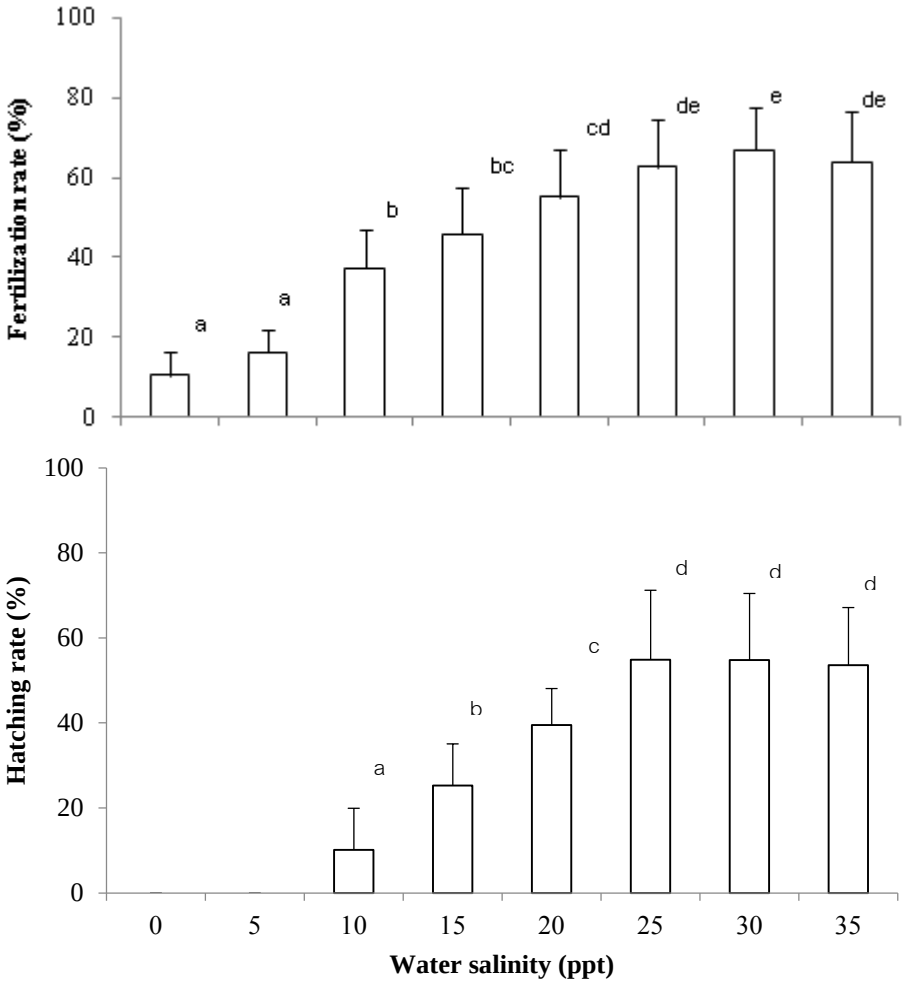

Figure 3.1 Fertilization rate and hatching rate (mean ± SD) of artificially inseminated spotted scat at different salinities (different letters indicate statistically significant means; $p<0.05$)

A low abnormality rate was obtained in the high water salinity treatments. The abnormality rates at salinities of 25 and 30 ppt (3.9-4.9%) were lower than those of 10 and 15 ppt (9.0-9.4%) ($p<0.05$) but not significantly different from those at 20 ppt (7.3%) or35 ppt (5.5%). Moreover apart from the 0 and 5 ppt treatments from which no larvae hatched, there were no significant differences ($p>0.05$) in the total length of the larvae in each treatment which ranged from 1.18-1.92 mm (Figure 3.2).

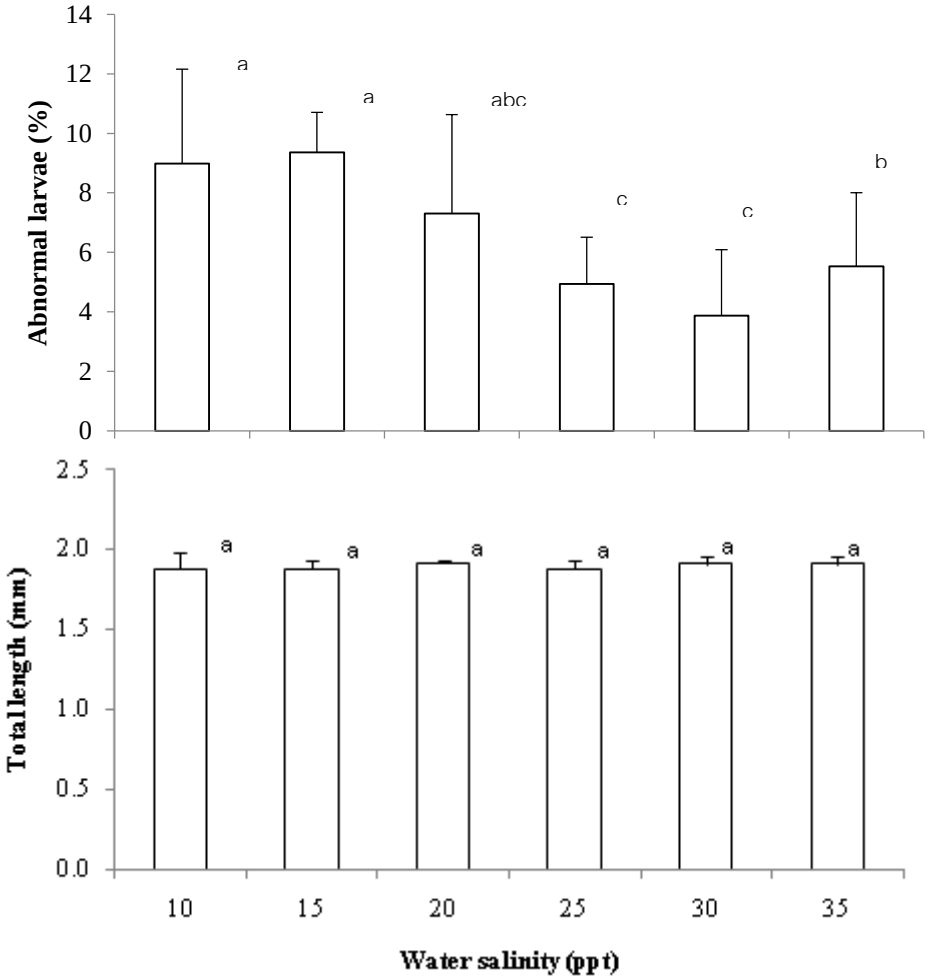

Figure 3.2 Abnormality rate and total length (mean ± SD) of spotted scat larvae hatched in different salinities (no larvae were hatched at salinities of 0 and 5 ppt; different letters indicate statistically significant differences; $p<0.05$)

Effect of water salinity on survival of the larvae

The survival rate of 3-day-old larvae at salinities of 15, 20, 25, 30 and 35 ppt (84.6-91.6%) were higher than those at 0 and 5 ($p<0.05$) but were not significantly different from the rate at 10 ppt (75%). However, the larvae at 7 and 15 days old tended to produce significantly higher survival rates than those reared in lower salinities. For the 0 ppt treatment, the survival rate for all ages of the larvae was low

and significantly different from all other treatments (p<0.05). The total length of the larvae in all treatments was not significantly different (p>0.05) (Figure 3.3).

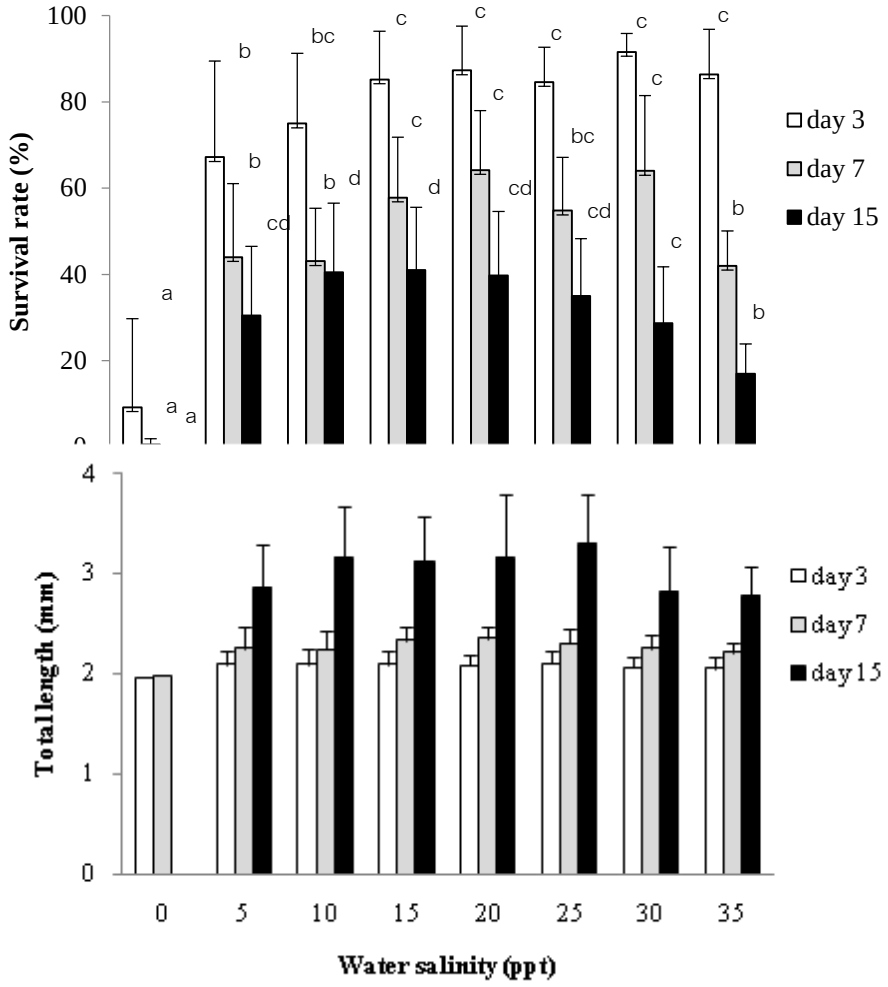

Figure 3.3 Survival rate and total length (mean ± SD) of spotted scat larvae reared in different water salinities (different letters in each age indicate statistically significant differences; p<0.05)

Discussion

The finding of this study showed that spotted scat area strongly euryhaline species which agrees with past reports relating to both mature fish (Barry and Fast, 1992) or early stage fish (Chang *et al*., 2005). Most of the results obtained from the investigation of artificial insemination and larval rearing found no difference among the water salinity levels tested. However, although spotted scat inhabit a wide water salinity gradient from freshwater, through brackish water to sea water, the ovulation rate of broodfish was low when they were kept in a water salinity of 0 ppt. This indicates that water salinity affects spotted scat spawning, since in freshwater conditions, the injected females did not respond to hormone inducement and/or delayed ovulation beyond the optimal period (i.e. the latency period was high). Generally, the fertilization rate is low in females demonstrating a high latency time (Black and Black, 2013). In contrast, the fertilization and hatching rates were high in high water salinity levels (>20 ppt). Khan *et al*. (2012) found that the highest hatching rate (43.3%) was presented at water salinity of 30 ppt. Thus, spotted scat do not appear to spawn in a freshwater habitat. Moreover, the fertilization rate at salinities of 0 and 5 ppt was relatively low, probably because the scat sperm quality at low when water salinity decreases (Chang *et al*., 2005; Ruensirikul *et al*., 2009b). At low water salinity, hatching was completely absent. In addition, there was a high larval abnormality rate at salinities of 10 and 15 ppt. This data supports the hypothesis that spotted scat do not spawn either in freshwater habitats or brackish water habitats where the water salinity is lower than 15 ppt. This finding broadly agrees with that of Barry *et al*. (1988) who stated that spotted scat did not spawn if the water salinity is less than 10 ppt and Cai *et al*. (2010),

who suggested that spotted scat need to migrate to high salinity waters for the purpose of spawning. The fertilization rate, hatching rate and abnormality rate at salinities of 10-35 ppt were not significantly different. Therefore, water salinity levels for spotted scat breeding should not be lower than 25 ppt while the optimal water salinity for suiting gonad development which is useful for producing broodstock in a hatchery should be investigated in the future.

The results obtained from this study confirm that the spotted scat is an excellent euryhaline species, since especially during the first week after hatching, since there are no differences in growth or survival rates of scat larvae in a wide range water salinities of between10 ppt and 35 ppt. Socorro *et al*. (1988) stated that during the early stage of scat larvae development, they could tolerate high water salinity up to 40 ppt. In the present study, only in a water salinity of 0 ppt could scat larvae not be raised for 15 days. This may be because in nature the spotted scat is not a freshwater fish. However, the survival pattern of the larvae at each age tended to be different. The growing larvae were likely to be favored by lower water salinity and the survival rate of 15-day-old scat larvae at a water salinity of 5 ppt was not significantly different from that at higher salinities between10 and 35 ppt. However, the findings of the present study differed from those of Ruensirikul *et al*. (2012), who found that water salinity (10-35 ppt) had no effect on the survival rate of scat larvae during the first 20 days. After that time, rearing scat larvae in gradually reduced water salinity as their increased age produced a higher survival rate than that with stable water salinity throughout the rearing period. Moreover, study in the past (Khanh *et al*., 2012) have revealed that 30-60-day-oldscat fry produced the best growth and survival when they were held in low salinity water of about 5 ppt.

The findings of this study reflect the ecology of the early stage of spotted scat development in their natural habitat in that the larvae may migrate to find lower salinity waters as they become older. The cause of this behavior may be osmoregulation adjustment by the larvae to balance their bodies with the surrounding environment because osmolality is positively correlated with water salinity (Herrera *et al*., 2009). However, further studies should be conducted separately into each stage of the life cycle of this species which would determine the applicable baseline information for broodstock establishment and seed production in hatcheries.

Conclusion

Spotted scat proved to be a strongly euryhaline species. A wide range of tested salinities supported artificial fertilization and seed production at all levels except pure freshwater. However, successful fertilization and hatching are favored by higher water salinity while larval rearing is favored by lower water salinity levels. This finding reflects the crucial importance of environmental requirements for the reproductive behavior of the spotted scat in the wild and the need for baseline data to be applied in seed production operations.

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CHAPTER 4

EFFECTS OF WATER SALINITY ON THE REPRODUCTIVE PERORMANCE OF FEMALE HATCHERY-REARED SPOTTED SCAT (*Scatophagus argus* Linnaeus, 1766) BROODSTOCK

Abstract

The effect of water salinity on the reproductive performance of female hatchery-reared spotted scat was investigated. Broodfish were reared in a hatchery for 14 months until maturation and selection. Selected broodfish were maintained in 1-m^3 tanks with holding water salinities of 5, 15, 25 and 33 ppt. The maturity of the female broodfish was monitored monthly for 4 months after the experiment was initiated. Each month, their reproductive parameters were monitored and their serum 17β-estradiol (E2) levels and osmolality were determined. The results showed that none of the reproductive parameters including the E2 profile and blood osmolality differed significantly between treatments except that the ovulation rate of the fish held in water salinities of 15 and 25 ppt were significantly higher than those of other salinities (p<0.05). This finding indicates that a holding water salinity of 15 - 25 ppt is optimal for the culture of spotted scat broodstock in hatcheries.

Introduction

The spotted scat, *Scatophagus argus* (Linnaeus, 1766) is a tropical finfish with the potential for coastal aquaculture. The advantage of this species is that it is both a valuable food fish and a popular ornamental fish. Many countries have tried to breed this species (Cai *et al.*, 2010; Chang and Hsieh, 1997; Ruensirikul *et al.*, 2008)

but the results of mass seed production have been limited (Khanh *et al.*, 2012). Nowadays, almost all the juvenile or mature spotted scat fish found in the market have been caught in the wild and the quantity is inadequate to support market demand. One of the most important elements of the successful mass seed production of marine fish is the development of hatchery-reared broodstock because the availability of broodfish from wild sources is not consistent in either quantity or quality (Mair, 2002).

Before the establishment of a hatchery-reared broodstock, suitable environmental conditions for broodfish should be investigated to ensure appropriate fish rearing conditions that may affect the achievement of gonad development, such as water quality and nutrition (Mylonas *et al.*, 2010). Water salinity is an important environmental factor which influences osmoregulation and ion-regulation in fish. These biological processes require energy, thus the broodfish has to make adjustments in order to maintain homeostasis balance with its surrounding environmental medium and to decrease osmoregulatory expenditure (Sampaio and Bianchini, 2002). Inappropriate external water salinity levels can cause fish mortality and stress and long-term stress can impair the fish's growth and its immune system as well as affecting its reproductive development (Campbell *et al.*, 1994; Castranova *et al.*, 2005; Davis *et al.*, 2002). The spotted scat is a euryhaline species (Chang *et al.*, 2005) that can live in a wide range of salinities, from fresh to full marine water. However, Cai *et al.* (2010) stated that spotted scat broodfish require a high water salinity level for spawning. The optimal level of water salinity for maintaining a normal reproductive rate in scat is still unclear. Haddy and Pankhurst (2000) found that gonadal maturation in black bream, *Acanthopagrus butcheri*, a euryhaline fish, was unaffected by water salinity. However, the number of

ovulations and egg volume of this species is low when reared in water with a salinity of 5 ppt.

Currently, there is no well-established hatchery-reared broodstock of spotted scat and little is known about the effect of water salinity on its gonadal development and reproductive performance. The only study that has investigated the effects of water salinity on the reproductive activity of spotted scat was that of Khanh *et al.* (2012). The aim of the study described herein was to evaluate the reproductive performance of hatchery-reared spotted scat broodstock maintained under different water salinities ranging from 5 to 33 ppt. This study will provide important information for the establishment of domesticated broodstock in hatcheries, especially hatcheries located far from a seawater source.

Materials and Methods

Broodstock Rearing and Conditions

Spotted scat broodstock were obtained from artificial propagation at the marine fish hatchery of the Coastal Aquaculture Research Institute (Songkhla, Thailand). About 200 fishes were reared together in a 28 m^3 cylindrical cement tank for 14 months at a density of 10 fish/m^3 (around 1 kg/m^3) at a sex ratio of 1:1 before further selection. The water salinity was maintained at 15-30 ppt by diluting natural seawater with freshwater. The fish were fed to satiation twice a day with a commercially formulated marine fish diet containing 35% protein until selection. Half of the water was changed every two weeks and the water quality was maintained and measured by the standard methods of the American Public Health Association (APHA, 1998) at a

temperature of 26 - 29 ºC, a pH of 7.4 - 8.2, dissolved oxygen at 5.4 - 6.7 mg/L, ammonia at 0.04 - 0.32 mg/L and nitrite at 0.01 - 0.02 mg/L.

Broodstock Selection and Experimental Design

The water salinity of the broodstock tank was raised to 33 ppt for a week before selection and the broodfish were starved for a day before selection. Sex differentiation was determined by snout shape (Barry and Fast, 1992). Only post-spawning or resting stage females (non-swollen abdomen: abdominal width less than body width, GSI = 1.85 ± 0.68, n=5) were selected for the experiment (106.4 ± 7.1 - 124.2 ± 23.3 g). Experimental males were selected with expressible milt present after gentle abdominal stripping (i.e. spermiating males) (47.7 ± 4.2 - 58.3 ± 3.8 g). The fish selected were randomly put into the experimental tanks and the water salinity of each was adjusted by about 2 ppt/day by diluting it with freshwater until the targeted water salinity was reached, before the experiment was initiated.

The experiment comprised four treatments in which the broodstock fish were reared in varying water salinities of 5, 15, 25 and 33 ppt. The experimental fish were reared in 1,000 liter plastic tanks at a density 10 fish/tank (6 females : 4 males). Each treatment was conducted in triplicate. Two females from each treatment were randomly tagged with passive-integrated transponder tags (PIT-tags) for broodstock identification, steroid hormone and serum osmolality investigation. The broodstock fish were fed with the same diet and feeding procedure as in the broodstock tank but the water in the experimental tanks was changed at the rate of 100% daily. Sediment in the tanks was removed every day, and tank cleaning was performed weekly. The water salinity of each tank was monitored daily and other aspects of the water quality were maintained and measured according to standard methods (APHA, 1998) at a

temperature of 26 - 29 ºC, a pH of 7.4 - 8.2, dissolved oxygen at 5.5 - 7.2 mg/L, ammonia at 0.04 - 0.41 mg/L and nitrite at 0.01 - 0.02 mg/L.

Maturation Monitoring and Reproductive Performance Parameters

All the female broodfish in each tank were monitored monthly for 4 months after the experiment was initiated. The morphology of the fish's abdomen was observed and females with swollen abdomen were classified as mature broodfish. Before hormone injection, the percentage of mature fish was calculated as the number of broodfish which had a swollen abdomen x 100/number of all females. The oocytes of each matured fish (>100 oocytes/fish) were sampled via cannulation using a polyethylene tube, 0.5 mm in diameter connected to a 0.1 ml syringe and inserted into the genital pore. Then, the percentage of post-vitellogenic oocytes was investigated with a compound microscope under 40x magnification and calculated as the number of post-vitellogenic oocytes x 100/total number of sampled oocytes. The post-vitellogenic oocyte diameter was monitored using an ocular and stage micrometer with a microscope. The 10 largest oocytes were measured for their mean diameter (Mylonas *et al.*, 2013). Thereafter, each mature female that demonstrated a mean oocyte diameter of more than 350 µm (Barry and Fast, 1992) was artificially inseminated.

Ovulation was induced using a single intramuscular hormone injection of luteinizing hormone-releasing hormone analogue (LHRHa; Suprefact, Sanofi-Aventis Deutschland GmbH, Germany) at 20 µg/kg (Ruensirikul *et al.*, 2008). The induced broodfish were monitored for ovulation every 30 min to 2 h, depend on the degree of external abdominal swelling, after 32 h post-injection, by gentle abdominal massage. The ovulation time was recorded as having occurred when the ovulated oocyte was first presented after massage. The ovulated oocytes were manually stripped and

fertilized immediately with fresh pooled milt collected from 3 - 4 spermiating males obtained from the broodstock tank, with good motility. The ovulation rate (the number of ovulated females x 100/number of injected females) at each breeding time was monitored. The latency time (time from injection to ovulation) of each ovulated broodfish was recorded. The buoyancy rate (ml of buoyant egg x100/total ml of eggs) of 5 ml of stripped egg was measured as the percentage of viable buoyant eggs that were completely separated from dead eggs which sank in a 500-ml cylinder with 30 ppt seawater within 20 minutes (Zakeri *et al*., 2009). Ovulated oocyte diameter and oil globule diameter was also measured as the post-vitellogenic diameter before hormone injection. The percentage of normal ovulated eggs (number of normal ovulated eggs x 100/total number of eggs sampled) was monitored under a light microscope for approximately 100 eggs. Ovulated eggs which were spherical in shape, had transparent cytoplasm and a single oil droplet located at the centre, were defined as normal ovulated eggs. After fertilization, the fertilized eggs of each fish were incubated separately. The fertilization rate (number of eggs at the 4 - 8 cell stage x 100/total number of eggs), the hatching rate (number of larvae x 100/total number of fertilized eggs), the total length of the day-0 larvae (6 - 9 h post hatch; n = 20), the larval abnormality rate (the abnormal larvae characterized by vertebral deformity; n = 100) and the survival rate of day-3 larvae (number of surviving larvae x 100/total number of larvae initially) were determined by counting the surviving larvae reared indoors in 2-liter plastic containers after 3 days post hatching (experiment performed in triplicate) at a density of 15 larvae/liter without aeration or feeding.

Sex Steroid Hormone Levels

At each time of investigating maturation, blood samples were taken from the caudal vein of four fish from each treatment. Serum was obtained from the blood by centrifugation at 6,000 rpm for 5 min at 4 ^{o}C and was then stored at −20 ^{o}C until the determination of the sex steroid hormone level (17β-estradiol: E2) by electrochemiluminescence immunoassay (ECLIA) using an Elecsys Estradiol III kit (Roche Diagnostics, Germany) with an immunoassay analyzer (Modular Analytics E170) according to the manufacturer's instructions. The sensitivity of the assay was 5.0 pg/mL.

Osmolality Measurement

Sub-samples of the serum of the fish in each tank were measured monthly for osmolality using a freezing point depression osmometer (Fiske Micro-Osmometer Model 210, Fiske Associates, Massachusetts, USA) contemporary with steroid measurement. Distilled water (0 mOsmol/kg) was applied as a control solution. The osmolality of the ambient water in each treatment was also determined but only once, in order to determine the isosmotic point (Sampaio and Bianchini, 2002).

Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare the reproductive performance parameters, sex steroid level and osmolality among the treatments, followed by Duncan's multiple range test to determine significant differences among means, with a significance level of $p<0.05$. The statistical differences in the monthly average sex steroid level for the different water salinity treatments for the ovulated and non-ovulated fish were separately established and the differences among ovulated and non-ovulated fish within each treatment were also

determined. Statistical difference in the monthly average serum osmolality among water salinity treatments was also analyzed. The differences in serum and water osmolality were established using t-test. Percentage data were subjected to logarithmic transformations and the oil globule diameter was square-root transformed prior to analysis.

Results

Reproductive Parameters

The reproductive parameters before hormonal inducement of each treatment collected monthly representing the percentage of mature fish (35.4 ± 17.2 - 45.6 ± 24.4%), the percentage of post-vitellogenic oocytes (79.1 ± 8.5 - 85.8 ± 7.6%) and the post-vitellogenic oocyte diameter (391.1 ± 38.6 - 415.1 ± 9.6 μm) (Table 4.1) showed no significant differences among the water salinity treatments (p>0.05).

After ovulation, no significant differences (p>0.05) were detected in the latency time (36.4 ± 5.4 - 40.0 ± 1.7 h), buoyancy rate (72.4 ± 14.9 - 85.7 ± 8.1 %), ovulated oocyte diameter (632.6 ± 11.4 - 661.3 ± 43.1 μm), oil globule diameter (237.0 ± 21.7 - 244.5 ± 23.1 μm), or percentage of normal ovulated eggs (61.1 ± 21.6 - 81.4 ± 11.4 %) between the female spotted scat broodstock in each treatment for all water salinity levels. Similarly, after insemination, there were no significant differences (p>0.05) found between the fertilization rates (61.0 ± 22.7 - 80.7 ± 13.0 %), hatching rates (40.5 ± 10.0 - 62.9 ± 16.7 %), total length of larvae (1.67 ± 0.14 - 1.75 ± 0.15 mm), abnormality rates of larvae (2.5 ± 1.1 - 3.5 ± 0.5 %) or the survival of day-3 larvae (65.9 ± 3.9 - 68.1 ± 8.8 %) obtained from each treatment. However, some significant differences (p<0.05) were found in the ovulation rate. The highest ovulation rate was

obtained from broodfish in the 25 ppt treatment (65.9 ± 16.7 %) which was significantly different from all other salinities except the 15 ppt treatment (48.9 ± 1.9 %). The females in 33 ppt water salinity produced the lowest ovulation rate (28.5 ± 17.0%) which was significantly different from all other treatments apart from the 5 ppt treatment (34.6 ± 13.8 %) (Table 4.1).

Sex Steroid Hormone Levels

The average serum E2 level of the ovulated-female fish (797.6 ± 95.0 - 1,546.0 ± 448.8 pg/mL) was significantly higher than that of the non-ovulated fish (206.3 ± 106.6 - 415.6 ± 198.7 pg/mL) ($p<0.05$) in every treatment, except those in the 15 ppt treatment where data was only collected for non-ovulated fish since none of the tagged fish ovulated. However, the E2 level among the treatments was not significantly different ($p>0.05$) either in the ovulated fish nor in the non-ovulated fish when they were separately analyzed (Figure 4.1).

Serum Osmolality

There was no significant difference in serum osmolality of the fish among any treatments. The serum osmolality of the fish in all the treatments remained stable and ranged from 323.4 ± 16.6 to 345.0 ± 4.0 mOsmol/kg and thus did not vary according to the holding water osmolality which increased along with the water salinity (Figure 4.2). The isosmotic point was 337.3 mOsmol/kg.

Table 4.1 Reproductive performance parameters of female spotted scat broodstock reared in different water salinities for 4 months in a hatchery. Data was averaged from monthly investigation, expressed as mean ± SE. Means sharing the same superscript indicate no significant differences (p>0.05) between water salinity treatments.

Reproductive performance parameters	Water salinity (ppt)			
	5	15	25	33
Mature female (%)	45.6±24.4[a]	42.5±15.7[a]	43.6±23.2[a]	35.4±17.2[a]
Before injection				
Post-vitellogenic oocyte (%)	85.8±7.6[a]	82.1±6.8[a]	79.1±8.5[a]	79.2±5.8[a]
Post-vitellogenic oocyte diameter (μm)	400.1±15.3[a]	391.1±38.6[a]	400.1±5.2[a]	415.1±9.6[a]
After ovulation				
Latency time (h)	36.4±5.4[a]	38.9±1.2[a]	40.0±1.7[a]	38.4±2.9[a]
Ovulation rate (%)	34.6±13.8[a]	48.9±1.9[ab]	65.9±16.7[b]	28.5±17.0[a]
Buoyancy rate (%)	83.9±8.2[a]	85.0±9.0[a]	85.7±8.1[a]	72.4±14.9[a]
Ovulated oocyte diameter (μm)	657.9±23.8[a]	642.5±14.6[a]	632.6±11.4[a]	661.3±43.1[a]
Oil globule diameter (μm)	244.5±23.1[a]	237.8±20.4[a]	237.0±21.7[a]	243.0±18.8[a]
Normal ovulated egg (%)	75.8±4.6[a]	77.5±12.9[a]	81.4±11.4[a]	61.1±21.6[a]
Fertilization rate (%)	62.9±10.7[a]	80.7±13.0[a]	61.0±22.7[a]	68.8±30.4[a]
Hatching rate (%)	48.4±16.6[a]	61.7±12.7[a]	62.9±16.7[a]	40.5±10.0[a]
Total length of day-0 larvae (mm)	1.75±0.15[a]	1.73±0.03[a]	1.67±0.14[a]	1.74±0.07[a]
Larval abnormality rate (%)	3.0±0.3[a]	2.5±1.1[a]	3.5±0.5[a]	3.1±1.7[a]
Survival rate of day-3 larvae (%)	65.9±3.9[a]	66.1±1.9[a]	68.1±8.8[a]	67.7±6.6[a]

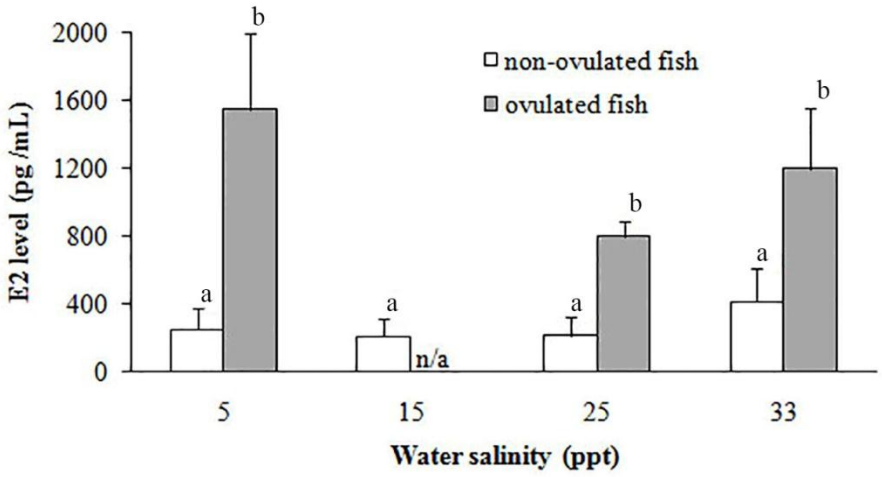

Figure 4.1 Sex steroid hormone level (17β-estradiol: E2) of female spotted scat broodstock reared in different water salinities in a hatchery for 4 months shown as overall average of each water salinity treatment (in 15 ppt treatment, no ovulated fish were observed in tagged females; means with the same superscript are not *significantly different* (*p*> 0.05); n/a=not available; expressed as mean ± SE).

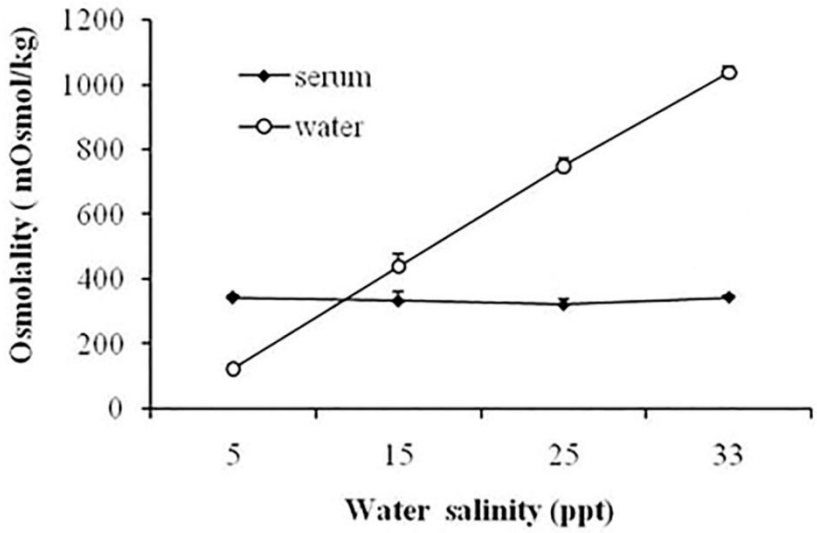

Figure 4.2 Serum osmolality of female spotted scat broodstock reared in different water salinities in a hatchery. Data were averaged from monthly investigation, expressed as mean ± SE. No significant differences were observed among serum osmolality of any water salinity treatment but at the same water salinity and serum osmolality are significantly different (p>0.05).

Discussion

Typically, water salinity can suppress the reproductive performance of fish but euryhaline fish are better adapted to environmental fluctuations. Although, this study clearly shows that the water salinity tested ranging from 5 to 33 ppt did not affect the reproductive performance of the female scat fish, a higher ovulation rate was obtained within the middle range of salinities tested at 15 and 25 ppt. Reproductive impairment when broodfish are subjected to low water salinity has been reported in previous studies. In a study of black bream (*Acanthopagrus butcheri*), a wide range of

holding water salinity (5 - 35 ppt) did not affect gonadal development or plasma steroid levels. However, the number of ovulations and egg volumes was lowest in fish held at 5 ppt (Haddy and Pankhurst, 2000). In other euryhaline marine fish such as Waigieu seaperch (*Psammoperca waigiensis*), holding broodfish in different salinities (5, 10, 20 and 30 ppt) during the breeding season affected gonadal development and spawning performance. Fish held at 5 ppt had 100% mortality and at 10 ppt, oocyte development or ovulation was diminished (Pham *et al.*, 2010) and similar results were found for mullet (*Mugil cephalus*) (Assem *et al.*, 2015) in which different water salinities affected gonadosomatic index values.

Thus, based on the present study, spotted scat broodstock can be reared for aquaculture in any water salinity (5-33 ppt) depending on available water sources and investment budget. However, to achieve higher ovulation rates in broodfish, salinities of 15-25 ppt are suggested as being the optimum range for hatchery-reared spotted scat especially in hatcheries where the number of broodfish available is limited. In Vietnam, Pham *et al*. (2010) noted that the production cost of Waigieu seaperch could be reduced by decreasing water salinity levels to 20 ppt through increased use of freshwater instead of the exclusive use of seawater. However, a certain degree of water salinity was still necessary during the process of artificial insemination because success in propagating this species was achieved only in high water salinity (>28 ppt) (Ruensirikul *et al*., 2008) which corresponds to the natural spawning behavior of spotted scat, which prefer to migrate to areas of high water salinity for spawning (Cai *et al*., 2010).

The pattern of the E2 level found in female scat was similar to that found in female black bream (*A. butcheri*) maintained at 5, 20 or 35 ppt water salinity after

being injected with 50 μg/kg LHRHa (Haddy and Pankhurst, 2000). The water salinity did not appear to affect the E2 level in this species although it was significantly elevated after induced ovulation and in ovulated broodfish at all three salinities. In contrast, the plasma E2 levels were observed to be significantly affected by different levels of water salinity during the spawning season of Waigieu seaperch (*P. waigiensis*). However, in that species, the E2 levels were high (308-629 pg/mL) during the spawning period and decreased in the off-season (6-54.8 pg/mL) (Pham *et al.*, 2010). This coincided with the E2 levels in spotted scat which were high in ovulated broodfish and low in non-ovulated fish. This pattern of E2 level profile has also been found in other fish, such as common snook, *Centropomus undecimalis* (Cruz-Botto *et al.*, 2018) for which the E2 level was not affected by different water salinity environments (estuarine and seawater), being high in the spawning season (300 - 400 pg/mL) and decreasing in the off-season (100 - 200 pg/mL). The similarity of the pattern of E2 profile of the spotted scat in each treatment indicated that the oocyte development of the broodfish held at each water salinity was similar. The level of E2 tends to increase before maturation when the oocyte size increases because estradiol regulates ovarian development through vitellogenin synthesis (Heidari *et al.*, 2010; Kagawa, 2013).

The isosmotic point of spotted scat was 337.3 mOsmol/kg which corresponded to a water salinity of 11.8 ppt. The serum osmolality of this species seems to be constant in any water salinity. Similarly, Urbina and Chris (2015) found that the plasma osmolality of inanga (*Galaxius maculatus*) reared in salinities ranging from freshwater to 43 ppt showed only minor changes and there were no significant changes in physiology such as in metabolic rate or energy expenditure. However, the period during which fish adapt following a change in water salinity may differ and for Urbina

and Chris's study of inanga, the period was only 16 days which was much less than in this study. Constant or almost constant variability of blood osmolality in fish has been commonly noted when they are maintained in different levels of water salinity, through ion and water regulation, mainly in the gills, kidneys and intestines (Varsamos *et al.*, 2005). In pure marine or pure freshwater fish that inhabit waters with a stable salinity, steady-state osmoregulatory mechanisms are adequate to sustain homeostasis. However, little is known about the mechanisms by which euryhaline fish adjust their osmoregulation to balance homeostasis in variable water salinity surroundings (Kultz, 2015). Shui *et al*. (2018) stated that the main mechanism for maintaining serum osmolality involves gill Na^+/K^+-ATPase. Both inanga and scat are classified as amphidromous fish that can move freely in any salinity gradient. Typically, the isosmotic water salinity and blood osmolality of adult teleost fish are about 10 - 12 ppt (Boeuf and Payan, 2001; Sampaio and Bianchini, 2002; Wada *et al.*, 2004) and 280 - 360 mOsmol/kg (Varsamos *et al*., 2005). The serum osmolality of the scat in this study was close to that reported by Mu *et al.* (2015) and was slightly lower than that of common estuarine fish such as Asian seabass (*Lates calcarifer*) (Sarwono, 2004) and rabbit fish (*S. rivulatus*) (Saoud *et al.*, 2007) when they were reared in seawater ranging from 0 - 30 ppt and 10 - 40 ppt respectively. Stable serum osmolality allows scat to exist in a wide range of salinities throughout their life cycle. The present study offers new insight into the range of holding water salinity for successful broodstock rearing for breeding purposes.

Conclusions

The spotted scat is a versatile euryhaline fish that can be cultured in a wide range (5 to 33 ppt) of salinities in hatcheries with no effects on growth, survival, sex steroid (E2) level or in its overall reproductive performance. However, water salinities of between 15 and 25 ppt are recommended to maximize its ovulation rate. This finding suggests that there is a good opportunity for the establishment of broodstock of this species in hatcheries or in natural water for the purpose of breeding and mass seed production.

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CHAPTER 5

TIMING FOR OOCYTE RECRUITMENT AND REPRODUCTIVE PERFORMANCE OF FEMALE HATCHERY-REARED SPOTTED SCAT (*Scatophagus argus* Linnaeus, 1766) AFTER ARTIFICIAL INSEMINATION

Abstract

Two year old spotted scat (*Scatophagus argus*) broodstock (selected mature females and males of 165.0 - 244.4 g and 55.2 - 122.4 g respectively) were reared at 10 fish/m^3 in cement tank and fed to satiation with 35% protein artificial diet twice a day until selection for 5 consecutive inseminations. The results showed that the timing for oocyte recruitment was 41.6±6.8 days. Most reproductive performance parameters of the first and the second artificial insemination were not significantly different (p>0.05), except for oocyte diameter before hormone injection, ovulation rate and viable egg of recruited females (381.3±16.0 µm, 37.5±26.2% and 54.8±21.2%, respectively) that were smaller/lower than those of the former matured broodfish (422.8±12.1 µm, 72.9±17.0% and 81.3±7.2%, respectively). Histological investigation of the ovarian development 1 day after stripping showed several residual hydrated oocytes occupying most of the area in the ovaries. After 15 days, most oocytes achieved the pre-vitellogenic oocytes. The vitellogenic oocytes were obviously present after 30 days, while post-vitellogenic oocytes predominantly located (45.1%) in the mature ovaries 45 days after stripping. This study provides considerable data for available broodstock planning for efficient mass seed production of this species.

Introduction

Spotted scat, *Scatophagus argus* Linnaeus, 1766 is a tropical finfish distributed widely in coastal areas of the Indo-Pacific region (Nelson, 1974). It is a euryhaline species that can inhabit any water salinity: fresh, brackish and sea water, as well as being highly tolerant and well adapted to environmental changes especially water salinity and temperature fluctuations (Chang *et al*., 2005). These biological properties make scat one potential candidate among finfish species for coastal aquaculture (Wongchinawit and Paphavasit, 2009). Spotted scat is one of the most highly valued food fish in some countries such as the Philippines (Barry and Fast, 1988) and a popular aquarium ornamental fish (Jayalal and Ramachandran, 2012). However, most of these fish are caught in the wild and are still inadequate to meet market demand whether in the past (Barry and Fast, 1988) or present as there are no reports of mass seed production of this species.

Researchers from many countries, such as the Philippines (Barry *et al*., 1988), Taiwan (Chang and Hsieh, 1997), China (Cai *et al*., 2010) and Vietnam (Khanh *et al*., 2012), have tried to study the reproductive biology and to breed the spotted scat. However, most of these studies applied artificial insemination for seed production because this fish has not spawned in captivity. Generally, after ovulation, the ovaries of broodstock enter into the recovery phase to regenerate steadily new oocytes for the next spawning season. The gonadal development was driven by sex steroids, especially estradiol that correlated with oocyte growth (Morehead *et al.*, 2000). Spotted scat are multiple-spawners (Cai *et al*., 2010) exhibiting group-synchronous oocyte development (Castanos and Barry, 1988) with at least two batches of oocytes in the ovaries. In wild fish, the larger oocytes are spawned naturally during that current breeding cycle while

the smaller oocytes will be developed and spawned in the next breeding cycle (Murua and Saborido-Rey, 2003). The stage of gonadal development of marine fish has been studied in several species (Imanaga *et al*., 2014; Bubner *et al*., 2012; Liu and Sadovy de Mitcheson, 2009) while information on the timing or period for oocyte recruitment after breeding and the reproductive performance of the newly matured oocytes compared with the previous clutch is still scarce. Understanding fish reproductive strategy information of each species, especially the timing of gametogenesis, is very useful for hatchery production planning. Accurate tallying of available broodfish and frequency of egg production is an important factor in reaching the highest efficiency of hatchery-reared broodstock (Mylonas *et al*., 2015; 2013).

The aim of this study was to determine the timing for oocyte recruitment, estradiol profile and the reproductive performance of females hatchery-reared spotted scat broodstock after artificial insemination, compared with the first brood. This study will provide significant data for artificial insemination of this species for mass seed production in the future.

Materials and Methods

Broodstock Rearing and Condition

Spotted scat fry, obtained from artificial breeding at the Coastal Aquaculture Research Institute (Songkhla, Thailand), have been reared indoors at a sex ratio of 1:1 in two 28 m^3 cylindrical cement tanks with a semi-recirculating system and constant aeration for 2 years. The fish were maintained at 10 fish/m^3 (around 1 kg/m^3) with at least 500 fish/tank for further selection. Fifty per cent of the water was changed once every two weeks. The fish were fed to satiation with 35% protein of commercially

floating artificial diet (Thai Union Feedmill Co., Ltd., Samutsakhon, Thailand) twice a day until selection. Water quality was monitored weekly following standard procedure of APHA (1998). Water salinity was maintained at 10-30 ppt, temperature at 26-28 ^{o}C, pH at 7.5-8.1, dissolved oxygen at 5.4-6.5 mg/l, ammonia at 0.05-0.13 mg/l and nitrite at 0.01-0.02 mg/l.

Broodstock Selection

Broodstock fish were starved for a day before selection. Sex differentiation was determined by snout shape (head profile in females has a relatively constant slope unlike that in males, in which there is an obvious curvature to the snout) (Barry and Fast, 1988). Female spotted scat broodstock were selected by combined criteria of external morphology (swollen abdomen, abdominal width was greater than body width) (García-López *et al*., 2006) and ovarian biopsy for oocyte sampling by inserting a polyethylene tube, 0.5 mm in diameter, connected with a 0.1 mL syringe, through the ovarian cavity into approximately the central portion of one of the ovaries. Then, the oocytes of each broodfish were sampled before hormone injection for stage development investigation and diameter measurement using an ocular and stage micrometer with a compound microscope under 40x magnification. The diameter of 10 oocytes of each female was measured (Mylonas *et al*., 2013). Females that achieved a mean oocyte diameter of more than 350 µm and males with expressible milt present after gentle abdominal stripping were identified as mature fish (Barry and Fast, 1988) and selected for artificial breeding. Selected female broodstock were 16.8-18.6 cm in total length and 165.0-244.4 g in weight, and were kept separately in 20-liter floating baskets located in a 1,000-liter plastic tank with constant aeration while male broodstock that

measured 12.1-16.1 cm in total length and 55.2-122.4 g in weight were kept freely in the same tank with the females.

Artificial Insemination and Reproductive Performance Parameters

Artificial insemination was conducted 6 times (the last time for histology investigation of oocyte recruitment) at intervals of 1-2 months. After mature broodstock were selected (maximum of 10 mature females each time), ovulation was induced in all selected females using single intramuscular hormone injection with lutenizing hormone-releasing hormone analogue (LHRHa; Suprefact, Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany) at 20 µg/kg. The broodfish were checked for ovulation every 30 min – 2 h, beginning 32 h after injection, depending on degree of external abdominal swelling, by applying gentle abdominal massage. The eggs of each ovulated fish were manually stripped and fertilized immediately with fresh pooled milt that was collected from 3-4 running males with good sperm motility. Latency time (time from injection to ovulation) of each broodfish was recorded. Ovulation rate (100 x number of ovulated females within 42 h/number of injected females) at each time of breeding was determined. The buoyancy rate (100 x ml of buoyant egg/total ml of eggs) of stripped egg was measured within 20 min after the eggs were transferred to a 500- mL cylinder containing 30 ppt seawater (Zakeri *et al*., 2009). Oocyte diameter was likewise checked before hormone injection. The percentage of viable eggs (100 x number of eggs perfectly spherical, translucent, without a perivitelline space/total number of sampled eggs) (Rasines *et al*., 2009) was determined by microscopic examination of approximately 100 eggs per fish.

Breeding performance variables were investigated comprising fertilization rate (100 x number of eggs at stage with 4-8 cells/total number of eggs)

that was checked under a binocular microscope 45-60 min after incubation, hatching rate (100 x number of larvae/total number of eggs) that was determined after a day of incubation at 26-28 ^{o}C, and absolute fecundity (number of eggs obtained from stripping per fish) that was determined by counting a subsample (1 mL of egg collected by a 1 mL syringe) in triplicates and then counting the eggs and multiplying by the total volume of ovulated eggs. The relative fecundity was calculated as the number of eggs obtained from stripping per g body weight. Larval quality comprising total length of the day-0 larvae (6-9 h post hatch) measured under the binocular microscope using an ocular and stage micrometer (n=20), larval abnormality rate (abnormal larvae characterized by vertebral deformity) determined under the binocular microscope (100 x number of abnormal larvae/total sampling larvae) (n=100) and survival rate of day-3 larvae (100 x number of survived larvae/total of initial number of larvae) (Zakeri *et al*., 2009) by counting the surviving larvae that were reared in 2 liter plastic containers 3 days after hatching (triplicates) at density 15 larvae/L without aeration and feeding, was monitored.

After ovulation and oocyte stripping, all broodfish were tagged with passive-integrated transponder tags on the dorsal musculature for broodstock identification before being moved back to the broodstock tank and kept separately in a 1-m^3 floating net cage with the same conditions as the first maturation. Tagged-ovulated females were reared under the same conditions as the first insemination until oocyte recruitment. Non-ovulated females in the first induction were discarded while ovulated brooders were checked for oocyte recruitment every 2 weeks using the same criteria as for broodfish selection. When each fish reached recruitment gravid stage, the second artificial insemination was conducted and all data were recorded applying the same

method and variables as the first artificial insemination. The timing of oocyte recruitment between the first and second maturations of this study was recorded as the period between two inseminations. The experiment was ended if all females were induced for breeding twice, except some broodfish that were injected only once because of death or observed oocyte recruitment failure (abdominal width did not increase within a month).

Sex Steroid Hormone Levels

After insemination, female broodfish were divided into 4 groups based on the period after egg stripping that were 1, 15, 30 and 45 days. Blood sample was taken from the caudal vein of 4 stripped females per group each time after insemination and centrifuged at 6,000 rpm for 5 min at 4 ^{o}C (Beckman, Avanti^{TM} 30 Centrifuge). Serum was separated and stored at -20 ^{o}C for determining the concentrations of sex steroid hormone levels (17β-estradiol: E2) by electrochemiluminescence immunoassay (ECLIA) using the Elecsys Estradiol III kit (Roche Diagnostics, Germany) with an immunoassay analyzer (Modular Analytics E170) according to the manufacturer's instructions. The sensitivity of the assay was 5.0 pg/ml.

Ovarian Histology

After the ovulated eggs were stripped for the last time, ten fish were maintained in the broodstock tank. Two fish were randomly sampled and sacrificed for ovaries collection at days 1, 15, 30 and 45 after egg stripping. Small fractions of the central part of the ovaries were removed from the body cavity and placed in 10% buffered formalin, dehydrated with gradient alcohol, cleared with xylene, before being embedded in paraffin. Sections were cut at 3-5 μm and stained with haematoxylin and eosin (H&E). Histological investigation divided the maturation of the ovaries into the

6 stages depending on diameter (Gandhi *et al*., 2014) combined with morphological features of dominant oocytes of hatchery-reared spotted scat (Ruensirikul *et al*., 2012) (Table 1). Subsequently, the diameter of the 10 largest oocytes (residual hydrated oocytes were not included) and percentage of oocytes in each stage of each sample from permanent slide were measured with an ocular micrometer using a 40x light microscope. Development of the oocytes was observed and photographs were taken using a digital camera. Four stages of spotted scat oocyte development were determined, as suggested by Shao *et al*. (2004) and Zhang *et al*. (2013) as follows: pre-vitellogenic oocyte (O1), vitellogenic oocyte (O2), post-vitellogenic oocyte (O3), and hydrated oocyte (HO).

Statistical Analysis

The reproductive parameters were expressed as mean±standard deviation (SD), timing for oocyte recruitment was presented as mean±standard error of mean (SE). Statistical differences in all variables in the first and second artificial inseminations were examined using Paired *t*-test, with a significance level of $p<0.05$.

Table 5.1 Maturity stage of ovaries of spotted scat in relation to oocyte size and development.

Ovary stage	Oocyte size (μm)[I]	Most advanced group of oocytes[II]
Immature	<20	pre-vitellogenic oocyte: chromatin nucleolus
Developing	21-100	pre-vitellogenic oocyte: perinucleolus
Maturing	101-300	vitellogenic oocyte: cortical alveoli
Mature	301-600	post-vitellogenic oocytes
Spawning	601-750	full of hydrated oocytes
Spent	<750	small number of hydrated oocytes

[I] modified from Barry and Fast (1988) and Gandhi *et al*. (2014)

[II] Ruensirikul *et al*. (2012)

Results

Timing for Oocyte Recruitment

The mean oocyte recruitment timing of a total of 19 female broodstock spotted scat after stripping obtained from 5 studies of artificial insemination ranged from 35.0±8.7 to 51.9±11.5 days (average 41.6±6.8 days) (Figure 5.1). The shortest and longest periods for development of new batch of oocytes in the ovaries were 21 days and 60 days respectively.

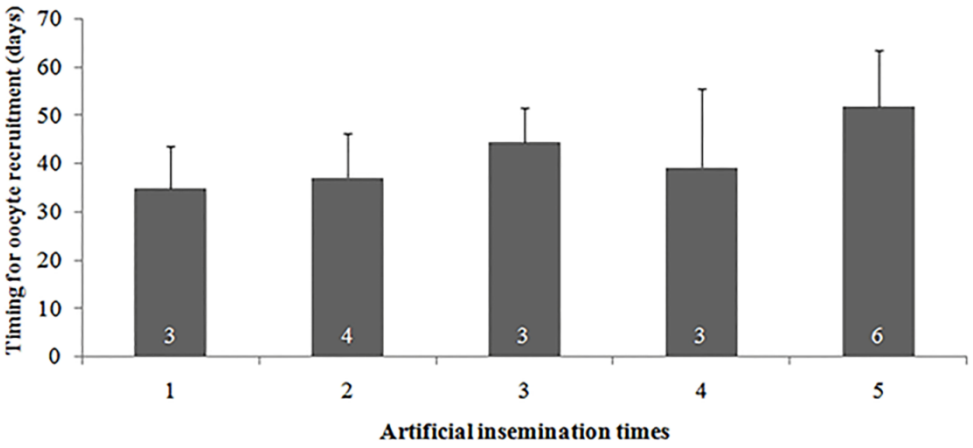

Figure 5.1 Timing for oocyte recruitment (the number inside the base of the bar indicates the number of fish that reached oocyte recruitment) of hatchery-reared spotted scat after 5 artificial inseminations.

Reproductive Performance

The reproductive performance variables are presented in Table 5.2 Almost all parameters in the first and second artificial inseminations (after oocyte recruitment) were not significantly different (p>0.05) except for oocyte diameter before hormone injection, ovulation rate and viable eggs of recruited females after ovulation (381.3±16.0 µm, 37.5±26.2% and 54.8±21.2% respectively) that were smaller/lower than the former matured broodfish (422.8±12.1 µm, 72.9±17.0% and 81.3±7.2% respectively). Other reproductive performance variables after ovulation that were not different were latency time (38.6±1.2 h and 38.3±0.6 h), buoyancy rate (65.0±8.9% and 64.1±37.3%), and oocyte diameter of viable eggs (622.4±11.7 µm and 624.4±23.2 µm) obtained from the first and second artificial inseminations, respectively. Breeding

performance after the first and second artificial inseminations was measured as follows: fertilization rate (50.7±14.1% and 48.2±21.2%), hatching rate (63.0±11.2% and 67.1±21.2%), absolute fecundity (197.2±43.7 $x10^3$ egg/fish and 177.5±15.6 $x10^3$ egg/fish) and relative fecundity (348.1±77.0 egg/g fish and 426.7±180.9 egg/g fish), respectively. Larval quality was assessed as follows: length of day 0 larvae (1.88±0.03 mm and 1.83±0.05 mm), larval abnormality rate (3.3±2.5% and 2.5±1.7%), and survival rate to day 3 (69.3±8.7% and 62.6±23.9%), respectively.

Table 5.2 Reproductive performance variables (mean±SE) of hatchery-reared spotted scat after first artificial insemination and when they were reared until oocyte recruitment (second insemination).

Reproductive performance parameters	First insemination	Insemination after oocyte recruitment
Before injection	*(n=32)*	*(n=32)*
Oocyte diameter (µm)	422.8 ± 12.1	381.3 ± 16.0*
After ovulation	*(n=12)*	*(n=12)*
Latency time (h)	38.6 ± 1.2	38.3 ± 0.6
Ovulation rate (%)	72.9 ± 17.0	37.5 ± 26.2*
Buoyancy rate (%)	65.0 ± 8.9	64.1 ± 37.3
Oocyte diameter (µm)	622.4 ± 11.7	624.4 ± 23.2
Viable egg (%)	81.3 ± 7.2	54.8 ± 21.2*
Breeding performance		
Fertilization rate (%)	50.7 ± 14.1	48.2 ± 21.2
Hatching rate (%)	63.0 ± 11.2	67.1 ± 21.2
Absolute fecundity (x 10^3 egg/fish)	197.2 ± 43.7	177.5 ± 15.6
Relative fecundity (egg g/fish)	348.1 ± 77.0	426.7 ± 180.9
Larval quality		
Length of larvae on day 0 (mm)	1.88 ± 0.03	1.83 ± 0.05
Larval abnormality rate (%)	3.3 ± 2.5	2.5 ± 1.7
Survival rate to day 3 (%)	69.3 ± 8.7	62.6 ± 23.9

* significant differences (p<0.05)

n: number of broodfish (data from the fish that did not have second breeding were discarded)

Sex Steroid Hormone Levels and Oocyte Size

One day after stripping, the average serum E2 level of brooder was low (55.1±19.5 pg/ml) and then significantly peaked 15 days after stripping (657.5±201.9 pg/ml). After that, E2 level gradually decreased over 30 days (355.9±147.5 pg/ml) and 45 days (192.7±47.3 pg/ml) after breeding respectively. However, the E2 levels of female fish after stripping for 15 days and 30 days were not significantly different (p>0.05) (Figure 5.2a). The oocyte diameter of female fish 1 day after stripping (46.1±7.9 µm) and 15 days after stripping (45.7±11.1 µm) remained unchanged and seemed to be stable. Thereafter, diameter increased gradually from 30 days (104.4±18.2 µm) until reaching its largest size at 45 days after stripping (319.8±76.5 µm), which was significantly different (p<0.05) from oocyte from fish at 1, 15 and 30 days after egg stripping (Figure 5.2b).

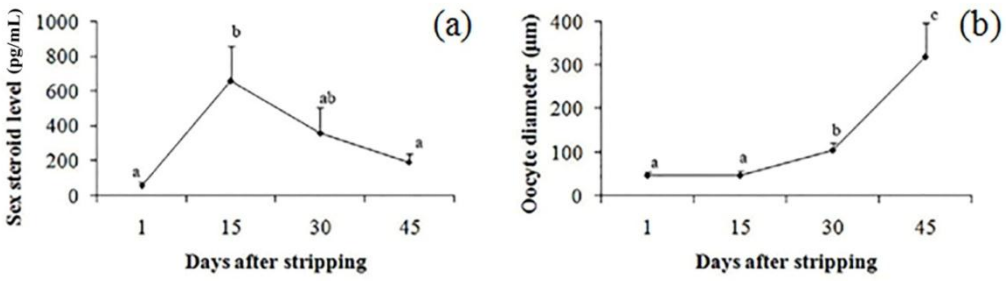

Figure 5.2 The serum sex steroid (17β-estradiol) level (left) and oocyte diameter (right) of hatchery-reared spotted scat after stripping. Error bars represented as the mean ± SD (n=4 for sex steroid, n=10 for oocyte diameter). Error bars with same lowercase letters are not significantly different (p>0.05).

Ovarian Histology

Histological investigation of the ovaries after stripping showed that the spent ovaries (post-stripping) contained several residual hydrated oocytes (6.8% by number) sparsely scattered within ovaries 1 day after stripping. In addition, most hydrated oocytes were irregular in shape. Pre-vitellogenic oocytes, mainly perinucleolus oocyte (93.2%), were located near the ovary wall while most of the inner area of the ovaries was empty (Figure 5a; Table 5.3). Post-vitellogenic oocytes (0.1%) and several post-ovulatory follicles were found in the ovaries of this period in one of two broodfish (Figure 5b; Table 5.3). After 15 days, the ovaries were classified into developing stage; however, the oocytes in the pre-vitellogenic oocytes, mainly perinucleolar (99.3%) still occupied most of the area of the ovaries. These oocytes located inside the ovarian lamellae were well developed throughout the ovaries in this period. Hydrated oocytes were still found (0.7%) in one of the two broodfish. In this period, post-ovulatory follicles were completely absent (Figure 5.3c, 5.3d). Thereafter, the oocytes were more developed, the proportion of pre-vitellogenic oocytes (71.7%) decreased by growing into vitellogenic oocytes (25.0%) that were present in the maturing ovaries 30 days after stripping (Figure 5.3e; Table 5.3), while some oocytes reached the post-vitellogenic stage (3.3%). At 45 days after striping, the post-vitellogenic oocytes were the dominant group (45.1%) occupying most of the area of the ovaries while pre-vitellogenic oocytes (52.9%) and vitellogenic oocytes (2.0%) continually decreased in number (Figure 5.3f) showing signs of maturation.

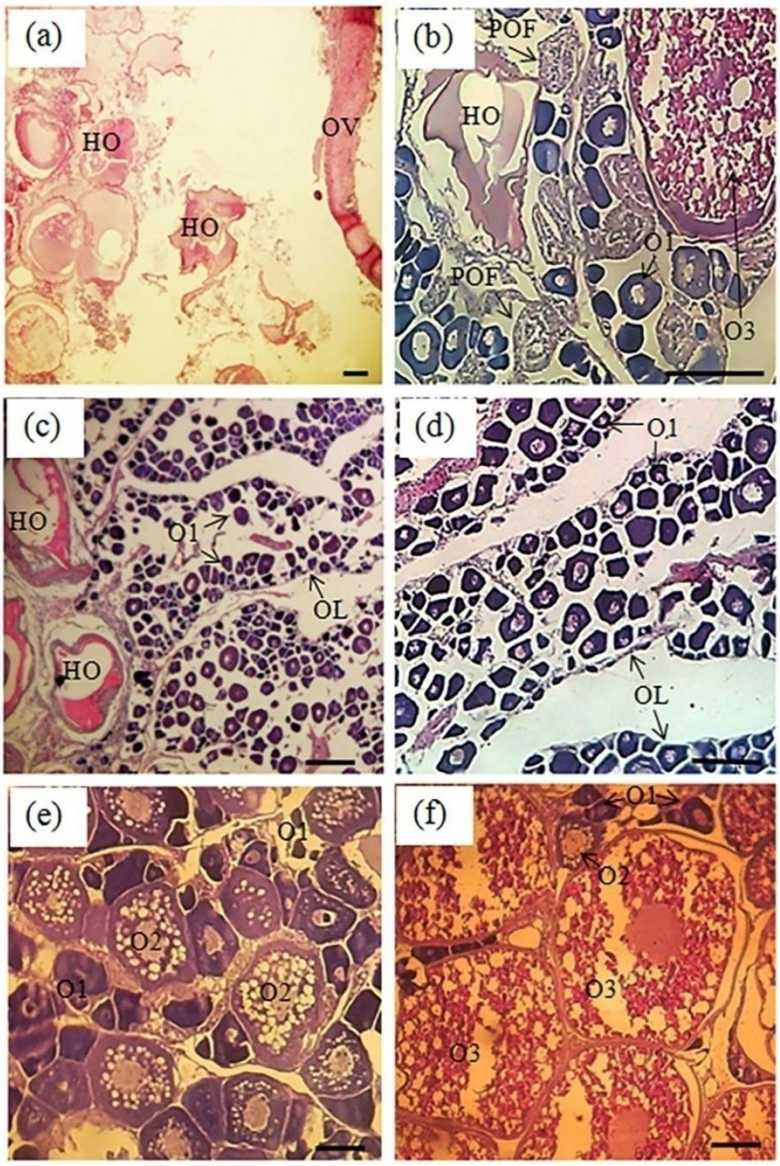

Figure 5.3 Histological section of hatchery-reared spotted scat ovaries 1 day after stripping: spent ovaries (a, b), 15 days: developing ovaries (c, d), 30 days: maturing ovaries (e) and 45 days: mature (f). HO=hydrated oocytes, OV=ovary wall, O1=pre-vitellogenic oocytes, O2=vitellogenic oocytes, O3=post-vitellogenic oocytes, OL=ovarian lamellae, POF=post-ovulatory follicles, HE staining, Scale bar = 100 µm.

Table 5.3 Oocyte size and percentage of oocytes (mean ± SD) at each stage of development of spotted scat females reared in hatchery after egg stripping.

Days after stripping	Ovary stage	Oocyte size[I] (μm)	Percentage of oocytes at each stage (%)[II]				POF
			O1	O2	O3	HO	
1	Spent	46.1 ± 7.8	93.2 ± 3.4	0.0	0.1 ± 0.03[III]	6.8 ± 3.4[IV]	present[III]
15	Developing	45.7 ± 11.1	99.3 ± 0.9[IV]	0.0	0.0	0.7 ± 0.9[III]	not found
30	Maturing	104.4 ± 18.2	71.7 ± 6.6	25.0 ± 7.5[IV]	3.3 ± 1.0	0.0	not found
45	Mature	319.8 ± 76.5	52.9 ± 8.1	2.0 ± 1.2	45.1 ± 9.4	0.0	not found

[I]oocytes of the largest size group in ovaries of one broodfish

[II]average from two broodfish per days after stripping

[III]found only in one (of two) broodfish

[IV]oocytes that occupied the most area in ovaries of two broodfish

O1: pre-vitellogenic oocyte, O2: vitellogenic oocyte; O3: post-vitellogenic oocyte, HO: hydrated oocyte; POF: post-ovulatory follicle

Discussion

The oocyte recruitment period of female broodstock was nearly one month and a half, an optimum time for seed production of this species because the timing for juvenile production from day-1 larvae to juvenile was approximately the same, namely, about 40-45 days (Chang and Hsieh, 1997). Hatcheries, especially small-scale hatcheries, may plan to sell or move juveniles produced after the first insemination to fish farmers before inducing ovulation of the next breeding. Data about timing for oocyte recruitment of hatchery-reared fish after spawning or after being stripped from artificial breeding are limited. Morehead *et al.* (2000) found that the timing of egg production of captured striped trumpeter (*Latris lineata*) was 22-37 days, slightly shorter than in this study. The study of hatchery-produced meager, *Argyrosomus regius* broodstock, was found to be similar in timing to the spotted scat, as vitellogenesis in

this species was completed within 2 months (Mylonas *et al*., 2013), while some species, such as bighead carp, *Aristichthys nobilis* took more than 2 months (71-107 days) for oocyte recruitment (Fermin *et al*., 1991). The annual reproductive period of fish depends on the reproductive strategy of each species. In the species with group-synchronous spawning, the reproductive cycle was short while the period was longer in asynchronous spawning fish. The germ cell renewal was more intense during the annual reproductive cycle which occurred during the regenerating phase and developing phase while renewal was sharply reduced during the spawning-capable phase (Wildner *et al*., 2013). The regenerating phase is the recovering phase when new oocytes are generated for the next spawning or the next stripping when artificial insemination is applied.

Some of the reproductive performance parameters of the two consecutive inseminations were significantly different. Thus, the result is beneficial for seed production planning of this species because the quantity and quality of the gonadal recruited broodfish did not decline when the same broodfish were re-inseminated. Although, presently, the spotted scat is not a domesticated species and broodstock still cannot spawn naturally in captivity, they can produce continuing reproductive cells under captive conditions. This provides evidence that spotted scat can adapt well to an artificial environment, similar to the findings of previous research (Wongchinawit and Paphavasit, 2009). However, in the present study, some parameters were significantly different such as egg size before hormone injection, ovulation rate and percentage of viable eggs which were inferior to those at the first breeding. This result may be due to accumulated stress of broodfish after first stripping because, after stripping, broodfish were kept in a small floating net cage inside a broodstock tank for convenience in further maturation monitoring. Fish stress clearly affects reproductive capability,

especially gamete quality such as decreased egg size or low hatching rate (Contreras-Sinchez *et al*., 2013; Sarameh *et al*., 2012). The captive stress can be the cause of oocyte development dysfunction. Imanaga *et al*. (2014) found that 60% of captive female failed to undergo vitellogenesis. Moreover, in the present study, the subsequent effect of decrease in egg size before hormone inducement caused inconsistent ovulation rates and viability of eggs of recruitment females. Thus, the average ovulation rate of recruitment broodfish was almost 2 times lower than that of the first bred. Spedicato *et al*. (1995) showed that an indicator of success of hormone-induced spawning in dusky grouper was initial oocyte diameter (vitellogenic oocyte) that should be larger than 420 μm. However, Mylonas *et al*. (2003) found that in multi-batch group-synchronous fish, egg size may decline with subsequent spawning events. Therefore, under optimum environmental conditions and nutrition, spotted scat broodstock rearing in captivity inside the hatchery can provide adequate eggs and has potential for sustainable seed production.

The result of 17β-estradiol (E2) level confirmed that the spotted scat has group-synchronous ovaries development because the highest level of E2 was observed in the post-ovulated broodfish, similar to the study of Utoh *et al*. (2013) that they studied in Japanese conger, *Conger myriaster*. However, these results contrasted to some studies, in which the E2 level was typically at its peak during the vitellogenesis phase (Nazari, 2010; Pramanick *et al*., 2010). In the present study, the E2 level nearly peaked at 15 days after stripping. At that time, the diameter of the recruited oocytes had not increased, and was similar to that found 1 day after stripping. However, the development of oocytes in the next period was initially observed after E2 level enhancement. Utoh *et al*. (2003) suggested that the early high of E2 was probably a

result of secretion from the remaining vitellogenic oocytes, but oocytes in this stage were rarely observed in the present study. Most of remaining oocytes at this period were perinucleolar and residue of hydrated oocytes. However, hatchery-reared broodfish easily undergo reproductive dysfunction affecting their hormonal profile (Imanaga *et al*., 2014). Thus, scat broodstock in captivity in this study may have captive stress affecting the E2 profile. This possibility is supported by the oocyte size not exceeding 400 μm in diameter even though mature, unlike the mature oocyte size of wild scat (Gandhi *et al*., 2014).The results obtained from ovarian histology clearly show that the new oocytes firmly established inside ovarian lamellae, while the former mature oocytes degenerated within 15 days after stripping. This is consistent with some reports suggesting that ovaries development of spotted scat is group-synchronous (Castanos and Barry, 1988) and as such it can spawn several times during the spawning season (Cai *et al*., 2010).

Conclusion

This study has shown that the timing for oocyte recruitment of hatchery-reared spotted scat was 41.6±6.8 days. The vitellogenic oocyte diameter, ovulation rate and percentage of viable eggs of oocyte recruited females were smaller/lower than the previous matured broodfish ($p<0.05$) while other reproductive performance parameters of the first artificial insemination and the second breeding with oocyte recruitment were not significantly different. This study provides basic information about this species that reveals interesting characteristics of brooder performance which could benefit mass seed production in the future.

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CHAPTER 6

THE MATURATION AND POSSIBLE PROTANDROUS SEX CHANGE OF HATCHERY-REARED SPOTTED SCAT (*Scatophagus argus* Linnaeus, 1766)

Abstract

Research into the maturation and the possible protandrous sex change of hatchery-reared spotted scat, *Scatophagus argus* was undertaken to verify that spotted scat is a protandrous species. Cultured spotted scat fry were reared in a hatchery until they reached maturation. Then mature fish were selected for 2 rearing regimes in different tanks: in tank 1, only 50 males (average body weight 70.7g) were reared for 18 months, and in tank 2, both males (average body weight 66.5 g) and females (average body weight 127.7 g) were reared together at a ratio of 1:2.5 (70 fish in total) for 12 months. Fish body weight, maturation and sex change were investigated every 3 months. The average final body weight of the male fish in tank 1 was 124.9 g and the average final body weights of the male and female fish in tank 2 were 117.2 and 262.0 g, respectively. The percentage of running males in both tanks, ranged between 74 and 100 % and 40 and 85 % in tanks 1 and 2 respectively, with a tendency to decrease gradually. Milt qualities were not significantly different (p>0.05) at any of the studied intervals. The sperm densities were 4.0-5.8 x10^9 and 3.7-5.3 x10^9 spermatozoa/ml, percentages of motile sperm were 68.8-80.1 % and 70.0-80.6 % and the sperm motility durations were 3.8-4.5 and 2.4-4.7 min in male fish in tanks 1 and 2 respectively. The milt volume of the male fish in tank 1 seemed to have decreased gradually, as the final volume was 81.7 µl/fish (54.6 µl/100 g fish). Moreover, evidence of sex change was

not found in either tank. The sex steroid hormone (E2), which ranged between 55.7-100.6 pg/ml and 40.0-68.0 pg/ml in the male fish in tanks 1 and 2 respectively, was not significantly different (p>0.05) throughout the studied periods. This study indicates the reproductive potential of two rearing regimes of hatchery-reared spotted scat with no finding of protandrous sex change in this species at the age and size of the broodstock studied.

Introduction

Spotted scat or "Kitang fish" (*Scatophagus argus*) is a teleost which is commonly distributed around the coast of the Indo-Pacific region (Nelson, 1976). It is a popular food fish and in the Philippines, scat is one of the most expensive fish (Barry and Fast, 1992) with a price higher than both grouper and sea bass. Moreover, scat is a popular aquarium fish which is traded in both domestic markets as well as being exported abroad (Jayalal and Ramachandran, 2012) due to its unique morphology and color pattern. Currently, almost all scat fry are captured from the wild and the available quantity is insufficient to meet market demand (Barry and Fast, 1992). There have been studies conducted in several countries attempting to institute mass seed production for this species including the Philippines (Barry *et al*., 1988), Taiwan (Chang and Hsieh, 1997), China (Cai *et al*., 2010), Vietnam (Khanh *et al*., 2012) and Thailand (Ruensirikul *et al*., 2008).

In Thailand, the Coastal Aquaculture Research Institute succeeded in breeding spotted scat in 2008 using artificial propagation (Ruensirikul *et al*., 2008) and subsequently, larval rearing and nursing were studied (Ruensirikul *et al*., 2009a) for the purpose of mass seed production. At Present, some fish farmers are fairly successful in

earning income by rearing spotted scat to a marketable size. However, almost all the scat broodstock used for breeding are captured from the wild with consequent uncertainty of both the quantity and quality of the available broodfish. This uncertainty represents a significant disadvantage of using wild broodfish. Moreover, captured broodfish or fish fry in the wild are only available in some seasons (Mair, 2002). Thus, there needs to be long term planning for the production of domesticated broodstock for commercial scale breeding (Duarte *et al*., 2007) in the same way as it has been successfully instituted for various commercial marine fish (Fuchs and Nedelec, 1989; Emata *et al*., 1992; Maynard *et al*., 2012; Ranjan *et al*., 2014). The establishment of a reliable supply of spotted scat broodstock is important for the mass seed production of this species in the future. Ruensirikul *et al*. (2012) found that spotted scat can be reared in hatcheries until they reach maturation and are able to breed. However, the long term maturation of cultured broodfish in hatcheries has not yet been studied.

A sufficient supply of fish broodstock in terms of both quantity and quality is one of the most important factors for successful fish breeding using hatchery-reared broodfish. Excess numbers of broodfish can affect production costs, while too few broodfish can affect the level of mass seed production. Therefore, the proportion of mature males and females should be maintained at the optimal level. However, sex change can affect the fish sex ratio (Allsop and West, 2004) which will subsequently affect broodstock planning. The sex reversal of cultured broodfish has been reported in several marine finfish species and a pattern of sex reversal in older fish is termed sequential hermaphroditism. This pattern has commonly been found in teleost fish (Todd *et al*., 2016). However, to date, there have been only limited reports of indications of sex reversal in spotted scat. Arrunyakasemsuke (1975) suggested that

spotted scat may be a protandrous sex change species in which fish are male in the early stages of development but then later change into females, since the number of wild male scat has been observed to be much lower than females. In addition, Shao *et al*. (2004) found ovotestis in some spotted scat.

The aim of this study was to investigate the maturation and possible protandrous sex change in hatchery-reared spotted scat in order to discover baseline data which will be important for the establishment of spotted scat broodstock in hatcheries.

Materials and Methods

Broodstock Rearing and Conditions

Spotted scat broodstock were obtained from artificial propagation at the marine fish hatchery of the Coastal Aquaculture Research Institute (Songkhla, Thailand). Both sexes (ratio 1 : 1) were reared together in a 28 m^3 cylindrical cement broodstock tank, for at least 12 months, until maturation, at a density of 10 fish/m^3 (around 1 kg/m^3). The fish were fed twice a day to satiation with a 35 % protein commercial diet for marine fish (Thai Union Feedmill Co., Ltd., Samut Sakhon, Thailand) throughout the period. About 50 % of the water was changed every two weeks and the water quality was measured by the standard method of the APHA (1998). It was maintained at a water salinity of 10-30 ppt, a temperature of 26-28 ^oC, a pH of 7.5-8.1, with dissolved oxygen at 5.4-6.5 mg/L, ammonia at 0.05-0.13 mg/L and nitrite at 0.01-0.02 mg/L.

Broodstock Selection

Spotted scat broodstock from the broodstock tank were selected using head (snout) shape (Barry and Fast, 1988) (Figure 6.1) combined with other signs of maturation. The milt of mature males was collected by applying abdominal massage and the oocytes of mature females was sampling with a cannulation tube (ovarian biopsy). The selected broodfish were reared in 2 tanks, which were the same size as the broodstock tank in two culture modes as follows: In mode 1, only 50 mature male (70.7±15.4 g in weight and 13.2±0.8 cm in total length (TL)) were reared in tank 1; and in mode 2: mature males (66.5±13.6 g, 12.9±0.9 cm TL) and females (127.7±32.4 g, 15.6±1.2 cm TL) were reared together in tank 2 with a total of 70 fish at the ratio of 1 : 2.5. All the broodfish in both tanks were tagged with passive integrated transponder (PIT) tags. The conditions and culture manipulation in the study tanks were the same as those for the broodstock tank. The water quality was monitored every 2 months by the APHA (1998) method and maintained at a water salinity of 5-30 ppt, a temperature of 26-28 ^{o}C, a pH of 7.4-7.8, with dissolved oxygen at 5.3-6.4 mg/L, ammonia at 0.10-0.80 mg/L and nitrite at 0.01-0.35 mg/L. All the fish were regularly weighed to monitor their maturation and their average daily growth (ADG) was calculated based on the formula: final weight in g - initial weight in g / culture period in days.

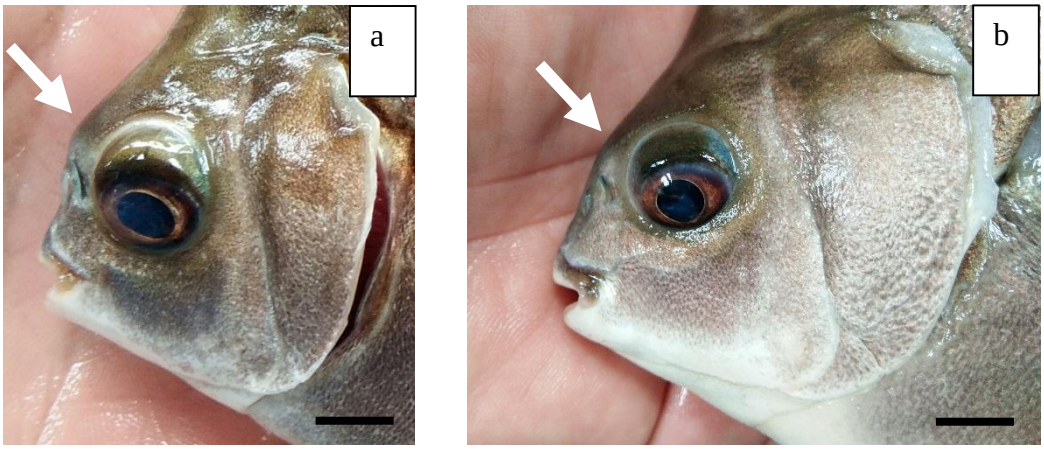

Figure 6.1 Snout shape (arrow) of mature male (a) and female (b)

(scale bar = 1.0 cm)

Maturation and Sex Change Monitoring

Sex change was monitored every 3 months by monitoring the maturation of every male fish. Sex change occurs when vitellogenic oocytes are present in mature males and the β-estradiol (E2) hormone level increases significantly (Todd *et al*., 2016). All the male abdomen were gently pressed to check the number of running males, and the percentage of spermiating males was calculated by comparison with the total numbers of males which were checked. After that, the milt quality of ten spermiating males was investigated. Milt samples were collected using a cannulation tube and the total milt volume was checked using a micropipette (Rottmann *et al*., 1991). The milt volume per weight of male fish was then calculated. The sperm density and percentage of sperm motility were checked by the method of Rainis *et al.* (2003). The maturity of females was checked by ovarian biopsy and the sampling of their oocytes Females determined to be mature by this method were classified as gravid

females. The percentage of gravid females was determined by comparison with the total number of females checked. The oocyte diameter and the percentage of completed post-vitellogenic oocytes (PVO) of fish with swollen abdomen (either mature females or sex changed males) were measured after sampling the oocytes with a cannulation tube. Oocyte size was measured under a light microscope with an ocular and stage micrometer (n=20). The percentage of completed PVO was monitored by counting the completed PVO that were spherical in shape and of the same size and black color when observed under a light microscope and comparing the number with the total number of oocytes observed (100 oocytes/individual). Changes in the external morphology of the head shape of males, especially the snout were monitored since if sex change occurred then the behavior and external morphology of the fish would also probably change (Warner, 1984). If sex reversal did occur, the timing, the number of fish, their snout shape, weight and the total length of the sex changed fish were recorded. This study continued either until sex change was observed or the culture period reached 1 year.

Sex Steroid Hormone Levels

At the time of each maturation investigation, blood samples from six fish per tank were taken from the caudal vein. The serum was obtained by centrifuging the blood for 5 min at 4 ^{o}C and 6,000 rpm and it was then stored at −20 ^{o}C until the sex steroid hormone level (17β-estradiol: E2) level was determined by electrochemiluminescence immunoassay (ECLIA) using the Elecsys Estradiol III kit (Roche Diagnostics, Germany) with an immunoassay analyzer (Modular Analytics E170) according to the manufacturer's instructions. The sensitivity of the assay was 5.0 pg/mL.

Statistical Analysis

All data were expressed as mean ± the standard error of the mean (SE). One-way analysis of variance (ANOVA) was used to compare the milt and egg quantity and quality parameters including the sex steroid hormone level, with a significance level of $p<0.05$. Duncan's multiple range test was applied to determine significant differences among culture period of each mode.

Results

Maturation and Sex Change of Culture Mode 1

After a culture period of 18 months, no evidence of sex change in any of the male scat was found. The weight of the male fish increased gradually to an average final weight of 124.9 ± 23.0 g (Figure 6.2a), and an average total length of 15.7 ± 0.6 cm, with an ADG of 0.10 g/day and a final survival rate of 62.0 % (Figure 6.2b). After maturation, the numbers of spermiating males gradually decreased from 100 % to 74.2 % (Figure 6.3a). The milt volume of each male ranged from 81.7 - 217.9 µl/fish and 54.6 - 220.0 µl /100 g (Figures 6.3b and 6.3c). The sperm density, sperm motility and motility duration were not significantly different throughout the culture period ($p>0.05$) and were within ranges of 4.0 - 5.8 $x10^9$ spz/ml, 68.8 - 80.1 % and 3.8 - 4.5 min, respectively (Figures 6.3d, 6.3e and 6.3f).

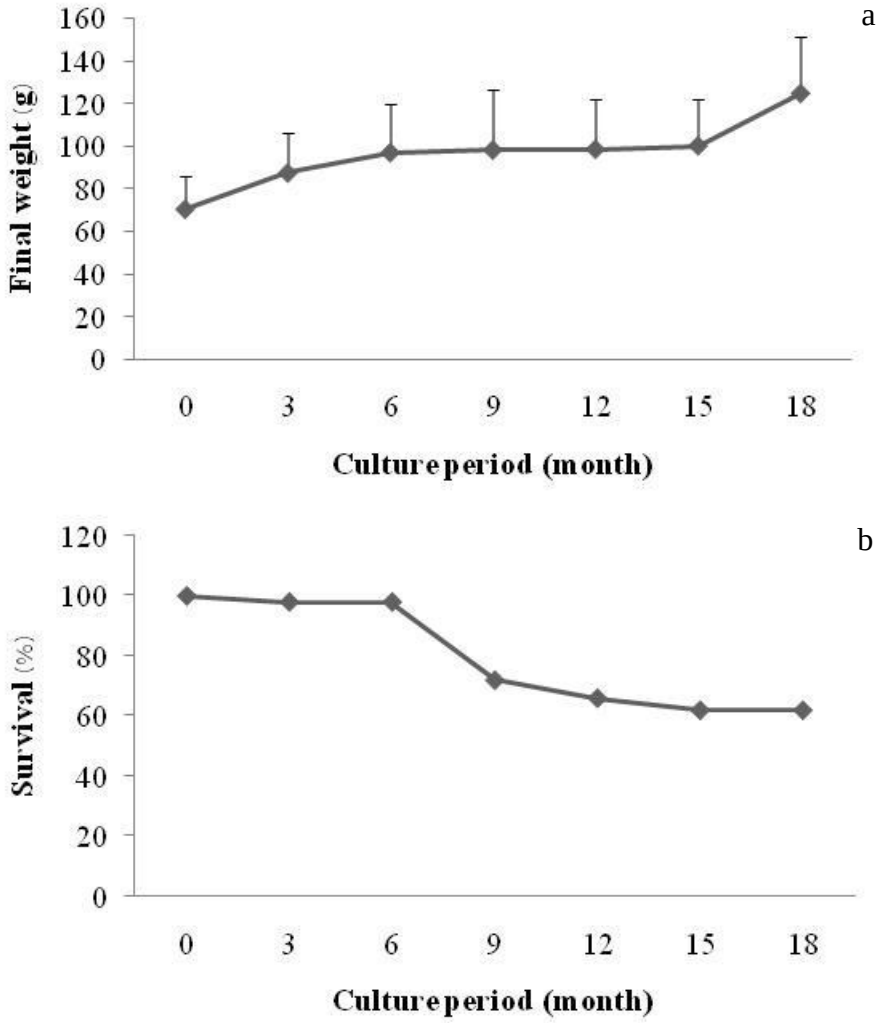

Figure 6.2 Final weight (a) and survival rate (b) of mature male scat reared in a mono-sex environment for 18 months

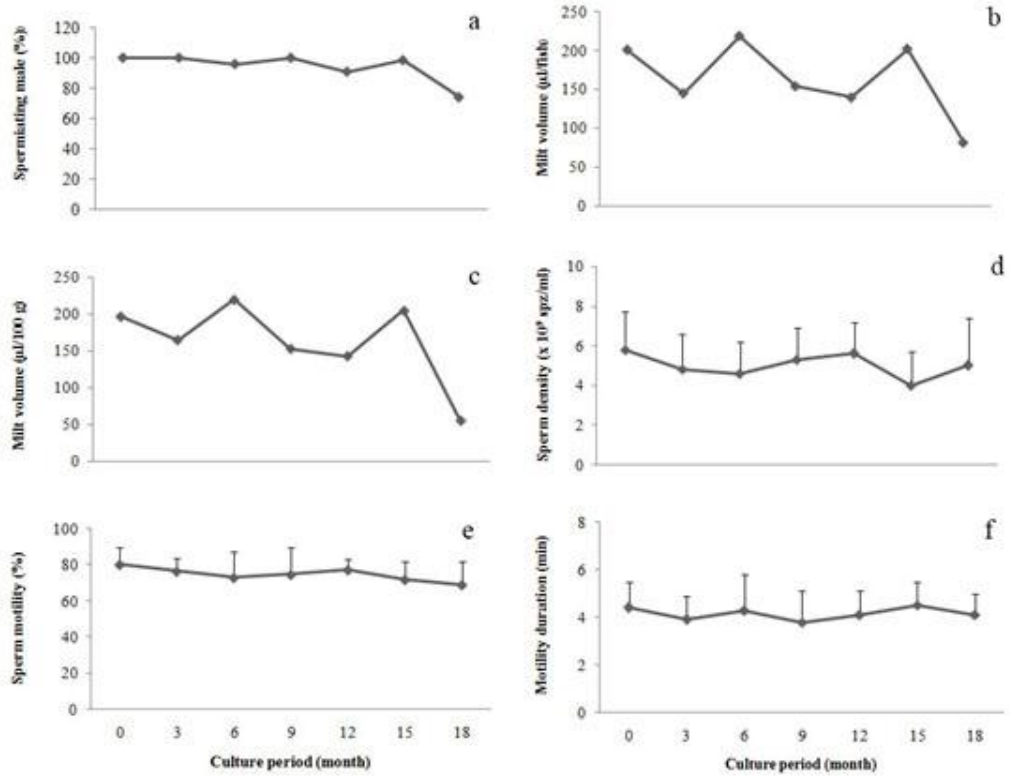

Figure 6.3 Percentage of spermiating males (a) milt volume per fish (b) milt volume per fish weight (c) sperm density (d) sperm motility (e) and sperm motility duration (f) of mature male scat reared in a mono-sex environment for 18 months

Maturation and Sex Change in Culture Mode 2

After rearing mature males and females together for 12 months, no protandrous sex change was found in any fish. However, the final weight of both sexes gradually increased with females growing faster than males. The final weight, total length and ADG of the male scat were 117.2 ± 28.5 g, 15.5 ± 1.6 cm and 0.24 g/day respectively while those of the female scat were 262.0 ± 36.6 g, 19.8 ± 0.8 cm and 0.37

g/day respectively. (Figure 6.4a). The survival rates were 50 and 56 % in male and female fish respectively (Figure 6.4b).

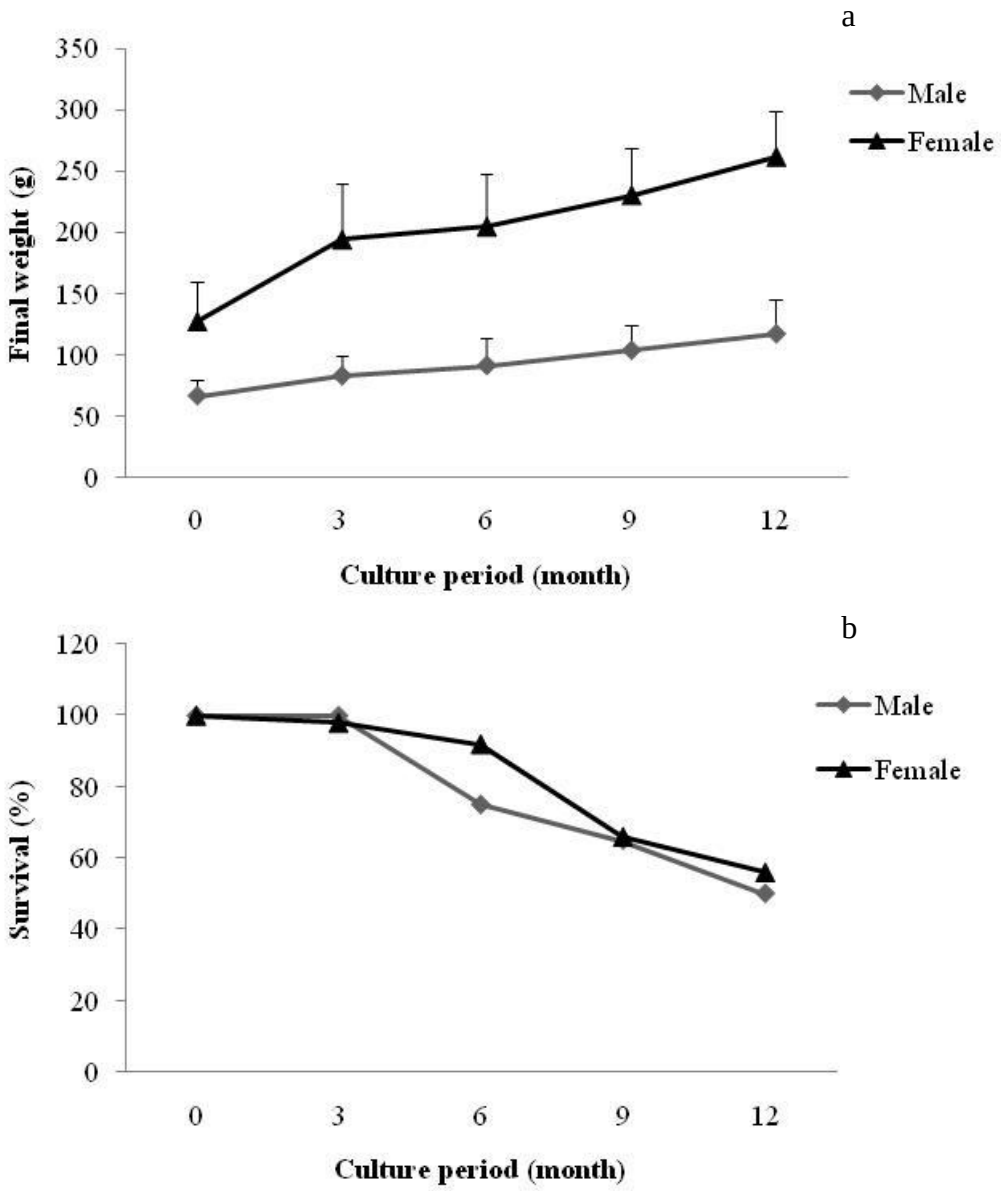

Figure 6.4 Final weight (a) and survival rate (b) of mature male and female scat reared together for 12 months

With regard to the maturation parameters of the males, the percentages of spermiating males varied and tended to decrease in a range of 85 – 40 % (Figure 6.5a) while the total milt volume of each male and the milt volume per male weight tended to increase in a range of 57.0-171.0 µl/fish and 2.4-167.0 µl/100 g, respectively (Figures 6.5b and 6.5c). The sperm density, sperm motility and motility duration tended to be stable and were not significantly different over all sampling intervals throughout the culture period (p>0.05) varying in ranges of 3.7 - 5.3 x 10^9 spz/ml, 70.0 - 80.6 % and 2.4 - 4.7 min, respectively (Figures 6.5d, 6.5e and 6.5f).

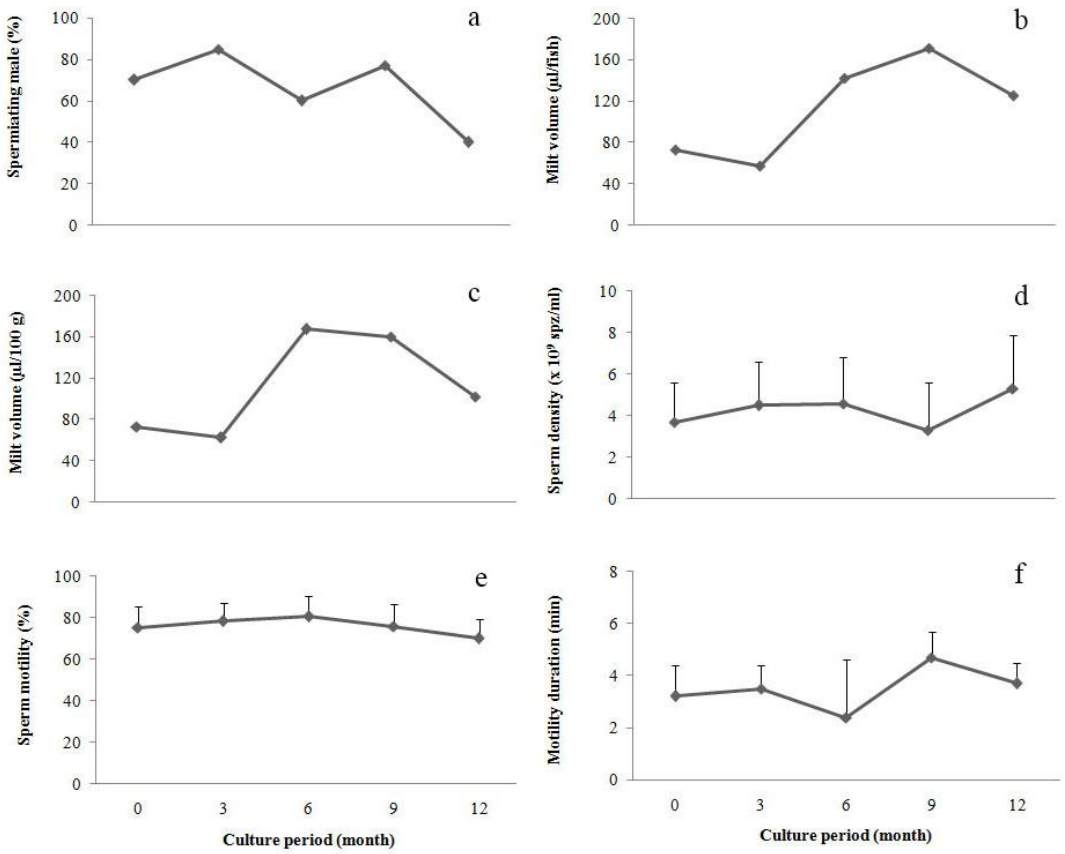

Figure 6.5 Percentage of spermiating males (a) milt volume per fish (b) milt volume per fish weight (c) sperm density (d) sperm motility (e) and sperm motility duration (f) of mature male scat reared together with mature females for 12 months

In regard to female maturation, gravid females were found throughout the culture period and the numbers increased in a range of 20.0 - 75.8 % (Figure 6.6a) while the egg size and the percentage of complete PVO whilst varying in ranges of 398.8 - 423.3 µm and 69.8 - 83.0 %, respectively tended to be consistent and were not significantly different throughout the culture period (p>0.05) (Figures 6.6b and 6.6c).

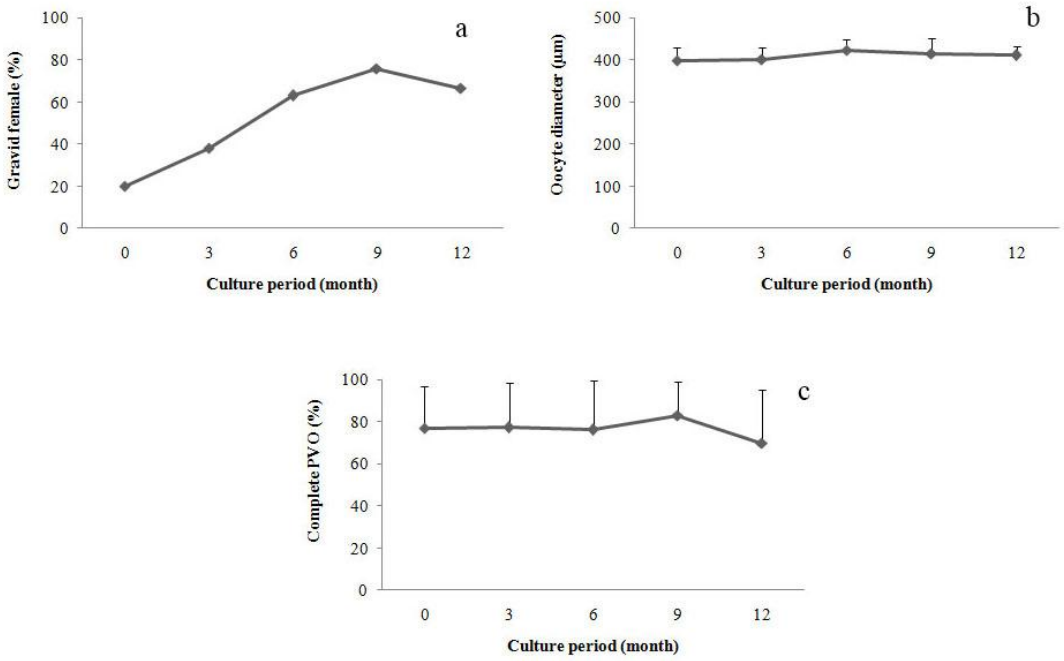

Figure 6.6 Percentage of gravid females (a) oocyte diameter (b) and percentage of complete post-vitellogenic oocyte (c) of mature female scat reared together with mature males for 12 months

Sex Steroid Level (E2)

The E2 levels for the fish in the two culture modes varied throughout the culture period in the ranges of 55.7 - 100.6 pg/ml and 40.0 - 68.0 pg/ml in tanks 1 and 2, respectively (Figure 6.7). However, the hormone levels measured at each time interval were not significantly different (p>0.05).

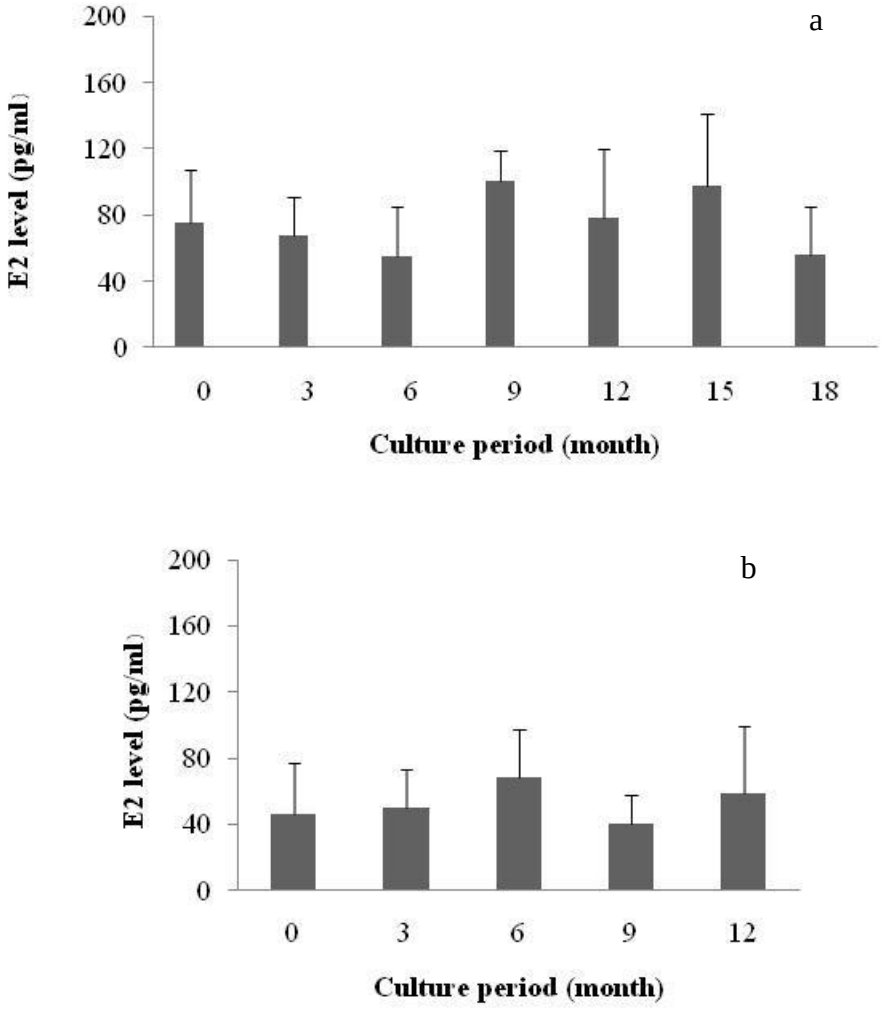

Figure 6.7 Sex steroid hormone (E2) levels of mature males reared in mono-sex mode (a) and both sex rearing mode (b)

Discussion

The findings of this study are important for the establishment of spotted scat broodstock in hatcheries. Normal growth and gonad development was found when compared with a previous study of scat broodstock culture in a hatchery (Ruensirikul *et al*., 2012). Male fish growth was lower than that of females which was consistent with the report of Gandhi *et al*. (2013). However, the survival rate in both culture modes was rather low because of fish mortality due to parasites and diseases during the observation periods. Overall, the quantity and quality of the sperm of the spotted scat did not significantly decrease and were not affected by the artificial breeding conditions except that the milt volume per fish and per weight of fish in mode 1 decreased to lower levels of 81.7 µl/fish and 54.6 µl /100 g, respectively. This is important because the ratio of the number of sperm to the number of eggs affects the success of the artificial breeding of fish (Chereguini *et al*., 1999; Gallego *et al*., 2013). Assawaaree *et al*. (2013) found that the ratio between sperm and eggs for the artificial fertilization of spotted scat should be at a minimum level of 1.5×10^6 spz/oocyte. However, the sperm volume of the fish in mode 2 in which males and females were reared together, tended to be higher and increased gradually. That was probably because male spermiation was activated by pheromones in the ovarian fluids of the females which may facilitate reproductive cells (De Fraipont and Sorensen, 1993; İnanan and Öğretmen, 2015). This is a natural mechanism in wild fish especially in the spawning season. Almost all the sperm quality measurements obtained from the present study were similar to those of a previous study of hatchery scat (Ruensirikul *et al*., 2009b). Although the egg size (average 409.5 µm) was larger than that found by Ruensirikul *et al*. (2012) (average 363.0 µm). This finding

should encourage artificial propagation because breeding success is likely to be higher with a bigger egg size (Ochokwu *et al*., 2016).

The present study did not investigate the success of artificial breeding because no mature females were bred. Thus, further research is necessary to determine the year-round breeding performance of mature females in a hatchery to ensure the availability of hatchery broodfish. However, the data relating to egg quality obtained from mode 2 reflected good performance by the female broodfish due to the high percentage of PVO and the larger egg size before hormone inducement, compared with previous studies of spotted scat. (Ruensirikul *et al*., 2008; 2012). Although the possibility of protandrous sex change in spotted scat has been previously reported (Arrunyakasemsuke, 1975; Shao *et al*., 2004) this study, in which spotted scat were reared to a mature stage for a minimum of 1 year in a hatchery, did not find any evidence of sex reversal of mature male fish, either in mono-sex culture or in mixed sex culture. No males produced eggs nor were any change in head shape (used for the sexual identification of scat) noted (Barry and Fast, 1988; Gandhi *et al*., 2013). In addition, the E2 level which is important for determining the sexual characteristics of fish was not significantly different throughout the culture periods. Typically, this sex hormone should abruptly increase when a male fish changes into a female (Todd *et al*., 2016) and the E2 level should also be higher at the vitellogenesis stage in the oogenesis process (Moyle and Cech, 2004). Zhang *et al.* (2018) found that The E2 level of mature female spotted scat was >200 pg/ml while the highest mean E2 level of male scat in the present study was only 100 pg/ml. That was probably the reason that no sex change occurred. Moreover, Zhang *et al.* (2018) also investigated the E2 level in mature males and found it to be only 6.1-7.8 pg/ml, which was approximately five times lower than

that found in this study. This level of E2 indicates that if the species is protandrous then mature male scat may only change into females after the age of one year or later. The E2 level found in the two different culture modes was not significantly different. Therefore, the appearance of female fish did not affect the sex reversal of male scat, This finding is similar to that of Kappus and Fong (2014), who found that the sex ratio was not affected by sex change in coral reef fish.

Nevertheless, Shao *et al*. (2004) found some mature scat which had both types of reproductive cells in their gonad (ovotestis). However the scat with ovotestis weighed about 143.0 g which was higher than the weight of the scat in both tanks at the end of this study. This suggests that the scat in the present study may not have been large enough to reach the optimal size for sex change. The age and size of fish are important factors in sequential hermaphroditism (Warner, 1984; Kobayashi *et al.*, 2013). Based on the average ADG from the two tanks (0.17 g/day), additional culture periods of at least 3.5 and 5.1 months would be needed for the fish in tanks 1 and 2, respectively, to reach the optimal size for sex change. Thus, further research on spotted scat sex change should focus on bigger broodfish than those used in this study and fish with an initial weight of at least 120 g should be cultured in a hatchery for 5 - 6 months to allow them to reach the size at which ovotestis were found by Shao *et al*.. In the present study, spotted scat initially weighing only 60-70 g were used and the slow growth rate of the male spotted scat rendered the data collection period quite long. The findings are in agreement with a study on sex reversal in black porgy (*Acanthopagrus schlegeli*), which revealed that this fish was male in the first 2 years of its development then at 3 years of age, about 50 % of the males changed into females (Lee *et al*., 2001).

Conclusion

The present study revealed no significant differences in most of the maturation parameters in terms of the quantity and quality of the broodfish reared in two different sex ratios in a hatchery, the exception being that the milt volume in males reared in the mono-sex condition seems to be lower than that of the males reared in a polysex culture. Moreover, sex change was not observed in either culture modes. This study however, highlights the importance of understanding the reproductive biology of spotted scat and the potential benefits of the two rearing regimes of hatchery-reared spotted scat.

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CHAPTER 7

SUMMARY

Because of the excellent characteristics of spotted scat and their potential as a subject for commercial aquaculture in the future, a breeding program for the mass seed production of this species needs to be established. Whilst breeding techniques have been developed in several countries, mass seed production has not yet been reported. In Thailand, the artificial insemination of spotted scat was successfully conducted in 2008 (Ruensirikul *et al*., 2008), although up to now, only broodstock captured in the wild have been used in induced ovulation for breeding. To exploit this species on a commercial scale, as is already done for well-known marine finfish such as sea bass and grouper, a hatchery-produced broodstock should be established accompanied by improved breeding techniques to facilitate mass seed production.

In spotted scat, hatchery-reared broodstock have only been introduced relatively recently (Ruensirikul *et al*., 2012). The breeding performance to date has, however, not been satisfactory because of a lack of basic information about both the environmental conditions under which spotted scat optimally breed or about the reproductive biology of the fish, which affects its breeding performance, thus rendering planning for commercial scale production of the fish difficult. A suitable environment is important for normal gonad development, as well as for spawning and the survival of the larvae. Spotted scat is a euryhaline species and migrates to a wide range of water salinity throughout its life cycle. Therefore, water salinity is the first environmental factor to be studied for its effect on breeding operations and reproductive development.

The effect of water salinity on breeding success and reproductive performance was dealt within Chapters 3 and 4. In **Chapter 3** it was shown that successful fertilization and hatching was favored by higher water salinity while lower water salinity levels were more conducive to the early stage of larval rearing. In **Chapter 4** it was shown that reproductive development is facilitated in a wide range of water salinities. However, water salinities of between 15 and 25 ppt are recommended to maximize its ovulation rate. No differences in sex steroid level or serum osmolality between treatments were found, indicating spotted scat's excellent adaptation to fluctuating water salinity. The findings obtained from those two chapters are summarized in Table 7.1.

Table 7.1 Recommended water salinity for each spotted scat breeding activity in a hatchery

Breeding activities	Range of tested water salinity (ppt)	Recommended water salinity (ppt)
1. Fertilization	0 – 35	25 – 35
2. Hatching	0 – 35	25 – 35
3. Larval rearing	0 – 35	10 – 15
4. Broodstock rearing	5 – 33	15 – 25

However, it is not only external factors such as water salinity which affect the establishment of hatchery-reared broodstock, since internal factors such as

some aspects of their productive biology of the broodfish are also important. In **Chapter 5**, the timing of oocyte recruitment was investigated and found to be 41.6±6.8 days. Moreover, the reproductive performance parameters of the first and the recruited oocyte were found not to be statistically different in their tendencies. This is important data relating to the availability of broodstock in hatcheries impinging on both their quantity and quality. The other aspect of reproductive biology investigated was sex reversal and in particular the possible protandrous pattern of development of this species, which was studied in the previous chapter. **Chapter 6** revealed that no sex change from male to female was found in hatchery-mature spotted scat, even though the fish were observed for a culture period of 1 year or more.

In conclusion, this thesis has revealed the potential for culturing hatchery-reared spotted scat broodstock for commercial mass seed production. The water salinity requirements for breeding and the reproductive performance of the broodfish was determined. The timing of oocyte recruitment and the quality of the recruited gonad was compared and the issue of possible sex change was also clarified.

Recommendations and Further Studies

The quality of hatchery-reared spotted scat should be improved in the future especially from the perspective of feed and nutrition since manipulating only the environmental conditions does not seem to be sufficient to promote good reproductive performance. In **Chapter 4**, percentage of mature females found was only about 35-45% and the ovulation rate was only about 40-50%. It was shown by Zhang *et al*. (2013) that broodstock nutrition is important for reproductive cell development in spotted scat broodstock.

In **Chapter 6**, the initial size of the mature males in this study may have been too small to successfully study sex reversal. Furthermore, the growth rate of male scat was much lower than that of females. Thus, a study employing a longer time to allow the fish to reach the target size for changing sex based on the report of Shao *et al*. (2004) is necessary. A further study of this topic should employ broodfish with an initial weight of 120 g or more.

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Publication

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