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Ontogeny of Embryonic and Yolk-Sac Larval Stage of the Sparid Sharpsnout Sea Bream (*Diplodus puntazzo* Cetti, 1777)

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ABSTRACT

In the present study the embryonic and yolk-sac larval growth and development of common sharpnout sea bream *Diplodus puntazzo* (Cetti, 1777), are described and illustrated. The eggs were obtained from captive broodstock (F_1 generation cultured specimens) in October of 2009. Egg incubation and yolk-sac larval development was performed in three laboratory tanks (of 35 l each) at temperature condition of 21°C. For the study of ontogeny, were sampled in the embryonic stage 25 eggs every 30 min and in yolk-sac larval stage, 10 specimens every 4 h. Eggs of *D. puntazzo* are typical sparid eggs whose presented a diameter of 0.868±0.009 μm and a wet weight of 0.349±0.014 mg, while contain a lipid globule of 0.237±0.007 μm diameter. At hatching (41 h after fertilization) the larvae of *D. puntazzo* measured 2.179±0.019 mm TL. The yolk sac resorted 64 h after hatching (autotrophic phase ends), when the larvae reached 3.102±0.046 mm TL. The aim of the present study was to acquire further knowledge on the autotrophic ontogeny of *D. puntazzo*, to help both commercial aquaculture and ichthyoplankton studies.

Key words: *Diplodus*, ontogeny, embryo, yolk-sac larvae

INTRODUCTION

Common sharpnout sea bream, *Diplodus puntazzo* (Sparidae), is a demersal sparid teleost marine fish, inhabiting at depths up to 150 m and widely distributed from the Black and Mediterranean Seas, to European and African coasts of Atlantic Ocean (from Bay of Biscay to Sierra Leone, the Canary Islands and Cape Verde). *D. puntazzo* is one of the target species of the commercial fisheries and a promising candidate for Mediterranean marine aquaculture (Abellan and Basurco, 1999; Favaloro *et al*., 2002), due to its high growth rate and food conversion efficiency (Hernandez *et al*., 2003).

This species is a rudimentary hermaphrodite fish with partial protandry (Pajuelo *et al*., 2008), whose puberty is reached at 2 years old (Georgiou and Stephanou, 1995). The spawning period extend from September to November (Marangos, 1995), with an optimum temperature at 21±0.5°C (Micale *et al*., 1996). Studies on egg and sperm production and quality (Lahnsteiner and Patarnello, 2005; Papadaki *et al*., 2008), larval rearing and development (Boglione *et al*., 2003; Palma and Andrade, 2002), growth and nutrition quality (Sara *et al*., 1999; Orban *et al*., 2000), digestive and pathology (Suzer *et al*., 2007; Pellitero *et al*., 2008) have provided information on the performance of this species in captivity. In addition, there is limited information on ontogeny of autotrophic stages.

The recording of growth and development (ontogeny), of a species provide major information's about the ecological patterns of habitats (Holmes and McCormick, 2010), behavior and swimming activity (Utne-palm and Stiansen, 2002; Georgalas *et al*., 2007), physiology (Zapata *et al*., 2006; Mazon *et al*., 2007), nutrition (Zouiten *et al*., 2008; Yang *et al*., 2010), while the morphological description offers taxonomic criteria for systematic diagnosis during the analysis of ichthyoplankton samples (Kimura *et al*., 2004; Turan *et al*., 2006). The ontogeny analysis, provide the possibility of identification on critical developmental stages and also can be used as a tool for developmental definition in morpho-anatomic abnormalities (Koumoundouros *et al*., 2002; Estevao *et al*., 2005). The above mentioned parameters can be used as section criteria of the type cultured manipulations while offer solutions on quality issues of fish production.

In the present study, described the embryonic and yolk-sac larval ontogeny (growth and development), of common sharpnout sea bream (*Diplodus puntazzo*), with aim of both giving solutions on commercial rearing systems and contribution to ichthyoplankton studies.

MATERIALS AND METHODS

Experiment was performed on October of 2009, when the natural temperature of the seawater was 21°C, with eggs from commercial hatchery of Nireus S.A. Company in Hiliadou Managouli Doridos, Greece. The eggs (52.500 in number) were collected from captive broodstocks, descended from culture specimens (F_1 generation). Maturation and spawning were performed spontaneously under natural photoperiod and temperature conditions. A total of 350 breeders were held in one outdoor polyester cylindroconical tank of 45 m^3 volume (with fish density: 8 kg m^{-3}), supplied with filtered (drum filter, UV) seawater, at a constant flow rate of 50% h^{-1}. Breeders fed on newly frozen fish and cuttlefish, *ad libitum*.

When eggs were sampled from natural mass spawning via overflow collectors, were disinfected with iodine and immediately removed in Technological Education Institution of Messolonghi. With density of 500 eggs L^{-1} (17.500 eggs per tank) the eggs were placed in three tanks (triplicate) 35 l water volume each. In these tanks applied water exchange rate of 50% of the tank volume per hour, via recycled system. Temperature was kept at 21±0.2°C and salinity at 38 ppt. The experiment was placed under darkness. Also, gently aeration was applied only in embryonic stage.

Three early life history traits were measured: egg diameter and lipid globule diameter (samples of 160 eggs) and number of eggs per gram of wet weight (6 samples of 84 to 200 mg). Definition of the stages followed by Cassie (1956).

The egg and yolk-sac larval development of *D. puntazzo* was studied *in vivo* using a stereoscopic microscope (Leica ICCA), while morphometric character with photographs via digital camera (Leica DM100) adapted at microscope. For the embryonic stage, 25 eggs were sampled every 30 min. Every sample was examined in respect to the developmental stage of the majority (›50%), of the individuals and to the related developmental events of each stage. In the yolk-sac larval stage, 10 specimens were sampled and photographed every 4 h. On these photographs, twelve morphometric characters (TL, NL, prOr, prAnl, pstAnl, prYs, pstYs, YsL, YsD, BD, ED and LD) were measured (with the program ImageJ) to the nearest 0.001 mm, while YsV and LV, were estimated (Table 1). All lengths were measured parallel to the longitudinal axis of the body and all depths, perpendicular to this axis.

During both embryonic and yolk-sac larval stage, all time intervals were measured from fertilization of approximately 50% of hatching, respectively (t_0 = 0 h). Also, estimated the relative

Table 1: Morphometric characters measured in the present study

Abbreviation	Character	Description
TL	Total length	From tip of snout to the posterior margin of body
NL	Notochord length	From tip of snout to the posterior margin of notochord
prOr	Pre-orbital length	From tip of snout to the anterior margin of eye
prAnl	Pre-anal length	From tip of snout to anus
pstAnl	Post-anal length	From anus to posterior margin of body
prYs	Pre-yolk-sac length	From tip of snout to the anterior margin of yolk-sac
pstYs	Post-yolk-sac length	From tip of snout to the posterior margin of yolk-sac
YsD	Yolk-sac depth	Maximum
YsL	Yolk-sac length	Maximum
BD	Body depth	Posterior to anus
ED	Eye diameter	(Maximum -minimum)/2
LD	Lipid globule diameter	Maximum
YsV	Yolk-sac volume	$(\pi/6)$ YsL x YsD^2 (Blaxter and Hempel, 1963)
LV	Lipid globule volume	$(4/3) \times \pi \times (LD/2)^3$

time (RT_i) of each developmental event i as $RT_i = (t_i/Tsd) \times 100$, were, t_i is the time interval from t_0 to developmental event i and Tsd is the total duration of the autotrophic stages.

RESULTS

Embryonic development: At 38 ppt salinity, the newly fertilized eggs of *Diplodus puntazzo* floated. These eggs were telolecithal, spherical in shape and transparent, with a homogeneous and un-segmented vitellus. They contained a single un-pigmented lipid globule of 0.222 to 0.248 mm (0.237±0.007 mm) diameter. Eggs presented a diameter of 0.845 to 0.885 mm (0.868±0.009 mm) and a wet weight of 0.326 to 0.363 mg (0.349±0.014 mg).

Soon after fertilization, a small perivitelline space developed. The first meroblastic cleavage occurred 1:00' h after fertilization (AF), the second 1:30' h, the third 2:30' h and the forth 3:30' h AF (Fig. 1a-d, respectively) (Table 2). The morula stage was attained 4:30' h AF (Fig. 1e), while the blastula stage 7:30' h AF (Fig. 1f, g). 9:30' h AF, pre-early gastrula stage was determined (Fig. 1h) and blastoderm started flattened. Early gastrula was observed 12:30' h AF, where blastoderm started to expand over the surface of yolk mass (epiboly) cover it about ¼ (Fig. 1i). Epiboly progressively advanced and the blastoderm covered the 1/3 (pre-middle gastrula) (Fig. 1j), the 1/2 (middle gastrula) (Fig. 1k) and the 3/4 (late gastrula) (Fig. 1*l*) of the yolk mass 14:00' h, 15:00' h and 15:30' h AF, respectively. Early neurula stage was observed 16:30' h AF (Fig. 1m) where, neural groove formation started with a mass of cells clearly visible in the anterior side of blastopore. Kuppfer's vesicle was formed near to the caudal end of the body (near to the blastopore) 30 min later (Fig. 1n). The blastopore was completely closed 18:00' h AF.

Following the series of events described in Table 1, the first somites developed 30 min to the blastopore closure. One hour after, the optic vesicles were developed in the head region, while the number of somites was 4 (Fig. 1o). 20:30' h AF embryo was covered the 1/2 of the yolk surface, while the number of somites reached the 6 (Fig. 1p). Pigment started forms in dorsal area of head 21:30' - 22:30' h AF (8 - 10 somites were observed) (Fig. 1q). When appeared 12 somites (23:30' h AF), the heart stared formation. Fourteen somites were determined 24:00' h AF (Fig. 1r) and sixteen somites 25:00' h AF. In the second time, disappearance of Kuppfer's vesicle was observed, while the primordial fin observed visible. After one hour and thirty minutes embryo was covered the 2/3 of the yolk surface, while 18 somites were observed and optic lens were formed in optic

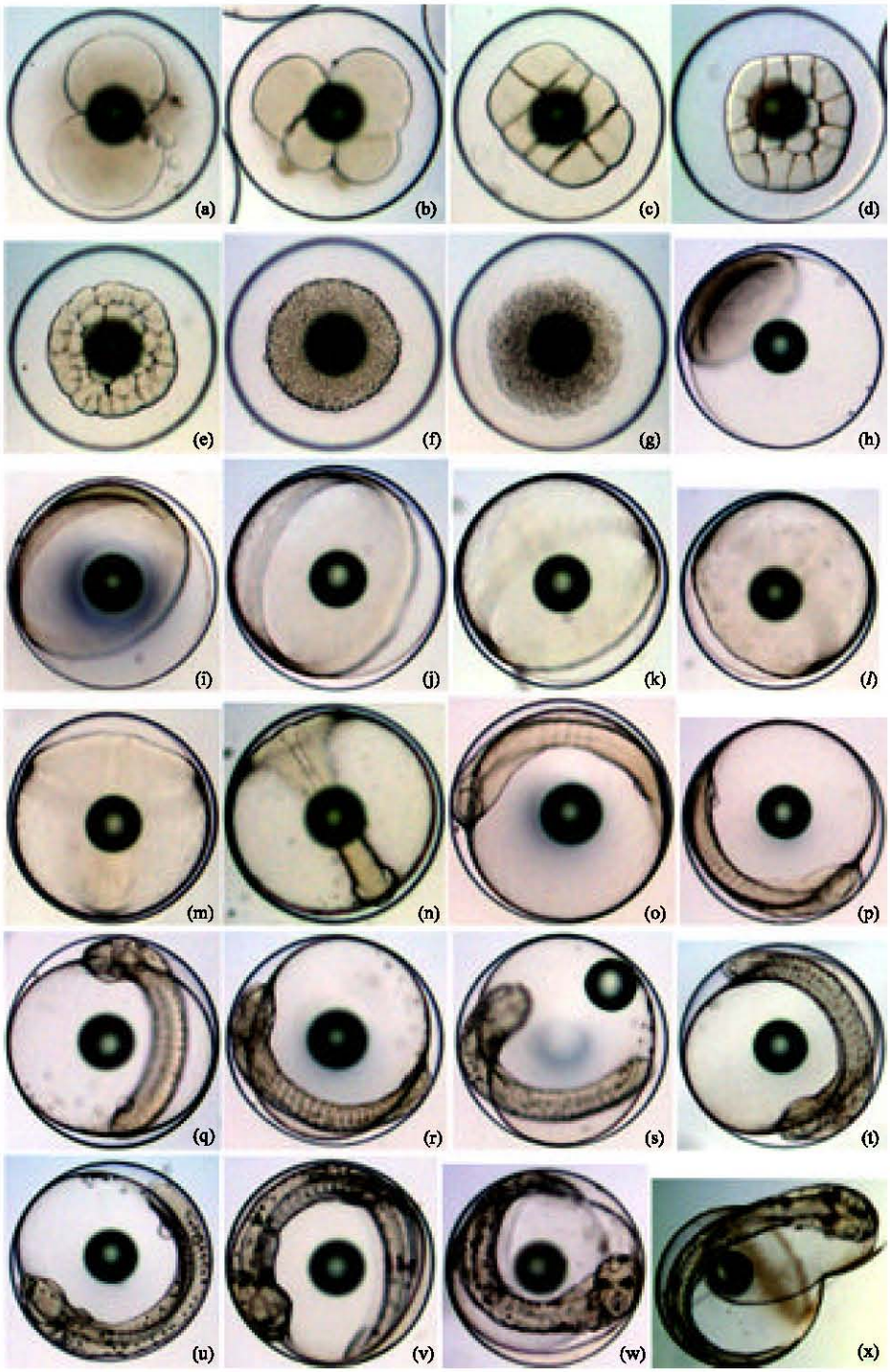

Fig. 1: Embryonic development of *D. puntazzo* at 21°C; (a) 2 cells, (b) 4 cells, (c) 8 cells, (d) 16 cells, (e) morula, (f) early blastula, (g) late blastula, (h) pre-early gastrula, (i) early gastrula, (j) pre middle gastrula, (k) middle gastrula, (*l*) late gastrula, (m) early neurula, (n) late neurula, (o) 4 somites, (p) 6 somites, (q) 10 somites, (r) 14 somites, (s) 18 somites, (t) 20 somites, (u) 22 somites, (v) ¾ embryo, (w) too active embryo and (x) hatching

Table 2: Chronological embryonic development of *D. puntazzo* at 21°C

Stage	Description	Hours	RT_i
Fertilization		0:00'	0.00
Cleavage	First cleavage, 2 cells	1:00'	2.44
	Second cleavage, 4 cells	1:30'	3.66
	Third cleavage, 8 cells	2:30'	6.10
	Forth-fifth cleavage, 16-32 cells	3:30'	8.54
Morula	Much more cells	4:30'	10.98
Blastula	Blastodisc formation	7:30'	18.29
Gastrula	Pre-early gastrula	9:30'	23.17
	Early gastrula, 1/4 epiboly	12:30'	30.49
	Pre-middle gastrula, 1/3 epiboly	14:00'	34.15
	Middle gastrula, 1/2 epiboly	15:00'	36.59
	Late gastrula, 3/4 epiboly	15:30'	37.80
Neurula	Early naurula, neural groove formation	16:30'	40.24
	Kuppfer's vesicle forms	17:00'	41.46
	Late naurula	17:30'	42.68
	Blastopore closed	18:00'	43.90
Embryo	2 somits, optic vesicle developed	18:30'	45.12
	4 somits	19:30'	47.56
	6 somits, embryo surrounds 1/2 of yolk	20:30'	50.00
	8 somits	21:30'	52.44
	10-11 somits	22:30'	54.88
	12 somits, heart formation	23:30'	57.32
	14 somits	24:00'	58.54
	16 somits, Kuppfer's disappears, primordial fin formation	25:00'	60.98
	18 somits, 2/3 embryo, optic lens formation	26:30'	64.63
	20 somits, otoliths formation, heart increased	28:00'	68.29
	22 somits, caudal fin independent from yolk-sac	28:30'	69.51
	3/4 embryo, activated embryo	34:00'	82.93
	Too activated embryo, head extremely developed	39:00'	95.12
Hatching	Start hatching	40:30'	98.78
	50% hatching	41:00'	100.00

vesicle (Fig. 1s). The pigmentation increased at all the surface of the body, with small mass of cells to be arterial and posterior of eyes. Otoliths were developed 28:00' h AF and the heart increased in volume (Fig. 1t). The caudal fin was determined separately from yolk-sac 30 min after, where 22 somites were visible (Fig. 1u). The body increase was continuous and embryo was covered the 3/4 of the yolk surface 34:00' h AF (Fig. 1v). At the same time, the activity of embryo started. Mass of pigment cells was observed in posterior area of otoliths, while three h later pigment cells increased near to the tail. After five hours this activity was very high and the head region show extremely increased (Fig. 1w).

Before the hatching, the pattern of pigmentation included big mass of pigment cells in the areas of arterial and posterior of eyes, posterior of otoliths, dorsal and vertical of body at 5-6th somites (near to the anus), dorsal and vertical of body at 12-13th somites and dorsal and vertical of body at 17-18th somites. The first hatching occurred 40 h and 30 min after fertilization, while after thirty minutes (41 h AF) the 50% of eggs were hatched (Fig. 1x).

Yolk-Sac larval development: The newly hatched larvae of *Diplodus puntazzo* were transparent and floated at the surface of the water. The total length was measured 2.179±0.019 mm (range 2.198-2.147 mm). They were characterized by a large yolk sac extending from the tip of snout to the middle of the body (Fig. 2a), with mean length of 1.065±0.014 mm (range 1.046-1.080 mm), mean width 0.802±0.016 mm (range 0.775-0.819 mm) and mean volume of 0.359±0.017 mm^3. A lipid globule of 0.219±0.006 mm mean diameter and 0.006±0.000 mm^3 mean volume was presented in the middle of the yolk sac. The head and anterior part of the body were curved around the yolk

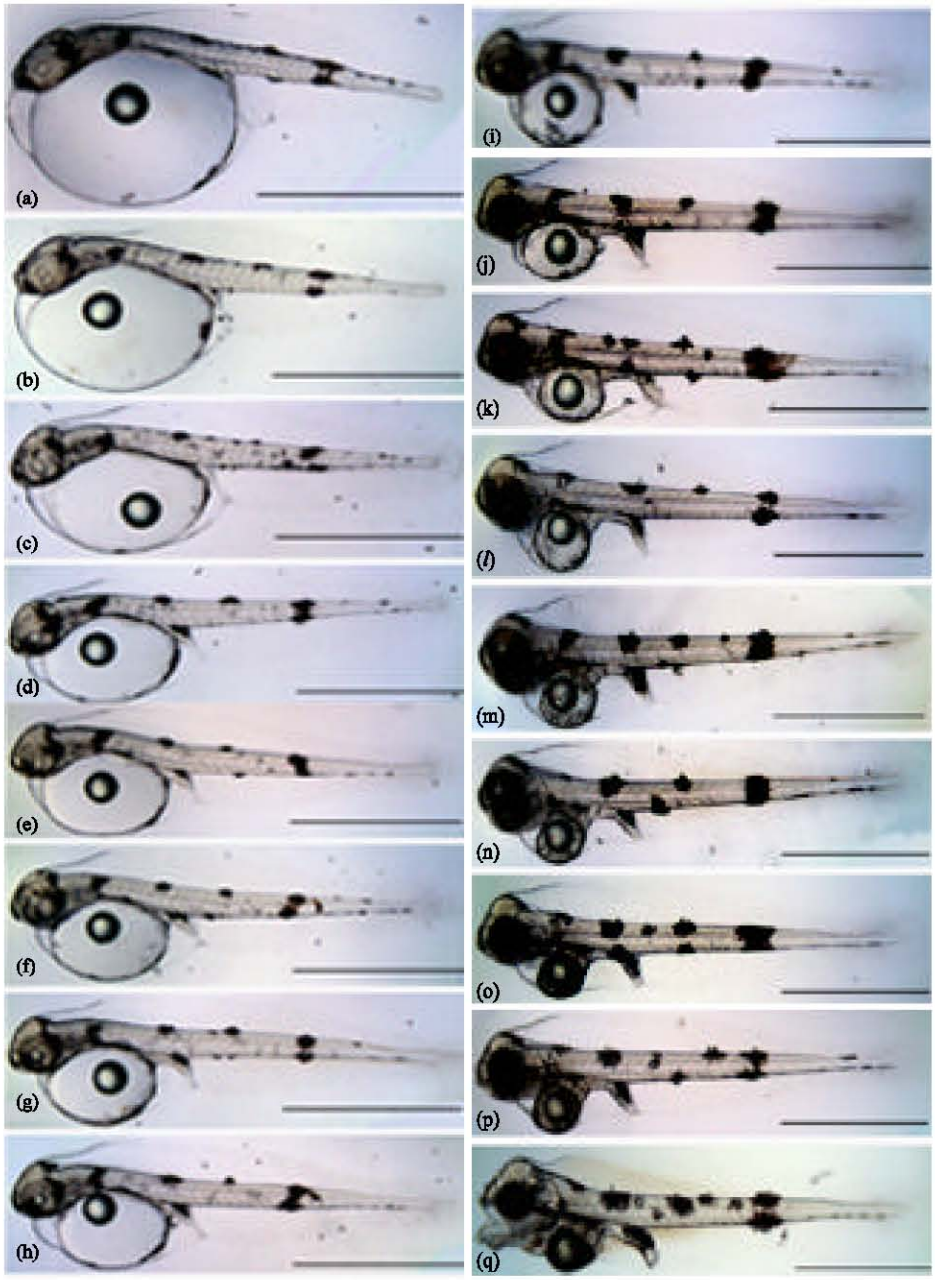

Fig. 2: Yolk-sac larval development of *D. puntazzo* at 21°C; (a) 0 (b) 4 (c) 8 (d) 12 (e) 16 (f) 20 (g) 24 (h) 28, (i) 32 (j) 36 (k) 40, (*l*) 44 (m) 48 (n) 52 (o) 56 (p) 60 and (q) 64 h after hatching

Table 3: Chronological yolk-sac larval development of *D. puntazzo* at 21°C

Developmental character	Hours	RT_i
Hatching	0:00'	0.00
Onset of eye pigmentation	4:00'	6.25
Intestinal canal	16:00'	25.00
Lipid glodule adherence	24.00'	37.50
Ureter	24.00'	37.50
Pectoral fin formation	32.00'	50.00
Intestinal loop	32.00'	50.00
Lower jaw appearance	36.00'	56.25
Liver formation	52.00'	81.25
Mouth opening	52.00'	81.25
Functional mouth	56.00'	87.50
Stomach formed	56.00'	87.50
Black eyes	60.00'	93.75
Anus opening	60.00'	93.75
Absorption of vitelline reserves	64.00'	100.00

sac and the primordial marginal finfold surrounds the body from the dorsal part of the head to the posterior margin of the yolk sac. The intestine was undeveloped, with mouth and anus (this is situated behind the 6-7th somites) closed, while a simple straight tubular gut was observed. Initially, this gut was in close contact with the posterior part of the yolk sac, but 4 h later it detached (Fig. 2b).

Eye pigmentation started four h after hatching (AH), (Fig. 2b) (Table 3), with the number of pigment cells increased gradually in eye surface (Fig. 2c, d). The intestinal canal started the formation 16-20 h AH (Fig. 2e, f). In the next stages, the lipid globule leaved from the yolk sac and was adherence on the body of the larvae, while the same time the ureter was observed (24 -28 h AH) (Fig. 2g, h). In this time, the pattern of pigmentation was the same with the end of embryonic stage, while the only different was the increased number of pigment cells at the same areas, especially at 17-18th somites, forming a spot. The formation of the pectoral fins completed at the middle of the stage (32 h AH) (Fig. 2i).

The development of the alimentary system started with the formation of the intestinal loop (32 h AH) and lower jaw (36 h AH) (Fig. 2j), continued with liver formation (52 h AH) (Fig. 2n) and finished with the stomach formation and faction of the mouth (56 h AH) (Fig. 2o) and anus formation (60 h AH) (Fig. 2p). At the middle of the yolk-sac larval stage (32 h AH), the intestinal canal was slowly covered with pigmentation cells. Near to the end of the yolk-sac larval stage (60 h AH), the eyes were pigmented black and also the oil globule was pigmented. The pattern of pigmentation is demonstrated gradually in all parts of Fig. 2a-q.

The autotrophic phase of *D. puntazzo* at 21°C ended 64 h after hatching (Fig. 2q), where the vitelline reserves were fully absorbed, while the oil globule was still present (with a mean diameter of 0.191±0.005 mm). The TL at feeding onset was measured 3.102±0.046 mm (range 3.045-3.160 mm).

During the yolk-sac larval stage, TL increased rapidly up to approximately 16 h after hatching, followed a slow growth rate to 44 h AH and then kept almost constant up to the end of yolk-sac larval stage (Fig. 3a-h). The same pattern of growth was followed from the NL also. The BD was presented constant for all the pre-larval stage. Specifically, the primordial marginal finfold at the anus area was observed thick at the begging of pre-larval stage and as the development occurred,

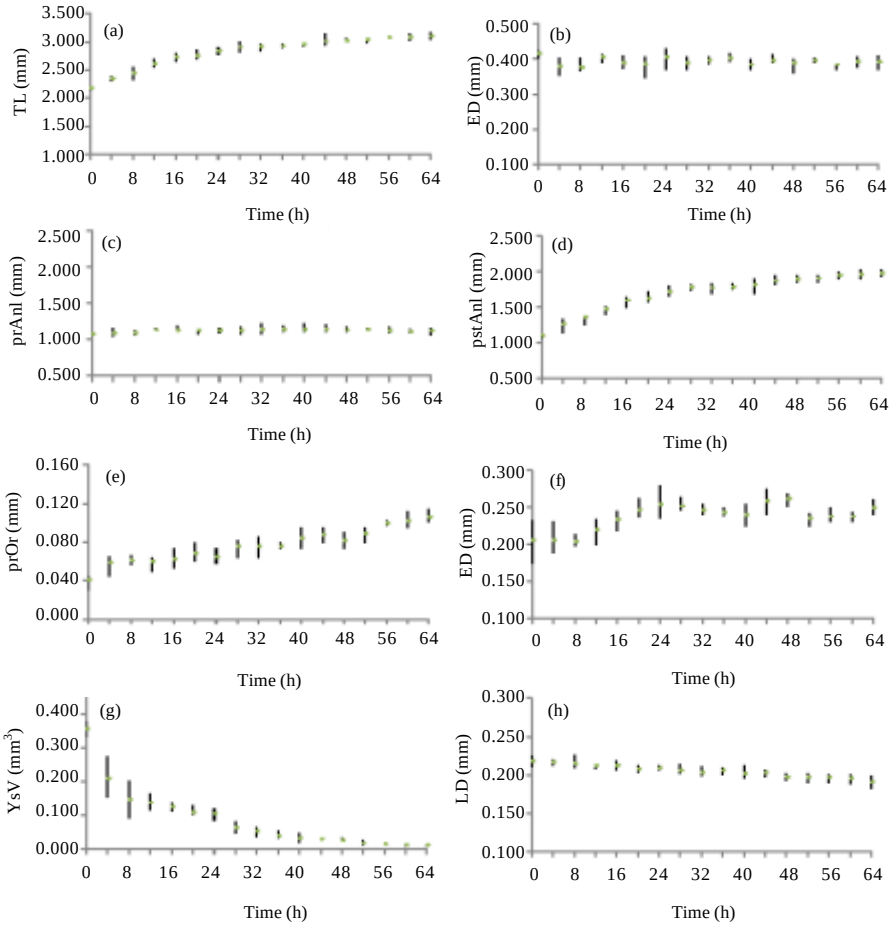

Fig. 3: Evolution of the (a) total length (TL) (b) body depth (BD) (c) pre and (d) post anal length (prAnl, (e) pstAnl), pre-orbit length (prOr) (f) eye diameter (ED) (g) yolk-sac and (h) lipid globule volume (YsV, LV), of *D. puntazzo* yolk-sac larvae, in relation to the time (hours after hatching)

Table 4: Egg and lipid globule diameter of different sparid species

Species	Egg diameter (mm)	Lip. glob. diameter (mm)	Reference
Sparus aurata	0.92-1.04	0.18-0.26	Kentouri (1985)
Diplodus annularis	0.71-0.81	0.18-0.22	Divanach (1985)
Diplodus sargus	0.90-1.16	0.18-0.26	Divanach (1985)
Diplodus vulgaris	0.88-1.04	0.18-0.26	Divanach (1985)
Dentex dentex	0.94-0.96	0.19-0.21	Jug-Dujakovic *et al.* (1995)
Dentex gibbosus	0.94-0.98	0.18	Fernandez-Palacios *et al.* (1994)
Lithognathus mormyrus	0.70-0.82	0.16-0.22	Kentouri (1985)
Pagrus pagrus	0.99-1.09	0.24	Mihelakakis *et al.* (2001)
Pagellus erythrinus	0.74-0.80	0.18-0.19	Klimogianni (2004)
Diplodus puntazzo	0.85-0.89	0.22-0.25	

this depth was reduced while at the same time the depth of body trunk increased. During this stage, the prAnl kept constant rate, while the pstAnl was continuously increased. prOr increased as pre-larval stage developed, while the ED increased with variation. This variation seemed to depend from the degree of the eye pigmentation. The consumption of vitelline reserves was rapidly increased the first eight h after hatching (the YsV was decreased approximately up to 60%), followed a slower rate for another 20 h (the YsV was decreased 28 h AH up to 80%), to consumed totally at the end of the stage. In contrast, the consumption of lipid reserves was slowly increased during the yolk-sac larval stage, while the lipid globule volume was decreased after 64 h approximately up to 35%.

DISCUSSION

In the present study, we studied the egg and yolk-sac larval growth and development of *Diplodus puntazzo*. As many studies have shown, the spawning period of this species extend from September to November, with an optimum temperature at 21±0.5°C. This is the reason for the chosen experimental period and temperature condition.

The ontogeny study of *D. puntazzo,* from fertilization to the full consumption of vitelline reserves seems a typical Sparidae. It is difficult to identify morphological characteristics by which the eggs of this species differ from those of *Sparus aurata* (Kentouri, 1985), *Diplodus annularis* (Divanach, 1985), *Dentex dentex* (Jug-Dujakovic *et al.*, 1995) and *Pagellus erythrinus* (Klimogianni, 2004). Actually, the comparison of the embryonic stage between *D. puntazzo* and the above sparid species showed same ontogenetic pattern, with similar estimated relative time of each developmental event.

Egg diameter and lipid globule diameter of sparid species are compared in Table 4. The *D. puntazzo* egg appeared with bigger diameter from *L. mormyrus*, *D. anularis* and *P. erythrinus* and smaller egg diameter from *D. vulgarys*, *D. sargus*, *S. aurata*, *D. gibbosus* and *P. pagrus*. Only theoretically the egg diameter can be identified on Sparidae species, because in practice the egg size can differ among females of the same species, while seems too dependent upon age, spawning time and geographical origin, nutrition (Kjorvik *et al.*, 1990).

The total length of newly hatched larvae of common sharpsnout seabream is significantly less than those of the rest studied sparid species, except of *Pagellus erythrinus*, with the total length at feeding onset being very close to these of common pandora (Klimogianni *et al.*, 2004). It is suggested that increase in total length is a biologically important aspect of the yolk-sac larval stages (Peterson *et al.*, 1996). In *D. puntazzo* this increase occurs mostly in post anal area. As TL is directly related to the mouth opening and the pray size that larvae are able to consume (Kentouri, 1985), the small size of first-feeding *D. puntazzo* should be taken into account for successful rearing of larvae, with the use of appropriate strains of small rotifers. The TL of newly hatched larvae of *D. puntazzo* may assist in identifying; however, it would be of limited value since during the first few hours after hatching this species show rapidly growth and length change immediately.

Because the rapidly increase of TL at the begging of yolk-sac larval stage, the consumption of vitelline reserves is damage higher in the same period. On the other side, the estimated volume of oil globule is not observed with high decreased. Indeed, the oil globule consumption in yolk-sac larvae stage of *D. puntazzo* seems lower comparative with other Sparidae species (Kentouri, 1985; Divanach, 1985; Klimogianni, 2004). A reasonable probable cause on this fact seems to be the delay of oil globule adherence from yolk-sac to the larvae body. In this study the adherence occurred to *D. puntazzo* 24 to 28 h after hatching, while to the rest searching sparid species found to occur 4 to 6 h after hatching.

Ranzi (1930) suggested the pattern of pigmentation and melanophores distribution as the starting point in diversification of early stages of sparid species. But attention is required as there are many examples of yolk-sac larval samples showed different pigmentation intensity in the same age and length. However, a valid display of pigment cells pattern and their distribution during early developmental stages, can offer previous idea of identification between species of the same family.

The sharpsnout seabream, *Diplodus puntazzo*, has been rearing in Mediterranean aquaculture for more than ten years appearing high levels on survival and growth rate. The next important step is to improve the accuracy on culture manipulation. The study of ontogeny offer knowledge to approach the previous view and the same time to advance the identification in ichthyoplankton studies.

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