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Allometric growth patterns and development in larvae and juveniles of thick-lipped grey mullet *Chelon labrosus* reared in mesocosm conditions

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Abstract :

Allometric growth and ontogeny were studied in thick-lipped grey mullet *Chelon labrosus* reared in mesocosms from 1 to 71 day post hatching (dph). Multivariate allometric analysis of morphometric growth distinguished three distinct developmental stanzas separated by two morphometric metamorphosis lengths (L_{m1} = 4.46 ± 0.06 mm; L_{m2} = 28.56 ± 1.04 mm). Body mass growth also showed three distinct episodes separated by two inflections, correlated with morpho-functional changes. First episode concerned pre-flexion larvae and ended around 4.5 mm-L_T (14-dph), coinciding with estimated L_{m1}. It was distinguished by reduced growth, but intense morphogenesis and differentiation processes. Organogenesis and allometric changes indicated that development priorities concerned feeding efficiency, by improving detection ability (sensory system development), ingestion capacity (head growth) and assimilation performance (digestive system differentiation), together with respiration efficiency (gill development). Second episode concerned post-flexion larvae and, ended around 8.6 mm-L_T (25-dph). It was distinguished by fast growth of trunk and tail, acquisition of adult axial muscle distribution and completion of gill filament development, improving locomotion and oxygenation performances. It corresponded to transition towards metamorphosing stage as indicated by later isometric growth, musculature maturation and acquisition of juvenile phenotype. Metamorphosis seemed to end at L_{m2}, suggesting to avoid zootechnic handling before this size.

Keywords : thick-lipped grey mullet ; *Chelon labrosus* ; larval development ; allometric growth ; mesocosm

1. Introduction

The grey mullets (family Mugilidae) are a group of fish distributed world wide in coastal temperate and tropical waters. Their most noticeable feature is that mullets are an important component in the flow of energy through the ecosystem because they feed on the lowest trophic level, utilizing either the direct grazing or plant detritus food chains as an energy source (Bruslé, 1981a). This feature places grey mullets as a very attractive group of fish species for developing sustainable aquaculture (Crosetti & Cataudella, 1995). The thick lipped grey mullet *Chelon labrosus* (Risso 1827) is among the species considered as having an interesting aquaculture potential (Cataudella, Crosetti, Massa & Rampacci, 1988; Boglione, Bertolini, Russiello & Cataudella, 1992) among others for its capacity to adapt to a wide range of salinities (Lasserre & Gallis 1975; Hotos & Vlahos 1998), including freshwaters (Chervinski, 1977). In addition, the species is also regarded as a candidate for stocking impounded waters (Ben Khemis, Zouiten, Besbes & Kamoun, 2006; Besbes, Besbes Benseddik, Ben Khemis, Zouiten, Zaafrane, Matouk, El Abed & M'rabet, 2010), notably where management of fresh water resources implies the construction of reservoirs in regions where native freshwater species are scarce or not valuable as harvestable fish.

Differences between wild and reared fish have been well documented for a variety of species (Marino, Boglione, Bertolini, Rossi, Ferreri & Cataudella, 1993; Sundell, Dellefors & Björnsson, 1998; Smith & Fuiman, 2004; Boglione, Marino, Giganti, Longobardi, De Marzi & Cataudella, 2009; Izquierdo, Socorro & Roo, 2010). These differences emerge early and are considered consequences of modified lifestyle conditions during larval development (Roncarati, Meluzzi, Melotti, & Mordenti, 2001). They may affect behavioural responses (Gisbert, Williot & Castelló-Orvay, 1999; Yamamoto & Reinhardt, 2003; Smith & Fuiman, 2004), morphological and skeletal traits (Boglione, Costa, Di Dato & Ferzini, 2003; Boglione *et al.*, 2009; von Cramon, Ling, Cotter & Wilkins, 2005; Izquierdo *et al.*, 2010) and/or physiological characteristics (Buckley, Halavik, Laurence, Hamilton & Yevich, 1985; Sundell *et al.*, 1998). Hatchery rearing technique and notably intensiveness markedly affect fish larvae development (Favaloro & Mazzola, 2003, Izquierdo *et al.*, 2010; Roo, Socorro & Izquierdo, 2010; Zouiten, Ben Khemis, Masmoudi, Huelvan & Cahu, 2011), however, mesocosm reared fish are recognized to present good similarity to wild fish with low incidence of deformities (Koumoundouros *et al.*, 1997; Boglione, Gagliardi, Scardi & Cataudella, 2001; Izquierdo *et al.*, 2010). Mesocosm could hence be considered the most appropriate larviculture technique for studying fish larvae development.

Important quantitative morphometric changes take place during the larval stage. They are responsible for a progressive transformation of recently hatched specimens from a larval body shape to a juvenile or adult form in a relatively short time, suggesting that growth functionally optimized for survival is a common feature among fish larvae (Osse & van den Boogart, 2004). This development, which is regulated by gene expression and influenced by the environment (Gilbert & Bolker, 2003), results in different phenotypes with differential relative growth defined as allometry. Allometry describes how the characteristics of an organism scale with each other and with the body size (Fuiman, 1983; Osse & Van den Boogart, 2004). For a morphological characteristic, allometry can be visualized as plots of character's size against the size of the body, reflecting ontogenetic shifts in morphology and phenotypic plasticity in specific environments and rearing conditions (Simonovic, Garner, Eastwood, Kovac & Copp, 1999; Shingleton, Frankino, Flatt, Nijhout & Emlen, 2007). Thus, comprehension of normal larval development and growth patterns might be considered useful tools for monitoring and optimizing hatchery production (Koumoundouros, Divanach & Kentouri, 1999; van Maaren & Daniels, 2000), evaluating the suitability of produced fish for stocking or further rearing (Gisbert, Merino, Muguet, Bush, Piedrahita, & Conklin, 2002) and assessing the quality of the produced young fish (Boglione *et al.*, 2001).

The ability to produce series of early-stage specimens provides an opportunity to enhance our understanding of the early development of fish larvae (Miyashita *et al.*, 2001). In present study, *C. labrosus* larvae and juvenile specimens, reared under mesocosm conditions, were studied to describe development and allometric growth patterns during early life of the species. The objective is to better understand the size related adaptations, as indicators of the priorities during early growth, and hence provide insight into fish biology, behaviour and ecology. For species considered candidate for aquaculture, this information may offer criteria for the proper synchronisation of zootechnic decisions (use of automatic feeders, releasing, *etc.*) with the stage of development of fish during early ontogeny.

2. Materials and Methods

2.1. Larval and juvenile rearing

Larvae and juveniles of *C. labrosus* examined in this study were sampled in 2004 at the Institut des Sciences et Technologies de la Mer (INSTM, Center of Monastir, Tunisia), during a mesocosm rearing trial with semi-extensive technology (Ben Khemis *et al.*, 2006). Eggs were obtained from a captive female (1.905 kg) hormonally induced to spawn with an injection of human chorionic gonadotrophin (HCG) at 10 000 IU kg^{-1} of fish followed five days later with a second injection of both HCG (10 000 IU kg^{-1}) and luteinizing hormone releasing hormone analog (LHRHa) at 100 µg kg^{-1} (Besbes, Fauvel, Guerbej, Benseddik Besbes, El Ouaer, Kraiem & El Abed, 2003). This female and two fluent males (1.290 and 1.450 kg) were then kept in a spawning tank in which a spontaneous spawn was obtained four days later (Besbes, personal communication). Eggs were incubated in UV sterilized sea water (T = 16°C, pH = 8.0, S = 37.5) during four days (Ben Khemis *et al.*, 2006). Two cylindrical (4 m diameter; 1.80 m depth) concrete tanks coated with a PVC liner and containing 20 m^3 stagnant sea water, filled a week earlier in order to promote the development of planktonic organisms, were used as mesocosms for larval rearing. Larvae were introduced into these mesocosms at the age of 1 day post hatching (dph) with an initial density of 2.5 larvae L^{-1}. Larval rearing carried on until 71 dph. Natural sea water filtered through a 300 µm mesh was used for enclosure filling and later for water renewal, which was adjusted to guarantee minimum dissolved oxygen contents superior to 5 mg L^{-1} (Ben Khemis *et al.*, 2006). Renewal rate increased progressively from 10% daily during the two first weeks of rearing to reach 40% on day 15 (dph), 100% on day 35 (dph), and finally 150% from day 55 (dph) and onwards (Ben Khemis *et al.*, 2006). Mesocosms were maintained under natural photoperiod (April to May at 35.7°N-10.8°E) and were shielded from direct sunlight with horizontal black curtains (75% shading factor).

Larvae began feeding at mouth opening (5 dph) and fed wild plankton naturally developing in the mesocosm enclosures and a complement of cultured zooplankton (rotifers and *Artemia* nauplii) added to maintain live prey densities between 0.3 and 0.5 individual ml^{-1} (Ben Khemis *et al.*, 2006). Diatoms dominated in the natural phytoplankton the two first weeks and then were progressively replaced by Chlorophycae and Cryptophycea. Rotifers of both *Synchaeta* and *Brachionus* genders dominated in the natural zoooplankton at first feeding of larvae and then copepods, at the nauplii and copepodite stages, became dominant from 17 dph. Rotifers *Brachionus plicatilis* enriched with DHA-PROTEIN SELCO® (INVE, Dendermond, Belgium) were added between 5 and 22 dph; newly hatched AF *Artemia* (INVE, Dendermond, Belgium) were added between 14 and 23 dph; and one day SELCO® (INVE) enriched EG *Artemia* (INVE) were added between 23 and 32 dph. Weaning onto commercial feed started at 23 dph with the microencapsulated diet (REPLACE II®, Rich SA, Athens, Greece). Weaned fish were then progressively fed with diets of growing pellets size : LANZY W3® and NRD 3/5® (INVE) and PERLA LARVA® 0.5-0.8 mm (Trouvit - Hendrix SpA, Mozzecane, Italy). Formulated feed rations were split into 12 to 20 lunches distributed

manually between 6 AM and 8 PM, and during co-feeding, four lunches were given prior any live preys distribution (Ben Khemis *et al.*, 2006). Daily rations increased progressively from 1g to 30 g per mesocosm between 23 dph and 32 dph. Afterwards rations were adjusted to satiation of fish (*i.e.* end of feeding demand, considered attained at dispersion of fish schooling during feed distribution). For instance, daily ration in each mesocosm was about 150 g at 40 dph, 220 g at 55 dph and 470 g at 70 dph.

2.2. Histological analysis

Samples for studying histological changes in *C. labrosus* larvae and juveniles during development were taken at 1, 5, 10 and 14 dph, then every five days from 20 to 30 dph and at 40, 55 and 71 dph. They consisted in 10 to 15 fish randomly captured within mesocosms, anesthetized in ice cold sea water and fixed for 24 h in Bouin"s fluid. fixed samples were then conserved in 70% ethanol until processing with a paraffin embedding automatic station Leica ASP300 (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Inclusion blocks contained either entire specimens or tail segments arranged to obtain sagittal or transversal sections, respectively. Serial sections of 6 µm thick were cut with a microtom Leica RM2125 (Leica Microsystems) and were stained with haematoxylin–eosin. Preparations were observed under a microscope Olympus BX 41 (Olympus Corporation, Tokyo, Japan) and photographed with a digital camera Olympus C-5050. From 25 dph to 71 dph, red and white muscle relative surface areas were estimated on histological preparations of cross sections at the anal region (Akster, Spierts, Berbner, Schmidbauer & Osse, 1995), using the image analysis software ImageJ 1.29 (National Institutes of Health, Bethesda, Maryland, USA), public domain open source software available at http://rsb.info.nih.gov/ij/.

2.3. Allometric analysis

Samples for describing the morphological development and allometric growth patterns in *C. labrosus* larvae and juveniles were randomly taken at the following ages: 1, 2, 3, 4, 5, 7, 10, 14, 20, 25, 30, 35, 40, 55 and 71 dph. Samples consisted in 15 to 30 specimens randomly captured from each 20 m^3 rearing enclosure. Fish were anesthetized with ice cold sea water, preserved in 4% formaldehyde in phosphate buffered solution (pH 7.4) and stored at 4°C until examination. Photographs of the fish were taken with a digital camera Nikon Coolpix 4500 (Nikon, Tokyo, Japan) mounted on a zoom stereomicroscope Hund-SZ (Hund-Wetzlar, Wetzlar, Germany) and using semi-darkfield and episcopic illumination. Fish were slightly stained with methylene blue solution to enable the transparent finfold to be clearly observed. A calibration micrometer was photographed with each set of photos to calculate magnification conversion factor avoiding errors due to the focus of the camera. Immediately after photographing, the drained masses (*M*) of fixed larvae were measured to the nearest mg onto an electronic analytical balance Mettler-AT 201 (Mettler-Toledo GmbH, Greifensee, Switzerland). Fish were weighed individually, except for specimens of less than 5mg for which pooled masses were taken.

Linear measurements were taken from the numeric photographs using image analysis software ImageJ 1.29. The body characteristics were measured along or perpendicular to body axis as follows: total length (L_T) from snout to tail tip; head length (HL) from snout to opercular edge; post-anal length (PaL) from tail edge of anus to tail tip; trunk length (TrL) by subtracting HL and PaL segments from L_T; head depth (HD) as the maximum depth between snout and opercular edge; eye diameter (ED); and body depth at anus (BDA) (Fig. 1). In yolk sac larvae, the volume of yolk was estimated using the equation for an ellipsoid: $V = (\pi/6)\ L\ H^2$ where L and H are length and height of the yolk respectively (Ben Khemis, de la Noüe & Audet, 2000) and the volume of oil globule was derived from the volumetric formula of a sphere, using the mean of 2 diameter measurements to derive the radius (Williams, Brown, Gotceitas & Pepin, 2004).

2.4. Data analysis

Yolk and globule depletion rates were estimated from slopes of linear regressions observed between corresponding volumes and ages of sampled pre-larvae (Williams, Papanikos, Phelps & Shardo, 2004). Multi-phasic growth in mean body mass was described by different regressions estimated from logarithm transformed data, inflexion points designating the *M* value where regression"s slope changed Thus, regressions were for M_{min} until $M_{intermediate}$, and for $M_{intermediate}$ until M_{max} where $M_{intermediate}$ was defined as the inflexion point and corresponded to the value that resulted in the largest *t* value when comparing growth coefficients by *t* test.Growth rates of mean body mass were compared with analysis of covariance (ANCOVA).

Length at morphometric metamorphosis (L_m) at which growth coefficients for measured morphometric characters became isometric in relation to L_T and fish larvae became morphometrically juveniles (Fuiman, 1983), was determined following the method described in Nikolioudakis, Koumoundouros, Kiparissis & Somarakis (2010), with minor modifications. Briefly, a Principal Components Analysis (PCA) was carried out, using logarithmic covariance matrix, in order to explore patterns of multivariate allometry among measured morphometric characters (L_T, HL, PaL, TrL, HD, ED and BDA). Kaiser–Meyer–Olkin's (KMO) measure of sampling adequacy was used to assess the usefulness of a PCA. KMO ranges from 0 to 1 and should be > 0.5 if variables are sufficiently interdependent for PCA to be useful (Tabachnick & Fidell, 2000). In a PCA of pooled logarithmic covariance matrix of groups of individuals with different growth patterns, PC1 summarizes shape variation resulting from growth allometry, while subsequent components summarize variation from divergent growth trajectories (Nikolioudakis *et al.*, 2010). Hence, growth patterns among different stanzas are reflected as divergent PC2 trajectories when plotted against the PC1 or L_T. Present multivariate allometry analysis was exclusively based on morphometrical characters (L_T, HL, PaL, TrL, HD, ED and BDA) and did not include morphological or osteological characters as in the study of Nikolioudakis *et al.* (2010). Piecewise linear regression, fitted with a non linear procedure, was used to estimate change in PC2 orientation: $PC2 = b_0 + b_1 L_T + b_2(L_T - L_m)$ ($L_T \geq L_m$); where b_0 is the intercept, b_1 is the slope during the „larval" stage, b_2 is the difference in slope between „larval" and „juvenile" morphotype, and L_m is the length of morphometrical metamorphosis (i.e. end of larval period), which corresponds to L_T at which slope changes and growth coefficients equals 1 (i.e. isometry) (see Nikolioudakis *et al.* 2010 for more details).

PC1 eigenvector from groups of individuals sharing a common growth patterns reflects the relative proportion of changes. Thus, the *i*th variable is isometric when its PC1 component score equals $1/\sqrt{p}$; where *p* is the number of variables in the analysis. Bootstrap method (Efron & Tibshirani 1986) was used to estimate standard errors and confidence intervals for components scores comparisons with the isometry theoretical value (see Nikolioudakis *et al*. 2010 and references therein).

For the specific allometric analysis of body characteristics, the data set was sorted according to increasing L_T and then, as generally achieved for simple mathematical illustration, linear regressions were performed on logarithm transformed data, using $\ln(L_T)$ as the independent variable (van Snik, van den Boogaart & Osse, 1997; Osse, van den Boogaart, van Snick & van der Sluys, 1997; Gisbert, 1999; Shingleton *et al.*, 2007). Robustness of the regression was measured by calculating r^2, which reflected the percentage of variation that corresponded to a linear relation between independent and dependent variables (Zar, 1999). Multi-phasic allometric growths were described by different regressions, inflexion points designating the L_T value where regression"s slope changed. Thus, regressions were for L_{Tmin} until $L_{Tintermediate}$, and for $L_{Tintermediate}$ until L_{Tmax} and inflexion point corresponded to $L_{Tintermediate}$,

was defined as the inflexion point and corresponded to the value that resulted in the largest *t* value when comparing growth coefficients by *t* test, as initially described in van Snik *et al.* (1997) and commonly used in allometric studies (Gisbert, 1999; Osse & van den Boogaart, 1999; Gisbert *et al.*, 2002). The significance of each regression was assessed by ANOVA and the comparison between regressions was assessed by ANCOVA (Zar 1999). Finally, each regression slope (*b*) was subjected to a *t*-test to determine if it differed significantly from isometric growth (*i.e. b* = 1).

3. Results

3.1. Morphological and histological development

At 1 dph [Fig. 2(a)], *C. labrosus* larvae weighed and measured 0.7 mg *M* and 3.7 ± 0.1 mm L_T, respectively. The head was aligned with the body axis but the mouth was not yet opened and the eyes were not yet pigmented. Larvae were bordered continuously by a primordial finfold which was widest on the dorsal posterior part of the trunk at the level of the anus. Larvae showed a large yolk sac (0.225 ± 0.028 mm^3, mean ± S.D., n = 25) and an oil droplet (0.050 ± 0.011 mm^3, n = 25) in its abdominal posterior region. At 5 dph (3.9 ± 0.1 mm L_T), eyes were well pigmented and the mouth was opened, allowing larvae to start exogenous feeding [Fig. 2(b)]. Between hatching and 5 dph, most of yolk sac was consumed, as remaining yolk (0.081 ± 0.014 mm^3, n = 27) represented only 36% of the initial volume; however, relatively large amount of oil globule (0.032 ± 0.007 mm^3, n = 27) was still available. Based on regression relationships between volumes of these reserves and larval age [Fig. 3], the daily depletion rates were 25% (r^2 = 0.975) and 12% (r^2 = 0.970) for yolk and oil globule, respectively.

At 10 dph, the larvae showed a slight growth since the beginning of the rearing (1 mg *M*; 4.0 ± 0.8 mm L_T). Nevertheless, the gill arches were well developed, the swimbladder was inflated and the intestine already showed its typical folded mucosa with a simple epithelium and differentiated enterocytes. At 14 dph (4.5 ± 0.3 mm L_T) most of the larvae showed beginning of primordial finfold differentiation as indicated by the drastic reduction in the abdominal part as well as the relative diminution on the dorsal posterior part of the trunk at the level of the anus [Fig. 2(c)]. At this stage, a residual part of oil globule was still recognizable in the histological preparations and the gut appeared already well developed with an intense folding of the intestinal mucosa, an enlarged esophagus with abundant mucous cells and a prominent liver and pancreas [Fig. 4(a)].

At 20 dph (6.6 ± 1.0 mm L_T) in all the larvae notochord flexion was accomplished, caudal fin rays (lepidotrichia) were visible and both dorsal and anal fins were separated from caudal fin which already showed the typical homocercal form for this species. At 25 dph (8.6 ± 1.2 mm L_T) [Fig. 2(d)], presence of scales was noticed on fish flanks. Regarding the digestive system, the most remarkable histomorphological characteristics were that the esophagus grew in length and folded longitudinally, the stomach was already visible and fat deposits were detected among acini in the exocrine pancreas. The secondary filaments of the gills were also clearly visible on sagittal histological preparations [Fig. 4(b)]. At this stage, red muscle fibres appeared distributed at the midline of the flank near the skin and their relative surface area represented about 18% of muscular area on transversal histological preparations just posterior to anus [Fig. 5(a)]. Afterwards, this ratio decreased progressively (Fig. 6) and attained 15% at 30 dph, 12% at 40 dph [Fig. 5(b)], 10% at 55 dph [Fig. 5(c)] and finally reached 7% at 71 dph.

3.2. Multivariate allometric growth

When considering growth in fish body mass, three different growth episodes (SGR-*M*) were detected (ANCOVA analysis, $P < 0.0025$; n = 15; Fig. 7). The first inflexion point was observed at 1.1 mg (*i.e*. 14 dph) and the second one was observed at 6.5 mg (*i.e.* 25 dph). The first episode of growth in fish body mass, exhibited a reduced growth rate (SGR-*M*) of 3.9% per day. SGR-*M* increased sharply reaching 16.3% of body mass per day during the second episode and then stabilized at 9.8% of body mass per day until the end of the experimental rearing (71 dph). Mean mass and mean length were related by a nearly isometric relationship ($y = 0.013\ x^{2.943}$; $r^2 = 0.994$; n = 15), but analysis of allometric growth showed considerable variation.

Principal component analysis conducted on log-transformed morphometric characters showed that, as expected, all characters were interdependent and positively correlated among them (Pearson's $r \geq 0.94$; $n = 474$; $P < 0.0001$, in all paired comparisons). The KMO's measure of sampling adequacy (0.756) indicated the usefulness of the PCA, with the first two axes explaining 97.8% and 1.5% of the total variation (99.3%), respectively. However, there were prominent changes in oblique orientation of PC2 scores when plotted against PC1 scores or L_T (Fig. 8), hence indicating shifts in relative growth of morphometric characters associated with development. The fit of the piecewise PC2 scores-L_T regression estimated a first point (i.e. knot) at $L_{m1} = 4.46 \pm 0.06$ mm and a second point estimated at $L_{m2} = 28.56 \pm 1.04$ mm (Fig. 8). Exclusively fish from the last sampling date (71 dph) corresponded to $L_T \geq L_{m2}$. Thus, all fish larger than 4.46 mm L_T were considered as a single group for practical purposes. In addition,, when allometric equations ($\log(Y) = \log(a) + b \log(L_T)$; where Y is the character and b is the allometric coefficient) for individual morphometric characters were applied to both fish groups separately (fish with $L_T < L_{m1}$ and fish with $L_T \geq L_{m1}$), residuals were normally distributed (Kolmogorov-Smirnov test, $P >> 0.05$ for all characters comparisons). Hence, the early growth in terms of body morphometrics of this species could be divided mainly into two distinct fish groups and bivariate allometric equations were appropriate to describe fish relative growth in both groups. The first group corresponded to fish of first growth stanza, which ended at 4.5 mm (L_T), whereas the second group corresponded to bigger fish; thus the intermediate phase between the two fish groups occurring around 14 dph.

PCA component scores were quite stable within groups (see standard errors in Table 1) and standard deviation of the morphometric characters were lower than those calculated for all individuals. When PC1 components scores were compared with the theoretical value of multivariate isometry (i.e. 0.378) we found that morphometric characters exhibited negative allometry (Fig. 9); and this was significantly higher in Group 1 ($L_T < L_{m1}$) than in Group 2 ($L_T \geq L_{m1}$). Overall, Group 2 characters were mainly related to body length and exhibited coefficients closer to isometry.

PC1 and PC2 explained 56.7 and 18.3% of the total variance of samples when computed for re-scaled component values (KMO statistic = 0.509). HD, HL, ED and BDA were the morphometric variables that loaded highly on the PC1, whereas PaL was the one that mostly loaded on the PC2 and L_T showed intermediate component loadings for both PC axes. Considering the morphometric developmental period comprised between 4.6 and 36.6 mm L_T (age period = 20-71 dph), 99.04% of the total variance of samples when computed for re-scaled component values was explained by PC1 and PC2 axes, from which 98.4% of the total variance was mainly explained by the PC1 and only 0.7% by the PC2 (KMO statistic = 0.785). During this period, all morphometric variables analysed showed high factor loadings for the PC1.

Bivariate allometric analysis of body characteristics showed that growth of head could be divided into two different periods [Fig. 10(a, c)]. Both HL and HD showed a strongly positive

allometric initial growth (b = 4.37 ± 0.41 and b = 5.35 ± 0.60, respectively) until their respective inflexion points at 4.2 and 4.1 mm L_T. After these sizes, relative HL growth became nearly isometric (b = 0.98 ± 0.01), while HD growth was negatively allometric (b = 0.81 ± 0.01). Allometric growth of ED [Fig. 10(b)] paralleled that of the head depth with a positive initial allometric growth (b = 2.77 ± 0.25) until the inflexion point at 4.3 mm L_T, followed by a negatively allometric growth (b = 0.73 ± 0.01) from 4.3 mm (corresponding to an age between 10 and 14 dph).

Allometric growth of BDA [Fig. 10(d)] was also biphasic with an initially strong positive allometric growth (b = 2.56 ± 0.44) from hatching to 4.1 mm L_T, followed by negatively allometric growth (b = 0.87 ± 0.01) until the end of the study.

Regressions of allometric relationships during initial phases showed moderate robustness (r^2 values between 0.304 and 0.417) for all of HD, HL, ED and BDA. Nevertheless, ANOVA analysis indicated that all of them were statistically highly significant (P < 0.001).

PaL and TrL segments showed triphasic allometric growth profiles [Fig. 10(e, f)]. The initial allometric relationship, until 4.3 mm L_T, was characterized by a regression with a very weak coefficient of determination for PaL (r^2 = 0.131; P < 0.001) but no significant regression for TrL (r^2 = 0.007; P = 0.243), emphasizing the negligible or absence of contribution of these segments to larvae linear growth. From the stage of 4.3 mm L_T, PaL showed a positive allometric growth until 8.9 mm L_T, after which it showed a nearly isometric growth (b = 0.95 ± 0.01). The TrL length showed first a negative allometric growth (b = 0.66±0.03) between 4.3 and 9.6 mm L_T, followed by a positive allometric growth (b = 1.15 ± 0.01) until the end of the study.

Existence of an additional inflexion point was tested in bivariate analysis of allometric growth around the size corresponding to L_{m2} (28.6 mm L_T) but it didn"t appear significant for any of body size characters HL, HD, PaL, BDA and TrL.

The larval total lengths at which the inflexion points of different body segments growth occurred were grouped within two narrow size intervals (Fig. 11). The first size interval (4.1 to 4.3 mm L_T) concerned changes in growth rates of the head (HD, HL and ED) as well as body thickness (BDA). This interval of sizes corresponded to larval ages comprised between 10 dph (4.0 mm L_T) and 14 dph (4.5 mm L_T). It coincided with the inflexion period separating the two first episodes of growth in fish body mass, characterized among others by beginning of differentiation of primordial finfold. The second size interval (8.9 to 9.6 mm L_T) corresponded to larval ages between 25 dph (8.6 mm L_T) and 30 dph (10.5 mm L_T) and concerned changes in the allometric growth of both PaL and TrL. It occurred few days later compared to inflexion of body growth but it coincided with development of scales and progressive decrease of red musculature relatively to white musculature.

4. Discussion

Like most marine finfish species from warm and temperate regions, *C. labrosus* have a high fecundity and produce numerous small eggs from which hatch tiny and poorly anatomically and morphologically developed larvae. Fish larvae, which often inhabit entirely different niches and present distinct body shape from juvenile and/or adult stages, are transitory forms possessing a dynamic and continuously changing morphology (Strauss & Bond 1990). In this sense, most functional systems of newly hatched larvae need to be differentiated and achieve its final function in order to acquire all the adult features by the end of the larval period (Fuiman, 1983). This period can vary substantially in duration within and among species (Pepin, 1995); nonetheless, fish larval stages generally grow at rates much faster

than any other developmental stage (Pedersen, 1997). However, in small eggs and newly-hatched larvae, not all organ systems can grow simultaneously (Osse *et al.*, 1997), and consequently, growth intensities are not distributed uniformly across the body (Fuiman, 1983). It is admitted that differences in relative dimensions of body parts and organs between newly-hatched fish larvae and post-metamorphic juvenile stages are due to the necessity of setting priorities during early larval growth to create at least primary conditions for survival (Osse & van den Boogart, 1995, 1999).

Under present mesocosm rearing conditions, growth of *C. labrosus* from 1 dph up to 71 dph showed three distinct growth episodes, in terms of body mass and length, which could be clearly distinguished by allometric inflexions that were related to different developmental and/or behavioural changes along larval ontogeny. Larvae of *C. labrosus* showed a negligible growth rate in fish body mass as well as length during the first days after hatching. Endogenous reserves were depleted moderately fast until mouth opening (64% of yolk and 36 % of oil globule in 5 days), hence a substantial amount of both yolk and oil globule existed when larvae started feeding on live prey. Thus, larvae showed a period of mixed nutrition based on exogenous and endogenous nutrients that extended from mouth opening at 3.9 mm L_T (at 5 dph) to around 4.5 mm L_T (at 14 dph), as a residual fraction of the oil globule was still detectable at this stage. This indicated the existence of a relatively long transitional feeding period in this species, in contrast to the generalized principle that the ontogenetic transition between the lecitotrophic and exotrophic larval stages is relatively short in this group of species (Bruslé, 1981a). Considering mesocosm rearing conditions, it seems doubtful that the reported slow growth rate observed in *C. labrosus* during the first days after hatching was consequence of an inadequate food and energy supply.

The growth results, showing an extended initial low growth phase, are in agreement with those determined from Cataudella *et al.* (1988) and Boglione *et al.* (1992) in which *C. labrosus* larvae reared under intensive hatchery conditions showed a SGR-L_T of 1.3% from 1 to 12 dph. They are also globally in agreement with those of Besbes *et al.* (2010), concerning comparative growth and development of larvae reared under intensive hatchery conditions with either green water or clear water technology. Even though these studies are not directly comparable since they differed in the rearing system and in the conservation and processing of the biological material, they emphasize lowness of growth during first days of early life history. Similar growth patterns could also be figured out in stripped mullet *Mugil cephalus* larvae according to growth curves presented by Nash & Shehadeh (1980), Eda, Murashige, Oozeki, Hagiwara, Eastham, Bass, Tamaru & Lee (1990) or Tamaru, Murashige, Lee, Ako & Sato (1993).

In a previous work (Ben Khemis *et al.*, 2006), we hypothesized that the extended initial low growth phase observed in *C. labrosus* larvae could be a typical feature in mullets early development, assuming that it might be due to the allocation of available energy and building materials in priority to functional changes. This pattern of development would allow larvae attaining earlier a better nutritional status that would enhance their growth performance at latter stages. Results from the present work strongly corroborated this hypothesis, since morphogenesis and differentiation were particularly intense during the first stanza of larval development even though linear and mass growth rates were minor. Histological observations showed that at as early as 4.0 mm L_T (10 dph), larval digestive system appeared well differentiated; suggesting that at that stage larvae had already developed their digestive capacities (alkaline digestion). This is in agreement with a recent study on digestive tract ontogeny in *C. labrosus* larvae, in which maturation of intestine appeared to be particularly precocious in the species (around 8 dph) according to pancreatic and intestinal enzyme profiles (Zouiten *et al.*, 2008).

In the same way, results of multivariate allometric analysis conducted by PCA on morphometric characters (L_T, HL, PaL, TrL, HD, ED and BDA) indicated that there were

shifts in the relative growth of these characters with larval development, since there were prominent changes in the oblique orientation of PC2 scores when plotted against PC1 scores or L_T (Nikolioudakis *et al*. 2010). Larval growth of *C. labrosus* in terms of body morphometrics distinguished a first length (L_{m1}) at which changes took place estimated at 4.46 ± 0.06 mm L_T (piecewise PC 2 scores-L_T regression); occurring around 14 dph in term of larval age and coinciding with first inflexion in growth of body mass.

Analysis of bivariate allometric growth in *C. labrosus* also indicated that during the first development stanza, growth concerned mainly the cephalic region, as both head measurements (HD and HL) showed strong positive allometric growth patterns. Different reasons may account for such a fast allometric growth of the head. Among priorities, there is enhancement of vision and most likely all sensorial system. This was notably supported by the positive allometric growth of ED during this period. Indeed, Packard & Wainwright (1974) evidenced that the linear eye dimension is a direct indicator of brain growth during early life history of fish. Moreover, the level of mechanoreceptive, visual development and larval responsiveness are shown to match developmental patterns observed in fish morphology (Higgs & Fuiman, 1998). It is hence admitted that the positive allometric growth of eyes is considered an indicator of development and differentiation of neural and sensorial structures; which would allow the larvae to react to light stimuli, detect zooplankton prey and potential predators in the water column (Gisbert *et al.,* 2002; Gisbert & Doroshov, 2006). In addition, this positively allometric growth of the head as a whole, also found in other fish larvae (see review in Osse & van den Boogart, 2004), would allow the larvae to improve their prey capture ability and successful. In this sense, those changes in head growth in carp larvae were linked to the positive allometric growth of mouth gap, hyoid and opercular length, and the synchronously changing position of the gill arches in order to improve the mechanism for suction feeding (Osse *et al.*, 1997).

Improvement of the respiratory capacity also seemed a priority during initial development. During most of the fish larval stage, cutaneous respiration is known to be more important than gill respiration (de Silva, 1974; Batty, 1984). Nevertheless, the shift from cutaneous diffusion to gill ventilation and gas exchange for oxygen supply is an important event (van Snik *et al.*, 1997; Gisbert, Cech & Doroshov, 2001). In Siberian sturgeon (*Acipenser baeri*) and green sturgeon (*Acipenser medirostris*) larvae, it is suggested to be accomplished when inflexion point of HL growth occurs (Gisbert 1999; Gisbert & Doroshov, 2006). Development of *C. labrosus* larval respiratory system seems in agreement with results in other fish species in which the inflexion in allometric growth of the head matched the development of the branchial apparatus, enhancing the oxygen supply of the organism. Indeed, inflexion points of head growth and presence of well developed gill arches were observed at the same stage, *i.e.* in larvae measuring 4.0 to 4.2 mm L_T (at around 10 dph).

There is a noticeable interspecific variability in relative energy allocation to growth or metabolic processes during the first developmental stages of fish (Parra & Yúfera, 2001). In *C. labrosus* larvae, it seemed that during the first growth stanza, development concerned in priority the structures and organs related with the feeding efficiency of the larva; by improving detection ability (development of sensory system), ingestion capacity (growth of head), and food assimilation performance (differentiation of digestive system); together with the respiration efficiency (development of gill). The first growth stanza ended at beginning of differentiation of primordial finfold shortly before the notochordal flexion stage, which indicated that it occurred just before larvae began to modify their swimming mode (Bone, Marshall & Blaxter, 1995).

During the second growth episode in fish body mass, which occurred between 4.5 mm L_T (*i.e.* 14 dph) and 8.6 mm L_T (*i.e.* 25 dph), growth concerned all body segments but was particularly fast for the PaL. In fact, this segment was the only one to show a positive allometric growth, suggesting that the enhancement of the locomotor function was the main

priority during this period. Relative development of tail implicitly indicates the enlargement of propulsive area and the increase of propulsive power (Fuiman, 1983). This growth of posterior part of body was concomitant with development of unpaired fins and fin rays. Under current rearing conditions, the homocercal tail was observed as early as 6.6 mm L_T (i.e. 20 dph) in agreement with an earlier description of this species made by Boglione *et al.* (1992). The morphological differentiation of the caudal fin and development of lepidotrichia rays closely parallels the progressive change in the larval swimming mode from the anguilliform motion, where locomotor waves pass along the whole body, to the subcarangiform swimming, where locomotion relies on oscillation of the caudal region alone (Bone *et al.*, 1995; Osse & van den Boogart 1995, 1999). This transition is a valuable strategy enhancing swimming efficiency (Müller & van Leeuwen 2006). Indeed, it allows reducing the high costs of fish larval locomotion and also attaining higher speed (Müller & van Leeuwen 2006; Koumoundouros, Ashton, Xenikoudakis, Giopanou, Georgakopoulou & Stickland, 2009). Development of propulsion is crucial for both food capture and predator avoidance (Hubbs & Blaxter, 1986; Williams *et al.*, 1996). In this sense, it is generally admitted that larval success or failure in feeding does not only depend on the abundance of suitable planktonic preys, but also on their searching power and ability to capture food items (Blaxter, 1963). Thus, the morphological development of the feeding apparatus coupled with behavioural changes over early ontogeny can profoundly affect the ability of an organism to obtain nourishment, and ultimately impacting survival (Lowry & Motta, 2007).

All the early changes observed in *C. labrosus* larvae, evidently allowed them to attain a better nutritional status during this second episode in growth of fish body mass, and hence may easily explain the drastic increase of larvae growth performance during this period. Overall, enhanced vision, improved digestive capabilities, and improved swimming ability might lead to changes in the prey type selected, shifting from small to larger more nutritious prey (Morote, Olivar, Pankhurst, Villate & Uriarte, 2008) and therefore, lead to a more favourable energetic balance between expended and ingested energy during feeding (Parra & Yúfera, 2001). In a previous paper (Ben Khemis *et al.*, 2006), it was pointed out that appearance of schooling behaviour and escapement reflex to moving shade were occurring around 23 dph (7.9 mm L_T) in young *C. labrosus*. These attributes also coincided with the silvering of flanks of the fish (*i.e.* development of scales) as confirmed with observations from the present study. In later rearing, we have noticed that automatic belt feeders could be used with great success from this developmental stage and it might be suggested to test self feeding strategy.

Nikolioudakis, *et al.* (2010) highlighted and emphasized the importance of multi-character approaches for defining transitions in fish ontogeny like the onset of juvenile period. All together, the observed changes in morphometric and morphologic traits as well as in swimming behaviour, may indicate that second growth stanza in body mass corresponded to the transition from post-larval to metamorphosing stage, and it could be indicative of the onset of larval migration from marine to coastal and estuarine areas that are used by this species as nursery habitats (Bruslé, 1981b). This is additionally supported by the completion of gill development, as indicated by the presence of secondary gill filaments as well as the myomer organization and distribution in larvae from 8.6 mm L_T (25 dph). Study of muscle distribution in cross sections through the anal region showed that red muscle fibres concentrated mainly at the midline of flanks near the skin. This organisation corresponds to the adult distribution and develops after the gills and the blood circulation become fully functional (Batty, 1984). In most teleost species, muscles are commonly stratified in layers with red muscle located superficially and white muscle located deeply (Akster *et al.*, 1995; Johnston, 1999; López-Albors, Ayala, Gil, García-Alcázar, Abellán, Latorre, Ramírez-Zarzosa & Vázquez, 2003). White muscles power high speed swimming using anaerobic metabolic pathways while red muscles utilise aerobic metabolism and are recruited for routine activity such foraging and migration (Johnston, 1999; Alami-Durante & Rescan, 2003). Thus, this second developmental period was also characterized by the organization

and development of axial musculature, which undeniably also reduced larval vulnerability to predation by improving their swimming performance. It is admitted that an increase in absolute body size also acts on predator-prey interactions, as larger locomotor muscles provide more power to escape predators, and size also limits the numbers of potential gape limited predators (Lundvall, Svanbäck, Persson & Byström, 1999).

During the last episode of growth in fish body mass, which began at 8.6 mm L_T (25 dph) and extended until end of trial at 36.6 ± 3.3 mm L_T (71 dph), growth rate decreased to a level approaching isometry. This is a typical feature of a juvenile growth profile (Fuiman, 1983), and was corroborated by the second shift in relative growth of morphometric characters (L_{m2}) which probably defined the end of metamorphosing period. It is also supported by the progressive maturation of the axial musculature as indicated by the decrease of the relative cross-section area of red muscle fibres and the increase of white ones. Fish have adapted the morphology of their axial musculature for high power output and efficiency (Müller & Van Leeuwen, 2006). Muscle tissue represents about 60% of total body mass in fish (Wilkes, Xie, Stickland, Alami-Durante, Kentouri, Sterioti, Koumoundouros, Fauconneau, & Goldspink, 2001). Relative amounts of red and white muscles changes at rates depending not only upon species and temperature, but also upon developmental stage (Stoiber, Haslett, Wenk, Steinbacher, Gollmann & Sänger, 2002). Cross-sectional area of white muscle increases slowly in eleutheroembryos, and up to four times faster in longer fish (Alami-Durante, Rouel & Kentouri, 2006). In mature axial musculature, red muscles are rarely exceeding 10% of the total cross-sectional area (Johnston, 1999) and myotomal growth is essentially due to growth of white muscle (Alami-Durante & Rescan, 2003). At this stage, blood flow is directed primarily to white muscle according to blood flow distribution studies (Schultz, Barron, Newman & Vick, 1999).

In conclusion, morphogenesis and differentiation were very intense processes in *C. labrosus* larvae during the first developmental stanza which ended around 4.5 mm L_T (14 dph) according to an inflexion in growth of body mass coinciding with a first shift in multivariate relative growth of morphometric characters. Feeding, sensory, digestive and respiratory systems grew and developed in priority, during this first developmental stanza, supporting the hypothesis of energy and building materials allocation in priority to functional changes rather than size increase. The following development episode was mainly characterized by the flexion of notochord, the fast growth of the trunk and tail segments, the acquisition of adult axial muscle distribution, and completion of gill filament development. These changes were related to improvement of the swimming and respiratory performances. They corresponded to the transition from post-larval to metamorphosing stage as corroborated afterwards by progressive maturation of axial musculature, isometric growth, and the acquisition of the juvenile phenotype. Optimal size for releasing young hatchery reared *C. labrosus* can not be determined with precision from present study, nevertheless it may be suggested to exclude all stressing zootechnic handling acts, including fish transfer for stocking, before reaching the second shift in relative growth of morphometric characters, estimated at 28.6 mm L_T, and which probably defined the end of metamorphosis period.

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Figures

Fig. 1. Morphometric characters measured in *Chelon labrosus* larvae and juveniles from 1 to 71 dph. Total length (L_T), head length (HL), post-anal length (PaL), trunk length (TrL), head depth (HD), eye diameter (ED), body depth at anus (BDA).

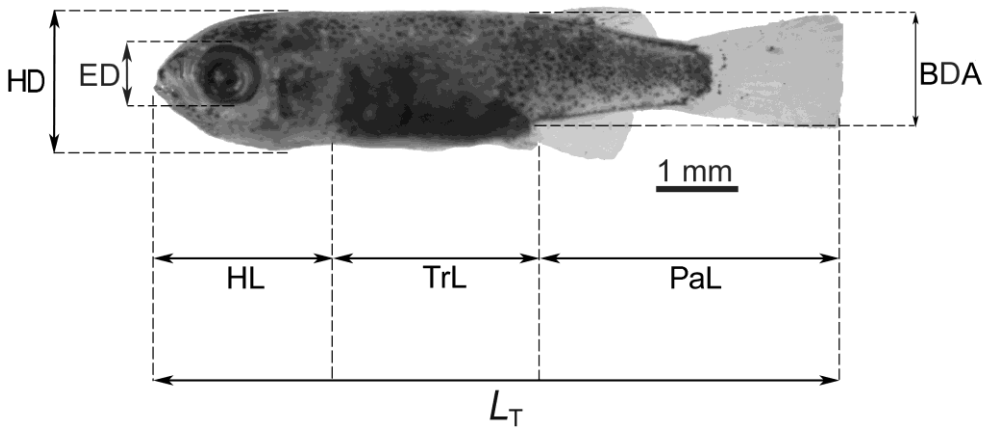


Fig. 2. Early life stages of *Chelon labrosus* larvae. (a) 1 dph, (b) 5 dph, (c) 14 dph, (d) 25 dph. Scale bar = 1 mm.

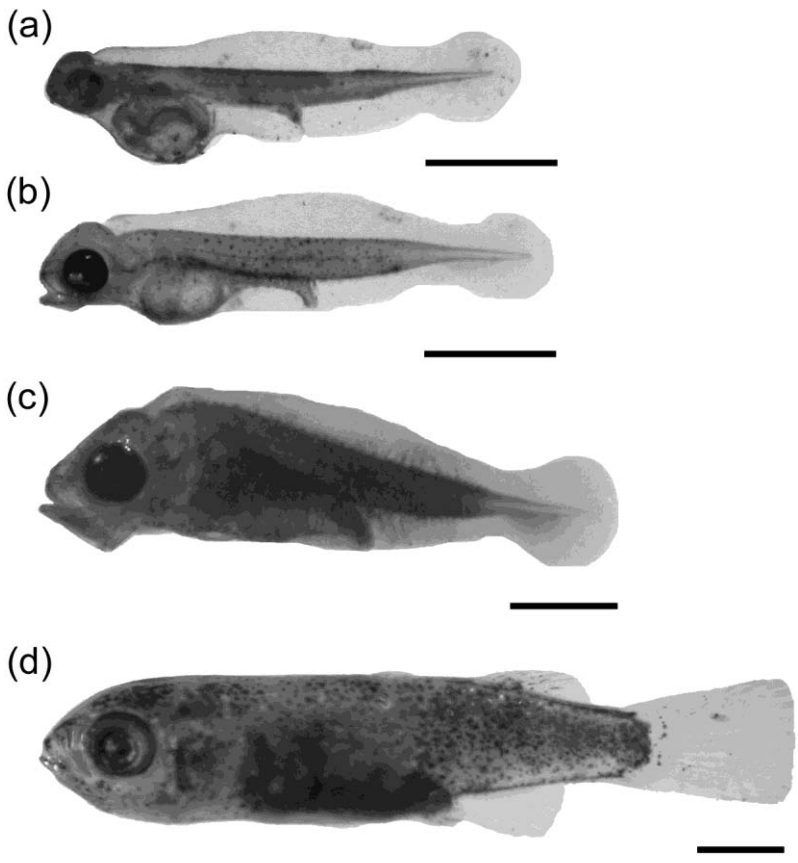


Fig. 3. Yolk-sac and oil-globule utilization in *Chelon labrosus* larvae between 1 and 5 dph. Data are plotted on a logarithmic ordinate grid. Bold lines represent linear regressions which equations were estimated by least squares method using log transformed data.

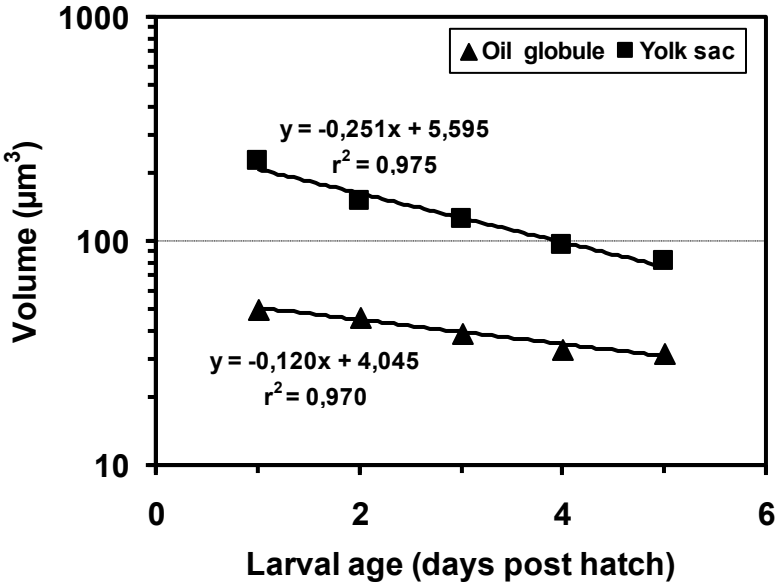


Fig. 4. Longitudinal histological sections of *Chelon labrosus* larvae at 14 dph (a) and 25 dph (b). Scale bar = 500 µm. SB, swimbladder; OE, oesophagus; L, liver; EP, exocrine pancreas; AI, anterior intestine; PI, posterior intestine; SC, spinal chord; V, vertebral column; FD, fat deposits; H, heart; G, gills; Asterisk, pyloric valve separating the glandular stomach from the anterior intestine.

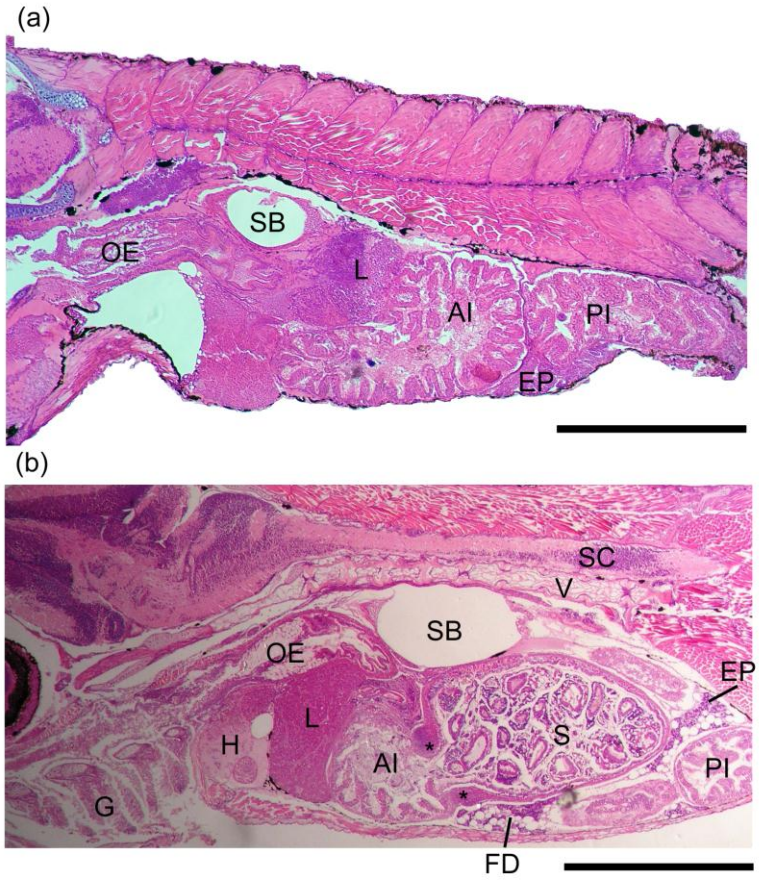


Fig. 5. Transversal histological sections of *Chelon labrosus* larvae at 25 dph (a), 40 dph (b) and 55 dph (c). Scale bar = 1 mm. WM, white muscle, RM, red muscle; SC, spinal chord; V, vertebral centrum; G, gut and abdominal cavity.

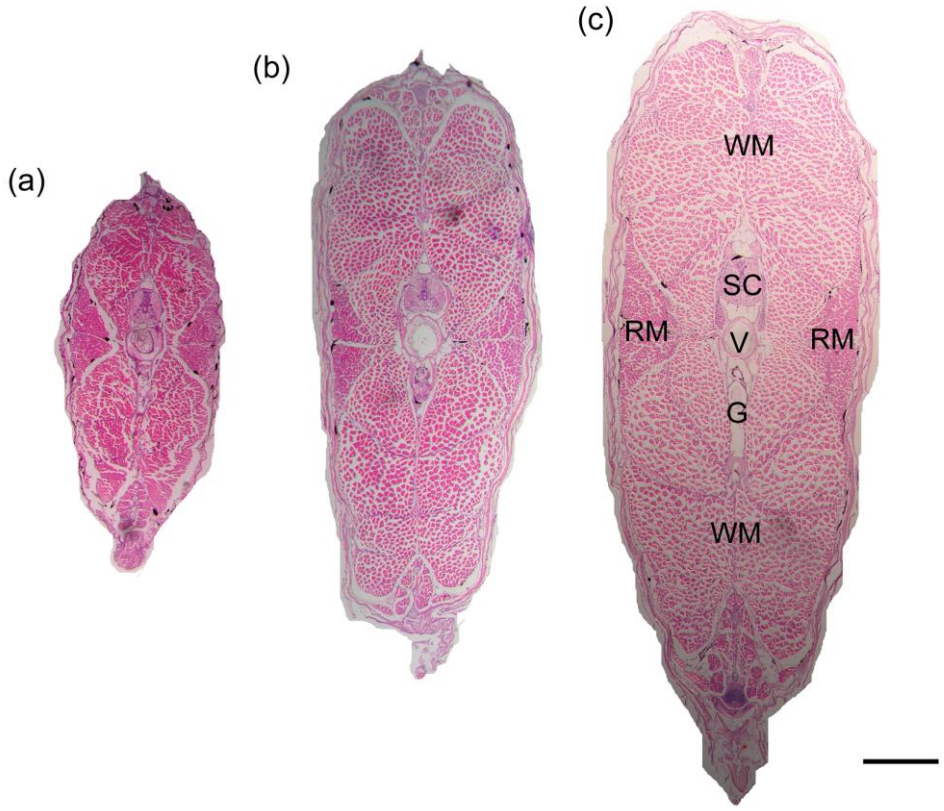


Fig. 6. Variation of mean percentage of red muscle to total cross-sectional area in young *Chelon labrosus* between 25 to 71 dph. Error bars represent SE.

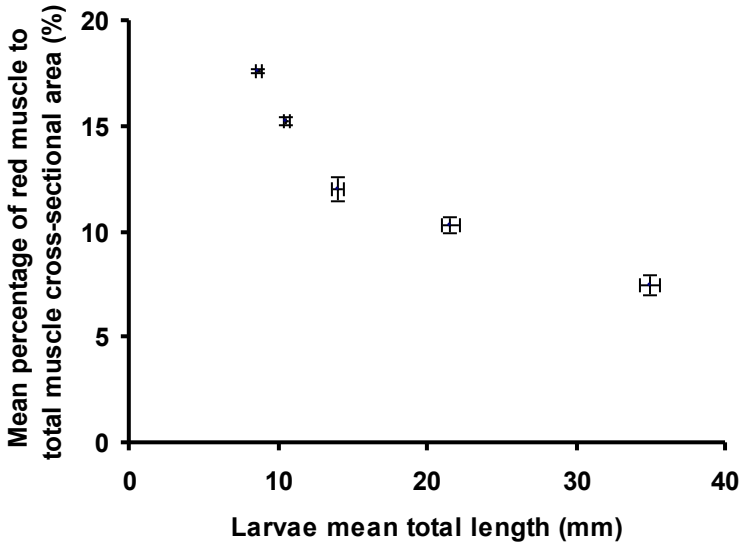


Fig. 7. Mean weight growth of young *Chelon labrosus* as a function of age from 1 to 71 days post hatch. Data are plotted on a logarithmic ordinate grid. Linear regression equation of each distinct growth stanza (bold dotted lines) was estimated by the least squares regression method using log transformed data. Vertical dashed lines indicate inflexion periods (i.e. change of regressions).

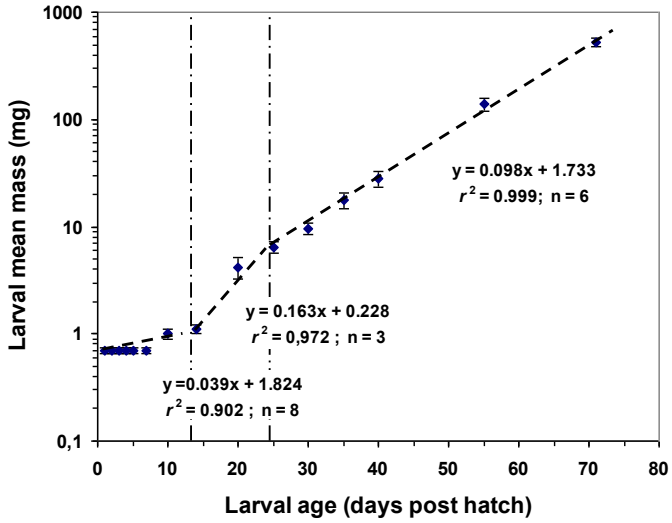


Fig. 8. Relationship of PC2 factor scores per days post hatching with fish total length in *Chelon labrosus* (Top) obtained by PCA. Relationship of PC2 factor scores per fish group selected by the piecewise regressions with fish total length (Bottom).

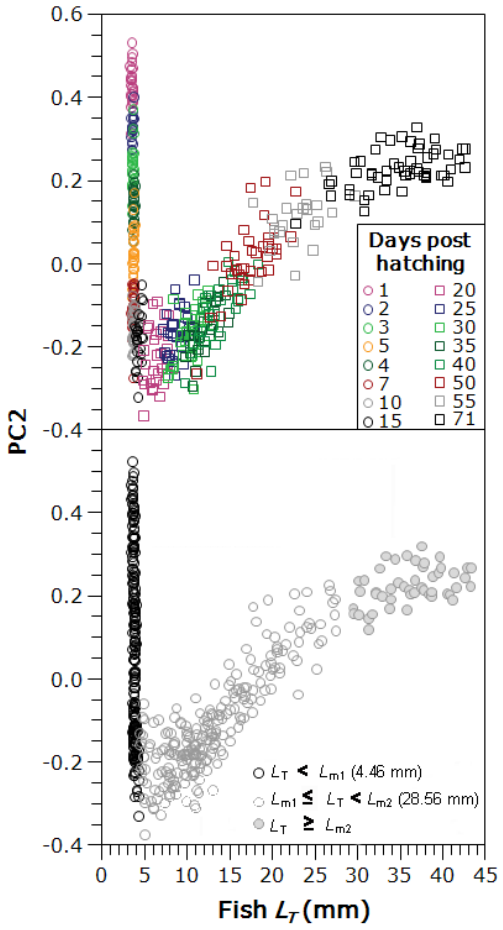


Fig. 9. Multivariate allometry in morphometric characters of *Chelon labrosus* (see methods for explanation of abbreviations). Dashed line indicates multivariate isometry (0.378).

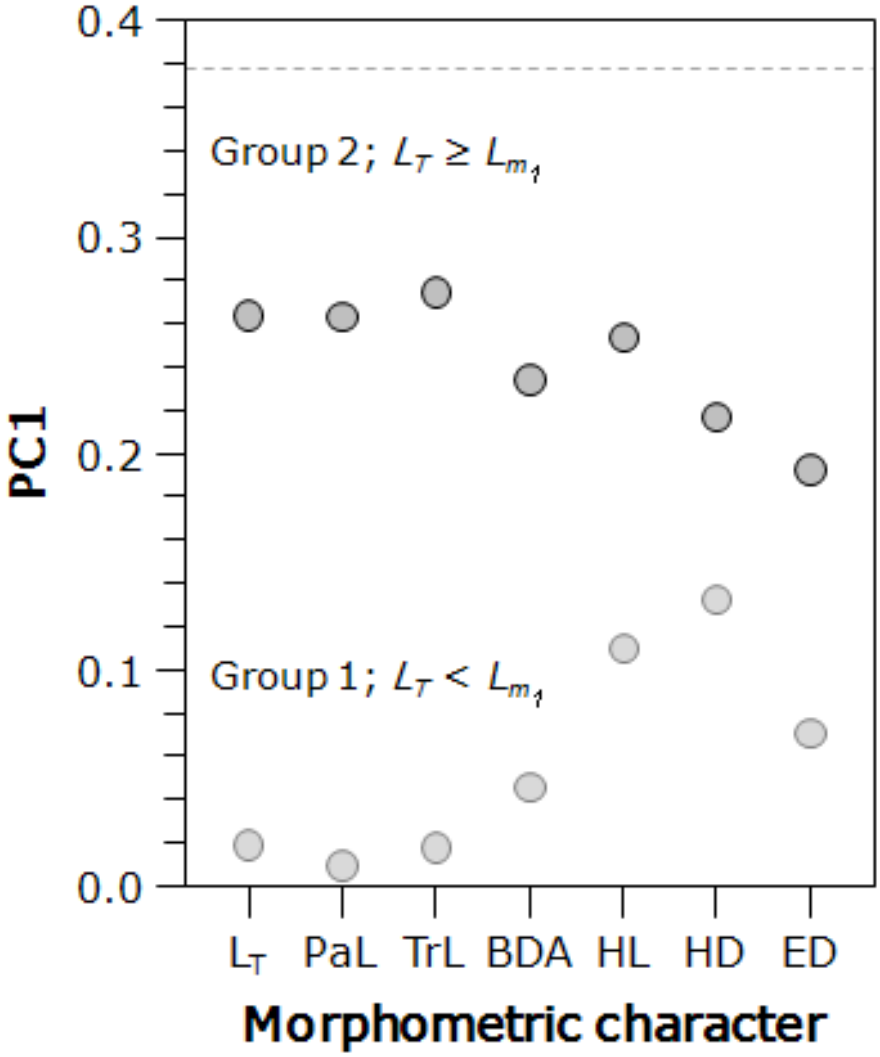


Fig. 10. Allometric growth relationships and regression equations of body morphological characters as function of total length in *Chelon labrosus* during early development between 1 and 71 dph. Data are plotted on double logarithmic ordinate grids dotted lines indicate total length at which inflexions of allometric relationships occurred. Linear regression equation of each distinct growth stanza was estimated by the least squares regression method using log transformed data.

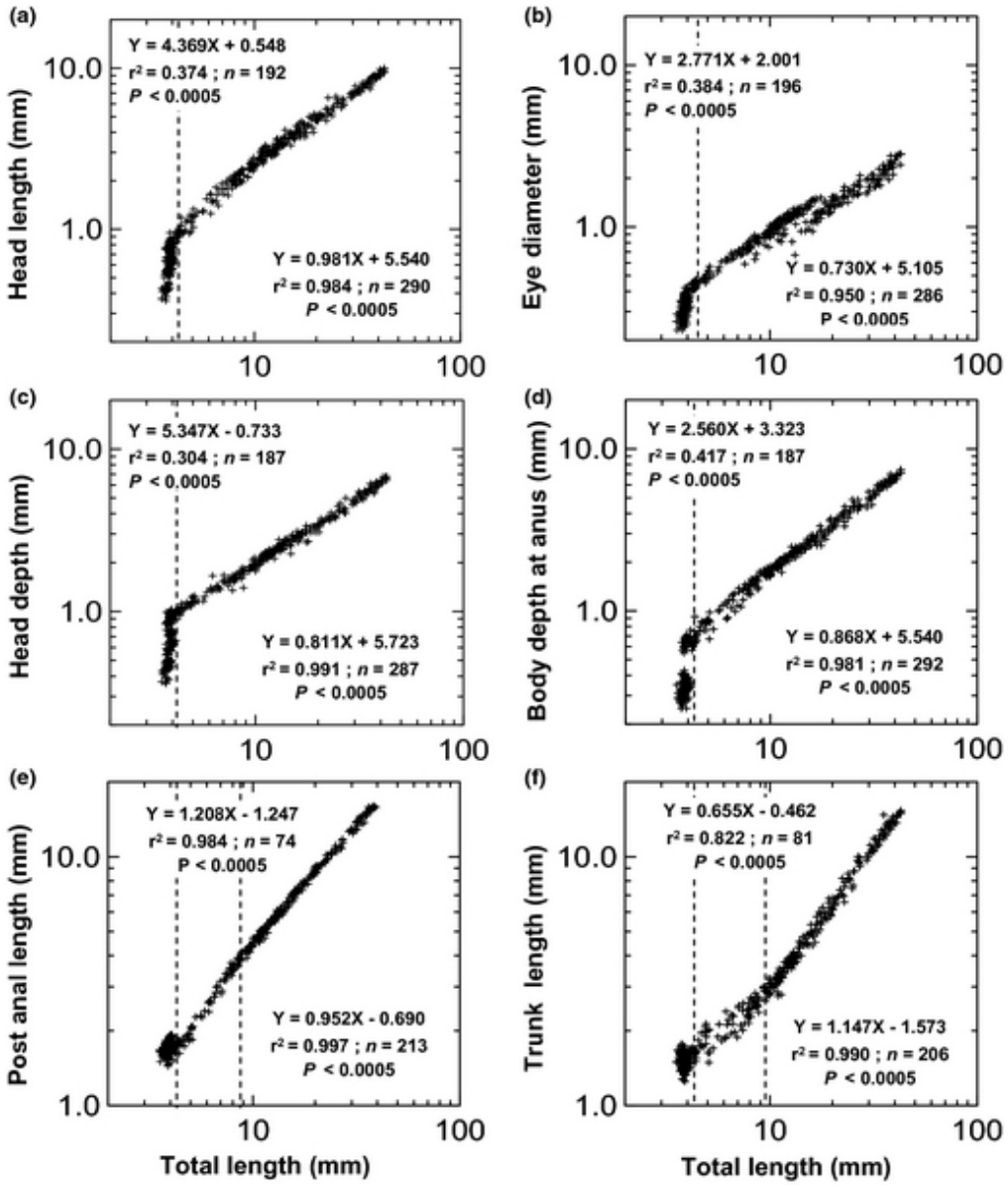
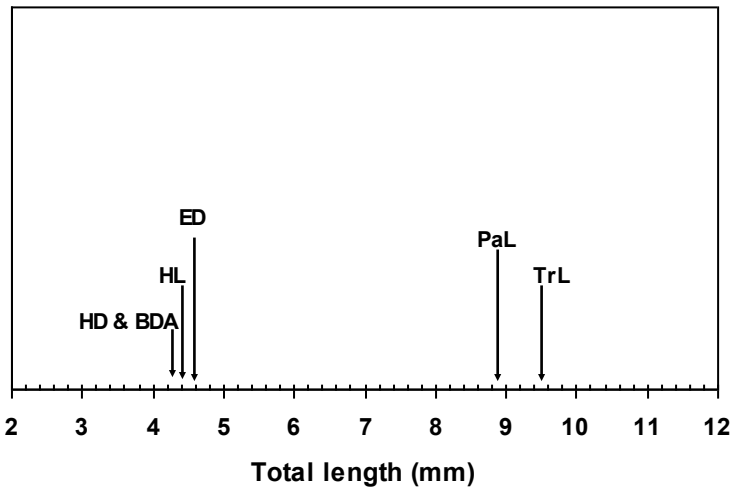


Fig. 11. Total lengths (L_T) at which inflexion points of allometric growths were found during early development of *Chelon labrosus* from 1 to 71 dph: HD, head depth; HL, head length; ED, eye diameter; BDA, body depth at anus; PaL, post-anal length; TrL, trunk length.



Tables

Table 1 First and second principal component scores from the PCA for the morphometrics characters studied. Rescaled components can be calculated by diving raw component scores with character standard deviation. Standard errors (bootstrapped) of the components scores (SE × 10^{-4}) and standard deviation of morphometric characters are also shown.

	All fish pooled					Group 1 ($L_T < L_{m1}$)					Group 2 ($L_T \geq L_{m1}$)				
	σ	PC1		PC2		σ	PC1		PC2		σ	PC1		PC2	
		Score	SE	Score	SE		Score	SE	Score	SE		Scor	SE	Score	SE
TL	0.340	0.339	2.414	0.028	0.415	0.017	0.012	0.411	-0.001	0.346	0.257	0.257	2.735	0.001	0.197
PaL	0.339	0.336	2.313	0.033	0.628	0.024	-0.002	0.496	0.013	0.819	0.258	0.257	2.919	-0.015	0.675
TrL	0.314	0.307	2.799	0.063	0.671	0.029	-0.011	0.713	-0.017	0.867	0.270	0.268	2.709	0.038	0.857
BDA	0.286	0.284	2.366	0.022	0.583	0.050	0.039	1.156	0.024	1.209	0.229	0.227	2.367	0.020	0.561
HL	0.410	0.407	2.415	-0.047	0.548	0.105	0.103	1.508	0.010	0.637	0.250	0.248	2.915	-0.026	0.977
HD	0.341	0.336	2.332	-0.050	0.710	0.127	0.126	1.336	-0.004	0.728	0.210	0.210	2.256	-0.001	0.279
ED	0.313	0.310	1.909	-0.032	0.621	0.071	0.064	1.042	-0.024	1.087	0.190	0.186	2.364	-0.022	1.248
% Var		**97.5**		**1.8**			**88.3**		**4.7**			**98.5**		**0.8**	