Induced Spawning, Embryonic and Larval Developmental Stages of *Solea vulgaris* in the Mediterranean Water

Samira, S. Assem[1], Alaa A.,El-Dahhar[2], Heba, S. El-Syed[1],
Mohamed, El. Salama[2] and Mona, M. Mourad [1]*

1. National Institute of Oceanography &Fisheries, Kayet Bey Alexandria
2. Animal & Fish Production Depart, Fac. of Agriculture, Saba Basha,
Alexandria University

*Corresponding Author

ABSTRACT

Spawning induction in the present study was carried out in two steps. The first step consisted of a priming dose of carp pituitary extract (CPE) with 40-70 µg/ fish, or from 2960 to 3100 IU of human chorionic gonadotropin (HCG) per fish, while the second step, which given 24h later, was consisted of the resolving dose of luteinizing and releasing hormone analogue (LHRHa) 200 µg/kg. After resolving dose, spawning occurred after 18-24h on average sex ratio was 1:1 female: male in all spawning trials to optimize fertilization rates. The total number of fertile ovae varied between 87,000 to 120,000 eggs/ spawn which represent 430±108 eggs/g.BW. The ripe unfertile eggs of *Solea vulgaris* were rounded, colorless and transparent with about 13-20 oil globules. The surface of the fertilized egg shell is smooth, however; the yolk was segmented. The fertilized egg appeared rounded, and was about 1.2x 1.04mm. The blastodisc appeared at mean age about 35±5 minutes from fertilization. Morula began to appear after 4h±15min., early gastrula occurred after 8h±15min., after 6h±30min. post fertilization (HPF), late gastrula occurred; epiboly took place and embryonic shield was enlarged. At 12±2 HPF, the late gastrula completed and the blastopore closed up. Organogenesis started at age of 18±5 HPF then fully formed embryo inside the egg was formed at age 38±12 HPF. After about 48±15 HPF hatching was started, at 15±2°C and water salinity of 34 ppt. The percent of fertilization varied between 82-88% and hatching rate was about 90%. At age 8 days after hatching (DAH), the yolk sac completely absorbed at body length 3.2 mm. At age of 33 DAH the larva reached 13.6 mm long. All primitive organ systems were representing at this stage. Endoskeleton was studied during the larval developmental stages, larvae, post-larvae and juveniles.

**Keywords: Induced Spawning, Embryonic Developmental *Solea vulgaris*
Mediterranean**

INTRODUCTION

Common sole is a demersal and sedentary species, living on sandy and muddy bottoms, mostly in rivers and near the river mouth and also digging into the sea bottoms (Jardas, 1996) and stated that the species is distributed from coastal water to depth of 250 m.

During the last decades, marine fish aquaculture in Egypt has been mostly concentrated on gilthead sea bream (*Sparus aurata*) and Sea bass (*Dicentrarchus labrax*). The common sole (*Solea solea* L.) is a species appreciated by the market and is a candidate for rearing in a commercial scale, although on significant productions presently exist in Egypt. Imsland *et al*. (2003), published a review of the culture potential of *S. solea* in comparison with *S. sengalensis*, and pointed more information about the commercial husbandry of *S. sengalensis*. Among the most promising candidates are solied flatfishes provided that effective culture methods and strategies for increasing market opportunities can be developed (Agulleiro *et al*., 2006).

On growing methods for sole, which include both the common sole (*Solea solea*) and Senegal (*Solea senegalensis*) have improved in the last decades (Ruedajasso *et al*., 2004).

Intensive farming of flatfish have been reported remarkable successes mainly in Europe with the Senegal Sole (*Solea sengalensis*) Dinis *et al* (1987), resent knowledge concerning these species in captivity is indispensable for developing effective farming on an industrial scale (Morais *et al*., 2005; Canavate *et al*., 2006; and Herrera *et al*., 2008) whom given these positive results in flatfish breeding and the need for diversification in aquaculture.

Sobrino *et al*. (1994), investigate the trial farming of other commercial pleuronictiformes such as the wedge sole (*Dicologoglossa cuneata*) considered as a target species of the demersal fisheries in the Gulf of Cadize, Spain.

Senegal sole is currently an important focus of research for flatfish aquaculture in Europe particularly in Spain and Portugal. However, before a reliable technology for mass production of Senegal sole can be transferred to the industry, several aspects of its culture still need to be solved and optimized (Agulleiro *et al*., 2006). The culture technology for Senegal sole is mainly impaired by the lack of methods to control reproduction in captivity (Cabral, 2000). This pattern of spawning is also observed in wild Senegal sole in captivity under natural temperature regimes (Anguis

and Canarate, 2005). However, natural spawning of wild caught fish require long periods of adaptation to captive conditions, and when they do occur they are often unpredictable and the quality of the egg produced is variable.

The application of hormonal therapies, mostly based on the administration of gonadotropin-relesing hormone agonists (GnRHa), which trigger the release of pituitary gonadotropins, are common strategies to ameliorate reproductive dysfunctions of cultured fish (Zohar and Mylonas, 2001).

In flatfish, GnRHa treatments are able to promote ovulation and spawning of females (Mugnier *et al.*, 2000), although in Atlantic halibut (*Hippoglossus hipoglossus*) administration of GnRHa in males has only a weak effect, at best, in including or enhancing sperm production (Vermeirssen *et al.*, 2000 and Tvedt *et al.*, 2001).

Senegal sole were treated with GnRHa during the natural spawning season, showed multiple ovulations (Agulleiro *et al.*, 2006).

The present paper reports studies on the acceleration of gonadal development in *Solea vulgaris* using hormonal injections and investigation of the embryonic and larval development in aquaria conditions and the descriptions of the early life history stags of *Solea vulgaris*. This study will improve the production of fingerlings in the hatchery to increase production of fish in aquaculture farms.

MATERIAL AND METHODS

Solea vulgaris broodstock (260-310 g/fish, body weight), used in the spawning trials were obtained from Damietta in north of Egypt. They were obtained during the middle of December 2009 and maintained at National Institute of Oceanography and Fisheries- Marine Hatchery in ($2m^3$) fiberglass rectangle tanks with stocking density of 4fish/ m^3. Females at a body weight of 290±30 gm (mean±SD), and males, at a body weight 280±10 gm.

System preparation

A flow through sea water 37±2 ppt system supplied salinity. The water temperature was about 16±1°C throughout the experiment. Fish were fed warms and shrimps, with daily rate of 1.5% of the body weight.

Induction of spawning

Two different strategies for spawning *Solea vulgaris* were attempted, the first strategy called "a priming" dose of carp pituitary homogenate (CPH) 200 μgm/Kg fish,

or human chorionic gonadotropin (HCG) 10,000 IU/ Kg fish (purchased from Argent chemical laboratories Lot #CP, 1408 R and #CG, 2304 R, respectively). Twenty four hours later a second "resolving" injection (200 µg/Kg) was giving consisting of luteinizing and releasing hormone analogue (LHRHa) (des Gly [10] [D-Ala [6]] LHRH ethylamide), (purchased from Argent chemical laboratories Lot # LH 2508 R, redmond, WA 98052). The appropriate dosage was dissolved in 0.9% saline and injected into the dorsal musculature, as it shown in Table (1).

About twenty four hours later, spawned eggs were fertilized naturally, collected and incubated in 1 m^3 open system fiberglass tanks. Fertilization rates, average spawned egg diameters, hatching rates and larval developmental stages were recorded.

Endoskeleton preparation (double staining procedure)

Skeletal development in *S. vulgaris* was previously described by (Gavaia *et al*., 2000) as follows:

Table 1: *Total body weight (g), dose of carp pituitary extract (CPE) µg/fish, Human Chorionic Gonadotropin (HCG) IU/fish, and Lutenizing and Releasing Hormone (LHRHa) µg/fish, number of batches, number of fertile ovae, percentage of fertilization and percentage of hatching, for each female Solea vulgaris throughout the period from January to March 2010.*

Fish No.	Body weight (g)	Priming dose		Resolving dose LHRH (µg/fish)	No. of batches per fish	No. of fertile ovae				Fertilizing (%)	Hatching (%)
		CPE (µg/fish)	HCG (IU/fish)			Min. (No./fish)	Max. (No./fish)	Total			
								per fish	Per Kg		
1	295	70	-	59	15	8,000	10,000	120,000	406779.67	92	90
2	285	50	-	57	14	5,000	12,000	100,000	350877.2	93	90
3	260	40	-	52	12	4,800	9,000	110,000	423076.9	91	89
4	310	-	3100	62	13	5,000	9,900	90,800	292903.2	81	89
5	296	-	2960	59	12	3,900	9,950	87,000	293918.9	80	89
6	300	-	3000	60	13	4,000	8,600	88,000	293333.3	82	90

Fixation Specimens in 4% formal saline, then keep it in 70% ethanol. After that it cleared in 2% sodium hydroxide (NaOH), until the bones become clearly visible. Cartilage staining with Alcian blue for 5-10 min., then the bone staining was placed into alizarin red then clearing.

Larval feeding regime

Larvae were transported to the larval rearing tanks, after the start of the exogenous feeding, at density 60±12 larvae per litter. *S. vulgaris* larvae were rearing from first feeding until 28 DAH, adopting a feeding regime based on live food only.

Larvae were fed Rotifer 10 ind./ml from 3 DAH until 12±2 DAH. From 14±1 DAH, larvae fed on *Artemia*, newly hatched nauplii 3-5 ind./ml. Insters I, II and III were gradually introduced to the larvae. By the age of 25 DAH, it fed on *Artemia* metanauplii 8-12 ind./ml.

Weaning trail performed on juveniles at age 28±2 DAH up to the ending of the study period 56 DAH, using weaning diet, in addition to the continuous supplemental of live food.

RESULTS

Solea vulgaris was acclimatized for 10 days in the spawning tanks accompanied by running ripe males. During 2009-2010 about 100% spawning success rate were achieved as indicated in Table (1), the first three tanks attempts using CPE, while in the second three tanks using HCG as a priming dose followed by LHRHa in the resolving dose. After 24±2 h from the resolving injection the female spawns, the mean total number of spawned egg varied between 87,000 minimally and 120,000 maximally per fish. The percent of fertilization varied between 80 and 93% at water temperature of 17±2 and salinity 34±1 ppt.

Embryonic developmental stages

S. vulgaris fish spawn pelagic eggs, which are fertilized externally and float individually near the water surface.

The ripe eggs of *S. vulgaris* appeared rounded and transparent, with a diameter of about 1.04 mm. The egg membrane is smooth, not separated from the yolk and the cytoplasm is reduced to a thin layer covering the yolk; about 13 to 20 oil globules were noticed in ripe ova. After a bout 20 min from fertilization the egg membrane swells up and separate from perivitelline space (space between the chorion and the yolk mass); the diameter of the eggs increases to

1.2±0.01 mm, then the stages takes place as follows:

Fertilized eggs appeared with active cytoplasm around the zygot nucleus as a disc shape cap of 0.65 mm width and 0.15 mm height at the animal pole (Fig. 1a). At age of 35±5 min. after fertilization the blastodisc were appeared as multicellular circular when seen from above. The height and width of blastodisc were about 0.2 mm and 0.7 mm, respectively (Fig. 1b).

After 4h± 15min. from fertilization, the peripheral periblast cells could be seen hardly. The blastoderm is expanding as a result of the peripheral periblast reduction to form morula stage (Fig. 1c,d&e).

After 8h± 15min., early gastrula occurred, blastoderm thins and interface between periblast and blastoderm curved (Fig. 1f). The embryonic layers were formed. The more than half of yolk covered by blastoderm occurred the late gastrula (Fig. 1g). At 12h the Epiboly took place and embryonic shield was enlarged (Fig. 1h); the late gastrula stage were completed and the blastopore closed up.

The organogenesis stage was started at the age from 18h± 30min. to 28±2h (Fig. 1,i&j). The embryonic fold began to differentiate into head and trunk region of the body. Yellow pigments started on both sides of the head and trunk region (Fig. 1k).

At the age of 30±2h fully formed embryo inside the egg was distinguishable; the yolk sac diameter was 0.7 mm, heart beat started with weak cardiac contractions (Fig. 1l); the blood was colorless. At the end of this stage (38±2h) the membrane began to loosen as a result of increased muscular and jerky trunk and tail movement (Fig. 1m).

Larval development stages

The total length of the larvae throughout the period of the study was increased gradually with the larval development. The relation between the larval total length and the age after hatching by days were illustrated in Fig. (2).

On the day of hatching (0DAH), the symmetric pre-larval hatched from the embryo and her tail flexure was retained, and the larvae swam in straight direction (Fig.3). Several incomplete organs were found; the eyes are not pigmented and still not functional. The mouth is not open and the larvae are endogenous feeding, it depends on the nutrients storage on the yolk sac.

56

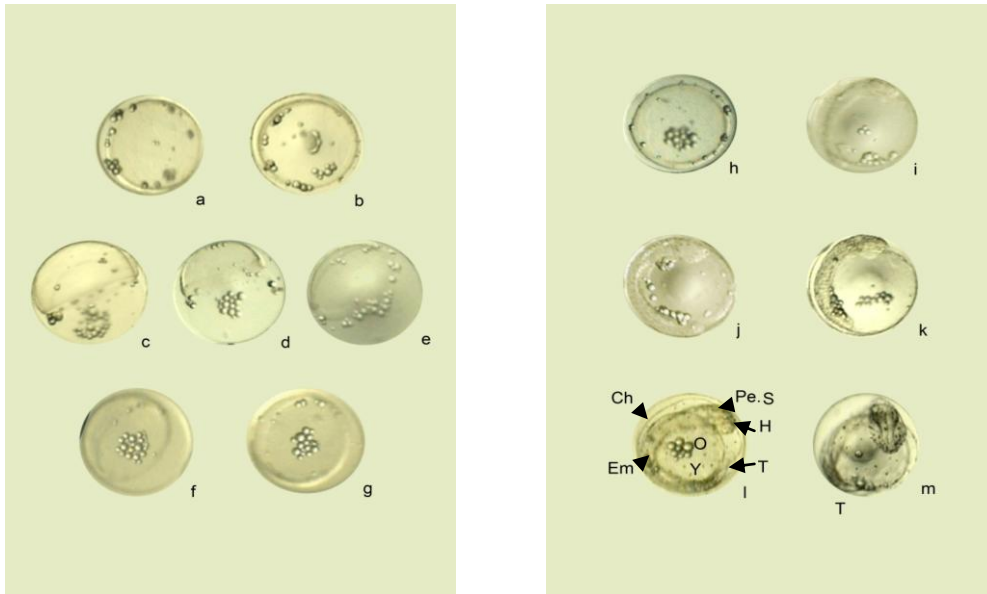*Fig. 1: Embryonic developmental stages inside ova of S. vulgaris. a).Fertilized egg; b).Blastodisc, 0.5h.; c).Early Morula, 2.25h. ;d).Intermediate Morula, 3h.; e)Late Morula, 4.3h.; f).Early Gastrula, 4.7h.; g).Late Gastrula, 5.5h., (4X). h).Epiboly, 6h.; i& j& k).Early full formed embryo,9, 11 and 14h, respectively; l& m). Late full formed embryo, 18& 25 h., respectively. Chorion (Ch), Embryo (Em), Oil globules (O), Tail (T), Head (H), perivitelline space (Pe.S), Yolk (Y), (4X).*
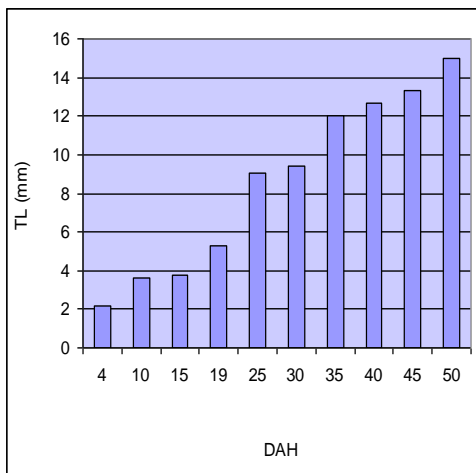

Fig. 2: *The relation between the larval total length (TL), (mm) and the age by days after hatching (DAH).*

Pre-larvae at age of 1 DAH, the body length was about 1.8 mm, the yolk was about 0.87x 0.62 mm. Eye development is in progress but the retina is not pigmented. The lower jaw begins to appear but still not fully formed (Fig. 4).

At the age of 2 DAH, the mouth is opened; the body length was about 2.1mm. The yolk sac was reduced to reach 0.71x0.58 mm, complete exogenous feeding had not yet started (Fig. 5). The lower jaw was moving and some of the larvae start feeding on live food. The pigmentation appeared on the skin.

57

Fig. 3: *Pre-larvae at (0 DAH), on the day hatching, the eyes are not pigmented and still not functional. The mouth is not opened. The yolk sac (YS), with oil droplets (OD), is very abundant and feeding is exclusively endogenous (4.5X).*
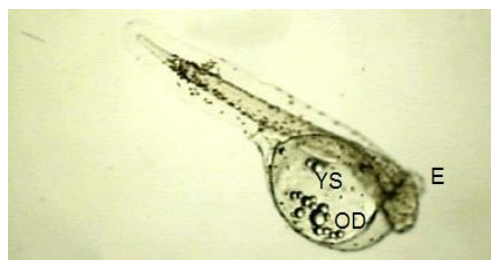

Fig. 4: *Pre-larvae at (1 DAH), most of the yolk sac (YS), with oil droplets (OD), has been resorted. Eye (E) development is in progress but the retina is not pigmented. The lower jaw begins to appear (4.5X).*

At the age of 2.5 DAH, swimming activity increased indicating that the swim bladder was functioning. The body length increased notably to reach 2.3 mm. The trunk region was about 0.7 mm and the tail 1.3 mm. The head height and length was about 0.4 mm and 0.31 mm, respectively. The eye diameter increased also to 0.15mm. The yolk sac decreased in size and was 0.6 X 0.4 mm in diameter.

Exogenous feeding stage was starting up with larval total length 2.57±0.06 mm. In this age (5 DAH), the yolk sac were 0.4x0.34 mm, both mouth and eyes are completed; the rudiments of the alimentary canal could be seen as narrow tube. The eyes were pigmented and completely functional, with diameter 0.09 mm. The head height and length were 0.49 and 0.39 mm, respectively (Fig. 6).

On the age of 8 DAH, the yolk sac completely absorbed; the body length was about 3.2 mm. The intestine was slightly convoluted inside the gut. The operculum covered the gill arches (Fig. 7). The head height and length were reached 0.9 and 0.7 mm, respectively, by age 10 DAH; and the body length reached to about 3.63±0.06 mm.

Dark yellow pigmentation started to deposit on the body. The larvae in this age are totally exogenous feeding (*Chlorella salina* and *Brachious placitilis*). The eyes were still on either side of the head. The fins fold didn't differentiation yet to specific fin.

At the age of 12 DAH, the abundant foods in the gut present the convoluted of the intestine. The exogenous feeding was on newly hatched *Artemia salina* (Fig. 8).
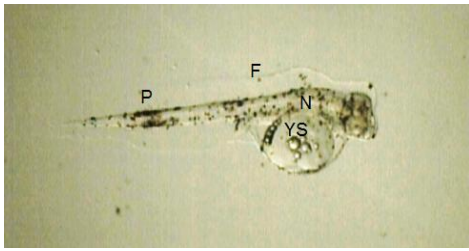
Fig. 5: *Pre-larvae at (2 DAH), the mouth is open and the pre-larval stage ends. Notochord (N) noticed, the larvae coated by fold (F) and still has yolk sac (YS). Pigmentation (P) is appeared in the skin (4.5X).*
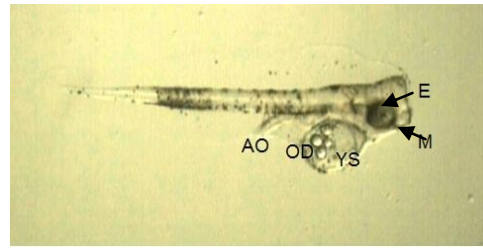

Fig. 6: *Larvae at 5 (DAH), the mouth (M) and the eyes (E) are completely functional and pigmented. Larvae are feeding exogenous but some yolk sac (YS) is still remains. Anus opening (AO) was indicated (4.X).*

At the age of 15 DAH, the larva was 4.20±0.07 mm long; the head was 1.2 mm in height and 1.05 mm in length. The trunk (pr-anal) and tail regions (post-anal to the tip of the tail) were 0.65 mm and 2.3 mm in length, respectively, and the eye reached 0.28 mm in diameter.

Metamorphosis in the eye and caudal fin were started and the larvae become asymmetric. The eye migration is beginning; the left eye is shifting to the dorsal midline of the head (Fig. 9).

At the age of 19 DAH, the caudal fin begins to assume its typical shape and caudal rays can be observed. The body reached to 5.30±0.06 mm and as a whole becomes stronger maintaining a flattened shape along the sagittal plane. The upper jaw is slightly curved down and takes a beaked shape. The most representative stages of

metamorphosis are when the left eye reaches the dorsal midline of the head; the larvae begin to change their swimming from vertical to benthic. The eye was 0.3 mm in diameter. The head was about 1.5 mm in height and 1.2 mm in length. Transparency of the body begins to reduce as skin pigmentation intensifies (Fig. 10).

At the age of 22 DAH ±20 h, the larva reached 5.8 mm long. The head was well developed, its length was 1.48 mm. from the tip of the snout to the posterior edge of the operculture, and its height was 1.54 mm. The trunk region reached 0.82mm long and the tail region was about 3.5 mm long.

The left eye began to migrate to the other side, in accordance with the normal changes which take place in this species. The dorsal and anal fins
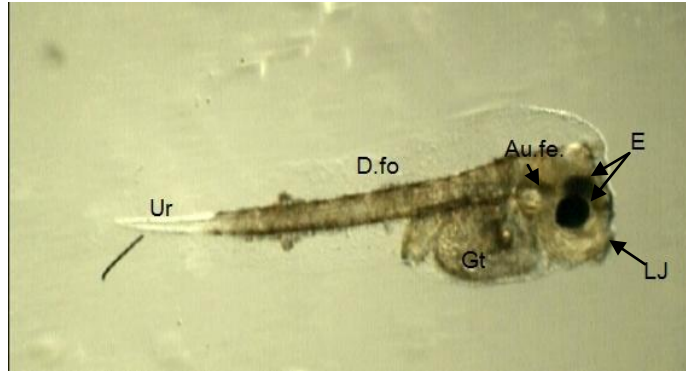
59

Fig. 7: *Larvae at 8 DAH, the eye (E) migration has not yet started. Lower jaw (LJ) and gut (Gt) are continued to development. Auditory vesicles (Au.fe.) were appeared in the head and dorsal fold (D.fo.) is formed. Urostyle (Ur) appeared without any rays on it (4X).*
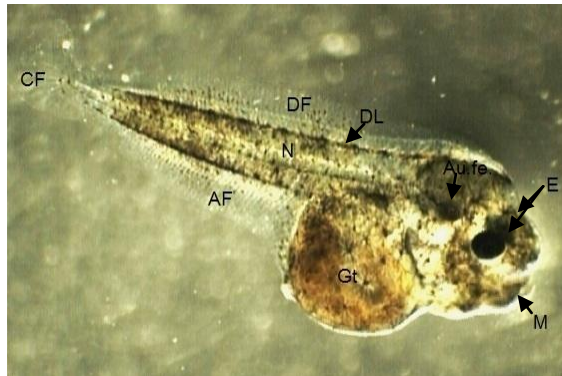

Fig. 8: *Larva at age 12 (DAH), fed with Artemia showing eye (E), mouth (M), auditory vesicles (Au.fe.), notochord (N), gut (Gt), dorsal fin (DF), dorsal line (DL), caudal fin (CF) and anal fin (AF) (3.5X).*
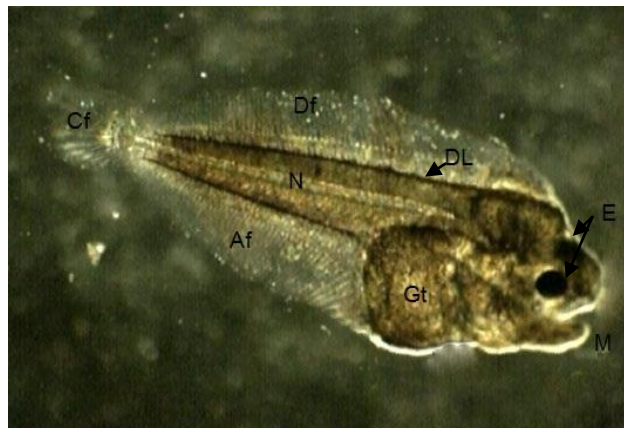

Fig. 9: *Larva at age 15 (DAH), showing eye (E), mouth (M), notochord (N), gut (Gt), dorsal fin (DF), dorsal midline (DL), caudal fin (CF) and anal fin (AF) (3.5X).*

begin to assume their definitive look, and the caudal rays appear stronger. This slow transformation of the *Solea vulgaris* larva to a dorsoventrally flat-bodied fish continued during the fourth week of its life (Fig. 11).

At the age of 25 DAH, the larvae transformed symmetric floating larvae to asymmetric benthic juvenile. Total body length was about 7.7±0.3 mm. Both eyes now in the right side of the body and the juvenile assumes the benthic behavior typical of flatfishes. The left eye was 0.3 mm in diameter, but the right eye reached 0.35mm in diameter. The head was about 2.4 mm in height and 2.2 mm in length (Fig. 12).

At the age of 31 days± 18h, the larva reached 9.40±0.80 mm. The pattern of pigmentation of sub-adult was becoming apparent. *The pre-anal length was 1.1 mm and the post-anal length was 5.6 mm to the tip of the tail.* The head was about 2.5 mm in height and 2.3 mm in length, with eye diameter reached 0.4 mm. All primitive organ systems were represented at this stage. At the age of 35 DAH, it is become morphologically typical the adult one; a concentration of the pigment is distributed in all over the body in just one side of the flatfish. The larvae were 10.5±0.58 mm long; the head was about 2.9 mm in height and 3.1 mm in length, with eye

diameter reached 0.5 mm. Eyes assume their position in the right side of the body, under the dorsal midline, the upper jaw complete his curved down and take the beaked form (Fig. 13).

Endoskeleton developmental stage

The procedure which improved to follow development of the sole fish skeleton was used for detecting simultaneously calcium deposition and cartilage formation. Specimens of Sole fish collected during the hatching period present an underdeveloped skeleton, composed only of cartilaginous elements, whereas specimens collected during the period (between 5 and 30, DAH) were calcified.

At 5 DAH, the notochord is the only axial support element. At these early stages, the only fin structures were the primordial marginal fin fold (Fig. 14).

No calcified structures were visible at the early stage, beside the notochord. At this stage a calcified cleithrum became visible. The first four cartilaginous hypural plates appeared ventrally at the posterior extremity of the notochord.

At 10 DAH the larvae presented a more advanced distribution of

Fig. 10*: Larva at age 19 (DAH), showing upper and lower jaw (UJ&LJ), notochord (N), auditory vesicles (Au.fe.),gut (Gt), caudal fin (CF) and anal fin (AF). The left eye (E) reaches the dorsal midline (DL) of the head. The larvae begin to change their swimming from vertical to benthic. Transparency of the body begins to reduce as skin pigmentation intensifies (2.5X).*

Fig. 11: *Larva at age 21±1 (DAH), showing eye (E), upper and lower jaw (UJ&LJ), operculum (OP) notochord (N), dorsal fin (DF), caudal fin (CF) and anal fin (AF) (2.5X).*
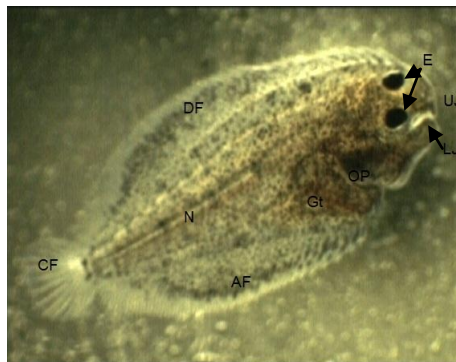

Fig. 12: *Juvenile at age 25 (DAH), showing eye (E), upper and lower jaw (UJ&LJ), operculum (OP) notochord (N),gut (Gt), dorsal fin (DF), caudal fin (CF) and anal fin (AF). Juvenile were fed with Artemia metanauplii. Both eyes are on the right side of the body and the sole assumes the benthic behavior typical flatfish (1.5X).*

Fig. 13: *Juvenile at age 35 (DAH), showing notochord (N) dorsal fin (DF), caudal fin (CF) and anal fin (AF). Appearance of the juvenile after one week of beginning the weaning stage, fed with Artemia metanauplii plus artificial diet (0.67X).*

skeletal elements (Fig. 15-a). Meckel's cartilage migrated forward and the mouth was already opened. At this stage calcification continued to increase in the cleithrum. The ceratohyal cartilage began mineralizing in a median region (Fig. 15-b) extending subsequently to both extremities.

The first forming vertebrae were observed at 10 DAH as a mineral deposition over the notochord, starting on the posterior zone with the caudal fin and then extending to the rest of fins. Vertebral formation continued towards the posterior end and all individuals showed vertebrae surrounding the notochord. The first calcified rays of the caudal fin were forming by intra-membranous calcification. At the age of 12 DAH, the dorsal fin were taking her form, with about 35 fin rays and the anal fin fold had 24 rays.
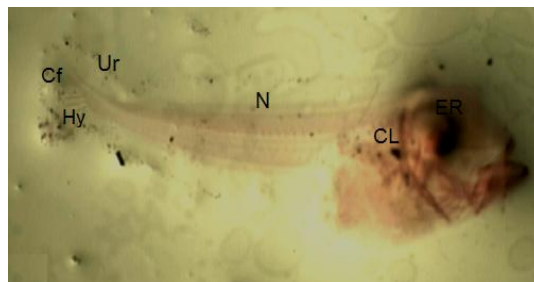

Fig. 14: *A normal endoskeleton of Solea vulgaris Larvae at 5 DAH. Symmetric larvae showing; Eye rings (ER), Notochord (N), Cletihrum (Cl) and Urostyle (Ur) with its Hypural (Hy). Stained with Alcian blue & Alizarin red double staining. Magnification (3).*
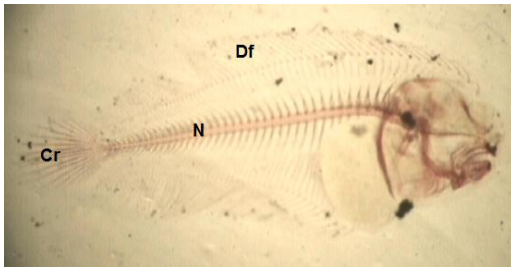
Fig. 15-a: *A normal endoskeleton of Solea vulgaris Larvae at 10 DAH. Larvae showing; Notochord (N), Dorsal fin (Df) and Caudal rays (Cr). Stained with Alcian blue & Alizarin red double staining. Magnification (2.5).*
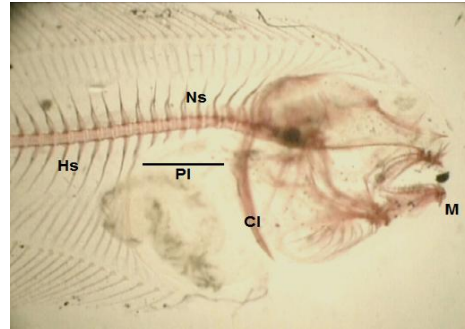

Fig. 15-b: *A normal endoskeleton of larval head, Solea vulgaris, at 10 DAH. Larvae showing; Cletihrum (Cl), Pleural vertebra (Pl), Meckels' Cartilage (M), Neural spine (Ns) and Haemal spine (Hs). Stained with Alcian blue & Alizarin red double staining. Magnification (4).*

The first mineral deposits appear in the Meckel's cartilage. The neural arches are already with visible calcification at this stage, while the almost completely formed vertebral column was visible at 23 DAH (Fig. 16, a-b) with all structures calcified.

The caudal fin achieved the total number of structures with all rays, hypurals and spines. The dorsal (40 rays) and anal (35 rays) fins were already formed and largely mineralized while in pectorals, mineralization of the rays just began.
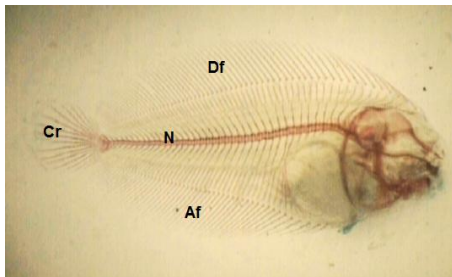

Fig. 16-a: *A normal endoskeleton of Solea vulgaris Larvae at 23 DAH. Asymmetric larvae showing; Notochord (N), Cletihrum (Cl), Dorsal fin (Df), Anal fin (Af) and Caudal fin (Cf) with it rays (Cr). Stained with Alcian blue & Alizarin red double staining. Magnification (2).*
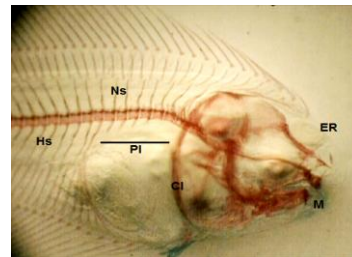

Fig. 16-b: *A normal endoskeleton of larval head, Solea vulgaris, at 23 DAH. Asymmetric larvae showing; Cletihrum (Cl), Pleural vertebra (Pl), eye ring (ER), Meckels' Cartilage (M), Neural spine (Ns) and Haemal spine (Hs). Stained with Alcian blue & Alizarin red double staining. Magnification (4).*

The head was largely mineralized although skull bones forming by intramembranous calcification and cartilage undergoing endochondral ossification continued to develop until late juvenile stages.

Development of vertebral column elements occurred in parallel with caudal fin structures and eye migration. By the time development of the vertebral column and formation of the caudal fin were completed, eye migration had also occurred.

Major morphological changes during metamorphosis imply acquisition of asymmetry and occur in parallel with changes in life style from pelagic to benthic. These changes are; eye migration from left to right side and concomitant bending of the urostyle and torsion of internal organs that starts during the process of eye migration.

In the largest individual observed (30 DAH), the vertebral and caudal skeleton were totally formed and mineralized.

The vertebral column was composed of vertebrae, separated in abdominal and caudal, including the urostile (Fig. 17, a-b). Each abdominal vertebra was equipped dorsally with a neural arch and neural spine and ventrally with a pair of parapophysis from the fourth to the eighth vertebrae. The first three neural spines were generally thicker than the others. The caudal vertebra was equipped dorsally with a neural arch and neural spine and ventrally with a haemal arch and haemal spine. The parapophysis of pleural vertebrae 1 and 2 were elongated.

A schematic representation of the early skeleton is given in (Fig.18) and centered on the structures focused in this study.

DISCUSSION

In this work, the gonadal acceleration recrudescence and induction of spawning in a local population of *Solea vulgaris* were presented. In the wild, *Solea vulgaris* are reproductively active throughout the period from December to late March (Assem, 1995). Spawning induction in the present study was carried out in two steps as commonly used in various fish species (lee *et al*., 1987; Marte, 1989 and El-Gharabawy and Assem, 2006). The wedge sole, from the Bay of Biscay to the western Mediterranean, their spawning season spans the months of December to March- April (Vila and Jimenez, 2002).
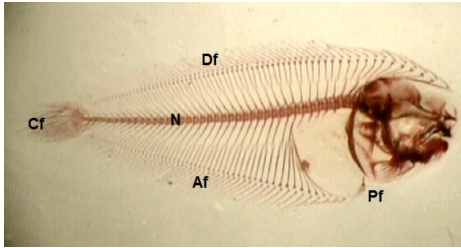
Fig. 17-a: A normal endoskeleton of Solea vulgaris Larvae at 30 DAH. Asymmetric larvae showing; Eye ring (ER), Cletihrum (Cl), Notochord (N), Dorsal fin (Df), Anal fin (Af) and Caudal rays (Cr). Stained with Alcian blue & Alizarin red double staining. Magnification (1.3).
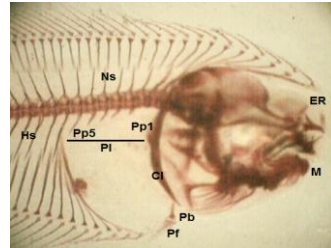

Fig. 17-b: A normal endoskeleton of larval head, Solea vulgaris, at 30 DAH. Asymmetric larvae showing; Cletihrum (Cl), eye ring (ER), Meckels' Cartilage (M), Pleural vertebra (Pl) containing Parapophysis (Pp), Pelvic bone (pb), Pelvic fin (Pf), Neural spine (Ns) and Haemal spine (Hs). Stained with Alcian blue & Alizarin red double staining. Magnification (2.5).

Herrera *et al*. (2008), studied reproduction and breeding of the wedge sole in captivity and indicated that the breeders adapt easily to captivity, and they can spawn in less than one year in captivity. Artificial spawning of S*olea solea* was recommended by (Zaki and Hamza, 1988; Assem, 1995 and Agulleiro *et al*., 2006).

Hormonal induction of ovulation for *Solea vulgaris* was successful with HCG, CPE and LHRHa. In present study, artificial spawning of *Solea vulgaris* were achieved using CPE from 40- 70 µg/fish or HCG from 2300 to 3000 IU/fish as a priming dose, followed by LHRHa about 200 µgm/kgm in the resolving dose.
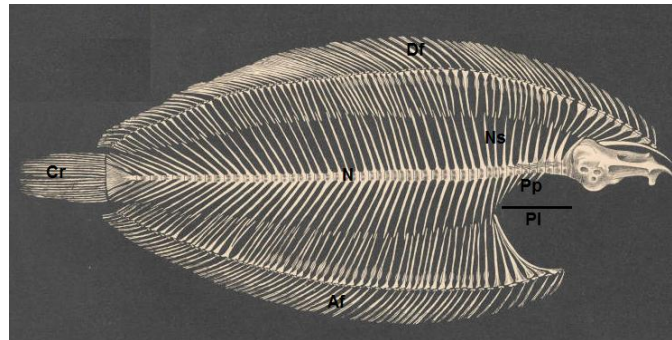

Fig. 18:*Skeletal structure of Solefish showing; Notochord (N), Neural spine (Ns), Haemal spine (Hs), Dorsal fin (Df), Anal fin (Af), Caudal rays (Cr), Pleural vertebra (Pl) and Parapophysis (Pp).*

66

Berlinsky *et al*. (1997) induced the ovulation of *P. dentatus* with HCG (total dose for ovulation equal to 500 IU Kg^{-1}) and CPE (total dose for ovulation equal to 16 mg Kg^{-1}). Ovulation of *p. tropicus* was obtained with HCG 2000 IU Kg^{-1} (Rosas *et al*., 1999). Bambill *et al*. (2006) achieved good results, inducing the spawning with 1000 IU Kg^{-1}.

Agulleiro *et al*. (2006), studied induction of spawning of captive-reared Senegal sole (*Solea senegalensis*) using different administration methods for gonadotropin- releasing hormone agonist and concluded that: female injected with a dose of 5 µg GnRHa/kg three times a week, or treated with a single GnRHa loaded implant (50 µg/kg) showed multiple ovulations and spawns within a period of approximatiple 30 days. Mylonas and Zohar (2001) listed nine flatfish species where ovulation and/or spawning was induced using different GnRHa delivery system ranging from 30 to 1000 µg/kg.

However, the responsiveness of individual fish, in terms of number of spawns and fecundity is variable. In present study, *Solea vulgaris* shows a group- synchronous ovarian development in which successive batches of vitellogenic oocytes are recruited into FOM and ovulation during the spawning season. This pattern of ovarian development, also observed in other flatfish species, often gives rise to multiple ovulations after GnRHa treatment (Mylonas and Zohar, 2001 and Agulleiro *et al*., 2006).

Assem (1995) reported that, in artificial spawning of *Solea solea*, the total number of spawned egg varied between 1.3x 10^6 egg per kg minimally and 2.1x 10^6 egg per kg maximally at water temperature 17±2.2°C and salinity 34±1 ppt. Herrera *et al*., (2008), indicated that for Wedge Sole (*Dicologoglossa cuneata*) a relative fecundity reached 1.06- 2.33x 10^6 egg kg per spawning season, gametes are released in a wide temperature range, 10- 21 °C. Similar result for relative fecundity were reported for other flatfish species cultured in Europe (*S. solea*, *S. senegalensis*, *H. hippoglossus*) except turbot, which presents similar or higher values (Howell, 1997; Dinis and Reis, 1995; Leclercq, 1994 and Tilseth, 1990).

In present study, hatching of *Solea vulgaris* was completed at the age of 38±2 h post fertilization at temperature of about 17±2°C. Whereas, incubation lasted 36- 48 h for wedge sole at 19° C by Herrera *et al*. (2008). In the present study, as the

spawning period progressed and the water temperature in the spawning tanks rose, the incubation time shortened, even to as low as 48 hours. The total number of spawned egg varied between 2.9x 10^5 eggs per kg minimally and 4.2x 10^5 egg per kg maximally.

The ripe unfertile eggs of *Solea vulgaris* were rounded, colorless, transparent with about 13- 20 oil globules. The surface of the fertilized egg shell is smooth; however, the yolk was segmented. In agreement with present study for ripe and fertile egg was described by (Zaki and Hamza, 1988 and Assem, 1995) for *Solea solea*. Herrera *et al*. (2008). Concluded that for the wedge sole (*Dicologoglossa cuneata*) the fertilized eggs measured 827±4 μm, and they contained 18- 22 oil drops of 19- 105 μm. Fertilized and non- fertilized eggs could not be distinguished until embryo formation (18- 24h).

Dinis and Reis (1995), studied the broodstock management and larval rearing of *Solea senegalensis* and concluded that after 7 months in captivity a wild broodstock spawned naturally at temperature ranging from 16.5±0.5 °C to 22±1.0 °C and salinity from 30 to 35 ppt. Egg with 100% fertilization presented viability ranging from 100% to 90%.

The fertilization rate of wedge sole (46.8- 73.8%) was discussed by Herrera *et al*. (2008), who concluded that the fertilization rates are slightly higher than those found for *Solea senegalensis* (39.8- 67.6%) by Anguis and Canavate (2005), and other flatfish species new for aquaculture as *Colistium nudipinnis* and *Colistium guntheri* by (Tait and Hichman, 2001).

In the present study, the percent of fertilization varied between 80 and 93%, whereas percent of hatching ranging between 89 and 90% at water temperature of 17±2 °C and salinity 34±1 ppt.

The hatching rate of wedge sole (58.3- 85.2%) were recorded by Herrera *et al*. (2008). There results were higher than those described by Anguis and Canavate (2005) for Senegal sole (55.4- 70.9%) and other cultured flatfish species as halibut (14- 51%) by Mazorra *et al*. (2003).

The newly hatched larvae In the present study were inactive and usually remained upside down suspended in water column. Thereafter, larval activity increased after the second day as having weak swimming activity with the posture of belly up and head down, sometimes moving with jerky motion up and down.

The newly hatched *Solea vulgaris* larvae was about 1.8 mm in total length which in agreement with (Zaki and Hamza, 1988; and Assem, 1995) who stated that the total length of *Solea solea* larvae at 5.5 days±12h and 2.5 days were 2.3 and 2.1 mm, respectively. Herrera *et al*. (2008) stated that the newly hatched wedge sole larvae were very small and fragile (2.34 mm) Jimenez *et al*. (2001) reported that the newly hatched wedge sole larvae total length in wild is 1.57mm. This value is smaller than that recorded in captivity (2.39 mm). This fact may be related to the large size of the captive breeders, bigger than those found in the wild.

In the present study, larvae began feeding 3- 5 days after hatching, in *Solea vulgaris* mouth opining and the well development of its upper and lower jaws allow the larvae to take food at three to four days. The time of eye compellation is nearly that of mouth opining and axis becomes straight, which helps the larvae to identify their food as internal- external feeding starts. These results are ingreement with study of Zaki and Hamza (1988), for *Solea solea*; Assem (1995), for *Solea solea*; El-Gharabawy and Assem (2006), for *Mugil cephalus*; and Herrera *et al*. (2008) for wedge sole. Kvenseth *et al*. (1996) stated that the development of functional eye at the time when the larvae halibut have been observed to capture prey and when the digestive system appeared histologically functional.

The behavior of benthonic *S. vulgaris* post larvae is completely different from that of other pleuronectiforms. It constantly swims throughout the entire water column, while only a few individuals remain calmly on the bottom. This behavior becomes less frequent when a fish grows, although it never disappears. A reduction in light intensity and in population density in the tanks might avoid stress problems. In agreement with present study Herrera *et al*. (2008) described the behavior of wedge sole post- larvae.

The alizarin red-alcian blue double staining method for cartilage and bone allows the visualization of the entire skeleton throughout vertebrate development and the easy detection of any deformities that may occur. Similar techniques have been used to localize bone and cartilage in froglets (Martı`nez et al., 1992), fetal mice (Webb and Byrd, 1994), and larvae and juveniles from sea bass (Boglione et al., 1993; Marino et al., 1993) and gilthead sea bream (Faustino and Power, 1998; Gavaia et al., 2000& 2002).

The double staining procedure presented in this study was carried out to identify the cartilage and bones during the larval skeleton development, since it allows the detection of small structures undergoing mineralization such as the jaw apparatus and branchial arches of sole larvae. This technique also demonstrates useful view for early detection of skeletal malformation in Sole Senegalese (Gavaia *et al*., 1999). Knowledge of the normal pattern of development of skeletal structures is essential prior to identification of the factors responsible for the onset of skeletal deformities (Gavaia *et al*., 2002).

In the present study, the first calcified structures to appear are the appendicular elements (cleithrum) at 2DAH that was in agreement with (Gavaia *et al*., 2000 & 2002). Development of both caudal complex and vertebral column begins at 12–13 DAH, accompanying the urostyle torsion and acquisition of asymmetry by migration of the left eye. At this stage, larvae progressively change to a benthonic life style. Similar observations were made in the Japanese flounder, where the flexion of the notochord is closely related to the development of hypuralia (Hosoya and Kawamura, 1998 and Gavaia *et al*., 2000 & 2002).

The caudal fin of Senegal sole is the first to develop, acquiring full meristic count after 6.1 mm Lst, followed by anal and dorsal and then paired fins. This same sequence of fin development has also been observed in this study on *Solea vulgaris*, also it indicated in the red sea bream (Kohno *et al*., 1983), the milkfish (Taki *et al*., 1987), the common dentex (Koumoundouros *et al*., 1999) and the Japanese flounder (Hosoya and Kawamura, 1998). In *Solea vulgaris*, there is a large variation in size and stage of development of larvae of the same age. This occurrence has also been reported for the gilthead seabream (Faustino and Power, 1998) and Senegal sole Gavaia *et al*., 2002).

REFERENCES

Agulleiro, M. J.; Anguis, V.; Cañavate , J. P.; Martínez-Rodríguez, G.; Mylonas, C. C.; Cerdà, J. (2006). Induction of spawning of captive-reared Senegal sole (*Solea senegalensis*) using different administration methods for gonadotropin-releasing hormone agonist. Aquaculture 257: 511–524.

Anguis, V.; Canˇavate, J.P. (2005). Spawning of captive Senegal sole (*Solea senegalensis*) under a naturally fluctuating temperature regime. Aquaculture 243:133–145.

Assem, S. S. (1995). Reprodctive physiology and induced spawning of *Solea* species. Ph.D thesis, Faculty of Science Alexandria University pp. 270.

Cabral, H.N., (2000). Distribution and abundance patterns of flatfishes in the Sado Estuary, Portugal. Estuaries 23, 351–358.

Canãvate, J.P.; Zerolo, R.; Ferna´ndez-Dı´az, C. (2006). Feeding and development of Senegal sole (*Solea senegalensis*) larvae reared in different photoperiods. Aquaculture 258(1–4):368–377.

Dinis, M.T.; Metallier, R.; Rodeia, J. (1987). Weaning of *Solea senegalensis* Kaup 1858 with rehydratable pellets. Nato Workshop on Nutrition, INIP Publ. 12, pp 139–147.

Dinis, M.T.; Reis, J. (1995). Culture of *Solea senegalensis*. In: Marine Aq. Finfish species diversification. CIHEAM—IAMZ. Zaragoza. Spain. Cahiers Options Mediterraneenne 16, 169 pp.

Dinis, M.T.; Ribeiro, L.; Soares, F.; Sarasquete, C., (1999). A review on the cultivation potential of *Solea senegalensis* in Spain and Portugal. Aquaculture 176, 27–38.

El-Gharabawy, M.M. and Assem, S. S.(2006): Spawning induction in the Mediterranean grey mullet Mugil cephalus and larval developmental stages. African Journal of Biotechnology, Vol. 5 (19): 1836 -1845

Herrera, M.; Hachero, I.; Rosano, M.; Ferrer, J. F.; Ma´rquez, J. M.; Navas, J.I. (2008). First results on spawning, larval rearing and growth of the wedge sole (*Dicologoglossa cuneata*) in captivity, a candidate species for aquaculture. Aquacult Int (2008) 16:69–84

Howell, B.R. (1997). A re-appraisal of the potential of the sole, *Solea solea* (L.), for commercial cultivation. Aquaculture 155:355–365.

Imsland, A.K.; Jonassen, T.M. (2003). Growth and age at first maturity in turbot and halibut reared under different photoperiods. Aquac Int 11:463–475.

Jardas, I. (1996): Jadranska ihtiofauna. Školska knjiga, Zagreb, 536 pp.

Jime´nez, M.P.; Pin˜eiro, C.; Sobrino, I.; Ramos, F. (2001). Studies on age determination and growth pattern of the wedge sole *Dicologoglossa cuneata* (Moreau,

1881) in the Spanish waters of the Gulf of Cadiz (southwest Iberian Peninsula). Bol Inst Esp Oceanogr 17(3 and 4):279–285.

Kvenseth, A. M, Pittman, K. and Helvil, J. V. (1996). Eye development in Atlantic halibut (*Hippoglossus hippoglossus*): differentiation and development of the retina from early yolk sac stage through metamorphosis. Can. J. Fish Aquat. Sci. (53):2524- 2532.

Lee, C.S; Tamaru, C.S; Miyamoto, G.T.; Kelley, C.D. (1987). Induced spawning of grey mullet (*Mugil cephalus*) by LHRH-a Aquaculture 62:327-336.

Leclercq, D. (1994), Turbot broodstock management: a key-point to mid-term progress. In: Lavens, P.; Sorgeloos, P.; Remmerswaal, R.A.M. (eds): Turbot culture: problems and prospects. Proc. of satellite workshop of World Aquaculture '93 (EAS Sp. Pub. N 22), Ostende, Belgium, pp 3–13.

Marte, C.L., (1989). Hormone induced spawning of culture tropical finfishs. Pp. 519-541. In: Advances in tropical Aquaculture. Feb 20-March 4, 1989, Tahiti (French Polnesia). IFREMER, Brest.

Mazorra, C.; Bruce, M.; Bell, J.G.; Davie, A.; Alorend, E.; Jordan, N.; Rees, J.; Papanikos, N.; Porter, M.; Bromage, N. (2003). Dietary lipid enhancement of broodstock reproductive and egg and larval quality in Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 227:21–33.

Morais, S.; Narciso, L.; Dores, E.; Pousão-Ferreira, P.; (2005). Lipid enrichment for Senegalese sole (*Solea senegalensis*) larvae: effect on larval growth, survival and fatty acid profile. Aquac Int 12:281–298.

Mugnier, C.; Guennoc, M.; Lebegue, E.; Fostier, A.; Breton, B., (2000). Induction and synchronisation of spawning in cultivated turbot (*Scophthalmus maximus* L.) broodstock by implantation of a sustained-release GnRH-a pellet. Aquaculture 181, 241–255.

Mylonas, C.C.; Zohar, Y., (2001). Use of GnRHa-delivery systems for the control of reproduction in fish. Rev. Fish Biol. Fish. 10, 463–491.

Rueda-Jasso, R.; Conceição, L.E.C.; Dias, J.; De Coen,W.; Gomes, E.; Rees, J.F.; Soares, F.; Dinis, M.T.; Sorgeloos, P., (2004). Effect of dietary non-protein energy levels on condition and oxidative status of

Senegalese sole (*Solea senegalensis*) juveniles. Aquaculture 231, 417–433.

Sobrino, I.; Jime´nez, M.P.; Ramos, F.; Baro, J. (1994). Descripcio´n de las pesquerı´as demersales de la regio´n suratla´ntica espan˜ola. Inf Te´c Inst Esp Ocenogr 151:1–79.

Tait, M.J.; Hickman, R.W. (2001). Reproduction, gamete supply and larval rearing of New Zealand turbot *Colistium nudipinnis* (Waite 1910) and brill *Colistium guntheri* (Hutton 1873): a potencial new aquaculture species. Aquaculture 32:717–725.

Tilseth, S. (1990). New marine fish species for cold-water farming. Aquaculture 85:235–245.

Tvedt, H.B.; Benfey, T.J.; Martin-Robichaud, D.J.; Power, J., (2001). The relationship between sperm density, spermatocrit, sperm motility and fertilization success in Atlantic halibut, *Hippoglossus hippoglossus*. Aquaculture 194, 191–200.

Vermeirssen, E.L.M.; Shields, R.; Mazorra de Quero, C.; Scott, A.P. (2000). onadotropin-releasing hormone agonist raises plasma concentrations of progestogens and enhances milt fluidity in male Atlantic halibut (*Hippoglossus hippoglossus*). Fish Physiol. Biochem. 22, 77–87.

Vila, Y.; Jime´nez, M.P. (2002). Reproductive biology of *Dicologoglossa cuneata* (Moreau, 1881) in three zones of Atlantic Iberian coast. Thalassas 18(1):19–29.

Zaki, M. I. and Hamza, A. K. (1988). Artificial fertilization and developmental stages of *Solea solea*. J. Folia morphological. Vol. (36): 229-242.

Zohar, Y., Mylonas, C.C., (2001). Endocrine manipulations of spawning in cultured fish: from hormones to genes. Aquaculture 197, 99–136.

التفريخ الصناعى والمراحل التطوريه الجنينيه واليرقيه لاسماك موسى (السوليا فولجارس) بالبحر الابيض المتوسط.

سميرة شحاته عاصم*، علاء عبد الكريم الدحار**، هبة سعد السيد*، محمد السيد سلامة**، منى محمد مراد*.

*المعهد القومى لعلوم البحار والمصايد، قايتباى الاسكندريه.

** قسم الانتاج الحيوانى والسمكى، كلية زراعة – ساباباشا، جامعة الإسكندرية.

الملخص العربى

فى هذه الدراسة تم تفريخ اسماك موسى على مرحلتين. تمت المرحلة الاولى بحقن الامهات بالجرعة الابتدائيه إما بخلاصة الغده النخاميه لأسماك المبروك من ٤٠-٧٠ مجم/ سمكه، أوالجونادوتروبين البشرى من ٢٩٦٠ الى ٣١٠٠ وحده دولية لكل سمكة. بعد الإنتهاء من الجرعه الثانيه وهى ٢٠٠ ميكروجرام/كجم من LHRHa ومراعاة نسبة الذكور الى الاناث ١:١ وذلك لتحسين معدل الاخصاب، وقد يتم التبويض بعد حوالى ١٨-٢٤ ساعة.

تم الحصول على عدد من البيض الملقح يتراوح بين ٨٧٠٠٠ و ١٢٠٠٠٠بويضة لكل انثى وذلك بمعدل ٤٣٠±١٠٨ بويضه لكل جرام من وزن الجسم مع ملاحظة ان البويضة الناضجه ، الغير ملقحه دائريه عديمة اللون وشفافه وتحتوى على حوالى من ١٣-٢٠ فقاعه زيتيه. يحيط البويضه الملقحه غلاف رقيق يظهر من خلاله المح البويضه الملقحه دائريه وقطرها ١,٢ x ١,٠٤ ملم. بعد حوالى ٣٥±٥ دقيقه من الاخصاب تصل انقسامات البويضه الى المرحلة الجنينيه. مرحلة المريولا تبدأ بعد ٤ ساعات±١٥دقيقه. تبدأ عملية الجاستريولا بعد حوالى ٨ ساعات وقد تنتهى بمرور ساعات±٣٠ دقيقه. بعد ٢٣±٥ ساعات من الاخصاب تبدأ عملية التخليق الجنينى وتم ظهور بداية الجنين داخل البويضة بعد مرور حوالى ٤٠±١٢ ساعة من الاخصاب. وقد اكتمل الجنين بعد حوالى ٣٨±١٢ ساعة من الاخصاب. تمت عملية الفقس بعد مرور ٤٨±١٥ من الاخصاب عند درجة حرارة الماء ١٥±٢°م ودرجة ملوحة ٣٤ جزء فى الالف بنسبة اخصاب حوالى ٨٢-٨٨% ونسبة فقس حوالى ٩٠%. بعد ٨ ايام من الفقس تم امتصاص كيس المح تماما وكان طول جسم اليرقه ٣,٢ مللى. عند اعمار ٣٣ يوم بعد الفقس وصلت اليرقه الى اطوال ١٣,٦ مللى وتتميز السمكه فى هذه المرحلة بوجود جميع اعضاء الجسم عند مقارنتها بالسمكه الناضجه. وقد تم دراسة الهيكل العظمى للأسماك خلال مراحل التطور اليرقى المختلفه.