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Variations in Gonadosomatic Index, Gonadal Development and Spawning Induction of Spotted Scat *Scatophagus argus* (Linnaeus, 1766)

Huy V. Nguyen*, Nghia V. Duc, Son K.H. Nguyen

University of Agriculture and Forestry, Hue University, Vietnam

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Abstract

This study evaluated variations in gonadosomatic index (GSI), gonadal development stages, and hormonal spawning induction of *Scatophagus argus* in captivity. Male and female fish were cultured separately in net cages in Tam Giang lagoon, Central Vietnam, from January to December 2020. Five fish of each sex were randomly sampled monthly. Gonads were collected, GSI determined, and subsequently prepared for histology. Gamete quality was assessed with a light microscope. Spawning was hormonally induced with different doses of human chorionic gonadotropin (hCG) and luteinizing hormone-release hormone (LHRH-A2). Gonadal development started to increase in March, peaking in July. The peak spawning period of the fish was from June to August, displaying the highest GSI value, sperm motility, and oocyte diameter. Only female GSI fluctuated significantly month-by-month ($P<0.05$). Histological examination indicated that *S. argus* is a multiple-spawner. Application of LHRH-A2 (70 µg/kg) stimulated spawning and resulted in better latency periods, fertilization, and hatching rates.

* Corresponding author: Huy V. Nguyen, e-mail: huy.nguyen@hueuni.edu.vn

Introduction

Aquaculture is the fastest-growing food-producing sector and an important contributor to poverty reduction and increased food security in Vietnam (FAO, 2016c). The Tam Giang-Cau Hai Lagoon is the largest brackish lagoon in Southeast Asia and has a surface area of nearly 22,000 hectares. It supports a high diversity of aquatic species, especially many highly valuable fish. The spotted scat (*S. argus*) is considered a typical species of this ecosystem and is one of the most economically important brackish water fish species in Southeast Asia; for human consumption and as an ornamental fish (Mandal et al., 2020). In the Tam Giang-Cau Hai Lagoon, spotted scat is considered the main target species for aquaculture because of its high adaptive capacity to environmental stress (Su et al., 2016) and its suitability to mono- and polyculture systems. However, the development of this species for aquaculture is limited by the availability of fingerlings because seed supply is currently highly dependent on natural collection. Several researchers have reported the artificial reproduction of this fish species in other countries around the world (Cai et al., 2010).

Nonetheless, the number of fingerlings produced has remained limited. Indeed, very few reports regarding the seed production of *S. argus* have been published (Barry & Fast, 1992; Cai et al., 2010). Factors that may have influenced the limitations in artificial seed production include a lack of scientific standards in broodstock management, the maturation of females in captivity, and insufficient quality and quantity of males and females for artificial production on a commercial scale.

The gonadal development of vertebrates is controlled by two gonadotropins from the pituitary gland (Yaron et. al., 2009; Mylonas et al., 2010). Correspondingly, in teleosts, the gonadotropin hormones, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) are considered key hormones in controlling steroid secretion through activation of specific receptors: follicle-stimulating hormone receptor (FSHr) and luteinizing hormone receptor (LHr) (Chauvigné et al., 2012). FSH is involved in vitellogenesis and oocyte development, while LH plays a role in final oocyte maturation and ovulation (Nagahama & Yamashita, 2008; Yaron et. al., 2009). The roles of FSH and LH in gonadal maturation in male fish remain unclear. A recent study by Xie et al. (2017) showed that a single signal (either FSH or LH) might be sufficient to stimulate spawning in male fish. Some fish caught from the wild and subsequently used for breeding in captivity exhibit reproductive dysfunction (Zohar & Mylonas, 2001; Mylonas et. al., 2004; Kokokiris et. al., 2005; Yaron et. al., 2009). This may be due to being in captivity and the resulting lack of a spawning environment or the absence of the maturation-inducing hormone after the accumulation of yolk proteins during oogenesis (Zohar & Mylonas, 2001; Mylonas et. al., 2004; Kokokiris et. al., 2005; Yaron et. al., 2009). Failures in the naturally spawning wild fish have also been observed in captive conditions due to reproductive dysfunctions (Zohar & Mylonas, 2001; Mylonas et. al., 2004; Kokokiris et. al., 2005; Yaron et. al., 2009). We also collected mature fish from the wild and induced breeding during the natural spawning season. This can be achieved by using artificial hormones to stimulate ovulation in fish (Zohar & Mylonas, 2001; Mylonas et. al., 2004; Kokokiris et. al., 2005; Yaron et. al., 2009). Unfortunately, larval survival in captured adults is very low when using this method. This is because it depends on the timing of intervention in the natural spawning season and on the hormone-inducing method used (Bourque & Phelps, 2007). Therefore, the broodstock needs to be well controlled, and the gonadal maturation process needs to be well examined before reproduction. Besides, monthly GSI values are also an important indicator for the maturation of fish and the spawning season of *S. argus* (Gandhi et al., 2014).

The hormones of human chorionic gonadotropin (hCG) and luteinizing hormone-releasing hormone analog (LHRH-A) are commonly used in fish artificial reproduction in fish farms; they have distinct differences in terms of bioactivity and physiological function. While LHRH-A stimulates gonadotropin secretion in teleosts as a result of ovulation and spawning, hCG

stimulates oocyte maturation. The hormonal administration of a low dose may result in failure to induce fish spawning. On the contrary, too high doses of hormonal induction may cause oocyte over-ripening or poor egg quality (Roy, 2016).

The present research aims to determine the gonadal developmental stages of fish in captive conditions, gonadosomatic index (GSI), and the effects of different doses of two hormones, (hCG) and (LHRH-A2, to induce spawning. These parameters were evaluated by studying oocyte diameters, latency periods, fertilization rates, and hatching rates of *S. argus*.

Materials and Methods

Broodstock management and sampling

A total of 300 healthy adult fish (>1+ year) were selected from the earthen pond and identified as male or female based on body size (Su et al., 2019) and head profile (Barry & Fast, 1992). Male (n=150) and female (n=150) fish were cultured separately in net cages of 4m × 5m × 1,5m (5 fish/m^3) in Tam Giang-Cau Hai Lagoon Central Vietnam (16°33'58.7^ N 107°38'01.9'' E) from January to December 2020. The water temperature and light intensity were recorded using automatic light/temperature data loggers (UA-002-08). The average temperature ranged from 23.7 ± 2 °C (in winter) to 29.8 ± 3 °C (in summer), and the average light intensity ranged from 500 lx (in winter) to 5,100 lx (in summer). The broodstock was fed with floating pellet feed (crude protein 55%, crude fat 8%) at a rate of 3% of body weight per day.

Five males (n=5) and five females were randomly sampled monthly. Fish were anesthetized with AquiS ® solution (20 ppm, Bayer Company, Vietnam). Subsequently, their total length and weight were measured and recorded before the gonads were removed by dissection. The individual gonads of each fish were weighed using an electronic balance with an accuracy of ±0.1 g to determine the gonadosomatic index (GSI) as 100 × gonads weight/total body weight (g). A small portion from three parts of the ovary (anterior, middle, and posterior) was fixed in a 10% buffered formaldehyde solution for histological examination. The central portions of the fixed gonads were extracted, dehydrated, and embedded in paraffin. The samples were then sectioned at 6 µm thickness using a semi-automatic Leica (RM2245) microtome and stained with hematoxylin and eosin for microscopic analysis (Alonso-Fernández et al., 2011). The development of ovaries was divided into five stages: immature (Stage 1), vitellogenesis (Stage 2), maturation (Stage 3), mature (Stage 4), and atresia (Stage 5) based on the oocyte frequency percentage, as described in (Mandal et al., 2020). Egg samples from each remaining ovary were kept in Ringer's solution for egg diameter measurement with a filar micrometer.

Broodstock selection for hormone induction

In addition to assessing the sperm through histological examination, sperm from individual males were carefully collected by gently massaging the abdomen. Since sperm motility is associated with higher fertilization capacity, sperm motility was evaluated under a light microscope after adding clean seawater to the specimen (Rurangwa et al., 2004). Male fish with highly motile sperm (**Figure 2**) were selected for the fertilization test (**Figure 2A**). A biopsy was performed using a polyethylene catheter to determine egg diameter and quality (Bardon-Albaret & Saillant, 2017). The eggs were preserved in Ringer solution for subsequent measurement. Egg quality was evaluated under a light microscope, as described by Mandal et al. (2020). Oocytes were scattered in a petri dish (**Figure 1**) in Serra's solution (ethanol-formalin-acetic acid, 6:3:1 by volume) to clear the cytoplasm and determine the oocyte maturation stage. Females with fully mature oocytes and diameters greater than 400 µm were selected for the hormone induction experiment (**Figure 2B**), as recommended by Barry and Fast (1988). In contrast, males with dead spermatozoids (**Figure 2C**) and females with spent oocytes in the ovary were not selected for the experiment (**Figure 2D**).

Administration of hormones

To determine the types and doses of hormones needed to stimulate spawning in spotted scat, two different doses of hCG and five different doses of LHRH-A2 were administered in two injection sessions at 24 h intervals (**Table 1**). Five females (n=5) were randomly selected using the biopsy method (**Figures 1** and **2**).

Figure 1 Egg collection from female *S. argus* with a catheter through a syringe.

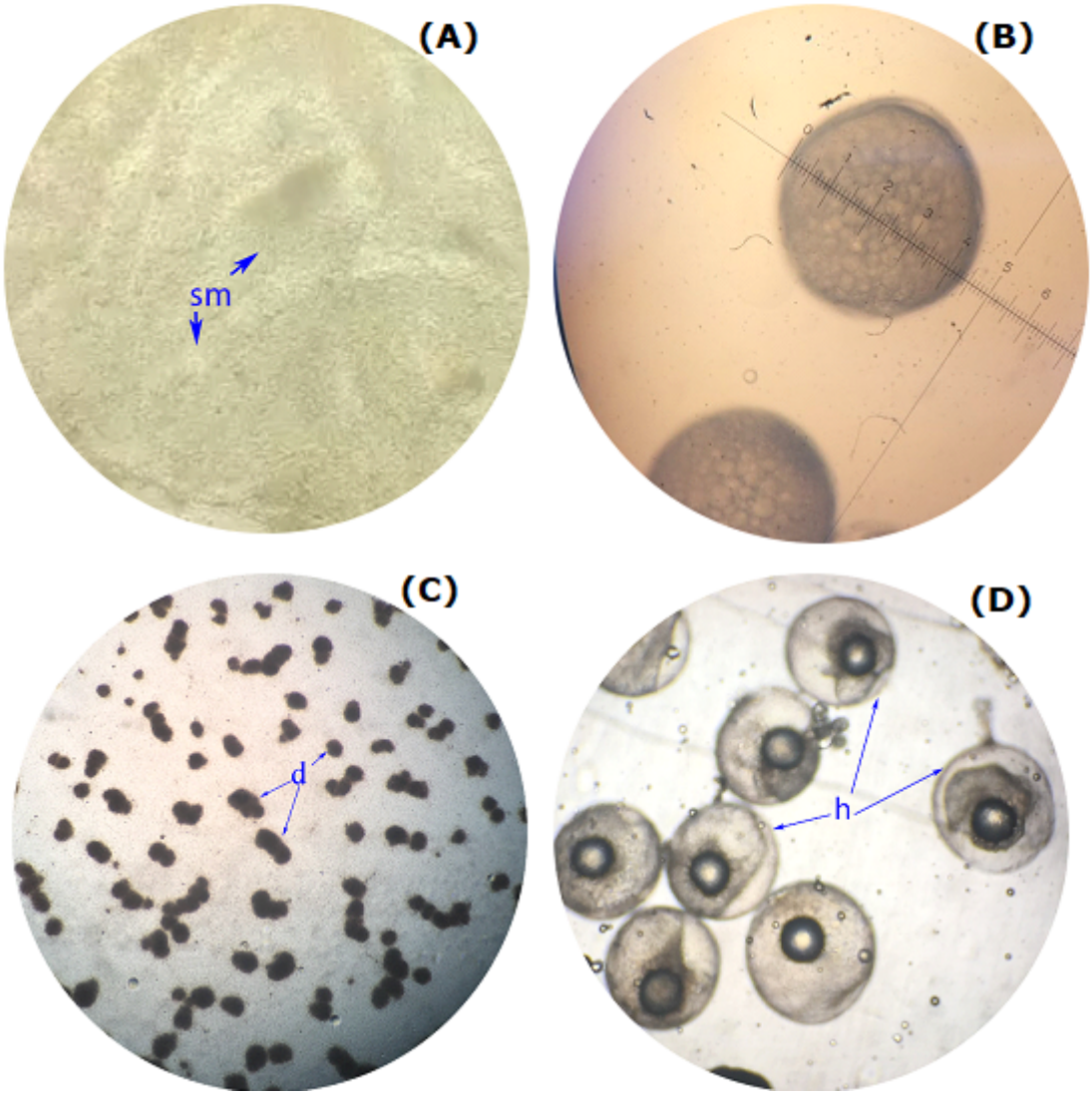

Figure 2 Observation of sperm and egg quality of *S. argus* under a light microscope:
(A): Maturing spermatozoa distributed uniformly and with high motility, collected in the early week of June: sperm motility (sm)
(B): Maturing oocytes filled with yolk granules and globules, oocyte diameter >400 µm, collected in the last week of June.
(C): Degenerated spermatozoa characterized by dead spermatozoids (d), collected in mid-August
(D): Degenerated oocytes characterized by the hydration (h) of the follicle wall and an oil drop concentrated in one at the central oocytes, sampled in mid-August.

Table 1 Hormone doses administered to randomly selected female *S. argus* in order to evaluate the effects of different levels of human chorionic gonadotropin (hCG) and luteinizing hormone-releasing hormone analog (LHRH-A2) on spawning

Treatments	*(control) 0,9%NaCl*	*hCG (IU/kg)*	*hCG (IU/kg)*	*LHRH-A2 (µg/kg)*	*LHRH-A2 (µg/kg)*	*LHRH-A2 (µg/kg)*	*LHRH-A2 (µg/kg)*
Fish weight (g)	415.3±2.9[a]	420.7±5.2[c]	408.3±2.3[b]	412±4.2[c]	416.4±5.0[c]	429.7±2.9[c]	420.7±4.7[ab]
First injection	-	500	500	10	10	10	10
Second injection	-	1,000	2,000	20	40	60	80
Total	**-**	**1,500**	**2,500**	**30**	**50**	**70**	**90**

After being anesthetized with AquiS ® solution (5 ppm), fish were injected at the dorsal fin with either hCG or LHRH-A2 at various doses. The control group was injected with saline water (0.9 % NaCl). The male brooders (see section broodstock selection) were injected once with 200 IU·kg^{-1} at the same time as that of the last injected female fish. At 24 h post-injection, females were biopsied to measure oocyte diameter. In addition, the females were evaluated every hour for signs of ovulation, such as courtship behavior and changing abdomen size. Both males and females were washed with clean seawater and immediately anesthetized with 20 ppm AquiS ® solution. The eggs and sperm were manually stripped into a graduated bowl containing 500 mL clean seawater (28 – 30‰), mixed well using a chicken feather, and then allowed to stand for 5 min.

Subsequently, the eggs were poured into a glass graduated cylinder to determine the total volume, floating fractions, and sinking fractions. Triplicate samples of sinking or floating eggs were randomly collected from the container and preserved in 10% formalin in a 2 mL Eppendorf. Sinking and floating eggs were counted in a Petri dish under a light microscope by multiplying the average eggs per milliliter of the total sample volume. The total number of eggs was the sum of the number of sinking and floating eggs. Sinking fractions of eggs from each female were discarded before incubation in a 500-liter tank with a recirculating system. Approximately 100 fertilized eggs from each female (floating eggs) were randomly collected and incubated in a plastic Petri dish containing 20 mL of clean seawater (28 ± 1‰) at 28 ± 1 °C. The mean fertilized and hatching rates were estimated for five replicates.

Statical analysis

The statistical analysis of GSI, egg diameters, latency periods, fertilization rates, and hatching rates was performed by analysis of variance (one way-ANOVA test) (multi-comparisons Tukey-Kramer HSD posthoc test) at a significance level of $P<0.05$, using SPSS software version 20.0. Data are expressed as mean ± standard deviation (SD).

Results

Monthly changes in GSI

Monthly changes in the GSI were observed for both sexes (**Figure 3**). The highest GSI was observed in July for males (1.89%) and females (9.09%). In addition, female GSI showed a significant difference among the delineated stages ($P < 0.05$). No significant differences were found in different stages of male GSI ($P > 0.05$). The annual change in male GSI was negligible, ranging from 1.02% in January to 1.89% in July. Meanwhile, the GSI of females increased gradually from January (0.98%) to March (2.04%) and then sharply increased from April (2.86%) to June (8.17%), reaching a peak in July (9.09%) before declining to the lowest value in December (0.88%). The increase in the GSI of female fish was related to ovarian stage development (**Figure 4**). The highest GSI value corresponded to Stage IV (June-August), and the lower values of GSI were in the pre-vitellogenesis (December-February) and atresia period of gonadal maturation (September-November) (**Figures 4** and **5**).

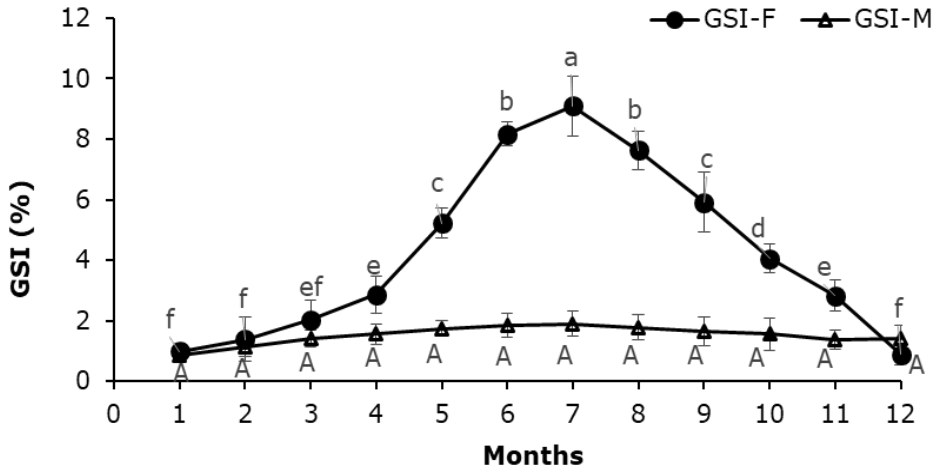

Figure 3 Monthly change in Gonado-Somatic Index of spotted scat *S. argus* denoted as: Male GSI (GSI-M); Female GSI (GSI-F). *Different letters a, b, c, d, e, and f show significant differences among monthly samples of female GSI at p < 0.05; the Group with the similar letter A in a bar is not significantly different among monthly samples of male GSI at P < 0.05.*

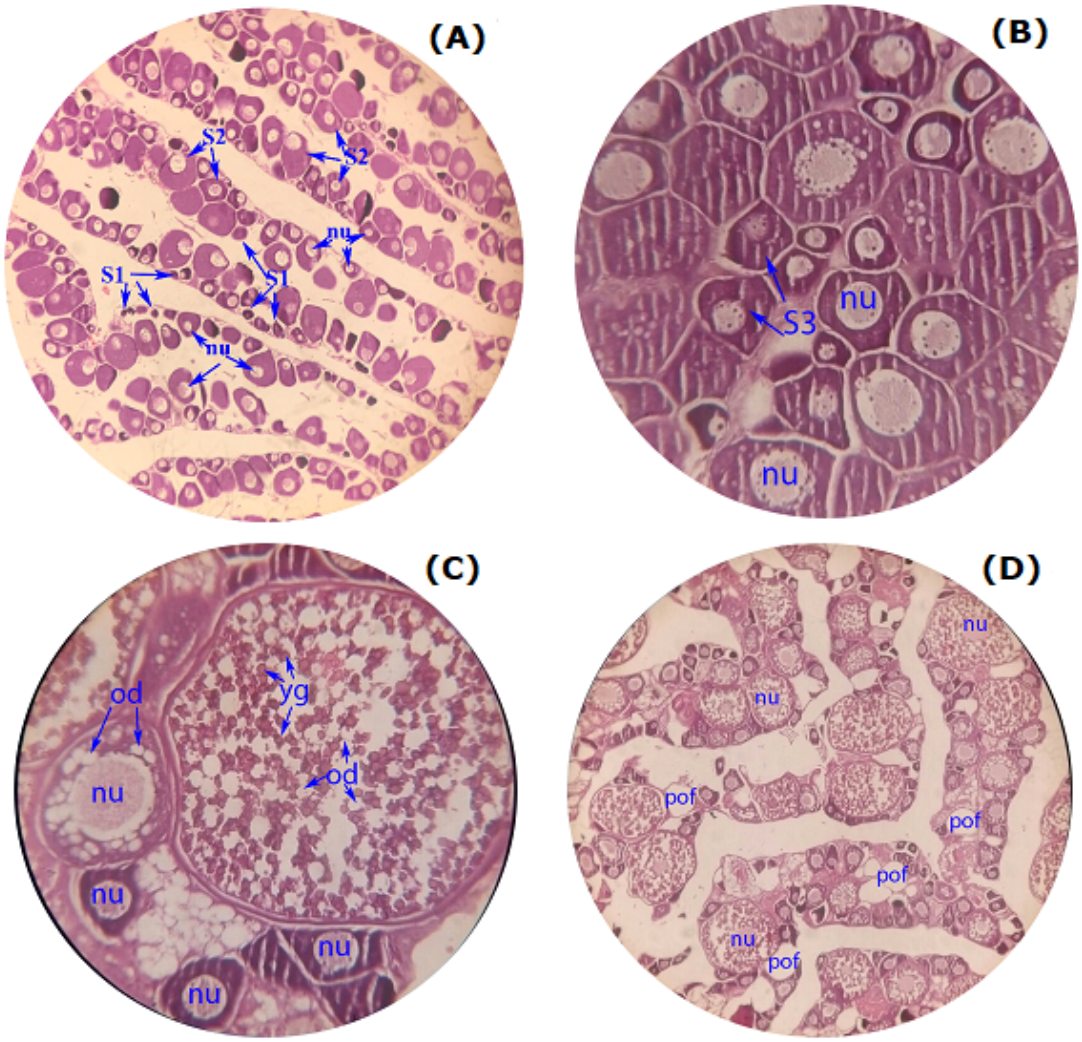

Figure 4 Histological observation of different stages of female *S. argus* ovaries: (**A**); Immature ovaries with mostly primary oocytes at S1 (Stage I) together with S2 (Stage II) with a large proportion of nucleus (nu) during November - February; (**B**) Maturing with dominant S3 (Stage III) together with S2 during March-May; (**C**) Mature – full growth oocytes with increasing in accumulation of yolk granules (yg) and oil droplets (od) during June - August; (**D**) Atresia during September – November is characterized by presenting post-ovulation follicles (pof).

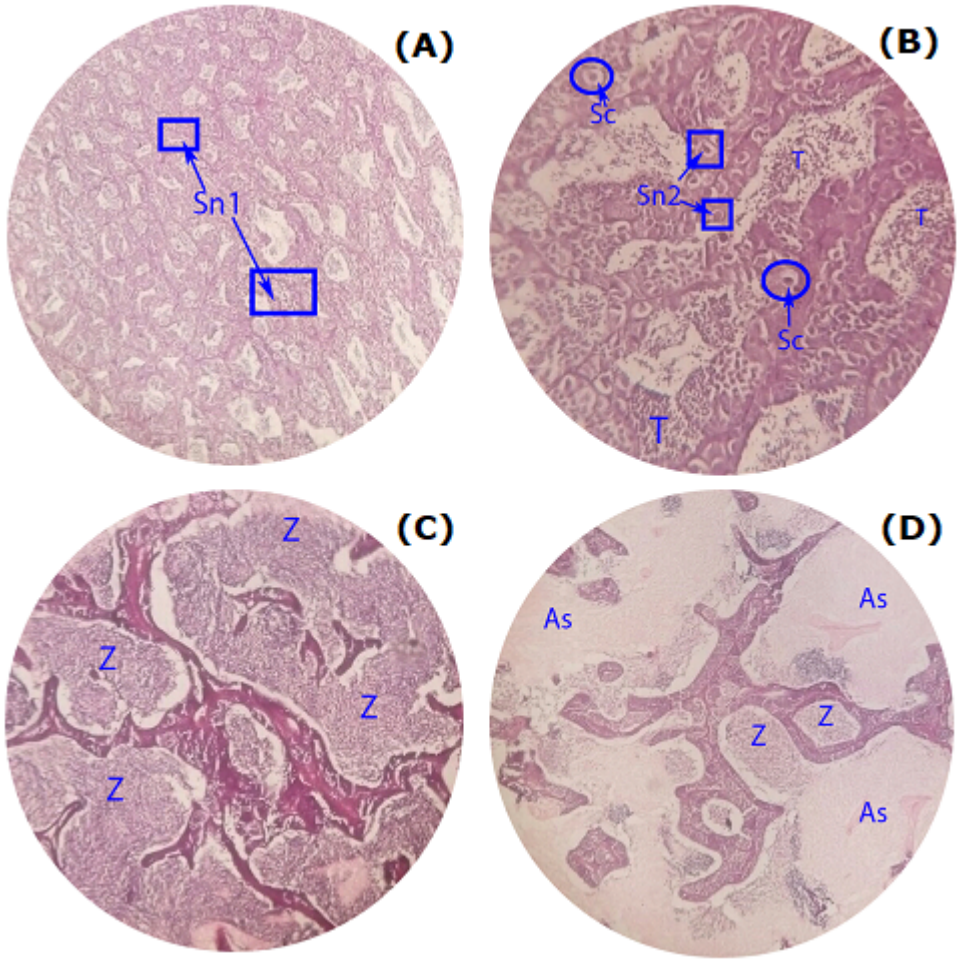

Figure 5 Histological observation of different stages of male *S. argus* testes***:***
(**A**) Immature primary spermatogonia (Sn1) were present in testis which are not visible; (**B**) Developing spermatogonia (Sn2) in spermatocytes (Sc), especially the appearance of many spermatids (T); (**C**) Mature with spermatozoa (Z); (**D**) Regression with remaining spermatozoa and mostly empty space after spent (As).

Stages of ovarian development

The development of different stages of ovarian and testicular was described in **Table 2** and **Figures 4** and **5**.

Table 2 The ovarian and testicular development stages of *S. argus*

Stages	*Ovaries*	*Testis*
Immature	Stage I (40–90 μm): The gonads are very small and transparent; they contain oocytes in the first growth phase. Cannot distinguish sex. Visible from December–January.	Stage I: The gonads are small, and transparent. Sex is indistinguishable. Only primary spermatogonia cells are present.
Vitellogenesis	Stage II (90–200 μm): Developing immature ovaries start to increase in size. While still translucent, sex can be distinguished. This ovarian stage shows oocytes with the appearance of cortical alveoli (**Figure 4A**). Visible from January–March.	Stage II: The testes are still slightly round in appearance. However, they begin to develop a pair of lobes. Secondary spermatogonia and spermatocytes are clearly detectable.
Maturing	Stage III (200 – 420 μm): In the ovaries, oocytes have irregular shapes in vitellogenic with clear appearance of yolk granules and oil droplets. The nucleus is in a central position. Visible in April and May.	Stage III: The testes are pinkish white in color and present in various ratios of testicular cysts including spermatogonia, spermatocytes, spermatids, and spermatozoa (**Figure 5B**).
Mature	Stage IV (420 – 570 μm): Ovaries with mature oocytes are characterized by the migration of the germinal vesicle from the central position to the periphery. Oocytes with a round shape and at maximum size with yolk granules and oil droplets are present.	Stage IV: The testis tissues presented spermatozoids. Milt and their quality are present (**Figure 5C**)
Atresia	Stage V The hydration of the follicle wall, irregular shape of oocytes and translucent appearance marked post-ovulation (**Figure 4D**). In this stage mostly atretic oocytes are present. Some mature oocytes are present as well as pre-vitellogenic and early vitellogenic oocytes, which are growing for the next breeding cycle.	Stage V: The testes are in degenerative process. They are irregular in shape with remaining spermatozoa which can be seen.

Effects of types and doses of the hormone on the spawning of S. argus

The effects of hCG and LHRH-A2 in stimulating *S. argus* were observed. Eggs exhibited a markedly larger size when fish had been injected with hCG or LHRH-A2, especially at higher concentrations (**Table 3**). This contrasts with the control treatment, which showed a negligible increase in oocyte diameter (from 437.3 ± 22.5 to 446.2 ± 21.2 μm). Fish injected with hCG did not ovulate or showed overripe eggs because they could not be obtained by applying gentle pressure on the abdomen and hydration of the follicle wall. In contrast, fish injected with LHRH-A2 (at all concentrations) showed more successful results. Different doses of LHRH-A2 resulted in different latency periods, fertilized rates, and hatching rates ($P < 0.05$). Induction with LHRH-A2 at a dose of 70 $\mu g \cdot kg^{-1}$ showed the best results among treatments in this study ($P < 0.05$). This result corresponded with changes in the oocyte diameter, which increased after LHRH-A2 administration.

Table 3 Effects of different doses of human chorionic gonadotropin (hCG) and luteinizing hormone-releasing hormone (LHRH-A2) on the change of oocyte diameters, latency periods, fertilized rate, and hatching rate of *S. argus*

Treatments	*Control (0.9% NaCl)*	*HCG 1,500 IU/kg*	*HCG 2,500 IU/kg*	*LHRH-A2 30 µg/kg*	*LHRH-A2 50 µg/kg*	*LHRH-A2 70 µg/kg*	*LHRH-A2 90 µg/kg*
Oocyte diameter before injection (µm)	437.3 ± 22.5^{a}	439.8 ± 19.2^{a}	434.5 ± 13.5^{a}	427.9 ± 18.1^{a}	432.2 ± 15.6^{a}	433.5 ± 34.0^{a}	429.3 ± 27.8^{a}
Oocyte diameter 24 h post-injection (µm)	446.2 ± 21.2^{a}	485.1 ± 21.7^{ab}	508.9 ± 17.0^{ab}	653,9 ± 28.7^{c}	671.0 ± 16.1^{c}	675.3 ± 26.2^{c}	662.5 ± 27.7^{c}
Latency period (h)	–	–	–	36.6 ± 2.3^{c}	31.0 ± 3.3^{b}	26.4 ± 2.8^{a}	24.7 ± 2.5^{a}
Fertilization rate (%)	–	–	–	81.7 ± 2.4^{a}	86.6 ± 2.5^{b}	88.9 ± 2.8^{b}	80.9 ± 2.8^{a}
Hatching rate (%)	–	–	–	82.4 ± 4.1^{ab}	85.6 ± 3.8^{ab}	87.6 ± 3.2^{b}	78.4 ± 3.2^{a}

The letter $^{a, b, c}$ in the same row indicates a significant difference among the treatments at $p < 0.05$

Discussion

The present study investigated variation in GSI, ovarian and testicular development stages of *S. argus* spawning under captive conditions and the effects of different doses of two hormones, human chorionic gonadotropin (hCG) and luteinizing hormone-releasing hormone analog (LHRH-A2) on the spawning of spotted scat. Female GSI values corresponded to ovarian development stages and increased GSI values corresponded with increasing oocyte diameter. Therefore, female GSI and egg diameter values are good indicators for determining the spawning season of *S. argus.* In contrast, male GSI did not show significant differences in the monthly samples. Semen analysis showed that it could produce gametes from April. This is a much earlier maturation than observed in females. Nevertheless, histological observation of the testes showed a similar trend in development as observed in the ovaries.

Histological observation of ovaries and the morphology of biopsied oocytes showed that previtellogenic and early vitellogenic oocytes were dominant during December-February. The oocyte diameter in this stage ranged from 40 to 90 μm. The oocytes started to develop and increase quickly from March to May and reached a mature size at the end of June and early July (420 – 570 μm). Vitellogenin increases the size of immature oocytes in the cytoplasm (Mandal et al., 2020).

GSI values declined from August with oocytes in atretic resorption until December, with oocytes developing for the next cycles (Stage 2); these observations suggest that the spawning season of spotted scat in captivity ranges from June to August. This observation agreed well with those of previous reports on this species. Barry and Fast (1992) reported that the spawning season of *S. argus* in the wild in the Philippines is from April to October, with a peak in August as the monsoons begin. Similarly, Gandhi et al. (2014) observed that the GSI of spotted scats from Mandapam coastal India peaked during the monsoon. Two peaks of GSI have also been reported in previous studies (Gandhi et al., 2014; Nguyen et al., 2004). However, a peak spawning season from May to July from the coast of South China has been reported for *S. argus* in cultured conditions, and spawning was not influenced by tidal cycles (Cai et al., 2010). The data obtained on the reproductive cycle of spotted scat in the present study may provide useful information for seed production in hatchery farming.

The application of hormones in spawning induction has been reported in wild and captive *S. argus* (Barry & Fast, 1988; Cai et al., 2010; Su et al., 2019). The present study indicated that LHRH-A2 at doses of 70 $\mu g \cdot kg^{-1}$ showed better results than other treatments, which was consistent with the suggestion by Barry and Fast (1988), who reported that the least effective dose of LHRH-A2 to stimulate the spawning of female spotted scat ranged from 40 to 70 $\mu g \cdot kg^{-1}$. These doses were much higher than those reported by Su et al. (2019). This was attributed to the different ages/sizes of broodfish or latitudinal factors of the duration of the spawning season (Hoque et al., 1998). Spotted scat did not respond to hCG treatments in the present study resulting in an absence of ovulation and overripe eggs. This could either be due to inadequate doses of hCG for stimulation (Lim, 2016) or to hCG being a large peptide hormone that may create antibodies that can react against itself (Zohar & Mylonas, 2001). It is possible that the combination of hCG with other hormones or the hormonal implant method might work to induce the spawning of *S. argus.* The present study indicates that *S. argus* was better adapted to LHRH-A2 than to hCG. The latency time (the time interval between the second injection and ovulation) varied from 24.7 ± 2.53 h to 36.6 ± 2.33 h. This period was much longer than the period reported by Su et al. (2019). It could be related to water temperature, or to shorter latency periods in higher temperature conditions (Rahdari et al., 2014), or to the oocyte diameter of the females (Barry & Fast, 1988). The present study showed that the fertilization and hatching rates were significantly different among different doses of injection of LHRH-A2 ($P < 0.05$). Fish injected at doses of 50 or 70 $\mu g \cdot kg^{-1}$ exhibited higher fertilization rate than those injected at doses of 30 or 90 $\mu g \cdot kg^{-1}$ ($P < 0.05$). The hatching rate was significantly higher in the treatment of 70 $\mu g \cdot kg^{-1}$ than in other treatments ($P < 0.05$). The

application of LHRH-A to induce fish spawning and achieve higher fertilization and hatching rates is commonly applied at doses of 20 – 70 µg/kg in India (Roy, 2016). Doses of LHRH-A significantly affecting fertilization and hatching rates of *S. argus* have been reported by Su et al. (2019). The present study suggests that administration of a too-low or too-high dose of LHRH-A resulted in poor reproductive performance and poor egg viability (e.g., fertilization and hatching rates in spotted scat.

In conclusion, under captive conditions, both males and females of *S. argus* started to develop gametocytes in March, reaching a peak in July. Fish spawned from June to August, when GSI values, sperm mobility, and oocyte diameter were highest. Hormonal tests indicated that applying LHRH-A2 at doses of 70 µg·kg^{-1} was optimal to stimulate *S. argus* spawning and obtain good results in latency periods, fertilization, and hatching rates.

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