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Quantification of muscle condition using digital image analysis in *Dicentrarchus labrax* larvae, and relationship with survival

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Digital image analysis was applied to quantify the degradation of muscle in sea bass *Dicentrarchus labrax* (Pisces: Moronidae) larvae. The measure obtained was the percentage muscle fibre separation (MFS). This measurement was compared to that obtained through classical scoring. The percentage MFS classified correctly a larger number of larvae from different feeding treatments (Fed, Starved and Delayed treatments) and paralleled nutritional condition, both by age and by size. It also yielded a significant correlation with the survival of the Starved larvae. The onset of the mass mortality of Starved larvae was used to define a critical value (M_{max}) of 6% MFS which would severely handicap survival. We regard MFS as a useful contribution to the determination of condition in fish larvae. It is a relatively fast quantitative method, and its use would reduce the bias caused by discrete grading (scores) and by differences in individual expertise.

INTRODUCTION

Histological analysis of nutritional condition is generally regarded as the most reliable method to detect severe starvation in fish larvae (Ferron & Leggett 1994). However, histological data are traditionally qualitative as they are based on the subjective scoring of the tissues (O'Connell, 1976; McFadzen et al., 1997). Starvation in teleost larvae is reflected in the condition of the trunk muscle. Typically, signs of muscle degradation include a pronounced separation of the muscle fibres (Shelbourne, 1957). In adult fish, the loss of parallel organization in the myofibrils was clearly shown by Gas (1972). In starving larvae, muscle degeneration occurs early after (or just before) yolk resorption and is due to the breakdown of proteins which produce the amino acids required to meet metabolic demands (Buckley, 1980). There are few data on quantitative measurements of muscle condition in fish larvae. Most of them regard chemical changes (e.g. percentage of water) along with starvation (Ehrlich, 1974). A quantitative property that is directly related to muscle condition is the variation of buoyancy or relative density and has been considered an indicator of condition ever since (Love, 1970). In the literature there are few attempts to quantify starvation-induced muscle degradation from histological sections. Green & McCormick (1999) measured muscle fibre separation by quantifying the presence/absence of muscle fibres in a Weibel grid, and Galloway et al. (1999) found an increased mean cross-sectional area of white muscle fibres in cod larvae subjected to diets enriched with fatty acids.

The goals of this work were: (i) to quantify starvation-induced muscle degradation; and (ii) to analyse the correspondence between muscle degradation and the survival of larvae in the laboratory.

MATERIALS AND METHODS

Larval rearing and sampling

European sea bass *Dicentrarchus labrax* L. larvae were raised from fertilized eggs coming from a natural spawning of a single female. Larvae were reared in a 500-l closed recirculation system at 19.5 ±0.85°C. Three batches of larvae were allocated to three feeding treatments: Fed, Starved and Delayed. Fed larvae were supplied with food *ad libitum* from five days after hatching (DAH). Food was supplied to Delayed larvae from day 13. The feeding regime consisted of rotifers, *Artemia* nauplii and 1 d-old enriched metanauplii according to Barnabé (1985). The daily collection of dead larvae from each treatment permitted the back-calculation of survival for each day, which was expressed as a percentage of the initial number of larvae at first day of feeding (5 DAH). Data on growth and survival of these larvae were analysed in a previous paper (Olivar et al., 2000) and will be referred to herein to explain some of the histological results. A total of 85 larvae were sampled for histological purposes. Fed treatment was sampled on days 6, 10, 14, 17, 19, 21 and 25 after hatching. Delayed treatment was sampled from day 14. Starved treatment was sampled similarly to Fed treatment except for last day (all Starved larvae died by day 21). Five ±1 individuals were drawn from each day and treatment.

Histological analyses

The histological variables were analysed by length and age. Two size-categories were chosen (4.5 to 6 mm and over 6 mm) based on the 2-cycle Gompertz growth curve which best fitted the length data (Olivar et al., 2000). The exhaustion of the oil globule coincided with the end of the

first Gompertz-cycle, a fact that reinforced the use of these two size-categories. This cutpoint corresponded to 13 DAH for Fed larvae, 16 DAH for Delayed larvae and comprised practically all Starved larvae (25 out of 29). These few Starved larvae over 6 mm were not sufficient to provide statistically valuable information hence were excluded from the analyses.

Larvae were dehydrated in an ethanol series graded up to 96%, embedded in Historesin (Leica) and serially sectioned at 3 μm in an approximate sagittal plane. They were mounted and stained with Lee's methylene blue–basic fuchsin (Benett et al., 1976). Sections were blow-dried and coverslipped with Eukitt mounting medium.

All sections of each individual larva were screened in order to choose those which best showed the different tissues. General observations on the response of several tissues to starvation were recorded, in order to complete the information obtained from the analysis of the muscle tissue.

Muscle scoring

Qualitative measurement of muscle consisted of grading two muscle characteristics, the degree of fibre separation (fibres densely packed, slight fibre separation or gaps as wide as fibres) and the amount of intercellular substance (abundant, scarce or almost absent). These traits have been used extensively in the literature to define the degree of muscle degeneration (O'Connell, 1976; Sieg, 1992). Depending on the degree of degradation, each characteristic was rated from 1 (bad) to 3 (good). For each larva, mean values were obtained from both characteristics. To establish the extremes of the scores, we compared the muscle tissue of severely emaciated larvae with well fed larvae. Larvae of days 19 and 21 were used for this purpose.

Determination of the percentage of muscle fibre separation

An image analyser (Optimas 6.0) connected to a Sony CCD video camera was used to calculate the percentage of muscle fibre separation (MFS). This measure was made on the central part of the trunk, in sections where myotomes and notochord were clearly visible. For several specimens examined, variability of MFS was high for areas comprising less than three myotomes. Therefore, regions comprising a minimum of three myotomes were selected. To determine the MFS, a macro from Optimas 6.0 was run within each selected area. This macro automatically detected the gaps between the muscle fibres against a luminance-corrected background.

Classification into nutritional classes

To evaluate the ability of each method to describe nutritional condition, each feeding treatment (Fed, Delayed or Starved) was assigned a range of values corresponding to a nutritional status (Healthy, Moderately Healthy or Emaciated, respectively). These ranges were established differently for the classical scoring of muscle or for the MFS. In the case of the scores, the three possible qualitative categories, 3 to 1, would have the following three classifying classes: 3–2.34, 2.33–1.67, and 1.66–1. As an example, a larvae showing a score of 1.5 would be classified as Emaciated.

In the case of MFS, the medians and 95% confidence interval (CI) for each feeding treatment were used to establish the correspondent nutritional categories. The range utilized was: $<1.35\%$ (Healthy), 1.36–3.95% (Moderately Healthy) and $>3.96\%$ (Emaciated). Although other ranges not based on medians were tested, the latter yielded the best division between treatments.

Relationship with survival

In order to analyse the degree of correspondence between each nutritional descriptor and survival, we compared the time-variation of each variable with the survival of the Starved larvae. Spearman's rank correlations with all data points were used.

The onset of the mass mortality of Starved larvae was used to define a critical value of muscle degradation which would severely handicap survival. This value was used to delimit larvae with high or low survival probabilities. Larvae were re-classified according to this criterion.

RESULTS

Yolk sac in *Dicentrarchus labrax* larvae lasted until 7 DAH and oil globule reserves were observed in larvae up to 13 DAH. Growth was similar for the three treatments until day 13. From day 11, mortality was near zero for Fed and Delayed individuals, while Starved larvae underwent a mass mortality on day 17 (Olivar et al., 2000). All Starved larvae died on day 21.

Tissular and cellular changes as starvation progressed were similar to those described for fish larvae of several species (O'Connell, 1976; Theilacker, 1978; Oozeki et al., 1989). In the case of the liver, the hepatocytes of Fed larvae exhibited a high degree of vacuolization after yolk resorption on 7 DAH. Starved larvae showed minimal vacuolization and a progressive accumulation of hepatocyte intracellular inclusions. The hepatocyte nuclei of Starved larvae appeared pycnotic and tended to occupy the centre of the cells. The pancreas showed high amounts of zymogen granules at hatching and showed high individual variability through development regardless of treatment. The procartilage of the Fed larvae showed a thick matrix enclosing the chondrocytes, which usually obliterated the capsular space. In severely starved animals, the chondrocytes appeared shrunk in the luminal space.

Muscle condition

In general, and for all age and size categories, Fed larvae showed compact myomeres, with straight muscle fibres and abundant interfibre substance. Starved larvae showed a high degree of MFS, which was more conspicuous towards the rear end of the body. Often, the Starved larvae showed an undulating pattern of the muscle fibres.

The mean MFS of the Fed larvae was $1.2 \pm 1.55\%$ (SD) along the experiment (Figure 1A). Neither muscle scores nor MFS differed significantly between treatments until day 14, in which Starved and Delayed larvae showed a higher MFS and lower muscle scores (Figure 1A&C).

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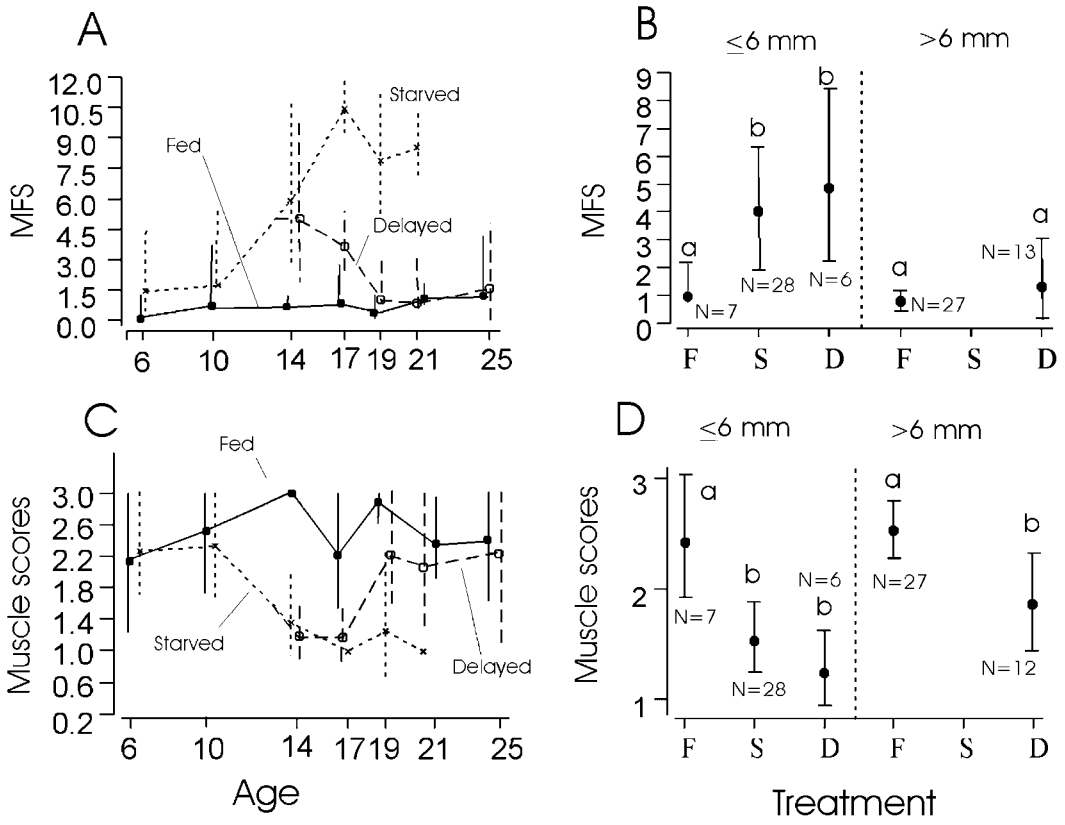

Figure 1. Means and 95% CI for each treatment of MFS (A) and muscle scores (C), along with age. (B&D) means and 95% CI of each length/food group for both measurements. Different letters for each size-class indicate significant differences (see text). In (C) points without error bars indicate coincidence in the scores. F, Fed; S, Starved; D, Delayed. For the sake of clarity, data of the three feeding treatments for a given day (A&C) are drawn slightly separated. Connecting lines were drawn to help in the visualization of treatments. All measurements are log(e)-transformed.

From day 14 onwards, Fed larvae were consistently in better condition than Starved larvae. Twenty-four hours after Delayed larvae began feeding, on day 14, the muscle showed no significant signs of recovery. Delayed larvae showed values significantly different (non-overlapping 95% CI) from Starved larvae on day 17 according to MFS, but not until day 21 according to muscle scores. The use of classical muscle scores presented less resolution than MFS.

Fed treatment was significantly different from both Starved and Delayed treatments at small sizes (Figure 1B&D; analysis of variance [ANOVA] for MFS: $F=3,69$, $P<0.05$; ANOVA for Scores: $F=6.25$, $P<0.01$). Values for both indices were similar in Fed larvae from both small and large size groups (Figure 1B&D). This indicated a lack of size effect on the utilization of these methods. Delayed larvae showed similar values to Starved larvae when they were small (⩽6mm) and approached the

Table 1. *Percentages of larvae correctly assigned to their feeding treatments by each histological variable.*

	⩽6 mm N=42						>6 mm N=37						All larvae					
	Scores			MFS			Scores			MFS			Scores			MFS		
Nutritional classes →	H	E	M	H	E	M	H	E	M	H	E	M	H	E	M	H	E	M
Treatment ↓																		
Fed	**70**	20	10	**86**	14	0	**72**	8	20	**84**	4	12	**71**	11	18	**84**	6	10
Starved	25	**61**	14	20	**66**	14	—	—	—	—	—	—	25	**61**	14	20	**66**	14
Delayed	0	100	**0**	0	60	**40**	20	40	**40**	40	13	**47**	15	55	**30**	30	25	**45**
%correct*			**44**			**64**			**56**			**65.5**			**54**			**65**

H, Healthy; M, Moderately Healthy; E, Emaciated. Larvae correctly assigned in bold. *, overall % larvae correctly assigned to their treatments.

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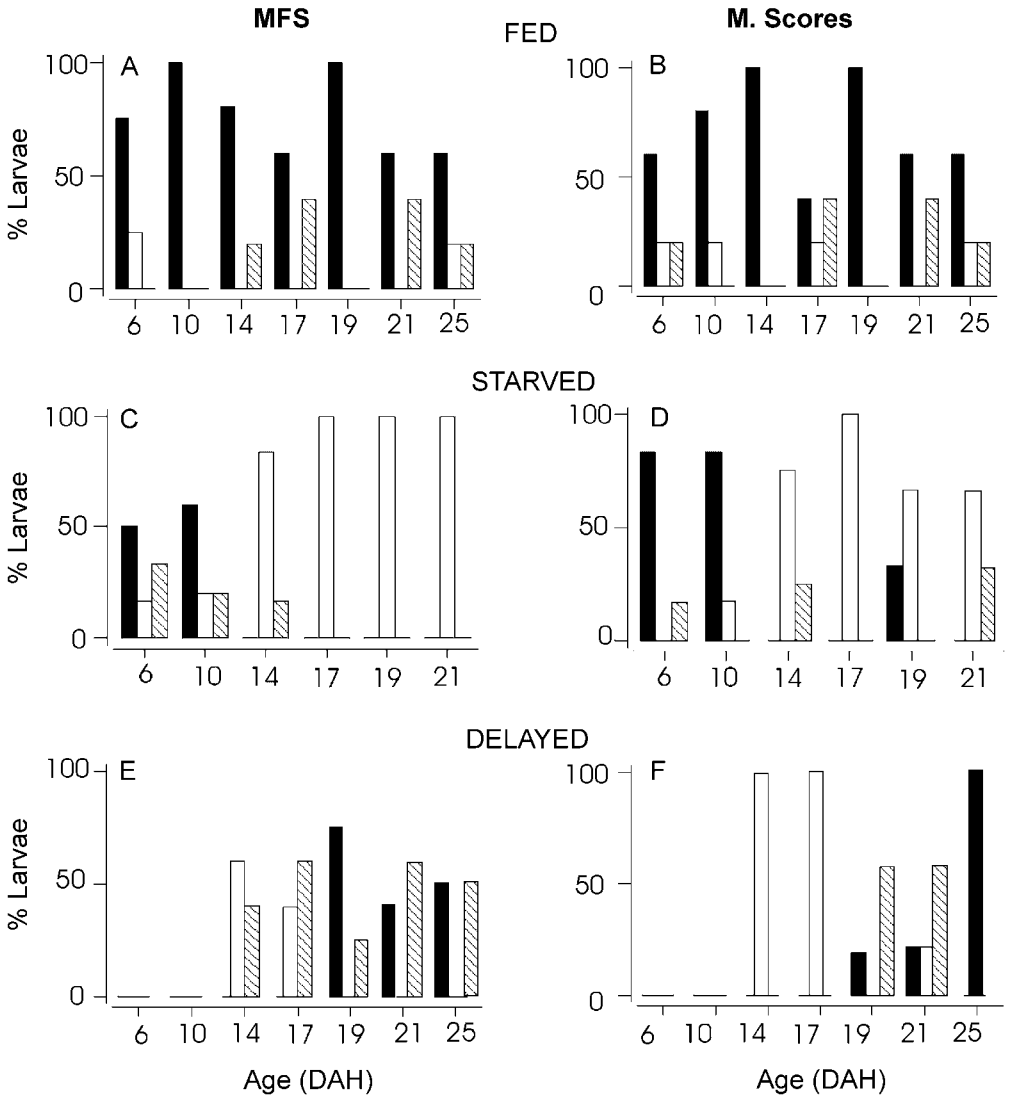

Figure 2. Age-group classification of Fed, Starved and Delayed larvae into nutritional classes by MFS and muscle scores. Black, Healthy; white, Emaciated; dashed, Moderately Healthy.

distribution range of Fed larvae when they were >6 mm (Figure 1B&D). The MFS of Fed and Delayed larvae was not significantly different at sizes over 6 mm. On the contrary, the larvae from the two treatments were still different according to muscle scores (*t*-test; $t=2.26$, $P<0.05$).

Table 1 shows the percentages of larvae of each treatment classified into the three nutritional categories (Healthy, Moderately Healthy or Emaciated) both by muscle scoring or MFS. The MFS yielded better classification than classical scoring regardless of size. It consistently classified over 80% of Fed larvae into the Healthy group and improved the discrimination of the small Delayed larvae (40% of Delayed larvae were classified as Moderately Healthy by MFS, while none using muscle scores).

Figure 2 shows the classification of the larvae of each treatment into nutritional classes by both methods at each sampled day. Noticeably, not all Fed larvae were classified as Healthy (Figure 2A,B). During the days prior to the exhaustion of the yolk and oil globule reserves (<14 DAH), scores and MFS classified most Starved larvae as

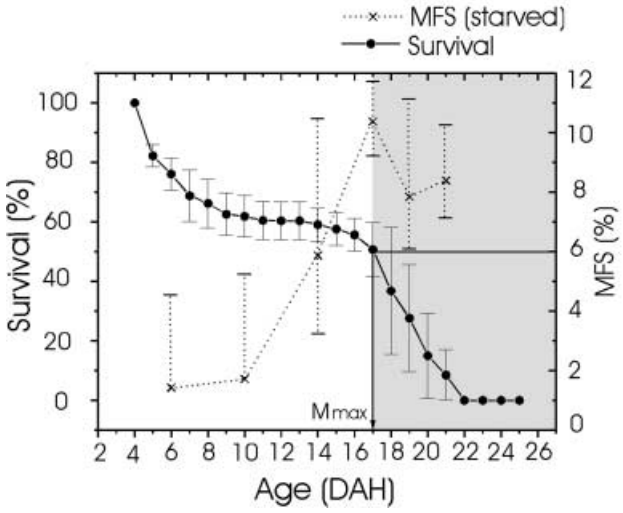

Figure 3. Double plot of MFS and mean % survival vs age. M_{max} is the value of MFS at the initiation of mass mortality. Bars for survival are ±SE. Bars for MFS are 95% CI for the mean.

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Healthy larvae (Figure 2C,D). Finally, MFS classified a greater percentage of Delayed larvae as Moderately Healthy (Figure 2E,F).

Relation between the indices and survival

Spearman's rank correlations showed that both muscle scores and MFS correlated significantly with the survival of the Starved larvae (MFS: $r_s = -0.668$, $t=4.66$, df=27, $P<0.001$. Scores: $r_s=0.697$, $t=4.95$, df=26, $P<0.001$). We chose the MFS to conduct further analyses for its best classifying properties and for the quantitative nature of the measurement.

The MFS value which corresponded with the initiation of mass mortality of Starved larvae (M_{max}) was 6%, and occurred at 17 DAH (Figure 3). This M_{max} was used to reclassify the larvae into pre and post M_{max}. The results of this dual classification substantially improved the initial classification into three categories. According to this, 93% of all Fed larvae were correctly classified as pre-M_{max}. From all the Starved animals, 55% would have gone over the M_{max} and would correspond to those 'severely emaciated' larvae over 16 DAH. Sixty per cent of Delayed larvae $\leqslant$6 mm would be classified as pre-M_{max}, leaving 40% of the larvae with values over 6% MFS (post-M_{max}). All larvae larger than 6 mm (thus Fed and Delayed) would survive. This result corresponds well with the null mortality observed in Fed and Delayed larvae over 16 DAH (Olivar et al., 2000).

DISCUSSION

Muscle condition

The use of digital image analysis enabled a relatively rapid determination of this variable and improved its resolving power with respect to the classical scoring of the muscle condition. Our results agree with most of the literature on larval condition which, for most of the species studied, report a gradual separation of muscle fibres and loss of interfibre substance in starving individuals (Gas, 1972; O'Connell, 1976; Martin & Wright, 1987; Green & McCormick, 1999). This has been explained by the ability of fish larvae to quickly mobilize protein by degrading muscle when glycogen and lipid reserves have been exhausted (Love, 1980).

Relationship with survival

Both MFS and muscle scores were significantly correlated with survival. We chose MFS as the best descriptor of survival, over muscle scores, for the quantitative nature of the data and for its best classifying performance (Table 1). According to the results based on the M_{max} criterion, high values of MFS can be used to assign a high susceptibility to starvation mortality in the laboratory.

In this experiment, we could not apply the 'necrotic criteria' used to define a point of irreversible starvation established for other species (Blaxter & Hempel, 1963; McGurk, 1984). Our results on susceptibility of survival based on the 'onset of mass mortality' criterion should not be interpreted according to the existence of a 'point of no return' (PNR) *sensu* Lasker (1970). There is evidence that several temperate species have either a very delayed PNR (Hunter, 1981) or do not show a true PNR at all (Eldridge et al., 1981). Instead, they can recover at any stage of starvation whenever food becomes available. In the case of species like sea bass, which require long periods to reach the PNR (Barnabé et al., 1976), the experimental correlation between a starvation index and mortality must be understood as an increased susceptibility to starvation mortality.

Also, according to the works on buoyancy (Neilson et al., 1986; Sclafani et al., 1993), muscle condition may be involved in the positioning of the larvae in the water column and hence in their interaction with prey and predators. The relationship of other nutritional indices (particularly those which have a short-time response to food deprivation) with survival in the wild seems less clear. There are other factors, apart from starvation, that can modify muscle characteristics of fish larvae. For example, increased temperature can favour muscle recruitment and hypertrophy (Johnston et al., 1998). In the wild, different batches of larvae having diverse thermal histories have been shown to differ, for a given age or body size, in the diameter and number of muscle fibres (Temple et al., 2000). These authors pointed out that probably both temperature and food availability interact to define the observed muscle pattern of the field-collected specimens.

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