Reproductive Characteristics and Gonadal Development of Asian Clam (*Corbicula fluminea*) Broodstocks in Recirculating Aquaculture System (RAS)

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Abstract

Asian clam populations in natural water bodies have been jeopardised due to overfishing and habitat loss recently. Consequently, the clam production for local consumption has declined and requires new avenues to compensate for this decline. Comprehending the reproductive characteristics of Asian clams (*Corbicula fluminea*) is important for seed production in aquaculture due to their hermaphroditic characteristics. This study investigates the reproductive characteristics and gonadal development of captive *C. fluminea* broodstocks reared for 24 wk in a recirculating aquaculture system (RAS). The condition index (CI), gonad index (GI), and histological analysis were completed to assess reproductive traits and gonadal development. The results show that broodstocks achieved optimal CI (>2.5) by the third month, peaking at 3.55 in the fourth month. Asynchronous spawning and heterogeneous gonad development patterns were observed, with active spawning during the conditioning phase. Histology reveals that *C. fluminea* is a hermaphrodite that possess ovo-testes at different stages regardless of the clam size. The body condition significantly influences gametogenesis, and the CI was correlated with GI. The reproductive cycle has four stages: resting/spent, development (gametogenesis), maturity, and spawning. Our comprehensive study of the reproductive characteristics and gonadal development underscores the substantial potential of *C. fluminea* in hatcheries to propagate and facilitate seed production for Asian clam aquaculture.

Keywords: condition index, gonad index, histology, hermaphrodism, *Corbicula fluminea*

Introduction

In Asian cuisine, the freshwater bivalve *Corbicula fluminea*, commonly known as the Asian clam, is a significant food source, particularly in East Asia. The production in China alone surpasses an annual production of 12 million tonnes within this region, underscoring its considerable food value (Zhu et al., 2018; Ramli et al., 2020). In addition, it can propagate across a range of habitats, from freshwater to brackish environments, in which *C. fluminea* shows an outstanding adaptability to various ecological conditions (Morden, 2015; Rak et al., 2022). Predominantly, it was popularised for consumption in China, and similar consumption trends have emerged in Asian countries such as Japan, Taiwan, Thailand, Cambodia, and Malaysia (Yamada et al., 2010; Zhu et al., 2018).

Moreover, consumption and trade have shown significant activity in Japan since the 1980s when *C. fluminea* stocks were imported, primarily from Korea and China to satisfy the local market (Okawa et al., 2016). Recent investigations highlight a growing trend of *C. fluminea* being transported from Cambodia and Thailand to Malaysia, highlighting its expanding global trade (Ramli et al., 2020). Despite its economic significance, the escalating demand for this clam simultaneously increases the incidence of overharvest from the wild, which poses a potential threat to its natural populations. However, attempts toward sustainable utilisation have generated fruitful results, such as successful farming in Hualien, Taiwan, where *C. fluminea* serves as a food and an eco-tourism attraction (Chang et al., 2016). Since the acquisition of

C. fluminea seeds from the wild remains crucial for aquaculture practices, there is also a need to explore the production of seeds in hatcheries.

Comprehending the reproductive characteristics of bivalves, including artificial breeding, spawning, and larval settlement periods, is crucial for effectively managing breeding strategies (Hamli et al., 2015). The reproductive biology of bivalves involves physiological processes such as gametogenesis, influenced by factors like water temperature, salinity, and food availability (Oyarzún et al., 2018). Earlier research on the reproductive biology of *C. fluminea* was conducted in seasonal climates, indicating a higher gametogenesis rate in summer but ceasing during winter (Byrne et al., 2006). Genetic and hormonal factors also play significant roles in bivalve reproductive biology (Thorarinsdóttir and Gunnarsson, 2003).

Previous studies have shown a correlation between the condition index (CI) and gonad maturity, as indicated by the gonad index (GI) (Hamli et al., 2015; Hafsaoui et al., 2016). The CI measures the shell mass ratio to living tissue mass in bivalves, indicating health, bioenergetic status, and reproductive readiness (Hafsaoui et al., 2016). Research has demonstrated that CI increases with progressing gonad maturity (Hamli et al., 2017). Conversely, GI is deemed the most appropriate metric for assessing gonadal development through histological analysis (Hamli et al., 2015). Histology remains the primary method for evaluating gonad development stages due to the difficulty of distinguishing between male and female *C. fluminea* individuals and assessing their spawning activity. Hence, this study examined the CI and GI in *C. fluminea* to reveal the reproductive characteristics and gonadal development of captive broodstocks, providing significant insights for seed production and enhancing the aquaculture of Asian clams.

Materials and Methods

Ethical approval

The clams were sampled, handled, and sacrificed following the approved protocols of the UMK Institutional Animal Care and Use Committee under code UMK/FIAT/ACUE/PG05/2019.

Corbicula fluminea *broodstock conditioning*

A total number of 1000 *Corbicula fluminea* adults measuring 10 mm in size and weighing from 0.8 to 3.0 g were harvested from Pergau Lake, Jeli, Kelantan, Malaysia (5°61'81.30"N, 101°68'08.55"E). Harvested clams were stored in ice and transferred to a hatchery for acclimatisation in a recirculating aquaculture system (RAS). They were reared for 24 wk in a connected polyculture RAS (3.0 m length × 2.0 m width × 0.5 m depth) located under a translucent roof. This RAS comprises two connected tanks: a clam tank and a tilapia tank. The clams were fed unidentified freshwater microalgae from the tilapia tank (which was filtrated at 60 μm). The concentration of microalgae in the recirculating system was maintained between 125 to 750 cells mL^{-1} with sunlight enriched the microalgae in both tanks. Therefore, the microalgae bloom was controlled by using ultraviolet light. The temperature within the system ranged from 24.58–25.81 °C in the morning (08:00 a.m.) and from 27.55–30.77 °C in the evening (05:00 p.m.). The overall condition of the clams and reproductive status was evaluated with the dry meat condition index (CI) assessed from a total of 30 clams (15–20 mm) from the cultured tank each month throughout the cultivation period, according to a previous study by Hafsaoui et al. (2016). The visceral mass of clams and shells were dried for 48 h at 60 °C. Then, the weights were calculated by the formula to estimate the fatness of the clam (Rahim et al., 2012; Hamli et al., 2017):

Condition index (CI) = [Weight of dry visceral mass (g) × 100 / Dry shell weight (g)]

where CI≤2.0 (Thin), CI 2.0–4.0 (Moderate), CI≥4.0 (Fat)

Estimation of gonadal development of Corbicula fluminea

Histological analysis

Every month, 30 individuals ranging from 10 to 30 mm in shell length were sacrificed to obtain the visceral mass and preserved in 4 % formaldehyde for 24 h. A total of 180 tissues underwent dehydration using various grades of alcohol (50 %, 70 %, 80 %, and 95 %) and displacement with xylene through an automatic tissue processor (TP1020, Leica, USA) before being infiltrated with paraffin wax (with a melting point of 57 °C) using a paraffin embedding section machine (EG1150, Leica, USA). Subsequently, the tissues were sliced into 5 μm sections using a manual rotary microtome (RM2235, Leica, USA) and stained with Harris haematoxylin and eosin utilising the autostainer program (ST 5010, Leica, USA). These sections were then examined under a compound camera microscope (Leica, USA), and the stage of gametogenesis was recorded i.e. before resting, development, maturity, and spawning (Smaoui-Damak et al., 2007; Hamli et al., 2015; Cao et al., 2017).

Gonad index

The gonadal developmental stages of *C. fluminea* broodstocks were examined as described by Cao et al. (2017). Subsequently, the calculation of each value for every stage was performed according to Hamli et al. (2015) as follows:

Gonad index (GI) = ns × r / N

where ns: number in each stage, r: number ranking of the stage, N: number of individuals in the samples

The gonad developmental stage was numerically ranked as follows:
1 = Resting / spent stages, 2 = Gametogenesis (development), 3 = Mature stage, 4 = Spawning stage

Data analyses

The histology photomicrographs were described in detail. A stacked column graph depicting the percentage of individuals at each developmental stage was constructed. Concurrently, the GI and CI were plotted to identify any correlation patterns using Spearman's rank correlation coefficient. Chi-square analysis was used to test for significant differences ($P < 0.05$) in gonad development between the sexes. All the statistical analyses were carried out using SPSS Version 26.

Results

Corbicula fluminea demonstrates hermaphroditic characteristics with descriptions primarily based on the dominant reproductive organ observed in photomicrographs. Histological analysis has verified the presence of these traits in *C. fluminea* (Fig. 1). Gonadal development stages were determined by examination of gonadic cells and storage tissue, primarily consisting of muscular tissue (MT). Ovotestes are dispersed organs with highly branched acini consistently found in all individuals. Gonadal development shows the simultaneous presence of various stages within the same individual or gonad.

While distinguishing between male and female gonads is relatively straightforward due to their distinct forms, defining the stages of gonadal cell development is intricate. A recent study introduced a gametogenic scale comprising four stages (rest/spent, gametogenesis, mature, and spawning) for both sexes, as illustrated in photomicrographs (Fig. 1 to Fig. 8). Male and female gonadal tubules may coexist or fragment, characterised by branched tubes forming acini laterally. Furthermore, Figure 1 highlights synchronous spawning activities, suggesting occasions of self-fertilisation in *C. fluminea*. However, the present study did not observe incubated larvae in the demibranches.

During the resting/spent stage (Stage 1), follicles appeared barren, with some exhibiting degradation (Fig. 3). There was a noticeable decrease in the presence of ova in the follicle lumen, accompanied by a reduction in follicle size. Connective tissue volume increased while unutilised gametes underwent degradation. Muscular tissue (MT) was notably dominant within the gonad, implying its role as the primary storage tissue. Furthermore, intrafollicular vesicular cells were frequently observed within the gonadal tubules in both resting and spent phases. Conversely, mature oocytes within the lumen exhibited elongation or detachment from the basal membrane, indicating the occurrence of spawning (Fig. 4).

Female gametogenesis in *C. fluminea* involved the development of oogonia and oocytes. Oogonia (Og)
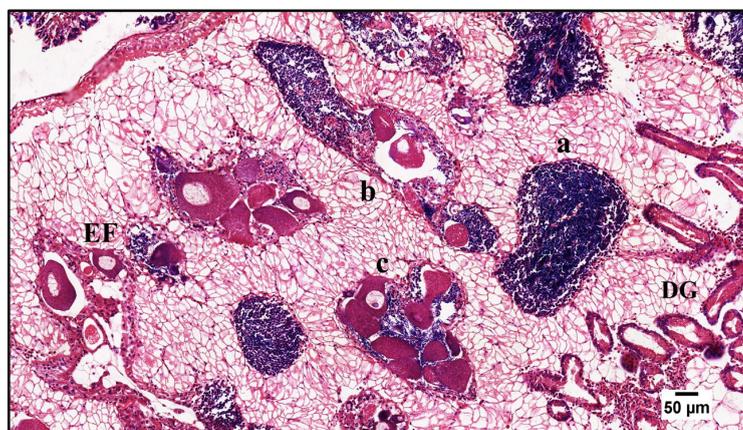

Fig. 1. Hermaphroditism in Asian clam *Corbicula fluminea*. General characteristics of the visceral mass showing spermatogenic follicles (a), oogenic follicles (b), mixed follicles (c), empty follicles (EF) and digestive gland (DG).
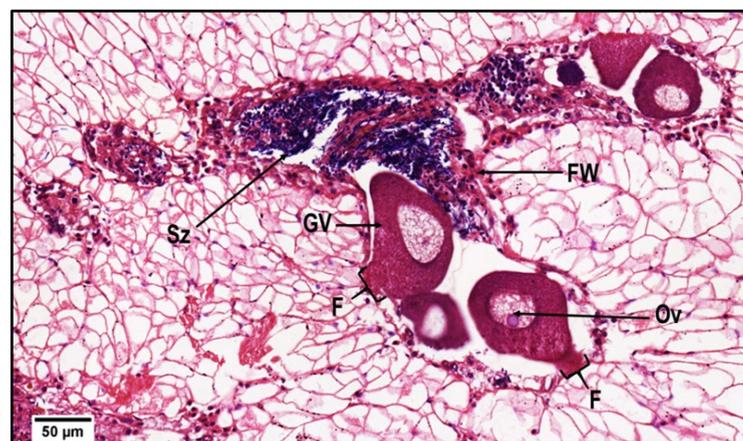

Fig. 2. Oogenic follicles of Asian clam *Corbicula fluminea* with distinct ova (Ov) could be seen in the lumen. GV: germinal vesicle, F: foot of germinal vesicle, Sz: spermatozoa, FW: follicle wall.
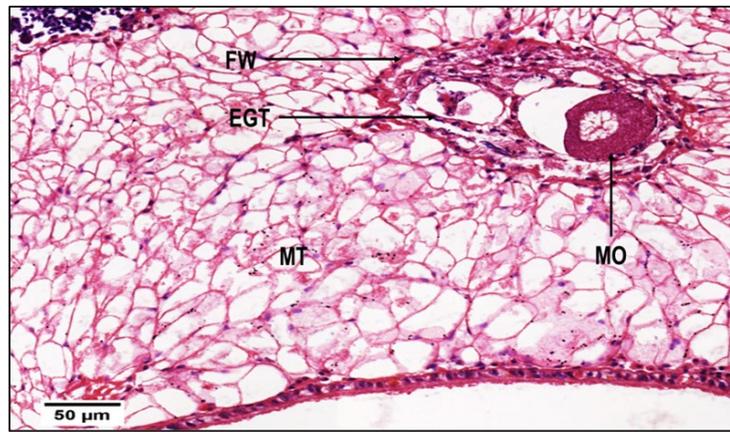
Fig. 3. The unspent mature oocyte (MO) and spent follicle at the spawning stage in Asian clam *Corbicula fluminea*. EGT: empty gonadal tubule, FW: follicle wall, MT: muscle tissue.
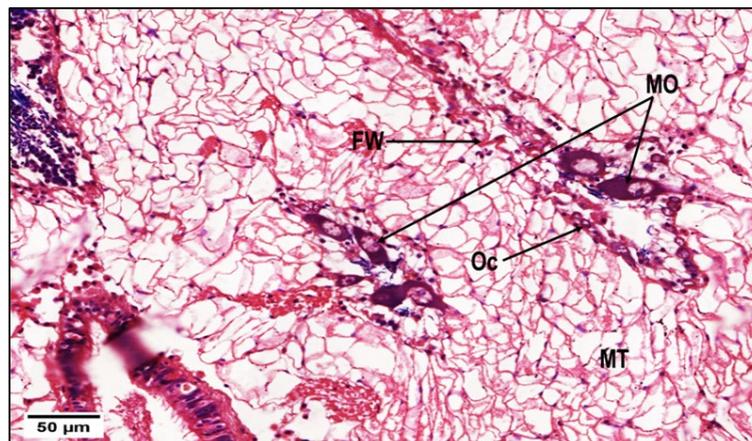

Fig. 4. Mature oocytes (MO) and reduced immature oocytes (Oc) during a spawning event in Asian clam *Corbicula fluminea*. FW: follicle wall, MT: muscle tissues.

appeared rounded and densely proliferated within the follicle, marking the initial stages of differentiation into female germ cells (Fig. 5). Within the gonadal tubules, immature oocytes (Oc) adhered to basal membranes at various vitellogenesis stages, with few mature oocytes found in the lumen. Mature oocytes displayed a height-to-width ratio greater than one, with a thinning and eventual disappearance of the connecting foot as they matured. Detachment of mature oocytes or ova (Ov) from gonadal tubules into the lumen was observed (Fig. 6). As follicle size increased, connective tissue diminished. In contrast, ripe oocytes with visible nuclei and germinal vesicles became abundant. Polygonal-shaped mature oocytes were predominant in the central region of the tubules, where the regression of muscular tissue facilitated the differentiation of oogonia into oocytes.

Histological examination revealed the presence of spermatogenic follicles in all individuals under scrutiny. During the second stage of gametogenesis, gonadal tubules were observed to be populated with spermatogonia (Sg) and spermatocytes (Sc) (refer to Fig. 7). Spermatogenesis commenced with the proliferation of spermatogonia, the male germ cells, initially displaying a rounded shape, adhered to the follicle wall (FW). As differentiation progressed, these attachments were lost. The cells nestled within the lumen of the gonadal tubule (Fig. 7). Concurrently, spermatogonia transformed into spermatocytes (Sc). At the same time, the muscular tissue (MT) exhibited a reduced presence compared to the preceding stage. Despite this reduction, both processes persisted continuously in the photomicrograph. The gametogenic process advanced as spermatocytes developed into mature spermatozoa, filling the lumen of the acini densely (Stage 3, see Fig. 8). Throughout this maturation process, spermatids gradually elongated, forming two distinct poles: the acrosome and the flagellum, positioned at the basal and apical ends, respectively. Figure 8 depicts the arrangement of spermatogenic follicles containing spermatogonia (Sg), spermatocytes (Sc), and spermatozoa (Sz) within both the apical and basal lumens. Mature spermatozoa were observed to be densely clustered, with their flagella directed inward towards the lumen. Additionally, an increase in follicle size was concomitant with a decrease in connective tissue size.

In Stage 4, the final stage of spawning, male and female gametes are released from the follicles, decreasing the number of spermatozoa and ova in the
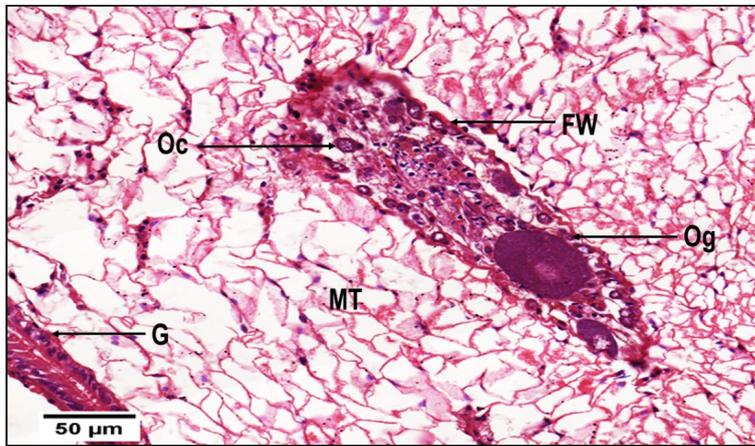
Fig. 5. Oogenic follicles with oogonia (Og) and immature oocytes (Oc) in Asian clam *Corbicula fluminea*. FW: follicle wall, G: gills, MT: muscular tissue.
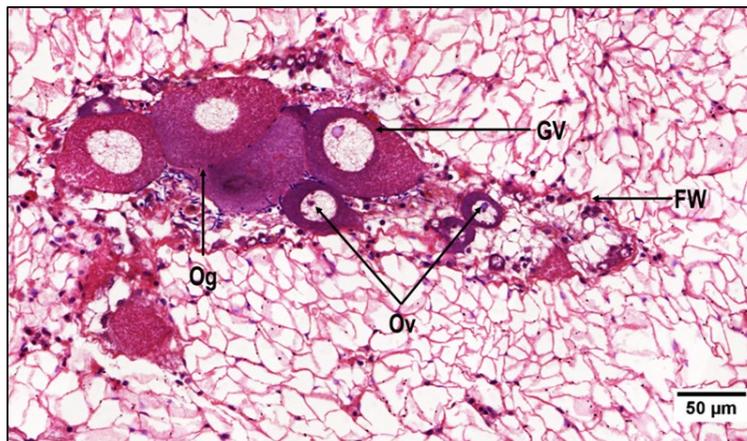

Fig. 6. Oogenic follicles (Og) comprise mature and growing ova (Ov) in the lumen of the Asian clam *Corbicula fluminea*. GV: germinal vesicle, FW: follicle wall.
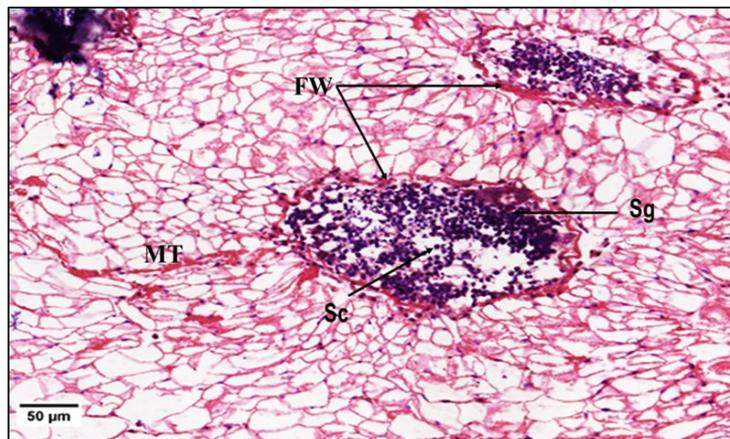

Fig. 7. Spermatogonia (Sg) and spermatocytes (Sc) at an early stage in the spermatogenic follicles of Asian clam *Corbicula fluminea*. FW: follicle wall, MT: muscle tissue.

lumen. Meanwhile, the follicles began to reduce in size, accompanied by accumulated loose connective tissue and degraded unutilised gametes. As a result of this process, some mature oocytes elongated from the basal membrane within the lumen. Consequently, the follicles predominantly appeared empty during spawning, with only a few mature oocytes remaining (Fig. 3 to Fig. 6). Therefore, empty follicles were a significant indicator of spawning activity throughout the study period.

Figure 9 illustrates the variation in gonad development stages of *C. fluminea* broodstocks undergoing captive rearing, presented as percentages. Monthly
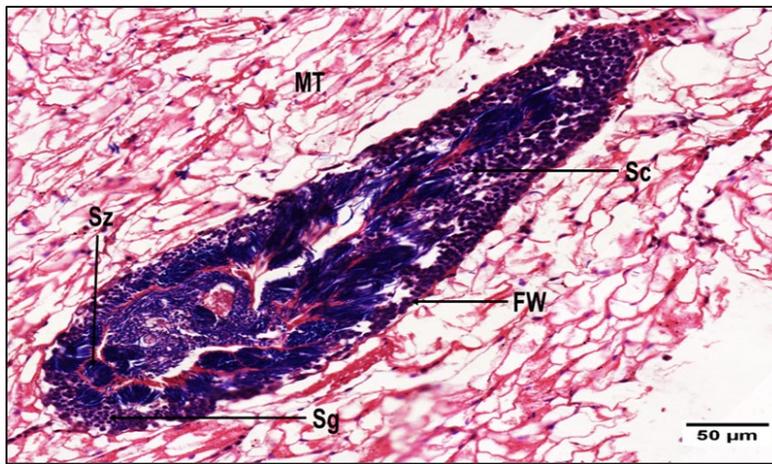
Fig. 8. Image of testis in the mature stage, showing the needle-shaped sperm clusters containing spermatogonia (Sg), spermatocytes (Sc), and spermatozoa (Sz) in the spermatogenic follicles of Asian clam *Corbicula fluminea*. FW: follicle wall.
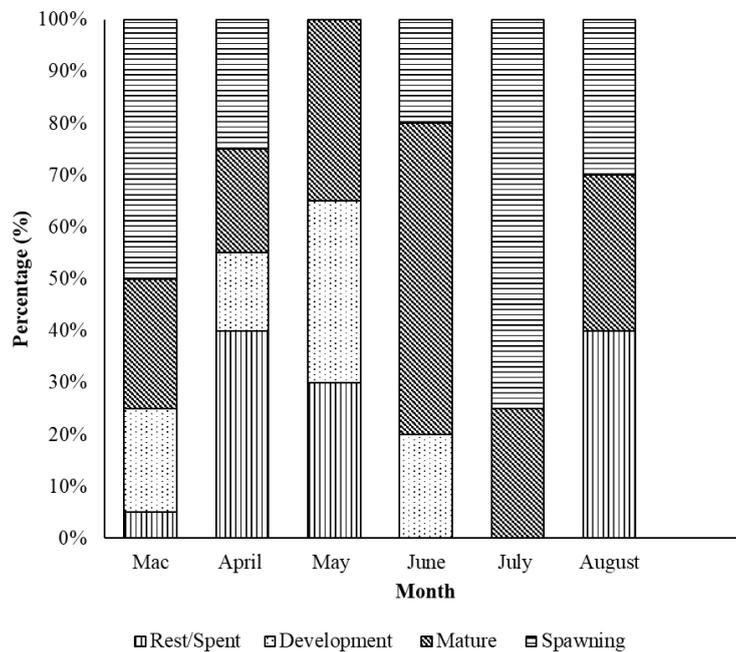

Fig. 9. Percentage of Asian clam *Corbicula fluminea* according to the gonad development stages.

assessments identified four stages of gonad development: rest, development, maturity, and spawning. The pattern of gonad development revealed heterogeneity. Active spawning by the broodstock occurred predominantly in April and August. The development of gonads among the broodstock was irregular, with each stage, including maturity, observed monthly (Fig. 9).

Throughout each assessment interval, gonad index (GI) values were recorded, correlating with the stages of gonad development in both male and female subjects, as depicted in Figure 10. Findings reveal the consistent presence of male gonads across all samples, notably exhibiting higher GI values than females. A peak GI value was concurrently observed in both genders during the fourth month of rearing (June), indicating a substantial maturity phase. Nevertheless, GI levels declined between the spawning and resting stages,

with the lowest GI value recorded in April, aligning with the resting time.

Table 1 shows the relationship between the condition index (CI) and variation of gonadal development stages (rest, spent, mature, and spawning) in *C. fluminea*. Significantly strong positive correlations (0.696) are observed to increase with the mature stage as the condition index increases. Clams in the rest stage have a strong negative correlation ($P < 0.05$). A weak correlation between the CI and spawning stage indicates no evidence that CI is reduced after spawning (spent stage).

Discussion

The gonad development for male and female *C. fluminea* follows gametogenic stages comprising resting or spent, gametogenesis (development),
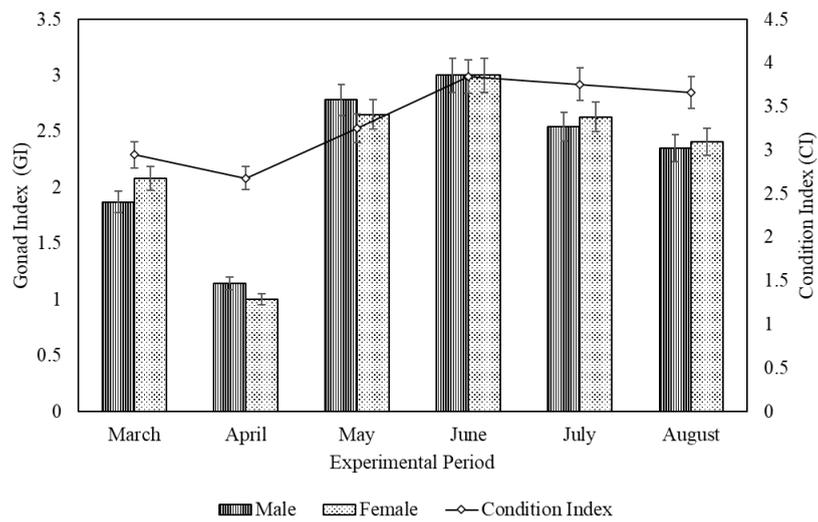
Fig. 10. Monthly male and female gonad index (GI) and condition index (CI) of the Asian clam *Corbicula fluminea* in captive rearing.

Table 1. The relationship between condition index (CI) and different gonadal development stages (rest, spent, mature, and spawning) in the Asian clam *Corbicula fluminea*.

	CI	Rest	Spent	Mature	Spawning
CI	1.000				
Rest	-0.677*	1.000			
Spent	-0.177	-0.136	1.000		
Mature	0.696*	-0.343	0.448	1.000	
Spawning	0.029	-0.235	-0.706*	-0.580**	1.000

*Correlation is significant at the 0.05 level, **Correlation is significant at the 0.01 level, n = 30.

maturity, and spawning. These stages elucidate the relationship between gametogenesis and the optimal conditions for the successful propagation of the current gametes in *C. fluminea*. Previous research has suggested that environmental factors such as temperature, nutrient availability, and geographic location play a substantial role in bivalve gametogenesis (Hamli et al., 2015). However, recent studies suggest an association between gonad development and the condition index (CI). Meanwhile, the previous literature has extensively described the gonad development of *Corbicula* spp. in natural habitats (Denton et al., 2012; Zeswita et al., 2016), but none of them has specifically addressed the captive environment, especially for *C. fluminea*. *Corbicula fluminea* exhibits spawning events throughout the year, which is relevant for hatchery culture. A recent study shows that this prolonged reproductive period leads to asynchronous development.

In general, the muscular tissue (MT) was found to occupy a significant portion of the gonad, which signifies that it is the primary storage tissue. Intrafollicular vesicular cells within the gonadal tubules were commonly observed during resting periods or post-expenditure, as found in this study. These cells are crucial for glycogen storage, providing energy (ATP) for initial gametogenesis and gamete ripening

stages (Smaoui-damak et al., 2007; Delgado et al., 2016). Additionally, they support surviving with low food availability or are utilised in subsequent gametogenic cycles (Delgado et al., 2016). The MT thickness in gonad tubules decreased as gametogenic development progressed. Furthermore, this study defined hermaphroditism in *C. fluminea*, in which oogenic, spermatogenic, and mixed follicles coexisted in all individuals inspected, indicating various gonadal stages and sex dominance. Cao et al. (2017) elucidated hermaphroditism during larval incubation in gill chambers and the lentic habitat. However, depending on environmental conditions, *C. fluminea* may exhibit dioecious characteristics rather than hermaphroditic traits.

In contrast, a recent investigation revealed a notable abundance of spermatogenic follicles compared to oogenic follicles, aligning with the observations made by Cao et al. (2017) in clams larger than 16 mm. This discovery contradicts earlier literature, in which Park and Chung (2004) reported abundant oogenic follicles in clams smaller than 20 mm. These contradictory findings highlight the inconsistent nature of gonadal development in *C. fluminea*, suggesting potential influences from environmental and demographic factors. Furthermore, there were divergences in the size of mature oocytes among the studied *C. fluminea*.

Earlier studies reported oocyte sizes ranging from 70–120 µm, while another study found sizes ranging from 70–170 µm (Park and Chung, 2004; Rybalkina et al., 2013; Cao et al., 2017). These variations in oocyte size appear to be linked to differences in broodstock sizes and maturity stages during analysis. Despite these variations, the recent findings do not significantly deviate from previously published works, indicating that the reproductive biology of *C. fluminea* demonstrates substantial plasticity irrespective of habitat conditions.

The present study found no evidence of intrafollicular embryos, despite the presence of both sperm and oocytes in the evaluated individuals. This contrasts with the findings of Park and Chung (2004), who observed intrafollicular embryos in *C. fluminea*, suggesting the possibility of self-fertilisation in hermaphroditic individuals. The reproductive strategy of *C. fluminea* appears flexible, as Cao et al. (2017) reported that embryos are primarily incubated within the inner demibranchs, with rare use of the outer demibranchs. However, this suggests that *C. fluminea* can utilise inner and outer gill chambers for embryo incubation, reflecting its reproductive adaptability. The heterogenous gonad development observed in the recent study further underscores the reproductive plasticity of clams, which is influenced by factors such as maturity and environmental adaptation. This reproductive plasticity aligns with broader patterns of reproductive diversity among freshwater clams, as different species exhibit unique brooding strategies suited to their ecological niches (Morton and Puljas, 2013). For instance, *C. madagascariensis* has been identified as viviparous, with its brooding strategy playing a crucial role in survival and recruitment in freshwater environments (Glaubrecht et al., 2006). Moreover, Cao et al. (2017) linked the reproductive plasticity of *C. fluminea* to its invasive characteristics, emphasising its ability to adjust spawning patterns in response to environmental cues rapidly.

Gametogenesis development varies among individuals in both males and females. In some occurrences, gametogenesis coincides with a trait like hermaphroditism. Matured gametes are released during fertilisation under favourable conditions, which recent research defines as 'stress' conditions induced by warm water temperatures, gonad dissemination, and salinity. Species spawn under stressful conditions to ensure the continuation of generations. Similar incidents, such as fertilisation in the hard clam genus *Mercenaria*, occur in the water column. The spawning process begins with the sperm or eggs releasing into the water when stimulated by high water temperatures (Hadley and Whetstone, 2007). Then, gametes in the water trigger spawning in other clams. A recent study observed a similar phenomenon where macerated gonads prompted clams to extend their siphons and discharge gametes.

Undischarged or unfertilised gametes in the broodstocks were observed to undergo resorption, characterised by cellular disintegration. Resorption, or self-cleaning, is a preparatory process for a new gametogenic cycle. This cleansing process involves the phagocytic activity of acinus walls (such as Sertoli cells) surrounding the seminiferous cysts of spermatozoa and spermatogonia lining the lobular lumen. Cellular disintegration is evidenced by the loss of crucial structural features: degradation of nuclei, rupture of plasma and vitelline membranes, and the presence of numerous phagocytes within follicles (Ceballos-vázquez et al., 2012). Resorption typically occurs at temperatures exceeding 25 °C, particularly in female bivalves like pinnids and *Atrina maura* (Ceballos-vázquez et al., 2012). Recent research has shown resorption during *C. fluminea* gametogenesis, although the precise temperature threshold remains undefined.

The condition index (CI) represents a health metric. It reflects the ability of bivalves to accumulate reserves for survival and reproduction. In this study, CI values ranged from 2.68 to 3.55, indicating a moderate condition. Previous research showed that *C. fluminalis* (CI= 1.2-3.0) and *C. fluminea* (CI=3.0-8.0), coexisting in the Loire Lateral Canal and the Roanne Canal, France, exhibited different levels of fatness (Mouthon and Parghentanian, 2004). The variance in CI values is strongly linked to habitat, species, environmental factors, food availability, and population density. Additionally, CI values offer insights into the reproductive cycle stages of bivalves without the need for histological analysis. Condition Index (CI) can rapidly evaluate large samples related to spawning events and shows fluctuations related to gamete production and reproductive cycles in clams like *Donax trunculus* and *Ruditapes decussatus* (Rifi et al., 2011; Yildiz et al., 2023). Wall et al. (2011) revealed that the CI reflects increased energy investment in gonads, with peak values corresponding to the spawning season. Therefore, evaluation CI reflects the reproductive cycle stages, which require no histological analysis.

The primary location of the gonad is within the mantle tissue, serving as a marker for gonadal index (GI) assessment. Additionally, weight loss can occur due to the absorption of gametes, coinciding with the gametogenesis process, which necessitates energy expenditure. A recent study observed weight loss in *C. fluminea* during April following a spawning event in Mac (Fig. 10). Both GI and condition index (CI) support this decline. GI was calculated to be at its lowest (GI = 1) during this evaluation period. Being sensitive to reproductive changes, CI typically correlates with GI (Mladineo et al., 2007). Previous studies coined the physiological relationship between bivalves' growth and reproductive stages (Hamli et al., 2017). Rifi et al. (2011) underscored that the high nutrient level influenced growth and significantly impacted gonadal activity and successful gamete regeneration in *Fulvia fragilis*. Hence, the energy required for growth and gonadal development is reflected in CI values, as they also signify nutrient availability.

Zeswita et al. (2016) categorised the gonad index (GI) into five developmental stages: GI I (<1.0), indicating immature gonads, GI II (1.0–5.0) indicating gonads primed for maturation, GI III (5.1–10.0) indicating early maturity, GI IV (10.1–20.0) indicating matured gonads and GI V (>20.0) indicating gonads matured and frequently spawning. In a recent investigation, male and female GIs were recorded within the ranges of 1.14–3.00 and 1.00-3.00, respectively. These values exceeded those reported in previous studies (0.78-1.30), corresponding to the early activity, spawning, and post-spawning phases in *C. Sumatrana* (Zeswita et al., 2016). *Corbicula fluminea* in tropical regions exhibited continuous spawning, contradicting the temperate population that has longer resting periods during winter and leads to an increase in the GI value (Mouthon and Parghentanian, 2004).

Conclusion

In conclusion, *Corbicula fluminea* shows potential to be produced in hatchery due to its adaptability to captive conditions and reproductive abilities. Selecting broodstock for artificial production in the hatchery aligns with the general principles of hatchery production. Additionally, recent research proposes potential guidelines for selecting gravid broodstock and determining optimal breeding periods. However, further study is essential to fully discover the potential of hatchery production for *C. fluminea* seeds.

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Author contributions: Mohd Zharif Ramli: Analysis, writing - review. Aweng Eh Rak: Sample collection, writing – review, project leader. Lee Seong Wei: Experimental set-up, analysis, writing.

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