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Induced Spawning and Stocking Density of *Tripneustus gratilla* for aquaculture Proposes in the Red Sea, Egypt

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ABSTRACT

Increasing fishing potential for edible sea urchins causes significant depletion in wild populations worldwide. Thus, induced spawning and larval rearing of *Tripneustus gratilla* were studied. Four induction agents were tested including injection with 0.5 and 1.0 M KCL, drying under direct light for 4 hours and mechanical shaking. The fecundity and responding times to induction agents varied significantly according to the treatment. The highest fecundity was estimated for the group treated with drying under direct light, while the lowest fecundity was recorded for the group treated with mechanical shacking, being 18.6 ± 1.1 and 0.94 ± 0.004 million oocytes/female, respectively. The quickest response was recorded for the group treated with 1M KCL, compared with other induction agents. It was noticed that the lower the abundance, the higher the survival rate, i.e. higher survival of larvae was recorded at a density of 3 larvae/ml. Embryonic development, larval metamorphoses and morphometric parameters were followed and measured.

INTRODUCTION

The demand for shellfish products has increased worldwide for last decades, and it is expected to continue increasing due to geometric human population growth **(Azra *et al.*, 2021)**. Many wild capture fisheries are already exploited at, or beyond, sustainable capacity **(Ricard *et al.*, 2012),** yet they are unable to satisfy the expanding market **(McBride *et al.*, 2015)**. Shellfish aquaculture may provide an economically and ecologically viable substitute for wild stocks **(Naylor *et al.*, 2000)**. Foreseeable, it will be intensified in the next few decades to bridge the gap between demand and supply **(Azra *et al.*, 2021)**. Sea urchins are a good example of this situation. Sea urchin's gonads (roe) are considered a culinary delicacy in France and Spain, Chile, North America, Asia, and

especially Japan, which accounts for around 90% of worldwide demand **(Sun & Chiang, 2015)**. The world production of sea urchins steadily increased through the later half of last century to a peak of 120 306 tons in 1995, and quickly declined, recording only 90,257 tons in 1998 **(Andrew *et al.*, 2002)**. Currently production is about 75,000 ton per year, and there is an unmet demand for sea urchins **(Stefánsson *et al.*, 2017)**. An increasing demand for sea urchins over the last decades has led to overfishing and the depletion of natural stocks **(Sloan, 1985; Keesing & Hall, 1998)**. However, the 99% of the production comes from wild fisheries and less than 1% from the aquaculture **(Carboni, 2012)**. Sea urchins is still being an unusual candidate for aquaculture **(McBride *et al.*, 2015)**.

Sea urchin aquaculture has mainly focused on slow growing temperate species as most of sea urchin fisheries are in temperate regions **(Dworjanyn & Pirozzi, 2008)**. One of few exceptions is *Tripneustes gratilla*. This echinoid is cultured in several places (Philippines and Japan) for restocking programs to enhance the recovery of natural populations previously depleted by fishing activities **(Shimabukuro, 1991; Andrew *et al.*, 2002; Juinio-Meñez & Bangi, 2010)**. *T. gratilla* is widely-distributed in tropical and subtropical waters of the Indo-Pacific region between 0 to 75 m depth **(Lawrence & Agatsuma, 2007)** from east Africa (Red Sea to Natal), the South Sea Islands (from the Norfolk and Kermadec Islands to the Marquesas and Hawaii), and from Australia (Port Jackson on the east coast and Sharks Bay on the west), to southern Japan (with the Bonin Islands) **(Lawrence & Agatsuma, 2007)**. It is an opportunistic grazer, with an omnivorous diet and can feed on a wide variety of macroalgae, seagrass, diatoms and coral tissue scraped from rocks **(Dafni, 1992; Klumpp *et al.*, 1993; Lawrence & Agatsuma, 2007)**. It is a fast growing species in both subtropical or temperate environments **(Steinberg & van Altena, 1992; Dworjanyn *et al.*, 2007)** and tropical **(Lawrence & Agatsuma, 2007)** environments, reaching sexual maturity **(Lawrence & Agatsuma, 2007)** between 60- 70 mm (diameter) during a year time. *T. gratilla* is collected by small-scale fisheries in several areas of its geographical distribution range (*e.g*., Japan, Fiji, Philipines, Mozambique and Egypt) **(Muthiga, 2005; Lawrence & Agatsuma, 2007)**. It is gathered to supply local markets and for export, particularly to Japan, Taiwan, Hong Kong and Korea where there is high market demand for this species **(Trinidad-Roa, 1987)**.

T. gratilla is commercially exploited along the Mediterranean coast of Egypt, where this species is gathered by hand from the coastline and by scuba diving from the coastline to a depth of 17m and sold at local markets but is not yet commercially exploited in the Red Sea, where it is among the most abundant sea urchin species **(AbouElmaaty *et al.*, 2023)**. *T. gratilla* is locally and internationally an economically valuable sea urchin species. Egypt is experiencing a rapid expansion of aquaculture sector but is mainly based on fish species **(Soliman & Yacout, 2016)**. Echino-culture may offer an opportunity not only to open supplying local markets, since there is a current gap between production and

consumption of fish and shellfish in Egypt **(Soliman & Yacout, 2016)**, but also to export for international markets.

T. gratilla reproductive cycle is highly variable along its wide geographical distribution range **(Lawrence & Agatsuma, 2013)**. Frequently, it shows a unique spawning season which occurs from mid-summer to autumn in Japan **(Kobayashi, 1969)** and Indonesia **(Radjab, 1997)**, in autumn in Australia **(O'Connor *et al.*, 1978)** and Taiwan **(Chang-Po & Kun-Hsiung, 1981)**, and in summer in Kenya **(Muthiga, 2005)**. However, it spawns twice (spring and autumn) each year on the Great Barrier Reef **(Stephenson, 1934)** and throughout the year in the Philippines **(Tuason & Gomez, 1978)**. Focusing on the Red Sea, *T. gratilla* spawning season has been recorded in spring **(Kidron *et al.*, 1972; Pearse, 1974)**; from autumn to spring **(Fouda & Hellal, 1990)** and from mid-summer to autumn **(AbouElmaaty *et al.*, 2023)**. Under controlled conditions (in a hatchery), sea urchins are relatively easy to spawn but difficult to breed **(Moe, 2019)**. **Shpigel *et al.* (2018)** observed that, under constant abiotic conditions and constant supply of nutritious, feed gonad growth and spawning events occurred throughout the year. Fertilization can be done using a dry sperm dilution insemination, with a success over 96% **(Parvez *et al.*, 2018)**.

The larval stage is typically long in period. The larvae of *T. gratilla* take between 30 and 40 days at 25°C under hatchery conditions **(Dworjanyn *et al.*, 2007)** and about 15 to 52 days before settlement in wild conditions **(Scholtz *et al.*, 2013)**. This period relies on specific factors, such as feed type and quality **(Byrne *et al.*, 2008)** in addition to rising temperature **(Rahman *et al.*, 2009)**. Increasing hatchery production by diminishing the long larval period and expanding survival of *T. gratilla* hatchlings will help improve generation, gainfulness and the potential achievement for the development of this species **(Scholtz *et al.*, 2013)**.

The main aim of this study was to inspect the possible use of *T. gratilla* in aquaculture in Egypt (Red Sea). To this end:

(1) We described the embryonic development and larval stages of *T. gratilla*, for the first time, under controlled hatchery condition in the Red Sea, and

(2) We conducted several experiments of larvae stocking density and survivorship.

MATERIALS AND METHODS

Sea urchin collection

A number (N= 40) of adult sea urchins *T. gratilla* were randomly collected from the Red Sea coast off Hurghada City by snorkeling from depths of 1- 2m. They were immediately transported in styrofoam containers, filled with sea water to hatchery facilities, belonging to the NGO Hurghada Environmental Preservation and Conservation Association (HEPCA) at the area of Port Ghalib, 70 km south of Qusier City.

Culture system

Samples were placed in fiberglass tanks of 1000 liters volume, each was filled with seawater for one day to dismiss the transportation's stress and excrete most of the gut content. In the second day, the samples were replaced in cleaned and disinfected tanks of 55L volume each and filled with filtered seawater through 1µm mesh size. In parallel, all equipment and tools were dis-infected using sodium hypochlorite (NaClO). Diameters and body weights of the sampled breeders were determined using a caliper and digital balance. The filtered seawater (FSW) was generally characterized with temperature between 18 and 28°C & salinity 38‰; it was kept in photoperiod cycle 12:12, Dark: Light (D:L) (no direct light).

Spawning and fertilization

Adult sea urchins were divided into four groups; 10 individuals each. The spines around the gonophore region were removed with a sharp blade, and the area was washed with filtered seawater and drained with tissue paper. Each individual of the 1st group was injected with 1- 2ml of 0.5 mole potassium chloride (M KCl) using 2 ml syringe. Injections for each individual were carried out at several sites of the soft membrane surrounding the peristome (peristiomal membrane) into the coelomic cavity. The Individuals of the second group were similarly injected but with 1-2 ml of 1 M KCl and shacked gently for few seconds after injection. Individuals of the third group were dried under direct sunlight for almost 4 hours. Individuals of the 4th group were shacked manually for few seconds.

Each induced individual was inverted upside down (the aboral side was down) on a disinfected 250ml conical flask containing FSW. After completion of gametes shedding, the urchins were removed from the flasks, and then the flasks were gently rotated in a clockwise and anti-clockwise directions for uniform mixing. Aliquots of discharged sperm and eggs were examined under microscope for the egg's maturation and sperms mobility. Oocytes were counted under a light microscope as total number of released oocytes per individual. Sperms from different individuals were mingled in a disinfected plastic box. Sperms were mixed with oocytes at ration of 100:1 ml "ova: sperm" **(Scholtz *et al.*, 2013)**, in a disinfected conical flask (1000ml). The flasks were covered with aluminum foil to prevent light penetration, stirred manually very gently with no aeration and examined every 15 minutes under light microscope to determine the start of fertilization. The mortality of brood stocks within the four groups was followed in the treated breeders.

Embryonic development and larval rearing

The fertilized eggs were placed into a 55-litre rectangular plastic boxes containing FSW, with mild aeration. Samples of 10ml each were collected at 15 minutes intervals to follow the embryonic development. At late prism stage, larvae were stocked at different densities in cylindrical polyethylene tanks of 400 liters each. Each tank was supplied with mild aeration and FSW maintained at 18– 22°C. Fifty percent of the rearing tank waters

were exchanged twice a day, at sun set and sun rise, by siphoning through a series of buckets of mesh sizes 100, 80 and 50 microns, respectively. Larvae were initially fed on *Nanochloropsis* species at a rate of 4,000 cells per larva and gradually increased to the highest of 10,000 cells per larva at the stage of eight arm echinopluteus.

Larval stages and morphometric measurements

Development stages and larval morphological measurements were determined. The four stages were recognized and photographed; namely, two-arm; four-arm; six-arm stage and eight-arm. To measure different morphometric characters at each developed stage, the larvae were collected before introducing algal food to ensure that the fullness of the stomach had no effect on the measurements. Three larvae were randomly collected to measure morphometric parameters using an Olympus BX51 microscope included: Total length (TL), total width (TW), post-oral arm length (PO-arm), stomach length (SL) and mid-body line length (MBL) according to **Soars *et al.* (2009)** as shown in Fig. (1).

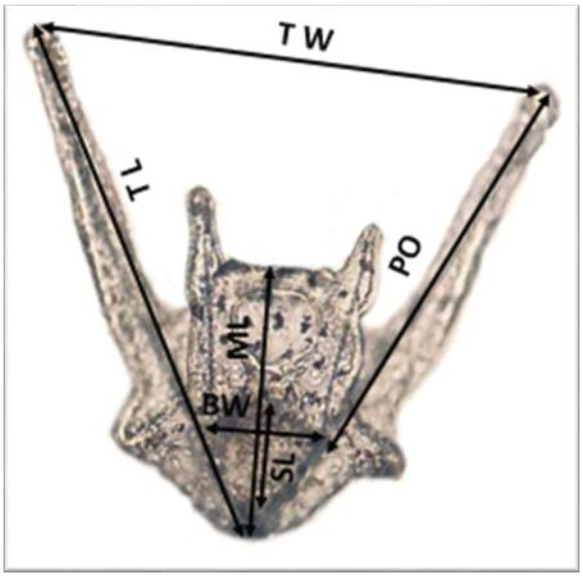

Fig. 1. Four-armed *T. gratilla* pluteus larvae depicting the morphometric characteristics used to compare larval growth performed by TL=Total length, TW = total width, ML=Mid-Body line length, BW= Width base, SL= Stomach length and PO = Postoral arm (40× magnification), as suggested by **Scholtz *et al.* (2013).**

Stocking density and survivorship

To study the survivorship at different stocking rates (treatments), larvae were stocked at three stocking rates of 9, 6, and 3-larvae/ml. Three cylindrical polyethylene tanks each of 400 L volume were used for each treatment. For the 1st treatment, each tank was stocked with a rate of 9 larvae/ ml (*i.e.*: 3,600,000 larvae/ tank). Second treatment tanks were stocked with 6 larvae/ml (*i.e.*: 2,400,000 larvae/tank), while for the 3rd treatment, each tank was stocked at a rate of 3 larvae/ml (*i.e.*: 1,200,000 larvae/ tank). On daily basis, each tank was mixed gently; three samples of 10ml each were collected, and larvae

were counted as number of larvae ml^{-1}. In addition, the percentage of occurrence of each larval stage was estimated.

RESULTS

Induce spawning and fertilization

T. gratilla showed separated sex, and they spawned once a year under the spawning induction condition. Sex determination in sea urchin was not possible by visual inspection, but it was based on the type of released gametes (*i.e.*: eggs orange and sperm white) (Fig. 2). The selected samples were closely equal in diameter; they have mean test length of 5.66 cm and mean weight of 335.64 g.

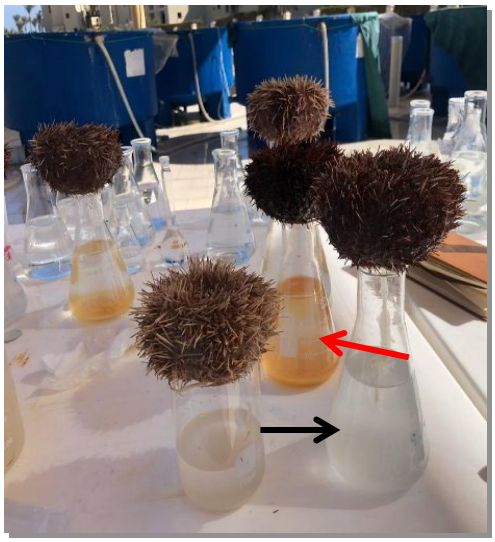

Fig. 2. The male *T. gratilla* shed its gametes as a creamy whitish (black arrow), and the female spilled an orange solution (red arrow).

The most successful induction agent was recorded in the third group, which was exposed to drying under direct sunlight as all individuals responded. In the 2nd group (injection with 1 mole KCL), 7 out of 10 breeders were induced to spawn. In comparison, out of the 10 *T. gratilla* breeders, 4 induced spawning in the 1st and 4th groups (injection with 0.5 mole KCL and mechanical shaking, respectively).

The total number of the released gametes/breeder (fecundity) and the time needed to spawn after induction varied according to the induction agent. The highest numbers of released oocytes were estimated for the 3rd group breeders averaged 18.6 ±1.1 million oocytes/female, while the lowest numbers were recorded for the 4th group averaged 0.94 ±0.00389 million oocyte/female. For the 2nd and 3rd groups, the total numbers of released oocytes estimated were 2.868±0.242 million and 14±0.0039 million, respectively. It was noticed that, females injected with double dose of KCl released 5 folds of oocytes rather than those injected with the lower dose (Table 1 & Fig. 3). The quickest response to induction was recorded in the 2nd group treated with 1M KCL (less than a minute). Other groups were responded within 2 to 5 minutes. There was no mortality recorded within the

breeders of the four treatment groups. The fertilization rate recorded almost 100% success, and 90% of the zygote stage entered the first two cell cleavage. Out of them, 95% reached the prism stage.

Table 1. Four methods tested to induce spawning of *T. gratilla* and the average number of oocytes and period in each trial.

Method	No. of oocytes x10^6 ± SD	Period
0.5M KCl	2.87± 0.242	3 -5 m.
1M KCl	14.00 ± 0.004	40-60 s.
Drying under direct light	18.60 ± 1.100	5-7 m
Mechanical shaking	0.94 ± 0.004	2-3 m

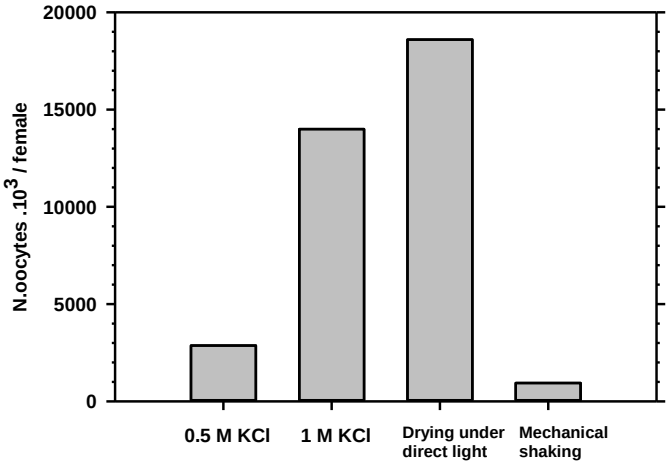

Fig. 3. The tested trials used to induce spawning of *T. gratilla* and the average number of oocytes in each trial, indicating the natural stress as the most effective trial

Embryonic development and larval description

The embryonic stages of *T. gratilla* were accomplished within 72 hours (Table 2). Division process occurred with no aeration. The fertilization happened within 30 minutes and was confirmed in the presence of complete fertilization membrane. No polyspermy cases were recorded. The zygote underwent several series of rapid complete cleavage to reach ciliated blastula within approximately 9hrs. The first division led to a two- cell stage, where the hyaline layer is visible. The four cells stage showed that, the first and the second cleavages are perpendicular to each other. The third cleavage gave the eight- cell stage. The fertilization membrane became invisible no more. The fourth cleavage resulted in sixteen-cell stage. The egg proceeded through sequential division, giving thirty-two, sixty-four, hundred twenty-eight cell stages and finally reaching morula. The plurocellular stage ends with the morula. It has 128- cell blastomeres and the same size as the fertilized egg (50 μm). Nine hours and 16 minutes after fertilization, the blastula was formed. Blastula appeared like a layer of cells surrounding a hollow cavity. There was a thickness of cells in one pole, which is the vegetable pole. Gastrulation started after six hours and

thirty minutes from fertilization. Late gastrula subsequently grew into a planktonic prismatic stage. The pyramid prism larva has a complete digestive tract and is characterized by its noticeable rapid motion and considered intermediate stage between gastrula and two armed echinopluteus larvae. Different embryonic stages of *T. gratilla* until reaching the planktonic prismatic echinopluteus are briefly illustrated in Fig. (4).

Table 2. A schematic elucidation for *T. gratilla* cell divisions. The embryo stages were completed within 72 hrs.

Stage	Period	Size
Fertilized egg	30 m	50 µm
2-cell pluteus	2 h	50 µm
4-cell pluteus	2 h 45 m	50 µm
8-cell pluteus	3 h 45 m	50 µm
16-cell pluteus	4 h 11 m	50 µm
32-cell pluteus	5 h 10 m	50 µm
64-cell gastrula	6 h 30 m	50 µm
Morula	7 h 45 m	50 µm
Blastula	9 h 16 m	50 µm
Early gastrula	11h 13 m	50 µm
Late gastrula	23 h 20 m	50 µm
Early prism	29 h 41 m	50 µm
Late prism	41 h 11 m	50 µm

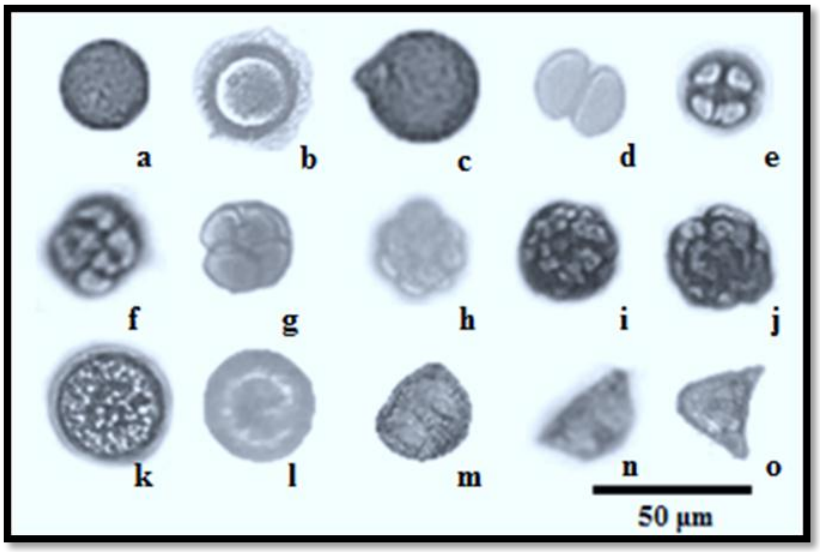

Fig. 4. Microscopic view of *T. gratilla* cell divisions starting from **a.** Mature ova, passing by **b.** Ova surrounded by sperms; **c.** Fertilization of oocyte, **d.** 2-Celled egg, **e.** 4- Celled egg, **f.** 8-Celled egg, **g.** 16-Celled egg, **h.** 32-Celled egg, **i.** 64-Cellled egg, **j.**128 Morula, **k.** Blastula, **l.** Early gastrula, **m.** Late gastrula, **n.** Early prism, until **o.** Late prism. (400X)

On day two after fertilization, the final gastrula gave rise to two- armed echinopluteus larvae. The body is bilaterally symmetrical. The postoral arms are formed, and pigmented spots were scattered on the arms. The digestive Tract is very distinctive. The size of the early larvae of the two-armed stage was TL: 82 ± 4.5 µm (Mean ±SD), TW: 63 ±7 µm, MBL: 45 ± 2.5 µm, PO: 54±2.3 µm, SL: 13 ± 3.6 µm and BW: 25 ± 2.8 µm.

The four- arm echinopluteus larvae were attained after twelve hours from the first stage. The anterolateral arms developed and extended within its simple rods that protruded a spine like structure at the arm's tip. They are connected basely forming oral hood above the mouth. The postoral arms became longer and thicker than the anterolateral arms. The feeding process started at this stage and confirmed by the presence of the algal remnants in the stomach and mouth. The pigment spots became increasingly concentrated. The mouth, esophagus and stomach became clearly evident. The size of the four-arm stage was TL: 180 ± 13.2 µm, TW: 138 ± 5.7 µm, MBL: 72 ± 2.8 µm, PO: 113 ± 2.8 µm, SL: 15 ± 0.5 µm and BW: 43 ± 2.8 µm. At the end of this stage, the base of the postoral arm was bluged and triradiate spicule was observed.

The next stage was marked by the formation of skeletal primordia in the anterior portion of the body between the anterolateral and postoral arm (triradiate spicule). On day 22 after fertilization, there was a noticeable development in the skeletal structure. The six-armed echinopluteus larvae completed. It was featured by the appearance of the posterodorsal arms. The dorsal arch became obvious. The reddish-brown pigmented spots increased on the surface of the larvae. In this stage, larvae could be recognized by naked eyes as forked spots swimming in the tank. The size of the six-arm stage was TL: 238 ± 12.5 µm, TW: 223 ± 15.2 µm, MBL: 134 ± 3.6 µm, PO: 171±17.9 µm, SL: 52 ± 2.8 µm and BW: 58 ± 2.8 µm.

By the end of this stage, the dorsal arch elongated anteriorly and protruded to give the preoral arms. The preoral arms showed the initiation of the eight-armed echinopluteus stage on the day number twenty-five after fertilization. By the end of this stage, the pedicellariae were formed and noticed at the base of posterodorsal and postoral arms. This indicated that the larvae were ready for settlement. The larval body extensions started to bend outside. The rudiment became clearly obvious. The size of the eight-arm stage was TL: 288 ± 2.8 µm, TW: 278 ± 10.4 µm, MBL: 158 ± 10.4µm, PO: 233 ± 12.5 µm, SL: 58 ± 2.8 µm and BW: 85 ± 8.5 µm.

Stocking density and survivorship

The survival rate in the previously illustrated stages varied according to the stocking density. In general, the survival rate possesses a noticeable decline in the day prior to the complete development of each stage. It was noticed that the lower the abundance, the higher the survival (Table 3 & Fig. 5). The lowest survival rate was reported in the first treatment (9 larvae/ ml). The density continues to sharply decline till reaching 1.26 ± 0.120 million larvae/ tank out of 3.6 million larvae/ tank (3 larvae/ml) at the four-armed stage. This experiment lasted for four days, then a mass mortality caught the attention,

and no larvae under this treatment entered further stages. While, the most successful treatment was the third one (3 larvae/ml) that ends up with 0.8 larvae/ml at the eight-armed stage. About 0.33 ± 0.002 million larvae/tank out of 1.2 million larvae/tank showed developing eight-armed stage plus pedicellariae (25% of the initial population). Regarding the second treatment (6 larvae/ml), the density shoed a somehow gradual decrease though in a higher rhythm than the third one. Comparing with the third treatment, only about 0.24 ± 0.056 million larvae/ tank out of 2.4 million larvae/ tank (10% of the initial population) completed the larval metamorphosis stages, which are about 0.6 larvae/ ml.

Table 3. The mean number of echinopleutus larvae (± SD x10^6) of *T. gratilla* under three stocking treatments

Treatment	Stage									
	Late prism		2- arm larvae		4- arm larvae		6- arm larvae		8- arm larvae	
	L/T	L/ml	L/T	L/ml	L/T	L/ml	L/T	L/ml	L/T	L/ml
1st treatment	3.60	9	2.52 ± 0.360	6.30	1.26 ± 0.120	3.00				
2nd treatment	2.40	6	2.28 ± 0.026	5.70	2.16 ± 0.028	5.40	1.40 ± 0.11	3.5	0.24 ± 0.056	0.6
3rd treatment	1.20	3	1.14 ± 0.018	2.90	1.10 ± 0.420	2.60	0.93 ± 0.013	2.3	0. 33 ±0.002	0.8

*L/T; number of larvae per tank (larvae/tank), L/ml; number of larvae per ml (larvae/ml)

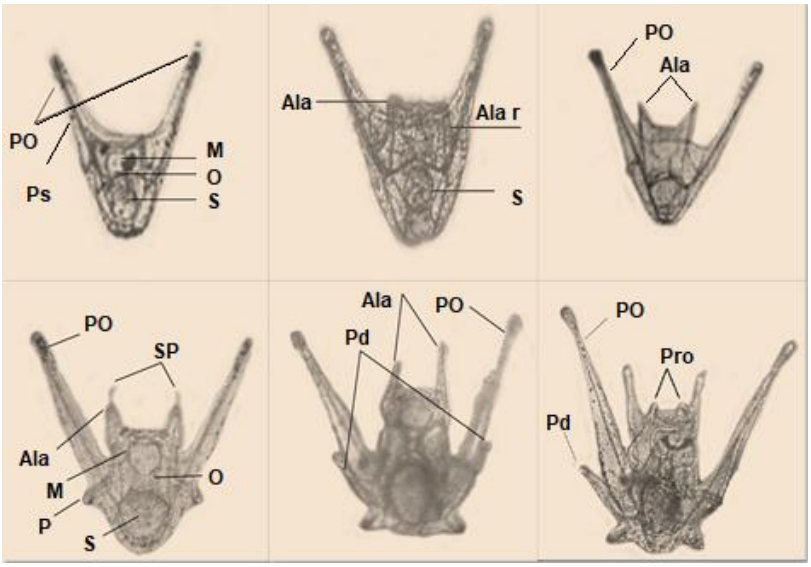

Fig. 5. Larval development of *T. gratilla*. (**A):** Early larvae of the two-armed stage *T. gratilla* showing postoral arms (PO); mouth (M); oesophegus (O); stomach (S) and pigmented spots (Ps). **(B):** Late stage of the two-armed larvae showing the projection of the anterolateral rod (Ala r), anterolateral arms (Ala) and stomach (S). **(C):** Four-armed stage showing postoral arms (PO) and anterolateral arms (Ala). **(D):** The late stage of four armed larvae showing postoral arms (PO) and anterolateral arms (Ala) with two spines protruding outside (SP) and new projection (P) for initiating the six- arm stage. Mouth (M); oesophegus (O), stomach (S). **(E):** Well-developed six-armed larvae showing postoral arms (PO.), and anterolateral arms (Ala) and posterodorsal arms (Pd). **(F):** The late stage of 8- armed larvae showing postoral arms (PO), posterodorsal arms (Pd) preoral arms (Pro), and two pedicellariae at the base of posterodoral and postoral arms indicated the readiness for settlement. (400X)

DISCUSSION

This study was carried out as an attempt to investigate the embryonic and larval development of *T. gratilla* from the Red Sea under laboratory condition. As the aquaculture of sea urchin in Egypt is still behind, it helps in putting an outline to the early life stage of one of the most commercial echinoid worldwide. Many factors affect gametogenesis in sea urchin, most likely temperature, photoperiod, food availability and water turbulence **(Gago *et al.*, 2010)**. This may explain the non-response of some individuals to induction in our study. Despite potassuim chloride (KCl) injection being the mostly used induction method for spawning, our study revealed that exposition to drying under direct sunlight was the most efficient and natural one for obtaining higher number of gametes within the sun set since all individuals responded; however, it took longer time to induce breeders. Besides, no mortality cases wee detected for the broodstocks after the fifth day of induction. The induction by change in the temperature degree and light intensity were also reported by **Sonnenholzner-Varas *et al.* (2018)** as the most effective method for obtaining the gametes of *Tripneustes depressus* in 20 minutes. The far difference in the timing of spawning in the two studies may be attributed to the method followed to rise the temperature as they used thermal shock (4-5°C for 10 minutes), while the current study used exposure to sunlight. Besides, **Wolpert (1958)** determined that, *Tripneustes esculentus* spawned in response to change in light intensity. In contrast, **Gago *et al.* (2010)** reported that, thermal shock induction must not be utilized as an induction technique for spawning echinoid *Paracentrotus lividis* since no spawning occurred on using it in their study. KCl induction came in the next ranking in the number of gametes after changes in temperature. Our study confirmed that, the concentration of potassuim chloride (KCl) is linked to the number of spawned gametes as when the concentration increased, the amount of oocytes increased. This coincides with the finding of **Gago *et al.* (2010)** who used three different concentrations of KCl (0.1, 0.2 and 0.5 M KCl) in inducing *Paracentrotus lividis* from the Ecuador. On the other hand, **Sonnenholzner-Varas *et al.* (2018)** in the previously mentioned study confirmed that, no spawning occur on induction of *Tripneustes depressus* with either KCl or manual shaking, which contradicts our findings. The manual shaking for three minutes gave the least number of gametes and showed no mortality for breeders, which coincides with **Sonnenholzner-Varas *et al.* (2018)**. KCl is considered the traditional used induction method for echinoid spawning; however, some studies reported its toxic effect on broodstock **(Hagen *et al.*, 2002)**. Thus, our results confirm that the non-destructive induction is much better than the classical peristomal injection of KCl. It gives a preliminary approach for substitutional induction methods for the KCl for further detailed investigation.

Cleavages and development of embryo and larva of *T. gratilla* in the Red Sea are similar to those reported in other *tripeneustids echinoids* in other locations of the world, with some modifications in both size and period of accomplish **(Sheppard Brennand *et al.*, 2010; Sonnenholzner-Varas *et al.*, 2018)**. The variation of size and timing of developmental stage may be attributed to the species, the nutritional facts and the rearing temperature but in general most echinoid species attain early pleutus in 2-5 days after fertilization **(Hodin *et al.*, 2019)**. The mean egg size of *T. gratilla* in the Red Sea is 50 µm, which is comparatively less than the recorded mean egg size of *tripeneustids*

echinoids. The egg diameter of *T. gratilla* from the Indo-Pacific is 85.2 ± 1.3 µm; *T. depressus* from the Ecuador is 78.4 ± 2.1 µm, and *T. ventricosus* from the Caribbean and Panamanian coasts is 80.0 ± 1.1 and *T. esculentus* from Barbados, British West Indies **(Wolpert, 1958; Lessios, 1988; Byrne *et al.*, 2008; Sonnenholzner-Varas *et al.*, 2018)**. The eggs underwent 100% fertilization under temperature of 20°C, which coincides with the results of **Rahman *et al.* (2009)** who reported 100% fertilization of eggs of *T. gratilla* at 20 to 30°C. No polyspermy was recorded which indicated that, the used amount of sperm for fertilization was sufficient as the eggs didn't suffer from repeated penetration attempts by numerous sperm.

The developmental timing of hatching blastulae took approximately 9 hours, which is a longer period compared to that (7 hours) consumed for *T. depressus* from the Ecuador **(Sonnenholzner-Varas *et al.*, 2018)**. In contrast, it is considered a short period when compared to *T. esculentus* from Barbados, British West Indies as **Wolpert (1958)** reported that, Blastulae were formed within twelve hours and swimming gastrulae within twenty hours. The larvae of *T. gratilla* in the Red Sea attain competency after 25 days at temperature from 18 to 22 °C. This coincides with the status in Anuenue Urchin Hatchery in Oahu, Hawai'i, where the collected urchin *T. gratilla* is cultured from egg to juvenile in about 23 days **(Hodin *et al.*, 2019)**. **Mos *et al.* (2011)** showed that the competent larvae appeared on the 19th day after fertilization. **Shimabukuro (1991)** also specified the metamorphosis of *T. gratilla* larvae from 20 to 30 days. **Shokita (2001)** stated that the larval cycle in the wild environment can persist for 25 days. **Sonnenholzner-Varas *et al.* (2018)** mentioned that *T. depressus* larvae need 21 days to reach competency. This slight change in the duration of larval competency may be attributed to the change in habitat's temperature as **Rahman *et al.* (2009)** reported that, the higher the temperature, the lower the time required by larvae to develop. *T. gratilla* is tolerant to a wide range of intermediate temperature within the range of 18 to 22°C. Our results are relatively close to those of these authors who reported that the maximum hatching and limits for normal embryonic development and 100% larval development to the two-arm stage of *T. gratilla* from Okinawa Island, Japan were from 22 to 29°C. Our results are less than the border mentioned by **Sonnenholzner-Varas *et al.* (2018)**. These authors experimentally confirmed that the optimum range of temperature for *T. depressus* was between 25 and 27 °C for normal development. This slight difference may attribute to the difference of inhabiting location. **Shokita (2001)** also reported that, larval cycle (prismatic larvae, 4 and 8 arms larvae) in wild environments can persist for up to 25 days, but their planktotrophic larvae are competent to feed at 2.5, 2.0 and 3.5 day post-fertilization, respectively **(Wolpert, 1958; Lessios, 1988; Byrne *et al.*, 2008)**.

The *T. gratilla* larvae was competent to feed once, reaching the four arm larvae after 2 days and 12 hours from fertilization. The same timing was recorded by **Sonnenholzner-Varas *et al.* (2018)**. While, a shorter period was recorded by **Lessios (1988)**, and a longer one was recorded by **Byrne *et al.* (2008)** as 2.5, 2- and 3.5-days post fertilization, respectively. The fed larvae have higher number of triglycerides in most echinopletus **(Sewell, 2005; Meyer *et al.*, 2007; Byrne *et al.*, 2008)**. The optimum stocking density during larval rearing is considered a critical factor because overcrowding can affect access to food resources, reducing both larval growth and survival rates; besides, it affects the number of organic wastes, rise the ammonia level, and in turn, affects the quality of the rearing water. Moreover, the larvae of sea urchin are planktonic

so that they require a sufficient surface area to swim **(Kalam Azad *et al.*, 2010)**. This interprets our findings as the stocking density of 3 larvae/ml showed high survival rate for *T. gratilla* and succeeded to complete the larval metamorphosis stages with higher survivorship. The reared larvae under the densities of 6 and 3 larvae/ml showed much lower rate of mortality, compared to that under 9 larvae/ml. Larvae managed to reach the 8 arm- stage under both densities. The lowest survival rates in both densities were recorded in the period prior to the settlement after completely developed eight-armed stage. Comparatively with *T. depressus* larvae studied by **Sonnenholzner-Varas *et al.* (2018)**, *T. gratilla* follow the same features of *T. depressus* staged, yet the divergence of the morphometries of different larval stages and the delay in some metamorphic stages. The type of supplied food influences the morphology, growth and duration of larval development **(Jimmy *et al.*, 2003)**. Consequently, the delay in metamorphosis, especially in the six-arm stage and the comparatively less size of larvae most likely be attributed to the nutritional status. A reasonable mortality rate occurred throughout the rearing experiment with excluding the mass mortality that occurred on entering the eight- arm stages and since then. Furthermore, this can be due to the food since uni-algal diets might lack one or more of the essential nutrients required for the development of larvae. Mixed algal diet provides better growth performance for *echinopluteus* than uni algal diet **(Ahmed *et al.*, 2016)**. This proposes a further much more research to examine the effects of other phytoplankton species and combinations of some species on larval development of this species.

CONCLUSION

Embryonic and larval developments of *T. gratilla* were investigated for aquaculture proposes in the Red Sea, Egypt. Four induction agents were tested on four groups of ten *T. gratilla* of the same size. The treatments included injections of 0.5 and 1.0 M KCL, 4 hours of drying under direct light, and mechanical shaking. The treatment had a significant different effect on both the number of oocytes released per female and the response times to induction agents. The group treated with drying under direct light had the highest fecundity, while the group treated with mechanical shacking had the lowest, with 18.6 ±1.1 and 0.94±0.004 million oocytes/female, respectively. Furthermore, the group treated with 1M KCL had the quickest response (less than a minute), compared to 2-5 minutes for groups treated with other induction agents. The lower the abundance, the higher the survival, *i.e.,* higher survival of larvae at a density of 3 larvae/ml compared to the other tested densities of 9 and 6 larvae/ml, all fed with the alga *Nanochloropsis* sp. The development of embryos, larval metamorphoses and morphometric parameters were tracked and measured.

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