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Induced spawning of *Liza ramada* using three different protocols of hormones with respect to their effects on egg quality

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Two intramuscular injection strategies were performed for females of *Liza ramada*. The first strategy was applied with two injections; priming dose (CPE, 20 mg per fish). Then, the resolving dose (200 µg/kg of LHRH-a) was given after 24 h later. The second strategy was applied with two injections; the priming dose (3500 IU HCG per fish). Then, the resolving dose (200 µg/kg LHRH-a) was given after 24 h later. The three successful spawning attempts occurred within 3 days when the female received an extra dose of LHRH-a 100 µg/kg (3rd injection) from the second breeding protocol. In both strategies, the males received a single dose of LHRH-a. Ovarian biopsy of hormonal treated and control females were taken at different times after each treatment in order to monitor oocyte development and to determine the time of ovulation, since voluntary spawning was not expected. Measurements of oocyte diameters were carried out at 24 h intervals (0, 24, 48, and 72 h). Diameters of oocytes were measured with a hemacytometer under a light microscope. Mature oocytes with a diameter of 600 ± 50 were more appropriate to injection and spawning. After the injection of the first strategy, the egg diameters ranged from 650 to 680 µm with clear oocyte center and final dose of the resolving dose of the egg diameters were 700 ± 50 µm. After 48 h from the second injection of the first strategy, only one fish spawned. The total number of the spawned eggs ranged from 1 to 1.2 million/fish with no signs of fertilization having a diameter that ranged from 700 to 750 µm. The spawned unfertilized eggs were rounded colourless and transparent. In the second strategy and after the final injection of the resolving dose, the egg diameters were 800±30 µm. After the resolving dose of the first strategy, there was no response of spawning. It showed more successful spawning rather than the first one which showed deformed unfertilized eggs. At the second breeding protocol, fish spawned during the 48 h after the third injection dose. After 48 h, the first three fish were successfully spawned with fertilization rates of 1, 1.8 and 1.6 million eggs/spawn and the percentage of fertilization were 52, 75 and 64%, respectively, but without hatching, and all the fertilized eggs reached the gastrula stage. Control non-injected females were subjected to the same rearing conditions but did not spawn. Two replicate samples of 1 ml of eggs were taken for both control and injected fish. Regarding fatty acids profile, the results reported that the mono-unsaturated fatty acid (MUFA), oleic acid, was highly recorded in the fertilized eggs of the treated females with the second strategy injection while in the first strategy this was not detected except in the unfertilized eggs. The females that was treated with the second strategy injection had more fatty acids particularly the saturated fatty acid, highly unsaturated fatty acid and polyunsaturated fatty acid, (eicosapentaenoic acid (20:5n-3,EPA), docosahexaenoic acid (22:6n-3,DHA) and arachidonic acid (20:4n-6,ARA). The most significant depletions were observed in polyunsaturated fatty acids. Our results suggested that CPE, HCG and LHRH-a promote ovulation and spawning process for both scheduled induction and the frequency of hormone injection influence the fatty acid composition of normal, injected gonad and fertilized eggs of *L. ramada* in relation to egg quality.

Key words: Induced spawning, luetunizing hormone releasing hormone analogue (LHRH-a), gonadotropin hormone (HCG), carp pituitary extract, fatty acids, *Liza ramada*.

INTRODUCTION

Liza ramada is an attractive species for farming in sea, brackish and fresh water (Pillay, 1990). It is considered as an excellent candidate for aquaculture because of the rapid growth rate, ability to efficiently utilize a wide range of natural and artificial foods, tolerance of a wide range of environmental conditions and resistance to disease and stresses. Thin-lipped mullet, *L. ramada,* is an economically important species of fish found in Egypt. However, its supply is almost dependent on the wild. Recently, numbers of wild thin-lipped mullet have been gradually declining. All farming is carried out using the fries in Egypt which increase fears of a further decline in this resource and sharp rise in price. Therefore, the establishment of a method of artificial propagation for thin-lipped mullet is needed to support the supply. Induced spawning using different hormonal injection is the common spawning induction technique in numerous fish species in aquaculture. Failure of the hormonal treatment has been observed in many cultured broodstock (Mylonas and Zohar, 2001), and has been attributed to the low levels of the GnRH-a in circulation due to the rapid metabolism and clearance of this peptide after injection (Gothilf and Zohar, 1991). Other studies reported that the presumed lack of gonadotropin in the circulation of captive fish could result from an insufficient amount of gonadotropin in the pituitary, inadequate secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus, or a combination of these reasons (Hill et al., 2005; Monbrison et al., 2003). To overcome possible failure along the endocrine axis controlling gonadal function, various hormonal treatments have been adopted to induce maturation and spawning. The effectiveness of injections to induce ovulation was evaluated by monitoring the percentage of ovulated fish, the ovarian maturation and ovulation after hormone treatment, fecundity and egg quality (Montchowui et al., 2011). The effect of injection on gonadal maturation was reported by Park (2002) in captivity. Higher or lower doses affected the egg quality, led to spawning failure or low output of hatching. Kagawa et al. (2013) documented variation in egg quality obtained from the female eels injected with exogenous hormones.

Lipids and their constituent fatty acids are utilized as energy sources throughout embryogenesis, and particularly in the later stages of development prior to hatching (Yanes-Roca et al., 2009). Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA) and Archidonic acid (ARA) are the major fatty acids in the total lipid of eggs of most fish and these fatty acids markedly influence the reproductive parameters. There was a marked relationship between egg quality and fatty acid content of eggs. Successful fertilization of eggs and subsequent development of the offspring depend greatly on the quality of gametes produced by the parent fish. Fatty acids and particularly polyunsaturated fatty acids (PUFA) are functionally essential for normal growth, development and reproduction in fish (Sargent et al., 2002). Hormonal induction of final oocyte maturation and ovulation, however, can result in reduced egg quality. Ovulated eggs from fish injected with high dose of GnRH-a had lower fertility, survival to eyeing (eye-pigment formation stage) and hatching rates compared to eggs from fish injected with lower GnRH-a doses or from control fish (Mylonas et al., 1992).

The aim of the present study was to induce spawning of *L. ramada* and study the effect of multiple hormonal inductions on oocyte diameter and spawning performance and also, to study the fatty acid composition of captive normal and injected mullet ovaries with special reference to its effect on egg quality at different stages.

MATERIALS AND METHODS

Broodstock and broodstock management

The broodfish of *L. ramada* were captured in natural water of Mallahat Port Fouad during their migration to the spawning grounds in the Mediterranean Sea in December and transported to the marine hatchery. The broodfish were acclimatized indoors and stocked in well circulating aerated fiberglass tanks (3 m³) with sea water supply of 15 L/min. Fifteen ripe broodfish samples (nine females and six males) were selected carefully. The total length of the females ranged from 34 to 47 cm, and total weight from 750 to 950 g. This study was carried out in the marine hatchery in the National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt. Throughout the experimental period, the water temperature was 15 ± 2°C and salinity of sea water was 32 ± 1.5 ppt. Control non-injected females were subjected to the same rearing conditions. Air and fresh sea water were introduced into the bottoms of the tank. Roofing sheets were added to serve as shelter. Fish were not fed during the entire period of the experiment. A ripe female is deemed as ready for hormone injection when the mean diameter of the eggs is at least 600 µm (when they are in prime spawning condition).

Experimental design and hormone injection strategies

Two intramuscular injection strategies were adopted for *L. ramada* females in the present work. The first strategy was applied by two injections; the first one (priming dose) was carp pituitary extract of 20 mg per fish. After 24 h later, the second dose (resolving dose)

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Abbreviations: **EPA,** Eicosapentaenoic acid; **DHA,** docosahexaenoic acid; **ARA,** archidonic acid; **PUFA,** polyunsaturated fatty acids; **HCG,** gonadotropin hormone; **LHRH-a,** luetunizing hormone releasing hormone analogue; **CPE,** carp pituitary extract.

Table 1. The dosage, number of injections and egg diameters in the two different induction strategies.

S/N	Body weight (g)	Initial Egg diameter (0 h)	First strategy				Second stategy						Total No. of spawnd egg Million	% of Fertilized egg
			Dose		Egg diameter after 1st injection (µm) (24 h)	Egg diameter after 2nd injection (µm) (48 h)	Dose			Egg diameter after 1st injection (µm) (24 h)	Egg diameter after 2nd injection (µm) (48 h)	Egg diameter after 3rd injection (µm)		
			CPE	LHRH			HCG	LHRH 2 nd	LHRH 3 rd					
1	950	650	20	200	680	700	-	-	-	-	-	-	deformed	-
2	900	660	20	200	700	750	-	-	-	-	-	-	1.2 million (unfertilized)	-
3	750	650	-	-	-	-	3500	200	-	700	730	-	-	-
4	850	700	-	-	-	-	3500	200	100	750	790	830	1.8 million	75
5	880	700	-	-	-	-	3500	200	100	760	780	820	1.6 million	64
6	860	720	-	-	-	-	3500	200	100	760	780	800	1 million	52

was given as 200 µg/kg of luteinizing hormone releasing hormone (LHRH-a) (Table 1). The second strategy was also applied by two injections; the first one (priming dose) was 3500 IU of human chorionic gonadotropin (HCG) / fish. After 24 h later, the second dose (resolving dose) was given consisted of 200 µg/kg of LHRH-a (Table 1) and due to signs of incomplete spawning process after the resolving dose of the second strategy. The three successful spawning attempts occurred within 3 days when the female received an extra dose of LHRH-a (3rd injection) from the second breeding protocol (Table 1), thus all females were injected with 100 µm/kg LHRH-a as an extra dose of hormone for performing spawning. During the time of the resolving dose in both strategies, the males received only single dose of LHRH-a (100 µg/kg). Before injections, fish were anaesthetized using phenoxy-2 ethanol (40 mg/L) Sigma. After the resolving dose of the two strategies, the females were transferred with spermiating males (sex ratio 1:3 males to females in all spawning trials to optimize fertilization rates) to an indoor spawning tank and divided into two groups according to the two different breeding protocols of the injected females. Afterwards, the females were maintained together with active males and then some females spawned after 48 h. Prior to stocking of the fertilized eggs, production tanks were set up with filtered seawater adjusted to a temperature similar to that in the broodstock tanks. The fertilized eggs were added to production tanks with very light aeration and no water circulation in order to reduce water movement to the minimum.

The ripe females were characterized by the swelling of the soft and red abdomen and the protrusion of the genital papilla. Ovulation was checked by applying gentle pressure to the abdomen of female broodstock at intervals. At this time, the males were characterized by slim body shape and when squeezed on its abdomen the semen appeared and swim in unison with the females or circle around them. After spawning, floating fertilized eggs were gathered by egg collectors which are placed on the outside of each tank and then removed and incubated in circular incubators with extra oxygen which can be provided by aeration and the different embryonic developmental stages were documented by compound microscope with camera. Five samples of 1 ml from the spawning tanks containing fertilized eggs were examined under a light microscope to assess their fertilization rate and egg diameter and embryonic development. The spent breeders were removed from the tank or aquarium as soon as the spawning is over.

Gonadal biopsy

After anesthesia, the ovary of hormonal treated and control females were biopsied at different times after each treatment in order to monitor oocyte development and to determine the time of ovulation, since voluntary spawning was not expected. Measurements of oocyte diameters were carried out at 24 h intervals (0, 24, 48, and 72 h). Diameters of oocytes were measured with a hemacytometer under a light microscope. Fully-grown females were selected by using a polyethylene cannula (small tube) of 0.85 mm diameter that inserted into the oviduct of the anaesthetized female and a sample of the eggs withdrawn from the middle zone of the ovary using gentle aspiration. The sample of the eggs were fixed in a clearing fixative solution of ethanol, formalin, and acetic acid (6:3:1) allowing follicle separation and observation of cellular content and examined under a compound microscope in order to select fully grown oocyte females for hormonal treatment.

Fatty acid analysis

Two replicate samples of 1 ml of gonads were taken for both control and injected fish. Samples of gonads were carried out at first and second strategy during 1st , 2nd and 3rd injection. All samples were immediately freeze dried at -40°C. Before the assay was performed, dried tissues were ground to a powder individually. TL of each sample was extracted with chloroform–methanol (2:1, v/v), according to the method of Folch et al. (1957). Fatty acid methyl esters were prepared by transesterification with 0.4 M KOH–methanol, and then detected by gas chromatograph (GC-6890A, USA) following Huang et al. (2010). Fatty acid

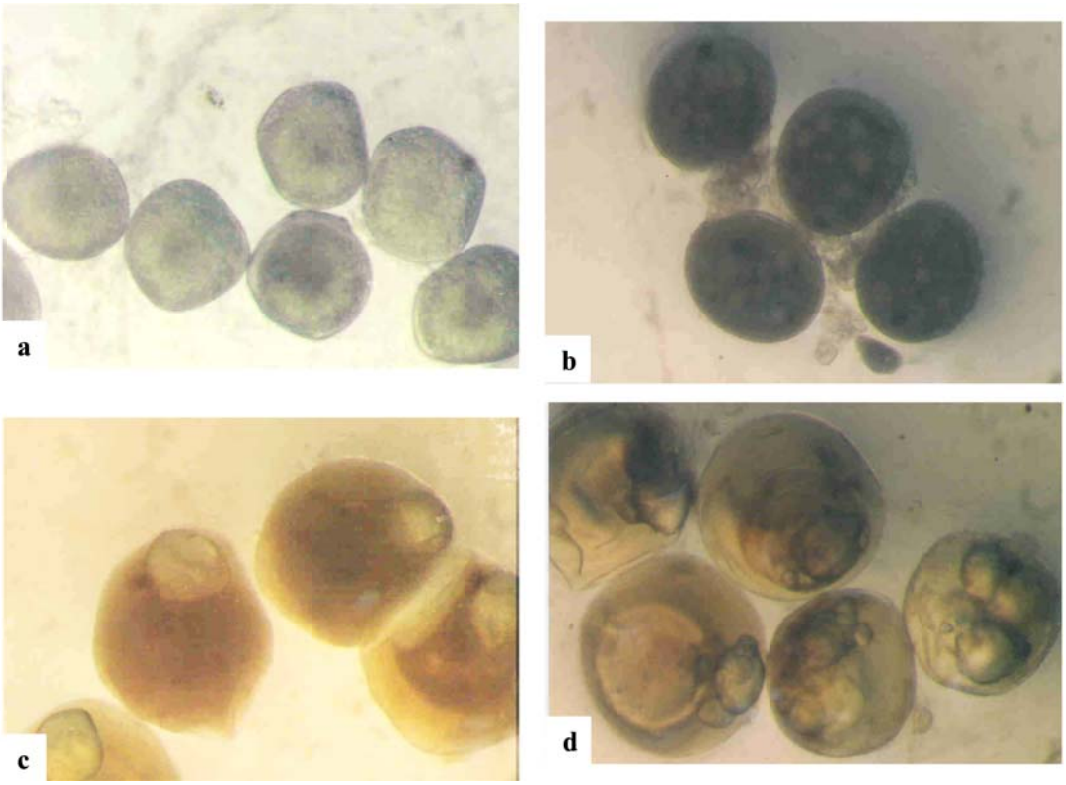

Figure 1. Photograph of cannulated eggs of *Liza ramada* from the ovary showing: a) cannulated eggs with diameters less than 600 µm, b) devoid eggs of control group, c) cannulated eggs of first strategy, and d) cannulated eggs of second strategy.

content was determined using the normalisation method. All measurements were taken in triplicate, and the fatty acids content was expressed as area percentage.

Statistical analysis

Statistical analysis was performed using ANOVA to detect significant differences between means of analytical data on first strategy and second strategy hormone treatment groups for both males and females. All results are presented as mean ± standard deviation.

RESULTS

At the beginning of the experiment, ovarian biopsy in some samples contained grown oocytes (post-vitellogenic stage) with diameters of 475 ± 40 µm having a central and opaque germinal vesicle and a granular cytoplasm (Figure 1a). In other fish samples, the biopsies contained oocytes with diameters of 560 ± 30 µm, having a heterogeneous cytoplasm and a coalescent central yolk vesicle and mature oocytes with a diameter of 600 ± 50 µm having a translucent cytoplasm, a migrating germinal vesicle, and a round yolk vesicle which are more appropriate to injection and spawning (Figure 1b). Accordingly, fish with egg diameters of 600 ± 50 µm were stocked in fiberglass aquaria of 3 m³ sea water, while those less than 600 µm were excluded. Females were injected according to the schedule shown in Table 1. After first injection of the first strategy, the egg diameters ranged from 650 to 680 µm with clear oocyte center. After 24 h of the resolving dose, all fish contained ovulating oocytes with egg diameter of 700 ± 50 µm. Also, it showed the appearance of large oil droplets (Figure 1c). No response of spawning was noticed in all broodstock, but signs of ovulation were started in the abdomen of the females. After 48 h of the second injection of the first strategy, numbers of fish were spawned. The numbers of spawned eggs varied between 1 to 1.2 million/fish with no singe of fertilization. The spawned eggs varied in diameter between 700 ± 30 to 700 ± 50 µm respectively (Figure 2a, b and c). During the next 2 h, the cytoplasm become dense and more or less yellow in colour, all eggs were demerit in the bottom of the tank.

In the 2nd strategy, the females were having a minimum oocytes diameter of 790 µm. After 24 h from the third injection of an extra dose of hormone, cannulated ova ranged from 800 to 850 µm. The ovulating oocytes were presented with a migrating germinal vesicle, and a heterogeneous cytoplasm showing the fusion of oil droplets to a single oil droplet and more or less a coalescent central yolk vesicle (Figure 1d). After 48 h,

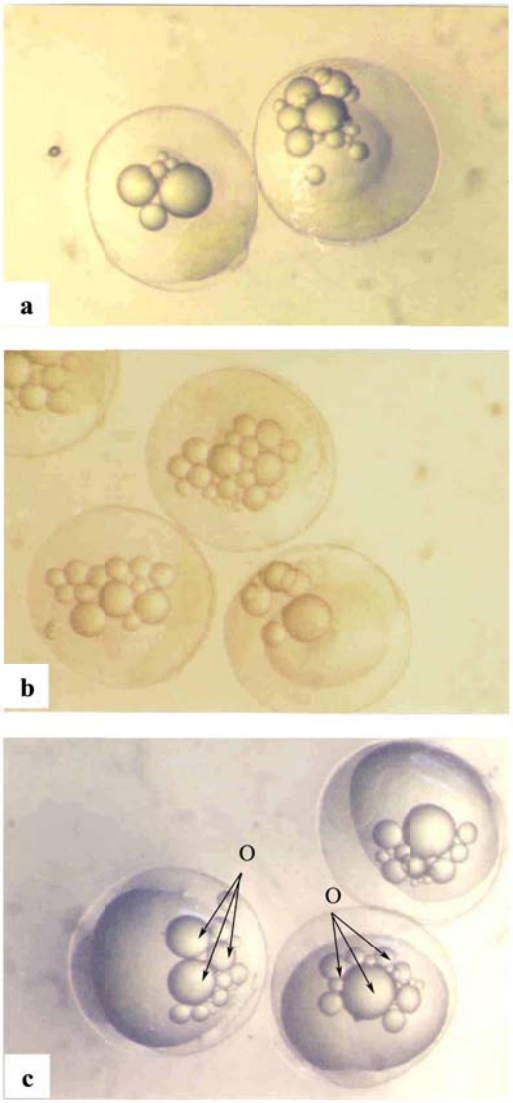

Figure 2. Photograph of spawned unfertilized eggs (a, b and c) having multi numbers of oil droplet (o) with egg diameter of 700 ± 50 μm (a, b) and of 700 ± 30 μm (c), after 48 h of the second injection of the first strategy.

the first three fish were successfully spawned with fertilization rates which were 1, 1.8 and 1.6 million eggs/spawn and the percentage of fertilization were 52, 75 and 64%, respectively, without hatching. All the fertilized egg reached the gastrula stage (Table 1). Control non-injected females subjected to the same rearing conditions did not spawn.

Embryonic development stages

The spawned fertile eggs of *L. ramada* were rounded, colorless and transparent with one oil globule. Thin-lipped mullet has a synchronous ovarian maturation and usually spawns only once a year. The surface of the fertilized egg shell is smooth, however; the yolk was unsegmented. After 20 min from fertilization the egg membrane swells up and separated from the previtelline space. The egg diameter was 800 ± 30 □m and water salinity was 34 ppt. The percent of fertilization varied between 52 and 75% without hatching. The stages take place as follows:

Germinal disc stage

After about 30 min from fertilization, the germinal disc (blastodisc) was in the form of cap-shaped at the animal pole and visible previtelline space.

Cleavage and morula stage

The first cleavage takes place at about 45 min from fertilization which divided the blastodisc into two blastomeres of approximately the same size (Figure 3a). After $1^{1/2}$ h, the second cleavage takes place at right angle to the first one to give four blastomeres. The third cleavage took place at age of about $2^{1/2}$ h after fertilization. This cleavage is parallel to the first one and producing eight blastomeres which were arranged in the 2 rows (Figure 3b). Cleavage continues but with irregular pattern forming a multicellular cap (morula stage (Figure 3c) at age of about 5 h after fertilization). The mean total diameter of egg was 850 ± 20 μ, the height of blastodermal cap was about 265 μm and the oil globule was 350 μ.

Blastula stage

After about 7 h of fertilization, the blastoderm was flattened out over the yolk forming a cellular cap (340 μm) and the total egg diameter was 865 ± 10 μm (Figure 3d).

Gastrula stage

This stage is characterized by cell movement by the epiboly process. The cells of blastoderm thickened forming embryonic shield and moved over the whole surface of the yolk (Figure 3e). The egg diameter was 880 μm, while at the end of gastrula stage, the egg diameter was 900 μm; the egg was recognized with a fold at the animal pole.

Organogenesis stage

At early stage of organogenesis the embryonic shield was clearly recognized with a thickening along the dorso-

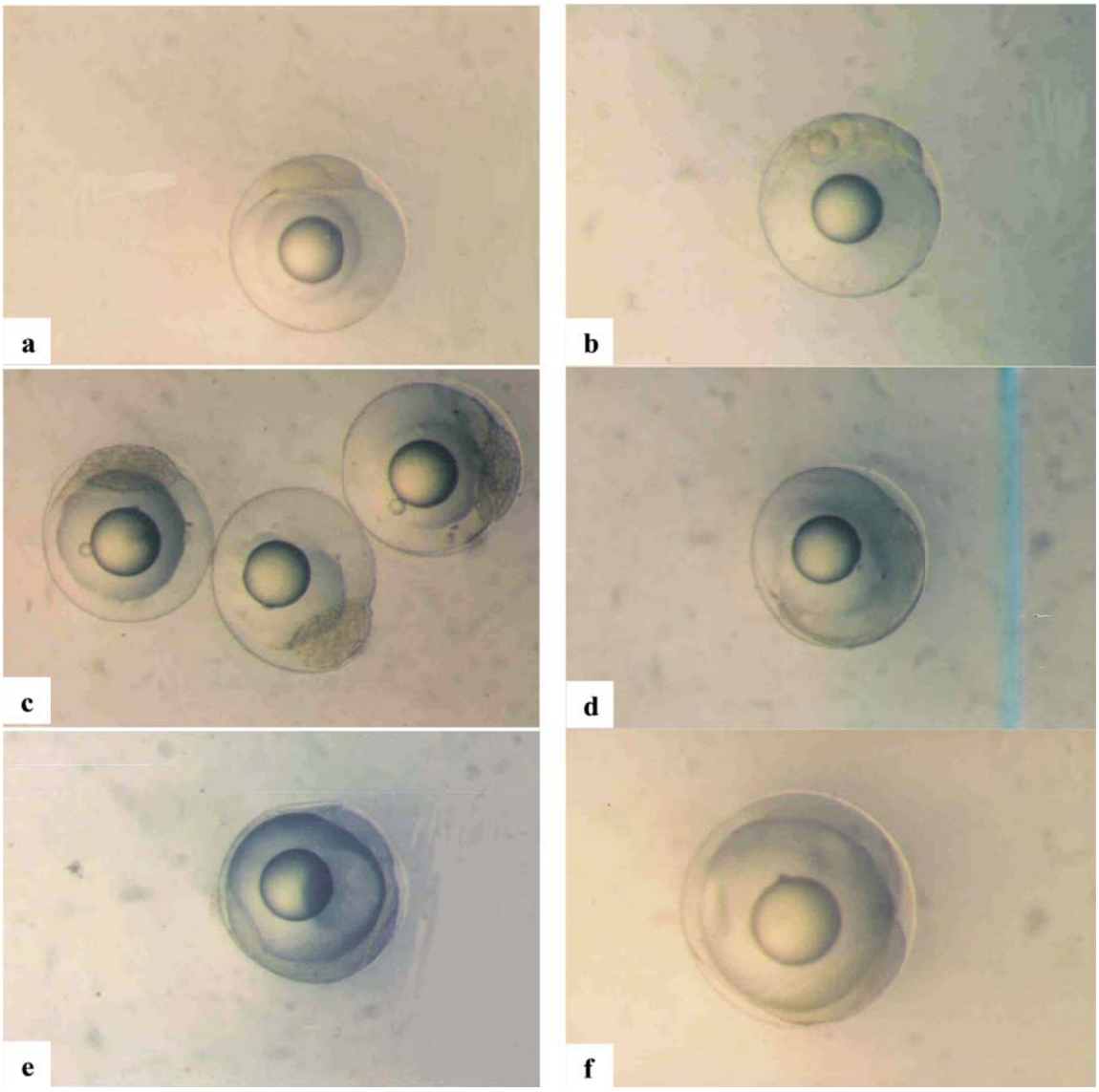

Figure 3. Photomicrograph of early embryonic stages in fertilized eggs with single oil droplet (until gastrula stage). **a,** First cleavage in fertilized eggs divided the blastodisc into two blastomeres of approximately the same size(x10). **b,** Third cleavage took place and producing eight blastomeres which were arranged in the 2 rows(x10). **c,** Cleavage continues with irregular pattern forming **a** multicellular cap, morula stage and blastomer formation (x10). **d,** Blastula stage: blastoderm was flattened out over the yolk forming a cellular cap(x10). **E,** Gastrula stage: The cells of blastoderm thickened forming embryonic shield and moved over the whole surface of the yolk.(x10). **F,** End of gastrula stage and beginning of early stage of organogenesis: the embryonic shield was clearly recognized. (x10)

lateral margins of the yolk after 8 h of post fertilization and with aggregation of cells but did not form any clear cut division or boundaries (Figure 3f). After this stage, shrinkage of the eggs and sinking at the bottom of the tank were observed.

Fatty acids profile in the gonads and eggs at the first and second strategy protocols in treated fish

Fatty acid compositions of *L. ramada* were determined during different stages of injected gonad (ovary and testes) and spawned eggs (fertilized and unfertilized eggs) at the first and second strategy as shown in Tables 2, 3, 4.

Tables 2 and 3 show the overall average fatty acid composition in ovary and spawned ova of *L. ramada* in the two injections strategies together with the range of individual fatty acids observed. Linolinic acid (ω3) and highest value of Σ ω6 fatty acid were recorded only in fertilized egg. The essential fatty acids (EPA, DHA, ARA, and PUFA) represented in un-respond ovary were

Table 2. Fatty acid composition of ovary and spawned ova of *Liza ramada* in the first injection strategies.

Fatty acid	Control (%)	1st Strategy (%)	Unrespond (%)	Unfertilized (%)
Leuric acid	0.15±0.005	0.25±0.006	ND	0.35±.02
Myristic acid	0.83±0.006	4.53±0.52	4.04±0.05	12.68±0.11
Palmitic acid	8.57±0.2	9.90±1.36	8.38±0.09	16.64±0.62
Margarinic acid	2.21±0.008	ND	0.92±0.02	3.97±0.40
Stearic acid	10.16±0.75	4.120.09	16.47±0.96	0.003±0.001
∑ Saturated acid	21.91	18.80$	29.81*	33.64**
Oleic acid	1.82	24.85	5.16	ND
∑ MonoSaturatedacid	1.82	24.85	5.16	ND
Linoleic acid ω6	ND	1.01	ND	2.58±
Linolinic acid ω3	ND	ND	ND	ND
Arachidonic acid ω6	2.17	ND	4.97±	1.98±0.40
Eicosapentaenoic acid ω3	ND	3.64±0.37	ND	1.06±0.14
Docosahexanoic acid ω3	0.23±0.003	0.24±0.02	0.05±	0.35±0.001
∑Polyunsaturated acid	2.41	4.90$	5.03**	5.97$
∑ω6	ND	1.01	4.97	4.56
∑ω3	0.23	3.89$	0.05*	1.41$
DHA/EPA	ND	0.07	ND	0.33
ARA/EPA	ND	ND	ND	1.87
∑ ω3 / ∑ ω 6	ND	3.85	0.01	0.31

$, Not significant; *significant; ** highly significant (p<0.05); nd, not detected.

Table 3. Fatty acid composition of ovary and spawned ova of *Liza ramada* in the second injection strategies.

Fatty acid	Control (%)	2nd strategy (%)	Spawn (%)	Fertilized (%)
Leuric acid	0.15±0.005	1.41±0.02	1.68±0.02	0.29±0.11
Myristic acid	0.83±0.006	ND	3.23±0.08	0.29±02
Palmitic acid	8.57±0.2	14.01±	26.27±1.08	8.62±0.73
Margarinic acid	2.21±0.008	1.60±0.05	3.13±0.2	2.24±0.66
Stearic acid	10.16±0.75	24.14±1.02	25.67	5.21±0.53
∑ Saturated acid	21.91	41.16**	59.97**	22.87*
Oleic acid	1.82	6.27	2.85	21.22
∑ MonoSaturatedacid	1.82	6.27	2.85	21.22
Linoleic acid ω6	ND	ND	1.37	0.87±0.02
Linolinic acid ω3	ND	ND	ND	0.17±0.0
Arachidonic acid ω6	2.17	3.07±	2.80	0.17±
Eicosapentaenoic acid ω3	ND	ND	1.24±	2.49±
Docosahexanoic acid ω3	0.23±0.003	0.35±0.009	0.23±0.3	0.39±
∑Polyunsaturated acid	2.41	3.42**	5.64$	10.90$
∑ω6	ND	3.07	4.17	7.86
∑ω3	0.23	0.35*	1.47$	3.04$
DHA/EPA	ND	ND	0.18	0.14
ARA/EPA	ND	ND	2.26	2.81
∑ ω3 / ∑ ω 6	ND	0.11	0.35	0.39

$, Not significant; *significant; ** highly significant (p<0.05); nd, not detected.

recorded as 1.06, 0.35, 1.98 and 5.97%, respectively (Table 2). Arachidonic acid recorded the highest value in un-respond ovary. The results show no significant in the un-respond ovary and unfertilized eggs in case of 1st

Table 4. Fatty acid composition of testes of *Liza ramada* in the second injection strategies.

Fatty acid	1st injection testes (%)	2nd injection testes (%)	Spawned (%)
Leuric acid	0.54±0.06	0.97±0.07	1.06 ± 0.47
Myristic acid	0.33±0.18	0.35±0.05	0.31 ± 0.10
Palmitic acid	33.14±2.77	12.22±1.07	1.55 ± 0.41
Margarinic acid	1.36±0.33	-	3.13 ± 0.81
Stearic acid	11.17±0.76	21.23±2.67	57.45 ± 5.62
∑ Saturated acid	46.51	34.77$^{\$}$	65.50$^{\$}$
Oleic acid	46.07 ± 1.02	-	3.48 ± 0.11
∑ Monosaturatedacid	46.07	-	3.82
Linoleic acid ω6	0.48 ± 0.07	0.43±0.07	-
Linolinic acid ω3	-	0.83±0.18	
Arachidonic acid ω6	2.91 ± 0.93	3.23 ± 2.55	-
Eicosapentaenoic acid ω3	2.24 ± 1.51	-	-
Docosahexanoic acid ω3	-	-	1.62 ± 0.62
∑Polyunsaturated acid	5.63	4.49$^{\$}$	1.62$^{\$}$
∑ω6	3.39	3.66$^{\$}$	-
∑ω3	2.24	0.83$^{\$}$	1.62$^{\$}$
DHA/EPA	-	-	-
ARA/EPA	1.29	-	-
∑ ω3 / ∑ ω6	0.66	0.22	-

strategy except in ARA and in MUFA (Table 2). In unfertilized eggs, the MUFA value was not detected while linoleic acid ω6 recorded the highest value (Table 2).

The results reported that essential fatty acids that affected egg quality such as EPA, DHA, ARA, PUFA and MUFA were recorded as 2.49, 0.39, 0.17, 10.90 and 21.22%, respectively, in the fertilized eggs of second strategy injection (Table 3). While fatty acid results revealed that mono-saturated fatty acid, oleic acid, was highly recorded than polyunsaturated fatty acid and highly unsaturated fatty acid. In the present study spawned ovary had a high record of saturated fatty acid (Table 3). Highly significant differences were recorded in first and second strategies between fertilized and unfertilized eggs particularly in saturated fatty acid, monounsaturated fatty acid, polyunsaturated fatty acids and Eicosapentanoic acid (EPA) ω3 (Table 2, 3 and Figure 4). There are no differences in the level of DHA during different injection periods. The highest value of Σ ω6 fatty acid was recorded in the fertilized ova, while it does not record in control fish (Table 3). The ratio of (n3) / (n6) PUFA in the fertilized eggs and unfertilized eggs were 0.39 and 0.31, respectively (Table 2, 3 and Figure 4). Fatty acid results in the first strategy treated females were lower than that in the second strategy. The lowest PUFA was (22:6n-3, DHA) in both strategies (Tables 2 and 3).

Fatty acid composition of testes in male fish were determined during different stages and spawning testes of the second injection strategy only as shown in (Table 4 and Figure 5). The present results report that the mono-saturated fatty acid, recorded in the 1st injection stage 46.1% and spawned stage only 3.8%, while saturated fatty acid results revealed high level in 1st, 2nd injection and in spawned testes as (46.51, 34.7 and 65.5%, respectively). After 1st injection, the major essential fatty acid that affected the quality of EPA, ARA, and PUFA were recorded as 2.25, 2.91 and 5.63% respectively. There was no DHA value detected in this treatment. Docosahexanoic acid ω3 just detected with a low level in spawned male. In the present study, the level of arachidonic acid in 1st and 2nd injection was 2.91 and 3.23%, respectively. There was no trace of arachidonic acid in the spawned testes (Table 4 and Figure 5).

DISCUSSION

The present study demonstrates the effect of different hormonal protocols injection on spawning of *L. ramada* and fatty acid composition in gonads and eggs during experiment. Hormonal injections have been adopted to induce ovulation and spawning in *L. ramada* fish. Human chorionic gonadotropin hormone (HCG), Luetunizing hormone releasing hormone analogue (LHRH-a) and Carp pituitary extract (CPE) were used in various combinations and tested as spawning agents to increase the final oocyte maturation (FOM) and ovulation in captivity as reported by Duncan et al. (2003). Dosage of HCG and LHRH-a used in the present study were based on results of previous investigations as indicated by Monbrison et al. (2003). Other experiments revealed that

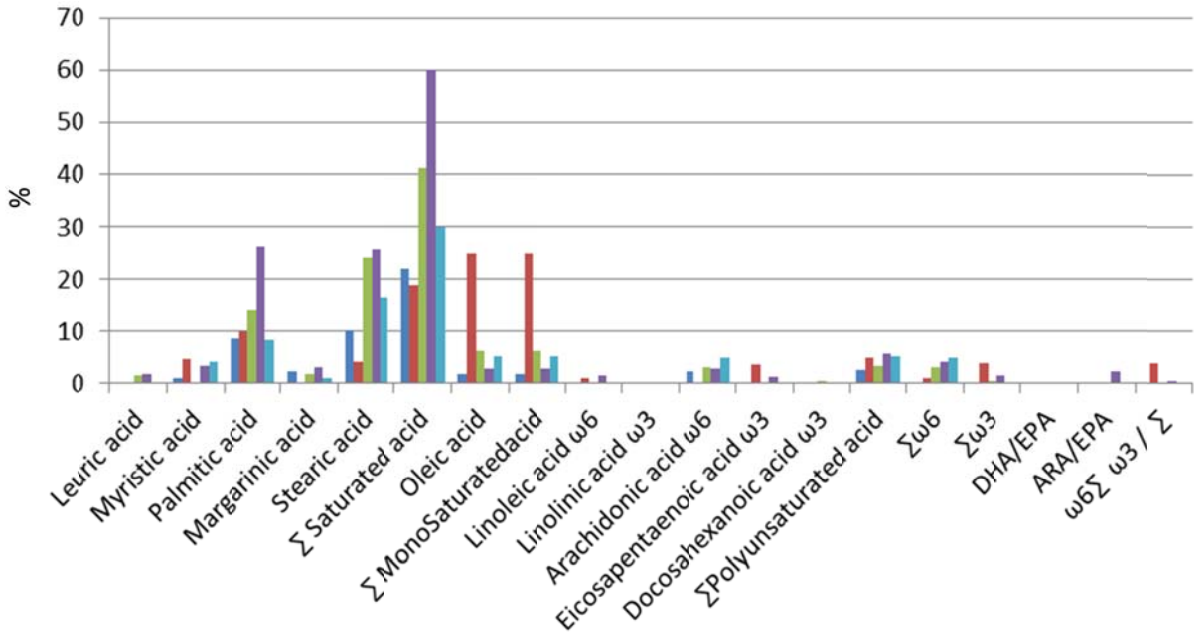

Figure 4. Fatty acid composition of ovaries of *Liza ramada* during different stages and spawned eggs in the two injection strategies.

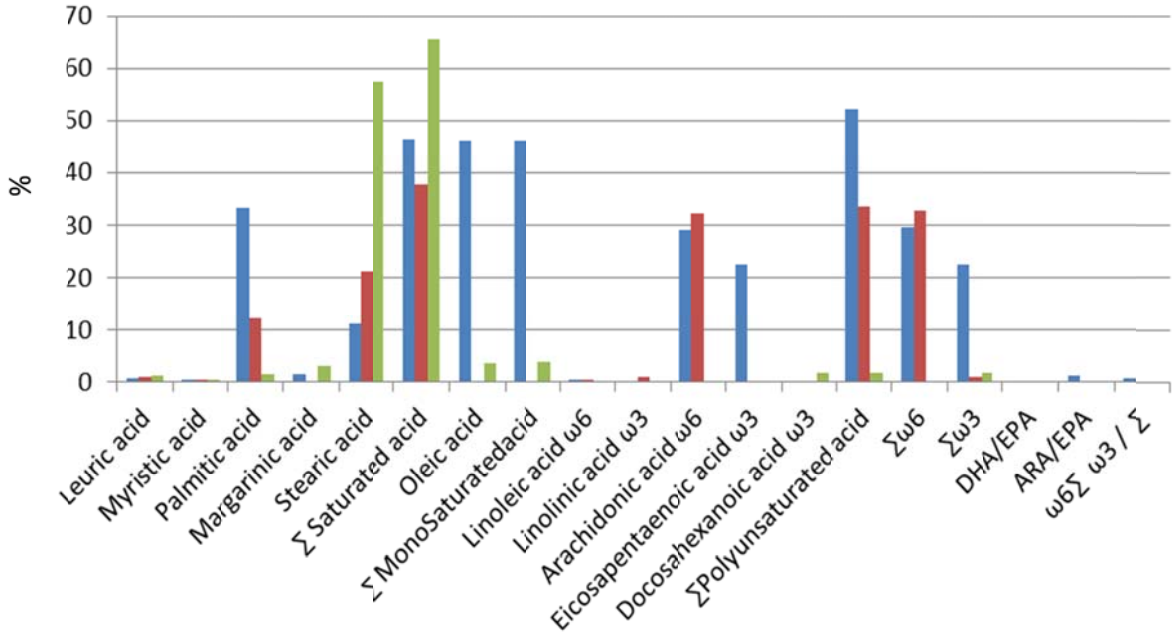

Figure 5. Fatty acid composition of testes of *Liza ramada* during different stages and spawning male in the second injection strategies only.

females grow faster than males because spermatogenesis occurs much faster than ooogensis during gonadotropin treatment (Ohta et al., 1997). Therefore, in our study, the males received only half dose of the females and it was necessary to start hormonal treatment in males later, after females have started.

Monbrison et al. (2003) indicated that the presumed lack of gonadotropin in the circulation of captive fish could result from an insufficient amount of gonadotropin in the pituitary, inadequate secretion of gonadotropin

releasing hormone (GnRH) from the hypothalamus, or a combination of these reasons to overcome possible failure along the endocrine axis controlling gonad function. The minimum egg diameter for successful induc-tion of spawning in grey mullet in our study was 600 µm as reported by El-Gharabawy and Assem (2006). They reported that female mullet were first observed to possess early vitellogenic oocyte in November and weeks later, vitellogenic had progressed to the point where spawning could be induced when mean oocyte diameter reach at least 600 µm. In contrast, Monbrison et al. (2003) indicated that once oocytes of grey mullet, *Mugil cephalus* reached the diameter of 500 µm females were induced to spawn with hormonal treatment.

In this study, the hormonal injection frequency had an influence on increasing the oocyte diameter particularly after 48 h of the last injection in the second strategy. The cannulated ova ranged from 800 to 850 µm and the ovulating oocytes were presented with a migrating germinal vesicle, with a single oil droplet. These results indicate that hormones injection frequency affects efficiency of nutrient transfer from liver to gonads (Naz, 2009). The nutrient reserves of the fish eggs are used by developing larvae both as substrate for energy metabolism and as structural component in membrane biogenesis (Sargent, 1995).

Marino et al. (2003), reported that in the induced breeding of the dusky grouper, the batch of the larger vitellogenic oocytes grew from 350-450 to 800-830 µm as a result of GnRH-a effect, and underwent completion of vitellogenesis, FOM and ovulation within 70 to 80 h, while a batch of smaller vitellogenic oocytes continued their vitellogenic growth and increased in diameter as a results of hormonal injection.

In this study, after 48 h from the second injection dose (resolving dose) in the first breeding protocol, only one fish spawned. The ovulated ova were about 1,000,000 their diameter varied between 700 and 730 µm. The spawned deformed unfertile eggs of *L. ramada* were rounded, colorless and transparent with more than one oil droplet. The survival of eggs which initially contained multiple oil droplets was always low (El-Gharabawy and Assem, 2006). After 48 h from the 3rd injection, the injected females were successfully spawned. The spawned fertile eggs were rounded, colorless and transparent with one oil globule with fertilization rates that varied between 1 and 1.8 million eggs/spawn and with average diameters of spawned eggs that ranged between 800 and 830 µm and the percentage of fertilization varied between 52 and 75 % but without hatching (until gastrula stage). In agreement with the present results, Lee et al. (1987) reported that some spawned egg diameter with more than 800 µm do not have big fertilization rate or no hatching rate. Ohta et al. (1996) showed that over-ripening after ovulation is one of the main factors affecting quality, but the percentage of buoyant eggs and fertilized eggs is usually low even if the eggs are stripped immediately after ovulation. These findings suggest that the cause of the poor quality is not only over-ripening after ovulation. In contrast with the present result, Lee et al. (1987) found that 94% of spawning success rate of fertilized ova was achieved with first strategy in which CPE is a priming dose followed by LHRH-a in a resolving dose. The spawned fertile eggs of *L. ramada* were rounded, colorless and transparent with one oil globule. The percent of fertilization varied between 52 and 75% but without hatching. The egg diameter was 880 µm, while at the end of gastrula stage the egg diameter reached 900 µm and the egg recognized with a fold at the animal pole. After this stage, shrinkage of the eggs and sinking at the bottom of the tank was observed then all eggs were died. This sudden egg mortality was believed to be due to excessive water turbulence in the incubation system (Main et al., 2004).

In the present work, during the time of the resolving dose in both strategies, the males received only single dose of 100 µg/kg (LHRH-a). In contrast with the present results, Shehadeh et al. (1973) concluded that grey mullet males were able to complete spermatogenesis without hormonal treatment. This information is very important for commercial hatcheries for optimum collec-tion of food quality eggs leading to higher larvae production (Sahoo et al., 2005). The present study demonstrates that the fatty acid compositions in gonads and eggs from second strategy group were significantly higher than the first strategy group ($p < 0.05$). The major fatty acid in the injected ovary that was treated with the second strategy injection were saturated fatty acid, monounsaturated fatty acid, polyunsaturated fatty acid, highly unsaturated fatty acid, (eicosapentaenoic acid (20:5n-3,EPA), docosahexaenoic acid (22:6n-3,DHA) and arachidonic acid (20:4n-6,ARA). It was determined that saturated fatty acid ratio is high in comparison with unsaturated fatty acid. Evans et al. (1998) suggested that the decline in the unsaturated to saturated fatty acids ratio, and total lipid indicates a reduction in egg membrane fluidity. Most significant depletions were observed in polyunsaturated fatty acids. Ballestrazzi et al. (2003), reported that the content of unsaturated fatty acids, particularly the ω3 fatty acid series (EPA and DHA) significantly decreased with increasing levels of saturated fatty acids. Among the PUFA, eicosapentaenoic acid (EPA, C20:5 n-3), docosahexaenoic acid (DHA, C22:6 n-3) and arachidonic acid (ARA, C20:4 n-6)), have been shown to play pivotal role in regulation of oocyte maturation and ovulation (Pickova et al., 2007).

In our study, the mono-unsaturated fatty acid was highly recorded in the fertilized eggs of the treated females with the second strategy injection. Results obtained in the study are supported by those of Bulut (2004). The high levels of the major fatty acids (mainly MUFA) found in the common snook shows their importance as energy store for embryonic development (Almansa et al., 2001). Fatty acid that results in the first

strategy treated females were lower than that in the second strategy and MUFA value was not detected in unfertilized eggs. In contrast, in the second strategy, fatty acid results revealed that the fertilized eggs contain more mono-unsaturated fatty acid and n-6 fatty acid than highly unsaturated fatty acid. Furuita et al. (2003), reports that higher levels of ω6 fatty acids have negative effect on egg quality. The ratio of (n3)/(n6) PUFA in the fertilized eggs and unfertilized eggs were 0.39 and 0.31, respectively. This ratio was very low compared to similar ratio in many other marine species (Naz, 2008; 2009). It is well documented that the (n3/n6) PUFA ratios in snook eggs is lower (2.52%) than the typical marine fish (2.9%) (Yanes-Roca et al., 2009). Deficiency or excess of ω3 highly unsaturated fatty acids (HUFA) has been found to depress egg quality of several species (Furuita et al., 2000; 2002). May be the hormonal manipulation impaired ovarian function that cause the result of the diminished of ω3 fatty acid particularly absence of linolenic acid which is the precursor of ω3 fatty acid series and reflecting the diminished ratio of ω3/ ω6 fatty acid. These fatty acids play an important structural role as components of phospholipids in fish bio-membranes and are associated with the membrane fluidity and correct physiological functions for bound membrane enzymes and cell functions in marine fish (Bell et al., 1986). Considering the relative low proportion of ratio of (n3)/(n6) PUFA, the low value should be attributed to the low value of 22:6n-3, DHA in the n3 PUFA in both strategies. Ferna´ndez-Palacios et al. (1995) observed poor hatching rates and survival in sea bream, linked to low fatty acid content. Also, Furuita et al. (2002; 2003) observed the same thing in Japanese flounder. In marine fish, EPA and especially, DHA are regarded as EFAs due to their necessity for good development and growth.

The results of the previous and the present experiment suggest that high levels of ω6 fatty acids, low levels of ω3 and low ratio of ω3/ ω6 may be the reasons of the negative effect on egg quality of *L. ramada* fish. In our results in males, mono-unsaturated fatty acid was recorded in the 1st injection and spawned stages. It was determined that monounsaturated fatty acid ratio is high in comparison with the spawned stage. In the same stage, the most significant depletions were observed in polyunsaturated fatty acids and DHA. Eicosapentanoic acid (EPA) (ω3) and DHA were recorded only in one different stage. In the present study, the level of arachidonic acid in 1st and 2nd injection were 2.91 and 3.23%, respectively and there was no trace of arachidonic acid in the spawned testes.

Izquierdo et al. (2001) reported that EPA and ARA modulate steiodogenesis in the testes and the timing of spermiation may be delayed and the fertilization rate reduced in the conditions of deficiency of these fatty acids. ARA stimulates testicular testosterone production in goldfish testes and ovaries by conversion to prostaglandin (Mercure and Van Der Kraak, 1996). In our study, the lower EPA, and DHA in both females and males of the 1st and 2nd injection strategy groups are indication of the negative effect of the embryonic development process. In general, a certain level and correct balance between the three essential fatty acids EPA, DHA and ARA seem to be important for successful reproduction and embryonic development. This finding can be used as an important and quick, diagnostic marker to predict the egg viability of wild *L. ramada* in order to save time and money (Bell et al., 1997).

Conclusion

In conclusion, in both strategies, human chorionic gonadotropin carp pituitary extract and LHRH can influence the developmental process and induce spawning of *L. ramada* and they have an effect on the biochemical composition during the development stages at different hormone injection protocols, spawning and fertilized eggs. The second intramuscular injection strategy (the priming dose (3500 IU HCG per fish and the resolving dose (200 then100 µg/kg LHRH-a) showed the best results for successful induce spawning with fertilized eggs and embryonic development but without hatching. Poor survival may be linked to low fatty acid content. In the second strategy, fatty acid results revealed that the fertilized eggs contain more mono-unsaturated fatty acid and n-6 fatty acid than highly unsaturated fatty acid that may have negative effect on egg quality. Deficiency or excess of highly unsaturated fatty acids ω3 (HUFA) has been found to depress egg quality of several species. The hormonal manipulation may be impaired ovarian function that cause the result of the diminished ω3 fatty acid particularly the absence of linolenic acid which is the precursor of ω3 fatty acid series and reflects the diminished ratio of ω3/ ω6 fatty acid. This finding can be used as an important and quick, diagnostic marker to predict the egg viability of wild *L. ramada.* Therefore, egg composition can be used as a possible index to evaluate broodstock physiological condition and subsequent egg quality. Further work may be needed to determine the whole fatty acid requirements of wild L. *ramada* fish at further production stages.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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