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Improvement of spawning in *Siganus rivulatus*: the impact of different $LHRH_a$ doses, spawning methods and sex ratio on spawning performance and egg quality

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ABSTRACT

Induced spawning is used to solve failure of complete oocyte maturation and fish spawning in captivity. Hormonal inductions with luteinizing hormone releasing hormone analogue ($LHRH_a$) were examined to induce synchronized ovulation and improve spawning of wild-caught rabbitfish, *Siganus rivulatus*. Three experiments were carried out to study their impact on egg quality. Experiment I: $LHRH_a$ intramuscular injections of 50 and 100 µg kg^{-1} were used for two different groups respectively with a third control group. Experiment II: $LHRH_a$ injection (100 µg kg^{-1}) was used to study the impact of both spontaneous spawning and stripping methods. Experiment III: $LHRH_a$ injection (50 µg kg^{-1}) was used to study the influence on sex ratios. Two successive injections ($LHRH_a$) were used for females in all experiments while male fish received only one dose. In experiment I, the response to higher dose showed a significant increase ($p < 0.01$) in egg diameters, latency time, total number of spawning fertilized eggs and hatching rate. In experiment II & III, a significant improvement ($p < 0.01$) was observed in all egg quality parameters with both spontaneous spawning and sex ratio (2 male:1 female). LHRHa injection is an adjunct to improve spawning performance and *Siganus rivulatus* culture production.

INTRODUCTION

Siganus rivulatus is the most dominant species in the Egyptian Mediterranean waters in the coastal areas of Alexandria and Abu-Qir Bay (Abdel-Maguid, 1997; Faltas and Ehkh, 2003). It belongs to family Siganidae and is considered as an excellent food, especially in the Eastern Mediterranean and Indo-Pacific region (Lam, 1974). Siganids are considered suitable for aquaculture and are economically important (Hara *et al.*, 1986). *Siganus* species are the most promising marine aquaculture fishes and have economic activity used as bio-control agents for aquatic weeds (Jaikumar, 2012). Under culture conditions, a number of fish species don't reproduce spontaneously in captivity due to the presence of reproductive difficulties or dysfunction of fish reproductive physiology (Mylonus *et al.*, 2011).

Environmental manipulation and hormonal therapies were demonstrated as efficient tools for inducing gonadal maturation and synchronization of ovulation, spermiation and spawning (Zohar and Mylonas, 2001).

As early as 1980's, Lam (1982) studied the induced spawning protocol with a single or multiple injection of HCG with different doses (250 to 300 IU Kg^{-1} body weight). While Harvey *et al.* (1985) induced spawning of some *siganus* species with $LHRH_a$ and silastic pellet implantation. The effect of different doses of hormone in captive breeding fish and consequently on latency periods was reported by Zalina *et al.* (2011). Rosenfeld *et al.* (2012) studied the role of gonadotropin releasing hormone agonist (GnRHa) to induce migrating germinal vesicle of oocyte stage, as a maturation-inducing steroid in Atlantic bluefin tuna. Lately, Fahmy and El-Greisy (2014) threw the light on carp pituitary extract (CPE), human chorionic gonadotropin hormone (HCG) and luteinizing hormone releasing hormone analogue ($LHRH_a$) in promoting ovulation and spawning process for *Liza ramada* fish. Some authors found that the spontaneous spawning method (SPT) optimized the spawning time that eventually affected spawning success and produced a higher quality of eggs (Okamura *et al.*, 2014; van Ginneken *et al.*, 2005). The superiority of spontaneously spawning method on manual stripping spawning method was observed in both quality and quantity of eggs fertility (Di Biase *et al.*, 2016). Cejko *et al.* (2016) studied the effects of different stripping methods of female and activation medium on fertilization success in northern pike fish (*Esox lucius*). The merits of the choosing spawning inductions method were pointed out and its consequences on gamete quality (Mylonas *et al.*, 2017). Several authors demonstrated the influence of sex ratio on reproductive performance, the variation in gamete quality and fry production (Adel, 2012; Spence *et al.*, 2008; Tahoun *et al.*, 2008). The present study aims to evaluate the efficiency of a new hormonal ($LHRH_a$) administration protocol on synchronized ovulation, spawning induction / performance and egg quality in *siganus rivulatus* fish. Moreover, evaluates the impact of different spawning methods and sex ratios on egg quality.

MATERIALS AND METHODS

Broodstock managements and Induced spawning

Adult wild rabbitfish, *Siganus rivulatus* {males (90±30 g) and females (130±25 g), were selected from capture stock at pre-spawning season (April and May months 2017) from Northwestern Mediterranean Coastal regions in Alexandria, Egypt. These broodstock samples were transported in tanks with well aerated sea water to the research unit in Marine Hatchery at National Institute of Oceanography and Fisheries (NIOF). Broodstock fish were stocked and acclimatized indoor in 100 liters (L) circular fiberglass aerated sea water holding tanks with a depth of 70 cm with ambient temperature (26 -29 °C), and at 39 g/l salinity. These tanks was filled with large rocks used for fish hiding and supplied with filamentous algae and seaweeds (Ulva) for daily fish feeding and preparation for an experimental spawning design. Forty of broodstock fish were selected according to a biopsy examination to recognize sexes and transferred to 150 L circular spawning tanks. All rabbitfish were kept in a circulating system and water was continuously exchanged and maintained in complete darkness. Prior to hormonal injections fish were anesthetized with clove oil (two drops in the bowel of 5L seawater) to examine the maturation either by gently pressing on the abdomen or using polyethylene cannula. The stage of oocytes maturation, assessed for all females using a biopsy probe, was characterized with only oocytes at the tertiary yolk globule stage on the late week of May. Small samples of oocytes from the middle of the ovary were taken by an ovarian biopsy probe to assess diameters of the oocytes and monitoring the oocytes developmental

stage. Only mature females with oocyte diameters over 450 µm were selected for hormonal injection. Induced spawning trials were carried out from 1st of June to the 1st of July 2017 and all injections were administrated at morning time between 9:00 to 9:30 Am. Female fish was placed with a healthy ripe spermiated male. The sperm motility was monitored by x40 light microscope. Only male fish with higher sperm motility were used in all groups to concentrate only on the state of female and its quality.

Spawning experimental design

Three experiments were designed to evaluate the best effect of doses, different spawning methods and sex ratio on spawning performance and egg quality. The hormonal dose used depended on the initial mean egg diameters. Injections were done when mean egg diameters of female fish was > 450 µm, and the final oocytes maturation was assessed. In all three experiments only females were injected with two successive same doses of $LHRH_a$ hormone at 24 hrs. intervals while males were injected with a single same dose of $LHRH_a$ hormone at the final dose of female injection.

Experiment I: The effect of different dose of $LHRH_a$ injection on spawning performance and egg quality

Twenty fish were selected and divided into two groups. The first group received high dose (100 µg kg^{-1}) of LHRHa injection while the second group received low dose (50µg kg^{-1}). Ten rabbitfish specimens in the control group were only injected with 0.9% saline injection. The three groups were stocked with control sex ratio (2 males: 1 female) and control spawning method (spontaneous spawning method-SPT) to optimize the fertilization rate. After the first injection, ovarian biopsies were repeatedly examined to assess progress in the oocytes diameter and monitoring the beginning of germinal vesicle breakdown (GVBD). The oocyte samples (20 oocytes) were treated with cleaning agent (absolute alcohol 6 ml: formalin 3 ml: glycerol 2 ml; glacial acetic acid 1 ml) (Rodriguez-Gutierrez, 1992). After one day of the last injection all females were undergoing final oocyte maturation (FOM) and ovulation was synchronized. Partial or complete ovulations were recognized with female swollen vent and a soft, rounded abdomen while males were characterized with freely flowed milt under light abdominal pressure from the urogenital pore. Spawning date were almost few days after new moon and spawning took place between midnight to early morning. All fertilized eggs spawned from hormonal gradient injection, latency time, fertilization and hatching rate were determined.

Experiment II: Effect of two different spawning methods (spontaneous and manual stripping- insemination methods) on spawning performance and egg quality.

A group of Ten fish was injected with high dose of $LHRH_a$ (100 µg kg^{-1}) and stocked with (2 males: 1 female) ratio as a control ratio using manual stripping insemination spawning (STP group). The results were compared to the first group from the experiment I (100 µg kg^{-1}) $LHRH_a$ which left to spawn spontaneously (SPT group). While in the STP group, ovulation was checked at 12 hours intervals by gentile abdominal pressure in a caudal direction. The artificial fertilization programme started when females were assessed for ovulation and collected into 5 liters plastic sterilized bowl. Stripped milt was added on the fleshly dry stripped eggs (dry method) and mixed using bird feather for 5 minutes. 100 ml of fresh sterile sea water was added to the mixture and stirred well for 10 minutes to allow maximum fertilization. The bowl was stocked into 150 liters tanks and maintained in the

ambient temperature and with optimized water quality. Spawning date occurred almost after few days of the injection. Egg quality parameters were determined as before.

Experiment III: Effect of different sex ratios on spawning performance and egg quality:

A group of ten fish was injected with lower doses of $LHRH_a$ (50 µg kg^{-1}), stocked with control spawning method (SPT) and 1 male: 2 females sex ratio. The results were compared to the second group from the experiment I (50 µg kg^{-1}) $LHRH_a$ with control sex ratio (2 males: 1 female). After hormonal injection, fish were stocked into 150 liter of two separated fiberglass tanks and maintained in the ambient temperature and with optimized water quality. Spawning date was almost after few days of the injections. Egg quality parameters were determined as above.

Egg incubation

In the three experiments, all injected fish were ovulated, spermiated and spawned. Transparent, demersal, fertilized eggs were siphoned from spawning tanks by an egg collector (mesh 100 µm), washed with sterile sea water and placed in 150 liters incubating hatching tanks at 25 °C ad 39 g L^{-1}. Unfertilized or opaque eggs were removed immediately to avoid any contamination. Latency times (the interval between time of injection and spawning) was determined to the nearest hour from 70 hrs. post injection. The total number of fertilized spawned eggs was estimated as follows: Firstly, the weight of the total fertilized spawned eggs was determined. Secondly, three small samples of (0.5) gram each were taken at random. Thirdly, the number of the fertilized eggs in each sample was counted under a binocular microscope. Lastly, the following equation was applied:

$$\textbf{The number of fertilized spawn eggs} = \frac{\text{No. of eggs in sub sample} \times \text{sample weight}}{\text{Weight of sub sample}}$$

After 2 hours post fertilization, the total fertilization rate (%) for each batch of spawned eggs was determined by calculating the percentage of eggs that reached the 8-cell stage hours. Three replicate subsamples of almost 200 fertilized eggs were used and examined under the light microscope at 40 x magnifications to determine the fertilized and hatching rate. Each sample was scored and averaged for each batch. The fertilization and hatching percentage were calculated as follows:

Fertilization (%) = number of fertilized eggs/total number of eggs × 100

Hatching (%) = number of hatched eggs/total number of fertilized eggs × 100

Statistical analysis

All data were expressed as mean ± SD and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Student t-test was used to compare two groups. Pearson coefficient was used to correlate between quantitative variables. Simple linear regression was assessed to find the equation of some parameters. Significance of the obtained results was judged at the 1% level.

RESULTS

Monitoring the ripping and ovulation of the oocytes

The oocytes of the control group were opaque and at the tertiary yolk globules stage (vitellogenic stage) and there was no progress in the oocytes stages in the ovary and didn't spawn along the time of the experiment while still at vitellogenic stage

even when used all environmental requirements (Figure, 1). While during the different experimental studies and throughout the exogenous hormonal injection, the mean egg diameter of oocytes increased pose an advanced reproductive condition lead to final maturation (GVBD) after first dose and ovulation after second dose of injection (Figure, 2A & B). The broodstock of the different experimental groups spawned after second hormonal injection ($LHRH_a$) with different latency times and fertilized eggs were spherical, transparent and demersal in nature (Figure, 3).

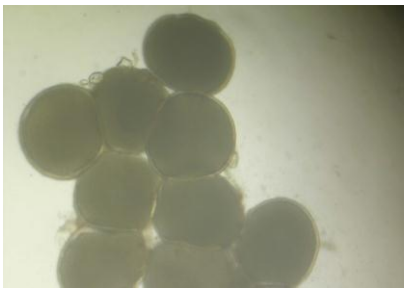

Fig. 1: Photomicrograph of vitellogenic control oocytes in captivity conditions.100 X

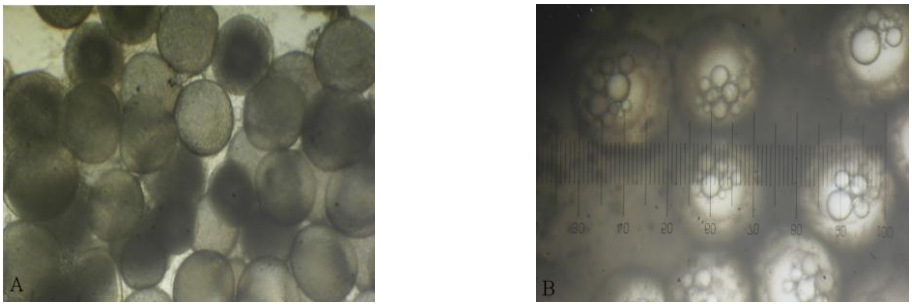

Fig. 2: Photomicrograph of injected oocytes in captivity conditions:
A: Final maturation germinal vesicle breakdown (GVBD), 40 X. B: Ovulated oocytes, 100 X

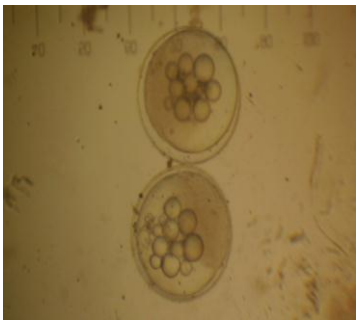

Fig. 3: Photomicrograph of spawned fertilized eggs in captivity conditions, after second hormonal injection of ($LHRH_a$) with different latency times.100 X.

Experiment I: Effects of different $LHRH_a$ doses on spawning quality

In this experiment, the response of broodstock exposed to hormonal injection was positive and produced viable eggs. High dose of hormonal injection (100 µg kg^{-1} $LHRH_a$) was more efficacious than low dose (50 µg kg^{-1} $LHRH_a$) with highly significant difference ($p < 0.01$) in egg diameters, total number of spawning and hatching rate; however, no significant difference ($p > 0.01$) were observed in the fertilization rate (Table 1). It was clear that the high dose of injection (group 1) had significantly ($p < 0.01$) reduced latency time by an average of 10.36 hrs. when compared to low dose of injection (Table 1). There was a strong negative correlation in both groups between egg diameter and both of the total spawned fertilized eggs (r

= -0.97) and latency time (r = - 0.95) (Table 2). The fertilization and hatching percentage rate increased with increasing egg diameters (Table 1). Moreover, strong negative correlations were observed between latency time with the fertilization and hatching percentage rate in both groups of injections (Table 3).

Table 1: Means (± SD) for egg diameters (µm), total egg spawned fertilized ($\times 10^3$), latency time (hrs), fertilization and hatching rate (%) for different doses, spawning methods and sex ratio in different experiments

Experiments	Egg diameter (µm)	Total Egg spawned fertilized ($\times 10^3$)	Latency time (hrs)	Fertilization rate (%)	Hatching rate (%)
Doses					
group 1 (100 µg)	0.64 ± 0.03	382.2 ± 8.1	95.78 ± 2.9	94.52 ± 1	90.41 ± 0.9
group 2 (50 µg)	0.58 ± 0.01	269.8 ± 7.7	106.14 ± 2.9	93.31 ± 1.1	83.5 ± 0.7
p	0.001*	0.001*	0.001*	0.191	0.0016*
Spawning methods					
(SPT group)	0.64 ± 0.03	382.2 ± 8.1	95.78 ± 2.9	94.52 ± 1	90.41 ± 0.9
(STP group)	0.57 ± 0.03	212.9 ± 10.1	77.14 ± 1.5	29.75 ± 1.1	78.35 ± 0.6
p	0.002*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
Sex ratio					
group A (2 males: 1 female)	0.587 ± 0.01	269.8 ± 7.7	106.14 ± 2.9	93.31 ± 1.1	83.5 ± 0.7
group B (1 male: 2 females)	0.527 ± 0.01	11.2 ± 1.8	71.57 ± 2.3	54.6 ± 2.7	12.28 ± 1.2
p	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*

Normally quantitative data was expressed in mean ± SE. and was compared using student t-
*: Statistically significant at $p \leq 0.01$

Table 2: Correlation of egg diameters with different egg quality parameters in the different experimental designs

Experiment		Egg diameter (µm)					
		Doses		Methods		Sex ratio	
		100 µg (group1)	50 µg (group2)	SPT group	STP group	2 males: 1 female (group A)	1 male: 2 females (group B)
Total egg spawned fertilized ($\times 10^3$)	r	- 0.9679*	- 0.970*	-0.967*	0.869*	- 0.970*	- 0.470
	p	0.0003	0.0002	0.003	0.01	0.0002	0.286
Latency time (hrs)	**r**	- 0.955	- 0.951*	-0.955*	- 0.703	- 0.951*	0.308
	p	0.0007	0.0009	0.007	0.077	0. 0009	0.502
Fertilization rate (%)	**r**	0.990*	0.941*	0.990*	0.570	0.941*	0.885*
	p	0.002	0.001	0.002	0.180	0.001	0.008
Hatching rate (%)	**r**	0.9831*	0.965*	0.983*	0.385	0.965*	0.911*
	p	0.006*	0.0004	0.006*	0.393	0.0004	0.004

r: Pearson coefficient
*: Statistically significant at $p \leq 0.01$

Table 3: Correlation of latency time with fertilization and hatching rate in different experimental designs.

Experiment		Latency time (hrs)					
		Doses		Methods		Sex ratio	
		(100 µg) group1	(50 µg) group2	SPT group	STP group	(2 male: 1 females) group A	(1male:2females) group B
Fertilization rate (%)	**r**	- 0.936*	- 0.875*	- 0.936*	0.094	- 0.875*	- 0.091
	p	0.001*	0.009*	0.001*	0.840	0.009*	0.84
Hatching rate (%)	**r**	- 0.968*	- 0.860*	- 0.968*	- 0.116	- 0.860*	0.476
	p	0.0003*	0.012*	0.0003*	0.803	0.012*	0.279

r: Pearson coefficient
*: Statistically significant

Experiment II: Effect of different spawning methods on spawning quality

In the spontaneous method group (SPT), a highly significant difference ($p < 0.01$) was observed in the egg diameters, total fertilized eggs spawned, latency time, fertilization and hatching proportion rate (Table 1). In manual stripping method group (STP), eggs were stripped after 77 hrs. from the final injection. An unpredictable positive correlation ($r = 0.869$) was found between egg diameter and total fertilized eggs spawned (Table 2). Although, strong negative correlations were observed between egg diameters and latency time of the two groups ($r = - 0.955$ and $- 0.703$ for SPT group and STP group, respectively). Moreover, in the SPT group, a positive correlation with statistically high significance ($p < 0.01$) was observed between egg diameters with fertilization and hatching (Table 2). Furthermore, strong negative correlations were observed between latency time with fertilization and hatching percentage rate (Table 3).

Experiment III: Effect of different sex ratios on spawning quality

Reproductive efficiency was varied among the different sex ratios; 2 males: 1 female (group A) and 1 male: 2 females (group B). Highly significant differences ($p < 0.01$) were observed in all egg quality parameters. The latency time was shorter (34.57 hrs.) in group A (Table 1). Inverse strong negative correlations were observed in group A between egg diameter with total fertilized eggs spawned ($r = - 0.970$) and latency time ($r = - 0.951$). In contrast, group B showed no significant differences ($P > 0.01$) in measured egg parameters (Table 2). In both group, a positive correlation was found in the percentage of fertilization and hatching rate with egg diameters (Table 2). Strong negative correlations were observed in group A between latency time with fertilization rate ($r = -0.875$) and hatching percentage rate ($r = -0.86$) (Table 3).

DISCUSSION

Herbivorous fish like rabbit fish are good candidate species for aquaculture, especially in Egypt. In captivity, Mylonus *et al.* (2011) emphasize on reproductive difficulties and physiological reproductive dysfunction of some fish species. The present work studied the effect of three methods on *Siganus rivulatus* spawning and egg quality in captivity.

The effects of different doses of $LHRH_a$ injection on spawning quality

The study shows that both doses of $LHRH_a$ injection protocol can be effectively stimulated the treated broodstock to final oocyte maturation, synchronized ovulation and improved spawning. Higher dose of hormonal injection (100 µg kg^{-1}) was more efficacious in inducing early ovulation and better egg quality when compared to low dose of injection. This is likely due to the effect of high dose of $LHRH_a$ led to increased endogenous secretion of Luteinizing hormone (LH) which controls the final oocyte maturation, synchronized ovulation induction and spawning. This exogenous hormone works on recouping the shortage or endocrine disruption of the endogenous hormone in the captivity through stimulation of sustained elevations of plasma LH. This is mirroring other authors as (Firat *et al.*, 2005; Forniés *et al.*, 2001) who used $LHRH_a$ to induce ovulation and spawning by releasing of endogenous LH from pituitary to stimulate and synchronized spawning. Also, Noori *et al.* (2010) induced ovulation by hormonal administration of gonadotropin releasing hormone agonist (GnRHa) to increase more secretion of pituitary luteinizing hormone (LH) in the blood stream which causes synchronization of spawning and onset of ovulation by reducing the of latency time. This was also echoed by Mateos *et al.* (2002). In contrast, the control group (saline injection) failed to ovulate in

captivity, possibly due to captive fish had low plasma gonadotropin hormone (GTH) levels, which is essentially necessary to initiate the final oocyte maturation and spermiation as reported by Fahmy and El-Greisy (2014). Cultured control fishes failed to undergo oocyte maturation might be due to negative signals on the pituitary gland that suppress the secretion of gonadotropin. From the present study, different doses of $LHRH_a$ treatment used had positive effects on fertility. As well, Zalina *et al.* (2012) found that increasing the dose of $LHRH_a$ hormone (200 µg kg^{-1}) give rise fecundity increased with no significant effect between hormone level on fertilization rate, hatching rate. They suggest that LHRHa hormone intensity could enhance fish spawning. Although, Alcántar-Vázquez *et al.* (2016) found that the highest percentage of fertilized and hatched egg produced at lowest concentration (12.5 µg kg^{-1}) of $LHRH_a$ hormone. Duncan *et al.* (2003) emphasized the important keys for successful induced spawning; doses, kind of hormones and timing of injections in relation to the stage of ovarian maturation. In the present study, shorter latency time was a clear indicator at high dose of hormonal injection. In the present work, the current LHRHa injection protocol showed that $LHRH_a$ are dose dependent. Higher concentrations of LHRHa were more effective with *Siganus rivulatus* in increasing the number of spawned eggs or proportion of fertilization and hatching rate of viable eggs.

The effects of two different spawning methods on spawning quality

The work proved that eggs quantity and quality were statistically significant in the spontaneously spawned group (SPT group) than the manual stripping insemination group (STP group). This was shown by Di Biase *et al.* (2016) who illustrated that spontaneous spawning method could display higher and better egg production in both quality and quantity term than the manual stripping insemination method. Moreover, (Okamura *et al.*, 2014; van Ginneken *et al.*, 2005) found that the SPT method produced a higher quality of eggs. The broodstock themselves, optimized the spawning time to effect spawning success. The time of egg stripping and free collection from spawners is the key to good egg quality. Therefore, it is important to accurately predict the actual time of ovulation for each species when using manual stripping technique. Rough handling of the female fish might retard ovulation or damage eggs and reduce spawning success. Dietrich *et al.* (2007) concluded that broken eggs from manual stripping increase turbidity of the ovarian fluid. Furthermore, there is an inverse relationship between ovarian fluid turbidity and the pH which has the ability to activate sperm motility and enhance the egg quality. Challenges facing this study in the manual stripping insemination group include: sperm competition, sperm: egg ratio, expert hand stripping and establishment of the accurate ovulation time. Optimization of these factors might improve the results. Further studies might prove it. Natural spawning alone has advantages over manual stripping in fish where egg quality deteriorates rapidly after ovulation.

The effect of different sex ratio on spawning quality

The work showed in group A (2 males: 1 female) sex ratio had a direct effect on spawning efficiency and egg quality. Increased competition between males may have improved the effectiveness of reproduction and encourage the spawning process. Black and Black (2013) found that the proportion rate of the fertilized egg spawned was improved when using 3:1 male to female spawning ratio. They was pointed that increasing the male numbers over a single female may supply essential environmental cues that have a great influence on spawning successful trials in yellow fin bream. Nevertheless, Meiri *et al.* (2002) illustrated the existence of an

endocrine response to male socio-sexual behaviors during the reproductive process and discussed the effects of this stimulus on spawning prosperity. Targońska and Kucharczyk (2012) referred that the reproductive efficiency may affected by male fertilization activity in different sex ratio. Tahoun *et al.* (2008) attributed the variation in sperm and the egg quality possibly due to sex ratio. The negative effect on egg quality which resulted, from the group B (1 males: 2 female) sex ratio is possibly due to males not capable to produce a suitable number of spermatozoa to fertilize spawned eggs. This led to the low percentage of egg fertilization. In the present study, lack of potential rivals in the group B may be associated with the lack of sufficient sperm release.

CONCLUSION

Improvement in egg quality of *Siganus rivulatus* was achieved by using higher dose of $LHRH_a$ injection (100 µg kg^{-1}), spontaneous spawning method and using sex ratio 2 males:1 female. *Siganus rivulatus* has an important economical and biological significance therefore, these factors considered to improve spawning activity and quality in its culture production.

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