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Gonadal Maturation and Artificial Spawning of the Grooved Carpet Shell Clam, *Ruditapes decussatus* from Timsah Lake, Suez Canal, Egypt

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ABSTRACT

The gonadal maturation and artificial spawning were investigated for the grooved carpet shell clam (*Ruditapes decussatus*) under experimental conditioning. In the current study, gonad stages, gonad index as well as induced spawning were carried out in a private fish farm in Abu- Sultan, Ismailia. Samples were exposed to different spawning induction methods, namely; thermal shock, chemical, microalgae and stripping methods. In both sexes of the clam, gonad stages (active, ripe, partially spawning and completely spawning) appeared throughout the conditioning periods. Findings showed that the highest values of male and female gonad index were 28.4 and 29.4, respectively in November 2015, while in December and September of the same year the lowest marked values were 18.4 and 19.3 for males and females, respectively. Notedly, among the four previously mentioned methods, the thermal shock and stripping methods proved their successful impact to induce spawning in the clam.

INTRODUCTION

Culture of clams is clearly limited by the availability of natural seed **(Ojea *et al*., 2008; Da Costa *et al*., 2020)**. Therefore, bivalve spat from hatchery is currently the only sustainable alternative for the support of aquaculture activities **(Ojea *et al*., 2008 ; da Costa *et al*., 2012).** Hatcheries are important to reinforce natural recruitment to restock natural beds which are threatened by over-fishing.

To improve the methods of cultivating this clam, detailed knowledge of the gonadal development and spawning periods is fundamental. Knowledge of gametogenesis would help hatchery managers to determine the best strategy to produce larvae with high quality and quantity. Thus, this information may help to regulate the collecting time, management and protection of natural clam stocks **(Serdar *et al*., 2010)**

Sexual maturity of clams depends on size rather than age or geographic distribution **(Ojea *et al*., 2004)**. The sexes are generally separate, and sexual maturity is generally until fully matured, but this development has been divided into several maturity stages, e.g. resting, developing, mature, partially spawned and spawned **(Delgado & Pérez-**

Camacho, 2007). Spawning can occur either once or twice each year depending on location and environmental condition. Remarkedly, it may extend throughout the year (**Kandeel, 1992)**.

Gametogenesis of clams relies on the combination of endogenous (neurosecretory control) and exogenous factors (temperature, salinity, light, availability of food, parasitic infestations) **(Abbas *et al*., 2018; Da Costa *et al*., 2020)**. The most important limiting factors in initiating this process are temperature, quantity and quality of food **(Meneghetti *et al*., 2004; Da Costa *et al*., 2020)**. Gametogenic cycles of clam vary from one location to the other. Many researchers have observed the gametogenic cycles (ripening, fecundity, spawning time and duration) of clams in different geographical areas **(Urrutia *et al*., 1999; Drummond *et al*., 2006)**.

Several studies of biochemical cycles in bivalves have been carried out in relation to reproduction **(Barber & Blake, 1981, 1985; Ruı´z *et al*., 1992)**. Generally, when food is abundant, energy is stored prior to gametogenesis in the form of glycogen, lipid and protein **(Matias *et al*., 2016)**. The storing process and the time of using energy vary among species, as well as among populations of the same species **(Sastry, 1979)**.

The period of spawning in natural populations differs within species and geographic locations. Spawning induction is important to the production of bivalve seed. In bivalve aquaculture, availability of broodstocks may be a limiting factor, so it is often prominent to obtain as many gametes as possible from selected individuals **(Southgate, 2003)**. Spawning is easier to induce in mature individuals than in non-mature **(Parwadani, 2011).**

Spawning may be triggered by several environmental factors including temperature, chemical and physical stimuli, water currents or a combination of these and other factors. The presence of sperm in the water frequently triggers spawning in other animals of the same species. Some bivalve species in tropical environments have mature gametes throughout the year, and limited spawning may occur continuously during the year **(Helm & Bourne, 2004)**. The current study aimed to determine gametogenesis stages of *R. decussatus* during conditioning periods. Moreover, it was conducted to induce the spawning of *R. decussatus* by different methods, and hence determine the morst efficient throughout the year.

MATERIALS AND METHODS

Experimental set up

One square fiber glass tank with the dimensions of 60 x 60 x 35 cm (length x width x height) was used for stocking the broodstock. This tank was supported with an outflow pipe and an anti-siphon air vent for the drainage and refilling water from a concrete rectangular tank with dimensions of 0.6 x 1.5 x 0.5 m. Sea water was pumped from the fish farm to the tank by an electric pump. The tank was disinfected with formaldehyde (38%), washed and then left to dry and filled with 80 liters by a peristaltic pump (in

flow–through circuit) and aerated using a blower (Model BOYU ACQ-009 Air compressor electromagnetic, China).

Two experiments were carried out on two phases between June and September 2015 (June conditioning), and between October and March 2016 (October conditioning). Both experiments were carried out in a small hatchery unit, specially designed for this study, and located outside the premises of a private fish farm in Abu- sultan (Fig. 1). The experiments were performed using 200 adult specimens placed in 80 L tank. Seawater was supplied from the neighboring fish farm ponds that contained natural food through a flow–through circuit at a rate of 1.25 l min^{-1}. During both experiments, tank water temperature ranged between 17.8 and 31.2 $^{\circ}C$. During the first phase, temperature was maintained to be 2-5 $^{\circ}C$ less than the temperature of the fish farm seawater using shades. Temperature at the second phase was left to match that of the atmosphere.

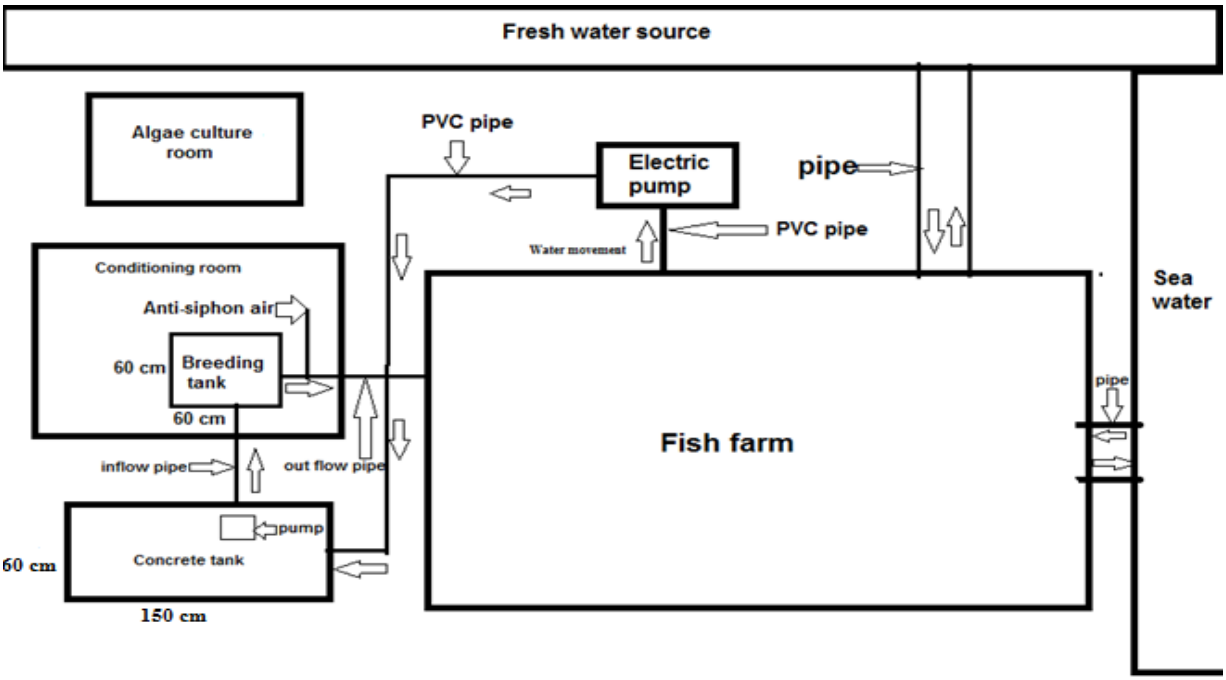

Fig. 1: Sketch showing the structure of the hatchery unit.

Broodstock collection

Specimens were purchased from clam collectors working at Timsah Lake during June and October 2015. They were measured for shell length by a precision vernier caliper with an accuracy of 0.1 mm. Only individuals ranging between 23 and 52 mm were selected to guarantee their maturity **(Kandeel, 1992)**.

Water quality parameters

Water quality in the conditioning tank was monitored twice a week between 1.00 pm to 4 pm for temperature, salinity and pH and once a month for chlorophyll- a and total suspended matter. Both pH and temperature were measured by means of Multimeter (Model AD1030, pH/mv). Salinity was measured using a Refractometer (Model Atago S/

Mill salinity meter 100%). Chlorophyll-a was measured using the spectrophotometric method **(Strickland & Parsons, 1972).** Fifteen liters of seawater were filtered using Whatman filter papers. It was then placed in an oven for 1 hour at 104 °C. The weight of the filter paper was recorded before and after drying, and the total suspended matter (TSM) was calculated as the weight difference between the two readings and expressed as g/l. Species diversity of phytoplankton in the natural sea water was determined by filtering an unspecified amount of water from the fish farm through a plankton net (Hydrolab, 20 µm). Formaldehyde solution (38%) was added to the filtered sea water for preservation of organisms. Water sample was then examined using a light microscope (Model Olympus BX-51), and the identification of phytoplankton was determined according to **Madkour (1992)**.

Broodstock sampling

The total conditioning periods for the first and second experiments were 4 and 6 months, respectively. Sampling was performed at a monthly interval at the beginning of each experiment. At each interval, one group of 10 individuals were sampled for determination of gonad weight and gonad index. Gametogenic condition of the gonads was determined after histological sectioning.

Calculation of Gonad index

The gonad was separated and weighed to the nearest 0.01 g. The gonad index (GI) was calculated for each sex as follows:

$$\text{Gonad index} = \frac{\text{Gonad wet weight}}{\text{Soft body wet weight}} \text{X100}$$

Microscopic examination of gonadal tissue

Preparation of gonadal smears

Five individuals from the broodstock tank under conditioning cases were randomly selected and used for the preparation of gonadal smears as well as histological sectioning. Gonadal smears were prepared by removing small portions of the gonads, and were spread over clean microscopic slides with a drop of water. Cover slips were laid down slowly and pressed gently. The smears were examined under electric microscope. Two or three smears were prepared from the different parts of the gonads for the assessment of the condition of the entire gonads.

Preparation of histological sections

Gonadal tissues were removed from the shells by a stainless steel scalpel and preserved in Bouin's fixative for 24 hours. Those tissues were dehydrated through a series of ethyl alcohol of ascending concentrations, cleared in xylene, embedded in paraffin wax and sectioned at 6 - 8 µm using a rotary microtome. The sections were then mounted on glass slides and stained with Ehrlich's Hematoxylin and Eosin. The prepared slides were examined under an electric microscope (Model Olympus BX-51) to determine both sex and stage of gametogenesis. Gametogenic stages of clam were categorized into six stages

(Stage 0: Inactive, Stage I: Early active, Stage II: Late active، Stage III: Ripe, Stage IV: Partially Spent and Stage V: Spent) according to **Delgado and Pérez-Camacho (2003)**. When more than one developmental stage was evident within a single individual, the clam was assigned to the reproductive stage that was observed with the majority of follicles **(Matias, 2013)**.

Spawning induction

Eighty individuals were gently cleaned and washed to remove sediments and debris**.** Broodstock tanks with dimensions of 25 X 20 X 15 cm (length X width X height) were prepared and filled with 3 liters of sea water sterilized by sodium hypochlorite solution (1ml/l). Individuals were placed in tanks for 30 min. Water temperature was kept similar to that of the surrounding atmosphere during the experiment. Samples were exposed to different spawning induction methods along the experimental period. The spawning induction methods were as follows:

Thermal shock method

Ten individuals were taken from the broodstock tank and placed on a refrigerator's shelf at 4°C for 12 hrs, then transferred back to the broodstock tank for 30 mins. Clams were moved to the spawning tank where water temperature was adjusted to range between 30 – 35 °C using an aquarium heater (Model MINJIANG HK-300, China). Individuals which did not spawn within the first 30 mins were returned to the broodstock tank for another 30 mins before being transferred to the spawning tank. The thermal shock procedure lasted for 6 hrs.

Chemical method

Two techniques were used, the first was carried out by placing the clams in a seawater tank with a capacity of 3 L with 20 ml of 3% potassium chloride (KCl) to trigger spawning. The second technique was performed by injecting 1 cm of the KCl solution into the mantle of the clams using a plastic syringe.

Microalgae method

Two algal solutions were prepared using the microalgae *Tetraselmis* sp. and *Nannochloropsis* sp. at he concentrations of 1.5 million cell/ml and added to the clam sample in static tank for 2 hrs. Afterwards, samples were returned to the spawning tank filled with 3 L sterile (with added chlorine: 1ml/L) sea water. This method was repeated for three times in each trial.

Stripping method

Ripe ovaries and testes were taken off the clams. Ovaries were placed in a container filled with seawater and 0.1 M ammonium hydroxide for 20 min to help with the disintegration of the follicular walls. The water was then filtered through a 40 µm nylon mesh sieve. Ova retained on top of the sieve were placed in 500 mL seawater beaker. The testes were placed in another container filled with seawater and cut into pieces to release the sperms. One mL of the sperm solution was then added to each 0.5 L of water with ova, then left for one hour for fertilization.

Fertilization process and embryonic development

Each clam that started to spawn was placed separately in a 500 mL seawater beaker to avoid self-fertilization. Once spawning is completed, ova and sperms were collected and placed in another 500 ml seawater beaker with a ratio of 1ml of sperm solution to 0.5 L of water with ova for 60 min. Fertilized eggs were sieved through a 45 µm nylon mesh sieve to discard excess sperms, divided into three equal amounts and incubated in 5 L triplicate tanks with a density of 100 eggs per ml. Temperature was kept similar to that of the surrounding atmosphere. For the next 26 hrs, one ml of water was examined underneath a light microscope for the examination and photography of different embryological stages using a digital camera (Model FUJIFILM FINEPIX JV100).

RESULTS

1. Water quality

1.1. Temperature and saliniy

Water temperature ranged between 17.8 °C in February 2016 and 31.2 °C in August 2015 while salinity ranged between 26 ‰ in June and July 2015 and 35 ‰ in November 2015 (Fig. 2).

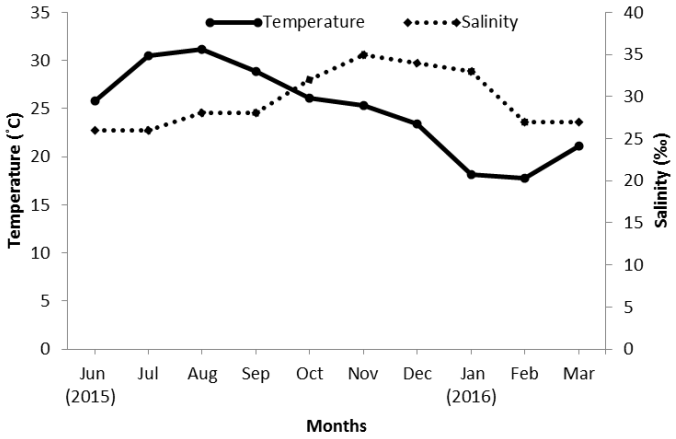

Fig. 2: Monthly temperature and salinity values in the conditioning tank during the experimental period.

1.2. pH, chlorophyll-a and total suspended matter (TSM)

Maximum value of pH (8.2) was recorded in July and August (2015), while the minimum (7.8) was in November, December (2015) and March (2016) (Fig. 3). Chlorophyll-a ranged between 6.5 µg/l in December 2015 and 12 µg/l in March 2016. The lowest value of TSM was 0.18 g/l in January 2016, and the highest was 0.55 g/l in October 2015 (Fig. 3).

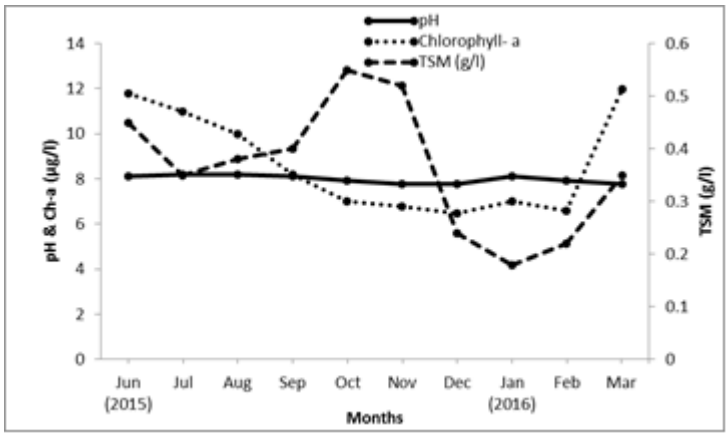

Fig. 3. Values of pH, Chlorophyll- *a* (Ch-a) and TSM recorded in the conditioning tank during the experimental period.

1.3. Species diversity of phytoplankton

A total of 16 taxa of phytoplankton were recorded in water sample from the conditioning tank (Table 1).

Table 1: Phytoplankton species in the water sample of the conditioning tank.

Family	Species
Bacillariophyceae (Diatoms)	*Nitzschia sigma* (Kützing)
	Pleurosigma sp.
	Melosira sp.
	Nitzschia sp.
	Navicula lyra, Ehrenberg
	Navicula sp.
	Gyrosigma sp.
	Rhizosolenia sp.
	Cymbella sp.
	Diploneis sp.
	Cyclotella sp.
	Surirella sp.
Dinophyceae(Dinoflagellates)	*Dinophysis* sp.
Chlorophyceae (Green algae)	*Staurastrum* sp.
Cyanophyceae	*Spirulina* sp.
Tintinnida	*Tintinnid* sp.

2. Gonad index (GI)

Monthly means of the gonad index of both clam sexes are represented in Fig (4). The highest values of male and female's gonad index were 28.4 ± 2.8 and 29.4 ± 4.1 in November 2015, respectively, while the lowest values were 18.4 ± 2.4 and 19.3 ± 3.2 in December and September for males and females, respectively.

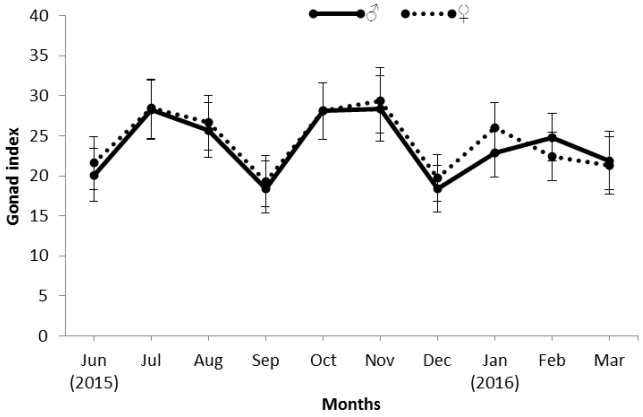

Fig. 4: Mean variations of the gonadal index in both sexes of *Ruditapes decussatus* during the experimental period.

3. Stages of gonadal development

3.1. Females

The different developmental stages of females' gonads were as follows:

1- Early active stage (Plate 1) was represented only in early June, September, late of December (2015), early January, February and March (2016).
2- Late active stage (Plate 2) was represented only in August (2015) and late January (2016)
3- Ripe stage (Plate 3) was represented in all months but showed high occurrence in May, July, August and November (2015). However, the lowest occurrence was in June and September (2015).
4- Partially spawning stage (Plate 4) was represented in June (2015) and March (2016).
5- Completely spawning stage (Plate 5) was observed in September (2015) January, February and March 2016 with high occurrence in December (2015).

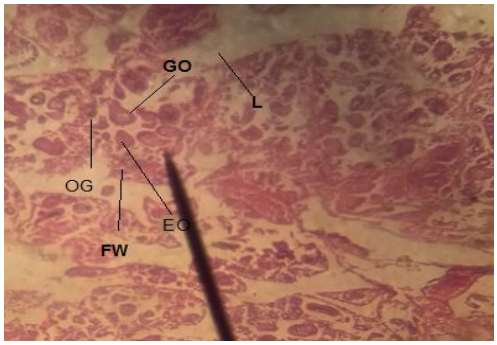

Plate 1: Histological photomicrograph of the early active stage of *R. decussatus* female gonads. FW (follicle wall), OG (ooginia), EO (early oocyte), GO (growing oocyte) and L (lumina). The arrow points to follicle wall (H&E; 800x)

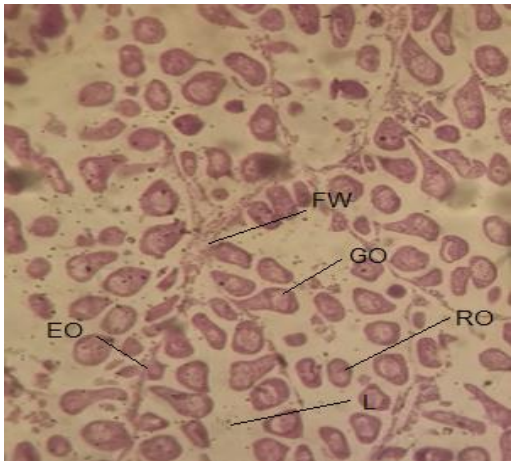

Plate 2: Histological photomicrograph of the late active stage of *R. decussatus* female gonads, FW (follicle wall), EO (early oocyte), GO (growing oocyte), RO (ripe ova), and L (lumina). (H&E; 1200x)

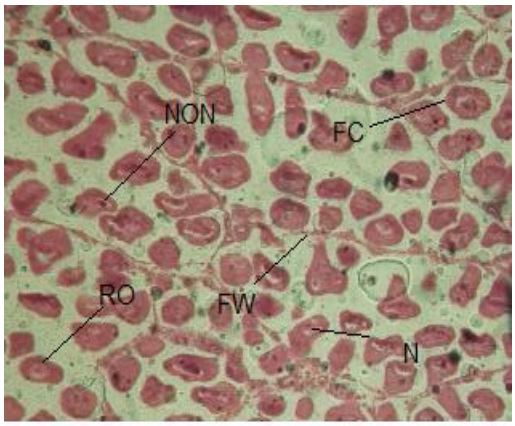

Plate 3: Histological photomicrograph of the ripe stage of *R. decussatus* female gonads. FW (follicle wall), FC (Follicle cell), N (nucleus), RO (ripe ova) and NON (nucleolus). (H&E; 1200x)

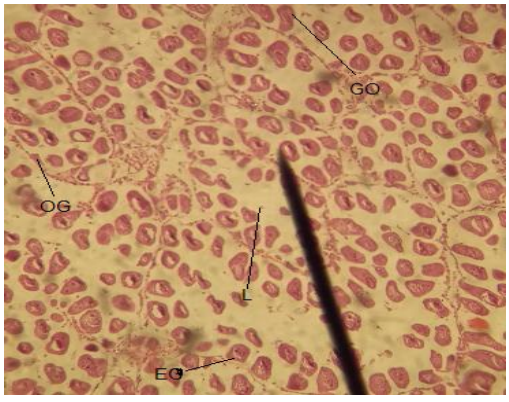

Plate 4: Histological Photomicrograph of the partially spawning stage of *R. decussatus* female gonads. OG (oognia), EO (early oocyte), GO (growing oocyte) and L (lumina). The arrow points to ripe ova. (H&E; 300x)

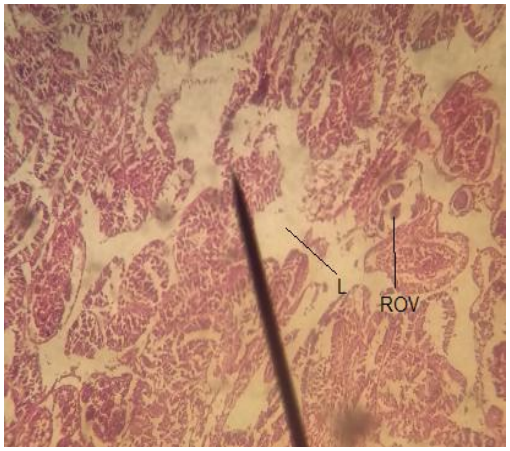

Plate 5: Histological Photomicrograph of the completely spawning stage of *R. decussatus* female gonads. ROV (residual ova) and L (lumina). The arrow points to connective tissues. (H&E; 300x)

3.2. Males

The different developmental stages of males' gonads were as follows:

1- Early active stage (Plate 6) was present for most of the experimental period (not observed in September, November and December, 2015) and with high occurrence in July and October (2015) and March (2016).
2- Ripe stage (Plate 7) was observed in all months with high occurrence in May, July, August and November (2015).
3- Partially spawning stage (Plate 8) was observed in June, September and December (2015).
4- Completely spawning stage (Plate 9) was observed in September and December (2015) and January and March (2016).

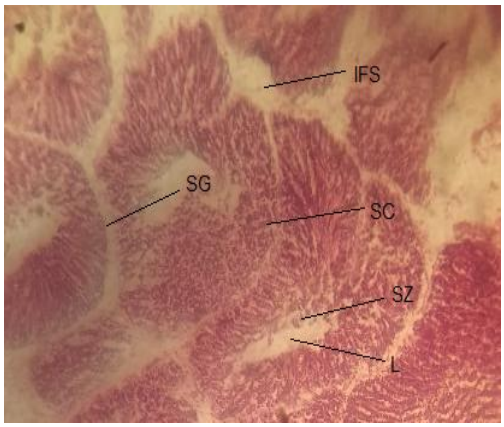

Plate 6: Histological Photomicrograph of the late active stage of *R. decussatus* male gonads. IFC (interfollicular connective tissue), SG (spermatogonia), SC (spermatocytes), SZ (spermatozoa) and L (Lumina). (H&E; 300x)

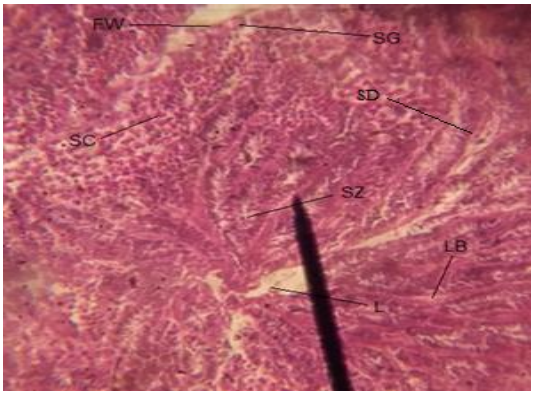

Plate 7: Histological Photomicrograph of the ripe stage of *R. decussatus* male gonads. FW (Follicle wall), SG (spermatogonia), SC (spermatocytes), SZ (spermatozoa), SD (spermatid), L (Lumina) and LB (longitudinal bundles of spermatozoa). The arrow points to spermatozoa. (H&E; 300x).

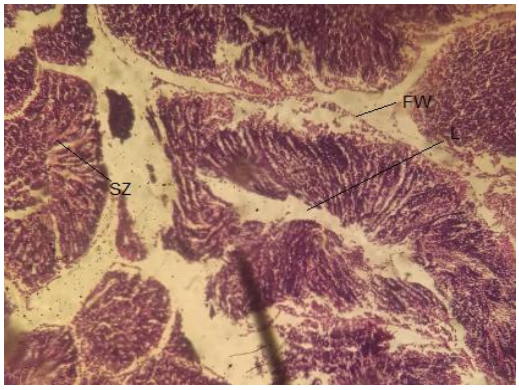

Plate 8: Histological photomicrograph of the partially spawning stage of *R. decussatus* male gonads. FW (follicle wall), SZ (spermatozoa) and L (Lumina). (H&E; 300x).

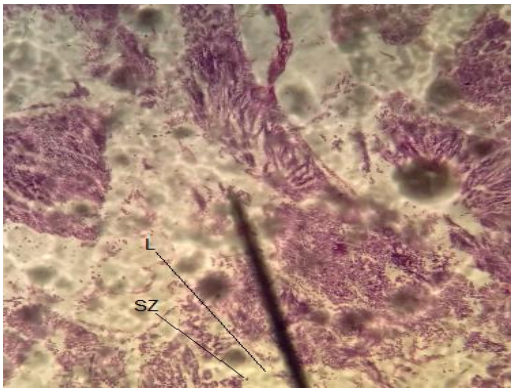

Plate 9: Histological photomicrograph of the completely spawning stage of *R. decussatus* male gonads. SZ (spermatozoa) and L (Lumina). The arrow points to lumina. (H&E; 300x).

4. Induced spawning

Of the four methods used to induce spawning in the clam, only two were successful; the thermal stress and the stripping methods.

4.1. Thermal stress method

Of the 35 trials, only 15 were successful. During the first conditioning period of 110 days, all 14 trials were unsuccessful. In the second conditioning period that lasted for 166 days, the six trials that were carried out during the first 53 days also failed. Successful trials started to take place 12 days after the last unsuccessful trial (Table 2).

Table 2: Successful and unsuccessful trials to induce spawning in *R. decussatus* by means of the thermal stress method.

Start of the conditioning period	Spawning date	Temperature range		Response
		Minimum	Maximum	
1/6/2015	16/6/2015	28.3	35	F
	21/6	28.8	35	F
	24/6	29.1	35	F
	30/6	30	35	F
	4/7	30.2	33	F
	7/7	30.4	33	F
	11/7	30.7	35	F
	19/7	30.5	35	F
	25/7	31	35	F
	4/8	31	35	F
	15/8	31.2	35	F
	25/8	31.7	35	F
	5/9	30	35	F
	20/9	30	35	F
7/10/2015	11/10	29.2	33	F
	12/10	29	34	F
	4/11	27.1	32	F
	14/11	27	33	F
	15/11	25	30	F
	30/11	18	29	F
	12/12	18	29	S
	29/12	13.6	28	S
	2/1/2016	16.4	28	S
	8/1	17	29	S
	16/1	16	28	S
	23/1	16	28	S
	31/1	12.2	28	S
	8/2	18.1	30	S
	9/2	14.2	30	S
	15/2	19.4	30	S
	18/2	21.6	30	S
	24/2	17.7	29	S
	5/3	17.3	29	S
	12/3	18	29	S
	17/3	18.3	30	S

Legend: S = successful trial; F= failed trial.

4.2. Stripping method

This method was successful during the two trials carried out during February and March 2016 (Table 3). The number of fertilized eggs obtained in the second trial was almost 2.5 times higher than that of the first one.

Table 3: Numbers of ova and fertilized eggs obtained by means of the stripping method.

Start of conditioning period (7/10/2015)	Month	Days after collection	Ambient temperature	Ova in gonads (NoX10^4)	Fertilized eggs (NoX10^4)
	February (2016)	135	19.4	1500	23
	March (2016)	161	18	1800	54

5. Embryonic development

Plate 10 shows the different embryonic stages observed through 26 hours post fertilization. The fertilized egg (Plate 10 A) showed the polar body at T_0+20 min. The first and third cleavage stages (2-cell, 8-cell) (Plate 10 B, C) were observed at (T_0+1 h 30 min and T_0+2 h 45 min), respectively. The Blastula stage (Plate 10 D) appeared at T_0+6 h, while the trochophore was observed at T_0+13 h (Plate 10 E). Finally, the D veliger larvae (Plate 10 F) was noticed at T_0+25 h.

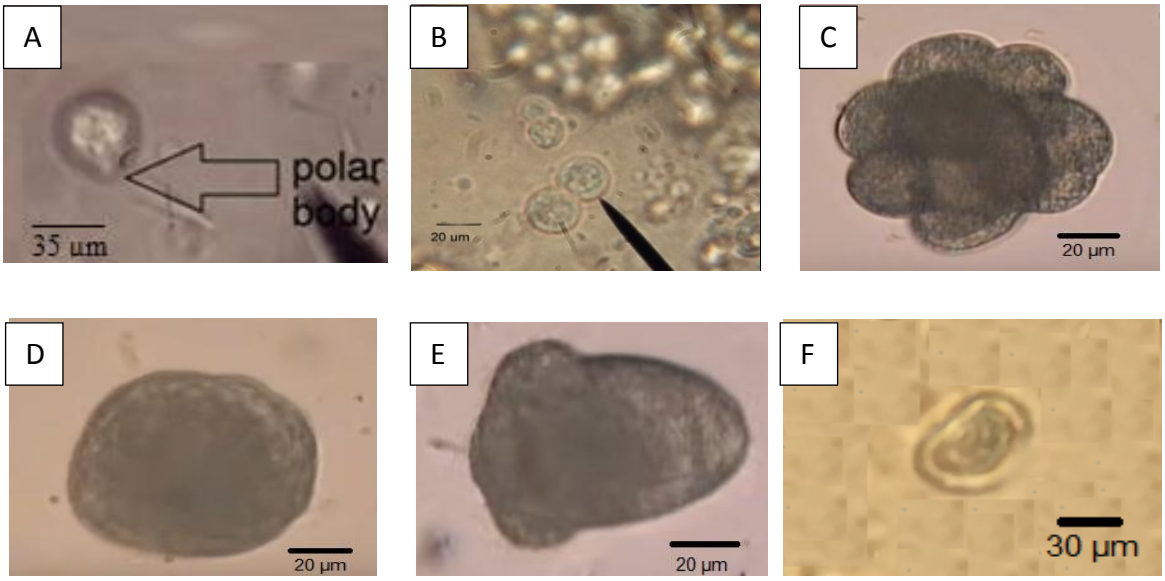

Plate 10: Embryonic stages observed through 26 hours post fertilization with details on the timing of appearance.

DISCUSSION

Knowledge of the gametogenic cycle of commercial species is important in aquaculture. Gametogenic cycles in bivalve molluscs are influenced by both exogenous factors such as temperature, salinity, light, turbidity, food availability and parasitic infestations **(Sastry, 1979; Daou; Goulletquer, 1988)** and endogenous factors such as energy demands for reproduction **(Normand *et al.*, 2008; Enríquez-Díaz *et al.*, 2009)**. **Partridge (1977)** previously concluded that in *R. decussatus* the duration of gonad maturation and, in particular, the duration of the spawning period is directly influenced

by the geographical latitude, because of the temperature of the area. **Da Costa *et al.* (2012)** concluded that temperature was closely linked to the geographical locations affecting indirectly the availability of food and/or consequently the timing and duration of the reproductive cycle and number of spawning per year.

In general, the high average values of GI were coincident with gonadal maturity, while minimal average values following the high average values were considered as an indicator of spawning **(Kanti *et al.*, 1993; Chung, 1997)**. In the present study, the gonad index of *R. decussatus* recorded several peaks in July, August and November (2015) for both sexes in addition to February (2016) for males and January (2016) for females. The lowest values were recorded in June, September and December 2015 for both sexes. **Kandeel (1992)** recorded peaks of GI of *R. decussatus* in Timsah Lake in all seasons with high peaks in May and July, August and November for both sexes,whereas the lowest values were recorded in June, September and December. His results agree with results in the present study. According to **Chung *et al.* (2001), Ojea *et al.*, (2004), Saba (2011), Ammar *et al* . (2013)** and **Matias (2013)** the higher values of GI were recorded for a limited period through the year, especially the spring and summer seasons. The present findings agree with results of those of the previous authors with reference to some months, though generally differ with the other. These differences among the results may be due to the differences in geographical distribution, temperature and nutritional values of *R. decussatus*.

Histological sections of the current work illustrated five stages of gonadal development of *R. decussatus* for both sexes. No resting stage was recorded in the present or previous studies **(Kandeel, 1992; Mohammad *et al.*, 2014)** in Timsah Lake. Nevertheless, it was recorded in other areas **(Ojea *et al.*, 2004; Saba, 2011; Matias, 2013)**. Notably, many studies have assessed the importance of geographical locations in defining and controlling gametogenesis **(Meneghetti, 2004)**. The continuous reproduction without resting stage may be explained due to the fact that the nutritional value is high and seawater temperature is suitable for gametogenesis. Thus, reproduction activities continued throughout the year in conditioning tank or experiment.

In the present study, the ripe ova were recorded in all experimental period in a phenomenon similar to that **Kandeel (1992)** and **Serdar *et al.* (2010)**. Some authors reported that ripe carpet shell clam was observed between June and September in Ireland **(Xie & Burnell, 1994)**, and in Çardak Lagoon in Turkey **(Gözler & Tarkan, 2000)**. In addition, it was monitored between April and June in both Galicia, Spain **(Ojea *et al.*, 2004)**, and Sufa Lagoon, Turkey **(Serdar and Lök, 2009)**. **Xie and Burnell (1994)** reported that the gametogenic cycle of the carpet shell clam showed one spawning period that occurred between August and September. In the present study spawning was observed all the year around. Moreover, **Serdar and Lök (2009)** mentioned that spawning period was between July and October in the Sufa Lagoon, Izmir Bay. According to **Matias (2013)** the reproductive cycle of *R. decussatus* comprises a ripe

stage in spring followed by spawning that begins in late spring and extends during summer until early autumn in both populations. However, other authors have deduced the occurrence of two major periods of spawning, in spring and then in summer or early autumn in different populations of this species **(Laruelle *et al*., 1994; Chryssanthakopoulou & Kaspiris, 2005)**. The differences observed between studies have been frequently associated to the influence of the geographical location and consequently the different environmental factors **(Da Costa *et al*., 2012)**.

Generally, it appears that two main factors (temperature and food supply) determine the acceleration of gonadal maturation **(Pérez Camacho *et al*., 2003; Matias, 2013)**. **Matias (2013)** recorded that the temperature was directly correlated with gonad maturation. He found that 18 °C was better than 14 °C. In the present study, the maturation rapidly continued through the conditioning periods. The continuous maturation resulted from the high and optimum temperature (17.8 to 31.2 °C) through the experiment. This agree with **Holland and Chew (1974)** and **Xie and Burnell (1994)** who recorded that low temperature is a limit factor for gonadal activity between (8-12°C) and for gametogenesis (14°C).

Feeding is another determining factor for gonadal development in bivalves, and if the abundance of food is enough in water, gametogenesis begins with continuous sexual maturation **(Pérez Camacho *et al*., 2003)**. In the present study, Chlorophyll-a ranged from 6.5 µg/L to 12 µg/L, and TSM ranged from 0.18 to 0.55 g/L. There is a direct relationship between food availability and acceleration of the reproductive process, provided that temperature is favourable **(Delgado & Perez-Camacho, 2005)**.

From the previous discussion, the geographical location can be considered as an important factor controlling timing and duration of gametogenesis and spawning. In the present study, the gametogenesis of clam continued throughout the year revealing that the temperature and food supply were suitable all year around. On the other hand, in the Mediterranean area, gametogenesis and maturity begins early **(Rodriguez-Moscoso *et al*., 1992)**, whereas in northern Europe gamete production starts in March and sexual maturity is reached in summer **(Xie & Burnel, 1994)**. **Urrutia *et al*. (1999)** reported that the reproductive cycle of the *R. decussatus* population in Mundaka Estuary (Spain) has a single gametogenic event per year. In Sufa Lagoon (Portugal), gamete release of *R. decussatus* continues from mid summer (July) to mid autumn (October). The previous results are in agreement with those of **Laruelle *et al*. (1994)** and **Ojea *et al*. (2004)**. All the previous studies concluded and confirmed the results of the present study.

The response of individuals for induced or artificial spawning was independent on timing of broodstock collection, site collection and conditioning temperature **(Matias, 2013)**. His results showed that the northern clams spawned much more often than the southern in both October and February experiments. Better performance was clearly exhibited by clams conditioned at 20 ± 1 °C and 22 ± 1 °C compared with those conditioned at 18±1 °C **(Matias, 2013)**. These findings agree with the present study

where clams in June conditioning experiment were not responding, compared with October experiment. All these observations indicated that the factor which triggers spawning is correlated to the temperature and the maturation state of the gonads (**Breber, 1980**; **Manzi *et al.*, 1985**). Thermal shock method was considered as the most effective method for releasing fertilized eggs without killing organisms through October conditioning. The failure of spawning during June conditioning could be attributed to that clams are perfectly adapted to high temperatures during this period. According to **Matias (2013)** and **Aranda-Burgos *et al.* (2014)**, all trials of induce spawning, by using of thermal shock method, succeeded under experimental conditioning.

On the other hand, stripping method succeeded in obtaining fertilized eggs using ammonium hydroxide to break the germinal vesicle of eggs of clam. According to **Brooks (1880)**, fertilizable eggs of many species, including those of O. *virginica*, can be obtained by stripping mature females. This method is possible only for eggs of those forms in which the germinal vesicle dissolves after stripping. In many species, *Mercenaria mercenaria*, *Pitar morrhuana and Tapes semidecussata* attempt to fertilize stripped eggs and usually fail because the germinal vesicles remain intact in these eggs, and as a result, fertilization does not occur. Recently, the researchers used a weak solution of ammonium hydroxide to break the germinal vesicle of eggs of certain bivalves. By employing this method they succeeded in raising normal larvae from eggs stripped from *Mercenaria mercenaria*, *Tapes semidecussata* and from several other species.

On the contrary, chemical methods and algal methods failed in triggering *R. decussatus*. This is similar with **Loosanoff and Davis (1963)** who carried out many trials to cause artificial discharge of reproductive elements by injecting weak solutions of Mn_4OH and other chemicals into the bodies of bivalves that could not be spawned by other means. Their results were usually unsuccessful, except in the case of *M. edulis*, when injection was in its adductor muscle.

All stages of embryonic development appeared through 25 hours. The required periods for each stage were similar to that recorded by **Aranda-Burgos *et al.* (2014)**.It was noted that D veliger larvae appeared 25 hours post fertilization in the present study. This result agrees with the previous study under the same condition. Then, mass mortality hit D veliger larvae. This mortality may be due to many reasons as parasites and bacterial contamination from broodstock. According to **Schulze *et al.* (2006)** and **Sandaa *et al.* (2008)**, broodstock constitutes one of the most important bacterial sources for larval cultures due to the vertical transmission of bacteria from adults to larvae. Microbiota may include opportunistic pathogens harmless to broodstock but proved harmful to larvae. Indeed, members of the genus *Vibrio*, including known larval pathogens, have been isolated in shellfish hatcheries from the gonad of broodstock **(Sainz-Hernandez & Maeda-Martínez, 2005; Prado *et al.*, 2014)**. Infection of bivalves with parasites may also represent an important reason for decreased production either by mass mortality (e.g.

mortality caused by *Perkinsus* sp.) or by slow growth, decreased fecundity or complete reproductive failure that can be caused by parasitism with digenean larvae sporocysts and cercaria of trematodes **(Taskinen *et al*., 1994; Hanafy *et al*., 1997; Khamdan, 1998; Nago & Choi, 2004; Cremonte *et al*., 2005).**

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