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FRY TILAPIA (*Oreochromis niloticus)* ANTIBODY IMPROVEMENT AGAINST *Streptococcus agalactiae* THROUGH BROODSTOCK VACCINATION

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Abstract

Maternal immunity through brood stock vaccination could improve antibody of seed to pathogenic infections. This study was aimed to determine the appropriate time to vaccinate tilapia brood stock using a combination of extracellular product and formaline-killed whole cells *S. agalactiae*. Therefore, experiments were divided into brood stock injected on day seven after spawning (Group 1), brood stock injected on day 14 after spawning (Group 2) and non-vaccinated brood stock as a control group. Vaccination was performed according to gonadal development stages. Macroscopic observation of gonadal development was conducted on the field using fresh specimen, and then analyzed using histological method. The second stage of gonadal development was observed on day seven, and the third stage (peak of vitellogenesis) on day fourteen post-spawning. The results indicated that vaccination time directly affects the antibody accumulation in fish eggs and seeds and the appropriate time to vaccinate broodstock was on the second stage of their gonadal development.

Key words: *Oreochromis niloticus*, *Streptococcus agalactiae*, vaccine, maternal immunity, gonadal development

Introduction

Nile tilapia is a well-known aquaculture fish species that is being intensively produced all over the globe. The intensification of its production is not only for its good taste but for its high market value as well. However, a major constraint in intensifying tilapia farming is the vulnerability to infectious disease outbreaks. A common infectious disease tilapia farmers are facing is streptococcal, which is commonly caused by bacteria. Fish Infected by *S. agalactiae* show some clinical symptoms such as septicaemia, exophthalmia, corneal opacity, melanosis, swimming abnormalities, swelling and hemorrhage. Moreover, a chronic type of streptococcosis caused by *S. agalactiae* was also identified in tilapia farming. Yuasa *et al.*, (2008), Sheehan (2009) and Jantrakajorn *et al.*, (2014) have reported that *S. agalactiae* bacteria is one of the most devastating bacterial infection in tilapia farming. It, consequently, causes high mortality in tilapia producing countries such as Indonesia, Thailand, Malaysia, and China.

Recently, an *S. agalactiae* outbreak has infected tilapia (*O. niloticus*) reported in Indonesia (Lusiastuti *et al.*, 2009; Lusiastuti *et al.*, 2012; Anshary *et al.*, 2014). *S. agalactiae* large scale outbreaks have tremendous impacts on tilapia farming in countries, resulting in important economic losses (Chen *et al.*, 2012). Li *et al.*, (2014) have reported that when fish are infected by *S. agalactiae*, death usually occurs within a short period.

Vaccine aimed to prevent bacterial infections (including *S. agalactiae)* has been successfully developed in previous researchers. Evans *et al.*, (2004a & b) demonstrated that a vaccine consisting of a combination of *S. agalactiae* formalin-killed whole cells and extracellular products (ECP) can significantly increase tilapia immune system response against bacterial infection. Furthermore, Pasnik *et al.* (2005) showed that a specific antibody, anti *S. agalactiae*, play a major role in fish immune system response against *S. agalactiae* bacteria. Antibody in brood stock blood stream was believed to enter oocytes along with vitellogenin during vitellogenesis (Swain & Nayak, 2009). Amrullah *et al.*, (2014) reported that specific vacci ne, ECP 89 kDa protein toxin, from *S. agalactiae* bacteria isolate $N_{14}G$, could be an important vaccine contender in enhancing tilapia immune system specific and non-specific responses.

An approach to support tilapia farming was to improve seeds quality. Optimal growth in early period could be achieved by increasing seed's immunity against infectious diseases through brood stock vaccination. Innate and adaptive immunity are derived from brood stock to seed as maternal immunity. They play important roles in protecting seeds (during early growth) before their immune system fully develop and actively produce antibody (Zhang *et al.*, 2013).

Maternal immunity transfer was developed as an alternative technique to anticipate high mortality in fry fish (at the age of less than one month). Hanif

et al. (2004) developed a vaccine, *Photobacterium damsel* subsp. *piscicida* SK7 (PhDP), for sea bream brood stock (*Sparus aurata* L.), which produces humoral immune parameters that were shown to be higher in eggs and larvae of the vaccinated brood stock compared to non-vaccinated one.

Nur *et al.* (2004) demonstrated that immune-globulin can be transferred to seeds through brood stock vaccination as maternal immunity against *S. iniae* infection, which is another pathogenic bacterium causing streptococcus in tilapia. However, more information is needed about tilapia brood stock vaccination time. Thus, this study aims to determine the appropriate vaccination (based on brood stock gonadal development) time using a combination of ECP and whole cells formalin-killed bacteria, to ensure that the seed inherit maternal immunity.

MATERIALS AND METHODS

Fish management and experimental design: Matured female's tilapia at an average body weight of 250g previously placed in individual ponds (hapa) and reared under conventional (12 hours of light and 12 hours dark) system, were used in this experiment. Feeding was done twice daily using a commercial feed (32% protein) and constant aeration was provided by an air blower.

Four treatments were applied, consisting of two brood stock groups, i.e. Estradiol-17β induced male, non-induced male, 17β-estradiol induced females, and non-induced female.

Vitellogenin Synthesis induction and Protein Fractionation: Estradiol-17β (E2) (Sigma, USA) was dissolved in an ethylene glycol solution and injected intramuscularly into brood stock at a dose of 10 mg per kg fish (Roubal *et al.*, 1997). Five days after injection, blood samples were collected from the caudal vein of each fish and placed in polyethylene tubes containing heparin (13.2 IU / ml of blood). Blood plasma samples were obtained after centrifugation at 4000 g for 15 min at 4° C. Fractionation of blood plasma protein was performed using SDS-PAGE (7.5% gel) based on Laemmli (1970) method.

Gonad Development Observation: Macroscopic observation of gonads was performed on the field using fresh sample, and placed in a Neutral Buffer Formalin (NBF) solution for histological analysis. Gonadal development stages were predicted according to previous research methods (Suwa and Yamashita, 2007; Mc Millan, 2007; Núñez and Duponchelle, 2008).

Blood plasma and gonad sample were collected shortly after spawning, on day 0, and continued every seven days until the next spawning with three replicates. Vitellogenin level (VTG) in blood plasma were qualitatively analyzed during the reproductive cycle using SDS-PAGE (7.5% gel) by Laemmli (1970) method.

Bacteria: *S. agalactiae* isolate $N_{14}G$ obtained from the Research Institute for Freshwater Aquaculture (Ministry of Marine Affairs and Fisheries, Bogor, Indonesia) was used in this research. Bacteria master seed pathogenic ability was improved using Koch's Postulate method and re-isolated from infected fish. Afterwards, the bacteria were characterized using API 20 STREP SYSTEM.

Vaccine and Vaccine Administration: A combination of whole cells and ECP (*S. agalactiae* bacteria) was used as vaccine in this research. The bacteria were grown in a brain hearth infusion broth (BHIB) and incubated using a shaker at a temperature of 27°C for 72 hours (Klesius *et al.*, 1999); Evans *et al.*, 2004a). The achieved concentration in this process was about 1 x 10^{11} colony-forming units (CFU) ml^{-1}, and NBF was then added at a concentration of 3%. Suspense centrifuged method was used to separate the whole cells and culture liquid and ECP was obtained by filtering the culture liquid using a sterile filter (0.22 µm Millipore, membrane syringe filter solution MS® CA). Concentrated ECP and whole cells were then stored separately in sterile containers at 4° C.

Brood stock were vaccinated at a dose of 0.4 ml kg^{-1} fish and a concentration of $1x10^9$ CFU ml^{-1}, which consisted of 50% whole cells and 50% ECP (v/v). Brood stock were injected on day seven after spawning (Group 1), and 14 days after spawning (Group 2) and non-vaccinated brood stock as a control group.

Indirect Enzyme-linked Immunosorbent Assay (ELISA) Method: ELISA antigen (from whole cells and ECP *S. agalactiae)* was prepared using the sonication method at 40 Hz for 5 minutes (on ice), followed by centrifugation at 3000 g for 60 minutes. The supernatant was collected as antigen and stored at -20° C.

Anti-tilapia globulin conjugate was prepared based on Swain *et al.* (2007) method, which was slightly modified using pooled serum (10–15 ml) from healthy tilapia brood stock (average body weight of 250–300 g). Ammonium sulphate was added to the pooled serum to obtain a concentrated serum, which was then placed on a magnetic stirrer (overnight) at 4°C. After the magnetic stirrer, process the serum was centrifuged and precipitated by

dissolving in 5 ml of carbonate bicarbonate buffer (pH 9.6).

The sample was centrifuged at 10.000 x g for 10 min at 4 °C. Then, the pellet was collected and mixed for 2 ml volume with carbonate-bicarbonate buffer (pH 9.6). The globulin solution was dialyzed using dialysis membrane against PBS (pH 7.2) for 72 h at 4 °C, after which the globulin was collected. The anti-tilapia globulin serum was raised in rabbit (New Zealand white bred) based on Lund *et al.*, (1991) method.

Blood sample were collected on the caudal vein of brood stock before and after 15 days of vaccination. Blood was then left to clot at room temperature for about 1 hour and placed at 4°C overnight. Serum was collected after centrifugation at 3000 rpm (10 minutes), divided into aliquots and then stored at - 20° C.

Eggs were collected directly after spawning while seeds (three groups) were collected on day 7, 14, 21 and 28 after hatching. Eggs and seeds were washed three times with sterile PBS at a pH 7.2, and homogenized for 4 times in phosphate-buffered saline solution, centrifuged at 3000 rpm for 5 min and stored at -20° C.

All tilapia serum samples were tested as antibodies against mix whole cells and ECP vaccine (indirect ELISA) based on Shelby *et al.,* (2002), Hanif *et al.,* (2004) and Pasnik *et al.,* (2006) methods with slight modification. Samples and reactive agents were added in the following sequence: (a) 100 µl of antigen was added to each well of a 96-well microtitre plate, which was incubated overnight at 4°C, (b) tilapia serum samples were diluted, 1:50 for serum and 1:4 for egg and larval extract samples in PBS-T, and 100 µl of the resulting solution was added to three replicates well of microtitre plate. Plates were then incubated at 25 °C for an hour and washed with PBS-T, (c) rabbit anti-tilapia IgM monoclonal antibody was diluted 1:200 in PBS-T and 100 µl of this solution added each well, the plate was incubited at 25°C for an hour, (d) peroxide-conjugated rabbit-anti rabbit IgG was diluted 1:5000 in PBS-T and added to each well, (e) after incubation at 25 °C for an hour, the plate was washed again, 100 µl of One Step Ultra TMB-ELISA was added to each well, (f) the ELISA reaction was stopped after 20 min with 50 µl 3 M H_2SO_4, and the ELISA optical density (OD) of the reactions read at 405nm. Relative amount of specific antibody was measured as OD value.

Data Analysis: Data on macroscopic and microscopic gonad development observations, vitellogenin and plasma protein fractionation were analyzed descriptively. Data on antibody level was analyzed using an ANOVA statistical test.

RESULTS

Gonad Development: Based on macroscopic analysis of gonads (shortly after spawning, day 0), gonadal development was divided into five stages (Fig. 1). First stage, gonads mostly contain whitish and/or yellowish oocyte. At that stage, matured eggs were slightly golden (color) indicate the non-release at the spawning time. The second stage after 7days gonad were characterized on green and/or yellow oocyte. On the third stage after 14 days, oocytes were yellowish colored. The fourth stage after 21 days' oocytes were distinguished by 'golden color. Spawning was done on the fifth stage (day 28), which was the end of the observation period. The reproduction cycle was repeated starting with the first stage.

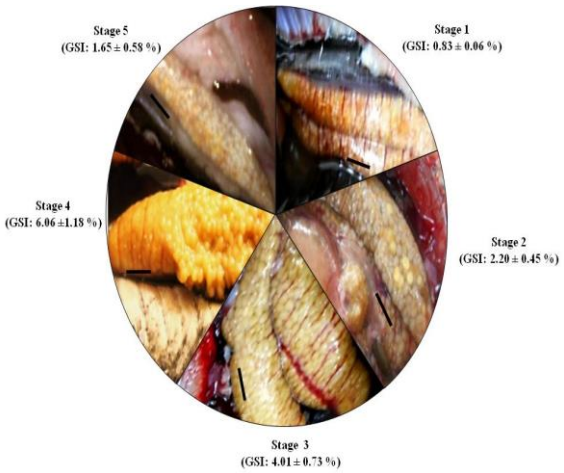


Figure -1: Gonad developmental stages and Gonadosomatic index (GSI, gonad weight/body weight x 100, %) during reproductive cycle of Nile tilapia brood stock. Data shown in mean ± S.E. Gonads were collected at 0 (Stage 1), 7 (Stage 2), 14 (Stage 3), 21 (Stage 4), and 28 (Stage 5) days post spawning respectively (Bar scale: 0.5 cm)

Gonadsomatic index: Gonadsomatic index increased throughout gonadal development stages, and decreased drastically after spawning (Fig. 1). Histological analysis of gonadal development (Fig. 2) indicated that pre-vitellogenesis predominantly occurred on the first stage, while primary growth was observed in the second stage. Germination of the vesicle (nucleus in the middle of the oocyte) was observed to occur in the third stage. The vesicle germination to the oocyte's side (germinal vesicle break down or GVBD) was observed on the fourth stage.

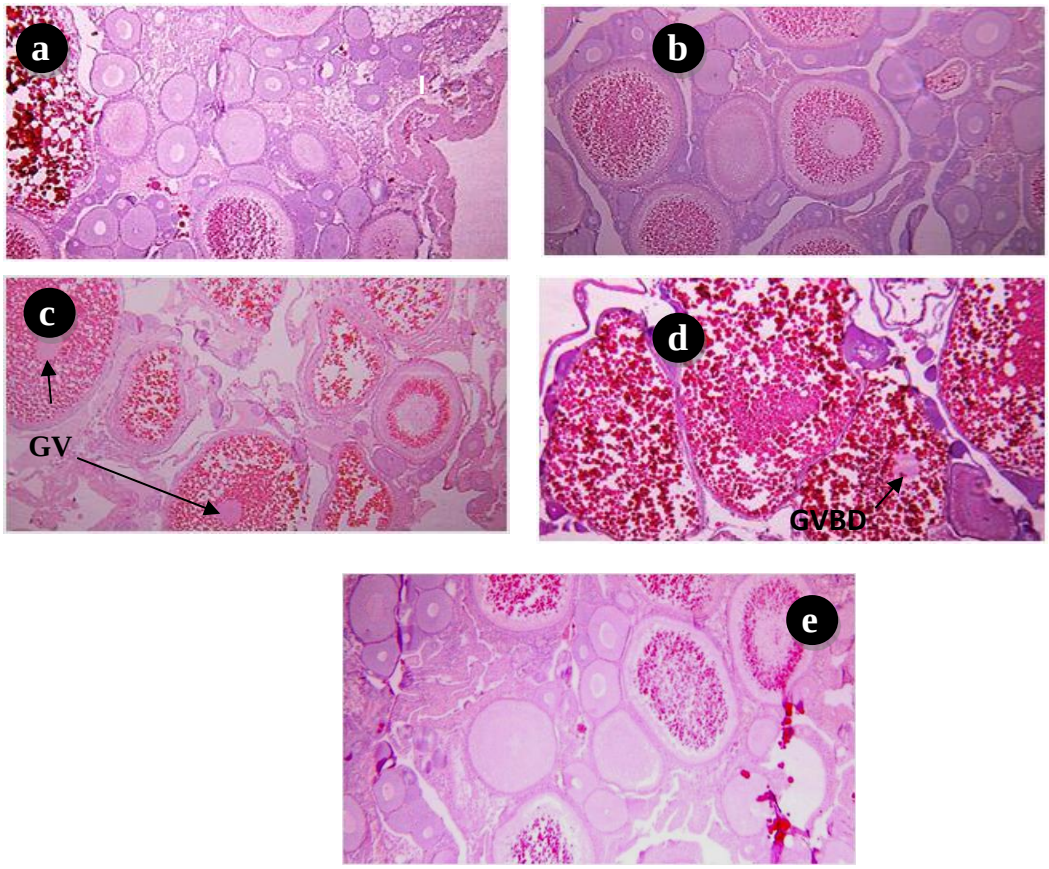


Figure- 2: Histological section of gonad developmental stages from Nile tilapia brood stock during reproductive cycle. (a) Stage 1, (b) Stage 2, (c) Stage 3, (d) Stage 4, (e) Stage 5; GV : germinal vesicle, GVBD : Germinal vesicle breakdown (x100)

Vitellogenesis: The SDS-PAGE (7.5% gel) results shown in Fig. 3 indicated that vitellogenin in blood plasma consisted of two different types of vitellogenin (VTG) protein: VTG 1 (molecular size and weighed about 120 kDa), and VTG 2 (molecular size and weighed about 195 kDa). However, these proteins were not found in the blood plasma of non-induced male. Based on qualitative analysis using SDS-PAGE (7.5% gel), VTG level in female's blood plasma (post-spawning) increased until day 14 and then decreasing up to 2 days (Fig. 4). The macroscopic observation (fish morphology), and eggs composition (fish gonads) showed that fish spawned twice (on day 28 and day 56) during the observation.

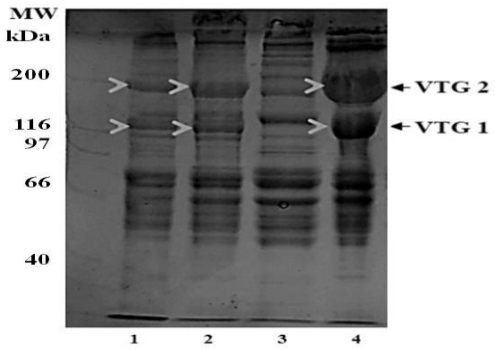


Figure- 3: Fractination of the vitellogenin protein (VTG 1 and VTG 2) from Nile tilapia using SDS-PAGE method;
1 : without 17β-estradiol treated female; 2 : 17β estradiol treated female; 3 : without 17β-estradiol treated male; 4 : 17β-estradiol treated male. Head arrow referred to VTG protein molecular weight (120 and 195 kDa)

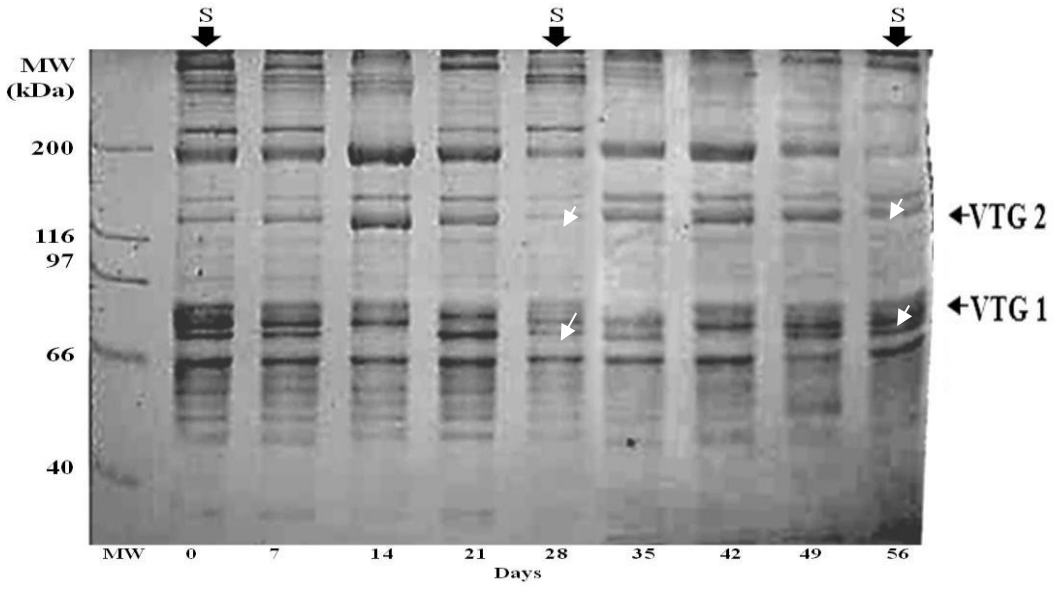
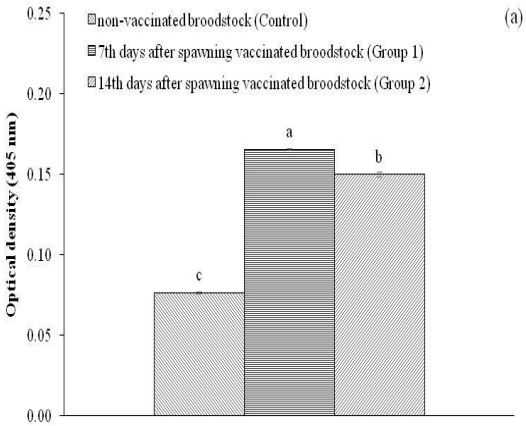
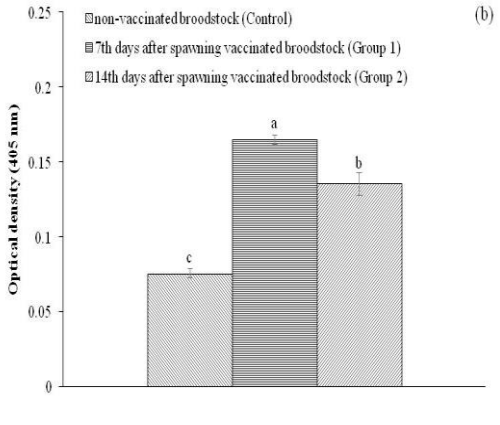


Figure-4: Fractionation of the vitellogenin protein (VTG 1 and VTG 2) from Nile tilapia brood stock during reproductive cycles using SDS-PAGE method. White arrow showed peak of vitellogenesis (on day 14 and 42) (S: spawning)

Specific Antibody in Serum Parent, Eggs, and Seeds: Specific antibody (ELISA OD) level analysis (Fig. 5) showed that antibody levels (7 days post-spawning) in vaccinated brood stock was significantly higher ($p < 0.05$) compared to vaccinated brood stock (14 days post-spawning), and non-vaccinated brood stock. The lowest specific antibody (OD) level was observed in non-vaccinated brood stock (0.076 ± 0.001). Specific antibody (ELISA OD) level of post-hatched seeds was like that observed in brood stock and eggs. However, specific antibody level in seeds and vaccinated broodstock (7 and 14 days post-spawning) continuously decreased until the end of the observation period.





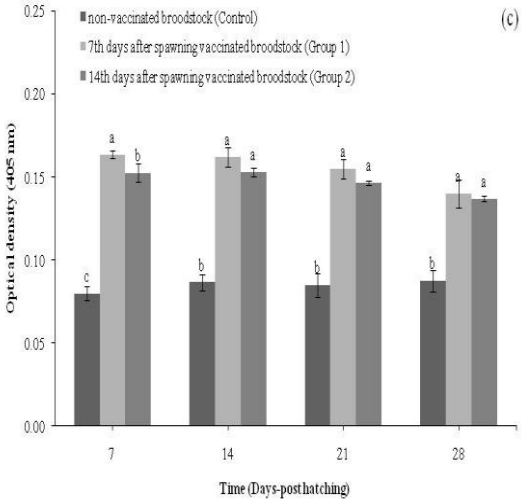


Figure -5: Spesific antibody titre by indirect ELISA method at 405 nm of the Nile tilapia. Data were shown in mean ± S.E. (a) OD ELISA of serum of brood stock, (b) OD ELISA of eggs extracts after spawning, (c) OD ELISA of seeds extracts during development.

DISCUSSION

Information on gonadal development stages are important in term of brood stock vaccination. The timeliness of brood stock vaccination was shown to improve seeds (during early life stages) quality through seeds' resistance to bacterial infection such as *S. agalactiae*. Maternal immunity (Antibody) can be transferred to seeds during early life period of fish through the yolk during the initial stage of vitellogenesis (Swain and Nayak, 2009). Furthermore, it was believed that the process occurred during the transportation of antibody into eggs through transcytosis by crossing follicle cells. During the process, follicle cells were actively involved since they were present in thecal and granulosa forms in cytoplasmic cells (pre-vitellogenesis and follicle vitellogenesis). Antibody transportation process was likely from the blood through follicles and depended on Ig concentration variations in the circulating blood.

According to this research, the brood stock can spawn every 4 weeks (28 days) and early stage and peak of vitellogenesis were observed on the seventh (stage 2) and fourteen days (stage 3) respectively. The matured eggs and brood stock ready to spawn were predominantly found on stage 4. Histological description of gonadal development (Fig. 2) stages (stage 3 and 4) was illustrated in previous research (Ndiaye *et al.*, 2006). Thus, the development of ovarian follicles reflects the accumulation of yolk in the primary oocyte. The third stage of gonadal development was shown to be the beginning of oocytes vitellogenesis, where vitellogenin began to enter the ovarian follicle with appearance of lipid globules and cortical alveoli (Le Menn *et al.*, 1999). Furthermore, it was observed that early accumulation of yolk into the oocyte (vitellogenesis) began along with the deposition process of external radiate zone. The yolk appeared in the oocyte on the third stage, but the accumulation of lipid was more dominant than the vitellogenin. The stage 4 indicated the end of vitellogenesis after oocyte maturation (Selman and Wallace, 1989).

Gonadosomatic index (GSI) increased according to the gonadal development stages (stage 1 to 4), which was explained by the growing gonad sizes through the accumulation of yolk in oocytes (during vitellogenesis). Decrease in GSI was observed after spawning (stage 5), signifying that matured eggs (gonads) were released during the spawning process. Similar results were reported by different researches, mainly on channel catfish (Pacoli *et al*., 1990) and rainbow trout (Bon *et al*., 1997).

VTG analysis in brood stock blood plasma using SDS-PAGE method indicated that proteins consisted of VTG 1 (approximately 120 kDa) and VTG 2 (approximately 195 kDa). According to Ndiaye *et al.* (2006), both polypeptide forms can be found in tilapia blood plasma using SDS-PAGE method. According to quantitative measurements of VTG in blood plasma, the VTG concentration continuously increased starting from stage 1 (Day 0) up to stage 3 (day 14); however, decreased at the beginning of stage 4 (day 21, a week before spawning). Similar results in rainbow trout (*Oncorhynchus mykiss)* showed that VTG concentration in blood plasma increased along with gonadal development stages and drastically decreased at the spawning time (Bon *et al.*, 1997). Based on these results, it could be supposed that vitellogenesis steadily increased during gonadal development (up to stage 3 or before the mature oocyte) and then decreased at the spawning time. Pacoli *et al.* (1990) reported that channel catfish reached their VTG peak a few months before spawning season (before ovaries reach their maximal size). Similar results were observed in Atlantic salmon (So *et al.*, 1985), where fish reached their highest VTG level a month before spawning (then decreased gradually before spawning and rapidly after spawning).

According to a few macroscopic analysis of gonadal development through color changes, micro -scopic through histology, GSI, and qualitative analysis of vitellogenin during the reproductive cycle, the vitellogenesis process predominantly occurred in the seven days following the spawning process and reached its peak 14 days post-spawning.

Based on specific antibody measurement results of eggs and *seeds*, it was observed that injecting the vaccine on the second stage of gonadal development increased antibody levels of eggs and seeds compared to the brood stock that was injected on the third stage. Therefore, antibody produce in response to a vaccination need longer time to enter the oocytes along with vitellogenin during vitellogenesis. Specific antibody levels in vaccinated brood stock were higher compared to the non-vaccinated one (control group).

The current study has successfully demonstrated that vaccinating the brood stock using a combination of ECP vaccine and whole cells from *S. agalactiae* bacteria could increase the specific antibody concentration in eggs and seeds as maternal immunity. It can be concluded that the

vaccination time directly affects the amount of antibody accumulating in eggs and seeds, and the appropriate time to vaccinate the brood stock was on the second stage of their gonadal development.

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