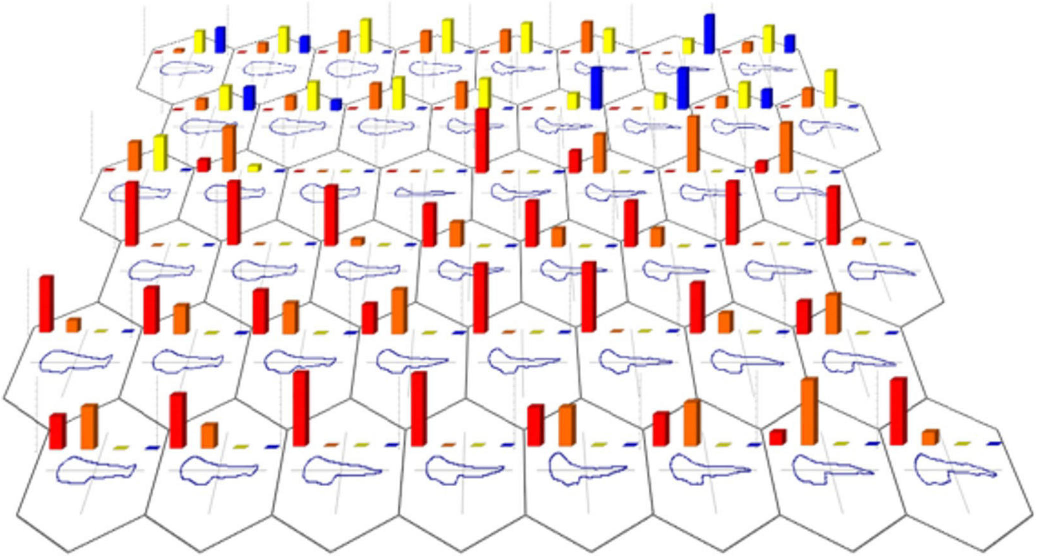
New insights into larval fish ecomorphology: shape and trophic habits changes during ontogenesis

Tommaso Russo



Laboratory of Experimental Ecology and Aquaculture

New insights into larval fish ecomorphology: shape and trophic habits changes during ontogenesis

Università degli Studi di Roma "Tor Vergata"
Corso di Dottorato in **Biologia Evoluzionistica ed Ecologia**
XVII Ciclo

Candidato: Tommaso Russo

Docente guida: Stefano Cataudella

An organism exists within the process of development,
it represents a unity of all other intervals of development.
This why any hereditary change of an organism is always
accompanied by changes of all intervals of its existence,
starting from an egg and up to adulthood.

Sergei G. Kryzhanovsky

The developmental hourglass poses a significant problem for evolutionary developmental biology. The neck of the hourglass presents us with a region of low probability of evolutionary changes: the phylotypic stage. Prior to that stage, development is far less constrained to evolutionary change. That leads us to an extraordinary phenomenon. The unconstrained upper portion of the hourglass allows a number of early developmental trajectory to reach a particular phylotypic stage. The evolutionary freedom of early ontogenetic stages is significant in providing novel developmental patterns and life histories. These stages also contain an unexplored potential for the discovery of mechanisms of evolution of the developmental features composing animal body plans.

Rudolf A. Ratt - The Shape of Life

Contents

Abstract

Fish larvae development is often characterized by the indirect acquirement of the morphological and ecological characters of adult stage. In this case, the ontogenesis of anatomical structures seems to go behind allometric and eterocronic patterns that make suddenly functional the structures involved. As consequence, the ecological relationships between organisms and environment could change following a saltatory sequence. This process has been historically investigated in studies of eco- and functional morphology, in order to understand the adaptative meaning underpinning the ontogenetic patterns of shape development. In this thesis, the ontogenesis of shape of two marine fish species (***Sparus aurata*** and ***Epinephelus marginatus***) was investigated. Changes of shape were described by means of two modern approaches: ***Geometric Morphometrics*** and ***Elliptic Fourier Analysis*** of outlines. At the same time, fish trophic habits were analyzed observing the stomach contents and the trophic niche was described with respect to the dimensional, ecological and biological characters of preys. The patterns of shape and trophic habits changes were then compared using a type of artificial neural network called ***Self Organizing Maps*** (**SOMs**).

The results obtained for ***E. marginatus*** allowed to understand the role of mouth in determining food selection during larval stage and, therefore, the shape analysis efficiently visualized the growth trajectory and the shape changes during ontogenesis. The entire developmental sequence, from hatching to adult stage, was studied for ***S. aurata***. Shape analysis tools provided exhaustive descriptions of shape changes at general and local scale. Several developmental stages were detected by shape analysis and defined by size limits ($\boldsymbol{T}_{\mathrm{L}}$). Afterwards, these stages were compared with trophic data. The matching of these features allowed us to infer the meaning of several shape changes during the development and to confirm the theory of ***saltatory ontogeny*** in the context of trophic adaptations.

Despite the goodness of these results, the comparison between shape (described by shape analysis tools) and ecological features like trophic habits or habitat (often surveyed as qualitative or quantitative discrete patterns) is affected by the fact that these aspects are inhomogeneous, precluding a direct visualization, abstraction and interpretation of their complex relationships. In order to solve this problem, SOMs were computed on the dataset obtained from Elliptic Fourier Analysis of outlines, and the trained maps were equipped with appropriate representations of shape prototypes, that were the abstraction of shape characters along the developmental series. Besides, SOMs were used and tested for the comparison of shape with the trophic niche. In this way, the feeding habits of each specimen were superimposed onto the maps as probability density. A good correspondence was observed, confirming the model of co-variation of these features during larval development.

This thesis added new important knowledge of the ecomorphological and ecological features of the two species studied and of the methodological aspects of ecomorphological investigations. Shape analysis tools (particularly Elliptic Fourier Analysis of outlines) and SOMs, were successfully applied in describing shape characters and related changes during ontogenesis, opening new perspectives in the investigation on early larval stages of fish.

Key Words: Ecomorphology, Fish larvae, Shape, *Sparus aurata*, *Epinephelus marginatus*, Self-Organizing Maps, Aquaculture.

Estratto

I primi stadi vitali dei pesci ossei sono spesso costituiti da forme larvali, caratterizzate da strutture che progressivamente scompaiono e vengono sostituite dai caratteri tipici delle forme adulte. Uno degli aspetti più interessanti di questo sviluppo indiretto è che i processi ontogenetici che portano alla formazione delle differenti strutture morfo-anatomiche mostrano dei rapporti allometrici ed eterocronici. Questo fa si che le parti diverse di organi (ed apparati) completino il loro sviluppo e divengano funzionali in maniera sincrona e, di conseguenza, gli individui divengono "competenti" per determinate performance in maniera intermittente. Per questo motivo, le relazioni ecologiche tra le larve ed il loro ambiente si modificano secondo una sequenza anch'essa saltatoria. Vari aspetti dinamici della biologia larvale dei pesci (tra cui le modificazioni della morfologia esterna) sono stati studiati, storicamente, sia dal punto di vista dell'ecomorfologia che della morfologia funzionale, al fine di comprendere il significato adattativo alla base delle traiettorie ontogenetiche.

Nel presente lavoro di tesi è stata analizzata l'ontogenesi della forma di due specie marine di pesci: l'orata (***Sparus aurata***) e la cernia (***Epinephelus marginatus***). I campioni utilizzati provenivano da lotti di larve ottenute da genitori selvatici in cattività ed allevate secondo una moderna tecnologia (Grandi Volumi) precedentemente testata per la sua capacità di produrre individui simili al selvatico per differenti aspetti morfo-anatomici e comportamentali.

I cambiamenti della forma sono stati descritti mediante due moderne tecniche: la ***Morfometria Geometrica*** e l'***Analisi Ellittica di Fourier*** sui profili laterali. Sugli stessi campioni è stata studiata l'ecologia trofica in condizioni di scelta polispecifica (con pabulum alimentare costituito da prede selvatiche ed allevate) mediante l'analisi dei contenuti stomacali. In

questo modo, è stato possibile descrivere la nicchia trofica dei vari stadi larvali e le sue modificazioni durante lo sviluppo, con particolare risalto per quanto concerne le dimensioni e le caratterictiche ecologiche delle prede. Infine, forma ed ecologia trofica sono state confrontate mediante un tipo di rete neuronale denominato ***Self Organizing Maps***.

I primi risultati ottenuti per *E. marginatus* hanno permesso di comprendere il ruolo della bocca nella selezione delle prede durante gli stadi larvali. Successivamente, su questa specie, sono stati messi punto e testati gli strumenti propri dell'analisi della forma che ha permesso di visualizzare efficacemente la traiettoria di crescita ed i cambiamenti della forma durante l'ontogenesi. Per *S. aurata* è stata studiata l'intera sequenza dello sviluppo, dalla schiusa fino allo stadio adulto. L'analisi della forma ha pemesso di ottenere una descrizione esauriente dei cambiamenti della forma alle scale locale e generale del corpo. Inoltre, è stato possibile individuare alcuni stadi nello sviluppo della forma, delimitati mediante misure di taglia e denominati in base allo stadio di ontogenesi raggiunto. Tali stadi sono stati comparati con i dati relativi all'ecologia trofica, e la precisa corrispondenza tra questi due aspetti dello sviluppo ha permesso di inferire ipotesi sul significato adattattivo di alcune variazioni della forma e di confermare la teoria dell'ontogenesi saltatoria nel contesto degli adattamenti propri dell'ecologia trofica.

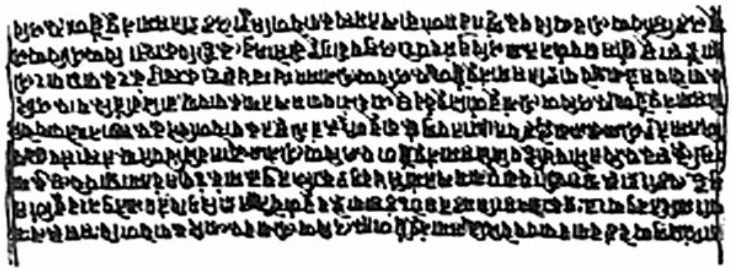
Nonostante la bontà di questi risultati, il confronto tra la forma e i descrittori ecologici come l'habitat o l'habitus trofico (descritti in maniera qualitativa o quantitativa) è risultata in prima istanza condizionata e limitata dal fatto che tali aspetti sono disomogenei, e questo preclude una visualizzazione diretta e quindi la generalizzazione ed interpretazione delle complesse relazioni tra di essi. Per superare questo limite sono state utilizzate le ***Self Organizing Maps*** (SOM). L'idea di base è stata quella di addestrate le SOM sui set di dati della forma (si è scelto di utilizzare i profili laterali utilizzati per Analisi Ellittica di Fourier). Le SOM sono state quindi adattate per visualizzare i "prototipi virtuali della forma", cioè astrazioni delle caratteristiche della forma lungo la sequenza dello sviluppo. Infine, le SOM sono state usate per confrontare i dati della forma con quelli dell'ecologia trofica, sfruttando la loro elasticità e potenza nelle applicazioni comparative tra set di dati disomogenei. In questo senso, l'habitus trofico di ogni individuo è stato sovrapposto alla mappa della forma come densità di frequenza. E' stata così evidenziata una buona corrispondenza che ha confermato il modello di covariazione tra questi due aspetti durante lo sviluppo larvale.

Questa tesi ha dunque apportato nuovi importanti contributi sia nel campo delle conoscenze ecologiche ed ecomorfologiche delle due specie studiate sia per quanto concerne gli aspetti metodologici delle investigazioni ecomorfologiche. Gli strumenti dell'analisi della forma, ed in particolare l'Analisi Ellittica di Fourier sui profili, si sono dimostrati potenti ed efficaci nel descrivere le caratteristiche della forma ed i suoi cambiamenti durante l'ontogenesi, come emerso anche dalla innovativa fusione con le reti neuronali artificiali (SOM). Questo potrebbe aprire nuove prospettive nello studio sugli stadi larvali dei pesci e nel campo più generale delle investigazioni ecomorfologiche.

1. General introduction

GETTING INTO ECOMORPHOLOGY: AN HISTORICAL AND THEORETICAL OVERVIEW

The necessity to describe, understand and catalogue the diversity of organisms and their distribution in the environment is a question that human beings have long been asking. Ecomorphology is a very ancient discipline: the first references to it can probably be found in the Hindu text "Susruta-samhita", where the link between body form and habitat in freshwater fishes is discussed, as well as in the writings of Aristotle (Motta *et al.,* 1995a).



The river fish are bulky in the middle because they move with their head and tail: the lake and tank fish are similar... but are characterized by a relative smaller head; the spring and pool fish... are extremely deep behind the head; the fishes of the torrents are traditionally well known by the possession of... greatly flattened body on account of their habitat of crawling with the chest, and a relatively reduced anterior part of the body.

A Sanskrit passage written between 600 B.C. and A.D. 500

Fishes historically represent the major animal group involved in those investigations, due to their incredible morphological and ecological diversity, their ability to colonize almost all aquatic environments, foraging on almost anything of energetic value, displaying a vast range of reproductive styles, and making a greater use of sensory modalities more than any other group of organisms (Matthews, 1998).

Ecomorphology was born and developed as a comparative science, combining the field of morphology and that of ecology. Nevertheless, only the recent developments in both these disciplines have allowed well-designed ecomorphological investigations to be undertaken, leading to the detection and understanding of the interactions between morphotypes and ecological features. While the roots of morphology can be traced to Plato and Aristotle, ecology is a relatively young discipline, officially starting in 1869, when natural historians such as van Leewenhoek, Darwin and Haeckel proposed the term "*oecology*" to refer to the study of organism-environment interactions (Motta *et al.*, 1995a). In particular, Darwin's publication of "On The Origin of Species" marks the beginning of scientific interest in the adaptation of organisms to their environments. In the theory of evolution, investigations of the relation between form and function represented the main thrust of the study of adaptation. For a long time morphologists and ecologists followed different, rarely overlapping, paths. It was only during the last two centuries that several fundamental ecological concepts were developed such as the niche, habitat, and competitive exclusion, which allow us to describe ecological communities or characteristics of the species in either qualitative or quantitative terms. The first studies dealing with ecological morphology date back to the beginning of the 20th century, but it is only after 1965 that Bock & von Wahlert specified the guidelines to follow in order to distinguish between functional morphology and ecological morphology. This coincided with a resurgent interest in vertebrate morphology, while ecologists began to see morphology as a powerful tool for addressing questions relating to niche, competition, resource partitioning and community structure. Finally, Karr & James in 1975 introduced the term "ecomorphology" in their study of avian communities. Since then, there has been a steady growth in interest in ecomorphology by both ecologists and morphologists, who benefited greatly from the remarkable development of experimental approaches, including tools for collecting and analysing data.

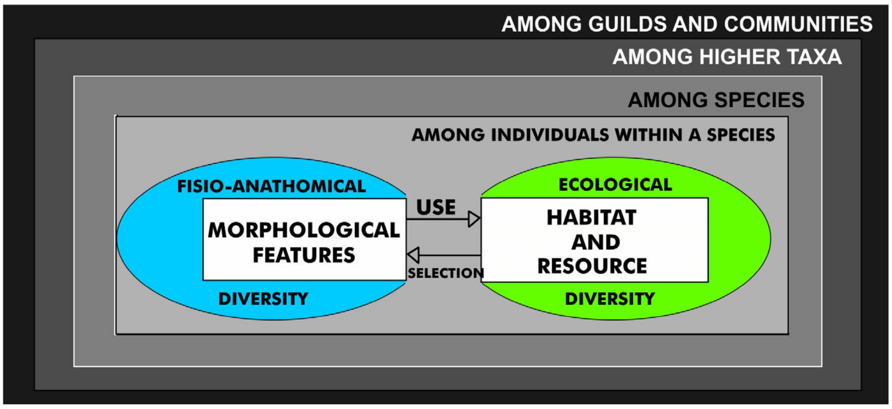
Matthews (1998) claims that three different approaches exist in linking the morphology of fishes to their ecology: (1) descriptive morphology, (2) functional morphology, and (3) "ecomorphology".

Descriptive morphology proceeds in an inductive way, starting from the morphological description of body shape, or of only one particular feature such as the mouth. By comparing these descriptions with ecological observations collected in the field or in the laboratory, this approach leads to an understanding of the role played by the organism or structure. In this approach, the scientist's most important tool is its intuition.

Functional morphology follows the same line of reasoning, but includes tests or quantitative measurements of the ability of the structures to perform in particular ways. This approach is based on the availability of appropriate technologies and instruments (see Chapter 1 of the present thesis).

"Ecomorphology", on the other hand, is characterized by a ***holistic*** approach. It is based on the observation of a large number of morphological features, that are often analysed by means of multivariate statistics, in order to evidence and describe relationships between multiple morphological features and specific ecological features.

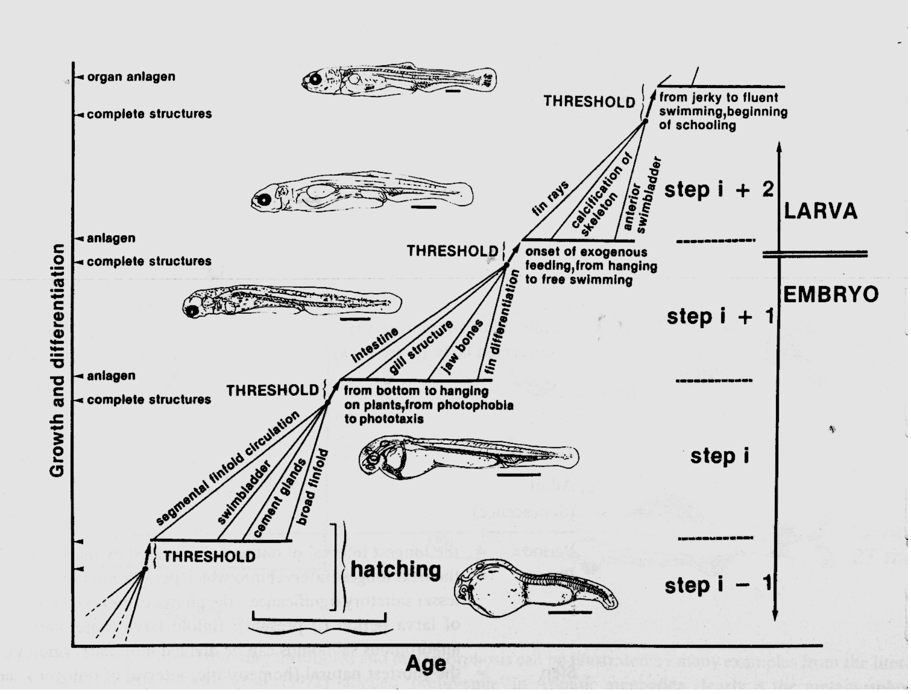
The ultimate goal of ecomorphology is to determine and quantify the relationship between variation in form (or only shape) and variation in resource use and environment selection among the organisms at a defined scale of diversity (Motta *et al.*, 1995a; Leisler & Winkler, 1985). This definition is very important because there is often confusion between ecomorphology and "functional morphology", the latter being more "adaptation" oriented and the former more "ecologically" oriented (Liem, 1991). Despite difficulties in setting up of experimental protocols, functional morphological studies play an important role in *a priori* identifying which morphological variables are likely to influence a particular ecological variable and *a posteriori* by providing a plausible mechanism for an observed ecomorphological correlation. Thus, functional morphology can be defined as a tool for ecomorphological investigations that can be performed at multiple levels: among individuals within a species, among species or higher taxa, and among guilds and communities.



Morphological features are related to patterns of resource use through the performance of important tasks. Habitat and resources mould the form (via selection and evolution) by establishing which tasks are important in increasing fitness, selecting phenotypes which show higher fitness.

Two types of constrains underlie the bi-directional relationship between morphological features and habitat selection/resource use: 1) morphological diversity (i.e. phenotype diversity) lead to functional and performance differences in environment and resources use by individuals, resulting in differences of fitness; 2) ecological factors influence further morphological changes both in evolutionary perspective, making a selection on morphological character and therefore changing gene frequencies in a population or through extinction/speciation of taxa, and over the life span of the organism through use-induced changes in morphological structures. Regarding the first aspect of the latter constrain, it was established that ecomorphology should become a framework for addressing adaptation, because morphological variation among individuals or among species can be *causally* linked to variation in resource use and, ultimately, in *fitness*. On the other hand, habitat selection and resource use by fishes can be influenced by a variety of factors operating simultaneously at different spatial scales, including abiotic and biotic factors. The niche realised by a species is delimited by its physical ability to use resources and morphology is one constraint on the use of resources (Matthews, 1998). Bolnick *et al.* (2003) claimed that there are three different ways to examine intra-population niche variation: (1) ecological sex dimorphism; (2) ontogenetic niche shift and (3) resource polymorphism. Considering the second, the majority of fish represent excellent case studies, because, during ontogenesis, fish undergo remarkable changes both of their fisio-anatomical features and of auto-ecological characteristics. A large number of fishes species actually display an indirect ontogenesis, in which the larval stage is therefore the transitory vegetative form, often inhabiting an entirely different niche than the definitive form, and equipped with numerous temporary organs and having a different body shape (Balon, 1985). In this way, the larval form requires a very complex series of anatomical changes to acquire adult juvenile characters. These changes (i.e. loss of temporary organs and development of new body structures) occur at different developmental rates (*heterochrony*), and this determines different relative growth of body structures (*allometry*), i.e. change of body shape.

Moreover, differences in morphology, use of resources, and survival are closely linked in this vertebrate group. It was consequently deemed of interest to investigate the correspondence between morphological and ecological features in fishes, which led to the development of important theoretical models of development, such as the Balon's theory of *saltatory ontogenesis* (Balon, 1985). In his classical studies, this author observed that structures that together form a system (organ) align their rates of development to become functional simultaneously and initiate a new vital function – e.g. photoresponse change of substratum, nutrition – at an accelerated rate. He called this the "switch" threshold, a rapid transition from one quality (homeostatic or steady state; more precisely Waddingtonian homeostasis) of the organs or organism-environment interaction to another.



from Balon (1985)

It follows that ontogeny is not a gradual phenomenon but a sequence of steady thresholds, through which the organism passes at accelerated rates in order to achieve the relative comfort of the next steady state. The external morphology was also observed to be subjected to developmental jumps. Sagnes *et al.* (1997) and Simonović *et al.* (1999) found developmental jumps in some biometric measurements during ontogenesis of two fish species. Osse & van den Boogart (1995) observed and reported a sequence of changes in allometry during the morphological development of some fish species. These authors found that patterns of development and growth are reflections of successive functional priorities at different sizes. Studies dealing with evolution generally focus on the adaptive nature of structural or genetic specialisations in adult organisms.

One fundamental question in contemporary biology is how to 'rejoin' evolution and development. Balon (1985) argued that evolution is a sequence of ontogenesis. In fact, selection operates at every stage of the life history of an organism, and afterwards, survival (or fitness) at an advanced stage depends on the survival of the previous stages. It follows that the adaptation of organisms must be the study of the adaptations along its ontogenesis. Besides, the dynamic processes underlying at the base of ontogenesis (*heterochrony* and *allometry*) are important features on which selection acts by producing evolutionary innovations. Some famous evolutionary "jumps" can be partially explained in terms of a re-arrangement of the developmental sequence, as postulated from the theory of punctuated-equilibria (Gould & Eldredge, 1977). Thus, to study evolution, ontogeny should be one of the crucial components under investigated because it lies at the basis of the pattern of evolution (Adriaens & Verraes, 2002). This path leads directly to the Evolutionary-Developmental (*Evo-Devo*) approach to the study of evolution. Evo-Devo reflects the long search to find and understand the relationships between the transformation of an organism within a single generation – development, ontogeny, ontogenetic change – and those transformations that occur between generations – evolution, phylogeny, phylogenetic change (Gould & Eldredge, 1977). This discipline is concerned, among other things, with discovering and understanding the role played by changes in developmental mechanisms in the evolutionary origin of aspects of the phenotype. In a very real sense, Evo-Devo opens the black box lying between genotype and phenotype, or more correctly, phenotypes as multiple life history stages arise a single genotype in many organisms from. This encourages a characterization of evolutionary developmental biology in the form of a marriage of evolutionary theory and embryology via developmental genetics. But there remains a largely untold story about the significance of morphology and comparative anatomy (also minimized in the Modern Synthesis). Functional and evolutionary morphology are critical for understanding the development of a concept that is central to evolutionary developmental biology, *evolutionary innovation*. Highlighting the discipline of morphology and the concepts of innovation and novelty provides an alternative way of conceptualizing the 'evo' and the 'devo' to be synthesized. Ecomorphology, due to this holistic approach to the study of phenotype/environment interactions, provide a key framework for this investigation. Nevertheless, these researches need series of samples which are complete and numerically exhaustive of all the developmental scale of ontogenesis. It is almost impossible to collect samples series in the field whilst the experimental context related to ecological aquaculture can provide suitable material. It should be stated that artificial rearing conditions can produce fish distinctly different from wild cohorts in behaviour, morphology and physiology. In fact, hatchery methodologies can impose different selective and adaptative pressures on fish, inadvertently determining selection for specimens adaptable to high densities and feeding levels, or in general to rearing in captive conditions. As the adaptability and diversity of wild fish have been shaped by interactions between a complex natural environment and natural selective forces, so are the characteristics of hatchery fish have been modified by their rearing environment and genetic changes imposed during the culture phase. Classical rearing practices purposely reduce individual variability: the genetic consequences of inbreeding, outbreeding and domestication selection are well documented for domestic livestock and other terrestrial organisms. Within an hatchery population this may be desirable but, all the same time, it prevent elaboration of model and abstraction from the observations in captive conditions. In this way, a rearing approach capable of producing stocks of fishes in meaningful numbers, but with several characters that allowed us to defined them as "wild-like", should be selected. A rearing approach of this type will operate on the concept that high quality fish, behaviourally and physiologically similar to wild cohorts, can be produced in conditions simulating the natural life history of each particular species under culture. The semi-intensive rearing technology of large volumes now represents the best practice to pursue this aim. Set up in the last two decades from empirical experiences collected in productive contexts (Cataudella *et al.*, 1988), under the boost of new concepts in responsible production and resource management (***FAO: Code of Conduct for a Responsible Fisheries and Aquaculture***), this technology was successfully tested as powerful in order to produce wild-like fry that shows morphological (Loy *et al.*, 1999), behavioural (Malavasi *et al.*, 2004), anatomical (Boglione *et al.*,1994; 2001) and physiological characters close to those of wild ones (AA.VV., 2001; Cataudella *et al.*, 2003). This result was obtained following several criteria:

- The use of locally adapted broodstock to maintain genetic diversity of wild populations in the reared cohorts, which always represent an F1 generation;

- The use of incubation and rearing vessels with options for habitat complexity to produce more natural environments in hatchery;
- The addition to rearing tanks of a particular aeration system that produces differential hydrodynamic regimes, thus determining differential hydrodynamic environment into tanks;
- The increase of foraging ability of young fish and improving the quality of their diet by adding natural live foods that were in general collected from an external natural source (i.e. a costal lagoon);
- The reduction of rearing density and augmentation of the rearing space to more natural distributions;
- The absence of drugs during all the productive cycle.

All these features globally provide more complex and wild-like habitats which approximate natural conditions in captivity, determining selective mortalities in rearing stocks and the recovery of wild-like characters. As consequence, fish reared with this technique have been proposed, i.e., for experimental trials aimed at identifying reliable indicators of animal welfare in marine fish since early stages of development (Cataudella *et al.,* 2003). Therefore, the samples used in this thesis were reared by means of this technique, in the framework of several productive experiences performed to supply some Coordinate Research Programs granted by ***Italian Ministry of Agricultural and Forestrer Politics***.

In this framework, in view of the need to obtain exhaustive data about the morphology of an organisms, it could be observed that classical studies have been based on a qualitative description of the development of much apparatus for and/or many survey of anatomical measurements (Motta *et al.,* 1995b; Luczkovich *et al.,* 1995; Wainwright & Barton, 1995). Meanwhile, the classic morphometric approach often resulted in a subjective and partial analysis that did not provide an effective and realistic overall representation of the form of the organisms. In fact, although selected morphological variables have known consequences, for example, on feeding performance, we cannot accurately determine and quantify the relative importance of inspected variables with respect to the overall complex of morphological adaptations. This leads us to the use of geometric morphometrics tools, that provide not only a realistic and complete representation of organisms (Kawamura & Hosoya, 1997) but also allow a quantitative description to be made of shape as well as statistical comparisons among different form (that is phenotype). This was the starting idea of the present thesis.

AIMS OF THE PRESENT THESIS

This research focused on the study of patterns of changes of shape and trophic habits during the development of two marine fish species: the dusky grouper (*Epinephelus marginatus*) and the sea bream (*Sparus aurata*). The aims were:

- To improve the knowledge of the trophic ecology of larval stages of two fish species that are very important for aquaculture;
- To set up methods for verify the feasibility of shape analysis tools to survey shape changes during ontogenesis of fishes, with special emphasis on new applications at early life stages;
- To match data sets of food item selection and shape changes in order to investigate any correspondences between them via an ecomorphological approach;
- To use SOMs, which are a kind of artificial neural network, to analyse, visualize and interpret the patterns of shape and trophic

habits changes, providing a new approach to ecomorphology investigations.

2. Feeding preferences and mouth gape limits of dusky grouper (*Epinephelus marginatus*, Lowe 1834) larvae reared in semi-intensive conditions using harvested and wild live food

INTRODUCTION

Domesticating fishes is often problematic because each species has developed specific adaptations of early larval stages (Brown *et al.,* 1997; Sánchez-Velasco, 1998; Shaw *et al.,* 2003), including feeding strategies, and our knowledge of larval ecology could be insufficient to perform a productive approach. In this view, the preliminary observation of feeding behaviour, in field or in experimental conditions, represents an important step to improve the understanding of trophic necessities and, in general, of eco-morphological adaptations of larvae. Actually, experiments are required which combine behavioural and performance data (i.e. gut contents, growth and survival) in developping protocols and culture technology (Brown *et al.,* 1997; Irwin *et al.,* 2002; Smith & Fuiman, 2004; Cox & Pankhurst, 2000). Inadequate food items determine lower growth rates with the consequence of high mortality already in the initial steps of rearing. Therefore, in larviculture conditions, the most important factors that must be taken into consideration to guarantee growth and survival are density, type and size of preys supplied (Puvanenedran & Brown, 1999; Planas & Cunha, 1999). In particular, the size of prey consumed by fish larvae is correlated to fish and mouth gape size (Krebs & Turingam, 2003; Shaw *et al.,* 2003). In this view, the relationship between mouth gape dimension (height and width) and prey dimension has been largely investigated (Planas & Cunha, 1999; Lemly & Dimmick, 1982; Luczkovich *et al.*, 1995). Surprisingly, any of this studies considers the prey as a three-dimensional object but, on the contrary, only the length and the width of the dorsal vision of the prey body are utilized.

The dusky grouper (*Epinephelus marginatus*, Lowe 1834) is a very important species, due to its relevance in sport and commercial fishery activities (Glamuzina *et al.*, 1998), its attractiveness for human consumption, but especially for the low standing of natural populations, as consequence of over-exploitation in most Mediterranean countries (Zabala *et al.*, 1997). Despite the high potential of this species to be used in aquaculture and restocking actions, investigations and rearing trials are still scarce (Glamuzina *et al.*, 2001), and only few studies have been published on reproduction and larviculture of *E. marginatus* (Glamuzina *et al.*, 1998a; Marino *et al.*, 1998). Considerable progress concerning reproduction has been made during last years, but larval rearing has stopped on preliminary trials, mainly due to the lack of data concerning feeding ecology of this species that limits remarkable results and survival of early-stage larvae. Previous studies on the dusky grouper reported low first feeding rates and high mortality of larvae (Glamuzina *et al.*, 1998; Spedicato *et al.*, 1995), particularly before or during the onset of feeding (Toledo *et al.*, 2002). In general, the first larval preys are the major problem in rearing all grouper species. (Glamuzina *et al.* (1998) reproduced artificially *E. marginatus* and described initial biometry of larvae (total length, gape of the mouth), time of mouth opening after fertilization, and they reported some problems occurring during the first feeding of this species. Doi *et al.* (1997), observed the foraging behaviour of dusky grouper larvae under different conditions of prey density and type, but their experiment stopped at day 10, the percentage of survival was reported only on day 6, and relationship between prey size and mouth size was not investigated. Moreover, these studies did not address neither the effect of a range of prey dimensions and type on larval growth and survival nor the feeding preferences of larvae during their development. As for many other species, exogenous feeding of *E. marginatus* larvae is limited by the size of the mouth, and many authors focused on the small dimension of newly hatched dusky grouper larvae: they seem to be the smallest of all the groupers investigated worldwide and of the other cultivated fishes in Mediterranean area (Glamuzina *et al.*, 1998). Thus, although the foraging behaviour hasn't been sufficiently studied, several authors suggest that gape limitation for live food greatly affects the survival of larvae.

The present work reports observations of feeding ecology of dusky grouper larvae reared in semi-intensive conditions and fed with harvested food items (rotifers and *Artemia*) wild zooplankton. Besides, it examines the relationship between the dimension of preys, defined as 3-D objects, and of fish. The aim of this study was to improve the knowledge of the larval feeding ecology of this species, investigating both changes of prey preference during ontogenesis and the effect of mouth gape size on prey selection.

MATERIALS AND METHODS

Experimental setup

Dusky grouper eggs from an artificial spawning, performed in a Mediterranean hatchery, using raised broodstock, were transferred to the hatchery of "SMEG" in Latina (Italy), near Rome. The age of larvae, measured in days post hatching (d.p.h.), was used as time indicator during the experiment and thoroughout the following descriptions. At the age of 3 d.p.h. yolksac larvae were stocked in 3 circular semi-outdoor tanks (about 60 m^3 of volume, diameter 8 m, water height 1.2 m), at the initial density of 6-7 larvae $\cdot l^{-1}$. Tanks were filled with sea water and several unicellular green algae species (*Clorella minutissima*, *Isochrisis galbiana*, *Nannochloropsis suecica*) were introduced into water tanks 2 days before fish larvae, in order to feed zooplankton and to improve environmental quality. Water temperature was maintained constant at 25°C and salinity at 35 during the experiment, so that no major differences between tanks were recorded. Tanks water was continuously changed from 5 d.p.h.: the flow rate equalled a daily change rate of approximately 20% until 17 d.p.h., after which it was increased gradually to 100% (final value at 35 d.p.h.). The experimental rearing was performed until larvae were 35 d.p.h. old, with the following experimental feeding regimes: (1) only wild zooplankton (W-tank); (2) wild zooplankton and enriched harvested live food (rotifers + *Artemia*) (HW-tank); (3) only enriched harvested live food (H-tank). For the HW regime, rotifers were supplied until 28 d.p.h. and then replaced with *Artemia* until the end of the experiment. For HW and H treatments, harvested live food was introduced into tanks in 2 daily supplies: the first at 8:00 and the second at 14:00. Both L-type rotifers, *Brachionus plicatilis*, and *Artemia* nauplii (AF cysts from *Artemia* Systems, Belgium) were enriched for 20 hours in a suspension of 0.3 gl^{-1} of Super Selco, in filtered seawater. For W and HW treatments, wild zooplankton was daily collected from coastal lake Fogliano, and the fraction separated between 50 and 300-µm mesh sizes was used. This zooplankton was dominated by nauplii, juvenile and adult stages of the harpacticoid copepod *Tisbe holoturiae*. Wild food was added daily at 8:00, at the same time of the first introduction of harvested live food. The food density in each tank was monitored at 8:00 in the morning and at 14:00 in the afternoon. Rearing tank HW and W were characterized also by autarchic internal production wild zooplankton. In this way, tanks HW and W represented a semi-intensive approach for rearing of larvae (van der Meeren & Naas, 1997).

Live food items

Planktonic food organisms in 50 litres of tanks water and zooplanktonic organisms siphon-sucked along a fixed area (15×10^{-2} m^2) of the tanks internal surface, filtered with a 45 µm net mesh, were preserved in 5% phosphate-buffered formalin solution. Specific composition and species abundance of these samples were examined. Each copepod species was counted and the specimens categorized into three groups: nauplii, copepodites (juvenile stages) and adults. At least 100 individuals from each zooplankton species/category were photographed and sizes (total length, width and height excluding spines and caudal rami) were measured as forward described for fish larvae.

Fish larvae

For each treatment, 20 specimens of dusky grouper were initially collected each day (at 3, 4, and 5 d.p.h.), and after every 3 days, always at 9:30 in the morning, 1.5 hour after the introduction of food. Larvae were photographed using a Polaroid digital camera connected to a Microscope. Survival of larvae was back-calculated from the daily removal of dead larvae, the number of sampled larvae during the experiment, and the number of survivors at day 35 d.p.h.. Standard Length (S_L), Total Length (T_L), Premaxillar Length (P_L) and Mouth Width (M_W) (Fig 1) of larvae were collected from images using a linear measurement morphometric software (TPSdig; Rolhf, 2001).

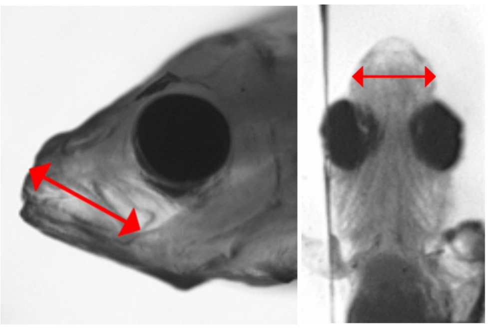


Fig. 1. Premaxillar length ($\boldsymbol{P}_{\mathrm{L}}$) and mouth width ($\boldsymbol{M}_{\mathrm{W}}$) as measured by TPSdig.

Mouth height ($\boldsymbol{M}_{\mathrm{H}}$) was calculated from $\boldsymbol{P}_{\mathrm{L}}$ as described in Marino *et al.*, 2001 (modified from Shirota, 1970). Mouth height and width were regressed on $\boldsymbol{T}_{\mathrm{L}}$ in order to examine the relative growth of mouth gape. Slopes were tested for difference from zero using F-test.

Each fish specimen was also examined for feeding incidence (frequency of larvae with gut content) and composition of food organism in the gut. Feeding selectivity of larvae toward each species/category was estimated by α electivity index proposed by Chesson (1983). The relationship was defined as:

$$\alpha = \frac{r_i / n_i}{\sum_{j=1}^{m} r_j / n_j}$$

where r_i is the percentage of a particular prey i in the larval gut in a mixture of m prey types, and p_i is the percentage of the same prey in the environment. The index may vary from 0 to 1, positive selection toward prey i is indicated when α values are greater than $1/m$, while values less than $1/m$ imply that prey i is avoided because it is used in lower proportion than its availability in the environment.

The length, width and depth (excluding spines and caudal rami) of the largest prey item in each gut was photographed and measured as described above. The relationship between prey size and mouth size of larvae was analyzed as described in Bremigan & Stein (1994): the smallest dimension of prey, that is width (**W_{LP}**) or depth (**D_{LP}**), was chosen as the discriminant parameter for ingestion, and was regressed on M_H, M_W, and T_L of larvae to examine the relationship between fish body size and prey size consumed. Slopes were tested for difference from zero using F-test.

RESULTS

Live food items

Harvested food (*B. plicatilis* and *A. salina* nauplii) was the main resource for dusky grouper larvae of treatment H and HW, while wild food represented the only available resource in W treatment (Fig 2). Rotifers availability ranged between 1-2.5 individuals ml^{-1} in tank HW and H, because this prey was given and maintained in order to ensure larvae requirements. For the same reason, rotifers abundance in water tanks was gradually increased thorough the experiment. *A. salina* nauplii, that replaced rotifers after 27 d.p.h., were progressively introduced in growing quantities (0.20-0.5 individuals ml^{-1}).

Total abundance of major wild taxa was reported, where each copepods species was separated in three life stages: nauplii, juveniles and adults. These stages are very different in size and, consequently, they probably represent different categories of prey for larvae. Among wild food, the harpacticoid copepod *T. holoturiae* always represented the most abundant taxon. Its juveniles and adults dominated the wild part of live food availability for most part of the time on W and HW treatment. The *T. holoturiae* (juveniles +adults) concentration ranged around 100-1000 indiduals/l^{-1} in tank W and around 100-700 in tank HW. The other wild taxa such as *Ameira clausi* and *Ameira parvula*, globally considered, never exceeded 500 indiduals/l^{-1}. Globally, the availability of live food, in all treatments, was relatively constant during the experiment. Besides, the contemporary presence of many taxa (with their relative life stages) in comparable concentrations determined a condition of polyspecific choice, giving the opportunity for studying trophic selection operated from dusky grouper larvae during growth.

Examining the smallest dimension of each species/categories (Tab 1), *B. plicatilis* appeared to be the smallest item (mean body depth=0.09±0.02 mm), followed by copepod nauplii, oyster larvae, *P. brevirostris* juveniles and adults, *A salina* nauplii, *T. holoturiae* juveniles and, finally, *T. holoturiae* adults. Body depth represented, in almost all cases, the smallest of three dimension: the only exception was represented by juveniles of *A. clausi*. Differences between depth and width were not negligible, because they ranged from 5 to 45%.

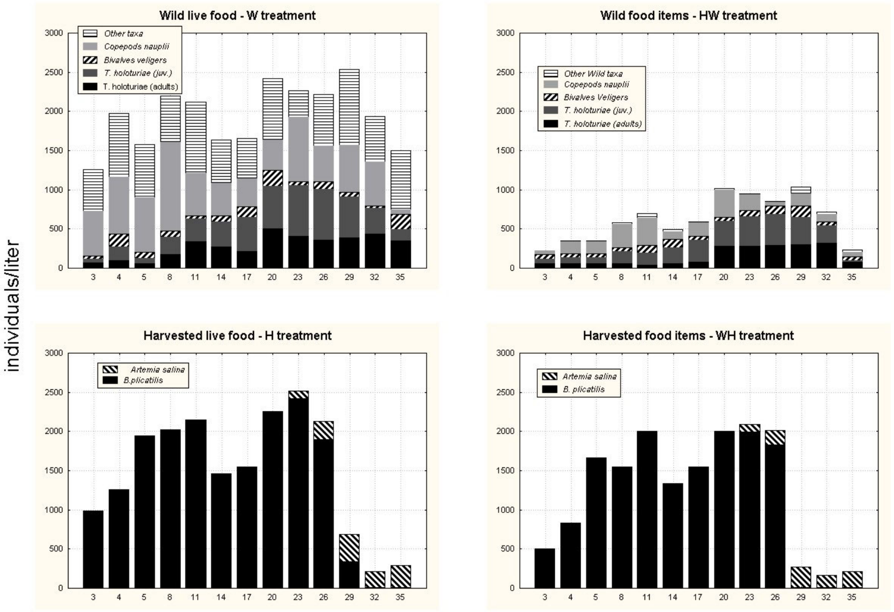


Fig 2. Abundance (individuals l^{-1}) of food organisms in tanks water.

	Body length (mm)	Body Width (mm)	Body depht (mm)	Biovolume (mm³ × 1000)
B. plicatilis	0.017±0.003	0.013±0.013	**0.009±0.002**	0.002±0.002
Copepod nauplii	0.023±0.003	0.014±0.002	**0.013±0.002**	0.004±0.001
Veligers of Bivalves	0.015±0.002	0.018±0.013	**0.014±0. 002**	0.004±0.003
P. brevirostris juv.	0.045±0.001	0.013±0	**0.012±0**	0.007±0
P. brevirostris	0.055±0.004	0.017±0.001	**0.015±0.005**	0.014±0.005
A. salina nauplii	0.07±0.006	0.02±0.002	**0.015±0.001**	0.022±0.006
T. hol juv.	0.066±0.006	0.021±0.003	**0.018±0.005**	0.025±0.015
A. clausi juv.	0.074±0.008	**0.021±0.002**	0.022±0.005	0.035±0.012
T. holoturiae	0.084±0.012	0.027±0.004	**0.024±0.008**	0.055±0.018

Tab 1. Mean biometric measures for each wild food species/category.

Survival and growth of larvae

Estimated percent survival of larvae at day 35 were 7% for treatment H, 2.5% for W and 17.5% for WH, respectively (Fig 3). The number of survivors in tank H initially decreased to 5 d.p.h., remaining constant until 7 d.p.h., and after starting to rapidly decrease to 25 d.p.h, when mortality rate was lower.

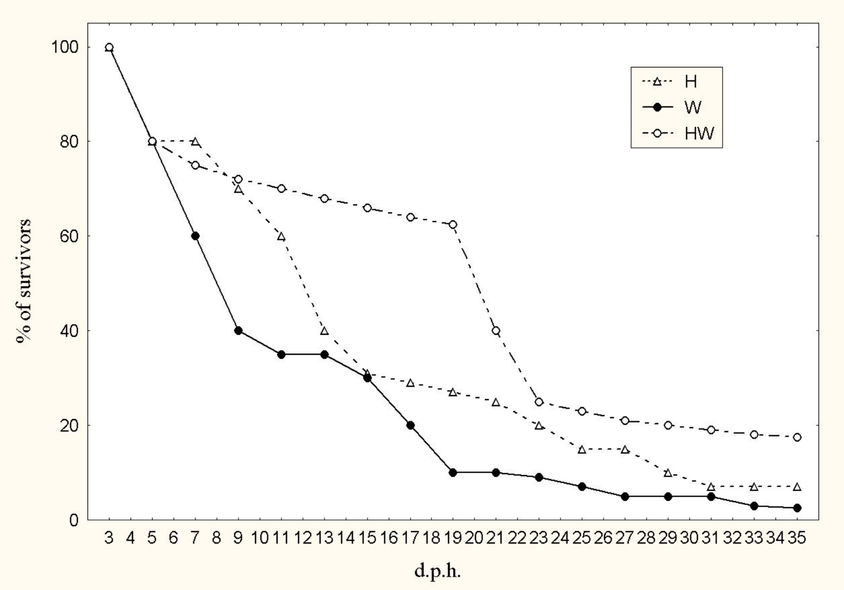


Fig 3. Trends of survivorship in the three rearing conditions.

In tank W mortality was continuous until 9 d.p.h., after which slowed down to 15 d.p.h. Another increase of mortality rate was recorded between 15 and 19 d.p.h., and then the number of survivors remained constant until the end of the experiment. After an initial decrease between days 3 and 5, survivorship of HW tank remained almost constant to day 20, after which mortality increased. Decrease stopped at 23 d.p.h., and any other mortality event was recorded until the end of the experiment.

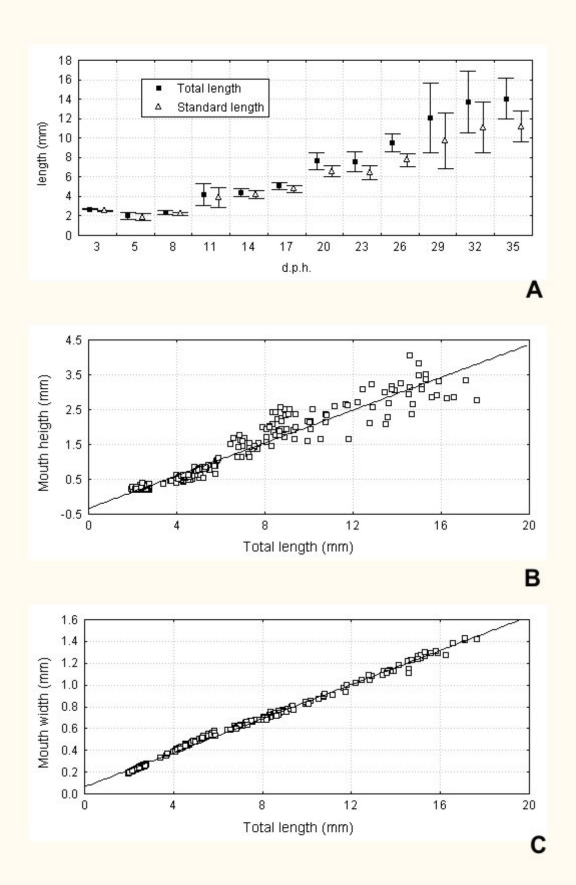


Fig 4. Larval growth (T_L versus d.p.h.) and relationships among T_L and B_H, M_H, M_W.

The larvae grew from an initial average T_L 2.68 ± 0.05 mm at release on tanks, to 11.05 ± 3.05 (H), 8.68 ± 3.52 (W) and 10.93 ± 2.28 mm (HW) at 35 d.p.h. (Fig. 4A). The mouth of all the larvae was completely functional after 96 h. The initial $\boldsymbol{M}_{\mathrm{H}}$ and $\boldsymbol{M}_{\mathrm{W}}$ were 214 ± 200 µm and 259 ± 6 µm, respectively. The relationships among $\boldsymbol{T}_{\mathrm{L}}$ and, respectively, $\boldsymbol{M}_{\mathrm{H}}$ (Fig. 4B) and $\boldsymbol{M}_{\mathrm{W}}$ (Fig. 4C) were found to be highly significant and were described by the following equations:

$$\boldsymbol{M}_{\mathrm{H}} = 0.2355 \cdot \boldsymbol{T}_{\mathrm{L}} - 0.3448 \qquad (r^2=0.92,\ p<0.001)$$

$$\boldsymbol{M}_{\mathrm{W}} = 0.0784 \cdot \boldsymbol{T}_{\mathrm{L}} + 0.0617 \qquad (r^2=0.99,\ p<0.001)$$

Trophic behavior of fish larvae

The trends of feeding incidence (f.i.) were reported in Fig 5. At 3 d.p.h. about 5 % of larvae had food items in the gut in all the three rearing conditions; these increased to 75 % (tank W), 77% (tank H) and 100 (HW) at 8 d.p.h. W and H still increased, both reaching 90% at 11 d.p.h. Larvae of treatment HW maintained 100 % until the end of the experiment, whereas those of treatment H showed a more discontinuous trend, resulting in lower mean value of f.i.. Finally, larvae of thank W, as well as showing the lowest values of f.i. (about 90%), were characterized by a strong decrease of f.i. that, from 29 to 35 d.p.h., reached the 50%.

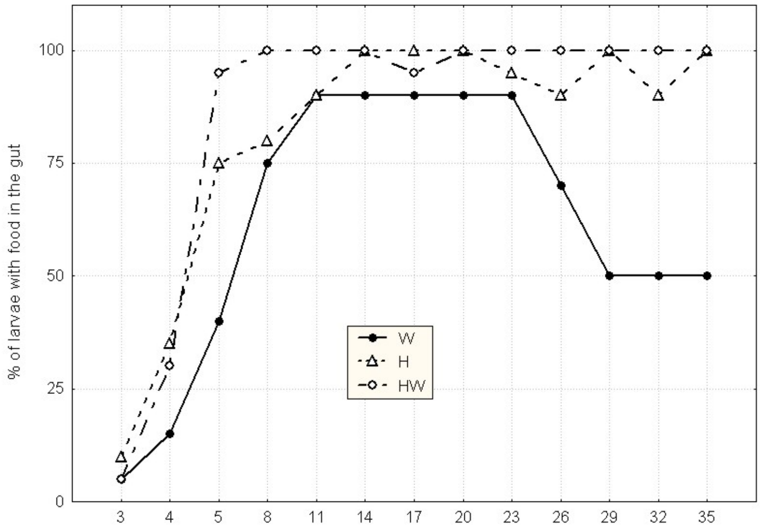


Fig 5. Feeding incidence observed in the three rearing conditions.

Larvae started to feed only on rotifers in all the three rearing conditions, but already at 5 d.p.h. those of HW and W fed also on copepod nauplii and veligers of bivalves and, at 8-11 d.p.h on copepod juveniles. Adults of copepod species (mainly *T. holoturiae*) entered the diet when larvae were 17 days old. Fig 6 reported the values of Chesson's α calculated on data of treatment HW. Chesson's index for veligers of bivalves, which were the smallest item in size among the live food available, resulted the highest (0.6-0.4) along the period from 8 to 17 d.p.h. (from 2.4 to 5.1 mm in T_L of larvae). This food item was present also at day 29 and 35, with α value smaller but bigger than $1/m$.

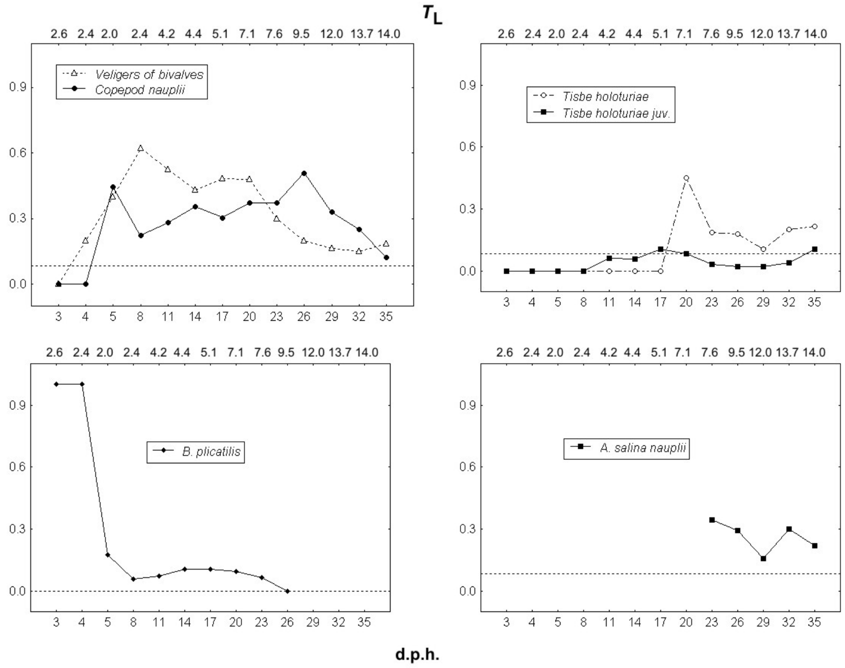


Fig 6. Chesson' α for main food items during experimental rearing (HW treatment).

This suggests that dusky grouper larvae preferred this prey type during all the experiment but particularly at the beginning of feeding. In the same way, Chesson' α for copepod nauplii showed high values from day 5 to day 29 (from 2.0 to 12.0 mm in T_L of larvae). The highest values were observed from day to day 29 but, in general, this prey was strongly selected by dusky grouper larvae in all the experimental rearing. It is interesting to underline that, from 8 d.p.h., Chesson's α for oyster larvae decreased, while for copepod nauplii it increased. Index value for juveniles of the main copepod species, *T. holoturiae*, was highest at day 17, but it remained close to *1/m* during the experiment, hence dusky grouper larvae showed a not selective behavior toward this item. The adult of the same species showed high values of the index from day 20 to day 26 (from 7.1 to 9.5 mm in T_L of larvae), and positive selection until the end of the experiment. Chesson's α for *B. plicatilis* showed that larvae exhibited a strong preference toward this item at the start of feeding, but this rapidly decreased and, from 8 to 26 days post hatching only weak preference characterized the behaviour for this prey. Thus, when *A. salina* nauplii were introduced in the tank, larvae actively selected this prey, and lessening of preference toward rotifers and copepods was observed. Standing the biometries reported in Tab. 1, we selected the minimum dimension of the largest prey ($\boldsymbol{M}_{\mathbf{LP}}$) detected in the gut of each larvae, and this value was plotted against $\boldsymbol{T}_{\mathbf{L}}$ of the correspondent specimens (Fig 7A). It seemed that there was an increase of prey size with fish growth until $\boldsymbol{T}_{\mathbf{L}}$ reached about 7.5 mm ($\boldsymbol{F}_{\mathbf{TL}}$), and after a steady state is reached. Fig 7B showed the plot of $\boldsymbol{M}_{\mathbf{W}}$ and $\boldsymbol{M}_{\mathbf{H}}$ against $\boldsymbol{T}_{\mathbf{L}}$, in which $\boldsymbol{F}_{\mathbf{TL}}$ was reported and correlated to the two correspondent values of $\boldsymbol{F}_{\mathbf{MW}}$ and $\boldsymbol{F}_{\mathbf{MH}}$. The slope of relationship between $\boldsymbol{M}_{\mathbf{W}}$ and $\boldsymbol{T}_{\mathbf{L}}$ was lower, and $\boldsymbol{M}_{\mathbf{W}}$ was ever the smallest of the mouth gape. Fig 7C showed the plotting of $\boldsymbol{M}_{\mathbf{LP}}$ against $\boldsymbol{M}_{\mathbf{W}}$, in which was reported the $\boldsymbol{F}_{\mathbf{MW}}$ values as graphically calculated from Fig 7 B. It seemed that the flexus in the relathionship between prey size and mouth gape corresponded, and the slope of the increasing phase was about 0.5.

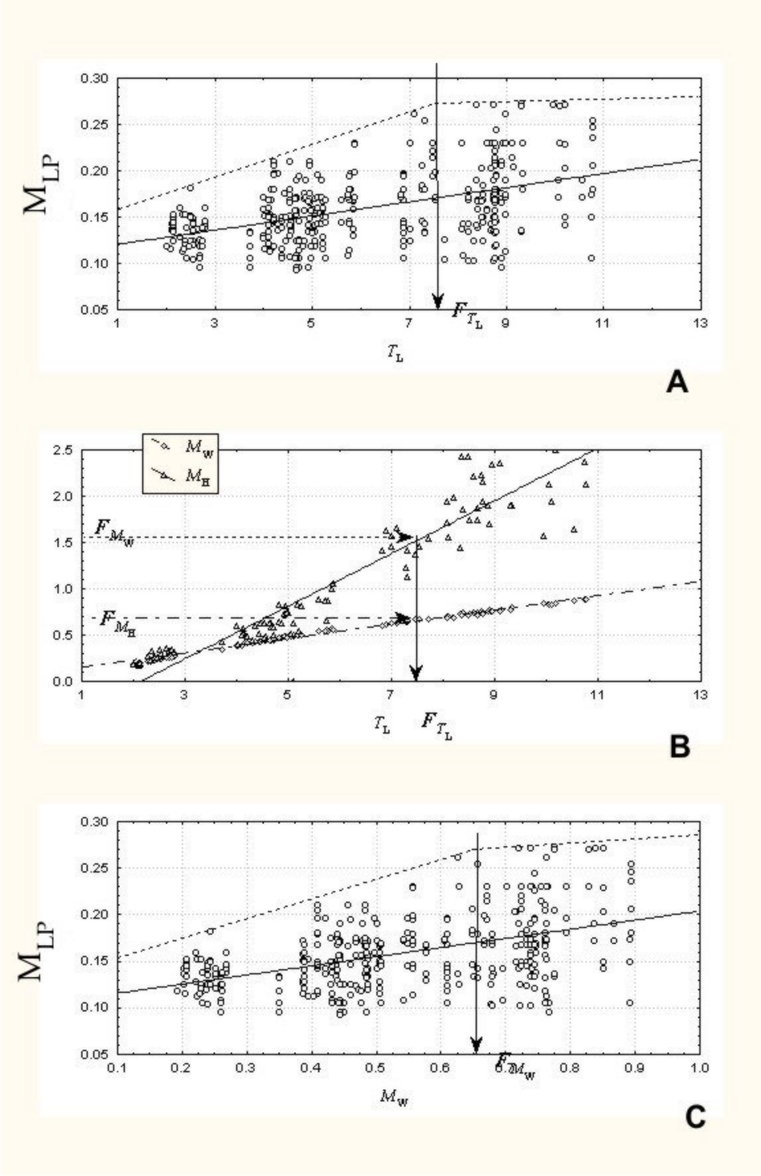


Fig 7. Relation between mouth size (height and width) and prey size during growth.

DISCUSSION

Food availability, feeding and survivorship of larvae

The setting-up of tanks resulted in three rearing conditions that differed for food availability, as defined in terms of composition, but not in terms of global quantitative availability of food. Treatment H resulted in a classic approach in the larviculture of marine species as sea bream and sea bass (Planas & Cunha, 1999), whereas W treatment simulated the natural conditions, using a mesocosms systems (that is "subunits of the natural environment" as reported in van der Meeren & Naas (1997)). This approach was been historically applied to the rearing of marine fish species (Cataudella *et al.*, 1988). Instead, HW treatment was an alternative way, joining the two extremes represented by H and W, developed both in order to enhance rearing performance and to investigate food habits of dusky grouper larvae in conditions of poly-specific choice. Other experiences of rearing in mesocosms with mixed diet suggested that this approach is indicated for domestication of species new candidate for aquaculture (van der Meeren & Jørstad, 2001) and that it is helpful in investigating larval ecology. Therefore, it was already detected as a powerful mean in order to obtain higher quality of fry production (Boglione *et al.*, 2001).

The success of rearing was pointed out by the high survival of larvae by treatment HW (17.5 % at 35 days post hatching) that represents the best result for this species. Precedent rearing trials of dusky grouper failed after not more than ten days, principally because inadequate preys were offered to larvae. Considering both the trends of survivors (Fig 3) and the *f.i.* (Fig 5) during experimental rearing, it could be suggested that there was, both in H and W treatments, a period of mortality due to the shortage of idoneous preys. This period started from 8 d.p.h. in H, whereas started from the beginning of the experiment in W. Another critical phase for feeding of larvae was detected, for W treatment, after 23 d.p.h.. Instead, on tank HW it was observed a period of mortality from 20 to 26 d.p.h. which was not directly associated with lowering of appropriate food. With respect to observed *f.i.*, HW treatment resulted in a more exhaustive, appropriate approach, whereas H and W gave not ever good food choice during experimental rearing.

The feeding preference and the role of mouth gape during ontogenesis

During the first days of exogenous feeding, fish larvae increase swimming activity and improve their feeding ability progressively (Cox & Pankhurst, 2000; Parra & Yufera, 2000; Shaw *et al.*, 2003). As in many other species (Gerking, 1994), dusky grouper larvae exercise an unusual degree of discrimination in selecting the organisms they capture for food, and until all capabilities in preying of the larva are entirely developed, the feeding efficiency is affected by different factors such as evasiveness of prey, previous experience of the larva, density of food and mouth dimension. The effect of mouth gape size was identified as critical in limiting preys size of fish larvae, and therefore it represented one of the major features that determine feeding habits of dusky grouper larvae (Planas & Cunha, 1999; Lemly & Dimmick, 1982; Luczkovich *et al.*, 1995; Krebs & Turingam, 2003; Shaw *et al.*, 2003). Some solutions have been proposed in order to avoid this problem in larviculture conditions: the use small rotifers (with a lorica width lesser than 90 µm, see Duray *et al.*, 1997), or the use of trochophores as first food either alone or in combination with small rotifers (Chen, 1990). Doi *et al.* (1997) affirmed that red-spotted grouper *E. coioides* showed higher survival and growth rates when they are reared with a diet based on mixed rotifers and copepod nauplii. Moreover, they evidenced that starting feeding larvae of this species preferred to feed on medium- and large-size nauplii of copepod, rather than on rotifers, as they grow. Finally, some authors underlined that survival of grouper larvae was improved by the use of large tanks (Duray *et al.*, 1997) and, in general, by the use of a semi-intensive approach. Analyzing results of food selection of larvae of tank HW, it was showed as the higher Chesson’ αvalues were obtained toward copepod nauplii from 5 to 29 d.p.h., and toward oyster larvae when they was available in the tank. These observations are consistent with those reported by Doi *et al.* (1997) about *E. coioides* and those suggested by Chen (1990) and Watanabe *et al.* (1996). Doi *et al.*, 1997 suggested that at the onset of feeding of *E. coioides*, the feeding success rate was higher for copepod nauplii than for rotifers. Instead in our experiment we found that *E. marginatus* larvae successfully fed on *B. plicatilis*, that was an idoneous prey at the start of feeding of this species as showed by *f.i.* values at 3-5 d.p.h.. Only a few selection was observed toward *B. plicatilis* except in days 3-5, and so, because large quantities of rotifers were available, the ingestion of this prey appeared to be determined only by the high degree of the encounter rate. Furthermore this observation was correspondent with Doi *et al.* (1997). Results for juveniles and adults of the main zooplankton species (*T. holoturiae*) showed that, while no selection was found toward juveniles, strong selection characterized adults from 20 d.p.h.. *T. holoturiae* is a zoobenthic copepod that colonized wall tank surface but they actively swim in water tank searching new patches. Laboratory experiments showed high fecundity rates for this species (Støttrup & McEvoy, 2003). His generation time is 7 days at 22°C, but food supply and salinity strongly influence development rates. Støttrup & McEvoy, (2003) reported that harpacticoids, particularly the genus *Tisbe*, are ideal candidates for cultivating in large cultures (due their short life cycle, ability to feed on a wide range of diets and to be cultured at high densities) and to provide food for marine fish larvae (i.e. when used as supplement to traditional feeds). In general, copepods species indigenous of coastal lagoon were detected as suitable food organism for fish rearing (Evjemo *et al.*, 2003; Shansudin *et al.*, 1997), and *T. holoturiae* was indicated on literature as a good integration for fish diet (Støttrup & Norsker, 1997). These authors also observed that , in cod larvae, first-feeding could be established solely on *Tisbe* nauplii, and that *Tisbe* can enhance feeding rates resulting in improved growth and survival if it are used in mixed diet (rotifers+*Tisbe*) compared to diet based only on rotifers and *Artemia* or only on *Tisbe*. Furthermore *A. salina* nauplii were actively selected when they were introduced in tank. These findings suggested that, at the beginning of feeding, copepod nauplii and oyster larvae are the most preferred food for dusky grouper larvae. Then, as they grew they seek for larger prey (as larger prey have higher energy contents), but preference toward some species/category remained unchangeable although other preys were added to the diet of larvae.

The size of the mouth gape, either measured as M_H or as M_W, was strong associated with the total length of each fish (Fig 4). This determined its progressive enlargement with the growth of the fish, but the two different value of slopes indicated that M_W grew slower, directly suggesting that this biometry was a limiting factor in prey ingestion. It was also evidenced that the maximum size of preys that were ate increase until mouth width is about 0.7 mm, and then it remained constant. Osse *et al.* (1997) suggested that, at this value of gape, fish larvae are capable to ingest the larger amount of natural available preys. Glamuzina *et al.* (1998) indicated an initial maximum gape of the open mouth, at the beginning of feeding, between 250 and 300 µm, while a range of 98-120 µm was estimated for the maximum functional mouth opening (based on the assumption that it represents only 40% of maximum mouth opening). In this study, it was detected that the rate between prey size and mouth size (M_W) was 50% while the initial mouth gape was about 250 µm. Many authors examined the role of mouth size during ontogenesis, and they assessed this structure set the upper size limit for prey, and particularly the mouth width that was closely correlated with the ability at capturing preys of different sizes (Hunter, 1981). Our results confirmed this hypothesis clearly indicating that (M_W) represented the limiting factor in ingesting preys. Otherwise also the size of preys was important in establishing which item could be eaten from fish larvae. Hunter (1981) and Hambright (1991) reported that the maximum width (or depth, if dorso-ventrally measured), including appendages, is the critical parameter determining selection in ingestion of copepods and all other oblong prey. This assumption is based on the fact that is possible to find in fish larvae gut some preys that are too large to be ingested if length was the critical factor. Also in this case, the results of this study confirmed that was the smallest dimension of preys that was critical in determining ingestion. Considering the food items as 3-D object, it was found that width and depth generally differed, and that depth was almost ever the smallest dimension. In this way, it could be assumed that preys were ingested so that their depth was opposed to M_W of fish. This conclusion are consistent with those of Hambringth (1991) for largemouth bass (*Micropterus salmoides*), of Blaxter (1965) for herring (*clupea harengus*), of Qin & Hillier (2000) for snapper (*Pagrus auratus*) and of Economou (1991) for five gadoids species.

Summing up, the present study evidenced that the larval rearing of dusky grouper can be realized with acceptable results in terms of fry production, but mouth gape limits (defined as mouth width) and feeding preferences of larvae must be strictly respected. In this way, rotifers can represent a food items only during first 6-8 d.p.h, however used in addition to veligers of bivalves and copepod nauplii, that are the better foods until 20 d.p.h.. After those, juveniles and adults of copepod, as genus *Tisbe*, and then *A. salina* nauplii could be supplied in order to efficiently fed larvae.

3. Larval shape development of dusky grouper, *Epinephelus marginatus* (Lowe, 1878): geometric morphometrics tools and elliptic Fourier analysis.

INTRODUCTION

The dusky grouper, *Epinephelus marginatus* (Lowe, 1834), a territorial and sedentary top-lever predator, is currently classified as a protected species in the Mediterranean, playing an important role in maintaining the ecological balance of hard-bottom ecosystems and other inshore environments of temperate areas (Morato *et al.*, 2000; Morris *et al.*, 2000; Heemstra & Randall, 2001; Sluka *et al.*, 2001; La Mesa *et al.*, 2002). Recently, some authors indicated this species as threatened (Bouchereau *et al.*, 1999) underlining the need for a wider knowledge before to take decision at management level. The range of *E. marginatus* extends from the English Channel to the Eastern Atlantic and Western Indian Ocean, throughout the Mediterranean Sea with highest abundances near African coast from Tunisia to Senegal (Bouchereau *et al.*, 1999; Froese & Pauly, 2003). Adults can reach a maximum size of 1.50 m TL (Heemstra & Randall, 1993) for a weight of 60 Kg (Göthel, 1992), are oval shaped with a robust and powerful body. Like other marine littoral fish species, dusky grouper has a life cycle that consists of a pelagic dispersal larval stage followed by sedentary benthic juveniles phase and, at least, adult are occasionally interested by a wandering activity (Bouchereau *et al.*, 1999; La Mesa *et al.*, 2002). In effect, as other centrarchid species, dusky grouper is well know as a model for the study important morphological changes during early development that affect its ecology and behaviour. These changes are followed by the acquisition of a different body shape, and the development of sensory structures (Boglione *et al.*, 1999).

Morphometric methodology are powerful tools in surveying and describing larval development in fishes (Kawamura & Hosoya, 1996). Although several studies examined biometry and growth of dusky grouper (Brusle & Prunus, 1980; Chauvet, 1988; Kara & Derbal, 1995; Bouchereau *et al.*, 1999) using traditional morphometry, these studies were limited to post-larval stages (>10 cm). Cavalcanti and collaborators (1999) analysed landmark based morphometry in selected species of serranid fishes (including *E. marginatus*), using only 5 specimen with standard length of between 68 to 190 mm.

In this paper we propose the use of geometric morphometrics (Bookstein, 1991) and elliptic Fourier analysis (Kuhl & Giardina, 1982) to study the shape changes of *E. marginatus* during larval development and the subsequent metamorphic transition to juveniles stage, from 2.5 mm to 42.0 mm in standard length. Larvae were obtained by inducing artificial spawning eggs from Marino and co-workers as described in Marino *et al.* (2001) and were reared using a semi-intensive aquaculture approach which has been shown to be effective in domesticating candidate species for aquaculture (Boglione *et al.*, 1994; Boglione *et al.*, 2001). Semi-intensive aquaculture also produces juveniles reminiscent of wild types.

Geometric morphometrics (Bookstein, 1991; Bookstein, 1996a;b; Rohlf & Marcus, 1993; Marcus *et al.*, 1996) is a landmark based technique currently considered to be the most rigorous morphometric technique. This is due to the viability of the statistical analyses the opportunity to observe the nature of shape differences through the visualization of splines. Landmarks are homologous points which bear information on the geometry of biological forms and which are the characters upon which geometric morphometrics are based. Elliptic Fourier analysis (Khul & Giardina, 1982;

Rohlf & Archie, 1984; Lestrel, 1997; Loy *et al.*, 2000; Loy *et al.*, 2001), is an alternative method to analyze shapes using few landmarks (Ferson *et al.*, 1985) based upon a series of coordinates along the body profile. Both methods allow the characterization of growth trajectories and the visualization of growth allometry (Alberch *et al.*, 1979; Klingenberg, 1996; Loy *et al.*, 1998a).

MATERIALS AND METHODS

Larvae of *Epinephelus marginatus* originated from artificial spawning eggs of brood stock raised in the Ittica Mediterranea hatchery (Sicily, Italy) using the protocols proposed by Marino *et al.* (2001). From 3 to 70 days, post hatching larvae were reared in a circular semi-outdoor 60 m3 tank (diameter 8 m, water height 1.2 m) using a semi-intensive approach in the hatchery of SMEG located in Latina (Rome, Italy). Mean water temperature was 25°C, salinity 35‰, density 6-7 larvae/l. Larvae were fed with a mixed diet composed of wild zooplankton (collected from an near coastal lagoon) and cultured live food (*Brachionus plicatilis* and *Artemia salina* nauplii). Experiment were started at 2 days post hatching (d.p.h.), and stopped at 70 d.p.h.. Final survival rate of larvae was 17,5 %.

A total number of 288 specimens were collected every 3 days from 3 to 35 d.p.h. and at the end of the experiment (70 d.p.h.). Individuals were preserved in buffered formaldehyde 4%. Images of each specimen were collected with a Polaroid digital microscope camera (24-bit true color images, 16 million colors, resolution 1600 × 1200). In vivo analysis of stomach contents were performed during the rearing.

Geometric morphometrics

For geometric morphometrics 3 different landmark configurations were collected using the software TpsDigit (Rohlf, 2001a). The first configuration, with 8 landmarks, is used for all the samples (288 individuals, 3-70 d.p.h.) (Fig 1A). After the 14th d.p.h. 6 other landmarks (n° 2, 4, 9, 12, 13, 14) can be added producing the second configuration of 14 landmarks; (Fig 1B). After the 20th d.p.h. it is possible to observe a total number of 18 landmarks (n° 3, 6, 7 and 8, Fig 1C). The increasing number of landmarks present with growth is due to the development of the anatomical structure The statistical analyses are conducted using groups with the same number of landmarks. Landmark configurations were aligned, translated, rotated and scaled to a unit centroid size (CS, square root of the sum of the squared distances from each landmark to the specimen's centroid; Tab 1) using the Generalized Procrustes Analysis (GPA; Rohlf & Slice, 1990; Bookstein, 1996a). Residuals from the fitting were analyzed with the thin-plate spline interpolating function (Bookstein, 1991).

		Biometry (cm)								CS					
Sample (day)	Number of specimens	TL		SL		MBH		ED		8 landmarks		14 landmarks		18 landmarks	
		Mean	St. dev.	Mean	St. dev.	Mean	St. dev.	Mean	St. dev.	Mean	St. dev.	Mean	St. dev.	Mean	St. dev.
3-5gg	44	0.25	0.03	0.23	0.03	0.05	0.01	0.02	0.00	0.22	0.02				
8gg	20	0.24	0.02	0.23	0.02	0.05	0.00	0.02	0.00	0.27	0.09				
11gg	15	0.44	0.04	0.41	0.03	0.08	0.01	0.04	0.00	0.38	0.04				
14gg	22	0.44	0.04	0.42	0.04	0.11	0.02	0.03	0.00	0.40	0.05	0.55	0.03		
17gg	26	0.52	0.04	0.49	0.04	0.14	0.01	0.04	0.00	0.48	0.09	0.57	0.12		
20-23gg	42	0.78	0.09	0.67	0.07	0.22	0.03	0.07	0.01	0.61	0.08	0.73	0.10	0.94	0.12
26-29gg	29	1.11	0.23	0.90	0.18	0.34	0.07	0.09	0.02	0.85	0.17	1.04	0.21	1.30	0.27
32-35gg	24	1.44	0.20	1.15	0.16	0.43	0.06	0.12	0.01	2.32	1.64	2.81	1.99	3.60	2.56
70gg	55	4.20	0.82	3.55	0.69	1.26	0.24	0.31	0.04	3.14	0.47	3.80	0.58	4.90	0.76

Tab 1. Number of specimens, biometries and Centroid Size values for each age classes

Shape changes during growth (allometry) were visualized as splines regressing the parameters of the function (W' matrix) on centroid size (software TpsRegrw; Rohlf, 2000). The correlation between shape variables and centroid size was tested with Permutation tests with 1000 random permutations (software tpsRegrw; Rohlf, 2000). Shape variation was decomposed through thin-plate spline into uniform and non uniform (localised shape changes) shape components; the statistical weight of each of these two components was computed to capture the overall shape variation using the software tpsRegrw (Rohlf, 2000). The 1st uniform component accounts for the stretching along the x-axis (antero-posterior axis of fish body) of the configuration, whereas the 2nd uniform component indicates dilations or compressions along the y-axis (dorso-ventral axis of fish body). Correlation and regression analysis (Cavalcanti *et al.*, 1999) was performed between centroid size and the uniform componets. In order to compute the grow trajectories, the W' matrix was used in a relative warp analysis (RWA - software TpsRelw; Rohlf, 2001b). RWA is analogous to a principal component analysis for this sort of data (Rohlf, 1993). The first axis of the relative warp analysis was plotted against CS representing the growth trajectory. The use of the Thin-plate Spline function allowed for the visualization of the shape change in the form of deformation grid (splines). Size related shape changes were then visualized as splines relative to the extreme values of the relative warp axis.

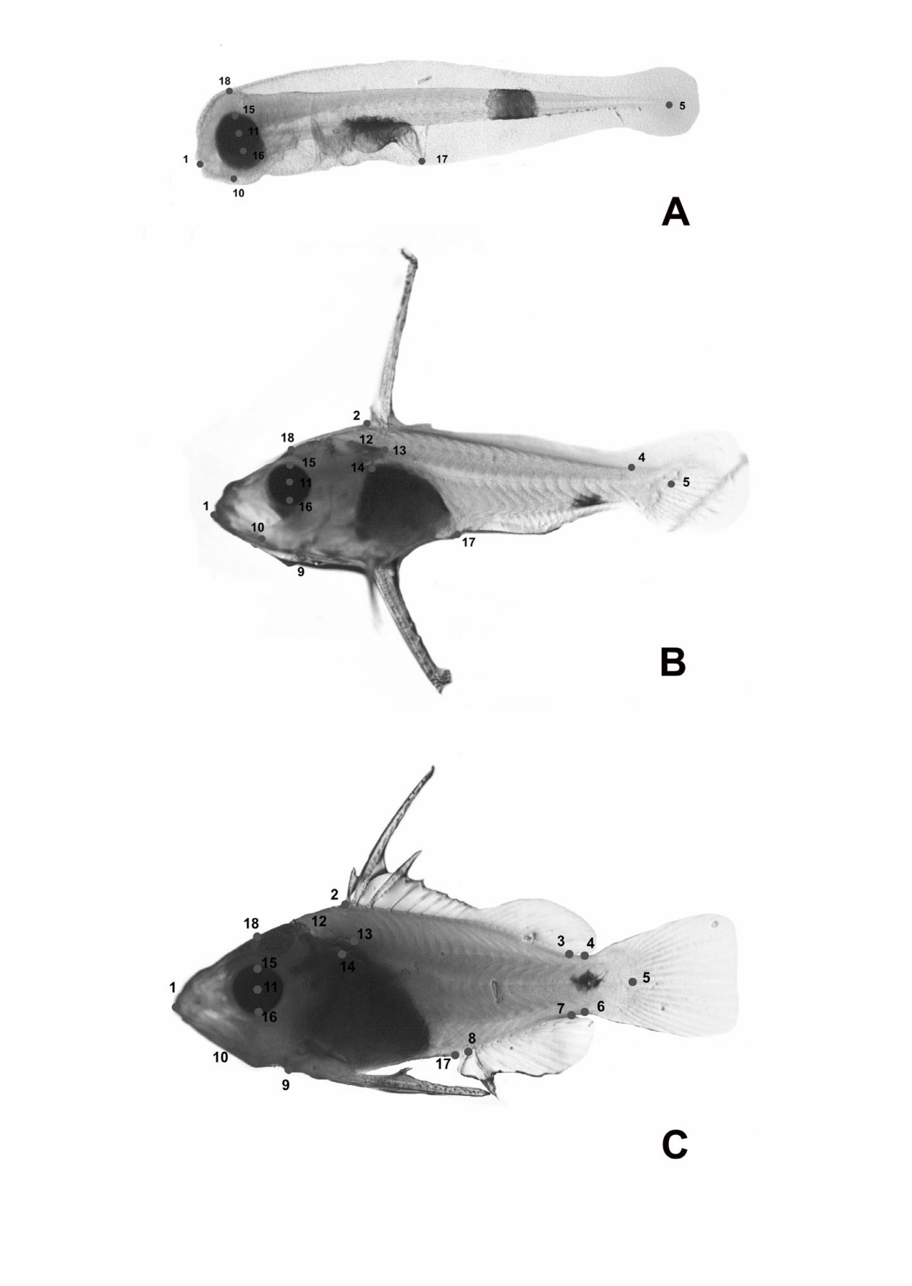


Fig 1. Different landmark configurations collected on the dusky grouper A: 8 landmarks configuration; B: 14 landmarks configuration and C: 18 landmarks configuration. Landmarks description: (1) snout tip; (2) and (3) anterior and posterior insertion of the dorsal

fin; (4) and (6) points of maximum curvature of the peduncle; (5) posterior body extremity; (7) and (8) posterior and anterior insertion of the anal fin; (9) insertion of the operculum on the lateral profile; (10) posterior extremity of premaxillar; (11) center of the eye; (12) superior insertion of operculum; (13) beginning of the lateral line; (14) point of maximum extension of operuculum on the lateral profile; (15) and (16) superior and inferior margin of the eye; (17) anus; (18) intersection between lateral profile and the axis passing through landmarks 11, 15 and 16.

In order to estimate the relative weight of the uniform and nonuniform components in determining overall size-related shape changes, we computed the following indices: CNU (the percentage of variability explained by the regression model without the uniform component); PCS (the percentage of variability explained by the regression model using the uniform component in addition to the nonuniform component); PNU (absolute percentage of variability explained by the nonuniform component) and PU (absolute percentage of variability explained by the uniform component). Finally, the same indices for the three landmarks configurations (8, 14 and 18) on the same common age range (20-70 days post-hatching) were calculated in order to compare the three models, exploring possible differences and the feasibility of determining a valid description using one or another of them.

Elliptic Fourier analysis

Elliptical Fourier function (Kuhl & Giardina, 1982) were used to fit specimens outline, that were collected as profile coordinates (software TpsDig, Rohlf, 2001a), excluding fins, on the same 288 specimens used for geometric morphometric analysis. The Fourier coefficents for a polynomial function of 18th degree were computed with the software Morpheus (Slice, 1998) using Elliptic Fourier analysis (Kuhl & Giardina, 1982; Fearson *et al.*, 1985; Rohlf, 1990). In order to standardise, coordinates were divided by length of the perimeter of the outline contour. For each specimen, 69 new variables were obtained by Fourier analysis.

In order to visualize size related shape changes, individuals were divided into groups according to their age. A principal component analysis was performed for each new set of variables (Software PcOrd for Windows version 3.09, MjM Software, Gleneden Beach, Oregon, U.S.A.). The first principal component (PC) axis was plotted against TL representing the growth trajectory. The mean configurations of the groups are presented.

RESULTS

General observations

Larvae opened their mouth during the second through third d.p.h. during this period, larvae are pelagic. They feed by utilizing a typical S-shape movement. Larvae of this size were capable of ingesting a limited number of organisms. From day 7 to 10 larvae developed elongated fin rays (two pectoral and the second dorsal spine). These structures reach the maximum relative-length between 18 and 20 d.p.h. (0,7 cm in T_L). At this stage in their development, as evidenced by stomach contents analyses, larvae preyed by waiting for prey to appear in front of them and subsequently ingest them with the S-shape movement. When larvae reach about 1 cm in T_L (Russo *et al.*, work in progress), dusky grouper larvae were capable of ingesting a larger class of prey (e.g. copepods such as *A. salina* nauplii). Larvae of this size class usually drifted in the midwater. At 30 d.p.h., when larvae were 1,2-1,5 cm in T_L, we observed the beginning of body pigmentation and the settling of larvae on the wall of the tanks.

Finally, we observed that when dusky groupers reached 2,5-3,0 cm in T_L, the body color changed to brownish and their behaviour shifted from swimming in the water column to settling to the bottom. According to observation on wild juveniles by La Mesa *et al.* (2002), we observed that *E. marginatus* juveniles choose microhabitat characterized by availability of shelter, and the transition from pelagic to benthic stage happens during metamorphosis.

Geometric morphometrics

Fig 2 shows the plot of RW1 versus CS obtained using the 8 landmark configuration. The top and bottom splines represent the shape of the extreme values of the RW1. Correlation between CS and the first RW is highly significant (r=0,78, p<0.001). The pattern of the scattering resembles the saturating growth trajectory documented by Alberch *et al.* (1979): the rate of shape change is very high for small sizes and decreases during growth. The rate of change decreases when centroid size values are approximately 2.3, correspondent to 1.15 cm in SL. The splines show the positive allometry of mouth region, a relative backward of the anus landmark and the relative shortening and upward movement of the eye that causes landmark 2 to overlapp with landmark 8.

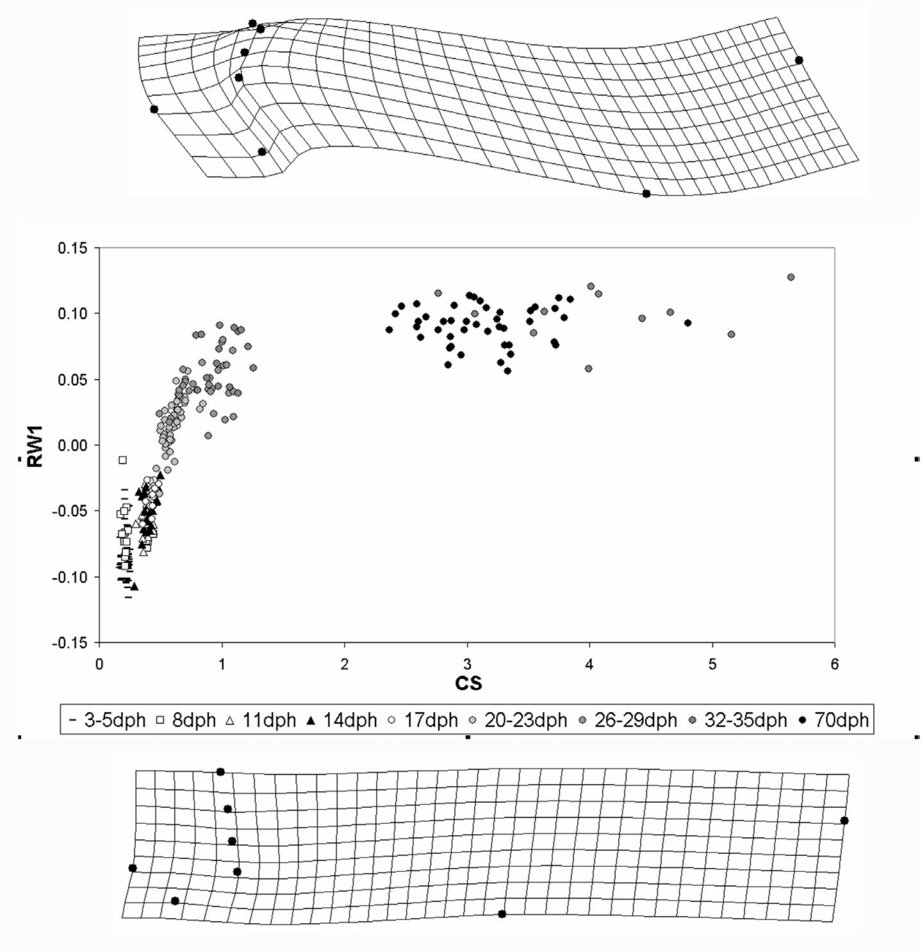


Fig 2. Growth trajectory obtained from 3 to 70 days post hatching (8 landmarks configuration): Relative Warps 1 (RW1) versus Centroid Size (CS). The splines represent fish shape variability along each relative warp axis.

Fig 3 shows the regression of RW1 versus CS obtained using 14 landmarks. The range of age considered in this case starts at 14 days post hatching.

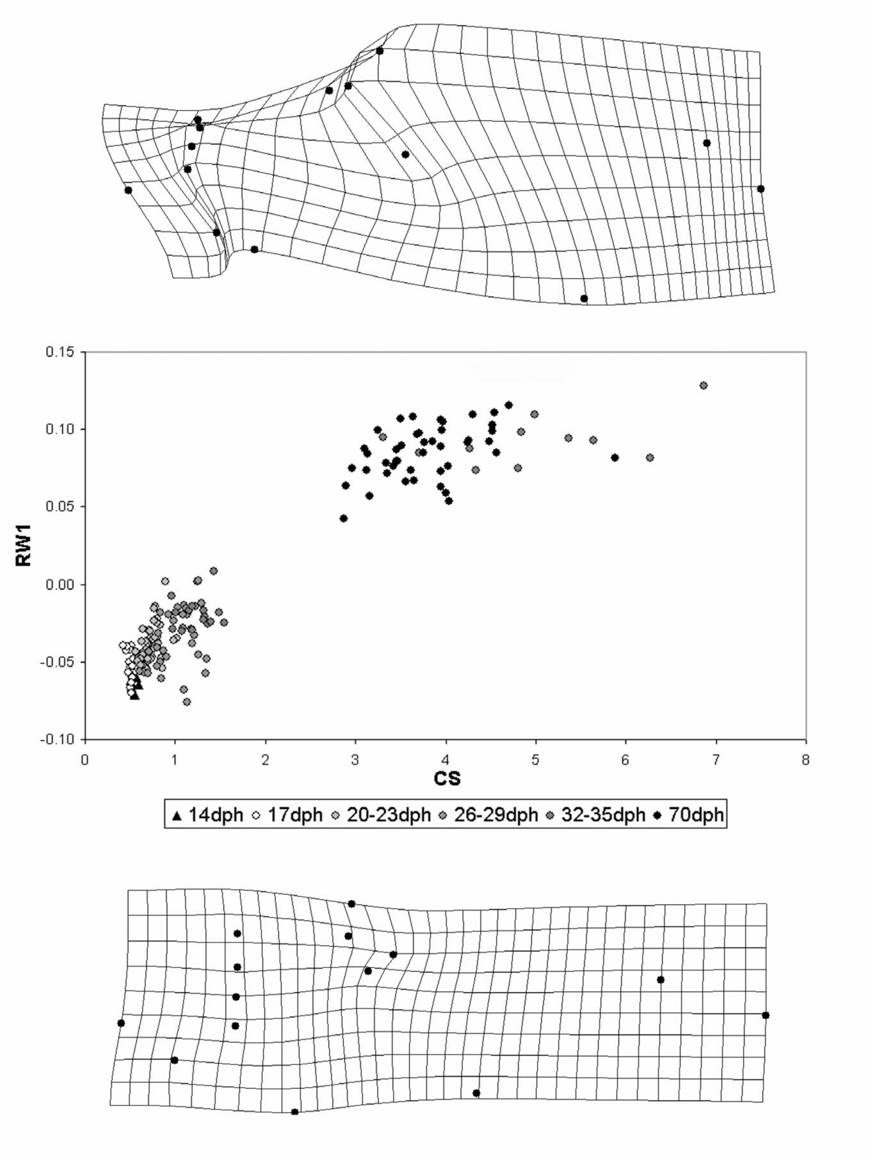


Fig 3. Growth trajectory obtained from 14 to 70 days post hatching (14 landmarks configuration): Relative Warps 1 (RW1) versus Centroid Size (CS). The splines represent fish shape variability along each relative warp axis.

Correlation between CS and RW1 is highly significant (r=0.94, *p*<0.001), and the growth trajectory appears to be more linear than that described for 8 landmarks, so the rate of shape changes is more homogenous during the this period. The splines show the negative allometry of head region (globally considered), the positive allometry of the mouth region, the widening of the central region of the body, a relative backward of the anus landmark and a relative upward movement of the eye that brings landmark 2 to overlap landmark 8.

Fig 4 shows the plot of RW1 versus CS obtained using the 18 landmark configuration ($r=0.94$; $p<0.001$). The range of age considered in this case starts from 20 days post hatching. With this configuration it is possible to observe the same growth changes of 18 landmark configuration and the changing of shape of the caudal peduncle and of the operculum. The caudal peduncle with growth tends to be shorter and higher and the operculum region wider. Centroid size was significantly correlated with either U1 or U2 uniform components for all landmark configurations (p < 1*10-6 in all three cases). Globally, all three analyses show that when SL values exceed 1 cm, dusky grouper post-larvae attain the adult shape.

Tab 2 shows values of CNU, PCS, PNU and PU obtained from the three configurations. Each index shows different values for the three configurations. The 8 landmarks model is distinct while 14 and 18 landmarks models are similar to one another. The 8 landmark model shows the highest value of PNU. Considering that models are correlated with different age ranges this index reflects the effects of inclusion/exclusion of the critical stage as the first ones represented by samples of smallest size. In order to compare the characteristics of the three models and analyse the accuracy of each configuration without the effect of the different age range, the indexes were calculated on the same age range (from 20 to 70 days post hatching) (Tab 3). All the values for 14 and 18 landmarks configurations are similar, differing from the 8 landmarks one. In particular the PNU value of 8 landmark configuration is lower than the otherconfigurations, underlining that this model has a lack of information about the non-uniform component in describing shape changes during growth.

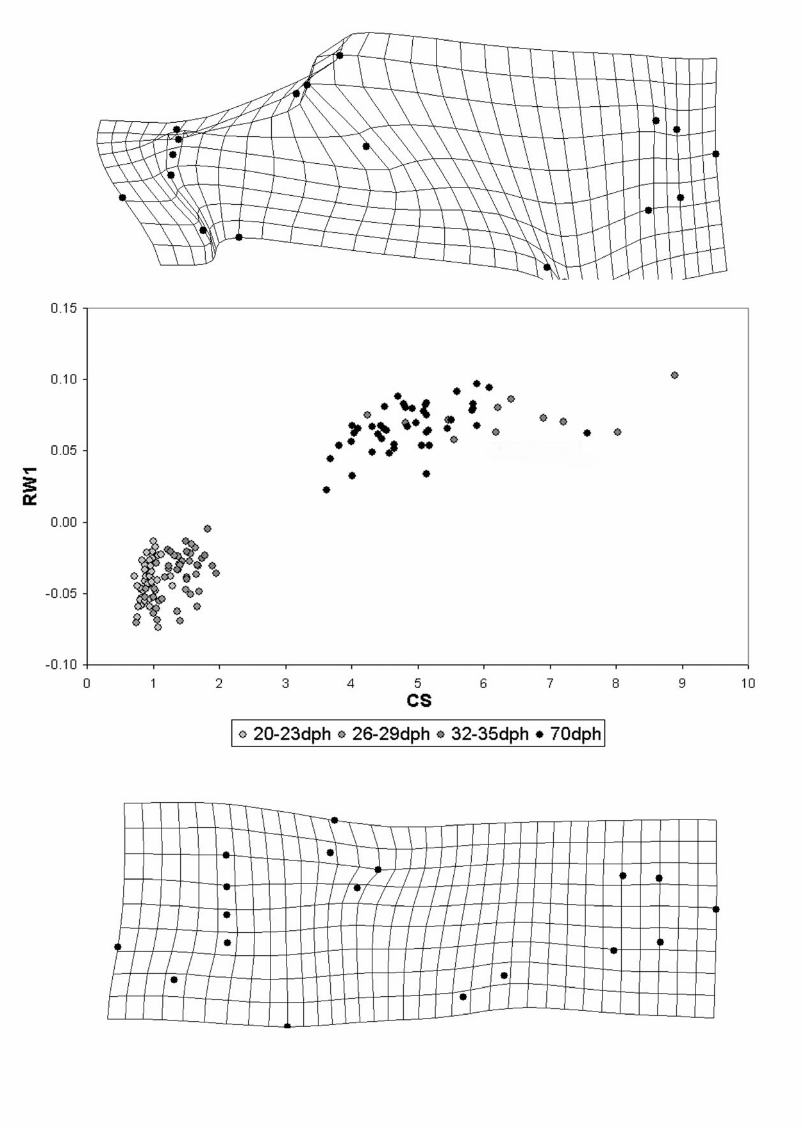


Fig 4. Growth trajectory obtained from 20 to 70 days post hatching (18 landmarks configuration): Relative Warps 1 (RW1) versus Centroid Size (CS). The splines represent fish shape variability along each relative warp axis.

Landmarks configuration	CNU	PCS	PNU	PU
8	**25.76%**	**41.55%**	**62.01%**	**37.99%**
14	**34.04%**	**37.33%**	**91.17%**	**8.83%**
18	**33.09%**	**40.00%**	**82.74%**	**17.26%**

Tab 2. Percentace of variability explained by the different components on the regression model (RW1 versus CS) for each landmark configuration calculated at different age range.

Landmarks configuration	CNU	PCS	PNU	PU
8	**21.492%**	**59.435%**	**36.160%**	**63.840%**
14	**35.837%**	**41.656%**	**86.030%**	**13.970%**
18	**33.092%**	**39.996%**	**82.739%**	**17.261%**

Tab 3. Percentage of variability explained by the different components on the regression model (RW1 versus CS) for each landmark configurations calculated on the same common age range (20-70 days post-hatching).

Elliptic Fourier analysis

A Principal Component Analysis (PCA) was performed on the 69 coefficients of the elliptic Fourier analysis. PC1 is significantly correlated both with S_L (Pearson r=0.76, P<0.001), and with TL (Pearson r=0.75, $p<0.001$) and MH (Pearson r=0.78, $p<0.001$). Fig 5 shows the plot of PC1 versus TL and shapes relative to the mean configuration of the groups. The growth trajectory for PC1 is very similar to the one obtained with the RWA of geometric morphometrics (8 landmarks), and it appears as a saturating curve: rapid morphological changes characterise small size values, after which a stable stage is approached. The same axis shows high morphological variability in small size individuals. Important shape changes during growth involve head, caudal and median regions. The head region becomes sharper during growth, and has a negative allometry. In contrast, the ventral region undergo a positive allometry. The anal fin region tends to decrease its area and move progressively backward, perhaps due to growth of the visceral cavity. The peduncle becomes higher and shorter and maximum body height increases relatively.

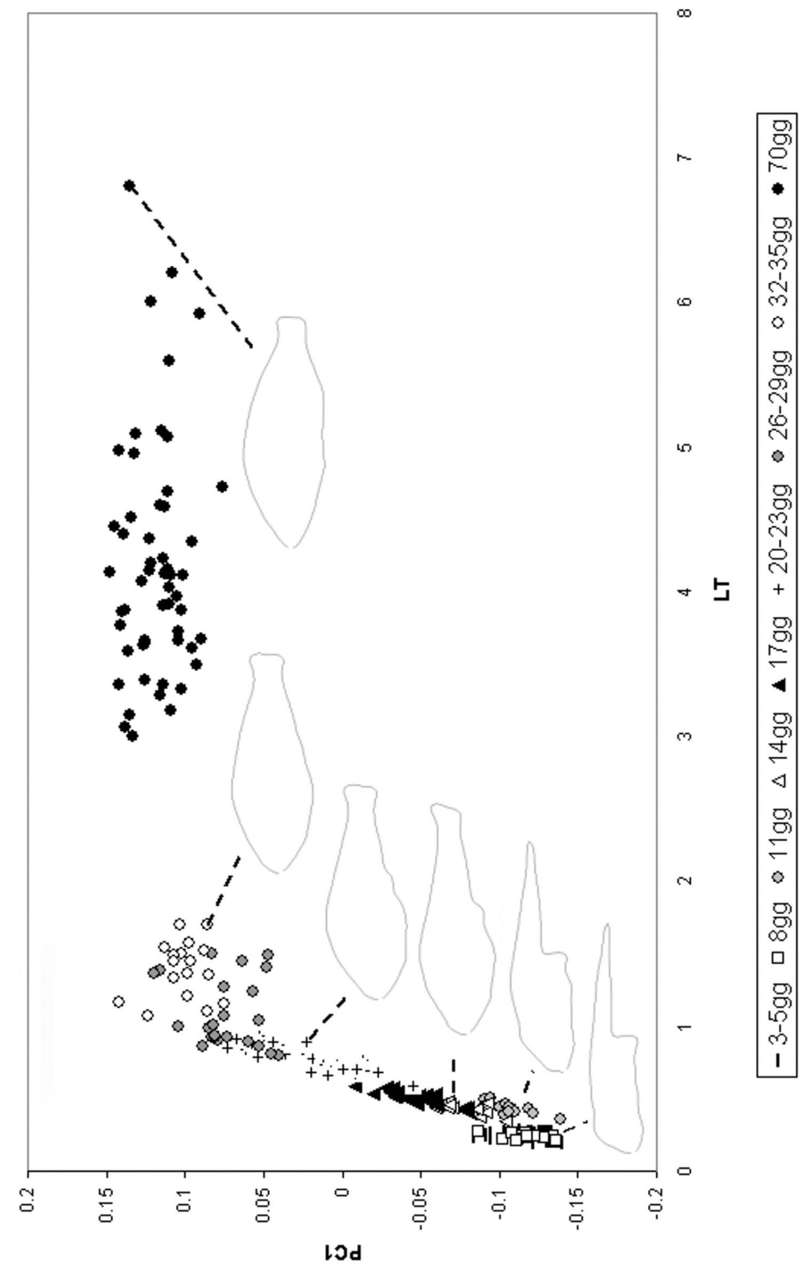


Fig 5. Growth trajectory obtained by means of Elliptic fourier analysis on the age range 3-70 days post hatching: PC1 versus Total length (TL). Near the curve are represented the mean configurations of some groups.

Globally, these changes result in a discoidal final body shape. In this analysis, the higher age groups are well separated. This result differs from the ones obtained for geometric morphometrics, where these groups always overlap.

DISCUSSION

Geometric morphometrics (Bookstein, 1991; Rohlf & Marcus, 1993; Marcus *et al.*, 1996) and elliptic Fourier analysis (Khul & Giardina, 1982; Rohlf & Archie, 1984; Lestrel, 1997; Loy *et al.*, 2001) has been used to characterize growth trajectories (Alberch *et al.*, 1979; Loy *et al.*, 1998b; Loy *et al.*, 2000) and to visualize size-related shape changes, i.e. growth allometry (Klingenberg, 1996; Loy et at., 1998b; Loy *et al.*, 2000), during orthogenesis. Dusky grouper larvae were obtained by aquaculture techniques using a semi-intensive approach already tested as capable of producing morphological and anatomical wild-like fry (see Boglione *et al.*, 1994; 2001; Costa, 2004), that could be used as an idoneous samples in biological experiments. These captive dusky grouper larvae provide the opportunity to study rapid shape changes of this species. The aim of this study was to survey morphological development of dusky grouper (starting from hatching) in order to: (1) initiate the use of morphometrics tools for describing larval stages; (2) verify that this approach should be a future tool for morphological quality assessment of fry for restocking programs and (3) widen the basic knowledge of dusky grouper biology and development using two different approaches, in order to compare results and, possibly, to identify a better system for describing shape changes during ontogenesis.

The dusky grouper represents an idoneous example for the study of changes in ecology, size and morphology during ontogeny and growth. Size-related shape changes during larval stages of *E. marginatus* seem to follow a precise pattern. The growth trajectories reported from the 8 landmark configuration and elliptic Fourier analysis closely reflect a saturating growth curve. Changes are very rapid for small size increments and decrease with growth up to an apparently stable relationship between shape and size. Early larvae have a stream-lined body shape, adapted to pelagic life, but rapidly acquire a rounded shape with a shorter and higher caudal peduncle, suitable for manoeuvring (Webb, 1984a,b; Costa, 2004). The rate of this shape change is high, suggesting the need for a rapid morphological shift. Reñones *et al.* (2002) reported that *E. marginatus* undergoes size-related changes in diet in exploited populations off the Balearic Islands. Although these authors didn't examined larval stages, they observed that juvenile dusky groupers (T_L < 300 mm) feed on rocky habitats in the upper infra-littoral, as adults. In our experimental conditions, we observed that body shape in early juveniles is very similar to adult ones, corresponding to a subcarangiformes shape as indicated by Webb (1984a,b). Their bodies are characterized by a discoid shape, a higher and shorter peduncle and the sharpness of head region, as seen from Elliptic Fourier analyses of outlines. These changes in body shape weren't as apparent from geometric morphometrics, reporting powerful of outlines fitting analysis when alternative model were characterized by few landmarks. The growth trajectory might be interpreted as a two stage growth curve (Bookstein, 1991), where the first stage is characterized by a dramatic morphological change for a small size interval, and the second by a change in size not accompanied by a change in shape (isometry). The steep portion of the growth trajectory corresponds to the size range in which the habitat transition occurs. The saturation of growth trajectory has been found to be related to changes in species feeding habits (Russo *et al.*, work in progress). The major inflexion point in the 8 landmark model corresponds to about 1 in CS. At this stage, larvae were about 1.5 cm in T_L, that is the value at which we can observe the inflexion point of the growing curve of larvae using outline analyses. The inflexion point occurs both in the 8 landmark and outline models at about 35-40 days post hatching. These results demonstrate a coherence in the two analyses. These observations persist when we examine both 14 and 18 landmark configurations, although the slope of the of steepest portion of growth trajectory is lower, and the transition between the two stages is not as marked. This effect can be explained by considering the different ranges of age and size examined for each configuration. Due to the high concentration of morphological changes in the relatively small size interval corresponding to early larval stages, inclusion or exclusion of these samples have a large affect on the curves obtained.

Comparing 8, 14 and 18 landmark configurations (Tab 2), it is possible to explain how PCS values are similar in all cases but PNU and PU values are different. This suggests that landmark configuration models could be characterized both by different degrees of goodness and fidelity to shape information. When 8, 14 and 18 landmark models are calculated on the same age range PNU and PU values for 14 and 18 are similar, however, the 8 landmark model is different due primarily to the relative weight of uniform component being strongest for the 8 landmark model. These results can be explained by considering that information about non-uniform components closely depends on the number of landmarks included in the model (Rohlf & Bookstein, 1988). When we compare growth curves obtained from 8 landmark configuration models with growth curves from elliptic Fourier analyses, the final portions of first curve are characterized by large superposition overlap of two samples (32-35 d.p.h. and 70 d.p.h.), the second clearly discriminate these stages, that results strongly separated at the beginning and at the end of the transitional phase.

Dusky grouper larvae are visual and olfactory predators, that recognize food items mainly by sight, selecting them principally by size (mechano-receptors) and secondarily by chemoreceptor (Boglione *et al.* 1999). Doi *et al.* (1991) reported that *E. suillus* larvae became bottom dwellers and their body colour became brownish when they are 35-40 days old. Consistent with these results, *E. marginatus* transitions from pelagic to benthic stage during metamorphosis, when larvae are about 35-40 days old acquiring juvenile characters regarding shape, trophic behaviour and microhabitat selection. Changes in these aspects of ecology and morphology are largely concurrent.

In this study for the first time we described the shape changes of *E. marginatus* from larvae to juveniles. Both methods of analysis, geometric morphometrics (8 landmark configurations) and elliptic Fourier analysis, statistically analyze and visualize these changes. The dramatic changes in ontogeny-related and shape variables in dusky grouper from larvae to juveniles are directly related to ecology, trophic habits and ecomorphological modification.

4. Shape and trophic habits changes, from hatching to adult stage, in the gilthead sea bream *Sparus aurata*

INTRODUCTION

At hatching, fish larvae present an incomplete and well-developed body that pass through very complex processes of morphogenesis and differentiation during growth. Development of organs and changes in morpho-anatomical characters happen one by one, resulting in a progressive transformation of specimens from larval to juvenile and then to adult form. Nevertheless not all body parts grow simultaneously at isometric rate, so that differentiate relative growth (allometry) is a common feature of fish development (Gisbert, 1999; Osse & van den Boogaart, 1995; 1999; Sala *et al.*, 2005). The allometric patterns observed in many species suggest that body structures develop in order of their importance for primary functions. These functions should be referred to given period of fish life, and the growth profiles of morphological features are related to subsequent functional needs of the growing individual (Gisbert, 1999; Osse & van den Boogaart, 1995; Sagnes *et al.*, 1997; Sala *et al.*, 2005; Simonović *et al.*, 1999; Stoner & Livingston, 1984). The development of body structures enable specimens to perform new abilities or to acquire more functional efficiency, and as consequence, patterns of shape development are closely linked to changes in habitats and resource use during ontogenesis, as evidenced in many species (Osse & van den Boogaart, 1995; Sagnes *et al.*, 1997; Webb & Weihs, 1986; Sala *et al.*, 2005). In the ecomorphological framework of investigations about causal connections between morphological diversity of the organisms and their resource use/habitat selection, studies on fishes examine often the relationship between body shape and feeding habits (Motta *et al.*, 1995a,b; Smirnov *et al.*, 1995). In this framework, it could be considered particularly interesting the studies about morphological changes during ontogenesis, following the ecomorphological developmental approach advanced by Kryzhanovsky, Vasnetsov, Disler and Soin (see Smironov *et al.*, 1995 and Luczkovich *et al.*, 1995 for a review) that directly preludes to the topic of Balon's theory of saltatory ontogeny, which affirms that ontogeny is a sequence of longer stabilized states punctuated by rapid changes in "integrative actions" or thresholds (Balon, 1985).

Despite the importance of these studies in improving the understanding of adaptations during fish ontogenesis, few reports are available in literature about the shape development of fish, and particularly scarce are works that correlate changes in shape with changes in habitat and resource use. Moreover, most of the reports use a classic morphometric approach, which consists into the survey of many measures along the body, giving subjective and incomplete descriptions of shape. Recently, development of shape analysis techniques (i.e. Geometric Morphometrics and Elliptic Fourier Analysis) allowed to survey form changes at all developmental stages (Kawamura & Hosoya, 1997), providing detailed and mathematically comparable description of shape and its changes between samples that differ from species composition (Linde *et al.*, 2004) or from age (Kawamura & Hosoya, 1997; Simonović *et al.*, 1999) or from genetic markers (Trapani, 2003) or from rearing techniques (Loy, 1996; 1998a, b). These tools also provide realistic and exhaustive representations that allow a direct visualization and understanding of findings (Bookstein, 1991).

In this study, the development of the gilthead sea bream *Sparus aurata* from hatching to adult stages is examined describing changes in shape and diet preference and then correlating these two aspects of biology. The gilthead sea bream is a sparid that plays an important role in fisheries and aquacultural activities in the Mediterranean Sea and North Eastern Atlantic Ocean (Bauchot & Hureau, 1986), where it commonly occurs on littoral sandy bottoms and Posidonia beds, up to depths of 30 m (juveniles) and 150 m (adults).This euryhaline and eurythermal species is characterized from an high trophic flexibility (Kraljević & Dulčić, 1997; Parra & Yúfera, 2000; Mariani *et al.*, 2002; Tancioni *et al.*, 2003) and from peculiar feeding adaptations throughout its life history (Cataldi *et al.*, 1987) that give to this species a wide adaptative range in terms of trophic habits. Even though the importance and the wide cultivation of this species that allows easy sampling of developmental samples series, there is a remarkable lack of published studies on development of this species (Faustino & Power, 1998), in particular covering all life history, from hatching to adult stages.

The aims of this work are: (a) to report the first description of the whole shape development, from hatching to adult stage, of a fish species; (b) to identify morphological stages during ontogenesis, with corresponding thresholds; (c) to examine correspondences between morphological development and trophic habits changes during growth, and (d) to describe allometric patterns of shape development within each stage, inferring its ecomorphological meaning.

MATERIALS AND METHODS

Larvae of *S. aurata*, originated from artificial spawning eggs of brood stock raised in “Valle Figheri” hatchery (Venice, Italy), were reared from 3 to 70 days post hatching (d.p.h.) in a circular semi-outdoor 60 m^3 tank (diameter 8 m, water height 1.2 m) using a semi-intensive approach (Boglione *at al.*, 1994; Boglione *et al.*, 2001; Shields, 2001). This method was already tested as suitable to produce wild-like specimens (Cataudella *et al.*, 2002; 2003) and provides daily supplies of cultured live food (*Brachionus plicatilis* and *Artemia salina* nauplii) on the rearing tanks, which were also fertilized by a connection to an external natural lagoon. Therefore, there was a natural build up of wild zooplankton (copepods nauplii, juveniles and adults, bivalves trocophores and polychaetes larvae) into tank water, resulting in a constant availability of natural food items that constituted an important trophic resource for fish larvae, both in terms of energy source and playing a fundamental role in learning of feeding behaviour (Zaragoza *et al.*, 1994). This determined polyspecific conditions in the diet of larvae and post-larvae and offered an idoneous substrate to analyze trophic selections of them until 40 d.p.h., when we started to use artificial dry pellets. Mean water temperature during larval rearing was 18°C, salinity was 25‰, density was 6-7 larvae/l. Newly hatched larvae were transferred into the tank at 4 d.p.h., and removed at 70 d.p.h. When they were settled in outdoor tanks for intensive rearing. Final survival rate was 17,5 %.

A total number of 559 sea bream specimens were collected, initially from 4 d.p.h. every 2 days, then at 76, 100, 123, 162, 279, 315 and 461 d.p.h.. Each specimen was photographed with a Polaroid digital camera (24-bit true colour images, 16 million colours, resolution 1600 × 1200) and images collected were analyzed with morphometric software (http://life.bio.sunysb.edu/morph/).

Geometric morphometrics

For Geometric Morphometrics (GM) 2 different landmark configurations were collected using the software TpsDigit (Rohlf, 2001a). The first configuration, with 8 landmarks, was used for all the samples (559 individuals, 4-461 d.p.h.) (Fig 1A). After the 40th d.p.h. other 8 landmarks (n° 2, 3, 4, 6, 7, 8, 9, 12, 13 and 14, Fig 1B, C and D) could be added producing the second configuration with 18 landmarks. The increasing number of landmarks associated with growth was due to the development of the anatomical structure. Statistical analyses were conducted using groups with the same number of landmarks. After digitalization, landmark coordinates were translated to align the centroid size (CS, square root of the sum of the squared distances from each landmark to the specimen's centroid) of each individual, then rotated and rescaled to produce Procrustes shape coordinates using the Generalized Procrustes Analysis (GPA) (Rohlf, 1988; Rohlf & Slice, 1990; Bookstein, 1996a; b). Residuals from the fitting were analyzed with the Thin-Plate Spline (TPS) interpolating function (Bookstein, 1991). Shape changes during growth were visualized as deformation grids (splines) regressing the parameters of the function (Weight Matrix, W') on total length (T_L) (software TpsRegr, Rohlf, 2000). The correlation between shape variables and T_L was tested with Permutation tests with 1000 random permutations. In order to compute the grow trajectories, the W' was used in a Relative Warp Analysis (RWA) (software TpsRelw, Rohlf, 2001b). RWA is analogous to a principal component analysis for this sort of data (Rohlf & Marcus, 1993). The first axis of the RWA was plotted against T_L representing the growth trajectory. The use of the TPS function allowed the visualization of the shape changes as splines. Size related shape changes were then visualized as splines relative to the extreme values of the relative warp axis. Consensus configurations, with their relative splines, were computed for each age samples.

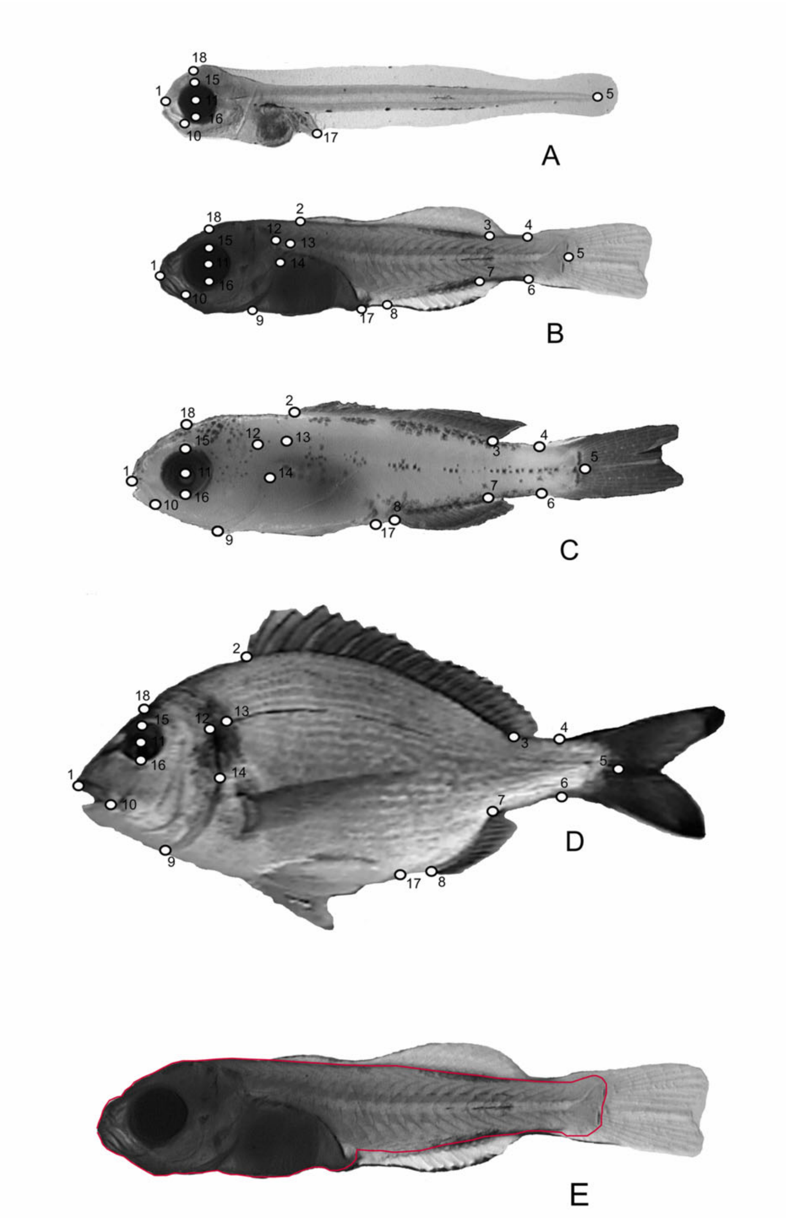


Fig 1.: Landmarks (A-D) Outline (E) collected on the gilthead sea bream at different developmental stages. A: 8 landmarks configuration; B, C and D: 18 landmarks configuration at different size. Landmarks description: (1) snout tip; (2) and (3) anterior and posterior insertion of the dorsal fin; (4) and (6) points of maximum curvature of the peduncle; (5) posterior body extremity; (7) and (8) posterior and anterior insertion of the anal fin; (9)

insertion of the operculum on the lateral profile; (10) posterior extremity of premaxillar; (11) center of the eye; (12) superior insertion of operculum; (13) beginning of the lateral line; (14) point of maximum extension of operuculum on the lateral profile; (15) and (16) superior and inferior margin of the eye; (17) anus; (18) intersection between lateral profile and the axis passing through landmarks 11, 15 and 16.

A Cluster analysis with complete linkage (NTSYSSpc Version 2.10j- Applied Biostatistic Inc.) was performed on Squared Euclidean distances computed on the 8 Landmarks configuration's W' data, using Ward's algorithm, in order to identify clusters between age/size groups. A Multi Response Permutation Procedure (MRPP) (Legendre & Legendre, 1998) was performed to test differences between development intervals. MRPP is a non-parametric procedure used to test the null hypothesis of no difference between two or more groups of entities that must be create *a priori* (PC-Ord for Windows, MjM Software, Gleneden, Oregon, USA).

Elliptic Fourier analysis

Elliptical Fourier function (Kuhl & Giardina, 1982) was used to fit specimens outlines, collected as profile coordinates (software TpsDigit, Rohlf, 2001a) (Fig 1E), excluding fins, on the same 548 specimens used for geometric morphometrics analysis. The Fourier coefficients for a polynomial 18th degree function were computed with the software Morpheus (Slice, 1998) using Elliptic Fourier Analysis (EFA) (Kuhl & Giardina, 1982; Ferson *et al.*, 1985; Rohlf & Slice, 1990). Standardization was obtained dividing coordinates by the length of the perimeter of the outline contour. For each specimen, 69 new variables were obtained by EFA. A principal component analysis was performed for each new data set (Software PcOrd for Windows version 3.09, MjM Software, Gleneden Beach, Oregon, U.S.A.). The first principal component (PC1) axis was plotted against T_L and age. The mean configurations of each age group are presented in the plot. In order to investigate the existence of developmental stages were selected the harmonics that best discriminate specimens following the procedure descript in Johnson (1997). Squared Euclidean distances computed on selected coefficients were used to perform a Cluster analysis (Ward's method based) in order to identify associations between specimens and groups. MRPP was used in order to validate groupings and Canonical analysis to visualize the between- and within-groups variability.

Gut contents analyses, food classification and feeding habits of different stages

Most data and observations were directly collected on the samples used in the present study, others compiled from scientific literature. To study the feeding ecology, all specimens of each sample from 4 to 40 d.p.h were analyzed, collecting contents of the stomach or, in the first stages, of the anterior 1/3 of gut. This material was inspected, using a stereomicroscope, and each food item was identified and counted. Samples older than 40 d.p.h. were not analyzed, and data about trophic habits were only collected from literature.

RESULTS

Geometric morphometrics

Fig 2 shows the regression betweens shape, represented by the first axis of the RWA (RW1), and size, expressed by total length ($\boldsymbol{T_L}$). Pearson correlation between T_L and RW1 scores resulted highly significant (r = 0,99, $p < 1\times10^{-5}$). The rate of shape change was very high for small sizes and started to decrease when fishes are 4 cm ($\boldsymbol{T_L}$). The splines showed the relative backward of the anus landmark, the downward movement of the mouth and the positive allometry of the head. No detail about development of tail region could be extract.

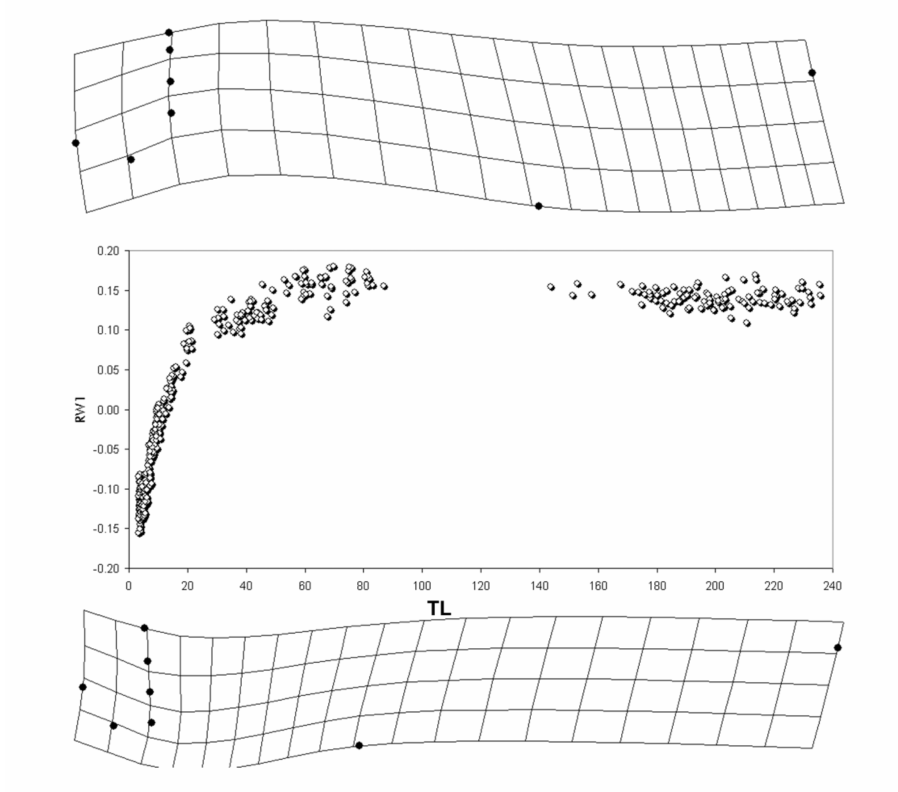


Fig 2. Growth trajectory obtained from 4 to 461 d.p.h. (8 landmarks configuration): Relative Warps 1 (RW1) versus Total length. The splines represent fish shape variability along each relative warp axis.

A more exhaustive description was represented by 18 landmarks configuration, that was the base of growth curve reported in Fig 3 (RW1 versus T_L). The range of age considered in this case started at 40 d.p.h.. Correlation between T_L and RW1 was highly significant (r = 0.99, $p < 1\times10^{-5}$), and the growth trajectory appeared to be similar to that described for 8 landmarks, except for the part characterized from rapid shape changes that was, in this case, shorter.

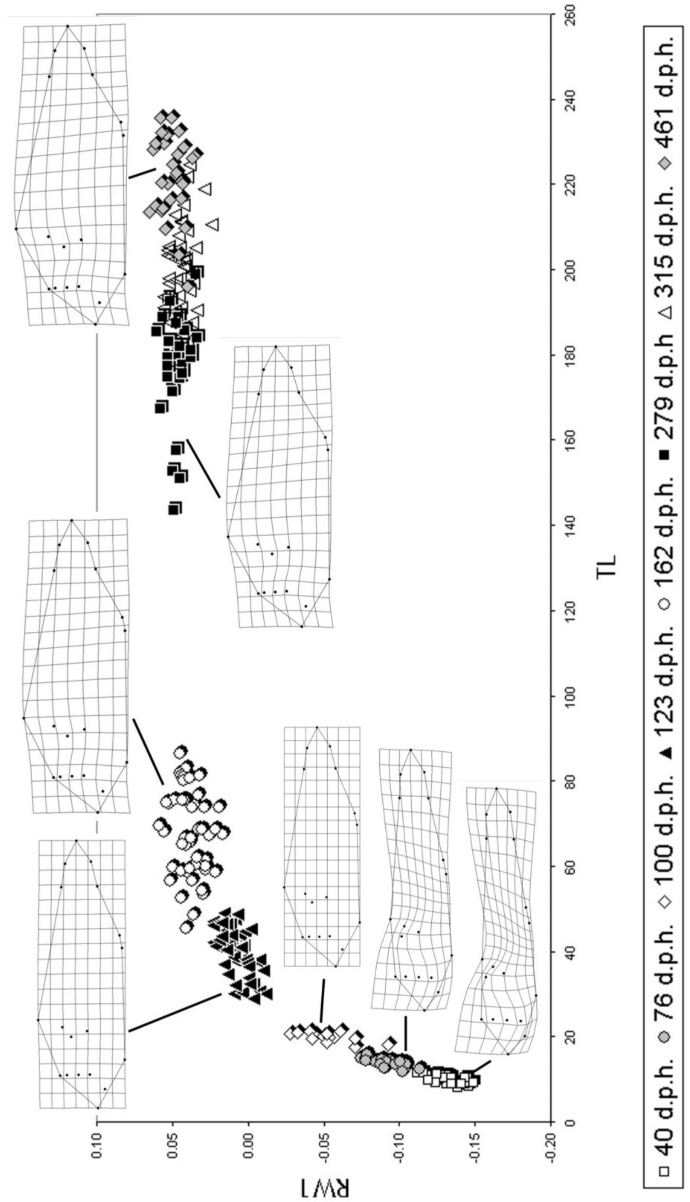


Fig 3. Growth trajectory obtained from 40 to 461 days post hatching (18 landmarks configuration): Relative Warps 1 (RW1) versus Total length (T_L). The splines represent fish shape variability along each relative warp axis.

The splines showed that the major changes involved only the period from 40 (1 cm in T_L) to 100 (2.5 cm in T_L) d.p.h., when the shape of fish became very similar to the adult one. After 100 d.p.h. it was only possible to appreciate the upward movement of landmark 2 that gives a more discoidal shape to the body. In the critical period (40-100 d.p.h.) shape changes concerned the negative allometry of head region, the positive allometry (widening) of the central region of body, the relative backward of the anus and of the anterior anal fin insertion, and the change in the orientation of mouth. Considering the orientation of an imaginary plan perpendicular to the longitudinal axis of the body and tangential to both lips of the mouth, it seemed that mouth keeps its median position (moving from frontal to horizontal). Globally, both analyses showed that when T_L values exceed 7 cm, gilthead sea bream attained the saturating adult shape.

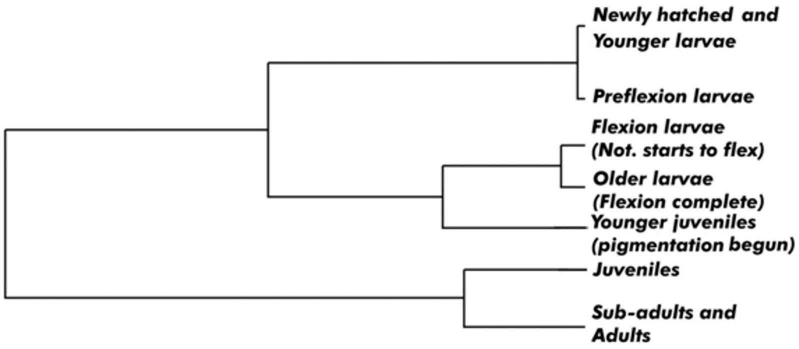


Figure 4. Dendrogram of cluster analysis performed on 8 landmarks configuration's Weight Matrix.

The cluster analysis applied on this data set discriminated seven groups (Fig 4), that were described and named according to the development of important organs and anatomical structures, as reported in Kendall (1984): (1) ***Newly hatched and younger larvae***, that included specimens with yolk-sack and without swim bladder; (2) ***Preflexion larvae***, that was referred to specimens with swim bladder but without notochord flexion; (3) ***Flexion larvae***; (4) ***Older larvae***, referred to specimens at the end of notochord flexion from the beginning of fin rays formation to initial appearance pigmentation; (5) ***Younger juveniles***, from the beginning of pigmentation to the beginning of squamation; (6) ***Juveniles***, referred to specimens more developed, that posses a complete scale cover and (7) ***Sub-adults and Adults***, referred to specimens approaching maturation and specimens sexually mature. MRPP applied to this data set reported significant differences between all groups (r = 0.6744, $p < 1x10^{-8}$, see table 2 for Intra-group distances), as shown by clustering. It seemed that larger distances ran between the group including sub-adults, adults and juveniles and the other stages. Instead, another remarkable gap divided early larval stages (before notochord flexion) from larvae that made metamorphosis and younger juveniles. Size-extreme measures (T_L) of each group are reported in Tab 2.

8 LANDMARKS						**OUTLINES**					
Developmental Stage	**TL: M±s (mm)**	**TL min (mm)**	**TL max (mm)**	**Intra-group distance**	**n**		**TL: M±s (mm)**	**TL min (mm)**	**TL max (mm)**	**Intra-group distance**	**n**
Newly hatched and Younger larvae	3.89±0.44	3.19	4.40	0.062	138	**Newly hatched larvae**	3.66±0.09	3.40	3.80	0.054	25
Preflexion larvae	5.43±0.47	4.46	5.50	0.049	80	**Younger larvae**	3.93±0.47	3.20	4.40	0.074	113
Flexion larvae	7.07±0.64	5.58	7.50	0.047	48	**Preflexion larvae**	5.43±0.47	4.46	5.50	0.058	80
Older larvae	8.34±0.92	7.55	10.50	0.043	60	**Flexion larvae**	7.07±0.64	5.58	7.50	0.051	48
Younger juveniles	14.15±2.45	11.10	25.00	0.055	36	**Older larvae**	8.34±0.92	7.55	10.50	0.044	60
Juveniles	45.73±12.91	25.20	71.50	0.044	100	**Younger juveniles**	14.15±2.45	11.10	25.00	0.047	36
Sub-adults and adults	168.32±17.80	125.60	210.00	0.032	97	**Juveniles**	45.73±12.91	25.20	71.50	0.029	100
						Sub-adults and adults	168.32±17.80	125.60	210.00	0.028	97

Tab II. Biometries, dimensions of samples and MRPP intra-group distance of developmental stages detected from geometric morphometry and Elliptic Fourier Analysis. T_L = Total length; M = mean; s = standard deviation; n = number of specimens in the samples.

Elliptic Fourier analysis

A Principal Component Analysis (PCA) was performed on the 69 coefficients of the EFA. PC1 was significantly correlated with $\boldsymbol{T}_{\mathrm{L}}$ (Pearson r = 0.75, $p < 1\times10^{-3}$). Fig. 5 showed the regression between PC1 and $\boldsymbol{T}_{\mathrm{L}}$ and the outlines relative to the mean configuration of some age groups.

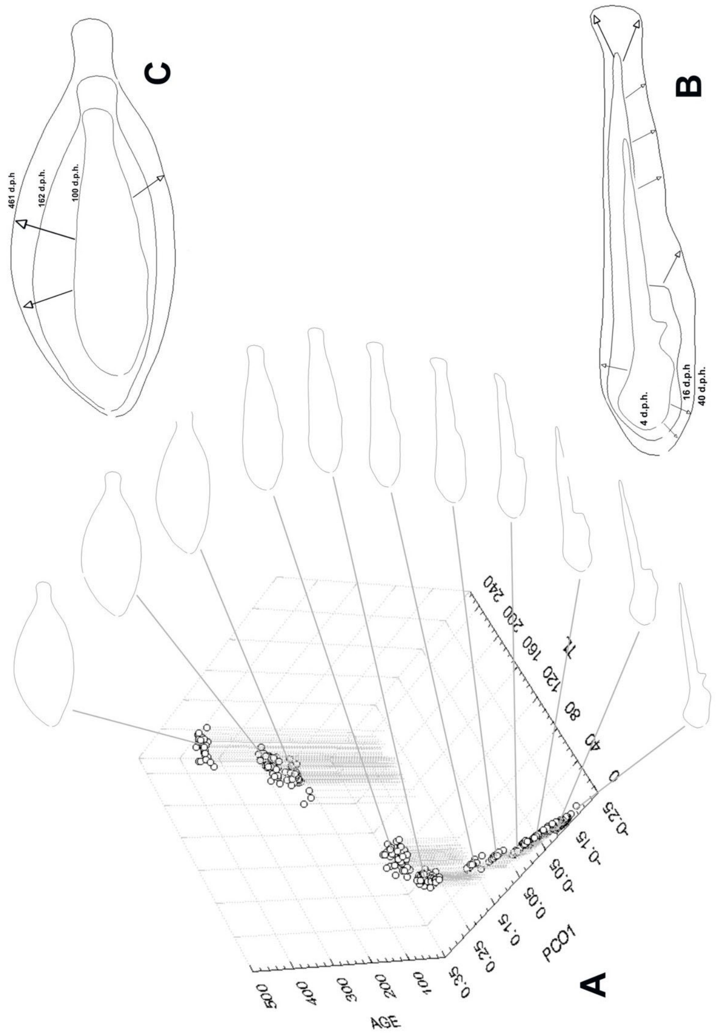


Fig 5. A: Growth trajectory obtained by means of elliptic Fourier analysis on the age range 4-461 days post hatching: PCO1 versus total length (TL) and versus age (days post hatching). Along the trajectory are represented the mean configurations of some groups. B and C: Representations of, respectively, major shape changes until 40 d.p.h. and after 100 dp.h..

The growth trajectory for PC1 was very similar to the one obtained with the RWA of geometric morphometrics (8 landmarks), and it also appeared to be a saturating curve: rapid morphological changes characterized by small size values, after which a stable stage is approached.

Morphological variability in small size individuals was high, especially in relation with fast development of anterior and posterior parts of the body (before 40 d.p.h.). The positive allometry of head and tail regions were clearly detectable in Fig 5B, which showed a remarkable strengthening of the tail and a widening of the head (with the correspondent development of organs located in this region, as eyes, mouth etc. (see Fig. 2)). Starting from this stage, the process of notochord flexion was observed, while the anal fin region tended to decrease its area and to move progressively backward, perhaps for the growth of the visceral cavity. Therefore, it was possible to observe the development of the ventral region, although limited to a lengthening corresponding to the backward movement of anus. Instead, the widening of the median part of the body gradually started after 100 d.p.h. (around 18 mm in ***TL***, see Fig. 5C) and became the major shape changes in the second part of the growth curve. At this stage, important shape changes involved also head region, that became sharper during growth, and was characterized by a relative negative allometry (reported to the ventral region that still underwent a positive allometry). Some more, the peduncle became higher and shorter and the body height relatively increased. Globally, these changes brought to a discoidal final body shape, which is reached about 160 days post hatching.

Clustering of Mahalanobis distances calculated on Fourier Coefficients (n=69 harmonics) revealed eight clusters (Fig 6), almost completely overlapped with those obtained from geometric morphometrics: (1) Newly hatched larvae, including only specimens with yolk-sack; (2) Younger larvae, referred to specimens without either yolk-sack nor swim bladder; (2) Preflexion larvae; (3) Flexion larvae; (4) Older larvae; (5) Younger juveniles; (6) Juveniles and (7) Sub-adults and Adults. Defining these groups by means of T_L, it resulted intervals reported in Table 2. Even in this case MRPP reported significant differences between all groups (R = 0.7195 $p < 1\times10\text{-}8$, see Table 1 for intra-group distances), and evidenced that larger distance ran between the group including sub-adults, adults and juveniles and the other stages.

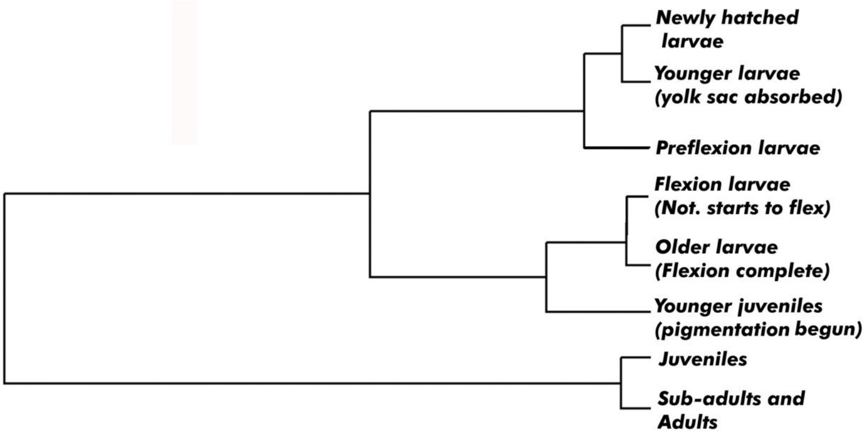


Fig 6. Dendrogram of cluster analysis on elliptic Fourier analysis coefficents.

Fig 7 showed the plot of canonical scores of specimens groupings into developmental stages. Stages seemed to be well defined and not much overlapped, although they were contiguous.

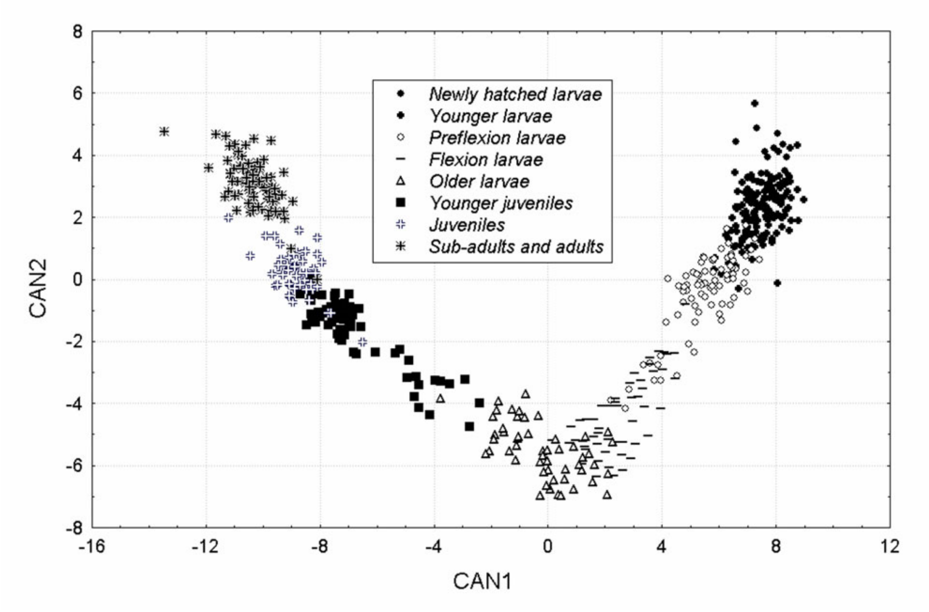


Fig 7. Plot of canonical scores for each developmental stage.

Development and trophic stages

It was directly observed that larvae opened their mouth during the second and third d.p.h. (about 3.6 mm in T_L) and started to feed at the fifth d.p.h. (3.6 mm in T_L). Larvae of this size were capable to ingest a limited number of organisms, mainly small rotifers (*B. plicatilis* S-type) and trocophores of bivalves. Yolk sac absorption was completed when larvae were 3.8 mm in T_L (7 d.p.h.). Near 28 d.p.h., at 7.5 mm in T_L, notochord started to flex and, at 30 d.p.h. (7.8 mm in T_L), flexion was completed. Fin ray formation begun at about 40 d.p.h., when larvae were 10.5 mm in T_L. Attainment of complete fin rays counts was not observed, because internal anatomy observation is necessary for this kind of information. Body pigmentation begun at 70 d.p.h. (13 mm in T_L).

Direct observations about feeding behaviour of larvae were reported in Tab. 3, integrated with literature data. Only small rotifers and trocophores of bivalves (a food items naturally developed in rearing tanks, together with copepods of groups calanoids, cyclopoids and harpacticoids) were detected in gut contests at the beginning of alimentation (*newly hatched* and *younger larvae* stage, 3.19-4.4 mm in T_L).

Growing up, larvae begun progressively capable to feed on larger rotifers and copepods nauplii. They became capable to ingest copepods nauplii and juveniles of Harpacticoids during preflexion stage (4.46-5.5 mm in T_L) and active selection shifted towards these items, while rotifers and trocophores became less important in their diet. At flexion stage (5.58-7.5 mm in T_L), copepods represented the main item and larvae extended their hunting spectrum including Cyclopoids and Calanoids juveniles and then, thought the older larvae stage (7.55-10.5 mm in T_L), adults of these groups, so that copepods became about their exclusive preys. Kentouri & Divanach (1984, 1986) evidenced that reared gilthead sea bream larvae eat selectively if exposed to polyspecific choice and that trophic selectivity changes with growth, although larval preferences could be influenced by the relative availability of different food items. Detecting stage in relation to fish size, these authors put the beginning of feeding at 4.1 mm in T_L, and the limits of first stage at 4.1 and 5.5 mm in T_L, respectively. During this stage, larvae feed almost only on small rotifers and trocophores of bivalves. The upper limit of following stage was detected, from the same authors, at 15.5 mm in T_L. Larvae started to show preference towards copepods juveniles and nauplii, but they still feed almost only on rotifers (small and large). The trocophores of bivalves were less selected. The copepods (nauplii, juveniles and adults) were the preys actively selected from specimens of 10.5-15.5 mm in T_L according to (Kentouri & Divanach, 1986). Tancioni *et al.* (2003) reported observations about wild trophic ecology of *S. aurata* juveniles, sub-adults and adults in the costal lagoons of Fogliano and Caprolace (Central Italy, Thyrrenian sea). These authors divided samples according to fish size class and feeding habits, detecting three groups characterized by different food preferences: younger juveniles ($T_L \leq 30$ mm); juveniles ($T_L > 30$ mm to $T_L < 70$ mm) sub-adult and adults ($T_L > 70$ mm). Younger juveniles fed principally on Nematods, Copepods, and Ostracods and also on polychaetes worms, as also observed by Wassef & Eisawy (1985). Globally, they showed a considerable trophic flexibility. Juvenile's stage corresponded to an intermediate behaviour between fry food preference and adult feeding profile, selecting Polychaetes, Mysidiaces, and Amphipods. Finally, sub-adults and adults essentially based their diet on decapods, bivalves, gastropods and polychaetes (Tancioni *et al.*, 2003), with a clear dominance of bivalves, gastropods and decapods as reported by Pita *et al.* (2002). It was possible to resume the trophic habits, starting from post-larval stage, in a gradual shifting toward larger benthonic preys where the final target of *S. aurata* adults (Decapods, Bivalves) represented the optimum food items due both to their nutritional value and to their accessibility (Pita *et al.*, 2002).

DISCUSSION

Shape changes and morphological stages

The availability of captive gilthead sea bream larvae, juveniles, sub-adults, and adults provided the opportunity to study, for the first time, shape changes of this species through all the development. Growth trajectories (Alberch *et al.*, 1979; Costa, 2004; Loy *et al.*, 1998a; b; 2000) of gilthead sea bream were characterized by means of GM (Bookstein, 1991;

Rohlf & Marcus, 1993; Marcus *et al.*, 1996) and EFA of outlines (Khul & Giardina, 1982; Rohlf & Archie, 1984; Lestrel, 1997; Loy *et al.*, 2001; Costa, 2004). Growth trajectories are aimed at the description of size-related shape changes as result of allometric growth of different part of the body during different period of ontogenesis (Costa, 2004; Osse & van den Boogaart, 1995; Klingenberg, 1996; Loy *et al.*, 1998a; 2000). Shape changes during ontogenesis of *S. aurata* seemed to follow a precise pattern (Fig 2, 3 and 5): both the growth trajectories reported from GM and from EFA closely reflected a saturating growth curve, with a bending zone ranging at about 60 mm in T_L of fish. Changes were very rapid for small individuals and decreased with growth until attaining an apparently stable relationship between shape and size. In effect, the growth trajectory might be interpreted as a two-stage growth curve (Bookstein, 1991), where the first stage was characterized by a strong morphological change for a small size interval, and the second by a change in size not accompanied by a change in shape (isometry). Early larvae have a streamlined body shape, adapted to pelagic life. The body parts initially characterized from a positive allometry were head and tail (Fig 2, 3 and 5), according with findings described for other species (Gisbert, 1999; Osse & van der Boogart, 1995; Sala *et al.*, 2005). These authors explained this pattern inferring that, during ontogenesis, the initial investments are allocated to completion of the most essential organs for primary functions (alimentations and escape from predators). Thus the growth of the head, with attendant growth of eyes, mouth gape and sensory surface, determine better ability in detecting, hunting and ingesting preys (Mérigoux & Ponton, 1998; Osse & van der Boogart, 1995). At the same time, development of tail increase swimming ability, either for feeding and for escaping predators. Together, positive allometry of posterior and anterior parts of the body reflected the need for the swimming at hydrodynamic regimes with high values of Reynolds's number, in order to reduce the drag forces on the body and the costs of transport (Gisbert, 1999; Osse & van der Boogart, 1995). In this way, development of median region appears to be secondary, because a planktonic diet, reported to a benthic or more complex diet, does not need a well-developed digestive apparatus. In the last part of steep portion of the growth trajectory (Fig 3 and 5), fish gradually acquired a rounded shape with a shorter and higher caudal peduncle, suitable for manoeuvring (Webb, 1984a;b). The final body shape was discoid, with a large, rounded, median region, a high and short peduncle and a sharp head, as seen from EFA of outlines. This shape appeared to be similar to the carangiform model (include swimming style) described by Lindsey (1978) and studied and classified by Webb (1984a) and Breder (1926). Some authors (Webb, 1984a, b; Ehlinger, 1990; Loy *et al.*, 2001) associated this shape to manoeuvre fish and were shape features that allowed for quick starts and rapid turns in complex environments such as reef or rocky shores. On the other hand the shape of larvae could be classified as sub-carangiform (Palomares, 1991), which were presumed to be less efficient and powerful than carangiform ones. This shape was associated by Winemiller (1991) to species that has hunt by sight, searching dispersal prays using a regular swimming. The growth of gut length and ventral region, that was partially responsible for attainment of discoid shape, seemed appropriate prior to switch from planktonic to more benthic feeding habits (Osse & van der Boogart, 1995). In this study, the bending zone of the growth trajectories corresponded to the size range (40-60 mm) in which the major habitat transition occurred: according to Bauchot & Hureau (1986) individuals of gilthead sea bream shifted from shallow coastal areas (until 30 m in depth) to deep open sea (until 150 m in depth).

Cluster analysis applied on both GM and EFA data detected two trees (Fig 4 and 6), which were completely overlapped with the only exception of first two stages (newly hatched and younger larvae). These two stages were united into GM tree and separated, but only from little distance, from EFA. Each of these stages seemed to match closely with stages based on very widespread, fundamental features of development, as described in Kendall (1984). In this way, GM/EFA stages have significance in the life history of the fish, both morphologically and functionally, such as a particular type of nourishment or locomotion. The plot of canonical scores (Fig 7) showed that stages were effectively separated and ordinated along growing sequence. Therefore, no jumps could be detected at transition, where instead superimposition of extreme specimens could be observed. This fact suggested that ontogenesis, as described from shape analysis, did not show a saltatory pattern but a sequence of stages that gradually follow each other. However, in many studies saltatory patterns in the development of some biometric measurement were described (Sagnes *et al.*, 1997), but in the same studies, authors also found that other biometry did not show jumps. Considering that shape analysis examined the overall shape variation during growth, it suggested that the effect of saltatory variables was weighted with non-saltatory ones. Therefore, it should be considered that different biometric variables showed not coincident jumps. Thus, our results were not in contrast with literature, rather saltatory patterns in the development of single-considered biometry were compatible with our model.

Correspondence between shape and trophic habits changes

The high flexibility of this species, a non-selective benthic feeder capable to use unusual resources (Andrade *et al.*, 1996), appear clearly at all stages. In spite of this, it could be identified some food items that are selected from individual of a size-class but are excluded to the previous ones and, in the same way, it could be defined several trophic stages, during life history, based on food items preferences (Tab III). Surprisingly, almost all these limits closely overlapped with that evidenced in this study from both GM and EFA and, so, we found an exact correspondence between shape development and trophic niche. This fact allowed us to detect, by size, several thresholds in shape and trophic habits during growth. These thresholds closely divided development, and growth trajectory, as in many stages (Fig 7). The shape changes occurring through each stage were represented by means of consensus of starting stage and vectors (one for each landmark) that described landmarks movement occurring during each stage (Fig 8). During *Newly hatched larvae* stage (Fig 8A), it could be observed the relative enlargement of mouth region, one of the major phenomenon occurring during larval development. It was also described a relative backward of landmark 18 (intersection between lateral profile and the axis passing through landmarks 11, 15 and 16). Both these trends, dealing with positive allometry of head region, increased during *Younger larvae* stage (Fig 8B), and the start of backward movement of the anus and the relative shortage of body (see vector of landmark 5) was also observed. During these first two phases, larvae fed almost exclusively on small rotifers (i.e. *S. triophthalma* or *B. plicatilis* S-type) and bivalves trocophores (see Tab III).

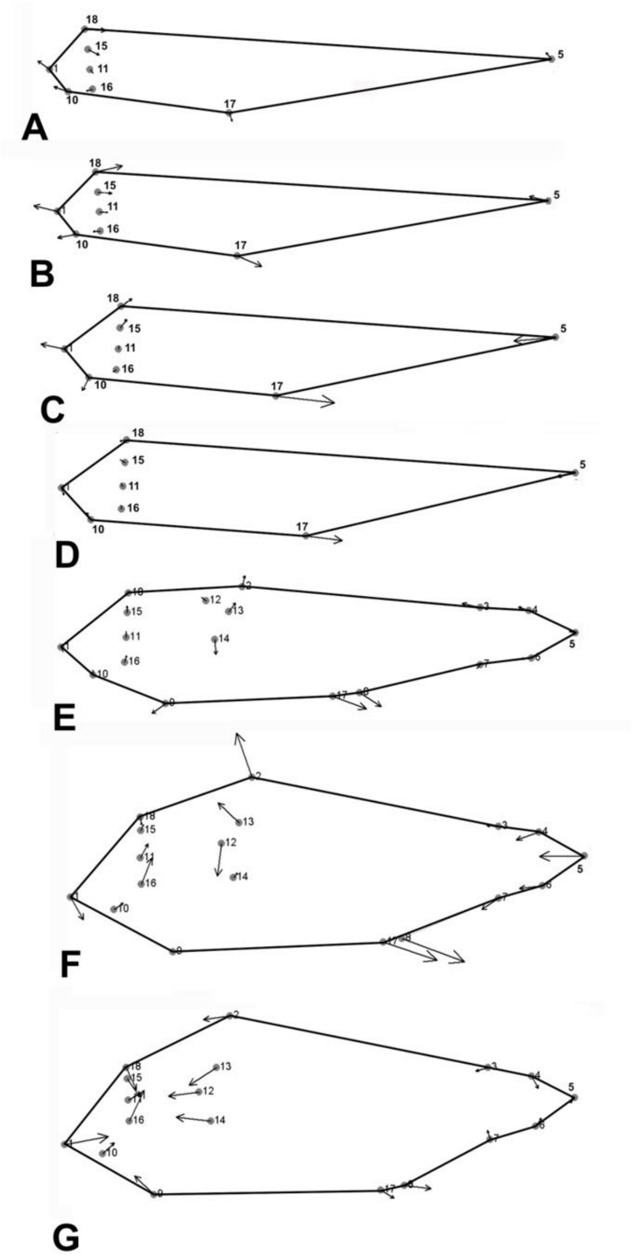


Fig 8. Scatter of landmarks vectors, after Generalized Least Square fitting, is reported for each developmental stage detected from cluster analysis (excepting the last): (A) ***Newly hatched larvae;*** **(B)** ***Younger larvae;*** **(C)** ***Preflexion larvae;*** **(D)** ***Flexion larvae;*** **(E)** ***Older larvae;*** **(F)** ***Younger juveniles*** **(G)** ***Juveniles*****.**

Last two trends in shape changes increased during transition into the *Preflexion larvae* stage (Fig 8C), but movement of landmarks 10 and 18 became oriented about dorso-ventrally, increasing their effect in head growth- although absolute length of vectors are shorter than during previous stage. Larvae showed a growing preference for copepods juveniles and large rotifers (*B. plicatilis* L-type) and a decrease for small rotifers and bivalve's trocophores. Only the relative backward movement of landmark 17 was detectable during *Flexion larvae* stage (Fig 8D), while shape changes involving head region stopped. At this stage, larvae preferred copepods but they still fed on rotifers. It is important to underline that, during previous stages, landmarks data did not provide any information about tail growth, due to absence of fin. Central region of the body represented the portion interested from major shape changes during the *Older larvae* stage (Fig 8E): the progressive upward of the region between the operculum and the anterior anal fin insertion clearly appears on movement of landmarks 2, 8, 9 and 17. Copepods are the only food item actively selected in this phase. Kentouri & Divanach (1986) already reported that hunting efficiency increased with growth, starting from stage I when is possible to observe a lot of overflow events and a decrease just in stage IV when all attack culminated with successful ingestion. Within copepods groups, Harpacticoids (nauplii, juveniles and adults) were the first item in the diet, probably because they represented the most accessible copepods group due to the fact that calanoids and cyclopoids have large appendices that exceeded mouth gape of larvae, as evidenced for other species (Economou, 1991; DeVries *et al.*, 1998; Bremigan & Stein, 1994). Assessment of adult shape character involved principally acquisition of a discoid body shape, as evidenced from vectors of landmarks 2, 8 and 17 during Younger juveniles stage (Fig 8 F). Cataldi *et al.* (1987) reported that several structure of sea bream alimentary canal, as mouth, undergo many important modifications from larval and post-larval stages (when the diet is mainly composed of plankton captured at sight) to the adult stage in which the food range is much wider and includes bivalves, mollusks, polychaetes, crustaceans etc. These authors evidenced that the digestive tract of adult sea bream, and particularly the dentition, are structured to perform a wide adaptative trophic range, in order to allow this species to colonize from coastal marine environments to confined lagoons. Belly length and growth of median/ventral region was the most prominent mensural character to discriminate stages during growth in the second part of growth trajectories reported in this study. This fact probably reflected an accelerating development of digestive organs, in particular the intestine and hence intensified feeding and growth. At the same time, new changes involved head and caudal region. The relative shortage of eye that moved up and a rotation of mouth extremes, that changed orientation and insertion of mouth, were observed. Meanwhile, caudal peduncle became shorter and thickset. Finally, during *Juveniles* stage, the extension tendency of ventral region decreased, such as modifications of caudal peduncle. Some important changes involved head region, that relatively contracted, both in terms of extension (relative forward of operculum landmarks, relative backward of landmark 1, relative upward of landmark 9, and relative downward of landmark 18) and in terms of dimension of some structures as eye and mouth, which became relatively shorter. Other authors (Sagnes *et al.*, 1997) previously found that shifts in habitat use or in resource use occur simultaneously with sudden morphological jumps, corresponding to predictions of the theory of saltatory ontogeny proposed from Balon (1985). Although this study did not examined systems and organ development but only shape changes during growth, several thresholds, that represent transitions from one quality of the organism-environment interaction to another, were found. In effect, passing through such thresholds, the organism achieved new ecological character as defined in term of trophic niche.

Thus, this study not only contributed to developments in the theory of saltatory ontogeny, but also would to establish a landmark, in the framework of ecomorphological study, for the analysis of relations between form (shape and size) and ecology in fishes, mainly considering the advantages offered from use of geometric morphometrics tools.

5. Applications of Self-organizing maps for patterning of changes in shape and trophic habits during larval ontogenesis of two fish species

INTRODUCTION

Ecomorphology is a comparative discipline that investigates relationships between shape (or form) and ecological features of the organism, species or community (Motta *et al.*, 1995a). At the scale of individuals, which are the object of research within a species, it looks for interactions between morphological type, as defined in a variety of ways, and ecological characters, as defined in qualitative or quantitative terms. The basic concept of this investigation is that inter-individual morphological variation leads to different performances, resulting in a different use of the environment and of the available resources and ultimately, that ecological constraints determine morphological changes over evolutionary time and over the life span of the organism through use-induced changes in the morphological characters (Motta & Kotrschal, 1992; Motta *et al.*, 1995a).

Fishes have historically been chosen as the subject of these studies due to the remarkable morphological plasticity of this group of vertebrates, and to their capability to colonize every kind of aquatic environment (Matthews, 1998). Besides, in contrast to other groups of vertebrates, during ontogenesis they often undergo important changes in body form and, in parallel, of their habitat, trophic habits, behaviour and physiology. It was consequently deemed of interest to investigate correspondences between morphological and ecological features in fishes leading to important theoretical models of development, such as Balon's theory of *saltatory ontogeny*. Nevertheless, classical studies have been based on qualitative description of the development of much apparatus for and/or many surveys of anatomical measurements (Motta *et al.*, 1995b; Luczkovich *et al.*, 1995; Wainwright & Barton, 1995). The classic morphometric approach often resulted in a subjective and partial analysis that did not provide an effective and realistic overall representation of the form of the organisms. In fact, although selected morphological variables have known consequences, for example, on the feeding performance, we cannot closely determine and quantify the relative importance of inspected variables with respect to the overall complex of morphological adaptations. This aim could be now pursued thanks to the development of shape analysis techniques, that have been tested as powerful tools both in the representation of organisms and in the quantitative description of shape, allowing statistical comparisons to be made between different samples, conditions, etc. (Simonović *et al.*, 1999; Trapani, 2003; Loy, 1996; 1998a). Thus, the comparison of shapes is performed in two steps: the description of shape, and the comparison of these descriptions versus each other or versus other selected descriptors, such as ecological variables. Although the commonest approach to the quantitative definition of biological shapes is the use of measurements or angles taken between defined morphological landmarks, an alternative landmark-free method might be preferred in some situations, e.g. when available landmarks are scarce or not well-defined (Johnson, 1997). Such an approach is the Elliptical Fourier Analysis of Outlines, which are recorded as a sequence of coordinates along the profile of the biological sample. The advantages and drawbacks of this approach are extensively reported in Lestrel (1997). Applications of this approach were recently made to the study of fish growth in several species (see other sections of the present thesis) which also confirmed the usefulness of this method in the investigation of the relationships between shape and ecological characteristics. All the same, the comparison between shape, as described by means of shape analysis tools, and ecological features like trophic habits or habitat, often surveyed as qualitative or quantitative discrete patterns, is affected by the fact that these aspects are inhomogeneous, thus precluding a direct visualization, abstraction and interpretation of their complex relationships.

A new approach to the identification and visualization of shape patterns versus ecology variation could be represented by the use of artificial neural networks, known as Kohonen's Self-organizing maps (SOMs). This method has proved useful in pattern recognition and classification (Kohonen, 1984; 1997), since it is based on the underlying variance structure in the input data (Dayhoff, 1990). In addition, SOMs are recommended in the analysis of ecological and biological data that are often non-linear, complex, and characterized by internal redundancy and noise (Park *et al.*, 2003). SOMs are particularly suited to the identification of characteristic patterns of continuous and dynamic processes in complex datasets containing high temporal variability, allowing the visualization of a meaningful two- or three-dimensional model of the input dataset and of the superimposition of all secondary variables (Park *et al.*, 2003).

The present work examines the application of SOMs to the data obtained by shape survey of two fish species from the time of hatching to the end of the larval stage, in which shape is described by the Elliptic Fourier Analysis (EFA) of outlines. In parallel, the trophic behaviour of the same specimens in polyspecific choice conditions was observed and analysed and the latter data were combined with the former. The aims of this study were to: 1) examine the feasibility of the use of SOMs in the visualization and analysis of shape data collected by EFA; 2) visualize and interpret the patterns produced by SOMs; 3) map the data of trophic habits upon SOM output and search for any correspondence between shape patterns and diet variations.

MATERIAL & METHODS

A total of 362 specimens of sea bream (*Sparus aurata)* and 199 of dusky grouper (*Epinephelus marginatus)* were used in the present study. Samples came from two rearing experiments, but all specimens, originated from artificial spawning eggs of brood stock, were reared with a semi-intensive approach using large volume tanks (60 m^3 in volume, diameter 8 m, water height 1.2 m), and were fed with a mixed diet of live food, either reared in parallel cultures or collected from natural sources, and supplied in the rearing tanks. Larvae of sea bream were allowed to hatch and thereafter were reared in the "Valle Figheri" hatchery (Venice, Italy). Larvae of dusky grouper originated from artificial spawning eggs of brood stock raised in the Ittica Mediterranean hatchery (Sicily, Italy) and reared in the hatchery of SMEG located in Latina (Rome, Italy). Specimens were collected at different ages. Each specimen was photographed with a Polaroid digital camera (24-bit true color images, 16 million colors, resolution 1600 × 1200) and the images collected were analysed with morphometric software (http://life.bio.sunysb.edu/morph/).

Shape reconstruction: Elliptic Fourier Analysis

The outline was collected on each specimen as a coordinates profile (software TpsDig, Rohlf, 2001a; Fig 1), excluding fins, and Elliptical Fourier function (Kuhl & Giardina, 1982) was used to fit specimen outline. The Fourier series can be expressed as:

$$F(\theta) = a_0 + a_1\cos\theta + b_1\sin\theta + c_1\cos\theta + d_1\sin\theta + a_2\cos 2\theta + b_2\sin 2\theta + c_2\cos 2\theta + d_2\sin 2\theta + \ldots$$
$$+ a_k\cos k\theta + b_k\sin k\theta + c_k\cos k\theta + d_k\sin k\theta$$

where a_0 is the constant, a_1, c_1 to a_k, c_k are known as cosine components, b_1, d_1 to b_k, d_k are known as sine components, and $F(\theta)$ is the magnitude of a polar radius r for the nth radius. The Fourier series can be considered as a series of instructions to deform a basic geometric shape, that is a circle, into a more complex one (that is the shape of an organism), where the first term, a_0, is the radius. Each of the successive harmonics, with their four coefficients, represents an additional deformation towards the final shape, which progressively becomes two-, three-, four-, five-lobed, etc. as the number of harmonics increases (Johnson, 1997). Before computation, outlines were standardized for size by dividing coordinates by the length of the perimeter of the outline contour. The Fourier coefficients for a polynomial function of the 15th degree were computed using Morpheus software (Slice, 1998) with EFA (Kuhl & Giardina, 1982; Fearson *et al.*, 1985; Rohlf, 1990). The number of harmonics for outline decomposition, that is the optimum length for the Fourier series, was chosen with the following procedure, according to Johnson (1997): 1) EFA with n harmonics was computed on outlines of specimens of all three species; 2) a cluster analysis on the harmonics coefficients was trained in order to agglomerate specimens and 3) the number of misclassified specimens was counted, considering the tree level corresponding to three clusters. This procedure was repeated ten times for different values of n (5-10-15-20-25) Fourier coefficients, and the number of misclassified specimens was plotted against the number of harmonics in order to find the minimum. This value was found to be 15 and so, after computation, each fish specimen was described by 60 new variables. The matrices obtained this way (one for each species) were used for training two SOMs.

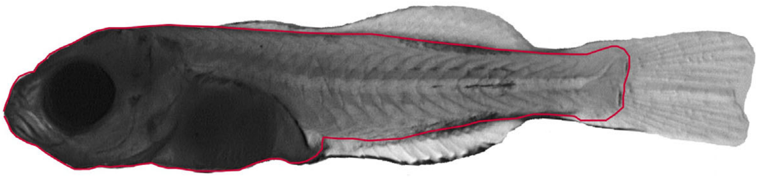


Fig 1 - Outline collected on sea bream specimens.

The self-organizing maps

SOMs were used in this study with the basic idea of displaying the high-dimensional datasets of fish larvae shape described by EFA in a 2-dimensional space: this implies a non-linear projection onto a rectangular grid laid out on a hexagonal lattice with *E* hexagons. The Kohonen neural network consists of two layers: the input layer, connected to each vector of the dataset, and the output layer, consisting of a two-dimensional network of neurons (the units of the map) arranged in a square grid. Each unit of the map is associated with a vector of weights, one for each input variable, i.e., in this study, the coefficients of the harmonics. During SOM training, only the input layer is used, so that this procedure is defined as "unsupervised". The SOM algorithm proceeds by generating a virtual shape unit (VU – the elements of output layer) for each hexagon of the map. The VUs are computed in order to put the sample units (SU - that is the shape of each specimen and constituted the input layer) on the map and preserve the neighbourhood, so that similar shapes should map close together on the grid. The learning procedure is an iterative sequence of instructions repeated for a fixed number of epochs, measured by the time parameter *t*. The procedure could be summed up in 5 steps: 1) t=0, when the VU_k are initialised with random samples drawn from the input dataset; 2) A sample unit SU_j is randomly chosen as an input unit; 3) the distance between SU_j and each VUs is computed using some distance measurement; 4) the VU_c closest to the input SU is chosen as the best matching unit (BMU); 5) the VU_js are updated by applying the rule:

$$w_{ik}(t+1) = w_{ik}(t) + h_{ck}(t)\lfloor x_{ij}(t) - w_{ik}(t)\rfloor$$

where w are the weights of the VU (in this case w are the coefficients of harmonics) and h_{ci} is the neighbourhood function; 6) t=t+1 and steps from 2 to 6 are repeated until t=t_{max} . The neighbourhood function defines the extension of the VU range that was updated at step 5 and, in this study, was chosen to be gaussian. Moreover, neighbourhood shrinking and learning rate decay were chosen to be exponential. The City-Block (Manhattan distance) was chosen as distance measure and several parameters, such as the number of training epochs and map sizes (number of output units, distributed in rows and columns), were established by training SOMs with different values of these parameters and choosing an optimum based on the minimum values of quantization and topographic errors (Park *et al.*, 2003; 2004). The number of epochs was established with respect to the stress of the grid and was set to1000 in all cases. Consequently the fine-tuning epochs were set to 500. At the end of the training, the weights of each VU, which corresponded to a series of 60 artificial harmonic coefficients, were transformed back into outlines that can be regarded as “shape prototypes”.

In order to distinguish subsets on the trained SOMs, the units of the maps were grouped according to the similarity of the weight vectors of the neurons. Two methods were used to divide the trained SOMs units into several subgroups. First, the unified distance matrix algorithm (*U*-matrix) was applied. The U-matrix calculated distances between neighbouring map units, which could be visualized as representing clusters using a grey scale display on the map. An agglomerative clustering algorithm (Manhattan distance - Complete linkage) was used to define groups of map units, which were visualized by means of different shades of grey and outlined by a thicker line (as boundaries). Then, a k-means method (Legendre & Legendre, 1998) was applied to the trained SOM maps to confirm the subgroups divided by the *U*-matrix. The best number of clusters was selected as input for *k*-means using the Davies-Boldin index (DBI; Park *et al.*, 2003). The lowest values of DBI defined the optimal number of clusters. The clusters of map units (i.e. of "shape prototypes") obtained in this way were then visualized.

Trophic ecology of fish larvae

To study feeding ecology, all specimens were analysed by collecting the stomach contents or, in the first larval stages, the anterior 1/3 of the gut. Each food item was identified and counted using a stereomicroscope. Therefore, when approaching the ecomorphological study of the correlation between diet and shape, we preferred to classify preys not in taxonomical units but by means of artificial, general groupings related to their ecology, habitat, vagility and escape response (Linde *et al.*, 2004). We established these groupings considering that, in this work, we were examining the relationships between fish shape and prey type during larval life, and so, the fish size /prey size ratio was a critical aspect. In this way, we divided preys into: (1) ***small planktonic***, including rotifers, bivalve trocophores and other small, less actively swimming items; (2) ***medium planktonic***, including copepods, nauplii and juveniles, and smaller ostracods (bosmins); (3) ***large planktonic***, including adults of copepods (either calanoids or harpacticoids) and large ostracods (such as *Dafnia magna*) (4) ***mobile benthic***, referring to the organisms living on the surface of the bottom, but not sessile, such as nematodes, crabs and larvae of chironomids. Thus, each fish specimen was defined by four values of as many binary vectors. Each vector reports whether some preys of the selected category were found or not in the gut of the specimen considered. These vectors were subjected to the trained SOMs, and then the mean value (E_V) of each food vector in each output neuron was calculated. The mean value was computed as:

$$E_V = \frac{1}{n}\sum_{i=1}^{n} e_i$$

where n is the number of input vectors assigned to each output neuron of the trained SOMs, and e_i is the value of each food vector of input vector i. If the neuron was not occupied by input vectors, the value was replaced with the mean value of neighbouring neurons. Finally, each vector was visualized upon the SOM in a grey-scale picture based on the distribution of specimens for each vector.

RESULTS

The shape

For each of the two species studied, Fig 2 shows the total length trend of the specimens. It seems that no elapses were present.

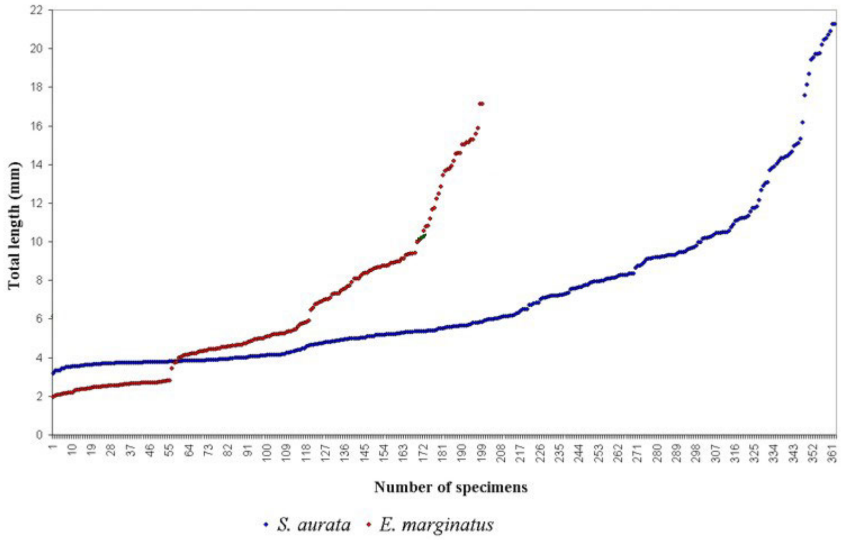


Fig 2 – Graph of T_L of specimens.

The number of SOM output units after preliminary trials with different map sizes was chosen as 48 (6 × 8) on a two-dimensional hexagonal lattice. The trained SOMs classified samples (specimens) according to the variation observed in harmonics coefficients. Three different types of SOM output were reported: the first showed the maps with the numbers of the specimens stored in each SOM unit, where the number was a label that identified each of the specimens along a series ordered by size (T_L); the second showed the virtual outlines computed for each SOM unit from weights calculated during training. The outline prototypes represented an abstraction from the information stored in the input data, and their use allowed the effective visualization of shape changes; the third showed the results obtained from the application of *U*-matrix hierarchical agglomerative clustering algorithm and partitive clustering using k-means. Fig 3 shows the linkage distance plotted against the number of steps, indicating the threshold levels at which the agglomerative procedures were stopped. Finally, four maps with the superimposition of food vectors were reported. To do this, the mean value of each food vector was calculated in each output neuron of the trained SOMs, and then each vector was visualized on the trained SOM map in which a dark area represented a high value, and a light one a low value.

Figures 4, 5 and 6 show the maps computed for the two species studied. Fig 4A shows the map of sea bream in which the specimens basically seem to be classified according to their size. It was possible to observe the smallest specimens in the upper right area of the map, whereas the largest specimens was located on the upper left area. A group of SOM units to which no specimens were assigned separated these two extremes. It was actually possible to detect on the map a trajectory that started from the upper right side, descended, then turned left and finally climbed up to the upper left side. Globally, the trajectory of size-ordered specimens has a **U** shape, in which the extremes are close together but separated from several empty units of the map. Fig 4B shows the virtual outlines computed from weights for the sea bream map. According to Fig 4A, the map reveals an effective pattern in shape development: the trajectory seems to be composed of shapes that have undergone a sequence of changes that is consistent on the whole with what we know about this species. The outlines in the upper right area are characterized by a less developed tail and median region of the body, whereas the head appears to be the relatively laegest part of the body. These characteristics are typical of newly-hatched and younger larvae of *S. aurata*. At the bottom of the map, moving from right to left, a series of shape changes involving the tail can be seen, showing a relatively positive allometry, while the gut has grown in length and the anus has moved backward. The beginning of the notochord flexion can be detected at the vertical boundary between the third and fourth column of the map. In the upper left area, the outline of a well-developed median region of the body is found, and the growth of this body part was actually the largest shape change detectable in the transition from the lower left side to the upper left one. Therefore, it is possible to observe the initial negative allometry of the head region and the shortness of the tail length, whereas the height of the peduncle increases. All these observations agree with those coming from cluster analysis as reported in Fig 4C. The *U*-matrix algorithm aggregative procedure merged map units with the same shape features and, in particular, the groups seemed to closely follow the shape changes described in the analysis of outline prototypes: the group I seems to merge map units with outlines corresponding to new and younger larvae, whereas groups II, III and IV correspond, respectively, to preflexion larvae, flexion larvae and older larvae. The visual intra-group homogeneity of the shape of the outlines appears clearly, and the clusters defined by the *U*-matrix and *k*-means agree with each other.

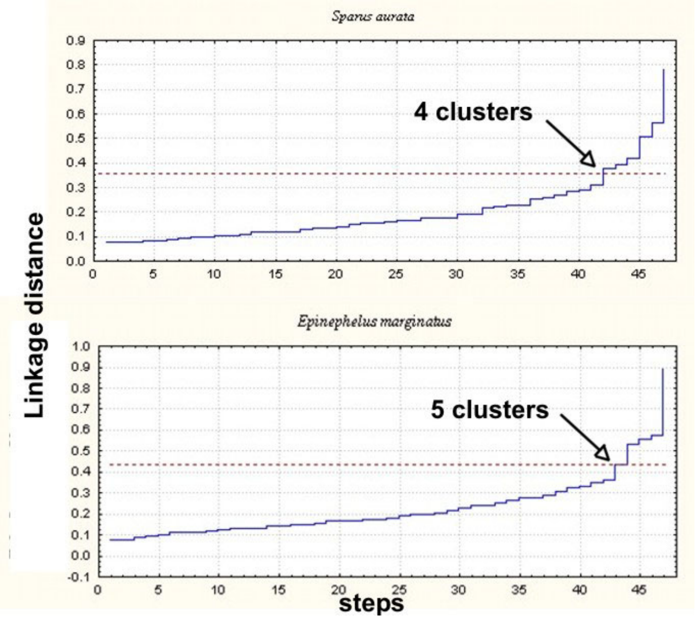


Fig 3 – Plot of linkage distance calculated during the training of *U*-matrix hierarchical agglomerative clustering algorithm.

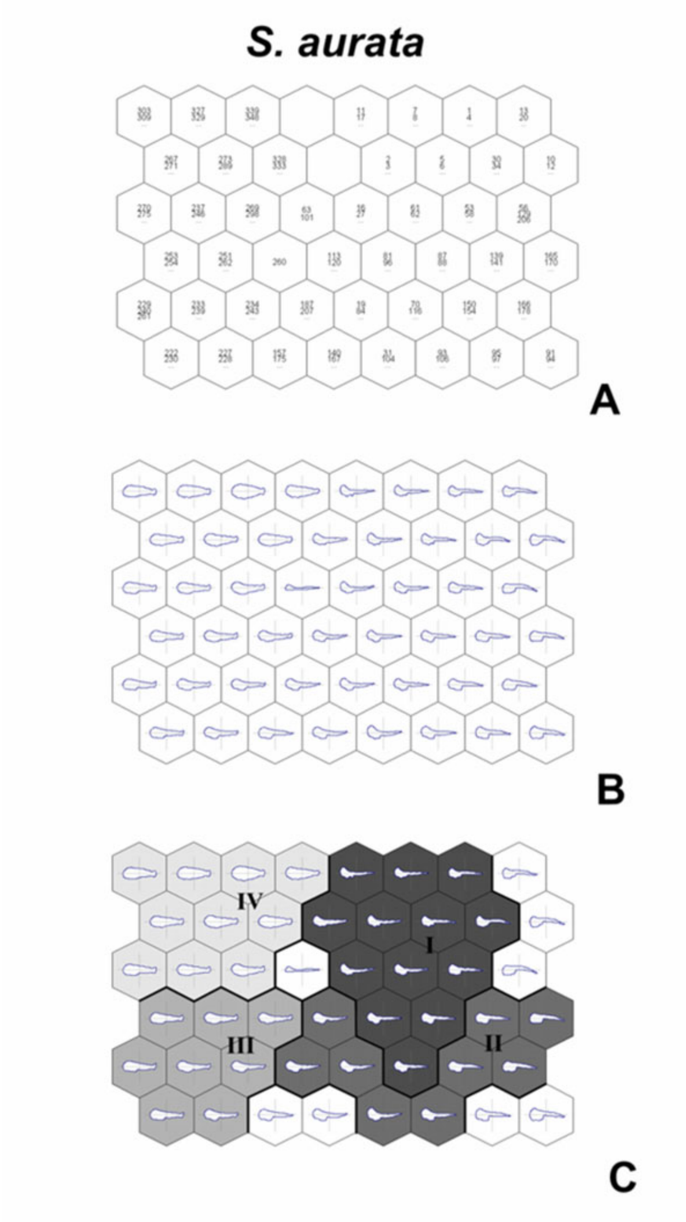


Fig 4 – A: Trained SOM for sea bream specimens, in which label indicated the number of input specimens along a growing size scale; B: The same map in which virtual shape prototypes were reported for each units; C: Clustering of SOM units. The Latin numbers (I-IV) in different grey scales displayed clusters.

Although the sequence started from a different side of the map, a pattern similar to that of sea bream is found in Fig 5A, which shows the output map of the dusky grouper specimens. In this case, the younger specimens are located in the lower left hand corner of the map, and the following stages are found by moving first right, then up and finally turning to the left. Thus, in this case the trajectory seems to have a left-rotated **U** shape, and even in this case, a series of empty units separate the extreme of the trajectory. Likewise, the analysis of outline prototypes (Fig 5B) allowed us in the bottom left area of the map to detect outlines with streamlined body, less-developed median and tail regions, and large head. Moving right, the shape changes begin, especially involving head and tail, which grow more than in the median region of the body. Then, turning left and moving up, it is possible to observe the start of the growth of the median region of the body, which became progressively higher, while the notochord started to flex. Starting from the upper right hand area of the map and moving left, the rearward position of the anus and the growth of the peduncle appear to be the most significant shape changes, together with the cessation of notochord flexion. Finally, as we approach upper left hand area of the map, it is possible to observe the negative allometry of the head, the increase in height and the shortness of the body and the further strengthening of the peduncle and the tail. Fig 5C shows the result of applying the clustering algorithm. Also in this case, as observed for sea bream, a close correspondence was found between the developmental shape changes described and the composition of the groupings. In group I, there were units, the outlines of which were reminiscent of the shape of younger larvae. The group II and III shape prototypes corresponded to those of preflexion and flexion larvae.

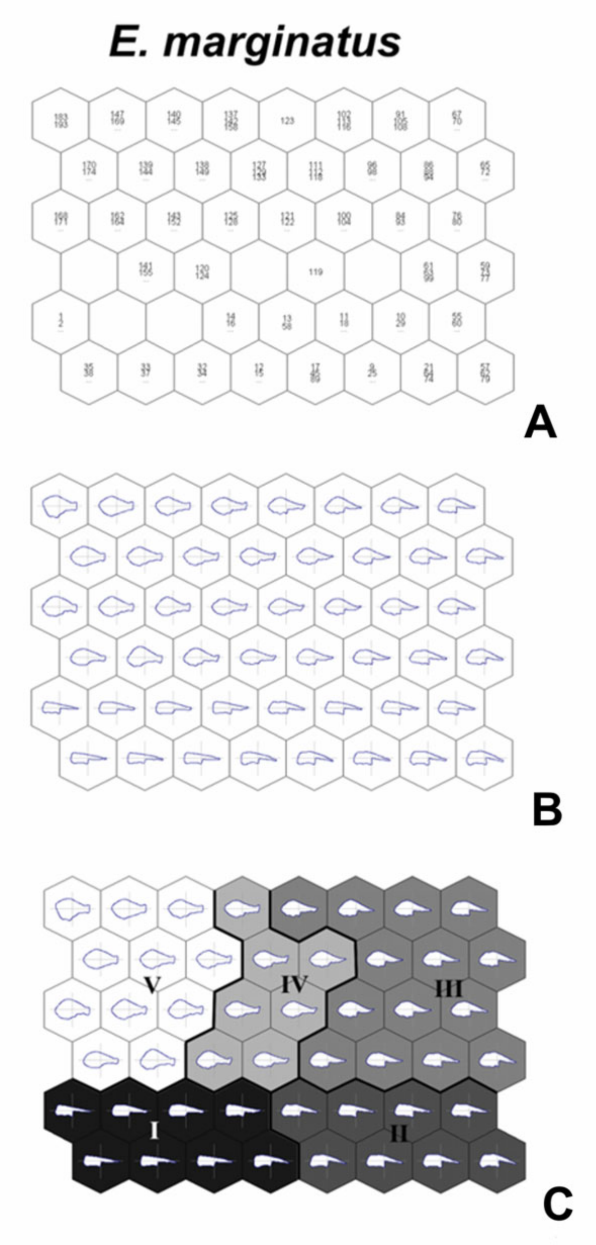


Fig 5 - A: Trained SOM for dusky grouper specimens, in which label indicated the number of input specimens along a growing size scale; B: The same map in which virtual shape prototypes were reported for each units; C: Clustering of SOM units. The Latin numbers (I-IV) in different grey scales displayed clusters.

Group IV contained outlines with almost complete notochord flexion and, finally, group V merged units that showed outlines, the shape of which closely resembled that of juveniles of this species. Also in this case, the clusters defined by the *U*-matrix and *k*-means agreed with each other.

The trophic habits

An evaluation of the approximate probability of trophic choices was calculated as the mean value for the specimens assigned to each SOM unit or as a weighted mean of the neighboring units in case no specimens were assigned to a particular SOM unit.

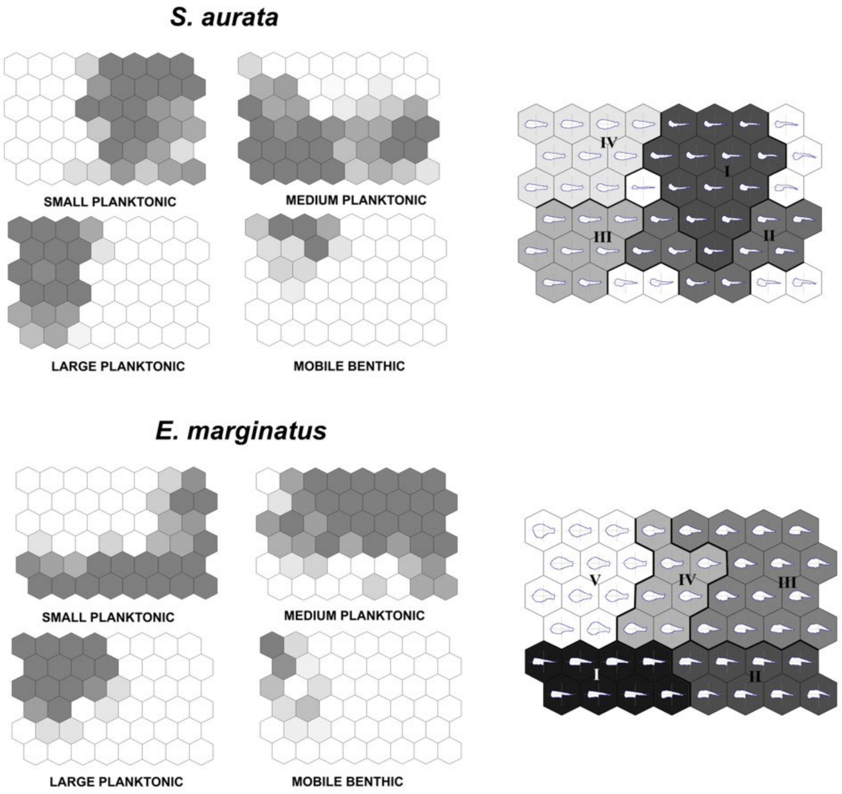


Fig 6 – Visualization of the food vectors on the trained SOM maps. The mean value of each vector was calculated in each output neuron of the trained SOMs. Dark represented a high value, while light was low.

These values were then represented on the SOMs by means of shades of grey. Darker units represented a higher probability of finding that particular category of food items in the gut of specimens assigned to the SOM units considered, whereas lighter units indicated a lower probability. Visualization was an efficient way to analyze correspondences between groupings (and related boundaries) constituted by shapes and changes in trophic selection during ontogenesis. Considering the sea bream samples (Fig 6A), a good matching was observed, respectively, between group I and small planktonic preys, group III and medium planktonic preys, group IV and large planktonic preys. Group II seemed to be characterized by specimens with a mixed diet based on both small and medium planktonic items, thus appearing to be a transitional group. Mobile benthic preys were selected from fishes stored at the upper right side of group IV. Fig 6B shows the data for the dusky grouper, in which small planktonic preys match groups I and II, medium planktonic preys groups III and IV and large planktonic preys group V. Mobile benthic preys are located on the upper left side of the map, in group V. Finally, the information shown in different visualizations of the same maps (Fig 6 – small multiple, see further on) could also be summarized in single 3D images (Fig 7 and 8). These are interesting results because they allow a direct visualization and understanding of patterns regarding both shape and food vectors. In addition, they are very easy to read and attractive.

DISCUSSION

As already evidenced in several other studies (Park *et al.*, 2003; 2004; Gevrey *et al.*, 2004; Hardman-Mountford *et al.*, 2003), SOMs are a powerful means to visualize and interpret the information underpinning complex datasets, and their adaptive learning algorithms provide an exhaustive (and easily readable) description of patterns. The SOM forms a low-dimensional map of training data, and the grid can be used as a convenient visualization platform for showing different features of the SOM (and thus of the data). The goal of this procedure is to present a large amount of detailed information in order to give a qualitative idea of the "properties" of the data. Typically the number of "properties" that need to be visualized is much higher than the number of usable visual dimensions, so that it is impossible to show them all in a single figure. The solution is to use a number of figures (each of which is a representation of a SOM map) that have been linked together so that one can immediately identify the same object from different figures. When several visualizations are linked in the same manner, scanning through them is very efficient because they are interpreted in a similar way.

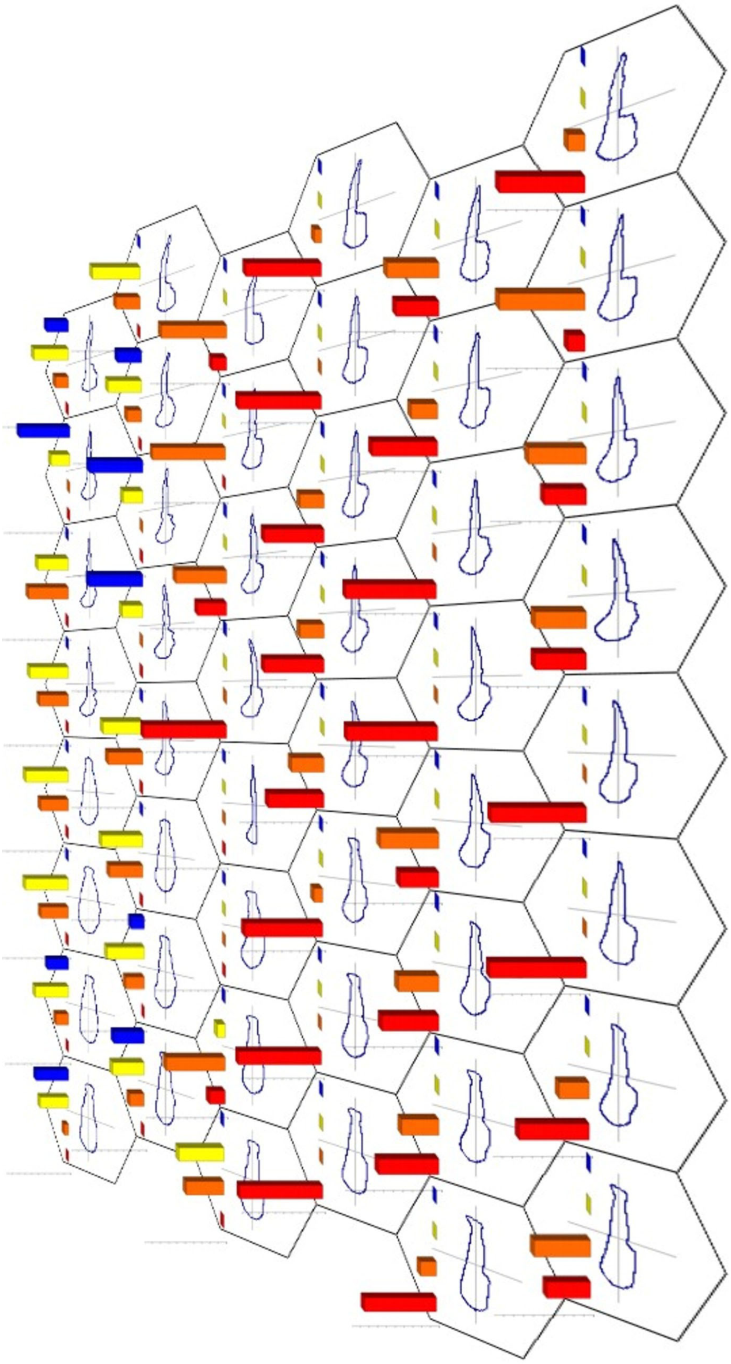


Fig 7. 3-D representation of small multiple for sea bream (*S. aurata*): each units of the trained SOM contains shape prototype and the probability density of feeding habits defined by food vectors.

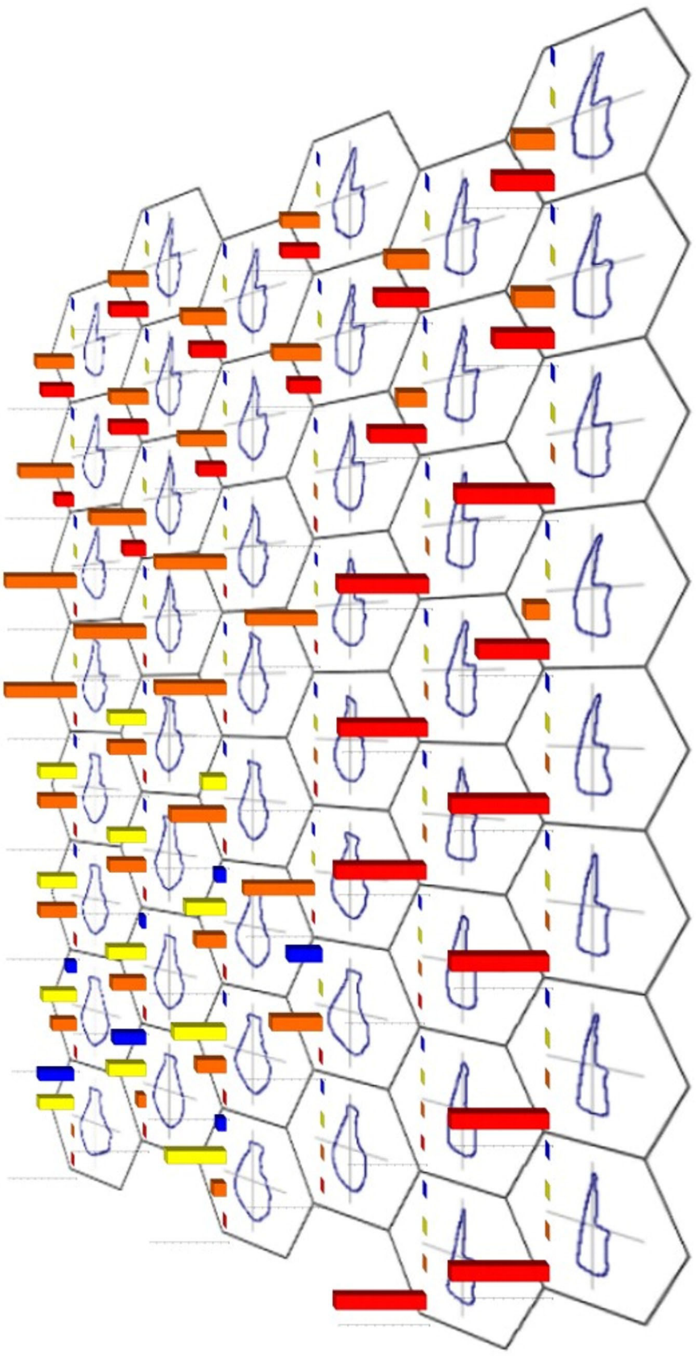


Fig 8. 3-D representation of small multiple for dusky grouper (*E. marginatus*): each units of the trained SOM contains shape prototype and the probability density of feeding habits defined by food vectors.

This kind of approach is called the "small multiple" approach. Objects in small multiples can be linked together using their similar position: each object is in the same position within each multiple (see Fig 6). Nevertheless, in classical applications of SOM to the classification of ecological datasets (Park *et al.*, 2004; Scardi, 1996; Scardi & Harding, 1999) no provision is made for the extraction and direct visualization of the information stored in each map unit. In this way, virtual species composition of each map unit is represented by means of grey scales in a small multiple approach, so that the descriptive meaning of each unit is still not directly readable and, therefore, paradoxically, cryptic. In this study we designed a type of SOM that, as well as storing data (fish specimens) in map units, also visualizes the virtual information of each unit as *shape prototypes*. This is possible because the input dataset contained the description of the shape (as an outline profile) of specimens, and then the starting data are themselves a visual representation of biological objects. The main advantage of this approach is that it not only allows us to efficiently describe such virtual shapes, and thus the properties of each map unit, but it also enables us to directly connect each map unit with a well-defined life-stage condition, that is a step along the ontogenetic trajectory of shape development. This approach resulted in a very exhaustive analysis of input data pattern, as described in the results. For both species studied, SOM training produced maps that organized specimens in a series of growing size (T_L) (Figs. 3 and 4). This allows a time/size sequence of units to be detected in the trained maps. The beginning and end of such sequences are located near each other, but are separated by empty units. Therefore, the elastic network formed by the weight vectors is oriented along with the probability function, so that the sequences are oriented in their horizontal directions in the rectangular maps.

Shape prototypes appear to be located on the map on the basis of their similarity. They seem to reproduce shape characters specific to each life-stage, but they also seem able to depict deformations and single characters of each specimen that are considered as noise. In this way, SOM analysis appears to be a robust approach to the abstraction of shape profile of each life stage, and a powerful means of summing up shape characters that have ecomorphological relevance. Analysis of shape prototypes efficiently allows the detection of several shape changes (e.g. the allometries of the different parts of the body). Therefore, SOMs were used and tested for the comparison of shape against the feeding preference of fish, which is one of the major ecological features in the framework of ecomorphological studies. Food vectors showed gradient distributions that are coincident with knowledge on the trophic ecology of these species. This fact confirms that SOM shows high performance in analyzing the relationships among variables used in the map training and external variables such as, in this case, food item preferences.

Finally, we further note the great power of the representation of shape prototypes, and suggest a similar approach should be followed in other cases in which input data can be visualized as figures or other explanatory representations.

6. General discussion

This research could be placed in the framework of ecomorphological and ecological investigations on larval stages of fish. In particular, this work focused on changes in feeding habits and body shape during the ontogenesis of two marine fish species. It is an overview of several features of the indirect ontogenesis in fish, and therefore represents a new chapter in the debate about the developmental processes of life history of fish and the adaptative meaning of changes in body shape (Alberch *et al.*, 1999; Balon, 1985; Blaxter, 1965; 1986; Boglione *et al.*, 1999; De Vries *et al.*, 1998; Gerking, 1994; Gisbert, 1999; Hunter, 1981; Kendall, 1984; Loy *et al.*, 1998b; 2001; Luczkovich, 1995; Mérigoux & Ponton, 1998; Morato *et al.*, 2000; Osse, 1990; Osse & van den Boogaart, 1995; 1999; Osse *et al.*, 1997; Sagnes *et al.*, 1997; Sala *et al.*, 2005; Simonović *et al.*, 1999; Stoner & Livingston, 1984). These studies were historically limited by the difficulties in collecting exhaustive samples series in the field, while the use of specimens from captive conditions should be adopted with caution because cultured fish could differ from wild cohorts in behaviour, morphology and physiology. All the same, recent progress in hatchery technology provide a new approach which seems to be a powerful means in order to produce "wild like" fish. The basic idea of this approach is that captive conditions should reproduce, as best as possible, natural conditions in the nursery area, with the exception of predators presence and food shortage (AA.VV., 2001). Thus, the use of F1 generation from local wild broodstock and the reproduction of appropriate environmental stimuli lead to the recovering of morphological and behavioural repertoires in cultured stocks. Recently (Cataudella *et al.*, 2003), the positive features of this technology - from the low incidence of skeletal anomalies to the high resistance towards common pathologies empirically observed by farmers - were proposed to support the hypothesis that fish reared by this approach could be used as model in biological investigations.

Feeding preferences and mouth gape limits of dusky grouper

Thereafter, the possibility to simulate several natural conditions, as the availability of wild preys and the constant presence of live food in the tanks, was the premise for performing feeding trials and observations on trophic ecology of larvae (see Chapter 1 of the present thesis), which are particularly important to improve our knowledge in order to domesticate species new candidate for aquaculture. The condition of polyspecific choice, and the survey of a large number of samples, allow the detection of the food preferences of larvae, and then of a sequence of live preys which should be supplied to obtain good survival. In addition, the observations on morpho-mechanical aspects of mouth function in larvae, via a functional morphological approach, lead to the understand of the role of this structure in determining food selection.

Larval shape development of dusky grouper: geometric morphometrics tools and elliptic Fourier analysis.

Chapter 3 reports the first work in which shape analysis tools were applied to describe larval shape development of a fish species. The larval development of *Epinephelus marginatus* (Lowe, 1878), a threatened marine fish species was surveyed using two types of morphometric approaches, geometric morphometrics and Elliptic Fourier analysis of outlines, in order to describe shape changes during growth and identify the correlation with ecological aspects. The two analyses realized growth trajectories and efficiently visualized the shape changes. The graphical representation of these fitting techniques showed how major shape changes were rapid for early larval stages and slowed down successively. Globally, the application of shape analysis tools resulted in a good and powerful approach to describe shape changes during ontogenesis, with special emphasis on early larval stages.

Shape and trophic habits changes in the gilthead sea bream

Chapter 4 reported the shape development of gilthead sea bream *Sparus aurata*, from hatching to adult stage, by means of geometric morphometrics and Elliptic Fourier analysis on outlines. In parallel, observations were reported about trophic habits collected on the same specimens until the end of the larval stage, and after implemented by data from literature. The final aims were to identify morphological groups, defined from different shape, during ontogenesis to compare these stages with corresponding trophic habits, and then to check for correspondences between morphological thresholds and trophic habits shifts during growth, focusing on the description of shape changes that occur within morphological stage and, so, affect diet shifts. The growth trajectory obtained reflected in both cases a saturating curve, made of different parts, which corresponds to different allometry of body region. Besides, morphometrics tools reporting modifications of allometric growth gave exhaustive descriptions of shape changes at general and local scale. Several groups, that constitute developmental stages, were detected by shape analysis. Comparing stage shape description with trophic data, close correspondences were found, identified by size ($\boldsymbol{T_L}$). The matching of these results allowed us to confirm the theory of *saltatory ontogeny* for gilthead sea bream in the context of trophic adaptations, although the pattern of shape changes, if globally considered, seemed not follow a classical saltatory plan.

Applications of SOMs for patterning of changes in shape and trophic habits during larval ontogenesis of two fish species

Despite the goodness of these results, the comparison between shape (as described by means of shape analysis tools) and ecological features like trophic habits or habitat (often surveyed as qualitative or quantitative discrete patterns) is affected by the fact that these aspects are inhomogeneous, precluding a direct visualization, abstraction and interpretation of their complex relationships. Chapter 5 reported a study in which a kind of artificial neural network, called Self Organizing Maps (SOMs) was tested in order to bypass this problem. SOMs was computed on the dataset obtained from Elliptic Fourier Analysis of outlines, and the trained maps were equipped with an appropriate representations of *shape prototypes*, that are the abstraction of shape characters along the developmental series. SOMs appeared to be a robust approach for the analysis, visualization and abstraction of shape profile of each life-stage, allowing the description of patterns of shape changes (e.g. the allometries of the different parts of the body) during the development of the two studied species. Several developmental stages, defined as groups of shape, were detected and identified by means of shape characters which have some ecomorphological relevance in the gradual acquiring of adult characters. Besides, SOMs were used and tested for the comparison of shape with the feeding preference of fish, that is one of the major ecological feature in the framework of ecomorphological studies. In this way, the feeding habits of each specimen was superimposed onto the maps as probability density of four categories of food items (small planktonic, medium planktonic, large planktonic and mobile benthic). A good correspondence was thus observed between feeding habits and shape, confirming the model of co-variation of these features during larval development.

The results presented in this thesis added important new knowledge both of methodological, ecomorphological and ecological features of the two species studied. Shape analysis tools, and particularly Elliptic Fourier Analysis of outlines, were successfully applied in describing shape characters and related changes during ontogenesis, opening new perspectives in investigations on early larval stages of fishes.

Ecomorphological investigations provided new interesting results in the framework of patterns of shape changes during ontogenesis. With respect to classical morphological studies, it was confirmed the model of *saltatory ontogenesis* of shape, but it was argued that the patterns of allometries should be examined via an holistic approach, as allowed by the use of shape analysis tools, in order to correctly detect and understand the adaptative meaning of shape changes. Finally, this thesis add new knowledge in the feeding ecology of *E. marginatus*, a species that is a new candidate for aquaculture, clarifying functional aspects and limits of prey ingestion. This knowledge is essential in the progress of fish cultivation, especially in terms of good practices needed for future development of more responsible forms of production, as pointed out by ***FAO Code of Conduct for a Responsible Fisheries and Aquaculture***.

7. References

Adriaens, D. & Verraes, W. (2004). An empirical approach to study the relation between ontogeny, size and age using geometric morphometrics. *Topics in Functional and Ecological Vertebrate Morphology*, pp. 293-324**.**

Alberch, P., Gould, S.J., Oster, G.F. & Wake, D.B. (1979). Size and shape in ontogeny and phylogeny. *Paleobiology* **5(3)**, 296-317.

Andrade, J.P., Erzini, K. & Palma, J. (1996). Gastric evacuation and feeling in the gilthead sea bream reared under semi-intensive conditions. *Aquaculture International* 4, 129-141.

AA.VV. (2001). Aquacultura responsabile. A cura di S. Cataudella & P. Bronzi, Unimar-Uniprom 2001, Roma.

Balon, E.K. (1985). Early life histories of fishes : new developmental, ecological and evolutionary perspectives. Dr. W. Junk Publishers, Dordrecht, 280 pp.

Bauchot, M.L. & Hureau, J.C. (1986). Sparidae. In : Whitehead, P.J., Bauchot, M.L., Hureau, J.C., Nielsen, J. & Tortonese, E. (Eds.), Fishes of the North-eastern Atlantic and Mediterranean, vol.2. UNESCO, United Kingdom, pp. 605-626.

Blaxter, J.H.S. (1965). The feeding of herring larvae and their ecology in relation to feeding. *California Cooperative Oceanic Fisheries Investigations Report* **10**, 79-88.

Blaxter, J.H.S. (1986) Development of sense organs and behavior of teleost larvae with special reference to feeding and predator avoidance. *Transactions of the American Fisheries Society* **115**, 98-114.

Boglione, C., Selmo, C., Contegiacomo, M., Spedicato, M.T. & Cataudella, S. (1999). Preliminary contribution to the knowledge of the sensory development and apparatus of the dusky grouper *Epinephelus marginatus* (Lowe, 1834). *Marine Life* **9(1)**: 9-17.

Boglione, C., Gagliardi, F., Scardi, M. & Cataudella, S., (2001). Skeletal descriptors and quality assessment in larvae and post-larvae of wild-caught and hatchery-reared gilthead sea bream (*Sparus aurata* L. 1758). *Aquaculture* **192**, 1-22.

Boglione, C., Marino, G., Ferreri, F., Finora, M.G., Scardi, M., Fresi, E. & Cataudella, S. (1994). Anatomical aspects for seed quality assessment in sea bass (*Dicentrarchus labrax*): hatchery and wild populations. Measures for Success. KESTEMONT P., MUIR J., SEVILA F., WILLIOT P. (Eds). CEMAGREF.

Bolnick, D.I., Svanba, R., Fordyce, J.A., Yang, L.H., Davis, J.M., Darrin Hulsey, C. & Forister, M.L. (2003). The Ecology of Individuals: Incidence and Implications of Individual Specialization. *The american naturalist* **161(1)**, 1-28.

Bookstein, F.L. (1991). Morphometric tool for landmark data. Cambridge University Press, Cambridge, p. 435.

Bookstein, F.L. (1996)a. Combining the tools of geometric morphometry. In Marcus, L.F., Corti, M., Loy, A., Naylor, G.J.P. & Slice D.E. (1996). Advances in Morphometry. NATO ASI Series. Plenum Press, New York, pp. 131-152.

Bookstein, F.L. (1996)b. Biometrics, biomathematics, and the morphometry synthesis. *Bull. Math. Biol.* **58**, 313-365.

Bouchereau, J.-L., Body, P. & Chauvet, C. (1999). Growth of the dusky grouper *Epinephelus marginatus* (Linnaeus, 1758) (Teleostei, Serranidae), in the Natural Marine Reserve of Lavezzi Islands, Corsica, France. *Scientia Marina* **63(1)**: 71-77.

Breder, C. M. (1926). The locomotion of fishes. *Zoologica* **4**, 159-297.

Bremigan, M.T. & Stein, R.A. (1994). Gape-dependent larval foragimg and zooplankton size: implications for fish recruiment across systems. *Canadian Journal of Fisheries and Aquat.* **51**, 913-922.

Brown, J.A., Wiseman , D. & Kean, P. (1997). The use of behavioural observations in tehe larviculture of cold-water marine fish. *Aquaculture* **155**, 297-306.

Brusle, J. & Prunus, G. (1980). Relation taille-poids chez les merous mediterraneens *Epinephelus aeneus* et *Epinephelus guaza*. *Cybium*, 3^{e} série, **11**: 15-21.

Cataldi, E., Cataudella, S., Monaco, G., Rossi, A. & Tancioni L. (1987). A study of the histology and morphology of the digestive tract of the sea-bream, *Sparus aurata*. *Journal of Fish Biology* **30**, 135-145.

Cataudella, S., Massa, F., Rampacci, M. & Crosetti, D. (1988). Artificial reproduction and larval rearing of the Thick Lipped Mullet (*Chelon labrosus*). *Aquaculture* **68**, 321-323.

Cataudella, S., Russo, T., Cataldi, E., Boglione, C. & Saroglia, M. (2003). Does the model of semi-intensive larval rearing of Mediterranean finfish contribute to the knowledge on animal welfare in aquaculture? – Oral communication at the International Aquaculture Conference "Fish farming in Mediterranean Europe: quality for developing markets" (Verona, Italy, 15-16 October) – Book of Abstract p.49.

Cataudella, S., Russo, T., Lubrano-Lavadera, P., De Marzi, P., Spanò, A., Fusari, A. & Boglione, C. (2002). An ecological approach to produce "wild-like" juveniles of sea bass and sea bream: trophic ecology in semi-intensive hatchery conditions –Aquaculture Europe 2002, "Seafarming today and tomorrow" – Extended Abstracts and Short Communications p. 177.

Cavalcanti, M.J., Monteiro, L.R. & Lopes P.R.D. (1999). Landmark-based Morphometric Analysis in Selected Species of Serranid Fishes (Perciformes: Teleostei). Zoological Studies **38(3)**: 287-294.

Chauvet, C. (1988). Etude de la croissance du mérou *Epinephelus guaza* (Linné, 1758) des côtes tunisiennes. *Aquat. Living Resour.***1**, 277-288.

Chen, L.-C. (1990). Aquaculture in Taiwan. Fishing News Books. Blackwell Scientific Publications Ltd. P.273.

Chesson, J. (1983). Estimation and analysis of preference. *Ecology* **64**, 1294-1305.

Costa, C. (2004). The study of shape in ichthyology: geometric morphometry and outline fitting methods. PhD thesis, University of Rome Tor Vergata, 172pp.

Cox, E.S. & Pankhurst, P.M. (2000). Feeding behaviour of greenback flounder larvae, *Rhombosolea tapirina* (Günther) with differing exposure histories to live prey. *Aquaculture* 183, 285-297.

Cunha, I. & Planas, M. (1999). Optimal prey size for early turbot larvae (*Scophthalmus maximus* L.) based on mouth and ingested prey size. *Aquaculture* **175**, 103-110.

Dayhoff, J.E. (1990). Neural network architectures – an introduction. New York: Van Nostrand Reinhold.

DeVries, D.R., Bremigan, M.T. & Stein, R.A. (1998). Prey selection by larval fishes as influenced by available zooplankton and gape limitation. *Trans.Am.Fish.Soc.* **127**, 1040-1050.

Doi, M., Mohd Nawi, M.H.J., Nik Lah, N.R. & Talib, Z. (1991). Artificial propagation of the grouper, *Epinephelus suillus* at the marine finfish hatchery in Tanjong Demong, Terengganu, Malaysia. Department of Fisheries Ministry of Agriculture, Malaysia.

Doi, M., Toledo, J.D., Golez, M.S.N., de los Santos, M. & Ohno, A., (1997). Preliminary investigation of feeding performance of larvae of early red-spotted grouper, *Epinephelus coioides*, reared with mixed zooplankton. *Hydrobiologia* **358**, 259-263.

Duray, M.N., Estudillo, C.B., & Alpasan, L.G. (1997). Larval rearing of the grouper *Epinephelus suillus* under laboratory conditions. *Aquaculture* **150**, 63-76.

Economou, A.N. (1991). Food and feeding ecology of live gadoid larvae in the larvae in the northern North Sea. *J. Cons. int. Explor. Mer* **47**, 339-351.

Ehlinger, T. J. (1990). Habitat choice and phenotype-limited feeding efficiency in bluegill: individual differences and trophic polymorphism. *Ecol.* **71(3)**, 886-896.

Evjemo, J.O., Reitan, K.I. & Olsen, Y. (2003). Copepods as live food organisms in the larval rearing of halibut larvae (*Hippoglossus hippoglossus* L.) with special emphasis on the nutritional value. *Aquaculture* **227**, 191-210.

Faustino, M. & Power, D.M. (1998). Development of the pectoral, pelvic, dorsal and anal fins in cultured sea bream. *Journal of Fish Biology* **54**, 1094-1110.

Fernández-Díaz, C., Pascual, E. & Yúfera, M., (1994). Feeding behaviour and prey size selection of gilthead seabream, *Sparus aurata*, larvae fed on inert and live food. *Marine Biology* **118**, 323-328.

Ferrari, I. & Chieregato, A.R. (1981). Feeling habitus of juvenile stages of *Sparus auratus* L., *Dicentrarchus labrax* L. and mugilidae in a brackish embayment of the Po river delta. *Aquaculture* **25**, 243-257.

Fearson, S., Rohlf, F.J. & Koehn R.K. (1985). Measuring shape variation of two-dimensional outlines. *Syst. Zool.*, **34(1)**, 59-68

Froese, R. & Pauly, D. (2003). FishBase. World Wide Web electronic publication. www.fishbase.org, version 29 January 2004.

Gerking, S.D. (1994). Feeding ecology of fishes. Academic Press.

Gevrey, M., Rimet, F., Park, Y.-S., Giraudel, J.-L., Ector, L. & Lek, S. (2004). Water quality assesment using diatom assemblages and advanced modelling techniques. *Freshwater biology* **49**, 208-220.

Giraudel, J.L. & Lek, S. (2001). A comparison of self-organizing map algorithm and some conventional statistical methods for ecological community ordination. *Ecological modelling* **146**, 329-339.

Gisbert, E. (1999). Early development and allometric growth patterns in Siberian sturgeon and their ecological significance. *Journal of Fish Biology* **54**, 852-862. doi:jfbi.1998.0924.

Glamuzina, B., Glavić, N., Skaramuca, B., Kożul, V. & Tutman, P., (2001). Early development of the hybrid *Epinephelus costae* ♀ × *E. marginatus* ♂. *Aquaculture* **198**, 55-61.

Glamuzina, B., Skaramuca, B., Glavić, N. & Kożul, V. (1998). Preliminary studies on reproduction and early life stages in rearing trials with dusky grouper, *Epinephelus marginatus* (Lowe, 1834). *Aquaculture Research* **29**, 769-771.

Göthel, H. (1992). – Fauna Marina del Mediterráneo. Ediciones Omega, S.A., Barcelona. 319 pp.

Gould, S.J. & Eldredge, N. (1977). Punctuated equilibria: the tempo and mode of evolution reconsidered. *Paleobiology* **3**, 115–151.

Hambright, K.D. (1991). Experimental Analysis of Prey Selection by Largemouth Bass: Role of Predator Mouth Width and Prey Body Depth. *Transactions of American Fisheries Society* **120**, 500-508.

Hardman-Mountford, N.J., Richardson, A.J., Boyer, D.C., Kreyner, A. & Boyer, H.J. (2003). Relating sardine recruiment in the northern benguela to satellite-derived sea surface height using a neural network pattern recognition approach. *Progress in oceanography* **59**, 241-255.

Heemstra, P.C. & Randall, J.E. (1993). FAO species catalogue. Vol 16. Groupers of the world (Family Serranidae, Subfamily Epinephelinae). An annotated and illustrated catalogue of grouper, rockcod, hind, coral grouper and lyretail species known to date. FAO Fisheries Synopsis 125. Rome FAO.

Hunter, J.R. (1981). Feeding ecology and predation of marine fish larvae pp.33-77. In Lasker, R. (eds.) Marine fish larvae: morphology, ecology and relation to fisheries Washington Sea Grant Program. University of Washington Press.

Irwin, S., O'Halloran, J.O. & FitzGerald, R.D. (2002). Mouth morphology and behavioural response of cultured turbot towards food pellets of different sizes and moisture content. *Aquaculture* **205**, 77-88.

Johnson, D.R. (1997). Fourier descriptors and shape difference: studies on the upper vertebral column of the mouse. In: Lestrel, P.E. (1997), fourier descriptors and their applications in biology. Cambridge University Press., Cambridge, United Kingdom, 466 pp.

Kara, M.H. & Derbal, F. (1995). Morphométrie, croissance et mortalité du Mérou *Epinephelus marginatus* (Serranidae) des côtes de l'est algérien. Cah. *Biol. Mar.* **36**: 229-237.

Kawamura, K. & Hosoya, K. (1997). Larval morphometry of the japanese flounder, Paralichthys olivaceus. *Ichthyol. Res.* **44(4)**, 389-398.

Kendall, A.W.Jr., Ahlstrom, E.H. & Moser, H.G. (1984). Early life history of fishes and their characters. In: Moser, H.G., Richards, W.J., Cohen D.H., Fahay, M.P., Kendall, A.W. & Richardson, S.L. Ontogeny and systematics of fishes. American Society of Ichthyologists and Herpetologists, Special Pubblications N° 1., Allen Press Inc., Lawrene, Kansas, pp 11-22.

Kentouri, M. & Divanach, P. (1984). Approache du comportment trophique des larves du sar (*Diplodus sargus*), de la daurade (*Sparus auratus*), du charax (*Puntazzo puntazzo*) et du marbré (*Lithognathus mormyrus*). In Barnabé, G., Billard, R. (1984). L'Aquaculture du Bar et des Sparidés. INRA Publ., Paris, 139-159.

Kentouri, M. & Divanach, P. (1986). Spectres alimentaires des larves de sparides en conditions contrôlées. Sélectivité spécifique de la daurade Sparus aurata. *Oceanol. Acta* **9**, 343-348.

Klingenberg, C.P. (1996). Multivariate allometry. In: Marcus, L.F., Corti, M., Loy, A., Naylor, G.J.P. & Slice, D.E. (1996) – Advances in Morphometry. NATO ASI Series. Plenum Press, New York, pp. 23-50.

Kohonen, T. (1984). Self-Organization and associative memory. Berlin: Springer Verlag.

Kohonen, T. (1997). Self-organizing maps. Berlin: Springer Verlag.

Kraljević, M. & Dulčić, J. (1997). Age and growth of gilt-head sea bream (*Sparus aurata* L.) in the Mirna Estuary, Northern Adriatic. *Fisheries Research* **31**, 249-255.

Krebs, J.M. & Turingan, R.G. (2003). Intraspecific variation in gape-prey size relathionship and feeding success during early ontogeny in red drum, *Sciaenops ocellatus*. *Environmental Biology of Fishes* **66**, 75-84.

Kull, F.P. & Giardina, C.R. (1982). Elliptic Fourier features of a closet contour. *Comp. Graph. Image Process* **18**, 236-258.

La Mesa, G., Louisy, P. & Vacchi, M. (2002). Assessment of microhabitat preferences in juvenile dusky grouper (*Epinephelus marginatus*) by visual sampling. *Marine Biology* **140**, 175-185.

Legendre, P. & Legendre, L. (1998). Numerical Ecology. Second English Edition: Elsevier Science B.V., Amsterdam, 853 pp.

Lek, S. & Guégan, J.F. (1999). Artificial neural networks as a tool in ecological modelling, an introduction. *Ecological modelling* **120**, 65-73.

Leisler, B. & Winkler, H. (1985). Ecomorphology. pp.155-186. *In:* Johnstonm R.F. (ed.) Current Ornithology, Plenum Press, New York.

Lemly, A.D. & Dimmick, J. (1982). Growth of young-of-the-year and Yearling Centrarchids in relation to zooplankton in the littoral zone of lakes. *Copeia* **2**, 305-321.

Lestrel, P.E. (1997). Fourier descriptors and their applications in biology. Cambridge University Press, Cambridge, UK. 466pp.

Liem, K.F. (1991). Toward a new morphology: pluralism in research and education. Amer. Zool. 31: 7597-767.

Linde, M., Palmer, M. & Gómez-Zurita, J. (2004). Differential correlates of diet and philogeny on the shape of the premaxilla and anterior tooth in sparid fishes (Perciformes: Sparidae). *J. Evol. Biol.* **17**, 941-952.

Lindsey, C.C. (1978). Form, function and locomotory habits in fish. In: Fish physiology Vol. VII, Hoar W.S. & Randall D. J. Eds., Academic Press, New York, 1-100.

Loy, A., Bertelletti, M., Costa, C., Ferlin, L. & Cataudella S. (2001). Shape changes and growth trajectories in the early stages of three species of the genus *Diplodus* (Perciformes, Sparidae). *Journal of Morphology* **250**, 24-33.

Loy, A., Boglione, C. & Cataudella, S. (1998)a. Geometric morphometry and morpho-anatomy: a combined tool in the study of sea bream (*Sparus aurata*, Sparidae) shape. *Journal of Applied Ichtyology* **14**, 1-7.

Loy, A., Busilacchi, S., Costa, C., Ferlin, L. & Cataudella, S. (2000). Comparing geometric morphometry and outline fitting methods to monitor fish shape variability of *Diplodus puntazzo* (Teleostea: Sparidae). *Aquaculture Engineering* **21(4)**, 271-283.

Loy, A., Cataudella, S. & Corti, M. (1996). Shape changes during the growth of the sea bass, *Dicentrarchus labrax* (Teleostesa: Perciformes), in relation to different rearing conditions. An application of Thin-Plate Spline Regression Analysis. In: Advances in Morphometry, Edited by L.F. Marcus *et al.* Plenum Press, New York: 399-405.

Loy, A., Mariani, L., Bertelletti, M. & Tunesi, L. (1998)b. Visualizing allometry: geometric morphometry in the study of shape changes in the early stages of the two-banded sea bream, *Diplodus vulgaris* (Perciformes, Sparidae). *Journal of Morphology* **237**, 137-146.

Luczkovich, J.J., Norton, S.F. & Grant Gilmore, R. (1995). The influence of oral anatomy on prey selection during the ontogeny of two percoid fishes, *Lagodon rhomboids* and *Centropomus undecimalis*. *Environmental Biology of Fishes* **44**, 79-95.

Malavasi, S., Georgalas, V., Lugli, M., Torricelli, P., & Mainardi, D. (2004). Differences in the pattern of antipredator behaviour between hatchery-reared and wild European sea bass juveniles. *Journal of Fish biology* **65,** 143-155.

Marcus, L.F., Corti, M., Loy, A., Naylor, G.J.P. & Slice, D.E. (1996). Advances in Morphometrics. NATO ASI Series. Plenum Press, New York, p. 587.

Mariani, S., Maccaroni, A., Massa, F., Rampacci, M. & Tancioni, L. (2002). Lack of consistency between the trophic interrelationships of five sparid species in two adjacent central Mediterranean coastal lagoons. *Journal of Fish Biology* **61**, 138-147.

Marino, G., Azzurro, E., Massari, A., Finoia, M.G. & Mandich, A. (2001). Reproduction of dusky grouper from the southern Mediterranean. *Journal of Fish Biology* **58**, 909-927.

Marino, G., Panini, E., Longobardi, A., Mandich, A., Finoia, M.G., Zohar, Y. & Mylonas, C.C. (1998). Induction of ovulation in captive-reared dusky grouper, *Epinephelus marginatus* (Lowe, 1834) with a sustained-release GnRHa implant. *Aquaculture* **219**, 841-858.

Matthews, W.J. (1998). Patterns in freshwater fish ecology. Kluwer Academic Publishers, Hingham, Massachusetts.

Mazzocchi, M.G. & Ribera d'Alcalà, M. (1995). Recurrent patterns in zooplankton structure and succession in avariable coastal environment. *ICES J. mar. Sci.* **52**, 679-691.

Mérigoux, S. & Ponton, D. (1998). Body shape, diet and ontogenetic diet shifts in young fish of the Sinnamary River, French Guiana, South America. *Journal of Fish Biology* **52**, 556-569.

Morato, T., Serrão Santos, R. & Andrade, J.P. (2000). Feeding habitus, seasonal and ontogenetic diet shift of blacktail bomber, *Serranus atricauda* (Pisces: Serranidae), from the Azores, north-eastern Atlantic. *Fisheries Research* **49**, 51-59.

Morris, A.V., Roberts, C.M. & Hawkins, J.P. (2000). The threatened status of groupers (Epinephelinae). *Biodiversity and Conservation* **9**, 919-942.

Motta, J.P., Norton, S.F. & Luczkovich, J.J. (1995)a. Perspective on the ecomorphology of bony fishes. *Environmental Biology of Fishes* **44**, 11-20.

Motta, P. J., Clifton, K. B., Hernandez, P. & Eggold, B. T. (1995)b. Ecomorphological correlates in ten species of subtropical seagrass fishes: diet and microhabitat utilization. *Environmental Biology of Fishes* **44**, 37-60.

Motta, P. J. & Kotrschal, K.M. (1992). Correlative, experimental, and comparative evolutionary approaches in ecomorphology. *Neth. J. Zool.* **42**, 400-415.

Ohtsuki, F., Uetake, T., Adachi, K., Lestrel, P.E. & Hanihara, K. (1997). Fourier analysis of size and shape changes in Japanese skulls. In: Lestrel, P.E. (1997). Fourier descriptors and their applications in biology. Cambridge University Press., Cambridge, United Kingdom, 466 pp.

Osse, J.W.M. & van den Boogaart, J.G.M. (1995). Fish larvae, development, allometric growth, and the aquatic environment. *ICES Marine Sciences Symposium* **201**, 21–34.

Osse, J.W.M. & van den Boogaart, J.G.M. (1999). Dynamic morphology of fish larvae, structural implications of friction forces in swimming, feeding and ventilation. *Journal of Fish Biology* **55**, 156–174. doi: 10.1006/jfbi.1999.1071

Osse, J.W.M. (1990). Form changes in fish larvae in relation to changing demands of function. *Netherlands Journal of Zoology* **40**, 362–385.

Osse, J.W.M., van den Boogaart, J.G.M., van Snik, G.M.J., & van der Sluys, L. (1997). Priorities during early growth of fish larvae. *Aquaculture* **155**, 249-258.

Palomares, M.L.D. (1991). La consommatíon de nourriture chez les poissons: étude comparative, mise au point d'un modèle prédictif et application à l'étude des réseaux trophiques. Institut National Polytechnique, Toulouse. 211 p. Thèse de Doctorat.

Park, Y.-S., Céréghino, R., Compin, A. & Lek, S. (2003). Applications of artificial neural networks for patterning and predicting aquatic insect species richness in running waters. *Ecological modelling* **160**, 265-280.

Park, Y.S., Chon, T.S., Kwak, I.S. & Lek, S. (2004). Hierarchical community classification and assessment of aquatic ecosystems using artificial neural networks. *Science of the total environment* **327**: 105-122.

Parra, G. & Yúfera, M. (2000). Feeding, physiology and growth responses in first-feeding gilthead seabream (*Sparus aurata* L.) larvae in relation to prey density. *Journal of Experimental Marine Biology and Ecology* **243**, 1-15.

Pita, C., Gamito, S. & Erzini, K. (2002). Feeling habitus of the gilthead seabream (*Sparus aurata*) from the Ria Formosa (southern Portugal) as compared to the black seabream (*Spondyliosoma cantharu*) and the annular seabream (*Diplodus annularis*). *Journal of Applied Ichtyology* **18**, 81-86.

Planas, M. & Cunha, I. (1999). Larviculture of marine fish: problems and perspectives. *Aquaculture* **177**, 171-190.

Puvanendran, V. & Brown, J.A. (1999). Foraging, growth and survival of Atlantic cod larvae reared in different prey concentrations. *Aquaculture* **175**, 77-92.

Qin, J.G. & Hillier, T. (2000). Live food and feeding ecology of larval snapper (*Pagrus auratus*). Hatchery Feeds: *Proceedings of a workshop held in Cairns*, 63-68.

Reñones O., Polunin, N.V.C. & Goni, R. (2002). Size related dietary shifts of Epinephelus marginatus in a western Mediterranean littoral ecosystem: an isotope and stomach content analysis. *Journal of Fish Biology* **61**: 122-137.

Richardson, A.J., Risien, C. & Shillington, F.A. (2003). Using self-organizing maps to identify patterns in satellite imagery. *Progress in oceanography* **59**, 223-239.

Rohlf, F.J. & Archie, J.W. (1984). A comparison of Fourier methods for the description of wing shape in mosquitoes (Diptera: Culicidae). *Syst. Zool.* **33(3)**, 302-317

Rohlf, F.J. & Bookstein, F.L. (1988). Proceedings of the Michigan morphometry workshop. Special Publications Number 2. The University of Michigan Museum of Zoology., pp. 380.

Rohlf, F.J. & Marcus, L.F. (1993). A revolution in morphometry. *Tree* **8(4)**, 129-132.

Rohlf, F.J. & Slice, D. (1990). Extention of the Procrustes method for the optimal superimposition of landmarks. *Syst. Zool.* **39(1)**: 40-59.

Rohlf, F.J. (2000). TpsRegr Ver. 1.24. Dept. of Ecology and Evolution, State Univ. of New York at Stoony Brook, Stony Brook.

Rohlf, F.J. (2001)a. TpsDig, ver. 1.31. Dept. of Ecology and Evolution, State Univ. of New York at Stony Brook, Stony Brook.

Rohlf, F.J. (2001)b. TpsRelw, ver. 1.24. Dept. of Ecology and Evolution, State Univ. of New York at Stony Brook, Stony Brook.

Sagnes, P., Gaudin, P. & Statznerm, B. (1997). Shifts in morphometry and their relation to hydrodynamic potential and habitat use during grayling ontogenesis. *Journal of Fish Biology* **50**, 846-858.

Sala, R., Santamaria, C.A. & Crespo, S. (2005). Growth of organ systems of *Dentex dentex* (L) and *Psetta maxima* (L) during larval development. *Journal of Fish Biology* **66**, 315-326.

Sánchez-Velasco, L. (1998). Diet composition and feeding habits of fish larvae of two co-occurring specie (Pisces: Callionymidae and Bothidae) in the North-western Mediterranean. *ICES Journal of Marine Science* **55**: 299-308.

Scardi, M. & Harding, L.W. Jr. (1999). Developing an empirical model of phytoplankton primary production: a neural network case study. *Ecological modelling* **120**, 213-223.

Scardi, M. (1996). Artificial neural networks as empirical models for estimating phytoplankton production. *Marine Ecology Progress Series* **139**, 289-299.

Shansudin, L., Yusof, M., Azis, A. & Shukri, Y. (1997). The potential of certain indigenous copepod species as live food for commercial fish larval rearing. *Aquaculture* **151**, 351-356.

Shaw, G.W., Pankhurst, P.M. & Purser, G.J. (2003). Prey selection by greenback flounder *Rhombosolea tapirina* (Günther) larvae. *Aquaculture* **228**, 249-265.

Shields, R.J. (2001). Larviculture of marine finfish in Europe. *Aquaculture* **200**, 55–88.

Shirota, A. (1970). Studies on the Mouth Size of Fish Larvae. *Bulletin of the Japanese Society of Scientific Fisheries* **36**, 353-367.

Simonović, P.D., Garner P., Eastwood E.A., Kovác V. & Copp, G.H. (1999). Correspondence between ontogenetic shifts in morphology and habitat use in minnow *Phoxinus phoxinus*. *Environmental Biology of Fishes* **56**, 117-128.

Slice, D.E. (1998). Morpheus *et al.*: software for morphometric research. Revision 01-30-98-Beta. Department of ecology and evolution, State University of New York, Stony Brook, New York.

Sluka, R.D., Chiappone, M. & Sullivan Sealey, K.M. (2001). Influence of habitat on grouper abundance in the Florida Keys, U.S.A.. *Journal of Fish Biology* **58**, 682-700.

Smirnov, S.A., Makeyeva, A.P. & Smirnov, A.I. (1995). Development of ecomorphology of fishes in Russia. *Environmental biology of Fishes* **44**, 23-33.

Smith, M.E. & Fuiman, L.A. (2004). Behavioural performance of wild-caught and laboratory-reared red drum *Sciaenops ocellatus* (Linnaeus) larvae. *Journal of Experimental Marine Biology and Ecology* **302**, 17-33.

Spedicato, M.T., Lembo, G., Di Marco, P., & Marino, G. (1995). Preliminary results in the breeding of dusky grouper *Epinephelus marginatus* (Lowe, 1834). *Cahiers Options Mediterraneennes* **16**, 131-148.

Stoner, A. W. & Livingston, R. J. (1984): Ontogenetic patterns in diet and feeding morphology in sympatric sparid fishes from sea-grass meadows. *Copeia* **1984**, 174178.

Støttrup, J.G. & McEvoy, T. (2003). Live feeds in marine aquaculture. Blacwell Publishing.

Støttrup, J.G. & Norsker, N.H., (1997). Production and use of copepods in marine fish larviculture. *Aquaculture* **155**, 231-247.

Tancioni, L., D'Ambra, R., Mamone, R., Mariani, A., Massa, F., Rampacci, M., Scardi, M. & Maccaroni, A. (1998). Lo studio del comportamento alimentare di pesci teleostei nel monitoraggio biologico delle lagune costiere: il caso dell'orata (Sparus aurata L.) nelle lagune di Fogliano e Caprolace (Parco Nazionale del Circeo, Italia Centrale). *Biol. Mar. Medit.* **5**, 2133-2141.

Tancioni, L., Mariani, S., Maccaroni, A., Mariani, A., Massa, F., Scardi, M. & Cataudella, S. (2003). Locality-specific variation in the feeling of Sparus aurata L.; evidence from two Mediterranean lagoon systems. *Estuarine, Coastal and Shelf Science* **57**, 469-474.

Toledo, J.D., Caberoy, N.B., Quinitio, G.F., Choresca, C.H. & Nakagawa, H. (2002). Effects of salinity, aeration and light intensity on oil globule absorption, feeding incidence, growth and survival of early-stage grouper *Epinephelus coioides* larvae. *Fisheries Science* **68**, 478-483.

Trapani, J. (2003). Geometric morphometric analysis of body-form variability in *Cichlasoma minckleyi*, the Cuatro Cienegas cichlid. *Environmental biology of Fishes* **68**, 357-369.

van der Meeren, T. & Jørstad, K.E. (2001). Growth and survival of Arcto-Norwegian and Norwegian coastal cod larvae (*Gadus morhua* L.) reared together in mesocosms under different light regimes. *Aquaculture Research* **32**, 549-563.

van der Meeren, T. & Lønøy, T. (1998). Use of mesocosm in larval rearing of saithe [Pollachius virens (L.)], goldsinny [*Ctenolabrus rupestris* (L.)], and corkwing [*Crenilabrus melops* (L.)]. *Aquaculture Engineering* **17**, 253-260.

van der Meeren, T. & Naas, K.E. (1997). Development of rearing techniques using large enclosed ecosystems in the mass production of marine fish fry. *Reviews in Fisheries Science* **5**, 367-390.

Wainwright, P.C. & Barton, A. R. (1995). Predicting patterns of prey use from morphology of fishes. *Environmental Biology of Fishes* **44**, 97-113.

Wasse,f E. & Eisawy, A. (1985). Food and feeding habits of wild and reared gilthead bream *Sparus aurata* L.. *Cybium* **25(2-3)**, 243-257.

Watanabe, W.O., Ellis, S.C., Ellis, E.P. & Lopez, V.G. (1996). Evaluation of first-feeding regimes for larval Nassau grouper *Epinephelus striatus* and preliminary, pilot-scale culture through metamorphosis. *Journal of the World Aquaculture Society* **27**, 323-331.

Webb, P.W. & Weihs, D. (1986). Functional locomotor morphology of early life history stages of fishes. *Transaction of the American Fisheries Society* **115**, 115-127.

Webb, P.W. (1984)a. Body form, locomotion and foraging in acquatic vertebrates. *Am. Zool.* **24**, 107-120.

Webb, P.W. (1984)b. Form and function in fish swimming. *Sci. Am.* **251**, 72-75.

Winemiller, K.O. (1991). Ecomorphological diversification in lowland freshwater fish assemblages from five biotic regions. *Ecol. Monographs* **61(4)**, 343-365.

Zabala, M., Garcia-Rubies, A., Louisy, P. & Sala, E. (1997). Spawning behaviour of the Mediterranean dusky grouper *Epinephelus marginatus* (Lowe, 1834) (Pisces, Serranidae) in the Medes Islands Marine Reserve, NW Mediterranean, Spain. *Scientia Marina* **61**, 65-77.

Zaragoza, V., Vioque, J.E. & Sanchez-Lamadrid, A. (1994). Influence of diet and experience in predator efficiency in gilthead seabream, *Sparus aurata* L.; restocking. *Journal of Fish Biology* **(45)** suppl. A.

8. Acknowledgements

I would like to acknowledge dr. Kurt Buck, dr. Eleonora Ciccotti, Antonella Guzzon, dr. Marco Lucarelli, Giuseppe Magnifico, Giulia Marsili and prof. Michele Scardi that edited and critically reviewed the manuscript.

Prof. Michele Scardi gave me a lot of statistical advices, and taught me the use of SOMs.

Prof. Stefano Cataudella introduced me to larval ecology, provided its scientific direction in my studies and allowed me to work profitably at the *Laboratory of Experimental Ecology and Aquaculture*.

9. Ringraziamenti

Questo lavoro di tesi viene alla fine di tre anni di studio e lavoro non sempre facili, ma belli, durante i quali ho conosciuto e lavorato con tante persone. Alcune sono state molto importanti per il mio lavoro perché mi hanno insegnato, aiutato, corretto, o sono state un modello per qualcosa che a me mancava. Altre mi hanno fatto crescere come persona, mi hanno fatto ridere e pensare, hanno dato voce a cose che sentivo, mi hanno spronato quando serviva e mi hanno smontato quando era il caso.

Prima di tutto ringrazio mia madre, perché in fondo c'è sempre stata e c'è ancora lei dietro tutti i giorni belli della mia vita. Ringrazio mio padre per avermi insegnato ad imparare sempre, a non smettere mai di migliorare, a non abbassare mai la testa e ad avere la presunzione di provarci sempre.

Poi ringrazio Antonella, per essere il muro impenetrabile di serenità e di amore che mi circonda dovunque vado.

Grazie anche a Sofia Ingrosso per avermi accolto nella sua bellissima famiglia, e a Roberto Olivieri per essere l'amico fidato che è, una persona con la quale ho passato giornate bellissime; ad Antonio Coppola & Co. per avermi sempre accolto nel posto da cui vengo come se fossi andato via il giorno prima, e per non avermi mai permesso di dimenticare chi sono; a Corrado Costa, perché è un ottimo collega ed una brava persona; a Paolo Nori per aver dato voce allo spirito ribelle e per le letture che hanno cambiato il mio modo di vedere il mondo; ad Ugo Cornia per aver ammazzato e sepolto l'ipocrisia; a Giuseppe Magnifico per essere uno che cerca di progredire e crescere, e per i discorsi che mettono la rabbia di non mollare; a Placido Lubrano Lavadera per un inverno niente male in valle; agli amici tutti che mi sono stati vicino durante questi anni;

a Stefano Cataudella per una lunghissima serie di motivi che io e lui conosciamo benissimo.

Da un punto di vista obiettivo, preoccuparsi del senso o del fine della nostra esistenza e di quella delle altre creature mi è sempre parso assolutamente vuoto di significato. Ciononostante ogni uomo è legato ad alcuni ideali che gli servono di guida nell'azione e nel pensiero. In questo senso il benessere e la felicità non mi sono mai apparsi come la meta assoluta (questa base della morale la definisco l'ideale dei porci). Gli ideali che hanno illuminato la mia strada e mi hanno dato costantemente un coraggio gagliardo sono stati il bene, la bellezza e la verità.

In singolare contrasto col mio senso ardente di giustizia e di dovere sociale, non ho mai sentito la necessità di avvicinarmi agli uomini e alla società in generale. Sono proprio un cavallo che vuol tirare da solo; mai mi sono dato pienamente ne allo stato, ne alla terra natale, ne agli amici e neppure ai congiunti più prossimi; anzi ho sempre avuto di fronte a questi legami la sensazione netta di essere un estraneo e ho sempre sentito il bisogno di solitudine; e questa sensazione non fa che aumentare con gli anni. Sento fortemente, ma senza rimpianto, di toccare il limite dell'intesa e dell'armonia con il prossimo. Certo, un uomo di questo carattere perde così una parte del suo candore e della sua serenità, ma ci guadagna una larga indipendenza rispetto alle opinioni, abitudini e giudizi dei suoi simili; ne sarà tentato di stabilire il suo equilibrio su basi cosi malferme.

Albert Einstein

Voglio quel che non c'è mai stato e che evidentemente non c'è; e che così continuando si fa meta sempre più lontana. Il che mi fa ancora e sempre apparire come un pessimista: e pare non sia permesso esserlo nemmeno di fronte al pessimo. Allegria, allegria.

Leonardo Sciascia