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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation　　**Studies of Egg Formation and Egg-Sperm Interaction in Teleost Spawn Pelagic Eggs**
（浮遊性卵を産む硬骨魚類における卵形成および卵―精子相互作用に関する研究）

学位論文要約：
Dissertation Summary

Teleost is one of the most diverse vertebrate groups having a wide range reproductive strategies including biological and behavioral system. Biological systems include various gender differentiation, final maturation, fertilization methods, and number of spawning cycles. Behavioral systems include mating systems and parental care. However, the most common reproductive method for teleosts is oviparity. Oviparous animals lay eggs with little or no embryonic development from the mother. The embryos are supplied with nutrition via yolk. Vitellogenesis is the process of the ovary sequestering yolk. It is the production and incorporation of egg yolk precursor into developing oocytes in oviparous animals. Vitellogenin (Vg) synthesized in the liver and transported to the ovary then incorporated into the growing oocytes via receptor-mediated endocytosis (Fig. 1). In teleosts, the yolk proteins (YPs) originating from Vg are

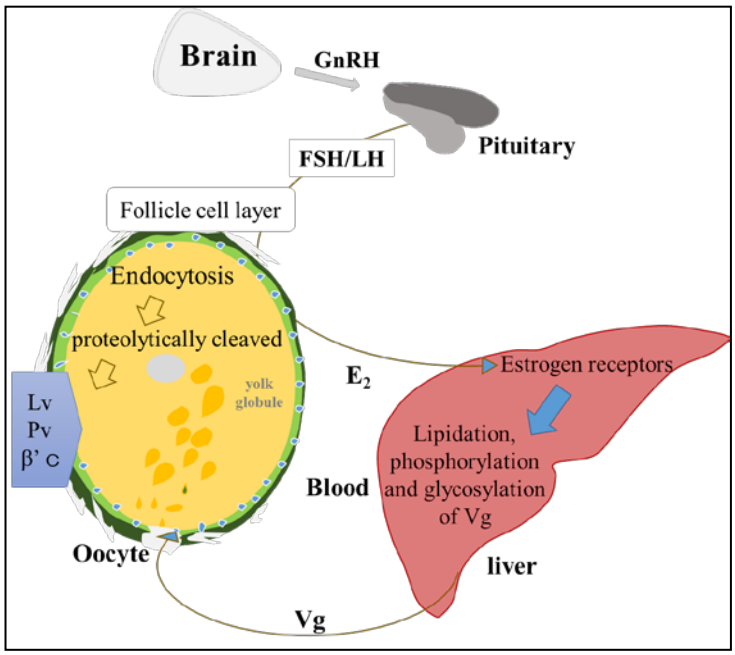

Fig. 1 Conceptual diagram of vitellogenesis. Note that many marine teleost have multiple vitellogenin genes that give rise to multiple lipovitellins being utilized during oocyte maturation. GnRH = gonadotropin-releasing hormone, FSH = follicle-stimulating hormone, LH = luteinizing hormone, E2 = 17ß-estradiol, Vg = vitellogenin, Pv = phosvitin, Lv = lipovitellin, and ß'c = ß-components.

classified as lipovitellin (Lv), phosvitin (Pv) and a minor Vg derived YP that contains neither lipid nor phosphorous namely beta component (β'c) and C-terminal (Ct), and various Lv-Pv complexes (Matsubara and

Sawano, 1995; Matsubara et al., 1999; Finn, 2007a; Reading and Sullivan 2011). The Lv is heavily lapidated, which carrying as much as 20% lipid by mass and Pv consists of ~50% serine residues, most of which are phosphorylated and bind to calcium and other ions that associated with Vg and are transported into the yolk to delivers essential minerals required for skeletal development and metabolic functions in developing embryo. The β'-c and Ct region contain conserved cysteine residues that form disulphide linkages, which may stabilize the folding and dimerization of Vg particles, play a role in cellular recognition or receptor binding, and protect Vg or its product YPs from premature proteolysis (Finn, 2007b; Reading et al., 2009). Generally advanced teleost members such as Paracanthopterygii and Acanthopterygii express three types of Vg transcripts, two of these are first designed as types *VgAa* and *VgAb* (Matsubara et al., 2003) and then renamed as types *VtgAa* and *VtgAb* (Finn and Kristoffersen, 2007) are referred to as complete Vg forms as they contain complete YPs domain (NH2-LvH- Pv-LvL-β'c-C-terminal-COOH) peptide, while the third type of Vg (*VgC*) was incomplete or Pvl Vg with lacks of C-terminal domain (Matsubara et al., 2003). Each type of complete Vg play disparate roles in oocyte hydration and embryonic and larval development. Until present, the complete sequence of multiple Vg cDNAs and their molecular and structural analysis has been analyzed in some fish species of tilapia (Ding et al., 1989; Lee et al., 1992), barfin flounder (Matsubara et al., 1999), haddock (Reith at al., 2001), medaka (Shimizu et al., 2002), Japanese goby (Ohkubo et al., 2003), mummichog (Lafleur et al., 2005) whereas complete sequence of multiple Vg (three forms of Vg protein) in white perch (Hiramatsu et al., 2002b), mosquitofish (Sawaguchi et al., 2005), red seabream (Sawaguchi et al., 2006), gray mullet (Amano et al., 2007), marbled sole (Amano et al., 2009), striped bass (Williams et al., 2014) and European sea bass (Yilmaz et al., 2016) have been distinguished. In contrast, information concerning Vgs and derived YPs in earlier teleosts during the evolutionary process is still very limited. To understand the diversity and commonality of vitellogenesis in all vertebrates, further studies on vitellogenesis in various teleosts are required.

In general, eggs of marine teleosts are divided into two types designated as pelagic eggs (pelagophils) and demersal eggs (benthophils). The pelagic eggs undergo a marked increase in water content during FOM compared with demersal eggs (Craik and Harvey, 1986). The higher water content of the pelagic eggs is thought to be a major reason for their buoyancy (Craik and Harvey, 1987; Fulton, 1898). Morphological indices such as germinal vesicle migration (GVM), germinal vesicle breakdown (GVBD), onset of lipid coalescence, yolk globule fusion and onset of transparency occur concomitantly with oocyte hydration during the maturation phase (Palstra et al., 2005). Investigations of the sequential progression of the events have been pursued in both fresh

water and marine teleost species (Craik and Harvey, 1984; Palstra et al., 2005; Lubzens et al., 2010); however, differences depended on the species, especially in marine species spawning pelagic eggs. Understanding the sequential progression and their time-course during FOM prior to ovulation is thought to contribute for the acquisition of high-quality eggs in aquaculture by the accumulation of information about reproductive ecology. In fish, egg YPs, which are nutrient stocks for embryonic development, are incorporated into oocytes during the phase of active oocyte growth (Specker and Sullivan, 1994; Wallace, 1985).

In addition, captive and wild population of fish are dependent upon the production of good quality of egg and sperm. Poor quality of egg and sperm is one of the major constraints in the expansion of aquaculture of both marine and many freshwater fish species. The good egg quality refers to the ability of the egg to be fertilized and subsequently develop into a normal embryo while sperm quality as its ability to successfully fertilize an egg and subsequently allow the development of a normal embryo (Bobe and Labbe, 2010). There are so many factors that affect the quality of egg and sperm like, hormone, the age of the fish, environmental factors, diet, photoperiod, water quality, pollutants, husbandry, sperm concentration, sperm morphology, and sperm motility. In this research, I mainly focuses on sperm morphology and viability as circadian rhythm in primitive teleost from lower taxonomic group namely Japanese anchovy, *Engraulis japonicus* (EJ).

Anchovies, a primitive teleost group, are small, pelagophil fish of Engraulidae family. There are about 145 species in 17 genera found in the Atlantic, Indian, Pacific Oceans and the Mediterranean Sea. *Engraulis japonicus* is a small (maximum size ~160mm) marine teleost that spawns pelagic eggs. This species is commercially important to fisheries in Japan, China, Korea and Taiwan, and plays an important role as a key member of marine ecosystems (Pandey et al., 2017a). The EJ is known as a multiple spawning fish having a long spawning season (Tsuruta, 1993; Funamoto, 2001) and spawn oval-shaped pelagic eggs. Females spawn periodically at two or more -day intervals during the season of spawning (Yoneda et al., 2013). In addition, EJ have several advantages over other saltwater species; (I) fecundity and photoperiod can control their ovulation, (II) multiple spawning species spawn oval-shaped pelagic eggs, (III) comparatively small in body size with short period maturation, suggesting that this species might be a model species in aquaculture research. In addition, I also used Kawakawa, *Euthynnus affinis* (EA) as a member species from advance teleost group. *Euthynnus affinis* is a species of tuna from Scombridae family. They mainly lives in the tropical and subtropical water of the Indo-West Pacific. They spawn pelagic eggs that develop very near the water surface. As EA has been introduced new aquaculture species from tuna family in Japan, especially in Ehime prefecture, research on complete

aquaculture system in this species is necessary, and research going on in South Ehime Fisheries Research Center (SEFRC), Nisuira Station. In this chapter, I also discussed the multiple Vg in EA as compare to EJ.

Key finding of the study

The aim of this study was to obtained fundamental knowledge of the biochemical and molecular biological characteristics of multiple Vgs (mechanism of egg yolk formation) in teleosts, morphological and biochemical characteristics of oocytes during FOM, and the circadian rhythm of spawning in EJ (spawning rhythm of male and female for successful fertilization). We hypothesized that the Vg is evolutionarily homologous among a large variety of animals from insects to chickens. We initially carried out the experiment in multiple Vg (molecular cloning and characterization, and quantification in liver). However, in this study, we observed the high similarities between sequence of complete type Vgs in EJ suggesting that complete type Vgs were unique in structure and might be different proteolysis as compared to other higher teleost. To elucidate physiological and biochemical changes in oocytes during final oocyte maturation (FOM), we designed the study (Pandey et al., 2017a). In addition, we also designed another experiment (Pandey et al., 2017b) in order to confirm sperm motility in circadian rhythm of spawning in EJ. To the best of our knowledge, no one has examined so far the sperm motility in circadian rhythm in any other species. In this research we also examine the ovarian fluid effect on sperm motility. And the overall key findings are listed below:

- Targeted both species (EJ and EA) showed multiple Vg.
- Species dependent differences in similarities of complete type Vgs were observed.
- In EJ, complete type Vgs were confirmed as major functional Vgs.
- The reproductive cycle in captive EJ was clarified and their spawning time was around 21:00.
- Final oocyte maturation completed within 8 hours in EJ.
- The size, weight and water content in oocytes changes remarkably due to hydration during FOM.
- Major YPs degraded and remarkable increased in the amount of free amino acids occurred during FOM.
- Circadian rhythm of sperm motility was demonstrated in EJ and this is the new finding among teleosts.
- Highly motile sperms at 21:00 is suggested to confirm the group synchronization in EJ.
- There is no significant effect of ovarian fluid on sperm motility and moving velocity.

Targeted both species (EJ and EA) showed multiple Vg:

Full-length cDNA clones encoding the three Vgs forms (a-type, b-type and c-type) of EJ were obtained from the liver cDNA library prepared from matured female. The cDNA sequence of a-type Vg (4893 bp ORF) encoding 1631 amino acid and b-type Vg (4902 bp ORF) encoding 1634 amino acid residue and characterized as *EjVgAα* and *EjVgAβ* respectively. These two obtained sequences (a-type and b-type) confirmed as complete type Vg consisting of LvH-Pv-LvL-β`c-Ct, While c-type Vg had 3771 bp ORF encoding 1257 amino acid residue and confirmed as incomplete type Vg consisting of LvH-LvL, characterized as *EjVgC*. Moreover, we cloned multiple Vgs in EA and characterized as *EaVgAa, EaVgAb* and *EaVgC*. Full-length cDNA clones encoding the three form of EA Vgs *EaVgAa* (ORF: 5115 bp), *EaVgAb* (ORF: 5160 bp) and *EaVgC* (ORF: 3816 bp) cloned from the liver cDNA of matured female EA. Obtained Vgs from EA was highly identical (90 to 95 %) with corresponding Vgs of bluefin tuna. Two complete type Vgs (*EjVgAα* and *EjVgAβ*) of EJ were estimated to have molecular mass of 177 kDa and 178 kDa respectively and the mass of the *EjVgC* was estimated to be 138 kDa. While, in EA *VgAa, VgAb* and *VgC* estimated to have molecular mass of 187 kDa, 189 kDa and 143 kDa respectively. Based on the results from sequence comparison between previously analyzed multiform of Vgs and also YPs (Matsubara et al, 1999), predicted domain structure of each type Vg of the EJ and EA were constructed (Fig. 2).

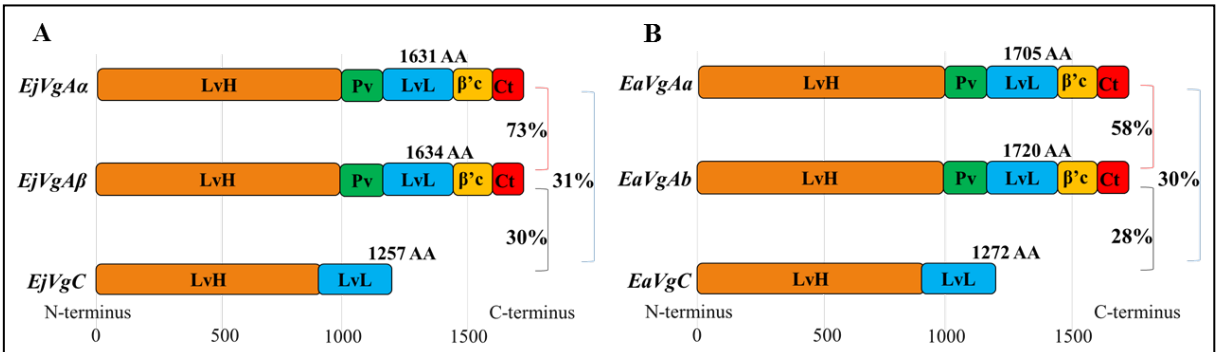

Fig. 2 General arrangement of domain structure of Vgs genes. A: *Engraulis japonicus* (*Ej*) B: *Euthynnus affinis* (*Ea*). The percentage value showing on right of each figures indicating sequence identity. The linear amino acid sequence of Vgs correspond to the YP domains as follows: N-terminus, lipovitellin heavy chain (LvH), polyserine domain (phosvitin, Pv), lipovitellin light chain (LvL), and β`-component (β`c) and C-terminus (Ct). AA, amino acid.

Species dependent differences in similarities of complete type Vgs were observed:

The obtained sequences of EJ A-types Vgs (*EjVgAa* and *EjVgAb*) aligned with each-other by 73% identity and 78% similarities. Those value is higher than that previously reported other species Vgs (*VgAa* versus *VgAb*) including the current finding of *EaVgAa* and *EaVgAb* (58% identity), suggesting that EJ complete type Vgs were unique in structure and might be different from other higher teleost. Therefore, we classified *EjVgAa* as *EjVgAα* and *EjVgAb* as *EjVgAβ* for EJ, while *EaVgAa* and *EaVgAb* for EA complete type Vgs.

In EJ, complete type Vgs were confirmed as major functional Vgs:

Expression of the Vgs (*EjVgAα, EjVgAβ* and *EjVgC*) mRNA in qPCR was abundant in the liver of mature females. However, expression level of *EjVgAα* and *EjVgAβ* were significantly higher than that of *EjVgC* suggesting that *EjVgAα* and *EjVgAβ* were main functional Vgs in EJ. The proportion of different Vg mRNA (*EjVgAα*, *EjVgAβ* and *EjVgC*) in the liver at late vitellogenesis phase of EJ is 23:25:1 (Fig. 3). Considerably

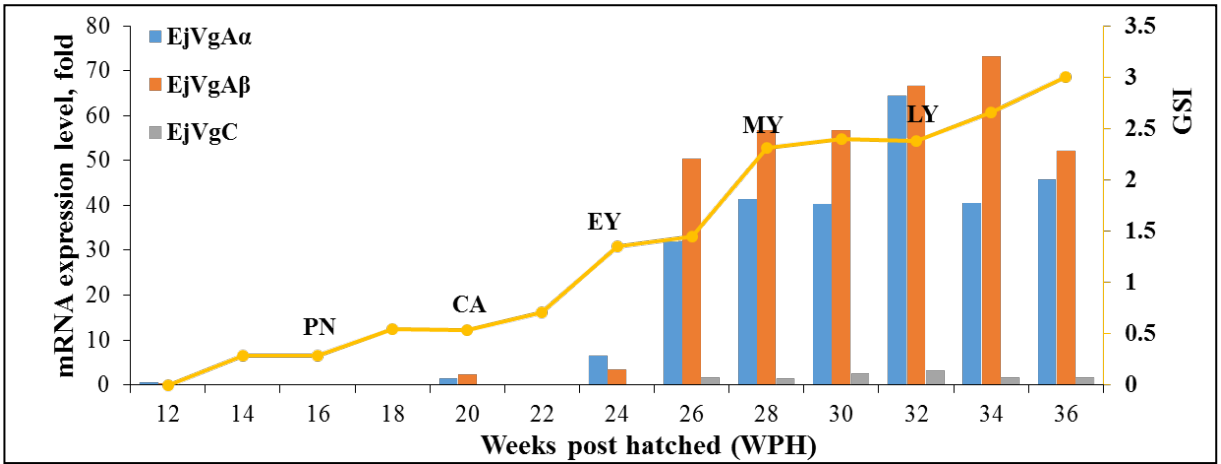

Fig. 3 Results of relative quantitative real-time RT- PCR used to quantify vitellogenin gene (*EjVgAα*, *EjVgAβ*, and *EjVgC*) transcripts present in *Engraulis japonicus* liver of different periodic sample (every 2 weeks interval). Data are shown as expression (fold) relative to reference gene elongation factor one alpha (*EF1α*). Gonadosomatic index (GSI) of sampled fish in different sampling point (starting sample at 12 weeks post hatched to until 38 weeks post hatched) showing along with the oocyte development and majority of maturational stages PN (Perinucleolus stage), CA (Cortical alveoli stage), EY (Early yolk globule stage), MY (Mid yolk globule stage) and LY (Late yolk globule stage).

lower levels of the *VgC* protein in serum compared to the complete type Vgs were reported in some species (Ohkubo et al., 2006; Sawaguchi et al., 2005), supports the present results of low Vg mRNA expression in *VgC*.

The reproductive cycle in captive EJ was clarified and their spawning time was around 21:00:

The gonadosomatic index of EJ increased due to the progression of oocyte growth and maturation and also increased in the mRNA expression in liver (Fig. 3). From the results, it is considered that vitellogenesis in EJ may initiate about 24 weeks after hatch and complete within 60-70 days. Overall, lifecycle may complete in 32 to 34 weeks, while spawning occurred at 21:00 to 22:00 at two-three day interval (Pandey et al., 2017a).

Final oocyte maturation completed within 8 hours in EJ:

In captive condition 14L:10D photoperiod in water of 19.5 to 20.9 °C, FOM in EJ complete in 8 hours and the process can be classified into five stages: stage-I, post-vitellogenic oocyte (PVO); stage-II, germinal vesicle migration (GVM); stage-III, germinal vesicle breakdown (GVBD); stage-IV, late hydration; and stage-V, ovulation, while FOM initiated from 13:00 to 15:00 and progressed to reach ovulation by around 21:00 (Pandey et al., 2017a).

The size, weight and water content in oocytes, changes remarkably due to hydration during FOM:

An egg is the final product of oocyte growth and differentiation. The physiological factors, size, weight and water content in eggs are responsible for its quality and significance for the production of high quality fish larvae. The average wet weight of post-vitellogenic oocyte was 69 µg with a longitudinal diameter of 728 µm and 75% water content and then increased to 244 µg (wet weight), 1,386 µm (longitudinal diameter), and 94% (water content), respectively, at the stage of ovulation (Pandey et al., 2017a).

Major YPs degraded and remarkable increased in the amount of free amino acids occurred during FOM:

The marked changed in molecular weight of major YP peaks, vitellogenic lipovitellin (vLv) 430 kDa in Post-vitellogenic oocytes to ovulated lipovitellin (oLv) 170 kDa in ovulated egg were confirmed in gel chromatography and result further supported in SDS-PAGE analysis indicated proteolytic degradation during the latter stages of FOM (Pandey et al., 2017a). In post-vitellogenic stage oocytes extract, the major YP peak was observed at an elution position of 13.7 ml, followed by minor peaks of 16.4 and 18.3 ml except for a peak of void volume (Vo) at 7.5 ml and a very small molecules at 20.2 ml. SDS-PAGE analysis showed that the major YP peak yielded major bands of 150, 110 and 57 kDa associated with minor bands around 25 kDa. On the other hand,

the minor peak at 18.3 ml generated 17, 16, 15 and 12 kDa bands. There were no marked changes in the elution and SDS-PAGE patterns in St-II. The elution position of the major peak in St-III changed to around 14 ml, which generated major bands of 150, 110 and 90 kDa with minor bands of 26, 24 and 20 kDa. The minor elution peaks of 16.4 and 18.3 ml disappeared; however, the YP bands of 17, 16 and 15 kDa were still observed around the elution position of 18.3 ml. In St-IV and St-V, the major elution peaks shifted to 17.3 ml, and the peak fraction yielded 90 and 65 kDa major bands with 26, 24, 20 and newly appeared 12 kDa minor bands (Pandey et al., 2017a).

Marine teleost species having pelagic eggs have been thought to share a common mechanism for oocyte hydration where partial hydrolysis of specific YPs to FAA creates a considerable part of the osmotic potential needed for the water influx (Thorsen and Fyhn, 1995; Fabra et al., 2005). The present results comparing FAA content before and after FOM showed that maturation-associated FAA production also occurred in this species, suggesting the change in FAA levels is the osmotic effector for oocyte hydration. However, the amount is lower compared with pelagic eggs of other reported marine teleost species (Pandey et al., 2017a). The total FAA amount was approximately 22 nmol egg^{-1} and the wet weight of the egg was about 0.24 mg in this species, whereas there was 200 nmol in 1 mg egg of Atlantic cod (Finn et al., 1995), 354 nmol in 2.9 mg eggs of barfin flounder (Matsubara and Koya, 1997) and 304 nmol in 1.6 mg eggs of walleye pollock (Ohkubo et al., 2006). Based on the same calculation manner (nmol/mg egg), the FAA content of EJ represents 75% less than FAA in barfin flounder and 48% less than FAA in walleye pollock but 30% greater than that FAA in Japanese eel. Thus, the total FAA content in the post-vitellogenic oocyte was 0.26 µg per individual (corresponding to about 2 nmol) and increased 11-fold that in ovulated egg was 2.8 µg per individual (corresponding to 22 nmol) except for taurine (Tau), which remained stable (Pandey et al., 2017a), suggesting that FAAs play a key role in raising osmotic pressure in the oocytes and inducing hydration during FOM in the EJ.

Circadian rhythm of sperm motility was demonstrated in EJ and this is the new finding among teleost:

The percentage of motile sperm significantly differed among the sample groups (circadian rhythm), highest motility was confirmed at 21:00 (75 ± 12%) and lowest motility was observed at 9:00 (4 ± 5%). Motility decreased in accordance with time (minutes post activation) and became almost inactive after 60 min post activation. On the other hand, the moving velocity of the motile sperm was not significantly different between the time sampling points, even though the values were slightly higher close to the spawning time. The average

moving velocity at 1 min post activation was 196 ± 26 µm/s at 21:00 and the moving velocity remained constant for 15 min post activation, and reached zero after 60 min post activation (Pandey et al., 2017b).

Highly motile sperms at 21:00 is suggested to confirm the group synchronization in EJ:

As EJ exhibits group synchronous in wild (Funamoto, 2001) and captive conditions (Pandey et al., 2017a). We have clarified the process in which spermatozoa acquire motility during a time period of approximately half a day. It is noteworthy that circadian rhythm in sperm motility was also observed in each sampled male (Pandey et al., 2017b), suggested that the process occurs concomitant with female FOM. Synchronous functionalization of both female and male gametes is suggested to ensure efficient fertilization in EJ. Moreover, long duration (> 60 min) of sperm moving seems likely to guarantee a group synchronous non-pairing spawning style. To show the rhythm is possible among teleost species having multiple spawning characteristics, further research is necessary to determine such a rhythm of sperm motility in other species.

There is no significant effect of ovarian fluid on sperm motility and moving velocity:

There are many factors that influenced the quality of sperm like, hormone, the age of the fish, environmental factors, diet, photoperiod, water quality, pollutants, husbandry, pH, temperature, ions, osmolality, sperm motility, sperm concentration, sperm morphology and ovarian fluid. In our research, we discussed about effect of ovarian fluid. As ovarian fluid is slightly viscous fluid found in the gonad cavity of oviparous fishes after ovulation. Ovarian fluid has been studied for its role in fish reproduction, its chemical composition, novel proteins and utility in testing for the presence of fish diseases (Sawyer et al., 2003) and sperm motility. Comparisons of two activation mediums, SW and SW + OF, on sperm motility and moving velocity showed no significant differences in EJ (Pandey et al., 2017b) suggesting the trigger for sperm activation in multiple spawning fish is possibly species dependent.

Conclusion

In conclusion, the majority of teleosts are oviparous animals that despite using a wide variety of reproductive strategies and tactics. In teleost, the formation of an egg is a complex process where the egg is self-sufficient to protect and sustain the developing embryo until hatching. All the content of an egg in the form of

mRNA and nutrition or hormones, must be incorporated into an egg during growth and maturation within the ovary. The present study of molecular evidence in identification and characterization of multiple Vg genes and proteins in EJ and EA, suggesting that vitellogenesis is similar processes in a wide range of marine teleosts. Obtained EJ Vgs, *EjVgAα, EjVgAβ* and *EjVgC* encodes 1631, 1634 and 1257 amino acids respectively, and residues contribute substantially to the pool of YPs present in oocyte and eggs. However, during oocyte maturation, the major product of each form of EJ Vg undergoes fairly limited degradation to smaller polypeptides, unlike the case of other marine pelagic fishes in which LvHA completely degraded to FAA. Quantitative expression pattern of multiple Vgs in real time mRNA expression analysis shows that, the *EjVgAβ* has slightly higher than that of *EjVgAα* and remarkably higher than *EjVgC* specific in matured female liver concludes that, *EjVgAα* and *EjVgAβ* are the main functional Vgs in in EJ.

In addition, the morphological, histological and biochemical changes along with time course progression of FOM indicating most developed clutch of anchovy oocytes initiate final maturation between 13:00 and 15:00, and completed at 21:00 in captive condition (14L:10D at water temperature 19.5–20.9˚C). *Engraulis japonicus* exhibits group synchronous and multiple spawning, and its FOM may complete in 8 hours. Result of longitudinal diameter of EJ oocytes changed from 0.73 to 1.37 mm with high water influx up to 94% suggesting that most of the water in the eggs is taken up during FOM as pelagic eggs of marine teleost acquire their buoyancy mainly by a large amount of water filled in the eggs (Craik and Harvey, 1987). Marine pelagic egg spawner are thought to share a common mechanism for oocyte hydration where by partial hydrolysis of specific YPs to FAA creates a considerable part of the osmotic potential needed for the water influx (Thorsen and Fyhn, 1996). The high water influx relates to the fact that the oocytes contain the large amount of osmotic effector mainly FAAs acquiring the buoyancy of eggs also in EJ. To clarify the mechanism of FAA production during FOM, time course of YPs degradations were examined by the combination of gel chromatography with SDS-PAGE analysis. In addition, the features of EJ sperm motility (75 ± 12%) and moving velocity (196 ± 26 μm/sec) were clarified, moreover the long duration of the swimming of sperm, more than one hour, seems likely to guarantee a group synchronous non-pairing spawning style activation in circadian rhythm. To show the spawning rhythm among teleost species having multiple spawning characteristics, further research is necessary to determine such a rhythm of ovulation (in female) and sperm motility (in male).

Thus, the multiple Vg and yolk proteolysis systems of the teleost species seems to vary among species or taxonomic groups despite of their similar egg types and reproductive environmental salinities. The unusual

pattern of maturational proteolysis and oocyte hydration found in this study in the EJ may be linked to their evolutional position. Moreover, short time course of final oocyte maturation and ovulation (female gamete) and circadian rhythm of sperm motility in addition to long duration of sperm moving seems likely guarantee a group synchronous non-pairing spawning style. The synchronous functionalization of both gametes (male and female) is suggesting to ensure efficient fertilization in EJ. Finally, the data from this study have helped to create techniques to control a variety of female reproductive events, and these techniques have significantly contributed to the aquaculture industry to improve new protocols for successful egg production and artificial fertilization in advance aquaculture species.

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