Alcántar-Vázquez, Juan Pablo; Pliego-Cortés, Hugo Skyol; Dumas, Silvie; Peña-Martínez, Renato; Rosales-Velázquez, Martín; Pintos-Terán, Pablo
Effects of a luteinizing hormone-releasing hormone analogue (LHRHa) on the reproductive performance of spotted sand bass Paralabrax maculatofasciatus (Percoidei: Serranidae)

Research Article

Effects of a luteinizing hormone-releasing hormone analogue (LHRHa) on the reproductive performance of spotted sand bass *Paralabrax maculatofasciatus* (Percoidei: Serranidae)

Juan Pablo Alcántar-Vázquez[1a], Hugo Skyol Pliego-Cortés[1b], Silvie Dumas[1], Renato Peña-Martínez[1] Martín Rosales-Velázquez[1] & Pablo Pintos-Terán[1c]
[1]Unidad Piloto de Maricultivos, Centro Interdisciplinario de Ciencias Marinas
Instituto Politécnico Nacional (CICIMAR-IPN). La Paz, Baja California Sur, México
[1a]Laboratorio de Acuicultura, Universidad del Papaloapan (UNPA)
Loma Bonita, Oaxaca, México
[1b]Departamento de Recursos de Marinos, Centro de Investigación y de Estudios Avanzados CINVESTAV-IPN
Unidad Mérida, Cordemex, Mérida, Yucatán, México
[1c]Instituto de Industrias, Universidad del Mar (UMAR), Distrito de San Pedro Pochutla
Oaxaca, México
Corresponding author: Juan Pablo Alcántar-Vázquez (jupasoul@hotmail.com)

ABSTRACT. Hormonal induction of spotted sand bass *Paralabrax maculatofasciatus*, was investigated in order to induce and synchronize spawning. Three experiments were conducted using wild fish captured in the Gulf of California: 1) LHRHa (luteinizing hormone-releasing hormone analogue) induced spawning with concentrations of 25, 50, 100 μg kg^{-1} and saline solution, 2) Effects of LHRHa on motility and sperm density, and 3) Induction and incubation of spawns obtained using LHRHa. In experiments 2 and 3 using concentrations of 12.5, 25, 50 μg kg^{-1} and saline solution. In experiment 1, a higher spawning ratio was observed for the concentration of 25 μg kg^{-1}; however, no significant differences were observed in the proportion of floating (viable) eggs. In experiment 2, significant differences ($P < 0.05$) were observed in duration of sperm motility and sperm density, with highest duration registered for wild fish (control group) and highest density registered for laboratory control group and concentration of 12.5 μg kg^{-1}. In experiment 3, significant differences ($P < 0.05$) were observed between treatments in volume of spawned eggs and proportion of fertilized eggs, with higher values shown for the concentrations of 50 and 12.5 μg kg^{-1}, respectively. Survival at hatching and proportion of live yolk-sac larvae were significantly higher ($P < 0.05$) for the concentration of 12.5 μg kg^{-1}. LHRHa was effective in inducing and synchronizing the spawning of spotted sand bass, a process which will be used for massive egg production.

Keywords: *Paralabrax maculatofasciatus*, LHRHa, spawn, survival, sperm cell density, sperm motility.

Efectos de un análogo de la hormona liberadora de la hormona luteinizante (LHRHa) en el desempeño reproductivo de la cabrilla arenera *Paralabrax maculatofasciatus* (Percoidei: Serranidae)

RESUMEN. Se analizó la inducción hormonal en la cabrilla arenera *Paralabrax maculatofasciatus*, para inducir y sincronizar el desove. Se desarrollaron tres experimentos utilizando peces capturados en el Golfo de California: 1) Inducción al desove con LHRHa, usando concentraciones de 25, 50, 100 μg kg^{-1} y solución salina, 2) efectos del LHRHa sobre la motilidad y la densidad espermática, y 3) inducción e incubación de desoves obtenidos con LHRHa. En los experimentos 2 y 3 se utilizaron concentraciones de 12.5, 25, 50 μg kg^{-1} y solución salina. En el primer experimento se observó una proporción de desoves más elevada utilizando la concentración de 25 μg kg^{-1}, pero, no se observaron diferencias significativas en la proporción de huevos flotantes (viables). En el segundo experimento se observaron diferencias significativas ($P < 0.05$) en la duración de la motilidad y densidad espermáticas, con la mayor duración en peces silvestres (grupo control), y la mayor densidad en el grupo control de laboratorio a una concentración de 12.5 μg kg^{-1}. En el tercer experimento se observaron diferen-

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cias significativas ($P < 0,05$) en el volumen de huevos desovados y proporción de huevos fertilizados, con los mayores valores en las concentraciones de 50 y 12,5 µg kg^{-1}, respectivamente. La supervivencia a la eclosión y proporción de larvas vitelinas vivas fue significativamente mayor ($P < 0,05$) en la concentración de 12,5 µg kg^{-1}. El LHRHa fue eficaz para inducir y sincronizar desoves de cabrilla arenera utilizados para la producción masiva de huevos.

Palabras clave: *Paralabrax maculatofasciatus*, LHRHa, desove, supervivencia, densidad de células espermáticas, motilidad espermática.

INTRODUCTION

Spotted sand bass *Paralabrax maculatofasciatus* (Steindachner, 1868), is a protogynous hermaphrodite species (Lluch-Cota, 1995) with a reasonable potential for culture on the northwest coast of Mexico due to its apparently suitable characteristics for aquaculture (Álvarez-González *et al.,* 2001), including its adaptability to environmental changes, low territoriality and potential for induced spawning under controlled laboratory conditions, all factors which allow for the mass production of juveniles almost all year (Avilés-Quevedo *et al.,* 1995; Rosales-Velázquez, 1997; Martínez-Brown, 2007). Additionally, several techniques involved in spotted sand bass cultivation have been successfully developed in our laboratory, including larval culture (Álvarez-González *et al.,* 2000), juvenile nutrition and grow-out in sea cages (Álvarez-González *et al*., 2001; Grayeb-del Alamo, 2001). However, when reared in captivity from larvae, sexual maturation occurs precociously before commercial size is reached (unpublished data). This is a major problem for fish farming because sexual maturation is accompanied, in many species, by a decrease in growth rate and survival (Felip *et al*., 2001; Piferrer *et al*., 2009). Production of triploid fish could be a solution to this problem, but first it is necessary to develop a protocol of hormonal induction in order to synchronize the spawning of spotted sand bass that normally occurs in captivity and be able to obtain male and female gametes which could be manually fertilized in order to induce a triploid treatment.

Environmental and hormonal manipulation have become reliable fish farming methods for inducing gonadal maturity and synchronizing ovulation, spermiation and spawning under culture conditions (Donaldson & Hunter, 1983; Nagahama, 1994; Zohar & Mylonas, 2001; Asturiano *et al.,* 2005; Bosak-Kahkesh *et al.,* 2010). Superactive analogues such as mammalian luteinizing hormone releasing hormone (LHRHa) are widely preferred alone or in combination with a dopamine receptor antagonist over native LHRH and other compounds in a number of freshwater and marine species (Ngamvongchon *et al.,* l988; Thomas & Boyd, l988; Alvariño *et al.,* 1992; Carrillo *et al.,* 1995;

Morehead *et al.*, 1998; Brzuska, 1999; Nayak *et al*., 2001; Duncan *et al*., 2003; Cek & Gökce, 2006). LHRHa has suitable characteristics such as high biological activity, low specificity, zero side effects and reasonable price (Zohar & Mylonas, 2001) which make it a perfect candidate for its use in the spotted sand bass.

The aim of this work is to study the effectiveness of the LHRHa administration for inducing and synchronizing spawning in wild, spotted sand bass broodstock in order to obtain gametes that will be used for the mass production of juveniles and in triploidy induction experiments.

MATERIALS AND METHODS

Experimental fish

A wild broodstock of spotted sand bass was captured in the southern part of the Gulf of California (Bay of La Paz, Mexico, 24°9′N, 110°19′W). In total, 52 females (150-250 g) and 90 males (300-500 g) were obtained. The broodstock was transported to the Unidad Piloto de Maricultivos at CICIMAR-IPN. Upon arrival, the fish were sexed by the shape of genital papillae and stocked separately for 14 days for acclimation in 600-L circular fiberglass tanks organized in a closed recirculating system under a controlled photoperiod (13L:11D) and constant temperature (22-23ºC). They were fed *ad libitum* once a day with squid and frozen fish. To avoid circadian variations, all experiments were initiated in the morning.

Experiment 1. LHRHa induced-spawning

Females were anesthetized with 2-phenoxyethanol (400 mg L^{-1}), and a 2-mm diameter polyethylene cannula (E.D. 2 mm; I.D. 1 mm) was used to retrieve egg samples. Mean diameter of eggs was obtained by measuring their major axis with imaging software (Image Pro Plus v4.5, Media Cybernetics, Jandal Scientific, Silver Spring, Maryland, USA). Only females that showed late-vitellogenic eggs with a diameter over 400 µm (Ocampo, 2002) were induced to spawn.

In total, 32 females were randomly divided into four groups of eight fish each. Each fish´s caudal peduncle

was individually marked with a color-coded plastic disc (Rosales-Velázquez, 1997). Three LHRHa (des-Gly10, [D-Ala6] LH-RH Ethylamide, Sigma-Aldrich, St Louis, Missouri, USA) concentrations were used: 25, 50 and 100 µg kg^{-1}. Injections of a physiological saline solution (0.7% NaCl; 0.5 mL kg^{-1}) were applied as control group. Fish were injected intramuscularly into the base of the lateral fin. LHRHa concentration for females was divided into two injections with a 24 h interval. Females were checked for ovulated eggs every 12 h after injection by applying gentle finger pressure to the abdomen from the anterior-to-posterior direction. Spawned eggs were collected by manual striping, placed in plastic bowls and then transferred to a graduated cylinder to volumetrically estimate the number of eggs. Floating eggs were considered viable, while eggs that sank were considered non-viable. Based on volumetric estimates made by Rosales-Velázquez (1997) on the number of eggs per mL (1800), the total number of spawned eggs per female was obtained.

Experiment 2. Effects of LHRHa on motility and sperm density

For this experiment, 64 males were randomly divided into eight groups of eight males each. Three LHRHa concentrations were used: 12.5, 25 and 50 µg kg^{-1} (two groups per concentration); and as control groups, two groups were injected with a physiological saline solution (0.5 mL kg^{-1}). The concentration was administered in a single intramuscular injection into the base of the lateral fin. Twelve hours after injection, one group per concentration was sacrificed, including a control group. Sperm was extracted by applying gentle pressure to the testes. Collected sperm was refrigerated at 4°C in a 2-mL test tube. Motility was evaluated immediately after milt collection with the technique described by Rodríguez-Gutiérrez (1992). In brief, using a disposable pipette tip, 10 µL of each milt sample was placed on a concave microscope slide and activated by adding 30 µL of filtered seawater (2-µm filter, UV-sterilized, chlorinated at 0.25 g 15 L^{-1} for 24 h, and then neutralized with sodium thiosulphate at 0.15 g 15 L^{-1}). Sperm motility was assessed within 5 s of dilution of semen. Each sperm sample was examined per triplicate under an optical microscope at 40x. Percentage of sperm cells demonstrating motility was recorded at different times after activation in each trial. Sperm cells were considered to be motile when forward movement was observed. All trials were conducted at 23°C and only with samples that exhibited initial motilities of at least 90%.

Sperm density was determined with a hemocytometer by the technique described by Coffin (1959). Milt was first diluted in a 20-mL test tube by adding 10 µL of milt to 10 mL of non-activating solution (5.0 g NaCO$_2$, 1 mL formaldehyde, 100 mL distilled water), then mixed for 30 s with a vortex mixer. Triplicate counts of five squares each were made on the hemocytometer for each dilution. The mean of the three counts was used to calculate actual sperm density. Samples were left undisturbed on the hemocytometer for five minutes prior to counting to allow sperm cells to settle. Counts were conducted using an optical microscope under a magnification of x40.

These procedures were repeated 24 h after injection in the four remaining tanks. In addition, two more control groups were used; one wild control group (WiC) that included three males sacrificed 24 h after their arrival from the field and one untreated control group (laboratory control, LbC) composed of seven males sacrificed at the time of the experiment (two weeks after their arrival from the field), three males sacrificed 12 h after injection of the LHRHa-treated groups and four males sacrificed 24 h after injection of the LHRHa-treated groups. Evaluation of sperm motility and density was performed by one observer (in each case) to avoid bias errors.

Experiment 3. Induction and incubation of spawns obtained with LHRHa

For this experiment, four groups of five males and five females each were stocked in a closed recirculating system composed of 1000-L fiberglass circular tanks equipped with egg collectors that received and filtered water from the top of the broodstock tank. The LHRHa concentrations for females were selected based on results obtained in the first experiment (12.5, 25 and 50 µg kg^{-1}). The concentration selected for males was 25 µg kg^{-1}. The LHRHa concentration was administered in a single intramuscular injection to both males and females. Control males and females were injected as described above.

Egg collectors were checked every day and the proportion of floating eggs was volumetrically evaluated. Three samples of 100 viable (floating) eggs were collected for each LHRHa concentration and incubated at 23°C in plastic cylinders with aerated, filtered seawater and a working volume of 2-L. Survival at hatching was calculated as the number of larvae with respect to the number of initial eggs and expressed as a percentage. Proportion of live yolk-sac larvae was calculated as the number of live yolk-sac larvae/hatched larvae.

Statistical analysis

Percentage data were logarithmically transformed (Log$_2$ and Log$_{10}$) before statistical analysis (Sokal & Rohlf, 1998). Normality was verified using a

Kolmogorov-Smirnov test and homogeneity of varian-ces using a Levene test. One-way ANOVA was used to compare the effects of different LHRHa concentrations in experiment 1. For experiment 2, one-way ANOVA was used to compare the effect of different concentrations of LHRHa on sperm density and a two-way ANOVA was used to compare the effects of different LHRHa concentrations and time on sperm motility. A Tukey test was used when significant differences were detected. For experiment 3, a Cochran´s Q test was used to compare the volume of spawned eggs and the proportion of fertilized eggs between days. A chi-square goodness-of-fit test was used to compare the total volume of spawned eggs and the proportion of fertilized eggs between groups. A one-way ANOVA was used to compare the effects of different LHRHa concentrations on survival at hatching and proportion of live yolk-sac larvae. The established level of significance for all analyses was $P < 0.05$.

RESULTS

LHRHa induced-spawning

Spawns were obtained 36 h after the second injection of LHRHa applied to females in all treated groups. No females injected with saline solution spawned. A higher spawning ratio was observed for the concentration of 25 µg kg^{-1} with a greater number of spawned females compared to the concentrations of 50 and 100 µg kg^{-1} (Table 1). No significant differences ($P > 0.05$) were found in proportion of live embryos showed between the three LHRHa concentrations (Table 1); however, a tendency was observed for a higher volume of spawned eggs in the lower concentrations.

Effects of LHRHa on motility and sperm density

The WiC group showed a significantly ($P < 0.05$) longer duration of sperm motility until 90 to 100% loss compared to the LHRHa-treated groups and the LbC group (Table 2). Between the LHRHa-treated groups, the concentration of 25 µg kg^{-1} showed a significantly longer ($P < 0.05$) duration of sperm motility until 100% loss compared to the concentrations of 12.5 and 50 µg kg^{-1} both at 12 and 24 h after injection (Table 2). The concentrations of 12.5 and 25 µg kg^{-1} showed a significantly longer ($P < 0.05$) duration of sperm motility until 10 to 50% loss at 12 and 24 h after injection compared to that observed in the concentration of 50 µg kg^{-1}. The LbC group showed statistically similar times of loss of sperm motility to that registered for the concentration of 50 µg kg^{-1}. The lowest times of sperm motility were observed consistently in the saline solution group, especially 24 h after injection.

Table 1. Spawning ratio, volume of spawned eggs (mL), floating eggs (mL) and percentage of live embryos (%) obtained 36 h after the second injection in the LHRHa-treated groups and the saline solution group (Sg).

	LHRHa (µg kg^{-1})			
	Sg	25	50	100
Spawning ratio	0:8	6:8	2:8	3:8
Spawned eggs	0	85	58	27
Floating eggs	0	79	56	25
Live embryos	0	93	96	96

Significant differences ($P < 0.05$) in sperm cell density were observed between the LHRHa-treated groups; however, the highest sperm cell density was observed for the LbC group at 12 h after injection. The concentration of 12.5 µg kg^{-1} showed a significantly ($P < 0.05$) higher sperm cell density compared with the concentrations of 25 and 50 µg kg^{-1} and the saline solution group (Table 3). No significant differences ($P > 0.05$) were detected between the LbC group and concentration of 12.5 µg kg^{-1} at 12 or 24 h after injection. The WiC group showed a statistically similar sperm cell density to that observed for the concentrations of 25 and 50 µg kg^{-1} at 12 h after injection. Sperm cell density showed a decrease 24 h after injection for all groups treated with LHRHa, LbC group and saline solution-injected group. The saline solution group showed significantly ($P < 0.05$) lower values of sperm cell density at 12 and 24 h after injection.

Induction and incubation of spawns obtained with LHRHa

Eggs were observed for three days in the egg collectors placed in each tank after injection of the LHRHa-treated groups and saline solution group. An increase in number of spawned eggs was observed 24 h after injection in all LHRHa-treated groups, including the saline group (Fig. 1a); however, the highest volume of spawned eggs and proportion of fertilized eggs were observed 48 h after injection in the concentrations of 12.5 and 50 µg kg^{-1} (Fig. 1b). Egg production decreased by day three in all groups (Fig. 1c). No significant differences ($P > 0.05$) were detected upon each daily observation in volume of spawned eggs and proportion of fertilized eggs. Significant differences ($P < 0.05$) between treatments were observed for total volume of spawned eggs and proportion of fertilized eggs. The highest volume of spawned eggs was observed for the concentration of 50 µg kg^{-1} (171 mL) compared with the concentrations of 12.5 (125 mL) and 25 µg kg^{-1} (50 mL) and the saline solution group (87 mL); however, the highest proportion of fertilized eggs was detected

Table 2. Time (s) of loss of different percentages of sperm motility obtained at 12 and 24 h after injection in the LHRHa-treated groups, wild control group (WiC), laboratory control group (LbC) and the saline solution group (Sg). Values expressed as mean ± standard error. Different superscripts in each column indicate significant differences ($P < 0.05$).

Time (h)	Group	n	Loss of sperm motility (%)			
			10	50	90	100
	WiC	3	60 ± 5^{ab}	132 ± 6^a	233 ± 8^a	943 ± 7^a
12	LbC	3	57 ± 5^{ab}	95 ± 6^{bc}	123 ± 7^{bc}	233 ± 22^c
	12.5	7	77 ± 5^a	118 ± 4^{ab}	161 ± 5^b	392 ± 26^c
	25	4	72 ± 6^a	116 ± 8^{ab}	160 ± 9^b	806 ± 15^b
	50	6	43 ± 2^b	78 ± 4^c	108 ± 5^c	187 ± 7^c
	Sg	4	40 ± 2^b	81 ± 6^c	114 ± 3^c	167 ± 8^c
24	LbC	4	34 ± 2^{cd}	72 ± 4^{de}	108 ± 5^{bc}	234 ± 21^{cd}
	12.5	7	56 ± 3^{ab}	97 ± 3^{bc}	129 ± 5^{bc}	351 ± 10^c
	25	6	69 ± 3^a	115 ± 5^{ab}	155 ± 7^b	513 ± 45^b
	50	4	43 ± 5^{bcd}	79 ± 4^{de}	125 ± 8^{bc}	230 ± 24^{cd}
	Sg	4	28 ± 5^d	64 ± 1^c	55 ± 5^c	152 ± 6^d

Table 3. Sperm cell density obtained at 12 and 24 h after injection in the LHRHa-treated groups, the wild control group (WiC), the laboratory control group (LbC) and the saline solution group (Sg). Values expressed as means ± standard error. Sp: sperm cells. Different superscripts indicate significant differences ($P < 0.05$).

Time (h)	Group	Sp x 10^7
	WiC	380 ± 44^b
12	LbC	665 ± 77^a
	12.5	590 ± 45^a
	25	360 ± 36^b
	50	380 ± 31^b
	Sg	200 ± 20^d
24	LbC	285 ± 29^{bc}
	12.5	265 ± 20^c
	25	170 ± 14^d
	50	225 ± 22^{cd}
	Sg	155 ± 15^d

for the concentration of 12.5 µg kg^{-1} (32%) followed by the concentrations of 25 µg kg^{-1} (18%), 50 (16.4%) and the saline solution group (4.6%).

Incubation of fertilized eggs differed between the LHRHa-treated groups. A significant ($P < 0.05$) increase in survival at hatching and proportion of live yolk-sac larvae were observed for the concentration of 12.5 µg kg^{-1} compared with the concentrations of 25 and 50 µg kg^{-1}. A tendency toward lower survival at hatching and lower proportion of live yolk-sac larvae was observed in the higher LHRHa concentrations, with a significantly lower ($P < 0.05$) value shown for the concentration of 50 µg kg^{-1} (Table 4).

DISCUSSION

LHRHa induced-spawning

Superactive LHRH analogues have been used with great success for inducing and synchronizing the spawning of several teleosts (Donaldson *et al.*, 1981; Zohar & Mylonas, 2001). With spotted sand bass, it has also proven to be effective in inducing and synchronizing spawning. In our work, 25 µg kg^{-1} was the most efficient LHRHa concentration for inducing spawning, with a ratio of 6:8 females. These results are similar to those obtained by Alvariño *et al.* (1992) for European seabass (*Dicentrarchus labrax*), whereby a concentration of 20 µg kg^{-1} was able to increase the quantity of eggs and ovulation rate (rate of eggs that have been released from the follicle and are ready to be expelled). Other studies have found similar results using low concentrations of LHRHa alone or in combination with other hormones (Arabaci *et al.*, 2001; Denson *et al.*, 2007; Bosak-Kahkesh *et al.*, 2010). Morehead *et al.* (1998) mention that for some species the application of low concentrations is more effective in inducing ovulation and spawning. However, for most teleost, LHRHa is usually applied in concentrations ranging from 1 to 600 µg kg^{-1} (Zohar, 1988; Zohar & Mylonas, 2001).

Higher concentrations of LHRHa were less effective with spotted sand bass in increasing the number of spawned eggs or proportion of viable eggs. It has been reported that higher concentrations of LHRHa can cause physical stress resulting in lower egg production due to inhibition of the maturation process (Garcia, 1993). However, it is important to point out that the decrease in egg production observed in our work
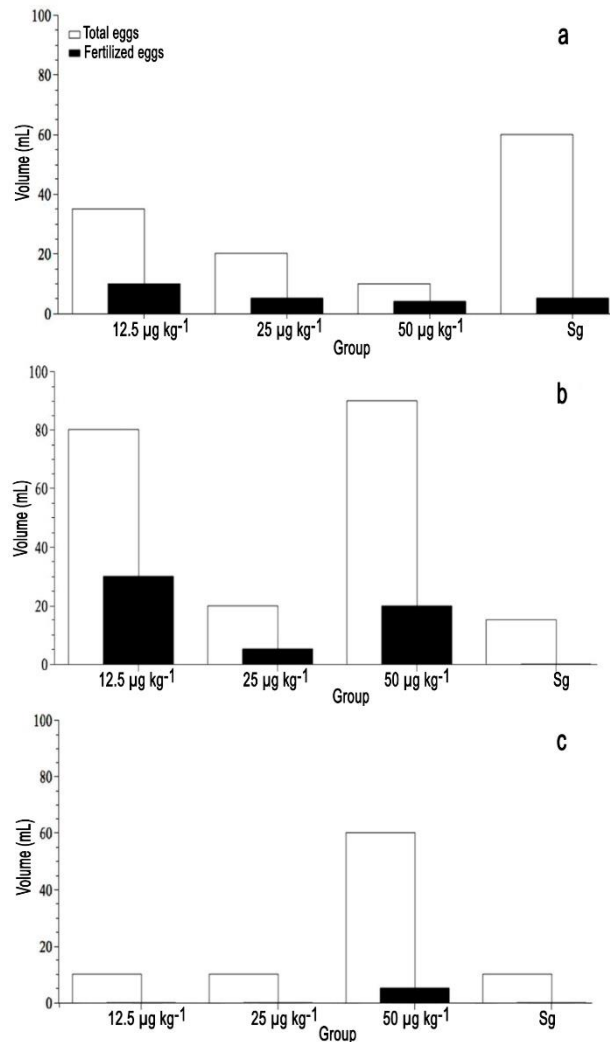
Figure 1. Volume of total and fertilized eggs obtained with a single injection of LHRHa in the spotted sand bass. a) volume of eggs obtained 24 h after injection, b) volume of eggs obtained 48 h after injection, c) volume of eggs obtained 72 h after injection. Sg: saline solution group.

was not accompanied by a decrease in egg viability. This could be explained by the fact that LHRHa does not cause significant side effects and manual spawning was carried out during the optimal time period, ensuring a high egg viability as stated by Zohar & Mylonas (2001). Females injected with saline solution did not spawn or show any effect caused from the injection. This agrees with the results obtained by Barry *et al.* (1995) for walleye (*Stizostedion vitreum*), whereby the control group reached neither ovulation nor spawning.

Latency period between final injection and initial egg release for spotted sand bass was approximately 36 h. Several studies have found latency periods as early as 14 h or as late as 48 h after injection. This period de-

Table 4. Percentage of survival at hatching (Sh) and proportion of live yolk-sac larvae (Ll) obtained from spawns collected 48 h after injection of the LHRHa-treated groups. Values expressed as mean ± standard error. Er: embryos per replicate. Different superscripts in each column indicate significant differences ($P < 0.05$).

LHRHa (µg kg^{-1})	Er	Sh	Ll
12.5	100	86.2 ± 2.3^a	77.0 ± 5.8^a
25	100	28.4 ± 7.0^b	22.0 ± 8.6^b
50	100	10.1 ± 2.2^c	5.0 ± 2.3^c

pends on water temperature, physiology of species and whether LHRHa is applied alone or in combination with other hormones (Arabaci *et al.*, 2001; Takushima *et al.*, 2004; Bosak-Kahkesh *et al.*, 2010).

Effects of LHRHa on motility and sperm density

Maximum duration of sperm motility recorded at 12 and 24 h was observed for the WiC group, which probably was related to the fact that spotted sand bass males were captured during breeding season (February-July) (Ocampo, 2002). This means that the fish were under optimal environmental conditions, including the presence of mature females. In LHRHa-treated groups, the concentration of 25 µg kg^{-1} showed the longest duration of sperm motility, followed by the concentration of 12.5 µg kg^{-1}. This appears to confirm that for spotted sand bass LHRHa is more efficient in low concentrations. On the other hand, the fact that the LbC group had higher sperm motility values than the group injected with saline solution shows the negative effect caused by handling techniques since both groups received the same treatment except for the injection.

The highest sperm motility values in the LHRHa-treated groups were reached 12 h after injection for the concentration of 25 µg kg^{-1}. Garcia (1993) and Mylonas *et al.* (1997) mention that the effect of a single injection of LHRHa has a short duration, possibly a few hours, but that the exact time depends on the particular species and reproductive state of the fish. For spotted sand bass, the effect of a single injection ended approximately 12 h after injection, since after 24 h the values of sperm motility decreased in the LHRHa-treated groups.

According to Nagahama (1994), it is probable that injection of LHRHa stimulates an increase of gonadotropin hormones (GtH), which results in a higher production of the steroid 17α20β-DP. An increase in plasma levels of this steroid causes an elevation of pH of sperm duct (~8.0) and accumulation of cAMP (cyclic adenosine monophosphate) inside sperm cells, stimulating the acquisition of sperm motility. The higher sperm motility values found in the LHRHa-treated groups compared to the LbC and saline

solution groups are probably the result of this process, which allows for a potential increase in sperm motility. However, for spotted sand bass, the increase in sperm motility caused by the injection of LHRHa was not enough to reach the levels observed in the WiC group. Tvedt *et al*. (2001) report that the application of a weekly injection of LHRHa in Atlantic halibut (*Hippoglossus hippoglossus*) produces an increase in sperm motility only after 40 days from the time of the first injection.

Sperm cell density after the LHRHa injection was higher at 12 h in all groups compared to 24 h. Mylonas *et al*. (1997) report that LHRHa administrated through injection produces an increase of the GtH II, therefore reducing sperm cell density due to the production of seminal fluid and testes hydration, which are processes associated with the final stages of testes maturity and spermiation. Similar results have been observed in yellowtail flounder (*Pleuronectes ferrugineus*) (Clearwater & Crim, 1998), plaice (*P. platesa*) (Vermeirssen *et al.*, 1998), winter flounder (*P. Americannus*) (Shaangguan & Crim, 1999), Atlantic halibut (Tvedt *et al*., 2001) and pejerrey (*Odontesthes bonariensis*) (Miranda *et al.*, 2005). The observed reduction in sperm cell density 24 h after injection in all LHRHa-treated groups is probably the result of the increase in seminal fluid caused by the LHRHa injection. However, it is difficult to explain the observed decrease in sperm cell density in the LbC group. Garcia (1993) mentions that this could be the result of physiological stress produced by handling techniques. Additionally, Kime (1993) mentioned that in closed recirculating water systems the pheromones of treated fish can affect control groups.

In spotted sand bass, the effect of a single LHRHa injection on sperm cell density is short-lived. This is consistent with that reported by Garcia (1993) for rabbit fish (*Siganus guttatus*) in which case semen must be collected 24 h after the first injection since beyond this time the sperm volume decreases back to normal levels. Mylonas *et al*. (1997) reported that the short duration of the LHRHa effect is not exclusive to this neuro hormone. Similar results have been obtained with different species by injecting HCG or other gonadotropin preparations. These authors also suggest that an increase in the production of seminal fluid is the first effect caused by the increase of plasma levels of GtH II, and that to observe an increase in sperm cell density, a sustained treatment is necessary through LHRHa delivery systems or repetitive injections.

Induction and incubation of spawns obtained with LHRHa

Using the results obtained in the first experiment, we decided to use a lower concentration of LHRHa with the purpose of increasing the number of spawned and fertilized eggs. However in this case, only the proportion of fertilized eggs was higher compared to the other LHRHa concentrations used. The higher volume of eggs obtained in all groups for several days was probably caused by the characteristics of the closed recirculating system used. The egg collectors of the system provided more suitable conditions for obtaining multiple spawns by eliminating the need to obtain spawns manually. The system was isolated, reducing human interference to a minimum; and the fish used were acclimated for a period of two weeks prior to the experiment and probably were in an advanced stage or even had completed the process of vitellogenesis at the moment of applying the LHRHa injection. It has been reported that degree of ovarian development and proximity to spawning will influence responsiveness to hormone preparations (Denson *et al*., 2007).

Although we obtained spawns for three days, the volume of unfertilized eggs was high in all LHRHa-treated groups, including the saline solution group. This was probably caused by the design used in the tanks to collect eggs. After eggs are expelled by females, they have only a few minutes to be fertilized before going from the tank through the main conduct to the recollection system. If this time is not enough to allow for proper contact between eggs and sperm, it can cause many viable eggs to die. Additionally, if the recirculation velocity of tank water is high, the contact time between gametes could be reduced. However, we cannot rule out that our results could be affected by other factors such as lack of synchronicity between females and males, age, nutrition and health status, and other environmental factors due to the wild origin of the broodstock used. The interaction of these factors can explain the variation observed between days 2 and 3 for the concentration of 50 µg kg^{-1}.

Another factor that could explain the higher number of fertilized eggs obtained 48 h after injection is the increase in seminal fluid produced by the LHRHa injection. This increase probably causes sperm to become less dense thereby increasing its capacity to fertilize mature eggs (Vermeirssen *et al*., 1998; Asturiano *et al*., 2005). The observed reduction in sperm cell density 24 h after injection in all LHRHa-treated groups supports this.

Use of LHRHa

One reason why LHRH analogues have become widely used is that in theory they cause no negative side effects, even in higher concentrations. Although in the first experiment the proportion of floating viable eggs was not affected by the LHRHa concentration, the use of higher concentrations in the third experiment provoked a significant decrease in survival at hatching

and proportion of live yolk-sac larvae after incubation. Alvariño *et al.* (1992) report that stress associated with handling and sampling or elevated hormonal concentrations can produce harmful effects on the reproduction process, hatching percentage and larval survival. This could explain the lower hatching percentages obtained after incubation for the concentrations of 25 and 50 µg kg^{-1}. King & Pankhurst (2004) report for Atlantic salmon (*Salmo salar*) a high hatching rate (>80%) but a lower survival rate of fish injected with LHRHa compared with control fish. However, Barry *et al.* (1995) found in experiments with walleye that LHRHa applied in a single injection was enough to induce final maturation and ovulation without causing negative effects to the fish, the percentage of fertilized eggs or larval survival. Additionally, Donaldson *et al.* (l981) report that survival until first stages of the larval period was higher (98%) in LHRHa-treated groups (0.1 and 0.2 mg kg^{-1}) compared to the control group (93%).

Hormonal stimulation is capable of inducing and synchronizing the spawning of several females for massive egg production, allowing for the planning and management of farming activities. In this case, LHRHa was highly efficient in inducing the spawning of wild females of spotted sand bass that presented eggs of a minimum diameter of 400 µm and for improving sperm motility in males. According to results obtained, the optimum concentration for spotted sand bass is 12.5 µg kg^{-1} for females and 25 µg kg^{-1} for males. With the use of these two concentrations it will be possible to obtain frequent spawns without any negative side effects on hatching rate or proportion of live yolk-sac larvae. This will allow for the production of gametes that will be used in triploidy induction experiments.

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