Revista de Biología Marina y Oceanografía
ISSN: 0717-3326
revbiolmar@gmail.com
Universidad de Valparaíso
Chile

Radonic, Mariela; López, Andrea V.; Oka, Masakazu; Aristizábal, Eddie O.
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Revista de Biología Marina y Oceanografía, vol. 40, núm. 2, diciembre, 2005, p. 9199
Universidad de Valparaíso
Viña del Mar, Chile

Revista de Biología Marina y Oceanografía 40(2): 91 – 99, diciembre de 2005

Effect of the incubation temperature on the embryonic development and hatching time of eggs of the red porgy *Pagrus pagrus* (Linne, 1758) (Pisces: Sparidae)

Efecto de la temperatura de incubación en el desarrollo embrionario y tiempo de eclosión de huevos del besugo *Pagrus pagrus* (Linne, 1758) (Pisces: Sparidae)

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Resumen.- La estimación de la calidad de huevos durante los estadios iniciales del desarrollo embrionario es un criterio predictivo útil para evaluar el potencial de la producción de juveniles para el besugo *Pagrus pagrus*.

El objetivo del presente estudio es describir en detalle los estadios del desarrollo embrionario del besugo y el tiempo de eclosión de larvas a tres temperaturas de incubación. Los huevos fertilizados obtenidos por desove natural fueron incubados a 15, 20 y 25°C. Los estadíos embrionarios fueron microfotografiados a las tres temperaturas. Se identificaron las divisiones celulares posteriores a la fecundación y la aparición de órganos durante el desarrollo embrionario.

La secuencia en las divisiones celulares y la aparición de órganos sensoriales y motores fue la misma para las tres temperaturas, aunque a mayor temperatura más rápido fue el desarrollo embrionario. La duración del desarrollo embrionario hasta la eclosión larval estuvo también determinada por la temperatura de incubación. A 25°C el embrión completamente formado eclosionó 26 h 25 min después de la fertilización, mientras que a 20ºC y 15ºC la eclosión se produjo a las 37 y 60 h post fertilización respectivamente.

Conocer los estadios embrionarios, la secuencia de segmentación, el diámetro del huevo, el número de gotas oleosas, el tiempo de formación de los órganos y la duración del desarrollo embrionario hasta la eclosión, es un requisito básico para la evaluación de la calidad de las camadas de huevos destinadas a la producción masiva de semillas.

Palabras clave: pez marino, organogénesis, calidad de huevos, producción de semilla, Argentina

Abstract.- Assessment of egg quality at early embryonic stages may be useful predictive criterion to evaluate the potential mass juvenile production in red porgy *Pagrus pagrus*.

The objective of the present study is to describe in detail the embryonic developmental stages in the red porgy and the time taken by larvae to hatch at three incubation temperatures. Fertilized eggs obtained from natural spawnings were incubated at 15, 20 and 25ºC. Photomicrographs of all the embryonic stages were taken at the three temperatures. Cell divisions after fertilization and organ appearance during the embryonic development were identified.

The sequence in cellular divisions and appearance of sensory and motor organs were the same for the three temperatures, although the higher the temperature, the faster the stages of development. Hatching time was also determined by the incubation temperature. At 25ºC the completely-formed embryo hatched at approximately 25 h 26 min after fertilization whereas at 20ºC and 15ºC hatching occurred 37 and 60 h post fertilization.

Knowledge of the normal embryonic stages, the sequence of cleavages, the egg diameter, the number of oil drops, the timing of organ formation and the hatching rate is a basic requirement for assessing the eggs batches quality during seed mass production.

Key words: marine fish, organogenesis, egg quality, seed production, Argentina

Introduction

The red porgy *Pagrus pagrus* (Linne) (Pisces, Sparidae), naturally found along the coast of the Buenos Aires Province, is a commercially important species due to its high quality and price in the international markets. Over-fishing over the last ten years determined drastic fluctuations in the captures of this species (Anónimo

1999). Therefore the Argentine Government supports the development of the culture technique for red porgy within the Mariculture Project of INIDEP as an alternative for the supply of juveniles of this species in the short term.

Several countries have successfully achieved the culture of other sparids such as *Pagrus major* in Japan (Foscarini 1988, Itagaki 1996) and *Sparus aurata* in Spain, France and Italy (Devauchelle 1984, Estévez García 1991, Zohar *et al*. 1995, Hernández Cruz *et al*. 1999). The culture of *Pagrus pagrus* has been successfully developed in Greece (Mihelakakis *et al*. 2001, Mylonas *et al*. 2004), and in Argentina the culture research for the red porgy started in 1994. Since then many advances have been accomplished in reproduction, larviculture and on-growing of this species (Aristizábal *et al.* 1997a, b).

Basic knowledge on the embryonic and larval development as well as the reproductive biology of *P. pagrus* was described for the first time in Argentina by Ciechomsky & Weiss (1973) and Ciechomsky & Casia (1974). Machinandiarena *et al*. (2003) described the red porgy eggs and in much detail the morphometric transformations that the yolk sac larvae undergo during the first 70 days after hatching. Kentouri *et al*. (1992) studied the embryonic development of the same species in Crete.

Although egg incubation and fry production at the Mariculture Experimental Station lasts from late October until early March, when the ambient temperature ranges between 18 and 20ºC, the objectives of the present study are to describe in detail the embryonic developmental stages and the time taken for larvae of the red porgy to hatch at three incubation temperatures.

Materials and methods

Broodstock management

Broodstock were caught between 1994 and 1998 and kept in an indoor fiberglass rectangular tank of 16.2 m^3 (4.5 m long, 2 m width and 1.8 m deep) supplied with a recirculated water system and subjected to natural photoperiod and annual controlled water temperature (April to August: 13ºC, September to March: 18ºC) (Aristizábal 2003). Sex ratio was 12 females: 15 males: 4 undetermined. Body weight of individual fish ranged from 1190 to 4660 g (2380 ± 0.70 g). Individual standard length ranged from 36.5 to 48 cm (39.76 ± 3.75 cm).

Fish were hand-fed once a day to satiation with fresh squid (*Illex argentinus*). Two months before the beginning of the spawning season they were given a moist pellet diet supplemented with vitamins and minerals (Vitafax Super Reproductores, Roche).

Egg collection

Eggs obtained from natural spawnings at 18ºC were collected daily in a plankton net suspended beneath the tank overflow outlet. Collected eggs were washed and placed in a graduated cylinder where they segregated themselves into two distinct layers: a top layer of buoyant eggs and a bottom layer of sinking eggs (Aristizábal *et al.* 1997a, Bromage 1994). The two layers were separated using a fine mesh net and the egg number estimated from three 1-mL subsamples. Egg diameter was measured from a subsample of 30 eggs using an image analyzer and calibre (Nikon Profile Projector V-12B, Japan).

Hatching rate and SAI

The quality of the different batches was evaluated by the hatching rate and the specific activity index (SAI). For the hatching rate, 50 fertilized eggs were placed in each of two 1000-mL beakers kept in an incubator at ambient temperature. After 48 h the number of live and dead hatched larvae and the remaining eggs were counted. The hatching rate was calculated according to the equation:

$$\text{Hatching rate (\%)} = \frac{\text{nº live hatched larvae}}{\text{nº live hatched larvae} + \text{nº dead hatched larvae} + \text{remaining eggs}} \times 100$$

For the SAI, 30 hatched larvae were placed in each of two 1000-mL beakers. Dead larvae were counted every 24 h until total larval mortality. SAI was calculated as follow:

$$SAI = \frac{1}{N} \sum_{i:1}^{k} (N - h_i) * i$$

where N: Total number of larvae, h_i: cumulated mortality by *i*-th, k: number of the days elapsed until all larvae died due to starvation (Shimma & Tsujigado 1981, Furuita *et al.* 2000).

Embryonic development

Broodstock behavior was recorded daily to determine the exact moment of egg release and therefore egg fertilization. The net was checked and fertilized eggs collected (time 0). Eggs collected at time 0 (n=300) were incubated in 1-L beakers at an initial density of 50 eggs and placed in an incubator (EYELA Multi Termo Incubator MTI-202) at 15, 20 and 25ºC. Temperature trials were achieved by duplicate.

Photomicrographs of the embryonic stages were taken at the three temperatures using a Nikon E600 microscope and an image analyzer (Digital High Definition Microscopy, Keyence, VH-7000C). Photomicrographs were taken as follows: every half hour during the first five hours of development, every hour until larval hatching at 20ºC and every two hours until the final hatching of larvae incubated at 15ºC.

Cell divisions after fertilization and organ appearance during the embryonic development were identified according to Oozeki & Hirano (1985) for the Japanese whiting *Sillago japonica*.

Results and discussion

This study was carried out with eggs obtained during the spawning season 2002-2003 which lasted from 3 November to 10 February. A total of 2,433,272 eggs were obtained from 68 batches, of which 37.6% were fertilized eggs. Mean egg diameter was 900 ± 30.0 µm (772-975 µm), mean hatching rate was 79.7% (6.9-100%) and mean SAI was 21.2 (3.4-29.8). Hatching rate and SAI over the spawning season are presented in Fig. 1. Hatching rate reached 70-80% eighteen days after the spawning began and got steady until the end of the season, whereas SAI did not show any pattern. No correlation ($P \geq 0.05$) was determined between hatching rate and SAI. In practical terms, good quality egg batches selected for seed production were those with hatching rates higher than 70%.

Broodstock behavior

Since the beginning of the reproductive season, natural spawnings occurred generally early afternoon (13 h-16 h). During that time broodfish swam vigorously making water movements called "splash". Few minutes after the "splash", the egg collector was sampled for fertilized eggs.

Embryonic development

After fertilization several cleavages were observed in the animal pole of the egg that determined the different developmental stages. The sequence of cell divisions and organ formation at incubation temperatures of 15, 20 and 25ºC is shown in Table 1. Embryonic development until hatching for the red porgy *P. pagrus* at 15ºC was divided into four main stages: cleavage stage, blastula stage, gastrula stage and embryo stage. Each stage might be divided into sub-stages depending on the number of divisions that occurred or the appearance of the different organs.

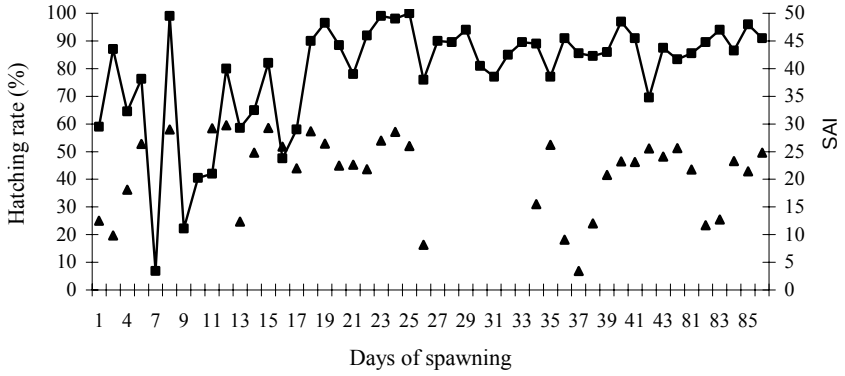

Figure 1

Hatching rate and specific activity index (SAI) throughout the spawning season 2002-2003 for *Pagrus pagrus*

Tasa de eclosión e índice de actividad específica (SAI) durante la temporada reproductiva 2002-2003 de *Pagrus pagrus*

Table 1

Chronology (in hours) of the embryonic developmental stages in *Pagrus pagrus* eggs at three incubation temperatures

Cronología (en horas) del desarrollo embrionario de *Pagrus pagrus* a tres temperaturas de incubación

Developmental stage	15°C	20°C	25°C
Spawning	0:00	0:00	0:00
Cleavage stage			
1. 1 cell	1.0	1.0	
2. 2 cells	1.5		1.0
3. 4 cells	2.0	1.5	1.5
4. 8 cells	2.5	2.0	
5. 16 cells	3.0	2.5	2.0
6. 32 cells	3.5	3.0	2.5
7. Early morula	4.0	3.5	
8. Late morula			
Blastula stage			
1. Early blastula	4.5	4.0	3.0
2. Middle blastula	6.0	4.5	3.5
3. Late blastula	8.0	7.0	4.0
Gastrula stage			
1. Early gastrula	13.0	8.0	5.0
2. Middle gastrula	16.0	12.0	8.0
3. Late gastrula	20.0	15.0	10.0
Embryo stage			
1. Appearance of embryo	21.0	16.0	11.0
2. Formation of optic vesicles	24.0	17.0	12.0
3. Appearance of Kuppfer´s vesicle	27.0	18.0	13.0
4. Seven somites	28.0	18.0	
5. Formation of a rudimentary heart	33.0	21.0	15.0
6. Elongation of tail	46.0	28.0	20.0
7. Motility of embryo	50.0	30.0	22.0
8. Ready for hatching	60.0	32.0	25.0
Hatching			
1. Start of hatching	60.0	37.0	26.25
2. 100% of hatched larvae	**65.10**	**43.40**	**29.40**

Cleavage stage

This stage is divided into 8 sub-stages according to the number of visible cells.

1.- One cell (Fig. 2A): Thirty minutes after fertilization, a narrow perivitelline space is observed due to the separation of the chorion from the cell membrane of the egg. This stage lasts thirty minutes until the formation of two cells.

2.- Two cells (Fig. 2B): One hour after fertilization, the first division occurs and two cells are distinguishable in the animal pole. This stage lasts thirty minutes until the formation of four cells.

3.- Four cells (Fig. 2C): One hour and thirty minutes after fertilization, the second division takes place and four cells are formed. This stage lasts thirty minutes until the formation of eight cells.

4.- Eight cells (Fig. 2D): Two hours after fertilization, the third division takes place and eight cells are formed. This stage lasts thirty minutes until the formation of sixteen cells.

5.- Sixteen cells (Fig. 2E): Two hours and thirty minutes after fertilization, the fourth division occurs and sixteen cells are formed. This stage lasts thirty minutes until the formation of thirty two cells.

6.- Thirty two cells (Fig. 2F): Three hours after fertilization, the fifth division occurs and thirty two cells are formed. This stage lasts thirty minutes until the appearance of the early morula.

7.- Early morula (Fig. 2G): Three hours and thirty minutes after fertilization, the morula stage is observed. This stage lasts one hour until the formation of the early blastula.

Blastula stage

Early blastula (Fig. 2H): Four hours and thirty minutes after fertilization the blastula is recognized. This stage lasts eight hours and thirty minutes until the appearance of the gastrula stage.

Gastrula stage

Early gastrula (Fig. 2I): Thirteen hours after fertilization, the gastrula is distinguished as a half circle inside the egg. This stage lasts eight hours until the beginning of the embryo stage.

Embryo stage

This stage lasts from the appearance of the embryo until hatching. The number of somites and the process of organogenesis are the two criteria considered for dividing this stage into 8 sub-stages.

1. - Appearance of embryo (Fig. 2Ja): Twenty one hours after fertilization, the embryo is distinguishable as a T-shape. From now on, the embryo's pigmentation increases and the organs begin to form. This stage lasts three hours until the formation of the optic vesicles.

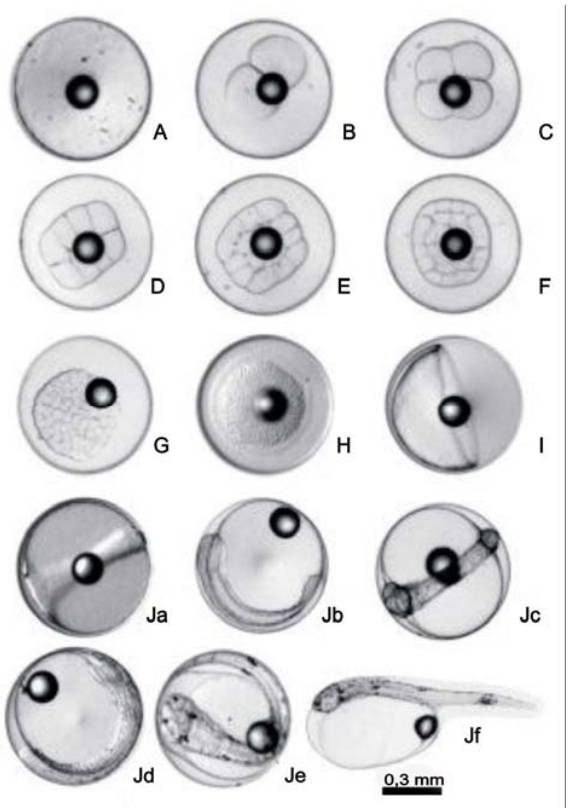

Figure 2

Photomicrographs of the embryonic development of *Pagrus pagrus* at 15ºC. A: 1 cell; B: 2 cells; C: 4 cells; D: 8 cells; E: 16 cells; F: 32 cells; G: Early morula; H: Early blastula; I: Early gastrula; Ja: Appearance of embryo and formation of optic vesicles; Jb: Appearance of the Kuppfer`s vesicle and seven somites; Jc: Formation of the rudimentary heart; Jd: Elongation of the tail; Je: Motility of the embryo; Jf: Hatched

Microfotografías de los estadios en el desarrollo embrionario de *Pagrus pagrus* a 15ºC. A: 1 célula; B: 2 células; C: 4 células; D: 8 células; E: 16 células; F: 32 células; G: mórula; H: blástula; I: gástrula; Ja: aparición del embrión y formación de las vesículas ópticas; Jb: aparición de la vesícula de Kuppfer y siete somitos; Jc: formación del corazón rudimentario; Jd: elongación de la cola; J e: movilidad del embrión; Jf: eclosión

2.- Formation of optic vesicles (Fig. 2Ja): Twenty four hours after fertilization the optic vesicles along the head sides are visible. No somites are distinguishable in this stage. This stage lasts three hours until the appearance of the Kuppfer`s vesicle.

3.- Appearance of the Kuppfer`s vesicle (Fig. 2Jb): Twenty seven hours after fertilization the Kuppfer`s vesicle is visible at the ventral side of the embryo's tail. This stage lasts one hour until the first seven somites are visible.

4.- Seven somites (Fig. 2Jb): Twenty eight hours after fertilization the first seven somites are seen in the dorsal side of the embryo. Pigmentation increases all along the embryo's body. One hour later ten somites are visible. This stage lasts five hours until the formation of the rudimentary heart.

5.- Formation of the rudimentary heart (Fig. 2Jc): Thirty three hours after fertilization a rudimentary heart is distinguishable. The number of somites increases from 11 to 24 and the Kuppfer`s vesicle disappears at exactly forty one hours after fertilization. This stage lasts thirteen hours until the elongation of the tail.

6.- Elongation of the tail (Fig. 2Jd): Forty six hours after fertilization the tail elongates together with the beginning of heart beating. This stage lasts four hours until the embryo starts to show motility.

7.- Motility of the embryo (Fig. 2Je): Fifty hours after fertilization the whole embryo starts to move and reaches 2/3 of the egg diameter. The oil drop and the embryo are pigmented with xantophores and melanophores. This stage lasts eight hours until the embryo bends itself violently and the heart beats regularly indicating that it is ready to hatch.

8.- Hatching (Fig. 2Jf): Sixty hours after fertilization 10% of the embryos hatches out and after sixty five hours and ten minutes 100% of the embryos completed hatching. At this time the alimentary canal is almost totally formed but the mouth is not yet opened.

The sequence in cellular divisions and appearance of sensory and motor organs mentioned above were the same for the three temperatures under study, although the higher the temperature, the faster the stages of development. Moreover, hatching time is also determined by the incubation temperature. At 25ºC the embryo completely formed, hatched at approximately 25 h 26 min after fertilization whereas at 20º and 15ºC hatching occurred at 37 and 60 h post fertilization, respectively. Even if embryos were ready to hatch, it

took few more hours to hatch out for any of the three studied temperatures (Table 1). At 15ºC hatching was completed after 65 h 10 min, at 20ºC hatching was finished after 43 h 40 min, and at 25°C hatching ended at 29 h 40 min.

Ciechomsky & Weiss (1973) made the first attempt to describe the embryonic and larval development of the red porgy *P. pagrus* from hand-striped wild broodfish. Eggs were incubated at 21-22.5ºC. In our study as well as in Machinandiarena *et al.* (2003), fertilized eggs were obtained from natural spawnings of wild individuals acclimated in captivity for several years. Machinandiarena *et al.* (2003) recorded the embryonic and larval development at 18ºC. These authors did not describe in detail the complete sequence and timing of organ formation (Fig. 3).

Ciechomsky & Weiss (1973) recorded that hatching occurred 38 h after fertilization (a.f.) for *Pagrus pagrus*, whereas Machinandiarena *et al.* (2003) mentioned hatching times for the same species at 16°C, 18°C and 20°C, being 48, 51 and 59 h a.f., respectively. In other sparids such as *Sparus aurata* hatching occurs 70 h a.f. at 16°C (Bedier *et al.* 1984). Kajiyama (1929) described for the first time the embryonic development and hatching times for the Japanese sea bream *Pagrus major* at 13.9ºC (86.9 h a.f.), 16ºC (66.8 h a.f.), 18ºC (53.6 h a.f.), 19.4ºC (45.6 h a.f.), 20.8ºC (40.6 h a.f.) and 21.8ºC (34.5 h a.f.). Hattori *et al.* (2004) studied hatching times for *P. major* at 16, 19 and 22ºC, being 65, 45 and 30 h a.f. respectively. Hatching times presented in this study were coincident with the ranges established by the authors mentioned above for sparid species (Table 2).

Temperature is one of the most important factors influencing the rate of development of eggs and fry (Herzig & Winkler 1986). It has been described for many fish species that increased temperature within an optimal range results in faster development and shorter time to hatch. The optimum range depends on the biological and ecological characteristics of the species under study therefore incubation temperature outside a species optimal range has negative effects on hatchability and survival (Braum 1978, Woynarovich & Horvath 1980, Marangos *et al.* 1986, Wallace & Heggberget 1988, Rana 1990, Legendre & Teugels 1991, Small & Bates 2001).

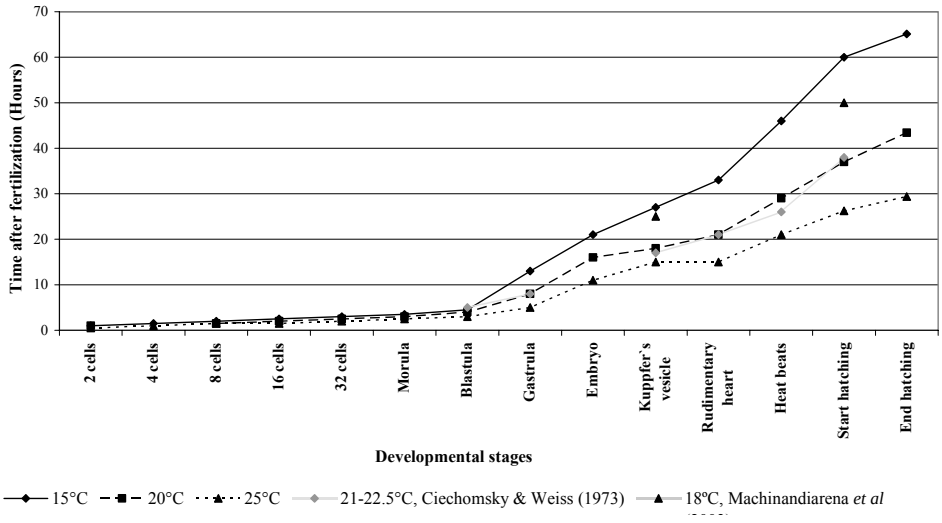

Figure 3

Sequence (time after fertilization) of the main stages in the embryonic development of *Pagrus pagrus* at different incubation temperatures

Secuencia (tiempo después de la fertilización) de los principales estadios en el desarrollo de *Pagrus pagrus* a diferentes temperaturas de incubación

Table 2

Hatching time (hours after fertilization) for *Pagrus pagrus*, *Sparus aurata* and *Pagrus major*

Tiempo de eclosión (horas después de la fertilización) para *Pagrus pagrus*, *Sparus aurata* y *Pagrus major*

Species	Temperature (ºC)											
	13.9	15	16	18	19	19.4	20	20.8	21-22.5	21.8	22	25
P. pagrus [1]									38			
P. pagrus [2]			59	51			48					
P. pagrus[3]		65.1					43.4					29.4
S. aurata[4]		70										
P. major [5]	86.9	66.8		53.6		45.6		40.6		34.5		
P. major [6]		65			45						30	

1- Ciechomsky & Weiss (1973) 2- Machinandiarena *et al.* (2003) 3- This paper
4- Bedier *et al.* (1984) 5- Kajiyama (1929) 6- Hattori *et al. (2004)*

Routine egg evaluation of each batch of eggs during the entire spawning season includes checking egg buoyancy, fertilization rate and stage of development in order to screen out dead or non-fertilized batches (Bromage 1994). Early egg stages until embryo formation are sensitive to physical and chemical changes in the environment such as handling, low dissolved oxygen concentration and pollutants (Kjørsvik *et al.* 1990, Shields *et al.* 1997, Hattori *et al.* 2004). Another egg quality parameter is the hatching rate expressed as the number of fertilized eggs to be incubated and the number of larvae appearing after hatching (24 h later for *Pagrus pagrus* at 18ºC). Mylonas *et al*. (2004) stated for *P. pagrus* that batches of eggs with >60% of 1-day embryo survival always had >80% hatching success. Therefore, decisions on whether to proceed with the incubation of eggs and larviculture can be made within one day from egg collection, before much effort is invested by the hatchery.

Any other rapid qualitative procedure to predict the survival of potential fertilized eggs is particularly important for those species with prolonged egg incubation periods, such as salmonids, the Atlantic halibut *Hippoglossus hippoglossus* and the red porgy *P. pagrus* (Shields *et al*. 1997). Blastomere morphology is a useful tool for routine fish egg checking in hatcheries as it was demonstrated that abnormal blastomeres yield low egg viability. For turbot *Scophthalmus maxumus* the quality of the produced juveniles is affected by the initial egg quality; surviving juveniles from egg batches with high rates of abnormal blastomeres have significantly less success with completion of metamorphosis and development of a normal pigmentation pattern (Kjørsvik *et al.* 2003). Cell division symmetry at the early blastula stage is considered a strong predictor of hatching and normal larval development in cod (*Gadus morhua*) (Kjørsvik 1994), halibut (Shields *et al*. 1997) and other marine species (Kjørsvik *et al.*1990).

Manooch (1976) first reported that red porgy eggs are transparent and spherical in shape, with a diameter of 800-900 µm and a centrally located oil drop of 190-210 µm. Kolios *et al*. (1997) recorded mean egg diameter of 840 µm for fish kept in captivity at 13-25ºC, whilst Mihelakakis *et al*. (2001) reported eggs diameter of 990-1090 µm. Machinandiarena *et al*. (2003) recorded eggs diameter of 890-930 µm, obtained in 1998 from natural spawnings of the same broodstock mentioned in this study. Mylonas *et al.* (2004) presented eggs diameter of 990-1070 µm for red porgy. In our study mean eggs diameter was 900 ± 30.0 µm (772-975 µm). The different eggs diameter recorded are not contradictory, considering that broodstock size, age and genotype, as well as the daily and seasonal feeding rates can influence the diameter of produced eggs (Bromage 1994). Egg and larval size are correlated. Larger larvae tend to survive longer without food that those hatched from smaller eggs (Kjørsvik *et al.*1990).

The nutritional status of broodstock is another important factor influencing egg quality. Watanabe *et al. (*1984) proved that female broodstock receiving essential fatty acid-deficient diets produced eggs with two small oil drops and low hatching capacity, whereas most of the hatched larvae showed body deformities. Since 1998, the red porgy broodstock used in this study has been naturally spawned by temperature manipulation. Neither eggs with two or more oil drops nor abnormal larvae were observed, indicating the

broodstock good nutritional condition and low stress levels during captivity (unpublished data).

Conclusion

At present, juvenile production of red porgy at INIDEP lasts from November to March when temperatures are high enough to allow rapid larvae development. The goal of the Station is to produce seed year-round, therefore the early assessment of egg viability is crucial for the onset of a successful seed production. The present study has demonstrated a close relationship between embryonic development and incubation temperature. The normal development of *P. pagrus* is possible at a wide range of temperatures, suggesting the possibility of culturing the species under different thermal conditions. The embryonic developmental stages for *P. pagrus* showed no difference for 15, 20 and 25ºC incubation temperatures although cells divisions and organ formation occurred faster as the incubation temperature increased with no visible abnormalities.

Knowledge of the normal embryonic stages, the sequence of cleavages, shape and size the blastomeres, number and size of the oil drop, and the timing of organ formation is basic for assessing abnormal egg batches. Hatching rate allows the selection of batches of eggs of good quality.

Acknowledgments

This research was funded by INIDEP and the Overseas Fisheries Cooperation Foundation (OFCF) of Japan. INIDEP Contribution Nº 1344. We would like to thank Lic. Mónica Müller (INIDEP), Dr. Gustavo Somoza (IIB/INTECH, Argentina) and Dra. Alicia García Alcázar (Instituto Español de Oceanografía, Spain) for their helpful comments on the manuscript. Lic. Daniel Hernández (INIDEP) contributed with the statistical analysis.

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Recibido en febrero de 2005 y aceptado en junio de 2005