IAMC-CNR UO di Capo Granitola

Engraulis encrasicolus (Linnaeus, 1758): technical report about extraction and recognition of the digestive tract contents in early stages of the life cycle

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Summary

1. Introduction

Among the most important fish stocks in many regions of the Mediterranean Sea there is the anchovy *Engraulis encrasicolus* (Linnaeus, 1758), a small pelagic fish belonging to the family of Engraulidae. IREPA data for the year 2009, shown that in Italy the fishing of *E. encrasicolus* represents on average the 26% of the total catch. This species is continuously monitored; thus it has been evidenced that pronounced inter-annual fluctuations happens (Cergole *et al*., 2002; Cingolani *et al.*, 2004). Several factors affect this scenario including human activities as the high fishing and natural factors (Borja *et al*., 1996). Grater attention should be given to the biological and environmental dynamics that influence the survival in the early life stages of this species and can affect the next recruitment. Therefore, these factors may represent a key cause of contractions and of the annual increases of the adult stock (Tikhonova *et al*., 2000; James *et al*., 2003; Cuttitta *et al*., 2003, 2006). The study of ichthyio-plankton stages and its relations with the environment and other organisms is therefore crucial for a correct use of fishery resources. In this context, the extraction and the analysis of the content of the digestive tract, is a key method for the identification of the diet in early larval stages, the determination of the resources they rely on and possibly a comparison with the diet of other species. Additionally this approach could be useful in determination on occurrence of species competition. This technique is preceded by the analysis of morphometric data (Blackith & Reyment, 1971; Marcus, 1990), that is the acquisition of quantitative variables measured from the morphology of the object of study. They are linear distances, count, angles and ratios. The subsequent application of multivariate statistical methods, aims to quantify the changes in morphological measures between and within groups, relating them to the type and size of prey and evaluate if some changes appear in food choices along the larvae growth.

Hence, the goal of this paper is to provide an appropriate methodology for the extraction of the digestive tract contents of *E. encrasicolus* in the larval stages. This species is the subject of study of the oceanographic cruises Ansic and Bansic, which are organized every year (since 1997) by IAMC-CNR of Capo Granitola in summer and conducted on board of the oceanographic ship Urania. Its aim is to study the relations between oceanographic structures on mesoscale (vertical and horizontal vortices, upwelling, etc.) and the spatial structures of the biological phenomena belonging to the first rings of the trophic chain (phytoplankton, zooplankton, distribution and abundance of small pelagic fish). Besides its economic value, from a biological and ecological point of view, this species is interesting because it shares the period and the reproduction area with *Sardinella aurita* (Valenciennes, 1847). That could create larval competition phenomena (Palomera *et al*., 1990; Morote *et al*., 2008). In this context, the analysis of the content of the digestive tract is a necessary method to determine the diet and to assess a possible competition with the similar species *S. aurita*.

1.1. Biology of the species object of study

The European anchovy (*Engraulis encrasicolus*), the only representative of the family of Engraulidae in the Mediterranean Sea (Tudela, 1999), belongs to the Order of Clupeiformes. It is widely distributed from the North Eastern Atlantic and the Central Mediterranean Sea to the southern coast of West Africa (Fig. 1). It represents a migratory and gregarious species, gathering in large schools near the coast for reproduction from early spring to late summer, and then leave toward deeper water in autumn (Patti *et al*., 2011). Through the evaluation of the gonadsomatic index and the evolution of the maturity stages, it has been determined that in the Strait of Sicily the reproductive period goes approximately from March to April until August-September and the greater reproductive effort is in July - August (Basilone *et al*., 2004, 2006). This period overlaps the peak of the zooplankton biomass (Garcia Lafuente *et al*., 2002). Indeed copepods are the main prey both for adult anchovies as well larvae during the reproductive period. Females produce

about 4,000 eggs at a time, issued in small lots in the surface layers, mostly at the sunset (Varagnolo, 1965; Ghirardelli, 1967; Regner, 1985). The issued eggs are floating, ellipsoidal, slightly longer than 1 millimetre (Varagnolo, 1967; Regner, 1972) and are entrusted to ocean currents.

Just open up, larvae are about 2 mm long and many of them are destined to be predated. They feed mainly on phyto and zooplankton, especially copepods, cirripedes, shellfish larvae, fish eggs and larvae (Catalàn *et al*., 2010; Morote *et al*., 2010; Bänärescu, 1964; Demir, 1963). From the morphological point of view they are elongated, have a subspheric eye and their gut typically ends at the dorsal fin, at approximately ¾ of their total length.

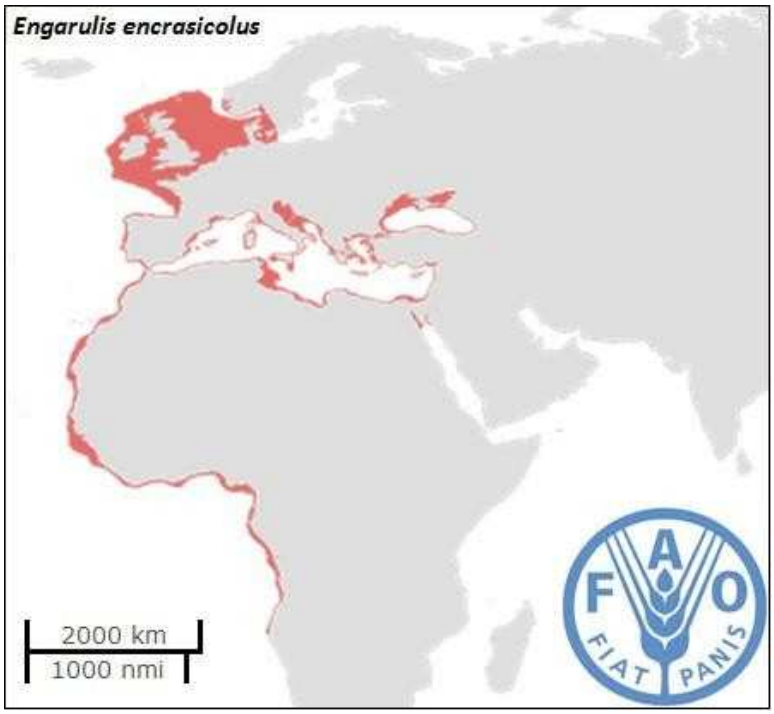


Fig. 1 – Distribution area of European anchovy

2. Materials and methods

2.1 – Image acquisition

From a methodological point of view, the first step is to obtain morphometric data to correlate the prey with the size of the larva and with the mouth opening, which involves the acquisition of images through the use of stereo-microscopes with integrated camera.

For this paper the stereomicroscope Zeiss stemi 2000- C with AxioCam ERC 5s camera was used (Fig. 2).

A.

B.

Fig. 2 - Stereomicroscope Zeiss stemi 2000-C (A) and AxioCam ERC 5s camera (B)

Photos of the larvae are acquired by the digital image processing software AxioVision SE64 Rel.4.8, which allows to store and manage files in a structured manner.

To facilitate the acquisition of the image, the larvae with the aid of a Pasteur pipet, constituted by a teat rubber and a thin glass tube, are taken from the test tubes and placed individually in a glass Petri dish of diameter 5 cm.

With the help of special needles, the larva is placed in an extended position on its side, taking care to remove any excess liquid used for storage (alcohol or water), which could disturb the image and prevent the adhesion of the larva to the bottom of Petri. The magnification to be used is the one that portraits the entire larva in the picture.

After taking a photo the magnification used in the acquisition phase and the name specifying the information of the larva are assigned (date, sampling point, species etc.) and reported on the test tube.

For this method two more pictures are needed, a detail of the head and another one taking the mouth in ventral position, that through measurements allow to relate the size of the prey with the mouth opening.

After the photo shoot, the larvae are stored individually in a test tube with 50% glycerol, indicating the parameters used when labelling the photo of the correspondent larva.

All tubes are placed in a rack and the position of each one is written and this facilitated the recovery for the opening phase. This methodology considers a limited manipulation of the larva and the housing of the glycerol allows to soften tissues and make easier to open the stomach and intestines. The racks are then stored in the refrigerator to prevent deterioration until the next step.

2.2 - Image analysis

The management software and image processing © Image Pro Plus (IPP) is used to acquire the morphometric parameters of the larvae from the photos. The method used is the one proposed by Torri *et al*., 2014.

The morphometric parameters used are those proposed by Diaz *et al.* (2009) (Fig. 3).

Fig. 3 - Morphometric parameters used for the study of morphometry. Pictures of *E. encrasicolus*

- Total Length (TL): from the extreme front of the upper jaw to the extremity of the caudal fin.
- Standard length (SL): from the extreme front of the upper jaw to the extremity of the notochord.
- Head length (HL): From the far front of the upper jaw to the pectoral fin.
- Body width to cleitra (BD), towards the pectoral fin.
- Eye diameter (ED).
- Anal length (AL): from the extreme front of the upper jaw to the end of the digestive tract.

Two other measures are taken from the photos of the head and the ventral mouth:

- Jaw length (JL): from the front end of the mandible to the point of the mouth terminal (Fig. 4)

- Mouth width (MW): from the ventral photos from one extreme to the other of the jaw (Fig. 5)

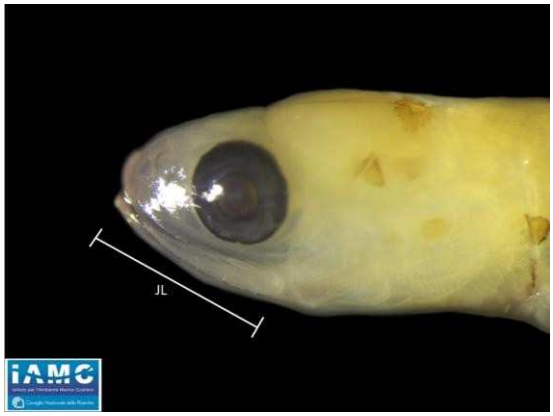


Fig. 4 Jaw length (JL)

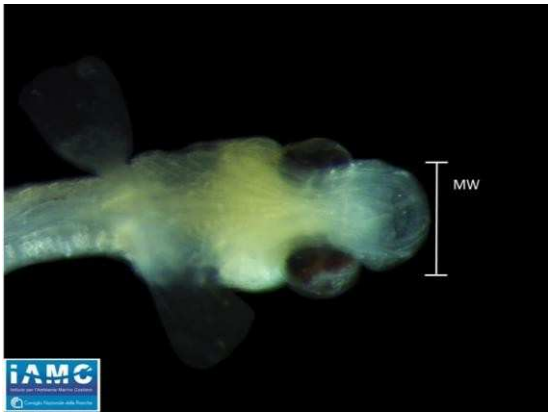


Fig. 5 Mouth width (MW)

2.3 Content analysis

After the data acquisition stage from the photos, size classes that are used for the study of the digestive tract content can be selected.

The larvae are taken from the test tubes and placed individually in a glass petri dish of diameter 5 cm, with the aid of a Pasteur pipet, constituted by a teat rubber and a thin tube glass.

Looking through the binocular and with the help of special needles, the larva is placed in extended position. By using ophthalmic scalpel the intestinal tract starts to detach from the anal portion, the relaying in glycerol facilitates this stage because it makes the tissue softer.

Once the digestive tract is disconnected, it is split into three sections:

1- From the opening mouth to the gastric cardia

2- From gastric cardia to the pylorus

3- From the pylorus to the anal opening

These three sections will allow to identify the position of the prey in the digestive tract, to be able to trace the supposed time of ingestion and check the levels of digestion according to the prey.

After that, the three sections are separately placed on a glass slide and immersed in 50% glycerol to maintain its moisture.

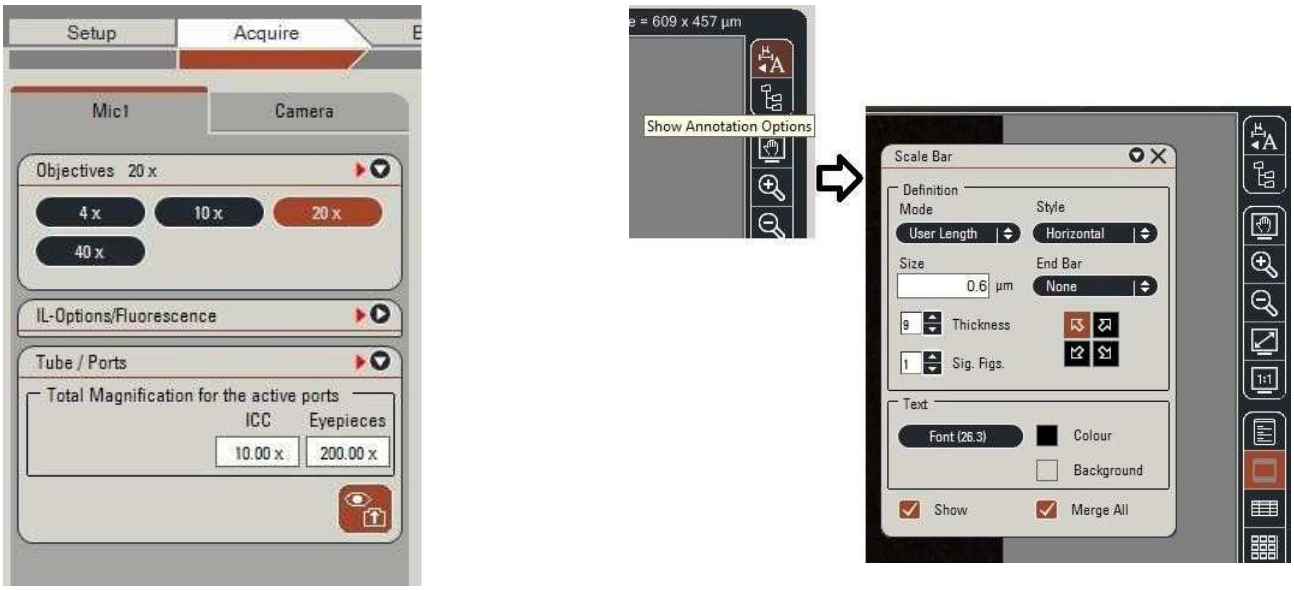
Positioned the three parts of the digestive tract, they are etched perpendicularly. This stage occurs on a glass slide and not before to avoid that smaller organisms get lost.

After the sectioning, they are observed by the inverted microscope Leica DMIL LED (Fig.6a), which allows a better view of the smaller prey thanks to the opposite light source on the objectives. Moreover, this microscope has the revolver in the lower part, so the content belonging to the phytoplankton is deposited at the base of the drops of glycerol and allows a better view of the sample.

A. B.

Fig. 6 Leica microscope LED DMIL (A) and camera Leica ICC50W (B)

This microscope allows a magnification from 4x to 40x, so also the smaller prey can be seen. It proceeds along the drop of glycerol, covering the entire surface occupied by it and thus avoiding that even the smallest prey can not be seen. As soon as the prey is framed, the highest possible magnification must to be set, so that all possible details can be photographed and measured. By using the integrated program Leica Application Suite (LAS), connected to the camera Leica ICC50W (Fig.6b), highly detailed photos can be taken, and can be measured immediately. With the "Objectives" program setting it is possible to select the magnification used and then to have a reference in microns useful for recognition (Fig. 7a).



A. B.

Fig 7. Objectives (A), Show annotation option (B) and Exposure Adjust (C)

Such reference must be set by selecting "Show annotation option" icon, selecting "scale bar" and typing the desired micron reference (Fig. 7b).

Before taking the picture, it is necessary to adjust the brightness and / or the exposure parameters, using the "Camera" function by selecting the "Exposure Adjust" panel (Fig. 8).

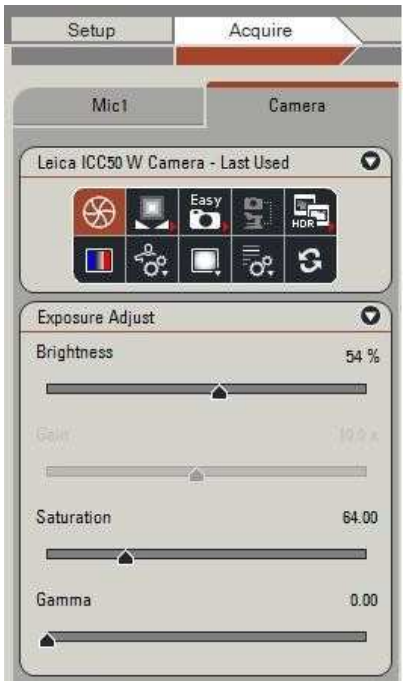


Fig. 8 Exposure Adjust

Once all parameters have been calibrated to get a good definition of the picture the "Acquire Image" command takes the picture. The file is saved without measures and reporting the data of the correspondent test tube (the identification number of the larva and the identification name of the prey). If the prey is too deteriorated or it is digested, insights may be necessary, in this case the picture is first listed as unknown and then the file name can be updated directly from the photo. The next step is for the measurement of prey, through the "Process" command it is possible to open a window for the measurements, and then "Annotate"→"show", from the drop down menu select "distance line" and "show" (Fig 9).

Fig. 9

Finally, it is possible to proceed to the measurement of the prey by using the cursor for drawing a continuous line from the summit to the extremity of the prey (Fig 10). If necessary, photos can be saved either individually for each size or having a single picture with all measures.

To get a photo for each measure at the end of each measurement the button “save” must be clicked, to continue on the same image use the "Merge all" command, proceed with the next measure and save (for each new measure "Merge all" should be first selected).

Once this phase is finalized, all the prey in the photo collections are recognized and catalogued and the measurements of each prey are reported on an Excel spreadsheet useful for subsequent statistical analysis.

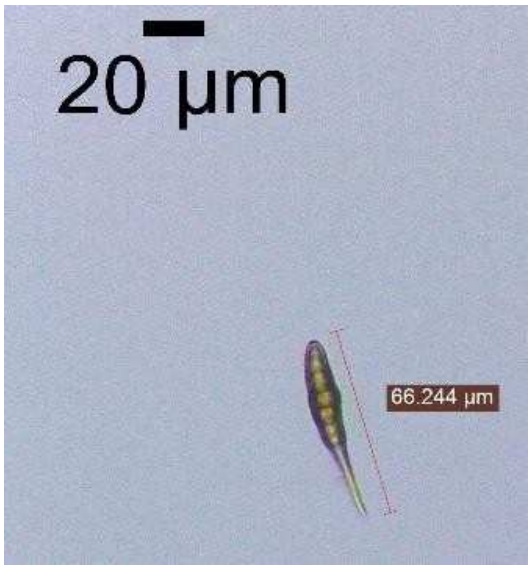


Fig.10 Representative cartoon of measure line

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