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Individual growth monitoring of European sea bass larvae by image analysis and microsatellite genotyping

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ABSTRACT

The aims of the present study were to develop non-lethal methods to identify individual fish larvae and post-larvae before tagging and accurately follow their growth characteristics. European sea bass (*Dicentrarchus labrax*) was used as a model species at four different ages ranging from 71 to 100 days post fertilization (dpf).
Two different methods were tested for non-lethal tissue sampling from each larva for DNA analysis: 1) using a sterile absorbent paper to sample mucus and/or epithelial cells by rubbing the fish skin and 2) fin-clip of the bottom part of the caudal fin. Whatever the age of the larvae, the genotyping rate (at 12 microsatellite markers) was low with the use of sterile absorbent paper but relatively high with fin-clip sampling at 80 and 87 dpf (on average 17 and 63% of the loci genotyped for sterile paper and fin clips, respectively).
Several measurements were performed on digital pictures of sea bass larvae to model body weight. Using area, perimeter, length, height and volume, it was possible to estimate body weight with a coefficient of determination $r^2 = 0.98$ on very small larvae (body weight ranging from 20.0 to 419.3 mg).
The present results suggested that individual monitoring of the growth of European sea bass larvae can be achieved by combining image analysis and microsatellite genotyping as early as 87 dpf or 236 mg mean body weight.

1. Introduction

The early growth of juvenile fish can have impact on key performance characteristics later in life such as body weight (Doupé and Luymbery, 2005; Saillant et al., 2007), survival (Diaz et al., 2011), behaviour (Nicieza and Metcalfe, 1999), reproductive success (Lee et al., 2012) or sex determination (Saillant et al., 2003; Vandeputte, 2012). In the cichlid *Cichlasoma citrinelum*, the largest juveniles become males whereas the smallest ones become females (Francis and Barlow, 1993). Consequently, monitoring the growth performance of very small fish is of considerable interest. However, weighing small fish is hazardous due to the need to remove excess water with absorbent tissue to improve accuracy, but potentially at the cost of a decrease in survival. There is no precise information available on the impact of this method on fish survival and growth, but it is supposed to be harmful to larvae or very small fish. Using image analysis could be a good way to reduce this problem and estimate the weight of fish without manipulation. Several studies showed that the body weight or length could be estimated using image analysis, as the structured light technology measuring the volume (Storbeck and Daan, 1991) or the stereo-video technology measuring the length (Costa et al., 2009; Shortis et al., 2013). In the same way, Costa et al. (2013a) demonstrated the utility of using shape analysis of digital images to monitor body weight of adult European sea bass *Dicentrarchus labrax* (around 250 g). The weight of the fish was estimated with a coefficient of determination $r^2 = 0.977$, higher than when using the log transformed body length ($r^2 = 0.944$), a more commonly used predictor. However, all these studies were performed on big fish (body weight higher than around 200 g) and the application of these techniques to very young fish faces a number of potential difficulties, as described by Costa et al. (2009) showing that the error of the weight estimation was inversely correlated by the size of the fish. The accuracy of the prediction of the body weight using digital photograph measurements could be limited in early life stages due to a strong allometric growth.

Furthermore, to individually monitor the growth performance of small fish, it is necessary to identify each fish at the smallest possible size or youngest age. Individual identification can be done in fish with the use of physical tags, such as passive integrated transponder (PIT) tags (Prentice et al., 1990) or RFID microglass tags (Nanotec RFID, Lutronic International, Rodange, Luxembourg, www.nanotec.net;

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6 mm long, 1 mm diameter, 10 mg weight), well described by Costa et al. (2013b). However, due to their size, the minimum tagging size was ranged between 300 and 450 mg in zebrafish (around 26 mm; Cousin et al., 2012) and in European sea bass (105 days post fertilization (dpf); 36 mm; Ferrari et al.,2013). Identification methods using individual tags are not available for fish smaller than this size.

DNA genetic markers such as microsatellites or SNPs (Carleton et al., 2002; Herbinger et al., 1995; Trinh et al., 2013) have proved reliable for individual fish identification, but they need a relatively high quantity of sample tissue. Non-lethal tissue sampling of small fish that can provide sufficient DNA for downstream analysis is a particular challenge. Mirimin et al. (2011) have recently developed a method to collect DNA of Atlantic cod post-larvae by rubbing fishes with a sterile paper, but these authors used fish for which RFID microglass tags are potentially applicable (330–1610 mg, 43–52 mm).

The present study aimed to develop: i) a non-lethal method to identify fish larvae and post-larvae individually, and ii) whether it is possible to estimate the body weight (BW) of very small fish only from digital photograph measurements, and whether BW can be predicted with a greater accuracy by measuring other dimensions than body length only.

2. Material and methods

2.1. Biological material

This study was carried out on European sea bass produced in March 2012 by artificial fertilization in a full factorial mating design between 5 dams and 30 sires from a West-Mediterranean population. Broodstock management and hormonal induction of spawning, artificial fertilization and incubation of eggs were done according to the protocols described in Saillant et al. (2001). Floating (live) eggs were separated from sinking (dead) ones 48 h post fertilization by decantation at a salinity of 38‰ (Chatain, 1994), and introduced in equal proportion for each dam into a single tank to reduce environmental effects during the larval rearing. The rearing procedures that were used are the standards developed for the first three months described in Chatain (1994).

2.2. Non-lethal methods to individually identify larvae

2.2.1. Tissue sampling

Two different methods were used to obtain tissue samples from each larva, previously anaesthetised with MS_{222} (2 min at 0.07 $g \cdot l^{-1}$). The first used sterile absorbent paper to sample mucus and/or epithelial cells by rubbing the fish skin as described in Mirimin et al. (2011). The second used a fin-clip of the lower part of the caudal fin stored in 70% EtOH. After sampling, larvae were maintained for five days in different tanks to estimate the mortality consecutive to each sampling method. The experiment was performed on five batches of larvae aged 71, 80, 87, 92 and 100 days old. At each age, 150 larvae were used among which 100 were tissue sampled, 50 with a sterile paper, 50 with a fin-clip, the remaining 50 being only anaesthetized to serve as controls for the anaesthetic procedure.

2.2.2. DNA extraction and genotyping

DNA was extracted from tissue samples using a proteinase K digestion, and the Chelex (Bio-Rad; www.bio-rad.com) extraction procedure. For each sample, 150 μl of 5% Chelex solution, 15 μl of 1× Tris-EDTA buffer (10 mM Tris, 1 mM EDTA) and 10 μl of proteinase K (10 $mg \cdot ml^{-1}$) were added to the fin or the sterile paper, then incubated at 55 °C for 2 h, then at 96 °C for 10 min. After that, the supernatant (containing DNA in aqueous solution) was transferred into a new clean tube. To verify the proper functioning of the extraction procedure, PCR was performed with sea bass specific primers (*amh* gene: 5′CCTAAGCTCCAG CTGACCAC 3′ and 5′ CTCCAACAGTGCAGGAGACA 3′, D'Cotta, personal communication). Amplification was performed in a 20 μl PCR mixture containing 2 μl of DNA, 2 μl PCR buffer, 200 nmol of each primer, 0.4 μl of dNTP mix and 0.11 μl of QiaGen Taq DNA Polymerase (ref 201207).

The following thermocycling procedure was used for PCR amplification (2 μl of DNA into 20 μl): denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 60 °C for 45 s and 72 °C for 1 min. A final extension step was then performed at 72 °C for 10 min. Negative (sterile water and sea water) and a positive control (sea bass purified genomic DNA) were included alongside each PCR run.

DNA for which the extraction procedure was a success, was precipitated with sodium acetate (Na-acetate, 1/10th of the DNA solution volume) and 100% EtOH, (2 fold of the DNA solution volume). Samples were then centrifuged for 15 min at 12,000 *g*, and the supernatant was removed. The pellet was washed with 1 ml of 70% EtOH, and a second centrifugation was performed (2 min at 12,000 *g*). The supernatant was discarded and the pellet was dried. The DNA resuspended with 50 μl of sterile water was performed.

To assess the possibility of identifying fishes, genotyping was performed by Labogena (Jouy en Josas, France) with a commercial suite of 12 microsatellite markers on 10 DNA samples obtained from sterile paper swabs and 10 DNA issued from fin of fishes at 71, 80 and 87 dpf.

2.3. Estimation of growth performances

At each sampling date, an additional 50 fish were sacrificed with an excess of anaesthetic (MS_{222}) to obtain the weight (after a careful drying with absorbent tissue) and length of each fish. Body weight (BW) was obtained using a precision scale (to the nearest 0.01 g), standard length (SL) was measured with a graduated ruler (to the nearest 0.1 mm) after magnification of the larvae. A digital picture (JPG format, 4272 × 2848 pixels) of each fish was taken using a stand with a Canon EOS 1100D digital camera (12.2 Mpixel). For these photos, each fish was placed on a numbered thin transparent plastic sheet to link the picture to the weight and length measurements, over a light table to increase the contrast, and beside a graduated ruler used as a reference.

Image analysis was performed with the ImageJ software (available at http://rsb.info.nih.gov; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). This software allows measurement of area, perimeter, length and height of each fish. The method allowed the use of fin-clipped animals without bias because it does not take into account the fish caudal fin in such measures. From length and height, the volume was also calculated as follows:

$$Volume = \pi * Length * Height * Height/6. \quad (1)$$

The different steps of image analysis (see Fig. 1) were: i) converting the image to 8-bit (black and white), ii) adjusting the brightness and contrast to distinguish the larva clearly from the background, iii) adjusting the threshold to blacken only the area of the larvae, iv) filling holes which may still exist, and v) analysing pixel particles. The ruler taken on each photo with the fish was used to convert all measurements from pixels to mm.

2.4. Statistical analyses

Statistical differences between survival rates between the tissue sampling methods and between genotyping results were performed with the NPAR1WAY procedure of SAS (Version 9.3, SAS Institute, Cary NC). Phenotypic correlations between the measurements done on the image those measured directly on the fish were estimated using the CORR procedure of SAS (Version 9.3, SAS Institute, Cary NC). Multiple regression models using length, height, perimeter, area and volume variables were tested using the REG procedure of SAS (Version 9.3, SAS Institute, Cary NC) to evaluate the efficiency of the different traits measured to predict BW. This last procedure estimated the coefficient of determination (r^2) and the Akaike information criterion (AIC) testing whether the BW is accurately estimated or not. To find the