A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Quantitative Assessment of the Reproductive Effort of *Tresus keenae* (Kuroda and Habae 1950) on the South Coast of Korea Using Egg Specific Polyclonal Antibody

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Abstract

Reproductive cycle and reproductive effort of *Tresus keenae* in Yeosu, South coast of Korea was determined using immune assay techniques combine with the histology. The polyclonal antibody developed against the egg proteins of *T. keenae*, only detected the egg proteins with a high sensitivity (0.64-10.24μg/ml) was used to quantification of the eggs using indirect ELISA. *T. keenae* in Yeosu spawned in late autumn (October and November) and ready to spawn female and spawning male observed in late winter (March) while first clam with oogonial mitosis also observed in March. The mean GSI of ready to spawn clam in October was 5.7±2.2 and March was 2.4±0.3. Condition Index (CI) from October to November was declined, however during March CI was not scaled down and potential fecundity of the *T. keenae* in Yeosu estimated to be varied from 121.92×10^6 to 22.07×10^6 (average 53.40×10^6) eggs per clam in October while it was ranged from 69.19×10^6 to 29.07×10^6 with average of 44.91×10^6 eggs per clam in March. The dry weight of siphon and carbohydrate of the siphon declined during the early development of the gametes suggesting that the carbohydrate stored in the siphon exploited for gametogenesis. A noticeable reduction of total carbohydrate in adductor muscle, siphon and body was observed during the spawning period suggesting that the carbohydrate fulfilled the energy demand during the spawning. The egg specific antibody developed in this study is sensitive enough to quantify the reproductive effort of the clam which is difficult to measure using the conventional methods and using developed antibody reproductive effort of *T. keenae* was successfully determined.

Keywords: *Tresus keenae*, clam, reproductive effort, ELISA, Polyclonal antibody

Introduction

The projected population growth to over 9 billion people by 2050 (Henry et al., 2018) while this geometrical expansion of the population size will be a reason for severe problems including shortage and inadequate food supply, environmental degradation and natural disasters. As the population expands, food supply will become inadequate and climate changes substantially affect to the agricultural productivity and shifted towards the animal base diets (Alexander et al., 2016). Among the animal base diet, the annual global sea food consumption has been doubled over the past 50 years, whereas protein derived from the seafood serve as essential nutrient components in many countries (Guillen et al., 2018). The capture fishery along is unlikely such an increasing demand for the sea food and aquaculture is possible solution for this shortage. Total marine and inland capture fish and shellfish landing was decrease from 92.2 million tonnes to 90.9 million tonnes from 2011 to 2016, however the aquculture production from both inland and marine fish and shellfish increased form 61.8 million tonnes to 80.0 million tonnes from 2011 to 2016 (FAO, 2018). The total global fish production peaked itself about 170.9 million tonnes in 2016 while the aquaculture accounting 47% of the total fish production (FAO, 2018).

The marine bivalves are appreciated by the consumers due to their nutrients values as well as their taste and recognized as an influential solution for the increasing protein demand of the growing world population in the future. The marine bivalve accounts about 14% of global marine production, although 11% of marine bivalve production comes from wild fishery, 89% of marine bivalve production derive from aquaculture (Wijsman et al., 2019). The marine bivalves are to be a sustainable type of production as they are herbivores that are in bottom of the food chain, requiring no additional feeding (Wijsman et al., 2019, Duarte et al., 2009) for this reason culture of bivalve is popular worldwild. To date, most of the bivalve

farmers are entirely rely on the wild seed, however the collection of wild seed is condemned because of the possible damages to the bottom habitat and resulting ecosystem collaps (Kamermans et al., 2013). On the other hand collection of wild seed is highly seasonal and production of hatchery breed seed is a possible solution while the hatcheries can produce year round seed under control conditions.

Understanding of the annual gametogenesis of marine bivalve is important to clarify the duration of gametogenesis, spawning and the quantity of gametes released during the spawning period because these data are crucial to manage and operate the hatcheries of the bivalves (Matias et al. 2013; Gribben 2005; Cross et al. 2014; Morsan and Kroeck 2005). Histology has been used to identify the reproductive cycle qualitatively (Howard et al. 2004) while quantification of the reproductive effort, that total energy devoted to the reproductive process, is not easy because in the most cases gonad of the bivalve is an integral part of the visceral mass as reported by Beninger and Lucas 1984 and Choi et al. (1993).

There are different methods to quantify the reproductive effort such as measuring the weight difference of the tissue just prior to and after spawning (Kautsky 1982; Pouvreau et al. 2000; Tirado & Salas 1998) and inducing the bivalve to spawn by thermal shock or chemicals and counting the number of gametes or weighing the gametes (Massapina et al. 1999; Choi et al. 1993). Instead of that gonadal weight can estimate from the histological preparation of gonadal tissues using stereology and planimetry (Morvan and Ansell 1988; Knigge et al. 2014). However, these methods may underestimate the true reproductive effort as the spawning is not always complete and it occurs several times during the spawning season with different intensities (Choi et al. 1993).

Alternatively, reproductive effort of bivalves can measure quantitatively using the immunological methods which is the most common method related to the production and use

of antibodies to detect specific proteins in biological samples and these assays are proven to be a great tool as fast and sensitive method to determine the reproductive effort of vertebrates and invertebrates such as shrimp (Tsukimura et al. 2000), fish (Daud et al. 2016) and insects (Dong et al. 2011) as well as to detect the pathogens in different hosts (Choi et al. 1991; Park et al. 2006). Immunological assays have been successfully utilized in several studies for the quantification of reproductive effort of bivalves including clams, mussels and oysters (Choi et al. 1993; Kang et al. 2003; Park and Choi 2004; Long et al. 2008; Uddin et al. 2012). This is known to be a highly sensitive, fast and affordable method to quantify the reproductive effort, once an antibody that specific for the egg protein is developed, target egg protein can quantitatively measure and visualize from the whole tissue using different immunological methods such as ELISA or immunofluorescence assay (Choi et al. 1993; Kang et al. 2003; Uddin et al. 2012).

As an attempt to understand the reproductive effort and the influence of the environmental condition such as temperature on the gonadal development of *Tresus keenae* distribute in Yeosu South coast of Korea, histology and immunological methods were used during this study. *Tresus keenae* is a clam with shell size of aproximatly 168.4 mm and total wet weight about 764.7 g (Fig. 2a, b) and can burrow in to as deep as 50-60 feet in to the sub-tidal soft bottom areas on the south coast of Korea (Kang and Kim 2018). They are belonged to family Mactridae and order Veneridae and distribute in North west Pacific ocean including Korea, Japan and China. In Korea, *Tresus keenae* landing decreased from 85,182 kg to 32,272 kg since 1999 to 2004 (Kim, et al., 2006). Because of the overfishing and habitat destruction of *T. keenae*, there is an obvious potential to decline the population or completely diminish the population. Switch to the aquaculture of *T. keenae* may help to alleviate the pressure on the wild *T. keenae* population.

The abundant reliable low cost seed is essential requiremnty for the sustainable bivalve cultivation activity. Currently most of the bivalve farmers collect seeds from natural beds by placing the seed collecting materilas. Hatchery breed bivalve seeds are the one of the best alternative sourse for the seeds that collected from the wild. A sound knowledge about the reproductive biology incluiding the reproductive cycle and reproductive effort of bivalves is paramount in order to optimize the performance of the hatchery (N Olivares-Bañuelos, 2018). For the countinous seed production in the hatchery, understanding of the gonadal development of the bivalve is vital for the stripping of the eggs and sperms as well as for the induce spawning (Solon, 1984). Thus understanding the reproductive biology of *T. keenae* is paramount while few studies have reported their reproductive biology (Kim et al. 1999; Kang and Kim 2018; Kim et al. 2005).

In bivalves, biochemical composition reflects the energy storage and utilization that closely related to the reproductive cycle (Barber and Blake 1981; Lee et al. 2015). Gametogenesis requires high input of energy and in general this energy requirement fulfill by the exogenous food supply, endogenous energy storage or combination of both (Vite-García and Saucedo 2008). Generally Bivalves stored the energy that derived from the food in different body parts prior to gametogenesis and subsequently utilized stored energy during the gametogenesis (Barber and Blake 1981). Chantler (2006) reported that the scallops entirely utilize the energy that stored in the adductor muscle for the reproduction while most of the other bivalves take-up energy from the muscle, mantle tissue, digestive gland and other cellular structures (Mathieu and Lubet 1993). The energy source, the place that energy store, the time that energy utilize in related to the reproductive cycle vary between the inter species and intra species (Bayne 1976; Sastry 1979).

During this study, polyclonal antibody that specific for the egg protein of *T. keenae* was developed and using the developed antibody, quantitative aspect of the reproduction of *T. keenae* was studied. In order to understand the energy storage and utilization for the gametogenesis and spawning, biochemical compositions of the adductor muscle, siphon and body of the clam was determined.

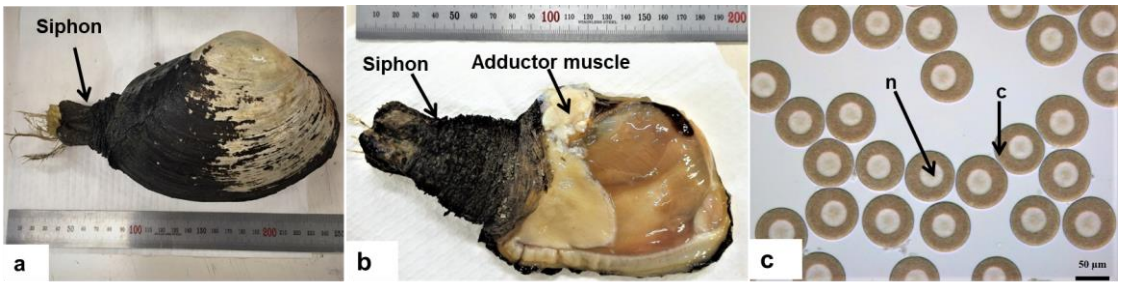

Figure 1: Photograph of *T. keenae*. a - adult live *T. keeane*, b - shucked *T.keenae* showing large siphon, c- Photomicrograph of purified *T. keenae* matured eggs. Ooplasm (c), nucleus (n)

2. Materials and methods

2.1 Study area and Sampling

Yeosu (34° 45' 46.01" N and 127° 39' 55.01" E) is situated in Jeollanam-do, south coast of Korean peninsula. The sampling site (Fig. 2) in Yeosu was located in the East China Sea while the annual sea temperature vary between 15.6℃ and the annual amplitude is 9℃ (Seong et al., 2014). *T. keenae* samples were collected by SCUBA on a monthly basis from October 2016 to September 2017. On arrival at the laboratory, shell length (SL, longest axis of the shell) and wet tissue weight of individual clams were recorded. Total 336 of clams with average shell length range between 115.4±8.1 and 139.5±10.0 mm and average tissue wet weight vary from 110.7±42.3 to 213.1±48.9 g were used for the analysis as summarized in the table 1.

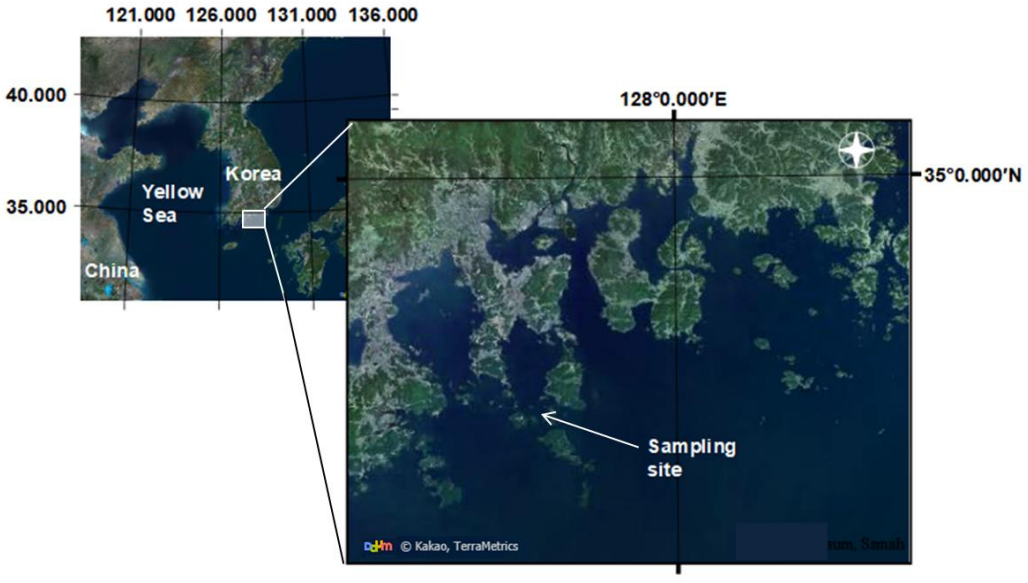

Figure 2: Location of sampling site of *T. keenae*, Yeosu, south coast of Korea

Table1: Number of clams (N) and their monthly mean shell length in mm (SL) and mean total wet weight in g (TWW)

Year	Month	N	SL (mm) ± SD	TWW(g) ±
2016	**O**	30	132.0 ± 11.0	146.98 ± 33.26
	N	16	139.5 ± 10.0	173.42 ± 41.20
	D	30	126.9 ± 15.5	110.72 ± 42.30
2017	**J**	25	138.7 ± 7.8	172.60 ± 37.26
	F	30	127.5 ± 8.8	136.19 ± 29.54
	M	30	137.4 ± 9.5	213.07 ± 48.95
	A	30	132.9 ± 12.2	195.65 ± 36.29
	M	30	127.8 ± 10.7	173.39 ± 33.06
	J	25	131.3 ± 6.3	207.93 ± 21.64
	J	30	127.9 ± 16.2	172.32 ± 60.00
	A	30	121.6 ± 24.6	135.45 ± 37.15
	S	30	115.4 ± 8.1	125.48 ± 29.59

2.2 Histology

To understand the reproductive cycle of *T. keenae* histologically, a thin section in the middle of each clam was cut and fixed with Davidson's solution. Remaining tissues were lyophilized and stored at –70℃ for further use. The fixed tissues were dehydrated with ascending series of ethanol as 70, 80, 90, 95 and 100% and embedded in paraffin. The paraffin embedded tissues were cut into 4 µm sections using microtome (Leica Rm 2125RT) and stained with Harris hematoxylin and counter stained with eosin Y. Finally, gonadal tissues were examined under light microscope. Reproductive stages of female and male were categorized into six stages as described in table 2 based on the appearance of the oocytes and

sperms in the gonads as resting, early developing, late developing, ripe, spawning and spent stages. Lyophilized clam tissues and dried shell were weighed and condition index (CI) was calculated as the ratio between the tissue dry weight and shell dry weight.

Table 2: Explanation of the different gametogenic stage

Reproductive stage	**Discription**
Resting	Gametes are absence, empty follicles are shrinked
Early developing	Few small follicles and gametes are clearly visible
Late developing	Follicles size increase and occupy the entire gonadal tissue. Gametes are large
Ripe	Follicles are almost full with ripe gametes. Follicles occupy entire gonadal tissue.
Spawning	Free ripe gametes are in the empty spaces. Follicle wall ruptured.
Spent	Degrading residual gametes are inside the shrinking partially empty follicles.

2.3 Purification of *T. keenae* eggs and development of the egg specific antibody

From the shucked clams sexually mature female clam was selected and gonadal tissue containing mature eggs were dissected and gently squeezed to release the eggs from the connective tissues and diluted with phosphate buffered saline (PBS, pH 7.0, 0.15M NaCl). The egg extract was filtered through 100 µm and 80 µm mesh screens to remove the tissue debris and washed with PBS several times. This cleaning procedure was repeated several times until pure eggs could be obtained. Fig1.c illustrates the photomicrograph of the purified matured egg of *T. keenae*. These purified eggs were stored at –70℃ until further use. To estimate the weight of a single egg, a known volume of purified eggs were taken and counted using hemocytometer. At the same time diameter of the mature eggs were measured by the image analyzing software (Image J, National Institute of Health, USA). Mean diameter of an egg is 76.17± 9.34 µm. Then eggs were lyophilized and weighed in order to determine the dry weight of an egg. According to that the average dry weight of a matured egg was 36ng. The purified *T. keenae* eggs were used to immunize the New Zealand White rabbit to develop the antiserum with egg specific antibody by following the method described by Park and Choi (2004).

2.4 SDS-PAGE and western blotting assay

To determine the egg specificity of the antibody, protein concentration of egg was measured using BCA Protein Assay Kit (PIERCE, USA) using bovine serum albiumin as the standard. Purified egg as positive control, female tissue homogenate, and male tissue homogenate as negative control were treated with 5% [beta]-mercaptoethanol (under reducing condition). Mercaptoethanol treated samples loaded into gradient 10% SDS-polyacrylamide gel with a protein marker (Precision Plus Protein Standards; Bio-Rad, Hercules, CA) and electrophoresed (SDS-PAGE).

For the Western blot assay, protein bands, which obtained from SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane at 30 V for 70 min using a Mini Trans- Blot Transfer Cell (ATTA EA 6675). The membrane was blocked in 5% skim milk in TBS-T (Tris-buffered saline containing 0.05 % Tween 20) and thereafter membrane was incubated with the 1/1000 diluted rabbit anti *T. keenae* egg IgG for one hour. After incubation, Membrane was washed with TBST and reacted with goat anti-rabbit IgG horseradish peroxidase (1:5000 dilutions) as the secondary antibody for one hour at room temperature. Finally membrane was repetedly washed with TBST and immune-reactive bands were visualized using BCIP/NBT premixed staining solution.

Western blotting results indicated that *T. keenae* egg specific antibody exhibited a weak but recognizable antigen-antibody reaction with the non-gonadal tissue protein. To eliminate those cross reacting antibody from the rabbit antiserum, the method that described by Fuchs and Sela (1973) was used with modifications. Glutaric dialdehyde treated male *T. keenae* tissue homogenate was used as an immunosorbent. Antiserum and immunosorbent

were incubated (1:1 v/v) for three hours at room temperature. Nonspecific antibodies were bound to the immunosorbent while the egg specific antibody remained unbound during incubation. Thereafter egg specific antibodies were separated by centrifugation. The egg specificity of the antibody was again tested using Western blotting assay.

2.5 Indirect ELISA and Immunofluorescence assay

To determine the sensitivity of the antibody methods described by Park and Choi (2004) and Jeung et al. (2014) was used. The purified egg sample (egg protein 40.98 μg/ml) was serially diluted on to a 96 well plate as the positive controls while PBS solution was used as the negative control and samples were incubated overnight. Threafter plate was washed with PBST (phosphate buffered saline containing 0.05% of Triton x-100) using ELISA microplate washer (Aquamax 2000). *T. keenae* egg IgG as the primary antibody was added to each well and incubated at the room temperature for one hour. Afterwards, plate was again washed with PBST and incubated with alkaline phosphate conjugate goat anti-rabbit IgG as the secondary antibody for one hour in room temperature. 4-nitrophenyl phosphate disodium salt hexahydrate, the color developing substrate (1:1 w/v dissolved in 0.1M Glysine buffer) was added to each well and incubated in a dark room for 20 minutes and finally the optical density was read at 405 nm using spectrophotometer (Versa max).

The anti-*T. keenae* egg IgG and *T. keenae* egg protein interaction was visualized by immunofluorescence assay using the method described by Kang et al. (2003). The paraffin embedded sexually mature female and male tissues were sectioned in to 6μm sections, deparaffinized using xylene and rehydrated using decending series of ethanol as 100%, 95%,

70%, 50% and distilled water. Tissue sections were blocked with 5% (w/v) BSA in PBST. Consequently, the tissue sections were incubated with the rabbit anti clam egg IgG (1:1000 dilutions) for one hour. The succeeding washing of the tissue was done using PBS and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:400 dilutions). Finally, antibody-antigen reactions of the female and male *T. keenae* tissues were examined under a fluorescence microscope.

2.6 Quantification of *T. keenae* eggs using indirect ELISA

Indirect ELISA was used for the quantification of reproductive effort according to the methods reported by Kang et al. (2003) and Park and Choi (2004). 100 µl aliquot of female clam homogenate to be analyzed and male homogenate as negative control were added to the 96 well- microplate. *T. keenae* egg IgG as the primary antibody and Goat anti rabbit IgG alkaline phosphate conjugate as the secondary antibody were used along with the nitrophenylphosphate (ρ-NPP) substrate. Finally, the optical density of each well in the plate was measured at 405 nm using a microplate reader. The quantity of egg protein in the *T. keenae* tissue homogenate was calculated from the standard regression curve plotted from the optical density and concentration of purified egg protein in the plate.

The quantity of the eggs in an individual clam was determined by multiplying the quantity of egg protein measured using ELISA by 2.44, as the protein accounts for 40.98% of the egg weight. The gonadosomatic index (GSI), the ratio of the estimated total dry weight of the eggs to the total dry weight of the clam tissue (GSI^1) was determined to investigate the seasonal variation of *T. keenae* reproduction. *T. keenae* is a large clam and dry weight of

siphon approximately account for the 25% of the total dry weight as the siphon is large in this species (Fig. 1b and Fig.4). Thus GSI was recalculated by deducting the dry weight of siphon from the total tissue dry weight of the clam (GSI^2).

For the determination of fecundity of *T. keenae*, ready to spawn female clams were selected based on the histological observation and select the female individuals with the highest GSI after determine the GSI from the ELISA. Potential fecundity, the number of mature eggs produce by a ready to spawn female was determined by dividing the estimated total weight of the eggs estimated from the ELISA by the weight of an average dry weight of the mature egg (36ng). Total 35 female clams collected in October, November and March were selected rely on histology and GSI, included in the analysis.

2.7 Tissue biochemical analysis

The adductor muscle and siphon were separated from the lyophilized tissues and weighed. The adductor muscle, siphon and rest of the body that contains visceral mass, foot and mantle were grounded separately. In order to understand the energy storage and utilization, the amount of total protein level in the siphon, adductor muscle and body was determined by the method described by Lowry et al. (1951) using BCA protein assay kit and bovine serum albumin as the standard. Total carbohydrate level was also tested for the adductor muscle, siphon and the body by the phenol-sulphuric method using glucose as standard (DuBois et al. 1956). In concisely 100mg of powdered lyophilized tissue were diluted with 15% of tricholoroacetic acid (TCA) and incubated for one hour in 4℃. The incubated samples were centrifuged and supernatant was reacted with 10% phenol and 95%

sulphuric acid. Finally absorbance of the samples were read at 490 nm using microplate reader.

3.0 Results

3.1 Annual gametogenesis

Figure 3 and 4 illustrates the temporal variation in the gonadal development stages of clams that observed from the histological preparation during the study period. *T. keenae* reproductive cycle has a distinct seasonality. Ready to spawn, sexually matured female clams recorded in October 2016 and March 2017 while sexually matured male recorded in October, November 2016 and March 2017 as 83%, 14% and 10% respectively (fig. 5). Although ready to spawned ripe female observed in March spawning female clams were common in October (72%) and November (40%) during 2016. However, spawning male clam was common in October (17%), November (86%), January (14%), February (11%) and March (40%).

All of the clams collected in December were in spent stage. Resting stage of female also observed in January, February, April and May while male only in January. It was difficult to differentiate the male and female gonads in this stage. In few samples, degraded gametes were still visible. The first early developing stage female and male clam observed in March 2017. Early development stage clams were common from March to September although 38% of female and 82% of male were in late development period in September suggesting that *T. keenae* has the long early development stage.

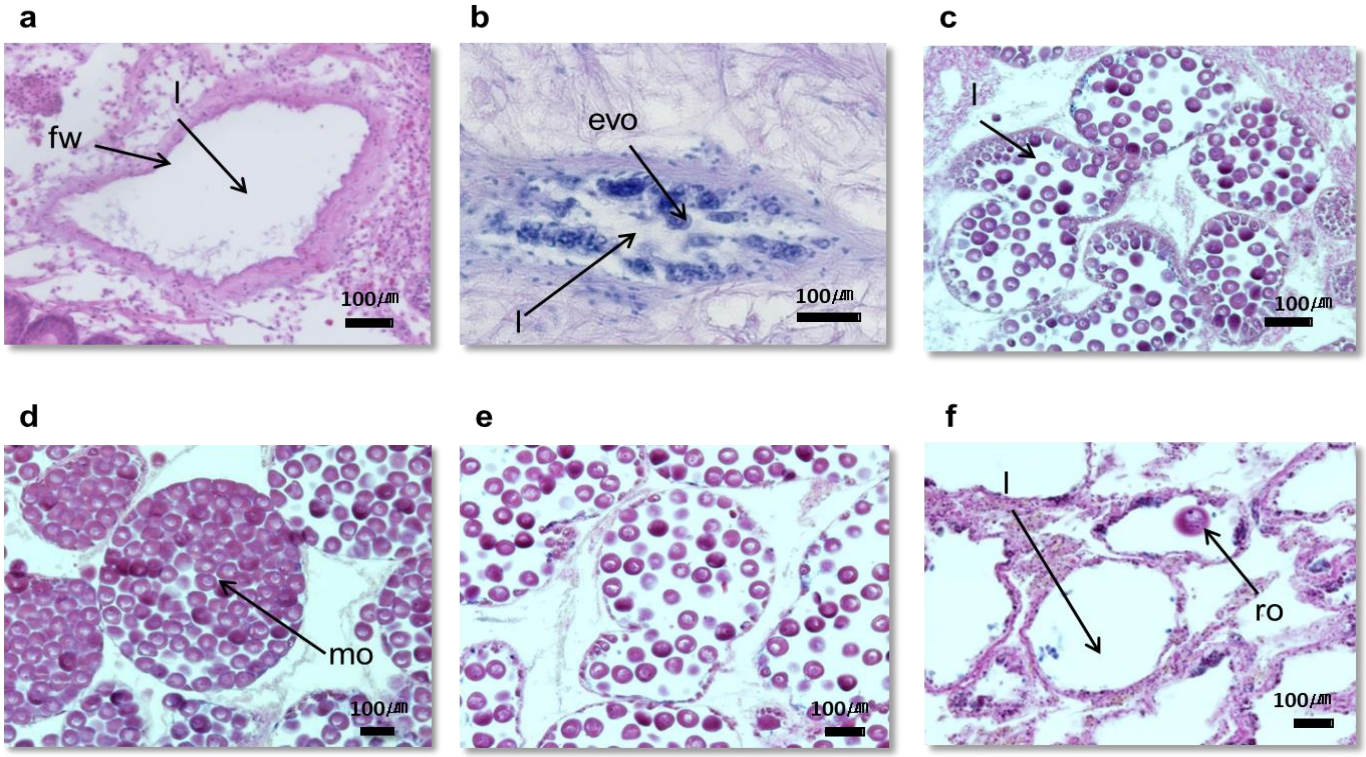

Figure 3 : Microphotograph of cross section of gonads of female *T. keenae* showing six different stages; a- resting stage; b- early developing stage; c- late developing stage; d- ripe stage; e- spawning stage; f- spent stage; fw- follicle wall; l- lumen; evo- early vitellogenic oocyte; mo- mature oocyte; ro- relict oocyte.

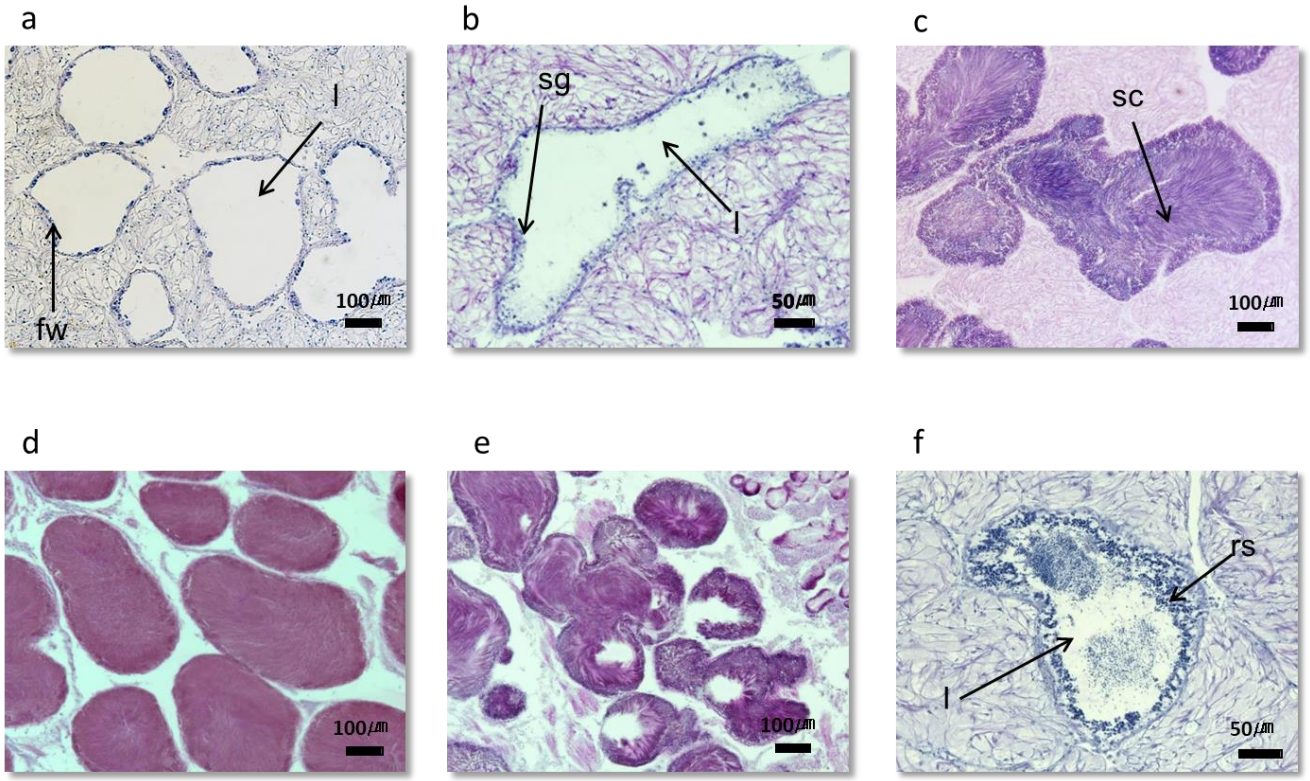

Figure 4: Microphotograph of cross section of gonads of male *T. keenae* showing six different stages; a- resting stage; b- early developing stage; c- late developing stage; d- ripe stage; e- spawning stage; f- spent stage; fw- follicle wall; l- lumen; sg- spermatogonia; sc- spermatocytes; rs- relict spermatozoa

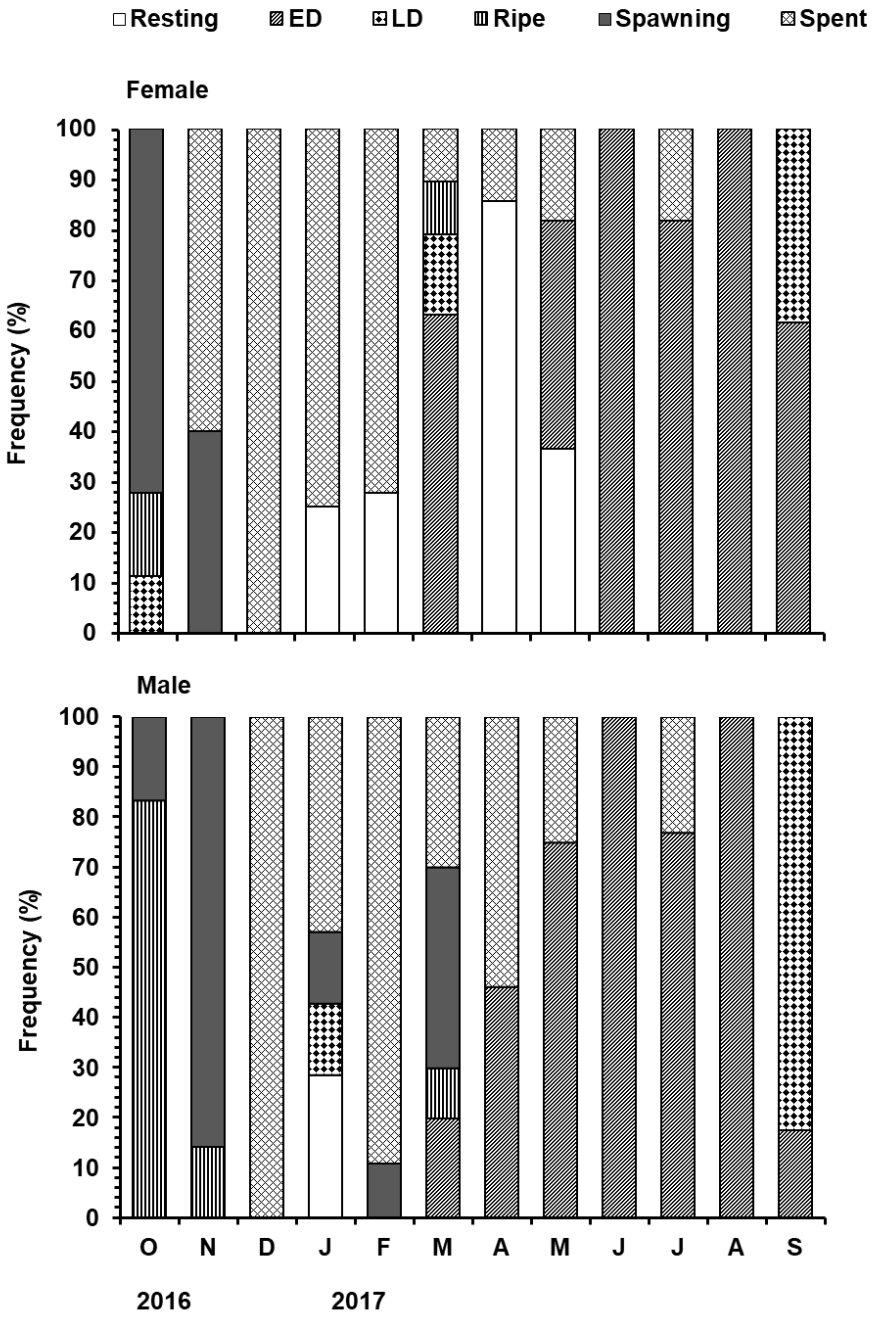

Figure 5: Seasonal variation of gametogenic stages of female and male *T. keenae* in Yeosu. Early development stage (ED), late development stage (LD)

3.2 Weight of siphon and adductor muscle

Figure 6 illustrates the percentage dry weight of siphon, adductor muscle and body. The weight reduction of the siphon shows a clear seasonality with the reproductive cycle of *T. keenae* while the prominent weight reduction was not observed in the adductor muscle. However dry weight of the siphon employs considerable weight from the total body weight than the adductor muscle. Peak percentage of siphon observed in November when lowest percentage of body weight observed. After spawning Observed in November, weight of the siphon continuously scaled down until June 2017. The minimum value of percentage dry weight of siphon (19.7%) and adductor (4.6%) recorded in June 2017 during the early development period of the gametes while percentage body weight recorded its maximum value (75.7%). Thereafter weight of the siphon and adductor started to upturn and the peak percentage of adductor muscle (5.7%) was observed in August.

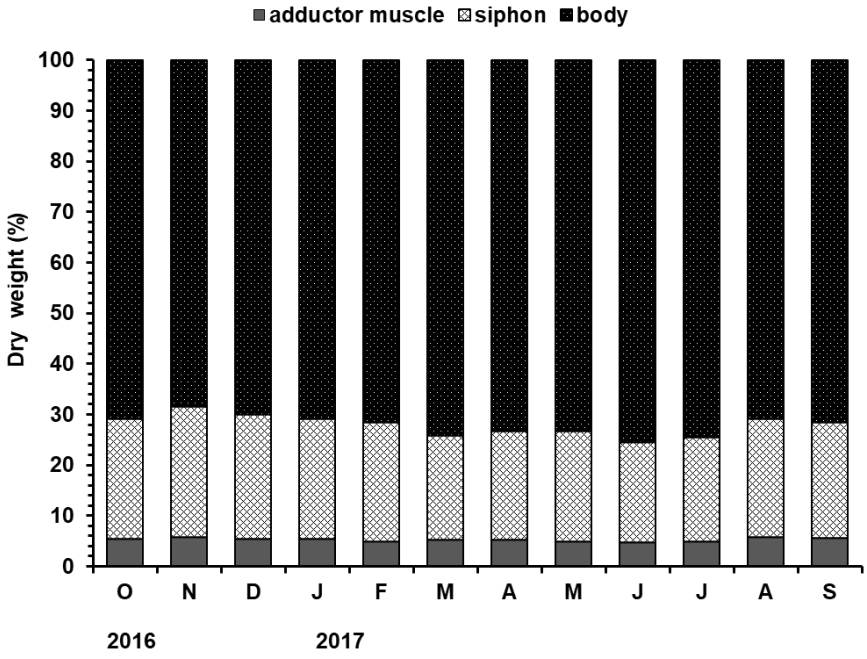

Figure 6: Temporal variations of percentage dry weight of adductor muscle, siphon and body

3.3 Biochemical composition of adductor muscle and siphon

Figure 7 shows the seasonal variation of protein of the siphon, adductor muscle and body of the Clams. Protein content of the all three parts was not followed by the reproductive cycle clearly. The highest amount of the protein (569.8 mg/g) was recorded in the siphon regardless of the seasons. Although sudden reduction of protein in siphon and adductor muscle was observed in November while most of the clam spawn. The lowest protein content in siphon (378.1 mg/g) observed in April while adductor muscle (316.4 mg/g) and body (186.6 mg/g) was recorded in May.

In contrast, total carbohydrate level in siphon, adductor muscle and the body was exhibited seasonality (Fig. 7). Spawning which occurred from October to November, caused a

considerable decline in the carbohydrate level in all three parts and reached to minimum in December. Carbohydrate content of the all three parts was rapidly increased from February and this was a common feature of all. The highest carbohydrate content was observed in body tissues than the adductor muscle and siphon. Carbohydrate content of the body was reached to its peak in March (279.1 ± 51.1 mg/g) while maximum carbohydrate level of siphon (153.9 ± 26.7) and adductor muscle (194.3 ± 49.6) was observed in April. However after April total carbohydrate in all three parts was started to decline.

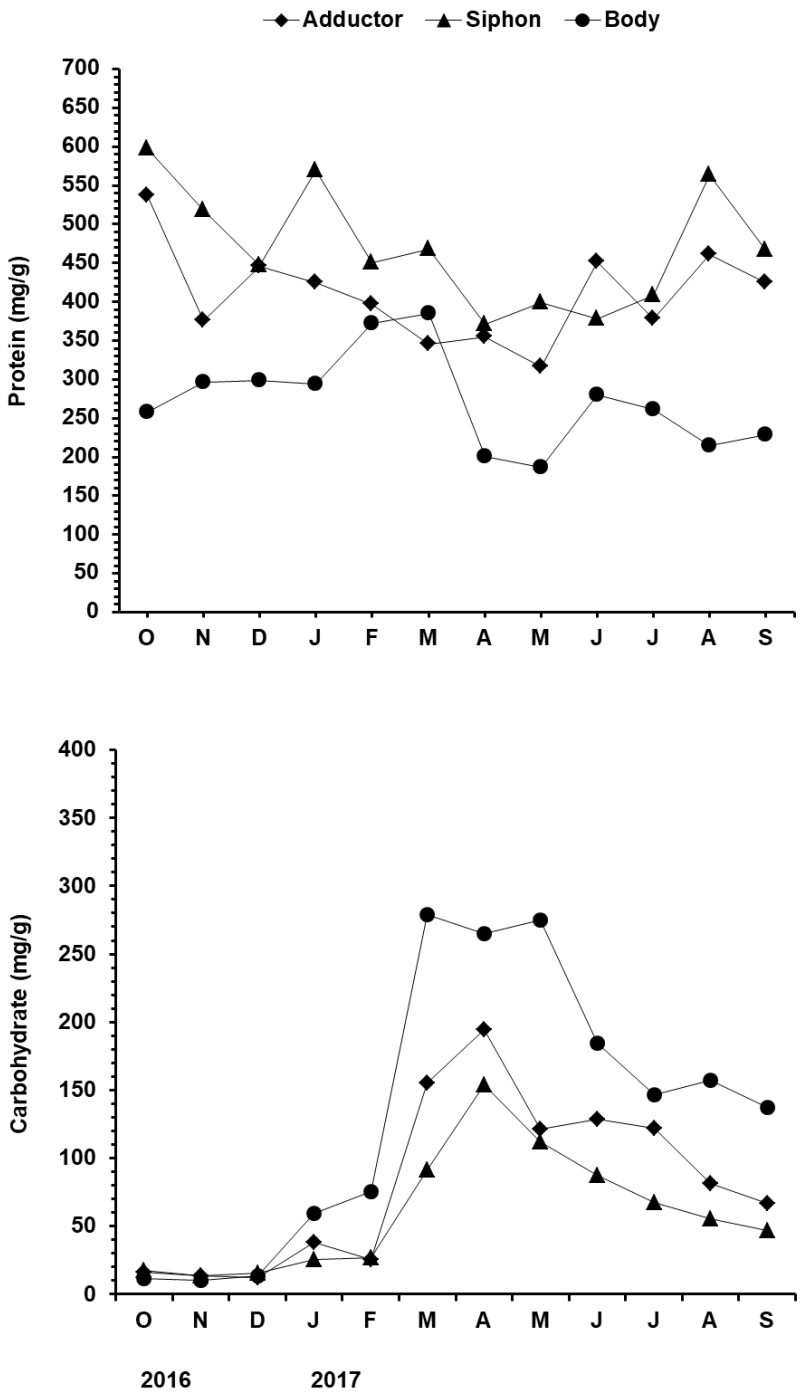

Figure 7: Monthly changes of the total protein and carbohydrate in the adductor muscle siphon and body

3.4 Specificity of the antibody

The antibody that developed from the purified *T. keenae* eggs initially illustrated strong binding to the *T. keeane* egg proteins while week but recognizable binding to the non-gonadal tissues protein in the western blotting assay (Fig. 8a). After the cross reacting antibody was successfully removed from the antiserum using immunoadsorbent assay, a strong antibody antigen reaction was observed between clam egg specific antibody and egg protein while no recognizable reaction was observed with male tissue homogenate and female somatic tissues (Fig. 8b) Western Blotting showed that the rabbit anti IgG recognized several peptides of egg protein with molecular weights of 244, 175, 109, 98, 62, 47, 38 kDa.

According to the immunofluorescence assay, highly specific interaction was observed between the egg protein and antibody. Oocytes were stained with fluorescent staining while gonadal and non- gonadal tissues of female, male and nucleus of oocytes was not stained with fluorescent staining (Fig. 9). The fluorescent staining was visible within the ooplasm only and this is suggesting that egg proteins which recognized by the antibodies are presented inside the ooplasm.

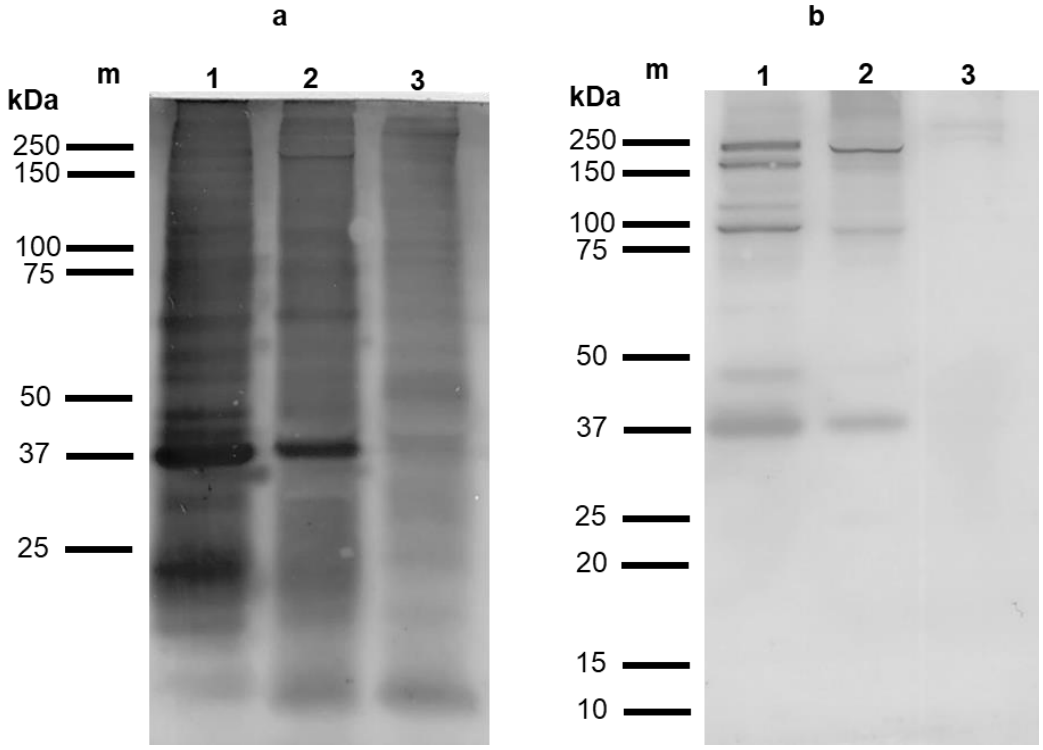

Figure 8: Specificity test for the rabbit antiserum by Western blot assay a: assay with the serum before immunosorbent assay. b- assay for the serum after immunorsorbent assay, m- marker, 1- purified *T. keenae* eggs, 2- female *T. keenae* tissue homogenate, 3- male *T.*

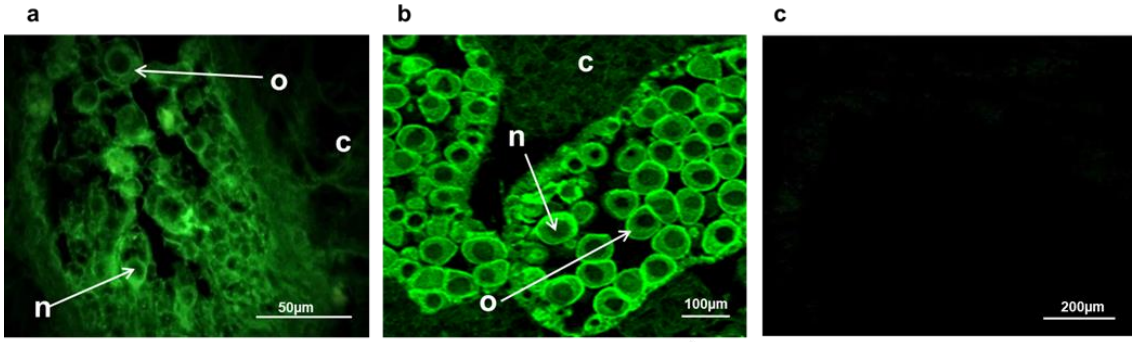

Figure 9: Fluorescence staining patterns of immune-stained histological sections of gonads of a- early developing stage of female. b- sexually matured female. c sexually matured male as negative control. s- somatic tissues, n- nucleus of oocyte, o- ooplasm of oocyte

3.5 GSI estimated from ELISA

T. keenae egg specific antibody is sensitive to quantify the eggs produced during the spawning season as the *T. keenae* egg IgG detected 0.64-10.24 µg/ml of the egg protein (Fig. 10). GSI is a ratio between the egg mass and total tissue dry weight, since siphon weight represents approximately 25% of total dry weight, we calculated GSI in two different ways as GSI^1 and GSI^2.

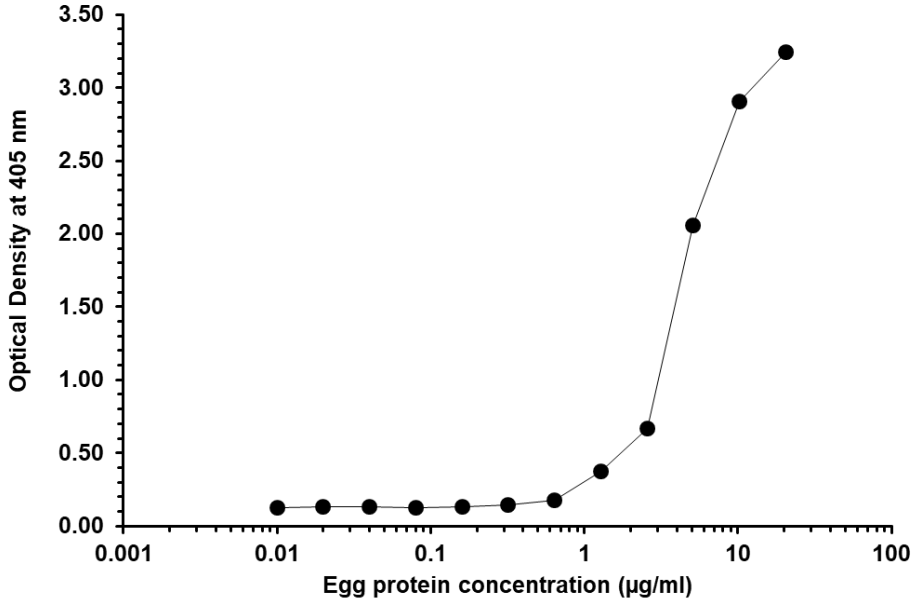

Figure 10: Titration curve of the indirect ELISA for *T. keenae* egg proteins using the rabbit anti *T. keenae* egg protein

GSI[1], the ratio between the egg mas and total tissue dry weight, was reached to peak in October (5.7) while sudden noticeable drop was observed in November indicates that the most of clams spawned during this period (Fig. 11). The decrease in GSI was continued until February. Resting stage of female clams was observed during December, January and February and this may be the reason for higher GSI as 1.6, 0.9, and 1.2 respectively. During the March 15% of late development and (11%) sexually matured female clams were observed and GSI recorded during the March is high as 2.4. After March GSI was again started to increase and peaked in May (3.6) and drop gradually. According to GSI[2] that calculated as the ratio of egg weight to tissue dry weight by excluding the siphon dry weight also follow the same pattern, however conspicuous three major peaks of GSI[2] can identify in October, March, June and July while all peaks are approximately similar as (8.6, 8.1, 8.8 and 8.1 respectively).

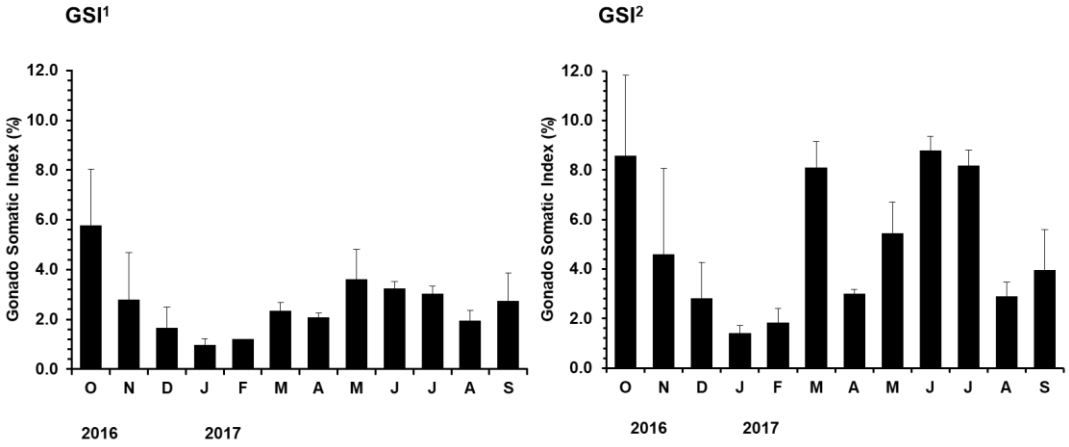

Figure 11: Temporal variations in the mean Gonadosomatic Index (GSI) of *T. keenae* GSI[1] ratio of the egg mass to total tissue dry weight, GSI[2] ratio of the egg mass to total tissue dry weight excluding the dry weight of siphon

3.6 Potential fecundity estimated from ELISA

According to the histology ready to spawn female clams were recorded during October 2016 and March 2017. The potential fecundity of female just before the spawning was ranged between 121.92×10^6-22.07×10^6 with the average of 53.40×10^6 of eggs per clam while it was ranged from 69.19×10^6 to 29.07×10^6 (average 44.91×10^6) of eggs per clam in March (Table 3 and 4) and this was lower than October. Although females are in early development phase during May according to histology, GSI^1 was high as 3.6 and fecundity was ranged from 76.16×10^6 to 15.10×10^6 with the average of 39.43×10^6. However recorded GSI^2 high as 8.8 and 8.1 in June and July, average fecundity was determined and it was 44.44×10^6 and 44.73×10^6 respectively.

3.7 Condition Index (CI)

Seasonal fluctuation of the CI is shown in Fig. 12. The lowest CI observed in November (16.5 ± 1.9) and this decline coincided with the mass spawning of the clams during November. CI began to increase from December to April and sudden drop of the CI observed in May. However thereafter CI reached to its peak in June (40.4 ± 5.0) and began to decline by following the early development stage of the gametes.

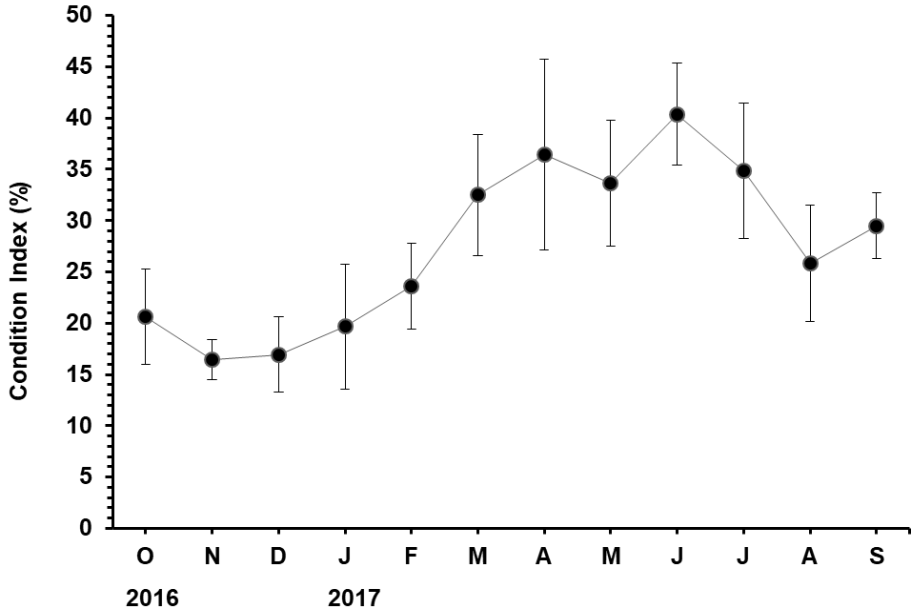

Figure 12: Monthly changes in the Condition Index (CI)

4.0 Discussion

4.1 Reproductive cycle of *T. keenae* in Yeosu

Reproductive strategies such as gametogenesis and spawning of the bivalves are influenced by the environmental factors such as temperature, salinity and food availability as well as the endogenous factors (Ahn et al. 2003).The spawning and gametogenesis periods of *T. keenae* in Yeosu were identified in this study using histology, Changes in the CI and the GSI. Since *T. keenae* is a large clam and as in Fig. 1, dry weight of the siphon is inversely proportionate to the dry weight of the body. Therefore two types of GSI was calculated as the ratio of egg mass to total tissue dry weight (GSI^1) and tissue dry weight excluding the siphon dry weight (GSI^2). In both cases peak GSI recorded at the time of October suggests that presence of ready to spawn clams and massive discharge of gametes appeared to be causing the sudden drop of the GSI and CI from October to November 2016 suggests that the spawning of the *T. keenae* occurred in this period. This peak spawning was also identified by histology. However GSI^2 clearly shows another two prominent peaks in March and July and June, that are highly noticeable than GSI^1. Histology also provides evidence of presence of ripe female and male as well as spawning male in March, suggests that *T. keenae* in Yeosu, spawn in March 2017 after October and November.

Although GSI^2 indicated peak value in June and July histology doesn't provide any clue of presence of ripe or spawning female during this period. As observed in histology 100% of female were in early development period in June while 82% and 18% were in early development stage and spent stage respectively during July. This highest recorded GSI^2 may be due to presence of vitellin in the early developing eggs as antibody was developed against

the vitellin, presence in the matured eggs.

Immunofluorescence images of different reproductive stages of female *T. keenae* also revealed that the vitellin like egg proteins present in the early developing eggs (Fig. 9a) and this observation suggest that vitellin synthesis initiated at the time of early development of the eggs. Similar observation in *Mercenaria mercinaria* was recorded by Eversole (2001). According to that Vitellogenesis of *Mercenaria mercenaria* was initiated in the time of the early development stage and progress with the development of the oocytes.

However in August also 100% female were in early developing stage but in this case GSI^2 was not increased as June and July. The reason behind this is unclear. Sometime recorded high GSI values may be associated with the presence of mature eggs in female and histology didn't give any clue about this.

In contrast, Kim et al. (1999) also observed two spawning pulses of *T. keenae* in Jangmok, South coast of Korea from April to June and September to November while first early development clam observed in December and second gametogenesis observed during July in the same reproductive cycle. The first early developing stage of *T. keenae* was observed in March during our study. This inconsistency in spawning and gametogenesis period may be due to the spatiotemporal variations in temperature, salinity and food availability in the water. Unfortunately environmental parameters such as sea water temperature, salinity and Food availability through the chlorophyll a concentration was not measured during this study. (Lee et al. 2009) reported that the average annual sea surface temperature is 16.4℃ in November and 6.8℃ in February while average annual salinity of the water is 31.27 psu during November and 31.81 psu in February in Yeosu. According to Mondol et al. (2016) chlorophyll a concentration of the water of Yeosu during October 2009 was 1.6µg/L and 4.06µg/L in March while during the initiation of the gametogenesis, water

temperature was 10℃ and chlorophyll a concentration started to increase from 4.0μg/L. Slight changes of temperature, salinity and food availability can alter the spawning and gametogenesis of bivalves (Herrmann et al. 2009). Furthermore endogenous factors like parasite infection can alter the duration of spawning and gametogenesis. Uddin et al. (2010) and Park et al. (2006) reported that parasite infection affect to *Ruditapes philippinarum* reproduction by changing the duration of gametogenesis and spawning. However during this study parasite infection in *T. keenae* was not identified histologically.

Although *T. keenae* spawning during the late autumn and winter period, many studies have shown that the spawning of the clams in temperate areas limited to late spring or summer as the environmental conditions favorable for the eggs and larvae (Ponurovsky and Yakovlen 1992) while the clam species that live in the tropical and subtropical areas often spawn year- round (Shpigel and Fridman 1990).

4.2 Energy storage and utilization related to the reproductive cycle

The prominent weight reduction in siphon after November and during March suggests that the energy stored in the siphon used for the recovery of the gonads after spawning as well as for the winter metabolism. Ahn et al. (2003) and Pazos et al. (1997) also reported that the clams and scallops utilize energy from different body parts after spawning and during winter period for the recovery of the gonads and winter metabolism.

However during June the lowest dry weight of the siphon was recorded. Unfortunately water temperature and food availability through the primary production data

were not available during this period. But as stated by Mondol et al. (2016) water temperature and chlorophyll a concentration in Yeosu 2009 increased during this season. However during this study period weight of the siphon was not increased and the reason behind this continuous weight reduction is unclear because in generally most of the bivalves accumulate the energy during the growing season after the spawning (Ahn et al. 2003).

Although according to histology in June and July female in early developing stage and spent stage, GSI^2 peaked in June and July suggests that those clams have high gonadal weight compare to the body weight. The conspicuous scale down of the siphon weight may be associated with the energy supply from the siphon for the gametogenesis. According to histology gametogenesis start from March and continue until September while a noticeable carbohydrate reduction also observed after April in siphon and carbohydrate that stored in the siphon may be exploited for the gametogenesis of *T. keenae*. In contrast Most of the studies revealed that bivalves are uptake energy from the adductor muscle for the gametogenesis. Vite-García and Saucedo (2008) reported that the energy requirement for the gametogenesis of winged pearl oyster supplied by the carbohydrate stored in the adductor muscle. However after July weight of the siphon start to increase. Bayne (1976) reported that sometimes gametogenesis obtains energy from the stored energy sources or directly from the food or sometime temporally overlaps those two cause to decline the energy sources in the tissues. At the time of the initiation of gametogenesis energy demand was supplied by the stored energy and this may be the reason of the decline of the weight of the siphon. Thereafter energy that derived from the food start to accumulate in the siphon and resulting weight gain can observe in the siphon. Although weight of the siphon decline during the gametogenesis period weight of the body increased and this may be the result of combine effect of active feeding and accumulation of gametes inside the body.

The conspicuous low level of carbohydrate observed during October to February in all three parts, suggests that the carbohydrate served as the energy source for the spawning as well as winter metabolism because external food supplies during the spawning and post spawning period may be limited and couldn't meet the energy demand of *T. keenae*. Under this condition, stored carbohydrate liberated for the metabolic process. Although GSI and histology suggested that the female *T. keenae* was ready to spawn and male clams are spawning in March carbohydrate reduction was not observed. Sometime reason behind this may be, at the time water temperature increased and food availability also increased cause to actively feed on the available food and clams utilized the energy that directly derived from the food rather than the stored energy. Ahn et al. (2003) and Gabbott (1983) reported that the carbohydrate is the main energy source of the marine molluscs and is varying widely as a function of environment and physical conditions.

Sudden drop of adductor muscle and siphon protein in November may be associated with the contribution of protein for the energetic maintenance in the periods of lack of carbohydrate level. Berthelin et al. (2000) also reported that the similar observation in *Crassostrea gigas*. Massive discharge of eggs, with 40.98% of protein from its weight that accumulated in the body may be the reason behind the noticeable protein reduction in the body after March. However after November this kind of conspicuous drop down of body protein was not observed and the reason behind this is unclear.

However after February carbohydrate content rapidly increased and this is a common feature of all body parts. The high level of carbohydrate indicating that active feeding during the nutrient rich period and accumulation of carbohydrate in the clams as observed by Barber and Blake (1981); Serdar and Lök (2009); Matias et al. (2013). Although weight of the siphon upturns after July, carbohydrate content in the siphon, adductor muscle

and body decline during the gametogenesis and the maturation period of the gametes. Barber and Blake (1981) also reported that glycogen stored in the adductor muscle of scallop, *Argopecten irradians concentricus* utilized glycogen to synthesize lipid during the development of the gametes.

CI reflects the changes in the body composition and energy reserved (Norkko et al. 2005) as well as the food availability and nutritional status of the bivalves (Orban et al. 2004). Although the reason for the sudden drops of CI during May and August unclear and this may be probably associated with the environmental condition of the water and decline of protein and carbohydrate in adductor muscle and siphon suggest that energy requirement during this period supplied by the carbohydrate and protein that stored in adductor muscle, siphon and body.

4.3 Specificity of the rabbit anti *T. keenae* egg IgG

Initially rabbit antiserum exhibited the immune-reactivity with female and male somatic tissue (Fig. 8a) and this result suggest that the common protein for the somatic tissues and eggs presents in the eggs. However immunosorbent that prepared from male *T. keenae* tissues helps to bind the antibody that specific for the somatic tissue proteins and separate the antibody that only specific for the eggs. Western blotting and immunofluorescence assay demonstrated that the antibodies show the immune-reactivity with the non-gonadal tissues proteins were successfully removed from the antiserum (Fig. 8b, Fig. 9). *T. keenae* egg protein consisted of seven bands with the molecular weight of 244, 175, 109, 98, 62, 47 and 38 kDa and these proteins are vitellin which is the main protein of the invertebrate eggs and mainly presents inside the ooplasm of the eggs (Shafir et al. 1992). Immunofluorescence

assay clearly suggest that antibody and antigen reaction only limited to the ooplasm (Fig. 9b). *T. keenae* egg specific antibody is sensitive to detect as 0.64 µg/ml egg protein present in the mixture of the somatic tissue proteins (Fig. 10).

4.4 Reproductive effort

Highest monthly mean GSI^1 (5.8±2.2) was observed in October suggests that female *T. keenae* in Yeosu heap together 5.7% of their body weight in eggs prior to spawning. In contrast, Park et al (2003) reported that *Saxidomus purpuratus* keeps 15.4% of eggs from their body weight as illustrated in table 3. *T. keenae* has relatively high body weight and this may be the reason for the comparatively low GSI recorded during the study since GSI is negatively proportionate to the body weight. The considerably large siphon of T. *keenae* occupy average dry weight as 25% from the dry weight of the body and this may be affected to the lowest GSI and to remove this effect GSI^2 was calculated and according to that female clams keep 8.6% of eggs from their body weight without siphon. Before the second spawning, female kept 2.4% of eggs from their body weight according to GSI^1 while 8.1% of eggs in the body as calculated by GSI^2.

As observed by the histology spent stage female clams were recorded throughout December, January and February cause to observe residual eggs and it is accounted for 1.7% to 1.0% of the total body weight of *T. keenae* according to GSI^1, while histology revealed that residual eggs were resorbed by the hemocytes that aggregated around the follicles. In contrary to this, Uddin et al. (2012) stated that *Ruditapus philippinarum* residual eggs account

approximately 5% of body weight of the clam and 3.6 ± 2.6% of residual eggs from the body weight observed in oyster, *Pinctada margaritifera* (Jeung et al. 2014).

Although gametogenesis commenced on March, comparatively high GSI^1 as 2.4 ± 0.3 and 8.1±1.0 was observed due to presents of 16% of late development stage and 11% of matured female in March. However thereafter GSI^1 peaked in May while GSI^2 recorded the maximum value in June and July. According to histology, there were no ready to spawn female clams in May, June and July only spent and early development stage of female clams were observed. This recorded high GSI may be related with the increasing the number and the size of the early developing eggs inside the follicles as observed in histology. *T. keenae* egg specific antibody was developed against the vitellin that presents inside the matured eggs. Relatively high GSI observed during the early development period is probably associated with the presence of low level of vitellin in the early developing eggs as observed in the immunofluorescence image (Fig. 9a) of early developing eggs. Park and Choi (2004) also reported that the similar type of observation during the early development stage of *Ruditapus philippinarum* eggs.

Potential fecundity which is defined as the number of eggs produced by a ready to spawn female clam was estimated by dividing the egg mass by the dry weight of a clam egg 36 ng. When determining the potential fecundity, we assumed that all eggs were with the same weight. The potential fecundity diversified from 121.92 to 22.07 with the average of 53.40 million of eggs per clam during October while it was ranged between 69.19×10^6 and 29.07×10^6 (average of 44.91×10^6) of eggs per clam. The potential fecundity of first spawning pulse is higher than second and therefore major spawning may be occurred in October 2016.

The potential fecundity observed during this study is higher than the fecundity values reported in previous studies (Table 3). Fecundity that estimated from ELISA is the

maximum fecundity as entire clam is homogenized and total gonadal protein of the clam measured. *T. keenae* is a large size clam (Table 1) and therefore their fecundity may be high as Hasegawa et al. (2014) reported. According to that the total number of eggs produced by an individual clam is a function of the body size of the clam. The other reason for the highest recorded fecundity may be, to ensure the survivorship of the larvae because *T. keenae* was spawned during late autumn and late winter as Honkoop et al. (1998) described winter temperature negatively correlated with the fecundity of clams. Dudas and Dower (2006) also revealed that high fecundity of the clams support the establishment and rapid increment of the abundance of the clams.

The average dry weight of the matured *T. keenae* egg was high as 36 ng compare to the dry weight of most of bivalves (Table 3). However diameter of *T. keenae* egg observed during this study was 76.17 ± 9.34 μm while slightly similar diameter (60-70 μm) was observed in *Tresus capax* eggs by Dudas and Dower (2006). Levitan (2006) suggested that large eggs have the ability to survive successfully than the small eggs. Largest eggs have the ability to fulfill the nutrient requirement of the larvae of the bivalves because lipid content in the largest eggs is higher than the small eggs (Gallager and Mann 1986). This is an advantage for the survival of the larvae of *T. keenae* because they are late autumn spawning clams. Generally fecundity of the most of the bivalves with large size eggs is lower than the fecundity of the bivalves with small size eggs (Honkoop et al. 1998; Smith and Fretwell 1974). In contradictory to this, fecundity of the *T. keenae* is higher. Factors such as food availability in the water and water temperature appeared to be involved in resulting differences of fecundity (Honkoop et al. 1998).

Table 3: Reproductive efforts of clams measured using different methods and size of the eggs of clams from previous studies

Species	Method	Mean GSI (%)	Fecundity	Egg weight (ng)	Location	Author
Ruditapus philippinarum	Immunological method	25	$4.1x10^6$	22	Gomso Bay of Korea.	Park and Choi (2004)
Tapes dorsatus	Induce spawning by serotonin	-	1.11×10^6	-	Sydney Harbour, New South Wales	Nell et al (1995)
Saxidomus purpuratus	Immunological method	15.4	$9\text{-}31\times10^6$	95	Geoje Island, Korea	Park et al (2003)
Tresus keenae	Immunological method	5.7(October)	121.92×10^6- 22.07×10^6 (October)	36	Yeosu, Korea	Present study
		2.4(March)	69.19×10^6- 44.91×10^6 (March)			

In present study total 28 clams collected were included in the analysis during October and March

Table 4: Summery of the reproductive effort of a few selected ripe (5 individuals) and spawning females (2 individuals) *T. keenae* in October, November 2016 and March 2017

Year	Month	Reproductive Stage	GSI^1	GSI^2	Fecundity
2016	October	Ripe	11.1	16.2	121.92×10^6
	October	Ripe	8.0	11.8	64.57×10^6
	October	Spawning	3.0	4.9	67.96×10^6
	November	Spawning	2.2	3.3	45.51×10^6
2017	March	Ripe	3.0	9.9	63.33×10^6
	March	Ripe	3.0	9.8	69.19×10^6
	March	Ripe	2.9	10.0	34.41×10^6

GSI^1, GSI^2 and fecundity values represent the individual values of the ripe (5 individuals) and spawning (2 individuals) female clams, GSI^1: the ratio of egg mass to total tissue dry weight and GSI^2: the ratio of egg mass to total tissue dry weight excluding the siphon weight

5.0 Conclusion

In this study, *Tresus keenae* egg specific antibody was successfully developed and reproductive effort of the clam which is difficult to measure using conventional methods was determined. At the same time histological studies also carried out with the combination of ELISA to understand the reproductive cycle of the clam. Gametogenesis of *T. keenae* in Yeosu, was initiated in March while spawning peak observed in October and November. Estimated potential fecundity of the clam was varying between 121.92×10^6 and 22.07×10^6 with the average of 53.40×10^6 of eggs per clam in October. However ready to spawn female clam was observed in March with the average potential fecundity diversified between 69.19×10^6 and 29.07×10^6 (average of 44.91×10^6) of eggs per clam. The spawning was fuel by the carbohydrate as energy source and the energy requirement for the gametogenesis supplied by the carbohydrate that stored in the siphon.

6.0 Reference

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