AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Reproductive performance and vitellogenin gene expression on female Bonylip barb (*Osteochilus vittatus*) during its reproductive cycle under culture conditions

Madihah Madihah[a,d], Sofi Andriani[b,d], Silmy Aulia Rufiatin Nisa[b], Indra Wibowo[c], Sony Heru Sumarsono[c,*]

[a] *Doctoral Program in Biology, School of Life Sciences and Technology, Institut Teknologi Bandung, Bandung 40132, West Java, Indonesia.*

[b] *Magister Program in Biology, School of Life Sciences and Technology, Institut Teknologi Bandung, Bandung 40132, West Java, Indonesia.*

[c] *Physiology, Developmental Biology and Biomedical Sciences Research Group, School of Life Sciences and Technology, Institut Teknologi Bandung, Bandung 40132, West Java, Indonesia.*

[d] *Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Sumedang 45363, West Java, Indonesia.*

Article Info

Article history:
Received 29 March 2021
Revised 23 July 2021
Accepted 31 July 2021
Available online 31 August 2021

Keywords:
Gonadosomatic index,
Hepatosomatic index,
Relative gene expression,
Spawning performance,
Vitellogenesis

Abstract

The Bonylip barb is a tropical fish indigenous to Southeast Asia and has high cultivation potential. The reproductive performance and vitellogenin gene expression were studied during the female brooders' reproductive cycle for 16 wk. Ovulation was induced in 30 fish using Ovaprim (0.5 mL/kg bodyweight) followed by artificial spawning. Every 2 wk, three subsamples were sacrificed. Ovary and liver samples were collected to observe the reproductive performance based on the gonadosomatic index (GSI), hepatosomatic index (HSI), ovarian histology and spawning performance at weeks 8, 12 and 16, and vitellogenin gene expression using real-time quantitative polymerase chain reaction. The GSI increased significantly ($p < 0.05$) towards week 16 (mean ± SD; 19.15 ± 3.88), whereas the HSI showed a constant pattern. Ovary histology showed asynchronous development of oocytes. The oocyte composition indicated the developing phase from week 0 until week 4 and the spawning-capable phase from week 6 onward. The relative *Vtg1* and *Vtg2* gene expression levels in the liver increased significantly in the developing (week 4: *Vtg1* 16.72 ± 7.08; *Vtg2* 20.02 ± 1.19) and beginning of spawning-capable phases (week 6: *Vtg1* 37.97±7.76; *Vtg2* 22.86±2.69) and declined from week 8 onward. The spawning performance, particularly mature egg diameter, fertilization rate, hatching rate, larval survival at 3 d post hatching (dph) and abnormalities until 10 dph were significantly higher at weeks 12 and 16 than at week 8. Despite the fecundity, the survival rate at 10 dph and abnormalities until 3 dph did not differ significantly at each observation time. Therefore, a relationship between the GSI, ovarian histology and *Vtgs* gene expression influenced the spawning performance of the female Bonylip barb.

* Corresponding author.
E-mail address: sonyheru@sith.itb.ac.id (S.H. Sumarsono)

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https://doi.org/10.34044/j.anres.2021.55.4.06

Introduction

Aquaculture is an important component of Indonesian fisheries which contribute to national food security and income; in 2017, the aquaculture subsector supplied 65% of total national fisheries production, even though land utility, cultivation management and productivity have not been maximized yet (Ministry of Marine Affairs and Fisheries, 2018) For sustainability of the aquaculture production, basic needs that support fish reproductive success must be fulfilled to ensure progeny availability from both consumption and conservation. The study of fish reproduction traits and early development of embryos is important to establish conservation measures and develop a fish farming protocol for higher efficiency egg production and enhanced progeny viability (Lubzens et al., 2010; Rizzo and Bazzoli, 2019). A major factor influencing egg quality is proper yolk formation during vitellogenesis, as most fish species are oviparous and any developing offspring are completely dependent on stored egg yolk as their primary nutrient source before the exogenous feeding period (Reading et al., 2018). Vitellogenesis is induced by estradiol-17β (E2) secreted by follicular cells in the developing oocytes and the circulating E2 binds to its receptor in liver cells and leads to initiation of vitellogenin gene expression and synthesis (Lubzens et al., 2017; Reading et al., 2017).

Vitellogenin (Vtg) is a large, homodimeric phospholipoglycoprotein with a molecular weight of approximately 350–600 kDa that functions as a major precursor of egg yolk protein (Reading et al., 2017). The linear yolk protein structure of complete teleost Vtg consists of five domains: NH2-lipovitellin (Lv) heavy chain-phosvitin (Pv)-lipovitellin (Lv) light chain-β component (β'c)-C-terminal component-COOH; however there are several distinct forms of Vtg in fishes encoded by different genes, with different nomenclature and domains (Sullivan and Yilmaz, 2018). After being taken up into the oocytes, teleost Vtg is cleaved into yolk proteins, including Lv, Pv and β'c and the contribution of Vtg-derived yolk to oocyte growth can be substantial, comprising up to 80–90% of the dry mass of an ovulated egg in some species (Reading et al., 2017). In addition to providing proteins for embryogenesis, Vtg also transports lipids and other nutritional resources, such as phosphorus and calcium, necessary for proper metabolism and skeletal development (Babin et al., 2007). The non-nutritive functions of Vtg include an immune-competent factor that can protect the host against attack by microbes, such as bacteria and viruses, the ability to suppress free-radical reactions in fish oocytes and to protect the host from oxidative stress (Sullivan and Yilmaz, 2018).

Bonylip barb (*Osteochilus vittatus* Valenciennes, 1842; Cyprinidae) is an endemic fish in Southeast Asia and widely distributed in Cambodia, Indonesia (Sumatera, Java, and Borneo), Lao's People's Democratic Republic, Malaysia (Peninsular Malaysia, Sarawak), Myanmar, Thailand and Vietnam (Lumbantobing and Vidthayanon, 2020). The fish has economic value and the potential to be cultivated. In Indonesia, particularly in Java, the fish and its eggs are popular for consumption (Farahita et al., 2012; Setyaningrum and Wibowo, 2016) and it is used to control water weeds (Syandri et al., 2015). In nature, the female brooders could be spawned three to four times a year, and under suitable environments, the spawning could be done 60 d after the previous one (Prayogo et al., 2018). However, information about their biological reproductive features with different lengths of the reproductive cycle in culture conditions has not been reported yet. This study aimed to determine reproductive performance based on the gonadosomatic index (GSI), hepatosomatic index (HSI), ovarian histology and *Vtgs* gene expression of the female Bonylip barb during its reproductive cycle for 16 wk, and to compare the spawning performance observed at different lengths of the reproductive cycle, specifically at weeks 8, 12 and 16.

Materials and Methods

Ethics statements

This study was approved by the Ethics Committee of the Medical Faculty, Gadjah Mada University, Yogyakarta, Indonesia (Approval no. KE/FK/0165/EC/2020).

Collection and acclimatization of broodstock

The immature female and male broodstock (65–100 g bodyweight (BW); age > 1 yr) were collected from the brooder pond in the Southern Regional Office of the Ministry of Marine Affairs and Fisheries at Singaparna, West Java, Indonesia. The broodstock were in healthy condition and devoid of any physical damage or malformation. They were acclimatized and reared in a concrete pond (2 m × 1 m × 0.5 m; 15 fish/pond) with groundwater flow-through at the hatchery facility of Kawasan Perikanan Darat Ciparanje, Sumedang, Indonesia until they became sexually mature and started their reproductive development phase. The cultivation conditions were under a natural photoperiod (14 hr light and 10 hr darkness) and ambient temperature (22–27°C) with acceptable water quality parameters, namely 4.0–6.0 mg/L dissolved oxygen

(DO; Sera O_2 test kit; Sera GmbH; Hamburg, Germany), 0.003 mg/L ammonia, 0.0–0.5 mg/L nitrite, pH of 7.0–8.0 (Sera aqua test kit; Sera GmbH; Hamburg, Germany), and salinity 0.0 g/100 g NaCl (Master 20T Hand-held Refractometer; Atago; Saitama, Japan). During rearing, they were fed to satiation with commercial feed (FF 888; CP Prima; Jakarta, Indonesia) twice daily.

Assessment of reproductive performance

First artificial spawning

The female brooders (age > 1 y, 120.5 ± 4.48 g mean BW ± SD) reached the first spawning capable phase indicated by secondary sex characteristics, such as plumpness of the abdomen and redness of the vent. Thirty female brooders were induced using an intramuscular injection of Ovaprim (Syndel Laboratories Ltd.; Vancouver, Canada) at a dosage of 0.5 mL/kg BW to synchronize and coordinate oocyte maturation. At 10 hr post-injection, the ovulatory response was assessed every 2–3 hr by gently massaging the abdomen. Females releasing ova after massaging were immediately stripped to empty the ovary. One week after the first spawning period, the female brooders were assumed to enter the second reproductive cycle and observed for this study for the next 16 wk.

Measurement and organ collection

Three subsamples of female brooders were anesthetized with clove bud oil (40 µL/L water) every 2 wk. BW to the nearest 0.01 g and total length (TL) to the nearest 0.1 cm were measured before the fish were euthanized by decapitation. Each fish was dissected from the vent to the operculum to collect organs. The ovaries and liver were removed and weighed. The gonadosomatic index (GSI; ovary weight × 100 / BW) and the hepatosomatic index (HSI; liver weight × 100 / BW) were measured (Nunez et al., 2011). The middle portion from both the left and right ovaries (±1 cm^3) was fixed in 10% neutral-buffered formalin for histological preparation. The liver tissue sample was immersed in RNA stabilization solution (ratio 1:10; Qiagen; Hilden, Germany), chilled in the refrigerator overnight and stored at -20°C until used for RNA extraction.

Ovarian histology preparation and imaging

After being fixed for 12 hr, the ovary tissue was dehydrated in serial ethanol solutions, cleared with xylene and embedded in paraffin at 60°C. Then, the paraffin blocks were cut (0.7 µm; MRM-RM, Medimeas; Haryana, India) and stained with hematoxylin and eosin (Merck; Darmstadt, Germany) according to standard procedures. Stained slides of the left and right ovaries from each subsample were viewed through a light compound microscope (CX-31; Olympus; Tokyo, Japan) and photographed using a digital camera (DP-27; Olympus; Tokyo, Japan). The oocytes were classified into four different developmental stages: primary growth (PG); cortical alveolus (CA); vitellogenesis (VTG) consisting of primary (VTG1), secondary (VTG2) and tertiary (VTG3) vitellogenic oocyte phases; and oocyte maturation (OM), consisting of early maturation (EOM) or pre-germinal vesicle breakdown (pre-GVBD) and late maturation (LOM) or post-GVBD phases. The classification was based on cytoplasmic inclusions and the characteristics of the nucleus according to Selman et al. (1993), McMillan (2007) and Brown-Peterson et al. (2011). The diameter of each oocyte was measured from micro-photography using image analysis software (cellSens™ Standard; Olympus; Tokyo, Japan). The relative abundance of oocytes at each developmental stage per observation time was counted based on 30 oocytes per subsample. The oocyte composition was used to define the reproductive phase in the reproductive cycle according to Brown-Peterson et al. (2011): developing phase, with PG, CA, VTG1 and VTG2 oocytes present, no evidence of post-ovulatory follicles (POFs) or VTG3 oocytes and some atresia may be present; spawning-capable phase, with VTG3 oocytes or POFs present in batch spawners, atresia of vitellogenic oocytes may be present, early stages of OM may be present and actively-spawning sub phase: oocytes undergoing late GVM, GVBD, hydration, or ovulation may be present; regressing phase, with atresia and POFs present and some CA, VTG1 and VTG2 oocytes present; and regenerating phase, with only oogonia and PG oocytes present.

RNA extraction, cDNA synthesis, and real-time quantitative polymerase chain reaction of vitellogenins gene

Total RNA was extracted using the SV Total RNA Isolation System (Promega; Madison, WI, USA) from the liver (±30 mg) according to the manufacturer's instructions. The purity and concentration of the RNA were assessed using spectrophotometry (NanoDrop Lite™ spectrophotometer; Thermo Scientific; Wilmington, DE, USA). Acceptable RNA (optical density at 260 nm (OD260)-to-OD280 ratio of 1.8–2.2) was reverse transcribed into cDNA using an Enhanced Avian first-strand synthesis kit (Sigma-Aldrich; St. Louis, MO, USA) using 250 ng of total RNA per reaction and the Oligo $(dT)_{23}$ primer according to the manufacturer's instructions.

To determine the expression of the *Vtgs* gene, the specific primer for real-time quantitative polymerase chain reaction (qPCR) was designed based on partial-length *Vtg1*, *Vtg2* (Nisa, unpublished data) and *β-actin* (*actb*; Madihah, unpublished data) genes as an internal reference gene of the female Bonylip barb (Table 1). Annealing temperatures were optimized using a temperature-gradient program. Primer specificity was confirmed using gel electrophoresis with 2% agarose in 1% Tris-Acetate-EDTA buffer and melt curve analysis. A standard curve of each primer pair for qPCR (calculated referring to the threshold cycle (Ct) to the logarithm of cDNA input by 10-fold serial dilution series) was performed to determine the detection range, linearity and real-time qPCR amplification efficiency (E; $E = 10^{[-1/slope]}$), according to Pfaffl (2011). The real-time qPCR process was run in a Quantstudio™ 1 Applied Biosystem Real-Time PCR System (Thermofisher Scientific; Madison, WI USA) and using a Sensifast SYBR no-Rox kit (Bioline; London, UK) according to the manufacturer's instructions, with 100 ng cDNA as the template. The qPCR reaction involved: 95°C for 2 min, followed by 40 amplification cycles of denaturation at 95°C for 5 s, annealing at 61.1°C for 10 s and extension at 72°C at 15 s. Template-minus negative controls were run for each subsample and all qPCR subsamples were performed in triplicate. The qPCR results were expressed as fold changes in the *Vtg1* and *Vtg2* genes, normalized to *actb* and relative to the expression at week 0, for data evaluation using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001).

Second artificial spawning and spawning performance observation

At weeks 8, 12 and 16 of the second reproductive cycle, three females and one male brooder were intramuscularly injected with Ovaprim (0.5 mL/kg BW) to induce ovulation and milt production. After 10–12 hr, the eggs and milt were collected from the females and males, respectively, by stripping the abdomen. The female BW before and after the stripping was weighed. The released ova were immediately collected and weighed. Then, a 0.25 g sample of the eggs was taken from each female and enumerated for absolute fecundity (fecundity = [number of eggs in a 0.25 g egg sample × total weight of eggs] / 0.25) and mature egg diameter. The milt was diluted with 0.9% saline buffer (1:100 volume per volume) and then poured into a glass containing the eggs and mixed by gently shaking for at least 1 min. The embryos resulting from this *in vitro* fertilization (±500 embryos per subsample) were placed in glass containers (100 cm × 80 cm × 60 cm) with 30 cm water depth and strong aeration to minimize clumping between the egg envelopes. Water temperature and pH during embryonic development were in the ranges 28–29°C and 7.0–7.2 and DO was kept at 4.0 mg/L. After hatching, the aeration was lowered to allow the larvae to move more easily. The developed embryos were monitored until 10 d post-hatching (dph) for calculation of the spawning performance.

The fertilized eggs were assessed by counting the embryos that developed into 16–64 blastomeres cleavage stage (2–3 hr post-fertilization) under a light stereo microscope (SZ-61; Olympus; Tokyo, Japan).The number of hatchlings was determined by counting the hatched larvae at 24–30 hr post-fertilization. The number of viable fry was determined by placing all hatchlings into new glass containers with normal aeration and counting the viable larva at 3 dph and 10 dph. Larvae with abnormalities of yolk sac formation and vertebral malformation were counted daily and the larva abnormality rate was accumulated at 3 dph and 10 dph. The spawning performance parameter was calculated using Equations 1, 2, 3 and 4 (Muchlisin et al., 2014):

$$Fertilization\ rate\ (\%) = \frac{Number\ of\ fertilized\ eggs}{Total\ number\ of\ eggs\ per\ subsample} \times 100\% \quad (1)$$

$$Hatching\ rate\ (\%) = \frac{Number\ of\ \ hatchlings}{Number\ of\ fertilized\ eggs} \times 100\% \quad (2)$$

$$Survival\ rate\ (\%) = \frac{Number\ of\ viable\ fry\ at\ observation\ time}{Number\ of\ hatchlings} \times 100\% \quad (3)$$

$$Abnormality\ rate\ (\%) = \frac{Number\ of\ larvae\ with\ abnormality}{Number\ of\ hatched\ eggs} \times 100\% \quad (4)$$

Table 1 List of primers used in real-time quantitative polymerase chain reaction

Primer code	Primer sequence (5'→3')	Amplicon length (bp)	Amplification efficiency
qF_actb	TTCCCTGTACTGCGTCCTGA	86	2.003
qR_actb	CCACGGTCTCCACACACAAA		
qF_Vtg1	GCTAATGGTGTGGTTGGAAA	99	2.017
qR_Vtg1	GAGGTTGAGCTGAAGGATGT		
qF_Vtg2	TGGTCCATGATGATGCTCCTCT	108	1.922
qR_Vtg2	GCCCTTTGTCCTTGAACTGAGC		

Statistical analysis

Data were presented as mean ± SD for all parameters. The variance of the data was analyzed using one-way analysis of variance. Significant F values were determined using Duncan's multiple range test and significance was tested at $p < 0.05$. The correlation between the GSI and relative *Vtg1* and *Vtg2* gene expression was analyzed using bivariate Pearson correlation. Statistical analyses were performed using the SPSS software (version 25.0, IBM Corp.; Armonk, NY, USA).

Results and Discussion

Gonadosomatic index and hepatosomatic index of mature female Bonylip barb

At the commencement of the study, the average size of broodstock was 106.79 ± 27.29 g BW and 18.33±1.40 cm (total length, TL). After 16 w of rearing, the brooder individuals averaged 162.48 ± 25.27 g BW and 21.1 ± 0.46 cm TL.

The GSI of the female brooders of Bonylip barb increased significantly during the reproductive cycle onward to week 16 (Fig. 1A). In fish, the GSI is a good indicator of reproductive activity, so the spawning period is often determined by an association between the GSI and the gonadal maturity levels (Rizzo and Bazzoli, 2019). The GSI value follows gonadal maturation, increasing in the developing phase and peaking in the spawning capable phase. In the spawning capable phase, the GSI value varies according to the species' reproductive strategies, and in general, total spawning fish have a higher GSI than those with multiple spawning (Rizzo and Bazzoli, 2019). The Bonylip barb is a batch spawner with indeterminate fecundity (Prayogo et al., 2018), and the GSI values during the spawning period at weeks 8, 12 and 16 were 11.42 ± 1.60%, 17.27 ± 0.75%, and 19.15 ± 2.24%, respectively. These results were consistent with other studies regarding the reproductive features of the female Bonylip barb. In a wild habitat, female with gonad maturation level IV had varying GSI values, such as 11.22 ± 4.94% (Omar, 2010), 13.05 ± 4.56% (Putri et al., 2015) and 45.32% (Rochmatin et al., 2014), while in fish farms, it varied in the range 20.31–41.24% (Rostika et al., 2017).

In the current study, the HSI values were similar ($p > 0.05$) throughout the 16 wk of the reproductive cycle (Fig. 1). The HSI is an indicator of liver participation in vitellogenesis and during gonadal maturation, Vtg and zona radiata proteins are synthesized by hepatocytes and then transported through the bloodstream to the ovaries by receptor-mediated pinocytosis (Babin et al., 2007; Reading et al., 2017). Therefore, the HSI value usually increases in the developing phase and decreases during the spawning season and shows a significant inverse correlation with the GSI. However, the cyclical variations of HSI are not always found in most fish species, probably due to the dual function of the liver for lipid metabolism and yolk precursor synthesis and also as a fish fecundity strategy (Nunes et al., 2011; Rizzo and Bazzoli, 2019). Perhaps in the current study of the female Bonylip barb, the HSI values indicated the fish fecundity strategy as a batch indeterminate spawner, in which the oocytes are continuously recruited for vitellogenesis during the spawning-capable phase and hepatocyte activity remains intense throughout this period.

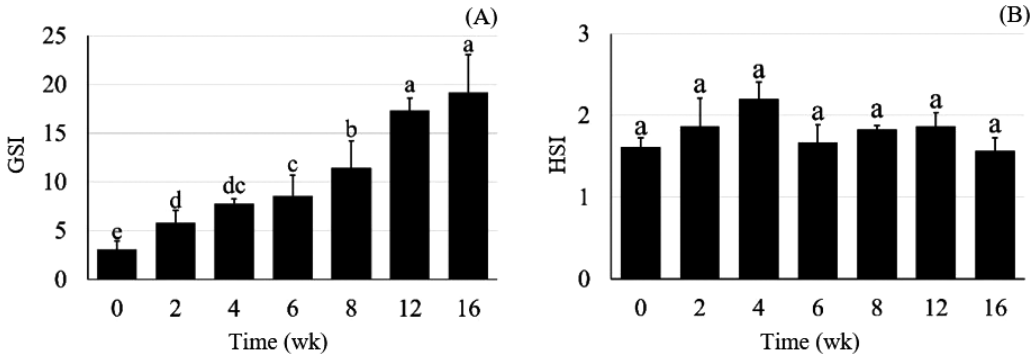

Fig. 1 Mature female Bonylip barb during 16 wk of reproductive cycle: (A) gonadosomatic index; (B) hepatosomatic index, where data are expressed as mean ± SD ($n = 3$ per observation time) and different lowercase letters above columns denote significant ($p < 0.05$) differences

Ovarian histology during reproductive cycle of female Bonylip barb

The major stages of oocyte development observed in the current study consisted of primary growth (PG), cortical alveolus (CA), vitellogenesis (VTG) and oocyte maturation (OM). Based on the histological features, the ovaries showed asynchronous development, where oocytes in several developmental stages were present at the same time (Figs. 2A and 2B). An oocyte in the PG stage was round or spherical (diameter < 161 µm), the nucleus or germinal vesicle was large and contained a few of large nucleoli or multiple peripheral nucleoli, the cytoplasm was dense, compact and strongly basophilic and surrounded by a squamous follicular epithelium (Figs. 2A and 2C). The CA oocyte was large and round (diameter 161–275 µm) with an enlarged nucleus which comprised numerous small nucleoli, the cytoplasm was basophilic and numerous cortical alveoli appeared in the ooplasm, with a distinct vitelline envelope and a cuboidal follicular epithelium around the oocyte (Figs. 2A, 2B and 2D). The VTG oocyte was characterized by extensive growth due to an increased number of cortical vesicles in the peripheral ooplasm and acidophilic yolk granules containing yolk protein derived from the vitellogenin, with the vitelline envelope a more prominent and cuboidal follicular epithelium. An oocyte in the VTG stage comprised three sub-stages of yolk deposition: (1) the VTG1 oocyte was round (diameter 276–380 µm), cortical alveoli was still prominent but were more densely stained yolk granules apparent, the nucleus was round, large and located in the center, the cytoplasm was slightly acidophilic (Figs. 2A and 2D); (2) the VTG2 oocyte was round (diameter: 381–560 µm) with cortical alveoli pushed to the periphery as the number of yolk granules increased in the ooplasm, the nucleus was round and large with a distinct nuclear membrane and located in the center, the ooplasm contained compact chromatin and small peripheral nucleoli (Fig. 2A); (3) the VTG3 oocyte was round or elliptical (diameter 561–800 µm), the cytoplasm was filled with yolk granules and the cortical alveolus was only in the periphery, the nucleus was large, round and centrally located, with a distinct nuclear membrane and a reduced number of nucleoli (Fig. 2A). In the OM stage, the oocyte was round or elliptical (diameter 801–1125 µm). At the beginning of the maturation phase, the oocyte had a large nucleus or GV located centrally or halfway between the center and the periphery. In the final OM phase, the GV migrated to the animal pole (germinal vesicle migration or GVM; Fig. 2E), with a decrease in size and an irregular shape, the envelope of GV was broken down (GVBD) and the nuclear contents scattered in the cytoplasm. This early OM phase (pre-GVBD) was characterized by a rapid increase in follicular volume due to hydration and the continued accumulation of macromolecules by endocytosis. In the late OM phase (post-GVBD), the oocyte continued to enlarge by hydration, the cortical alveoli vesicles were peripherally aligned, fused and became larger and the yolk granules filled up the ooplasm (Fig. 2F). The oocyte was surrounded by a cuboidal follicular epithelium and the vitelline envelope that became thinner and had almost completely disappeared by the end of the maturational stage (McMillan, 2007).

The relative abundance of each oocyte stages during the 16 wk of the reproductive cycle is shown in Fig. 3. At week 0, the ovary was dominated by the PG stage (65.56±22.69%); otherwise, CA, VTG1 and VTG2 oocytes were also seen. The number of PG oocytes decreased gradually during the reproductive cycle due to oocyte growth and maturation, while CA and VTG oocytes fluctuated in number. The highest numbers of CA and VTG1 oocytes were observed at week 4 (20.00±8.82% and 20.00±3.33%, respectively) followed by a declining trend onward to only 5.56±1.92% and 7.78±1.92%, respectively, at week 16. The number of VTG2 oocytes was highest at week 6 (28.89±5.09%) before declining from then onward (15.56±5.09%, 21.11±1.92% and 18.89±18.89% at weeks 8, 12 and 16, respectively). The VTG3 oocyte was first apparent at week 6 (17.78±9.94%) and numbers were highest at week 8 (31.11±1.92%) before declining at weeks 12 and 16 (26.67±8.82% and 26.67±3.33%, respectively). Oocytes in the early OM (EOM) stage, particularly the GVM phase first appeared at week 8 (17.78±8.82%) and numbers were highest at weeks 12 and 16 (23.33±8.82% and 22.22±7.69%, respectively). The late OM (LOM) stage or post-GVBD phase was observed at week 16 (4.44±1.92%). Differences in oocyte composition in the ovary at each observation time were related to the GSI value, with more numerous larger eggs corresponding with higher GSI values. The GSI values at weeks 12 and 16 were higher than at week 8 coincident with the numerous oocytes in the vitellogenesis and maturation stages. This trend was in line with the report by Rizzo and Bazzoli (2019).

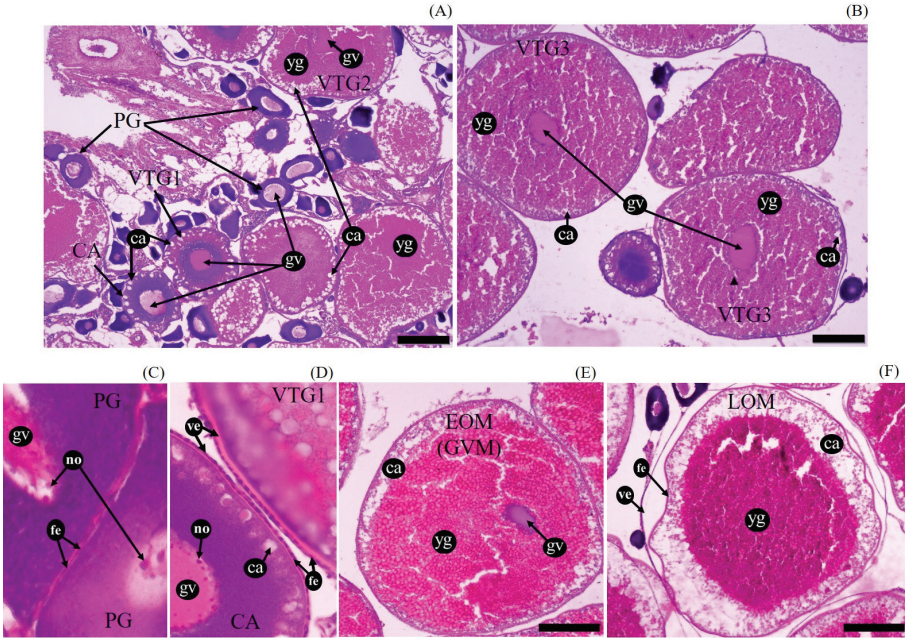

Fig. 2 Ovarian histological appearance (hematoxylin-eosin staining) of mature female Bonylip barb showing different oocyte developmental stages: (A) four oocyte stages comprising 1) PG (primary growth) oocyte, with spherical or round, large nucleus or germinal vesicle (gv) with multiple peripheral nucleoli and basophilic cytoplasm, 2) CA (cortical alveolus) oocyte, with round germinal vesicle (gv) and numerous cortical alveoli (ca) in the basophilic cytoplasm, 3) VTG1 (primary vitellogenic) oocyte, with yolk granules (yg) in addition to cortical alveoli (ca) and slightly acidophilic cytoplasm, 4) VTG2 (secondary vitellogenic) oocyte, with increased number of yolk granules (yg), germinal vesicle (gv) located at the center of oocytes; (B) VTG3 (tertiary vitellogenic) oocyte, with cortical alveoli on peripheral ooplasm due to increased size and number of yolk granules; (C) squamous follicular epithelium (fe) surrounding the PG oocyte, with nucleolus (no) in germinal vesicle; (D) vitelline envelope (ve) and cuboidal follicular epithelium (fe) in CA and VTG1 oocytes, with ve in VTG1 thicker than in CA oocyte; (E) EOM, early oocyte maturation at GVM (germinal vesicle migration) phase, with small-sized nucleus located near periphery (gv), oocyte completely packed with yolk granules (yg), cortical alveoli (ca) only at peripheral ooplasm; (F)LOM, late oocyte maturation at post-GVBD (germinal vesicle breakdown) phase, with nucleus invisible, cortical alveoli on periphery (ca) fused and larger and yolk granules (yg) coalesced. Scale bar = 200 µm

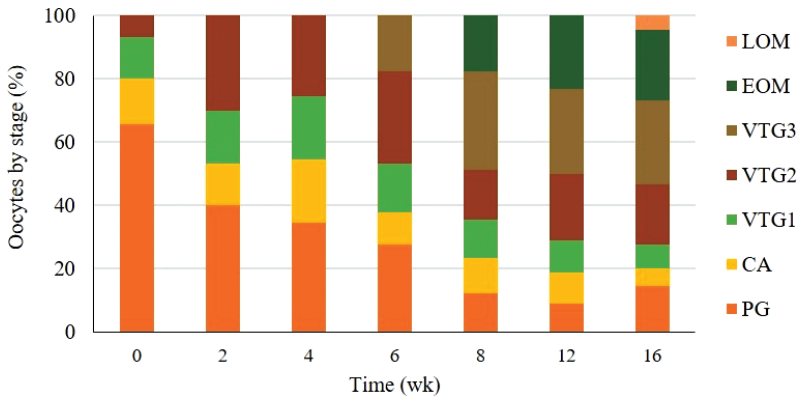

Fig. 3 Relative abundance of each oocyte stage in ovary of mature female Bonylip barb during 16 wk of reproductive cycle, where data are expressed as mean number of each stage from 30 oocytes examined per subsample (n = 3)

Ovarian histology is commonly used to examine gonadal development and the reproduction phase during the reproduction cycle (Brown-Peterson et al., 2011). The ovary's histological features suggested that the developing phase in the second reproductive cycle started at week 0 (in the current study, this was 1 wk after the previous spawning), characterized by the production of vitellogenic oocytes, primarily by VTG1 and VTG2. The female brooders remained in the developing phase as long as the ovaries contained CA, VTG1 and VTG2 oocytes or a combination thereof. The spawning capable phase was suggested to start at week 6 in this species, when VTG3 oocytes first appeared and have developed the receptors for maturation-inducing hormone and thus are able to progress to oocyte maturation. However, in asynchronous oocyte development, which is common in a batch spawner, CA oocytes continue to recruit into VTG by vitellogenesis throughout the spawning-capable phase (Brown-Peterson et al., 2011). Thus, the ovaries may have CA oocytes and various vitellogenesis oocyte stages in the spawning-capable phase, as observed in the ovarian histology of the female Bonylip barb.

Vtgs gene expression during reproductive cycle

In the female Bonylip barb, there were similar relative *Vtg1* and *Vtg2* gene expression levels during the reproductive cycle (Fig. 4). The relative *Vtg1* gene expression started to increase significantly at week 2 (3.57±1.09 folds compared to week 0) onward to week 8 ($p < 0.05$). The peak was observed at week 6 (37.97±7.76 folds); however, this was significantly different from week 8 (24.38±2.54 folds). The relative *Vtg2* gene expression started to increase significantly at week 4 (20.02 ± 1.19 folds; $p < 0.05$); however, there was no significant difference between weeks 6 and 8 (22.86±2.69 folds and 11.43±4.91 folds, respectively). During week 4, relative *Vtg2* gene expression (20.02 ± 1.19 folds) was higher than for *Vtg1* (16.72±7.08 folds); however, at weeks 6 and 8, the relative *Vtg1* gene expression was higher than for *Vtg2*. Furthermore, the values significantly decreased at weeks 12 and 16 compared to week 8. The relative *Vtg1* and *Vtg2* gene expression levels at weeks 2, 12 and 16 did not differ significantly between observation times.

In the current study, the *Vtg1* and *Vtg2* genes were expressed throughout the observation periods and concomitantly with ovarian histology observation. Increased relative *Vtg1* and *Vtg2* gene expression levels occurred in the developing phase (week 0 until week 4) and peaked at the beginning of the spawning-capable phase (week 6), when VTG3 oocytes first appeared. Pearson correlation (r) analysis showed moderate, positive correlations between the relative *Vtg1* and *Vtg2* gene expression and the GSI value during week 0 until week 8 of observation time ($r = 0.779$, $p < 0.01$ and $r = 0.668$, $p < 0.01$, respectively). It seemed that significantly higher relative *Vtg1* and *Vtg2* gene expression levels from week 4 until week 8 were related to the higher numbers of vitellogenic oocytes (VTG1, VTG2, VTG3) at 45.56%, 62.22% and 58.89%, respectively. Furthermore, the relative *Vtg1* and *Vtg2* gene expression levels were significantly decreased at the end of the spawning-capable phase (weeks 12–16) that was related to slower vitellogenesis due to oocyte maturation, as GVM oocytes started to appear at week 8 and reached their maximum number in weeks 12–16. This result was consistent with other studies regarding *Vtg* gene expression in fish. The relative *Vtgs* expression in Atlantic bluefin tuna was higher for *VtgA*

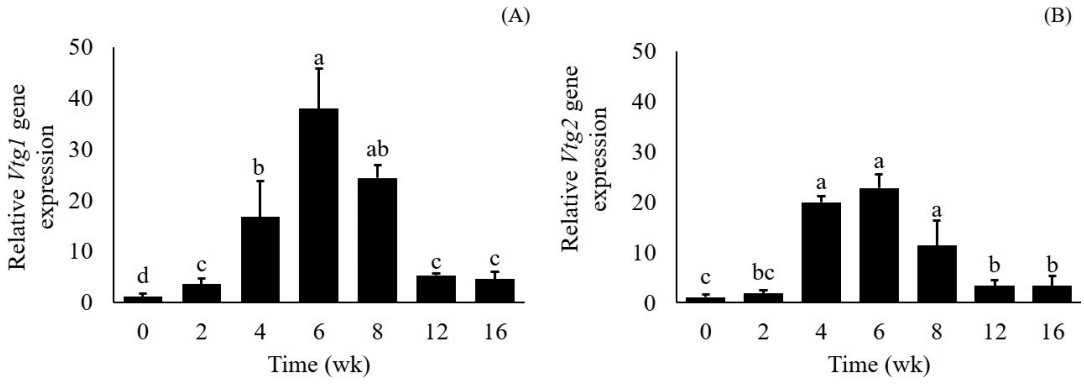

Fig. 4 Relative expression in mature female Bonylip barb during 16 wk of reproductive cycle of: (A) *Vtg1* gene; (B) *Vtg2* gene, where data are expressed as mean ± SD ($n = 3$ per observation time) and different lowercase letters above columns denote significant ($p < 0.05$) differences

than *VtgB*, where the highest expression was observed during the vitellogenesis period (Pousis et al., 2011). In Zebrafish, the relative gene expression of type-I *Vtgs* was higher than for *Vtg2* and *Vtg3*, although the values were not significantly different (Yilmaz et al., 2018).

Proteomics analysis has shown the incidence of vitellogenin (Vtg) products is significantly higher in good quality eggs (Yilmaz et al., 2017). Furthermore, each Vtg had a different role in embryo development, with VtgA and VtgB being subject to proteolytic cleavage during OM and were responsible for generating a pool of free amino acids to produce nutrients for the developing embryo (Reading et al., 2017). The type-I Vtgs, the most abundant in zebrafish, appeared to have essential developmental and nutritional functions in both embryos and larvae, and showed regulatory effects on the physiology of maternal females of zebrafish, including a limitation on fecundity (Yilmaz et al., 2019). Thus perhaps the different levels and timing of relative expression of the *Vtgs* gene could be used as markers to screen broodstock for potential spawning success and egg quality in fish.

Spawning performance at different reproductive cycle times

In the current study, the spawning performance was observed at weeks 8, 12 and 16 based on differences in spawning period occurring naturally (Table 2). The range of absolute fecundity at weeks 8, 12 and 16 were 16,398–37,153 eggs/individual; 36,720–46,222 eggs/individual and 27,923–86,883 eggs/individual, respectively. However, the mean values were not significantly different. These values were comparable with female brooders collected from three locations of Bonylip barb farming in West Java (Rostika et al., 2017), or captured from wild habitats, such as Rawa Pening (Rochmatin et al., 2014), and higher than for fish captured from Talaga Lake (Putri et al., 2015) or Singkarak Lake, Antokan River and the Koto Panjang Reservoir (Syandri et al., 2015).

The fertilization rates were very high (94.14%–99.89%) at all observation times. However, significant differences were observed for fertilization rates at weeks 12 and 16 that were higher than for week 8. The hatching rates ranged from moderate (48.53% and 52.36% at weeks 8 and 12, respectively) to relatively high (74.32% at week 16). However, a significant difference was observed only between week 8 and week 16. Survival rates at 3 dph were very high for all observation times. However, a significant difference was observed between week 8 (94.52%) with both week 12 (98.15%) and week 16 (99.02%). The survival rate at 10 dph and the abnormality rate until 3 dph did not differ significantly for any observation times. The abnormality rate until 10 dph was nil at week 16 and only 0.11% at week 12 ($p > 0.05$), but was significantly different from week 8. Furthermore, the fertilization rate, hatching rate and survival rate at 3 and 10 dph in the current study were higher than for fish captured from the wild in Nagan Raya District, Indonesia and then reared in captivity for 8 wk (Muchlisin et al., 2014). The shortest period of gonad re-maturation (spawning-capable phase occurrence after the previous spawning period) in the current study was assumed to be 8 wk, with the spawning performance comparable to weeks 12 and 16. However, the female brooders treated with Oodev™ at a dosage of 0.25–1.00 mL/kg BW had a shorter period of gonad re-maturation (17–29 d), according to Fitriatin et al. (2018).

The fertilization rate, hatching rate, survival rate and abnormality rate in weeks 12 and 16 were higher due to the

Table 2 Spawning performance at different times in reproductive cycle of female Bonylip barb

Spawning performance	Observation time (wk)		
	8	12	16
Absolute fecundity (eggs/individual)	23,739.15±11,634.01[a]	39,889.07±5,484.67[a]	59,413.25±29,684.99[a]
Fertilization rate (%)	94.12±0.70[b]	99.89±0.19[a]	99.06±1.13[a]
Hatching rate (%)	48.53±9.47[b]	52.36±15.59[ab]	74.32±10.31[a]
Survival rate at 3rd dph (%)	94.52±2.56[b]	98.15±0.68[a]	99.02±0.42[a]
Survival rate at 10th dph (%)	95.74±1.50[a]	97.28±1.58[a]	98.33±0.48[a]
Abnormality rate until 3rd dph (%)	1.89±0.70[a]	1.09±0.60[a]	0.88±1.17[a]
Abnormality rate until 10th dph (%)	7.21±2.08[a]	0.11±0.19[b]	0.00±0.00[b]

dph = days post hatching;
Values (mean ± SD) within each row with different lowercase superscripts denote significant ($p < 0.05$) differences.

larger diameter of the mature egg than at week 8. The mature egg diameters at weeks 12 and 16 were 975.50 ± 10.58 µm and 989.68 ± 11.60 µm, respectively, and differed significantly from week 8 (914.73 ± 6.17 µm). The range in the mature egg diameter observed in the current study was corroborated by other studies involving Bonylip barb (Omar, 2010; Putri et al., 2015; Syandri et al., 2015). The larger egg diameter resulted from the longer period of vitellogenesis and oocyte maturation. The increased egg size could provide distinct developmental advantages during the early stages of fish life because of the greater egg yolk reserves and more suitable larvae (Reading et al., 2018). Although large eggs tend to produce larger larvae, egg size as a criterion for evaluating egg quality in cultured fish is controversial. The intraspecific variability of egg size is associated with age, size, the female's physiological condition and the nutrition they received, spawning times, variations in environmental conditions, and chemical and physical factors in hatcheries that can significantly change reproductive performance (Valdebenito et al., 2013). Furthermore, the gamete quality in cultured fish results from the interaction of environmental and genetic factors that determine the ability to develop and the survivability of embryos, larvae and fry (Bobe, 2015).

Understanding the female reproductive cycle for individual species is important for identifying the critical time frame to administer spawning-inducing drugs or hormones. Such inducement must be properly administered for successful captive reproduction and high egg quality (Reading et al., 2018). Several studies have been conducted to induce gonad maturation, ovulation and spawning, with the aim to improve egg quality in Bonylip barb based on feeding (Santo et al., 2014; Tarigan, 2016; Setyaningrum et al., 2017), photoperiod (Prayogo et al., 2018) or hormonal treatment (Santo et al., 2014; Fadhillah, 2016; Semidang et al., 2018). However, a study on the reproductive aspects is necessary to improve the cultivation protocol and develop assisted reproductive technologies to achieve a reliable supply of high-quality gametes and their conservation aspects, especially for newly cultivated fish species. It is important to compare captive or cultivated individuals to their wild and naturally spawning counterparts when developing assisted reproductive technologies, especially in terms of drugs or hormonal therapy and their impacts on gamete quality (Bonnet et al., 2007; Bobe, 2015).

The current study investigated the relationship between the GSI, relative abundance of VTG oocytes in the ovarian histology and the relative *Vtg1* and *Vtg2* gene expression levels, in order to define the ongoing reproductive phase in the female Bonylip barb. The GSI value increased along with an increased number of oocytes in the vitellogenesis stage and relative *Vtg1* and *Vtg2* gene expression levels in the developing phase (weeks 0–4). Furthermore, during the spawning-capable phase (weeks 6–16), the GSI value increased due to oocyte maturation, whereas the number of oocytes in the vitellogenesis stage and the relative *Vtg1* and *Vtg2* gene expression levels seems to remain constant until week 8. At weeks 12 and 16, the number of vitellogenesis oocytes decreased in conjunction with a decrease in the relative *Vtg1* and *Vtg2* gene expression levels. The culture conditions in which the female brooders were reared allowed for a normal vitellogenesis process, with the shortest reproductive cycle being 8 wk. The spawning performance increased by extending the spawning-capable phase until 12 wk or 16 wk, allowing extensive vitellogenesis and oocyte maturation in one reproductive cycle. Therefore, the results of this study clearly added important information on the reproductive biology of Bonylip barb. Furthermore, the knowledge gained from the study should be useful for improving artificial breeding of this species which in turn would benefit not only aquaculture development but also relieve pressure on natural stocks and thus indirectly promote conservation.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the Research and Community Service Program (P3MI) 2020, funded by the Institut Teknologi Bandung (No. 1505/I1.B02/KU/2019). The Head of the Conservation Division of the Southern Regional Office of the Ministry of Marine Affairs and Fisheries at Singaparna, West Java, provided the Bonylip barb fish samples, the Head of Kawasan Perikanan Darat Ciparanje, Universitas Padjadjaran gave permission to use the rearing facility. Mr.R. Sugandhy and Mr. E. Harditama provided technical assistance.

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