CONDITION INDICES AND THEIR RELATIONSHIP WITH ENVIRONMENTAL FACTORS IN FISH LARVAE

IGNACIO A. CATALÁN ALEMANY

2003

CONDITION INDICES AND THEIR RELATIONSHIP WITH ENVIRONMENTAL FACTORS IN FISH LARVAE

IGNACIO ALBERTO CATALÁN ALEMANY

TESIS DOCTORAL

UNIVERSITAT DE BARCELONA
DEPARTAMENT D'ECOLOGIA

BARCELONA 2003

TESIS DOCTORAL

UNIVERSITAT DE BARCELONA

FACULTAT DE BIOLOGIA
DEPARTAMENT D'ECOLOGIA

Programa de doctorado: Ciencias del Mar
Bienio: 1998-2000
Título: " Condition Indices and their Relationship with Environmental Factors in Fish Larvae"

Memoria presentada por Ignacio Alberto Catalán Alemany para optar al grado de Doctor en Biología por la Universitat de Barcelona, Facultat de Biologia, Departament d'Ecologia, bajo la dirección de la Dra. María Pilar Olivar Buera, Científico Titular del C.S.I.C.

Ignacio Alberto Catalán Alemany,
Barcelona 2003

Dirección de tesis:
Dra María Pilar Olivar Buera
Científico Titular del C.S.I.C.
Instituto de Ciencias del Mar
CMIMA-CSIC
Barcelona

AGRADECIMIENTOS/ACKNOWLEDGEMENTS

A Gustavo y Luisa, mis padres, y a Lilas y Polo, mis hermanos, les dedico esta memoria, por fin.

A mi directora de tesis, María Pilar Olivar, le agradezco toda suerte de consejos y ayuda y, sobre todo, que haya dedicado mucho más tiempo del esperado. Ah, y cómo olvidarlo, su excelente humor durante todo este tiempo.

Quiero expresar mi gratitud a todos aquellos que me han apoyado a lo largo del tiempo transcurrido desde que llegué al ICM. Han sido muchas personas del Instituto, y absolutamente todas las del departamento de Recursos quienes, de una u otra forma, han colaborado proporcionándome información o intercambiado pareceres y críticas. Y, por supuesto, doy las gracias a todos aquellos con los que me divertí en las campañas a bordo del García del Cid.

Agradezco muy especialmente la colaboración de Elisa Berdalet y Cristina Roldán por sus análisis de ácidos nucléicos, y a Isabel Palomera y Belén Aguilera por sus datos sobre los otolitos de sardina. A Anna Bozzano, Guiomar Rotllant y Mercé Durfort por echarme una mano con la histología al comienzo. A Batis y Pachi, alias Joan Batista Company y Alicia González, por su ayuda con los enzimas y las proteínas. De Jordi Lleonart, Juan Pablo Pertierra, Toni Lombarte, Marta Rufino y seguramente muchos otros, recibí consejo sobre estadística, y Mikhail Emelianov y Jordi Salat fueron cruciales con los datos oceanográficos. Muchos de los citados y otros (Ana Sabatés, Francesc Piferrer, Pedro Pablo Ambrosio, Roger Villanueva etc.), ayudaron en el cultivo de lubina, o en la revisión de capítulos.

Estando fuera del Instituto, he recibido consejo de varias personas. Ya antes de comenzar el doctorado, Beatriz Morales, M. Luz Fernández y Pere Oliver me orientaron

decisivamente. En Cádiz, M. Carmen Sarasquete y Manuel Yúfera me enseñaron “la de cosas que puede mirarle uno a una larva”.

Now shifting to english: I want to thank Bob Batty (Dunstaffnage Marine Lab –SAMS-) for his help with the metabolic enzymes and for the herring samples. Special thanks go to Ian Johnston and his team from the Muscle Research Group (Gatty Marine Laboratory, Univ. St. Andrews), who helped in the analyses and instructed me on the possibilities offered by the muscle growth patterns. In the States I had the pleasure of meeting Jeff Govoni (Beaufort Laboratory, NOAA), to whom I am extremely grateful for his advice on larval fish biology and his hospitality.

A Jacopo y Quim, amigos de largo recorrido y ex compañeros de piso, tesis, y paridas sin cuento, les agradezco demasiadas cosas, al igual que a otros/as que desgraciadamente he visto menos; Tavo, Secre, Isra, Gemma, Xose, etc. etc. Last but not least, I want to specially thank Debs and Lesley Ann for having gone through some of this stuff for the affordable price of a dinner!.

La realización de esta tesis ha recibido el soporte económico de la Comisión Interministerial de Ciencia y Tecnología (Proyecto MAR 97-0902 CICYT).

INDEX

ORGANISATION OF THE THESIS / ORGANIZACIÓN DE LA TESIS	1
1. GENERAL INTRODUCTION AND OBJECTIVES	2
1.1. Rationale of the Thesis and General Objectives	3
1.2. Factors Affecting Larval Survival at Sea	5
1.3. Condition Indices	9
1.4. Early Life History of Mediterranean Pilchard	16
1.5.Interactions Between Pilchard Larval Ecology and Oceanography in the Catalan Sea	17
2. LABORATORY STUDIES	21
2.1. Experimental Rearing, Growth and Survival	22
Abstract	22
Introduction	23
Material and Methods	24
Results	27
Discussion	30
2.2. Morphometric Indices	34
Abstract	34
Introduction	35
Material and Methods	36
Results	40
Discussion	53
2.3. Histological Indices	60
Abstract	60
Introduction	61
Material and Methods	62
Results	68
Discussion	81

2.4. Biochemical Indices 92

Abstract 92
Introduction 93
Material and Methods 95
Results 102
Discussion 111

2.5. Comparison of Condition Indices and Discussion 120

3. FIELD STUDIES 134

3.1. Early Stages of *Sardina pilchardus* and Environmental Anomalies in the Catalan Sea 135

Abstract 135
Introduction 136
Material and Methods 137
Results 139
Discussion 148

3.2. Relationships Between Environment, Growth, and Condition of *Sardina pilchardus* in the Catalan Sea 153

Abstract 153
Introduction 154
Material and Methods 157
Relationships with environmental data 157
Growth 163
Mortality 167
Histological Indices 168
Biochemical Indices 174
Results 176
Growth 176
Mortality 184
Histological Indices 185
Biochemical Indices 192
Discussion 196
General Comments 196

Growth 198
Mortality 204
Histological Indices 205
Biochemical Indices 210
Summary 213

4. CONCLUSIONS 219

LITERATURE CITED 224

RESUMEN 243

Organisation of this thesis

This thesis is organised in four main chapters. The first chapter is devoted to a general introduction and objectives. Number two is devoted to all the work developed in the laboratory. Chapter three embraces all the work that was done with field data. Each of these two central chapters collects a mixture of published and row material. In the sake of clarity, all the thesis has been written in the same format, regardless the state of publication of the information. If the information of a particular section has been published, it is indicated at the beginning of the section. Information which is considered important but which was not included in a particular published section, has been inserted after authors' approval. A brief introduction and extensive discussion is given in each of the sections conforming chapters two and three. In chapter four, the main conclusions from each part are summarised. Provided that the thesis is organised in a similar way that a compendium of publications, and as a guide to the reader, the repetition of some material at the introduction was unavoidable.

Organización de esta tesis

Esta tesis se organiza en cuatro capítulos principales. El primer capítulo ofrece una introducción y objetivos generales. El segundo contiene la información obtenida de la experimentación en laboratorio. El tercer capítulo está dedicado a los estudios de campo. Cada uno de estos capítulos centrales se organiza en secciones. La información que contienen son una mezcla de material publicado e inédito. Todas las secciones se han escrito en un formato similar para facilitar la comprensión. Si una sección o parte de ella ha sido publicada, la referencia se señala al principio de la sección correspondiente. En los casos en que la información publicada ha sido ampliada, se ha obtenido el consentimiento de los autores de la publicación. En cada sección de los capítulos centrales se ha añadido una introducción y una discusión. En el capítulo cuarto se recogen las conclusiones generales de la tesis. Dado que la estructura de las seccciones es similar a la de un artículo, fue inevitable incluir, en algún caso, información redundante.

1. GENERAL INTRODUCTION AND OBJECTIVES

1.GENERAL INTRODUCTION AND OBJECTIVES

1.1. RATIONALE OF THE THESIS AND OBJECTIVES

Populations of marine fish undergo strong fluctuations in the number of young of the year recruits. These fluctuations are extremely difficult to predict and pose decisive problems for fishery managers (Koslow 1992). In many species of fish, only up to 0.01% end up surviving to the adult stage. It is therefore believed that small fluctuations in mortality at early stages of fish are responsible for recruitment variability.

It is also known that factors affecting larval survival interact closely but can, in terms of understanding, be divided into biotic (mainly feeding success, predation and disease) and abiotic factors (mainly hydrography). Studies on the relative importance of each factor have varying degrees of difficulty, either in terms of the technology needed, the labour involved or the money required. Whereas accurate understanding of the link between hydrodynamics and survival requires complex models (e.g. Brickman and Frank 2000; Hinrichsen et al. 2001), and *in situ* studies of predation have proven difficult (Bailey 1989), the study of nutritional condition (after Shelbourne 1957, meaning nutritional status or “health”) enables the individual analysis of the physiological state of a larva and, as a proxy of growing state, it is still believed to be a useful method to aid in the estimation of larval recruitment (Ferron and Leggett 1994; Theilacker 1996; Suthers 1998). Although severe starvation can cause direct mortality, this is seldom observed in the wild. More probably, and according to the current line of thought, unhealthy larvae will experience suboptimal growth, and will have an increased susceptibility to mortality by predators, starvation, unfavourable larval transport, etc. (Houde 1987; Leggett and Deblois 1994; Hare and Cowen 1997).

In the last decades, there has been extensive literature on the use of condition indices applied to fish larvae of several species, both in the laboratory and in the wild (Ferron and Leggett 1994, Suthers 1998). Most of these works can be divided into two main types. One

regards the development of indices and the study of their properties under controlled conditions. The other deals with the application of existing indices (prior species-specific laboratory calibration or directly for achieving relative comparisons in the field) to study the mean condition of wild larval populations, and relate it to environmental conditions in order to infer potential survival.

This thesis contributes to the body of knowledge of these two main fields by focusing on the following general objectives:

A) **To deepen the study of multiple condition indices in a well-known laboratory-reared species (sea bass *Dicentrarchus labrax*).** There is a need to test the maximum number of condition measurements in individuals with accurately known past feeding history, in order to establish meaningful comparisons between condition methods and properties. Whereas the bulk of laboratory works deal with only one or a few indices, there are not many works that compare various indices in a species obtained from a single culture (e.g. Martin et al. 1985; Bisbal and Bengston 1995). One requirement for conducting valid studies of condition indices in the laboratory is that the effect of the factors to be tested (e.g. food ration) do not vary unpredictably. For this, it is desirable that the chosen species has well established rearing parameters. We decided to choose *sea bass* for the experimental studies because of its well-known rearing requirements. Bearing in mind that the absolute values of some indices are species-specific, the focus of this experimental work was placed on the comparative responses of the indices, the improvement of some classical measurements, the relationship with larval survival and the theoretical usefulness of these indices for field studies.

B) **To study the relationships between environmental variability, nutritional status and growth of *Sardina pilchardus* (Walbaum 1792) larvae in an area of the Catalan Sea (North-Western Mediterranean).** Pilchard is the most abundant pelagic species in the NW Mediterranean (Martín 1991), and supports the major fishery (in terms of biomass) off the Catalan coast. Despite its economic importance and the known decline of the population biomass since 1993 (Anonim 2000), the studies on possible links between environmental conditions and pilchard early life histories are not yet developed in this area. Although the

oceanography of the Catalan Sea is well known (Salat and Cruzado 1981; Font et al. 1988; Pinot et al. 1995; López-Jurado et al. 1996; Millot 1999; Salat et al. 2002), the only studies on pilchard larvae deal with its horizontal and vertical distribution patterns during the peak spawning season (winter) (Olivar et al. 2001). There are no other studies on pilchard larval ecology for this area, and there is none dealing with nutritional status and its relationship with environmental conditions. Within this context, the second part of this thesis is devoted to offering a number of elements in response to this problematic.

1.2. FACTORS AFFECTING LARVAL SURVIVAL AT SEA

Prior to any description of potential factors determining survival, we consider it necessary to define a series of concepts and terms that characterise early life history of fish.

In general, marine fish spawn pelagic eggs that hatch into undeveloped larvae whose energy source is of maternal origin (yolk-sac and sometimes oil globules) until they develop the morphological, behavioural and physiological sufficiency that allow them to obtain external planktonic food. Important events during this development are the opening of the mouth and the development of a functional digestive system. In optimal environmental conditions, larvae develop at fast rates until they reach the juvenile form after a gradual or abrupt process called metamorphosis, by which larvae acquire most morphological and physiological characteristics of the adults (including ossification, scale formation, etc.). We will use the terminology of Kendall et al. (1984) to describe these phases, for its simplicity and wide-spread use: egg (fertilisation to hatching), larva (hatching to metamorphosis) and juvenile (beyond metamorphosis). Within the larval period, development is divided into yolk-sac, pre-flexion, flexion and post-flexion. In more general terms, we may refer to “young larvae” or “older larvae” (referring always to non yolk-sac larvae) when the studied processes do not necessarily follow the categorisation made by the urostyle flexion. All these phases are collectively referred to as “Ichthyoplankton” (Ahlstrom and Moser 1976). Ichthyoplankton, by definition, is considered to be dependent on horizontal currents for displacement, although active migration may be performed by larvae even in the pre- flexion stage. Ichthyoplanktonic distribution will thus be an result of the spawning site, hydrodynamics and vertical motility.

The factors determining final larval survival, and probably recruitment, are multiple and interact in a complex way. Acknowledging the fact that any division of putative sources of mortality is arbitrary and biased, we next briefly review the main biotic and abiotic factors that may condition larval survival according to the reviewed literature.

Abiotic factors

Abiotic factors like **water temperature, salinity or light** can influence individual larval survival by directly affecting developmental rates (Frank and Leggett 1981,1982; Miranda et al. 1990; Koumoundouros et al. 2001; Sawant et al. 2002), developmental patterns (Johnston et al. 2001), adult fecundity (McGurk 2000) or spawning time (Shelton and Huthchings 1989; Ré et al. 1988; Mihelakakis et al. 2001; Sánchez-Velascoa et al. 2002), among others. Temperature and salinity are conservative in nature and integrate to define the water masses in which larvae are immersed. It is generally believed that physical processes that cause water movement and structure of the water column at different scales of space and time have a bigger influence in final larval survival. We therefore will comment on some of these aspects.

Hydrodynamics may operate on survival at scales from less than a metre to thousand of kilometres. Unfavourable **larval transport** was already postulated to be one of the major causes of recruitment variability in the work by Hjort (1914). For example, in upwelling areas, the associated offshore transport of larvae to less productive zones due to Ekman transport is well documented (Parrish et al. 1981; Olivar and Shelton 1993). On the other hand, in species whose larval development is dependent on successful transport to defined nursery areas (i.e. defined areas where larval and juvenile survival is enhanced, like estuaries), favourable advective processes associated with the force of wind or pressure gradients may be crucial (e.g. Govoni and Pietrafesa 1994).

In species whose larvae develop in relatively confined areas, or with high mesoscale variability, survival may be linked to physical forces and features spanning a scale of metres to kilometres and days to weeks. Within these, the existence of **marked pycnoclines**, **frontal zones** (shelf-slope fronts, plumes), **upwelling** and the effect of **eddies** may contribute to the concentration of larvae and their prey in aggregates where survival is

promoted. The concentration of planktonic organisms is enhanced at frontal zones, which can be thought of as the surface expression of sharp gradients in the physical properties of two water masses. Aggregation of plankton results from its accumulation along horizontal hydrodynamic convergence (Kingsford 1990). Whereas there is evidence that maximum concentrations of larvae can appear associated to shelf-break fronts or river plumes (Sabatés 1990 a, b; Sabatés and Masó 1992; Govoni and Pietrafesa 1994), frontal zones appear to be the direct cause of larval retention and enhanced production, therefore contributing to final larval survival (Kiørboe et al. 1988). Also, the surface enrichment with nutrients caused by upwelling generally causes enhanced productivity that has been related to enhanced larval survival (Cushing 1990; Bakun 1998). **Wind stress** can disrupt these structures and contribute to the scattering of larvae and their prey lowering their survival probabilities, as expressed in the “stable ocean” hypothesis by Lasker (1981). Also, wind stress can generate highly dynamic conditions that require larvae to invest more energy in feeding or maintaining a position in the water column, producing physiologically unfitted larvae that will have a higher mortality potential (e.g. Gallego et al. 1996, Kloppmann et al. 2002). Small-scale wind-generated **turbulent forces** play a crucial role here. Their direct relation with individual feeding success will be discussed below.

Biotic factors

Within the biotic factors affecting survival, feeding success and predation are thought to be the most important.

Feeding success, though an intuitive concept, involves multiple inter-related factors such as larval and prey patchiness, turbulent forces, density-dependent mechanisms, behavioural aspects, size and age, prey composition, etc. (see Leggett and Deblois 1994). Feeding success must be differentiated from food abundance, that had been hypothesised as the main field variable determining recruitment (Hjort’s (1914) Critical period hypothesis and subsequent Cushing’s (1975) Match-Mismatch hypothesis). McKenzie et al. (1991) showed that larvae and their prey appear in patches, these being influenced by vertical water structure, active vertical swimming, etc. Indeed, larvae are not completely passive. They display active vertical migration, following species-specific patterns, in order to maximise feeding success and reduce mortality by predators (Neilson and Perry 1990).

Feeding success can also be fostered by wind-generated turbulence. Within certain values, small-scale turbulent forces are known modify the rate of encounter between larvae and food items in a dome-shaped way (Rothschild and Osborn 1988, modified by MacKenzie et al. 1994). More recently, the turbulence-avoidance hypothesis has been suggested as an alternative mechanism generating increased larval-prey patchiness and feeding success (Franks 2001). It suggests that both larvae and prey migrate vertically to calmer waters to avoid unfavourable turbulent mixing, hence concentrating at particular depths. At the individual level, interactions between development, predatory capacities and prey density may also play an important role (Laurel et al. 2001; Puvanendran et al. 2002).

The current view is that feeding success may have a more determinant effect on survival in young pre-flexion larvae, as at this stage they cannot withstand suboptimal feeding or high energy expenditure for too long due to their limited energy stores. For older larvae or yolk-sac larvae predation will be the dominant cause of mortality. However, a complete understanding of the importance of feeding success and the mechanisms governing it requires the integration of multiple parameters in field studies (e.g. Werner et al. 2001).

Predation on fish eggs and larvae is thought to be perhaps the final cause of most mortality, and can be exerted by numerous organisms (Bailey and Houde 1989). The numerous factors affecting predation include larval size (Pepin et al. 1992), age (Litvak and Leggett 1992), level of starvation (Yin and Blaxter 1987, Elliott and Leggett 1998, Green and McKormick 1999), density of predators (Sheperd and Cushing 1980, Steele and Forrester 2002), ontogeny (Fuiman 1994; Sheperd et al. 2000), temperature (Elliott and Leggett 1997) or developmental rate (Houde 1987).

The mechanisms by which individual larvae are removed through predation are understood in a frame of vulnerability to predators, which increases if larvae are small (the "bigger is better" concept), or develop more slowly (the "stage-duration" concept). These mechanisms are not mutually exclusive and fall into the broader frame of the "growth-mortality hypothesis". Although literature results are sometimes contradictory, there is an increasing number of papers that support the claim that faster-growing and/or larger larvae experience higher survival rates (e.g. Hare and Cowen 1997). Suthers (1998) analysed the

usefulness of studies of condition in the frame of the growth-mortality hypothesis ("Is fatter better"?). He concluded that having a better body condition is only advantageous if it is related to a faster growth. In addition, suboptimally feeding larvae may lose buoyancy control, being more vulnerable to unfavourable advective transport (Neilson et al. 1986; Sclafani et al. 1993, 2000).

Disease and parasitism are thought to be important biotic factors affecting larval survival, and could be enhanced in unfitted larvae. There is a paucity of information regarding disease and its incidence in wild populations, although in clupeoids recent evidence suggests that resulting mass mortality in juvenile and adults due to disease may be high (Gaughan et al. 2000; Ward et al. 2001). On the other hand, parasites are known to affect eggs and larvae (e.g. Crespo et al. 2001) and could be a potential source of mortality, though this is usually unquantified in the field.

1.3. CONDITION INDICES

From the above explanations, survival is believed to be a result of favourable hydrographic conditions, low predation, high feeding success and, with less certainty, incidence of disease.

Condition indices were extensively reviewed by Ferron and Leggett (1994) in terms or reliability, sensitivity, time response, size and age specificity, field *vs* laboratory estimates, processing time, costs and requirements. They divided condition indices into three main categories according to the main organisation levels: organismal, tissular and cellular. These indices operate at different time scales. Typically, the higher the organisation level of the index, the longer it takes to respond to an environmental change (i.e. food, turbulent mixing rate etc.) for a given developmental state, species, set of environmental conditions and specific nutritional status of the individual at the moment of study. Some indices can serve directly to estimate mortality (typically necrotic tissues, e.g. McFadzen et al. 1997), whereas others are a reflection of growth or immediate feeding status (e.g. some biochemical indices). As concluded by Ferron and Leggett (1994), no single index is "the

best index", and usually a specific combination must be chosen in order to respond to a particular question.

In the next paragraphs, a brief description of each category of indices is offered, focusing on those investigated in this thesis.

Organismal level

Condition indices at the organismal level are typically studied through morphometrics for its integrative character (changes in cellular properties cause histological variations that may end up provoking shape changes). The term morphometrics applies to the study of the shape, or changes in shape, of an organism or part of it. In fish larvae, morphometrics has been utilised to detect the effects of suboptimal feeding for many years (Shelbourne 1957; Theilacker 1978). The use of these indices is based on the premise that, in response to food deficiency, some body parts (e.g. body depth) are modified with respect to some less starvation-sensitive parts (e.g. eye diameter or length). Much of the success of these indices is attributable to their short processing time, low cost and ease of obtainability. Although differences between seasons, sites and years have been found in the nutritional status measured by some sort of morphometric measurement (Ferron and Leggett 1994), they have been criticised for having low sensitivity to short-term events (less than a week), such as upwellings, etc. Other criticisms are based upon their sensitivity to shrinkage (both due to preservatives and speed net collection), size and age dependence, the high differences between calibration data *vs* wild specimens, or the high species specificity.

Another criticism to morphometric indices is based on the usually neglected effect of allometry. With the term allometry we refer to the differential growth of some body parts along ontogeny. Allometry is highly species-specific, and can be easily confused with the effects of feeding ration or quality. Whereas several standardisation methods have been used in order to remove the effect of size, few have dealt with allometric effects.

In Section 2.2. of this thesis, this question is addressed by applying a method for totally removing size effects whilst accounting for allometry, and interpret the results in the frame of other previously used methodologies.

Tissular level

Histological indices have a tradition of determining condition both in the laboratory and in the wild (O'Connell 1976; Theilacker 1978; Oozeki et al. 1989; Theilacker and Watanabe 1989; Sieg 1992; Ferron and Leggett 1994; Bisbal and Bengston 1995; McFadzen et al. 1997; Sieg 1998). The success of histological indices is partly attributable to the high amount of information that can be derived from their study (even if slides were kept for a long time) and because they are thought to be the only true starvation indices (Suthers 1998).

The technique usually consists of the examination of cells and organs and the establishment of a grading system. Each organ is examined and the cellular aspect or tissular cohesion evaluated in order to obtain a measure of the general condition of a larva. A crucial advantage of histological indices measured through multiple grading is that the general pattern of tissular degradation is relatively independent of size and, to an extent, species (Ferron and Leggett 1994; Grioche 1998).

There are, however, some questions that require further investigation in the laboratory. One of them regards the need of objective methods for tissue grading. Indeed, a great amount of studies used qualitative or semi-qualitative measurements and relied on the experience of the histologist (O'Connell 1976; Sieg 1992; McFadzen et al. 1997). Until recently (Catalán and Olivar 2002), quantitative data have been restricted to the measurement of cell heights of few tissues, mainly gut and liver, and have proved useful for the early larval stages of some species (Ehrlich et al. 1976, Oozeki et al. 1989, Theilacker and Watanabe 1989, Theilacker and Porter 1995). However, some of these measurements are only obtainable from species with elongated digestive ducts (Theilacker and Watanabe 1989), or have been restricted to particular larval stages. Lastly, some characteristics of muscle cellularity and muscle fibre growth patterns have been pointed out as possible indicators of condition. Several workers have proved that temperature can modify the

patterns of hypertrophic or hyperplastic muscle growth (Stickland et al. 1988; Vieira and Johnston 1992; Johnston et al. 1995). The effect of food, however, has never been tested and only in one field study it was pointed out as a possible source of variation in the muscle growth patterns (Temple et al. 2000).

In Section 2.3, we compared the advantages of classical histological scoring *vs* quantification of several organs through digital image analysis. Also, much of the work on the condition of pilchard larvae was based on histological methods (Section 3.2.), some of which were derived directly from our laboratory experiments. The possible effect of temperature and food on muscle growth patterns in pilchard was also explored in Section 3.2.

Cellular level

This level is studied through biochemical indices and some histological indices. There is not, to date, a general frame that enables the establishment of a general pattern of mobilisation of biomolecules during suboptimal feeding conditions in fish larvae. From the reviewed literature, it seems that suboptimally-fed young larvae soon deplete liver glycogen stores (Govoni 1980) and endogenous lipids (Hakanson 1989), and rapidly rely on catabolism of muscle proteins, which are the main energy source of starved young larvae (Pedersen et al. 1990). As the larvae grow bigger, lipid and glycogen stores can cope with starvation for longer before muscle proteins are utilised. The establishment of biochemical condition indices in specimens of unknown age (typically wild-collected), given the large sources of variability other than nutrition, needs to relate the variation of a particular starvation-sensitive variable to a less-sensitive variable.

Measures of condition at the molecular level have had enormous success in the past years (Ferron and Leggett 1994, Suthers 1998), partly due to the increased availability of molecular techniques and the progressively lower costs. We focused this section of the introduction on the indices that we examined in this thesis (some widely used, some largely untested), which involved the measurement of relative RNA and protein contents, metabolic enzyme activities and cell proliferation rates. Section 2.4. is devoted to the comparative study of these indices.

One of the most popular condition indices in fish larval studies is the RNA/DNA ratio, which is a reflection of protein synthetic potential (Buckley 1979; Ferron and Leggett 1994; Bergeron 1997; Buckley et al. 1999). Fish growth is accomplished primarily by protein synthesis (Love 1970), which can make up over 50% of total dry weight. As RNA content per cell vary according to the amount of protein synthesis, but DNA is relatively constant within a cell, the RNA is usually standardised by DNA (e.g. Clemmensen 1987). RNA/DNA has been successfully used as a proxy for growth and condition in fish larvae (Buckley 1979; Westerman and Holt 1994; Clemmensen 1996).

Although several problems in the use of RNA/DNA have been identified in the laboratory (dependency on developmental stage, size, tissue, temperature or analytical method), the use of RNA/DNA is still recommended for field studies in relevant and relatively recent reviews (Bergeron 1997; Buckley et al. 1999).

Taking the clupeiforms as an example of fish group, several works found increased RNA/DNA ratios in larvae collected in frontal or coastal areas *vs* offshore areas (*Sardina melanostichus*, Shimizu et al. 1989, Nakata and Zenitani 1996), or in areas with higher zooplankton biomass (*S. pilchardus* Chícharo et al. 1998 a,b).

From the aforementioned reviews, several important recommendations arise for field studies: 1) RNA/DNA is best suited for the analysis of larvae that do not depend on maternal reserves. In the youngest stages, ontogenetic effects may be confused with the effect of food ration; 2) the use of single tissues (preferably musculature, as it is the most abundant tissue and is a particularly good indicator of condition) is recommended to reduce variability; 3) only similar size-ranges and temperatures should be compared; 4) analytical methods must try to minimise the error inherent in the classical RNA determination, usually obtained by subtraction from total nucleic acid estimates after RNA-specific enzyme digestion. The need to reduce this uncontrolled variability becomes evident after Houde (1989), who showed that only a 5% decrease in daily larval growth (variability in RNA determination is often larger) could reduce by an order of magnitude the individuals surviving through larval stage.

Another type of biochemical condition indices are those related to the activity of some metabolic enzymes. There are several works on juvenile and adult fish showing that starvation and lowered growth rates can be reflected in the activity of some metabolic enzymes (Lowery et al. 1987; Yang and Somero 1993; Mathers et al. 1992, Couture et al. 1998). In fish larvae, studies are scarce and are mostly based on the study of two enzymes, lactate dehydrogenase (LDH), from the anaerobic glycolisis cycle, and citrate synthase (CS) from the Krebs cycle.

Whereas LDH is associated primarily with the potential for anaerobic catabolism, CS is a reflection of feeding-induced aerobic metabolism (Sullivan and Somero 1980). The activities of both enzymes are usually standardised by unit protein.

Lactate dehydrogenase transforms pyruvate to lactate to produce energy in environments of low oxygen availability, typically in muscle (Fig. 1.1). LDH was found to be positively correlated to food quantity and quality in *Sciaenops ocellatus* and *Lutjanus synagris* (Clarke et al. 1992) and negatively related to swimming ability under suboptimal feeding regimes in the clupeid *Brevoortia tyrannus* (Fiedler et al. 1998).

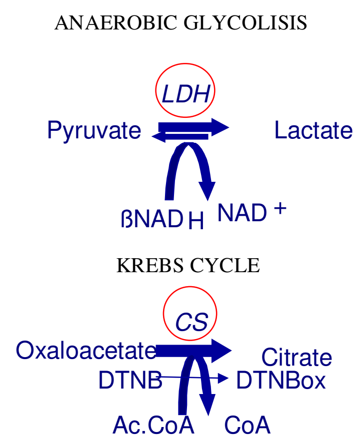

Figure 1.1. Schematic of the metabolic reactions measured in the laboratory belonging to either the anaerobic glycolisis or Krebs cycle

Citrate synthase transforms oxaloacetate to citrate in the Krebs cycle to obtain reducing potential plus some ATP (Fig. 1.1). This purely aerobic pathway is thought to be more conservative as it plays a basic role for cell metabolism. CS activity has been found to be lower in starved *vs* fed larvae, but not in larvae fed different rations (Mathers et al. 1992).

A general consideration applying to the use of both enzymes is the strong dependence on size. Relative enzyme activities (activity protein^{-1} by unit body mass) increase with size in large larvae, juveniles and adults (Somero and Childress 1980; Power and Walsh 1992; Overnell and Batty 2000). Metabolic scaling with size is not well studied in early larvae and it has been suggested that comparisons among individuals should be restricted to narrow size ranges (Brightman et al. 1997).

A method that is still less exploited in the realm of biochemical condition indices is based on the calculation of cell proliferation rates using Flow Cytometry (Theilacker and Shen 1993 a, b; Bromhead et al. 2000; Theilacker and Shen 2001). This method relies on the hypothesis that growth is related to increased cell division rates. During a normal eucharyotic cell cycle, cells that start DNA synthesis will have increased DNA contents until the end of phase S (Fig. 1.2.). The populations of dividing cells (S,G2 and M) can be distinguished from those of non-dividing cells (G0 and G1) by measuring the DNA-specific fluorescence of each cell using flow citometry (Fig. 1.2 A, B). Studies of *in vitro* cell cultures have shown that extracellular factors such as nutrient deprivation determine that normal growing cells in the G1 stage revert to quiescence (Pardee 1989; Murray and Kirschner 1989; Alberts et al. 1994).

The few available studies on cell proliferation rates using Flow Cytometry in larval fish have been conducted on brain or muscle tissue. Cells of these two tissues are known to divide throughout most of the life cycle in fish (Greer-Walker, 1970; Bierse et al., 1980). It is known that muscle growth has a high plasticity in responding to environmental changes

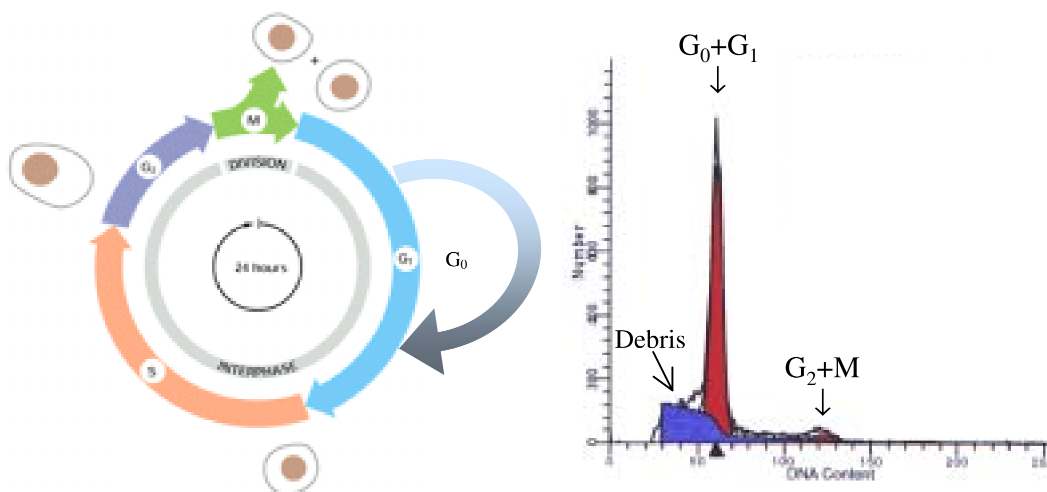

Figure 1.2. A; the main phases of the eucharyotic cell cycle. During interphase the cell grows continuously; during M phase it divides. DNA replication is confined to the part of interphase known as S phase. G1 (high biosinthesis) and G0 (quiescence) will show constant DNA. S phase will show higher DNA contets, peaking at G2 and M. B; output of a flow citometry analysis. The y axis shows the number of cells, and the x axis shows the amount of fluorescence, proportional to DNA contents per cell, in relative units. Fluorescence identified as "debris" is shown in blue.
A, modified from: http://www.ncbi.nlm.nih. gov:80/books/data/cell/pictures/ch17/ch17f3.gif
B, results from analysis performed on brain cells of *D.labrax* larvae (present study).

(Weatherley 1990; Johnston 1999), which makes it a particularly good candidate for these types of studies. Theilacker and Shen (2001) revealed the suitability of this tissue for studying growth and condition in fish larvae.

Works conducted on brain cells of larval fish also showed that the DNA synthetic activity was sensitive to different factors such as the nutritional status or the environmental temperature (Theilacker and Shen 1993 a, b, Bromhead et al. 2000). Despite the promising results obtained, all authors agree that further research is required in order to apply the fraction of dividing cells as an indicator of larval growth or condition.

1.4. EARLY LIFE HISTORY OF MEDITERRANEAN PILCHARD

The pilchard *Sardina pilchardus* is a clupeid distributed in the North-West Atlantic, Mediterranean Sea, Sea of Marmara and Black Sea. Within the Mediterranean, it is more common in the western part and Adriatic Sea than in the eastern part (Whitehead 1984). In the NW Mediterranean, it is the most abundant small pelagic species (Martín 1991). It supports the largest fishery of small pelagics in terms of biomass, although economically it is less important than anchovy.

Pilchard, like most clupeids, is a indeterminate, serial (batch) spawner that release batches of pelagic eggs at intervals within the spawning season (Hunter et al. 1985). Iberian pilchard has a protracted spawning season, with some differences in the spawning peaks depending on the area and temperature regime (Ré et al. 1990; Solá et al. 1992). For the Atlanto-Iberian pilchard, the main spawning months occur in winter (Portugese coasts) or spring (Cantabric coast) (García et al. 1992). For the North-Atlantic Spanish coast, two main spawning seasons have been identified, namely between January and June and a less marked peak between September and October (Riveiro et al. 2000).

In the Mediterranean the spawning season extends from October to May, peaking in January-February (Gómez-Larrañeta 1960; Palomera and Rubíes 1979; Palomera and Olivar 1996). This period is similar to that described for other Mediterranean areas like the Adriatic Sea (Dulcic and Grbec 2000) and embraces the most unstable months of the year.

An important aspect for the survival of the species is the reproductive strategy which, in the case of the small clupeids, is strongly coupled to environmental conditions (see below).

Eggs and larvae are usually found within the continental shelf. Eggs are around 1.5 mm, spherical, and are normally found in the upper 70 m of the water column, with maximum concentrations between 10 and 40 m (Olivar et al. 2001). The time to hatching is around 67 hours at 15 ºC (Miranda et al. 1990) and larvae can remain in the plankton for longer than a month (Ré 1984). Larvae feed during daytime at depths between 10 and 40 m., whereas at night they show a wider vertical distribution (Olivar et al. 2001). Most food ingested by larvae are copepod eggs, nauplii and copepodites (78-89%). The amount of nauplii is high at all ages, decreasing with larval length and being compensated for by an increased number of copepodites (Conway et al. 1994).

1.5. INTERACTIONS BETWEEN PILCHARD LARVAL ECOLOGY AND OCEANOGRAPHY IN THE CATALAN SEA

The major aspects of the dynamic conditions and the typical water mass structure in the Catalan Sea are well known and have been described by several authors (e.g. Salat and Cruzado 1981; Font et al. 1988; Pinot et al. 1995; López-Jurado et al. 1996; Millot 1999; Salat et al. 2002). The cyclonic circulation in the surface layer around the basin is controlled by two permanent density fronts: the Catalan shelf-slope front located over the Iberian Peninsula slope and the Balearic front located over the insular slope (Fig. 1.3).

The Catalan front separates the saltier old Atlantic Water (AW)[1] in the central part from fresher shelf waters near the Catalan coast. The Balearic front separates the old AW from the less saltier and warmer new AW coming into the basin through the Balearic Island channels (Pinot et al. 2002). The circulation is subjected to mesoscale variability, which has been attributed to the action of open sea eddies (Tintoré et al. 1990; García et al. 1998).

[1] Water mass acronyms in this paper follow the recent recommendations of the Round table on Mediterranean Water Mass Acronyms, 36th CIESM Congress, Monaco, 2001. (http://ciesm.org/events/RT5 WaterMassAcronyms.pdf).

Some variability in the horizontal distribution of pilchard and anchovy larvae in this area is known to be associated with the variability in the position of the shelf-slope front, the anticylonic circulation in the shelf and the effect of surface advection of fresh water occurring in the northern part of the Catalan Sea in spring (Palomera 1992; Sabatés and Masó 1992; Sabatés and Olivar 1996; Salat 1996; Olivar and Sabatés 1997; Olivar et al. 1998). These works have focused mainly on the spring-summer seasons, away from the period when the bulk of pilchard spawning takes place.

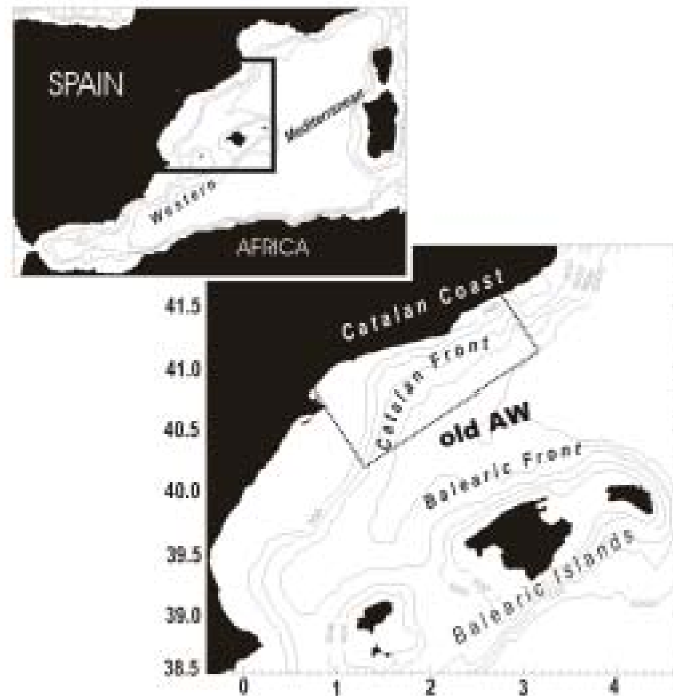

Figure 1.3. Western Mediterranean basin with indication of the sampling area along the Catalan coast shelf and slope.

Unlike in summer (when strong thermal stratification occurs), the winter period in this area is characterised by the fertilisation of all the water column over the continental shelf, caused by local vertical mixing and shelf intrusions of slope water (Salat et al. 2002).

The reproductive strategy in small clupeids is strongly linked to favourable environmental conditions. Whereas spawning seasonality in clupeids may be genetically-driven, the relative timing of egg production may have a strong environmental influence (Pitcher 1995). In agreement with this, pilchard larvae are known to distribute horizontally on coastal and central areas of the shelf, where productivity is high and currents are less pronounced than in the shelf-slope front (Olivar et al. 2001).

There are no data on the relationships between environmental variables and the condition of pilchard larvae for this area. The closest information related to this issue for this species was obtained from the north Atlantic Iberian Coast, where condition has been assessed by histological (McFadzen et al. 1997), RNA/DNA (Chícharo et al. 1998 a, b) and

C/N ratios (Coombs et al. 1999). All these indices showed that percentage of suboptimally fed larvae was low. Only RNA/DNA correlated positively with zooplankton biomass. Also, a relatively recent work was conducted on the growth of pilchard larvae in the South-Western Mediterranean Ramírez et al. (2001). Although they associated larval growth with increased RNA/DNA and protein/DNA contents, no relationship with differing environmental variables or periods was made.

Within the last part of this thesis (sections 3.1 and 3.2.), new information on the possible environmental relations between distribution patterns, abundance, growth and body condition of pilchard larvae in an area of the Catalan Sea is offered.

2. LABORATORY STUDIES

2.1. EXPERIMENTAL REARING, GROWTH AND SURVIVAL

Most of this section has been published in: "Olivar MP, Ambrosio PP, **Catalán IA** (2000) A closed recirculation system for ecological studies in marine fish larvae: growth and survival of sea bass larvae fed with live prey. Aquat Living Resour. 13:1-7."

ABSTRACT

This section describes the suitability of a closed recirculation system to study the development of fish larvae in a strictly controlled environment, where only feeding was varied (treatments Fed, Starved, Delayed, and short-term late-fast and re-feeding). The system served both as an incubator and hatchery. The time-variation of physical and chemical parameters together with survival and growth of reared sea bass (*Dicentrarchus labrax*) larvae were studied over the first month of life. Special attention was paid to nitrogen compounds. The recirculation design allowed for the maintenance of levels of ammonia, nitrite and nitrate below those cited as responsible for mortality or decreased feeding ability in other marine fish larvae. Almost no larval mortality occurred in the Fed larvae from day 9 after hatching. The Starved group of larvae showed a sharp decline in survival after 17 days of food deprivation. Larvae to which feeding was Delayed until day 13 ceased dying four days after food was supplied. Growth in length was similar during the first two weeks of larval life regardless of feeding treatment, and followed a Gompertz curve, with an asymptotic length of 5.3 to 5.7 mm. A second Gompertz cycle was observed in Fed and Delayed treatments some time after food was supplied. Growth differences (growth rate and total body length) between larvae in Fed and Delayed treatments were especially evident during the third week of life. Nevertheless, after two weeks of normal feeding, differences in size and growth rates were reduced. No changes in length-growth or survival were observed in the short-term late-fast or re-feeding treatments.

Growth patterns of Fed larvae in this study were similar to those reported for larvae reared in flow through systems. We believe that survival and growth of the reared larvae was a direct function of diet, and that the type of rearing system did not adversely affect these parameters. The results obtained suggest that future studies on nutritional condition may benefit from this rearing design.

INTRODUCTION

Low food concentrations in marine systems may result in direct or indirect larval mortality due to both starvation or reduced growth, which, in turn, may result in increased predation (Hunter 1981). However, numerous environmental factors affect both development and survival of fish larvae in the wild. Culturing techniques developed in aquaculture, where most variables can be controlled, have become useful for these type of studies.

This work is part of a broad- multidisciplinary study that deals with the factors influencing survival of fish larvae in the North Western Mediterranean. Accurate laboratory experiments which discriminate factors potentially affecting survival (e.g. starvation) are crucial to understand the relative importance of such phenomenon in the wild. As a first step, the effect of food deprivation on larval survival and growth was studied in larvae reared under controlled conditions. In order to ensure that these parameters were only dependent on feeding regime, it was necessary to set up an experiment in which, in a strictly controlled environment (closed recirculation system), only the feeding ration varied. The sea bass, *Dicentrarchus labrax*, was chosen for the experiments because of its wide distribution around Europe and the relatively well-known rearing requirements.

Most studies using closed recirculation systems focus on particular aspects of the reared species, but it is difficult to find detailed descriptions for short term experimental cultures. Previous studies reported on acceptable levels of several physical and chemical parameters that produce good levels of growth and survival for *D.labrax* (Barahona-Fernandes 1978; Corneille et al. 1989; Johnson and Katavic 1986). None of these studies, however, described the time-variation of all these parameters along with data on growth and survival at different feeding levels in a closed recirculation system.

The goal of the present work is to analyse the suitability of a short-scale integrated incubation-hatchery recirculation system to help in future studies of field collected larvae (in this case, for calibration laboratory experiments to study nutritional condition).

MATERIAL AND METHODS

The basic design was taken from Amat et al. (1991) and adapted to a closed system. These changes were made to enhance the water circulation in order to maintain a uniform water quality and enhance the elimination of non-consumed food.

Figure 2.1.1 shows the main components of the recirculating system. It was composed of a 310 l rearing tank and a reservoir of 220 l. A biological filter (BF) was placed on top of the reservoir tank and a mechanical filter of 85 l (MF) was located between the two tanks. Twelve grey polyvinyl chloride (PVC) rearing cylinders of 4 l (RC) were set in the rearing tank. A 500 µm mesh size was glued to the bottom of the cylinders. A PVC ring with a 100 µm mesh glued to it was used as a lid for the bottom of the cylinders (FC, Fig. 2.1.1 b). These lids were placed during feeding hours to prevent the escape of living prey. The total water flow through the system was kept constant at 17 l min $^{-1}$ (1020 l h^{-1}) during the whole experiment. This meant a water renewal in the rearing tank of 4.8 h^{-1}. Renewal rate in the rearing cylinders fluctuated between 0.37 and 2 h^{-1} (Table 2.1.1.).

Table 2.1.1. Controlled parameters of the water quality for laboratory-reared sea bass larvae. WF, water flow. Turnover refers to the rearing tank.

Age	Light ux)	Temp. (°C SD)	Salinity (±) psu	Oxygen g l^{-1})	PH (± SD)	$N\text{-}NO_2$ (± SD) g l^{-1})	$N\text{-}NO_3$ g l^{-1})	WF in cylinders h $^{-1}$)	Turnover h^{-1}	Aerat. (ml n $^{-1}$)
Eggs	11-15	16	37	9.2	8.2	0.004 ± 0.001	<5	4-6	1-1.5	40
L_0L_2	11-15	16	37	9.6 ± 0.2	8.0 ± 0.1	0.004 ± 0.001	<5	1.5-1.6	0.37-0.40	20
L_3L_{10}	15	17.5 ± 0.6	35.6 ± 1.3	7.9 ± 0.7	8.1 ± 0.1	0.02 ± 0.019	0-5	1.5-1.6	0.37-0.40	20
$L_{11}L_{18}$	15	20.1 ± 0.3	30.9 ± 1.0	6.9 ± 0.5	7.8	0.12 ± 0.019	10-15	1.5-1.6	0.37-0.40	20
$L_{19}L_{25}$	40	19.7 ± 0.2	30.1 ± 0.3	7.3 ± 0.2	7.9 ± 0.1	0.181 ± 0.025	15-40	1.5-1.6	0.37-0.40	40-60
$L_{26}L_{28}$	40	20.5 ± 0.9	29.9 ± 0.3	7.3	7.9	0.216 ± 0.013	15-40	2.4-2.6	0.60-0.65	40-60

Initially, the system was filled with water that had been filtered to 0.2 µm and sterilised by UV radiation. Water loss due to evaporation was replaced with distilled water,

Figure 2.1.1. A) Schematic diagram of the enclosed recirculating system for rearing marine fish larvae. BF, biological filter. DP1 and DP2, distribution pipes. FR, flow regulators. H, heater. IP, inlet pipes. MF, mechanical filter. OP, outlet pipes. MP, main pump. S, skimmer. SP, secondary pumps. RC, rearing cylinder. WL, water level. B) Detail of one of the rearing cylinders. Letters as in A, plus: A, air pipe. DP, distribution pipe. FC, feeding cap. WI, water inlet. Arrows indicate water flow.

Not to scale.

which was added to the reservoir on a daily basis. The whole system was set in an isothermal chamber at 15°C. Water temperature was adjusted with thermostats immersed in the reservoir (H, Fig. 2.1.1. A). Salinity was adjusted during the different rearing periods, varying from 37 ± 0.1 at incubation and progressively decreasing to 30 (Table 2.1.1.). Temperature was gradually raised throughout the experiment from 16 to 20°C (Table 2.1.1.). Photoperiod was established at 9 h light: 15 h dark.

Eggs were placed into the cylinders 43 days after the filter start up. Sea bass eggs in good condition have positive buoyancy at salinities over 34.5 psu (Barnabé 1991); thus they could be separated following the procedure of Felip et al. (1997) and evenly distributed in each cylinder using a Pasteur pipette. The effective number of individuals (eggs or larvae) by cylinder was obtained by adding the number of survivors at the end of the experiment to the number of dead and sampled larvae every day. The accumulated survival throughout the experiment is given as percentage of the living larvae in each cylinder at first day of feeding.

The food consisted on live prey according to Barnabé (1991) (rotifers, *Artemia* nauplii and one day old enriched metanauplii). Initially, two feeding treatments were established: the Fed treatment (8 cylinders) and the Starved treatment (4 cylinders). The Fed treatment was supplied with food *ad libitum* from day 5 after hatch. From day 13, one of the cylinders from the Starved group was supplied with food, to set the Delayed treatment. On day 23, a short-term fast and re-feeding scheme was introduced into 4 of the 8 cylinders of the Fed treatment to establish the Late-Fast treatments. The detailed scheme of these treatments will be shown in further sections because they were not used in this chapter. All food was suplied at the same hour every morning. We sampled 3-4 larvae per day and per cylinder. The methods of sample preservation depended on the later condition analyses and will be detailed in the corresponding Sections. For the present Sections, larvae were preserved in 10% phosphate-biffered formalin.

Larval length used for growth analyses refers to total body length (TL), measured as the distance along the midline of the body from the tip of the snout to the end of the caudal fin. Growth was analysed by fitting a Gompertz model to the data on TL. This model has

been used to describe early larval development in marine fish larvae (McGurk 1985 a; Polo et al. 1991; Zweifel and Lasker 1976).

Gompertz equation: $$TL = a\, e^{-be^{-ct}},$$

where TL is the total body length (mm), a is the asymptote (mm), c the instantaneous growth rate at the inflexion point ($days^{-1}$); t the age in days and b is a dimensionless parameter, such that $b \times c$ is the instantaneous growth rate when t=0. The Hotelling's T^2 test (Bernard 1981) was used to screen for differences between growth curves of different treatments. Absolute growth rate at an age t (dTL) was calculated from:

$$dTL = c\, TL\, (\ln a - \ln TL)$$

RESULTS

Incubation and hatching

Eggs were allocated in the rearing cylinders when they already exhibited an advanced stage of development. The embryo, already differentiated, had the caudal region separated from the yolk. All the eggs hatched on the same day. During incubation time and for the next 4 days after hatching concentrations of total ammonia ($N\text{-}NH_4^+ + NH_3$ in mg l^{-1}), unionised ammonia ($N\text{-}NH_3$), nitrite ($N\text{-}NO_2^-$) and nitrate ($N\text{-}NO_3^-$) concentrations were very low (Fig. 2.1.2., Table 2.1.1.).

Despite the attempted initial even distribution of eggs among cylinders, there was some variability in the actual number of eggs per cylinder (mean = 130 eggs l^{-1}, SD = 30). The hatching success, calculated as number of living larvae at day four after hatch, was 64.1% (SD 14.8%). Daily mortality in this period was highly variable both within and among cylinders. There was no correlation between the initial egg densities and the daily mortality observed during this period.

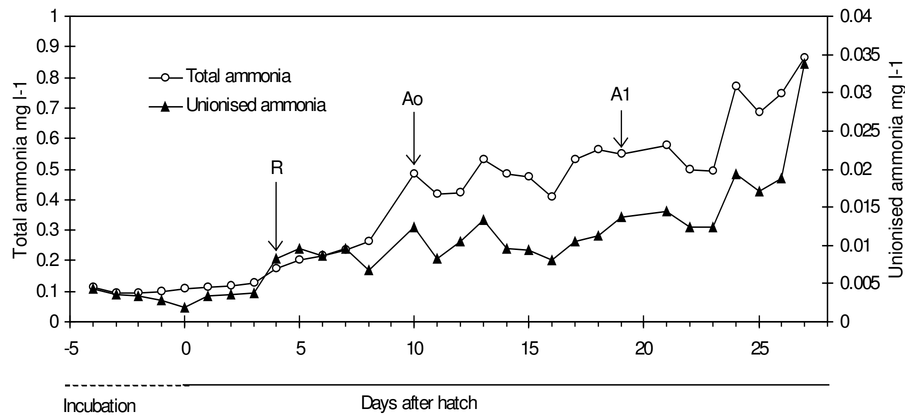

Figure 2.1.2. Concentrations of total ammonia and unionised ammonia, from five days before hatching until the end of the feeding experiment. Arrows indicate the initiation of feeding with rotifers (R), *Artemia* nauplii (A0) and one day old *Artemia* metanauplii (A1).

Larval rearing

During the experimental period, total ammonia, unionised ammonia and nitrite concentrations fluctuated within a range considered as non toxic for fish larvae (EPA 1989). However, the values tended to increase towards the end of the experiment (Fig. 2.1.2., Table 2.1.1.). The first clear increase in ammonia concentration (total and unionised) occurred on the first feeding day, when rotifers were introduced into the system (4 days after hatching) (Fig. 2.1.2.). The later introduction of *Artemia* nauplii (10 days after hatching) also produced an increase of total ammonia levels. However, concentrations of unionised ammonia could be kept below 0.014 mg l^{-1} until the introduction of enriched *Artemia* nauplii (19 days after hatching). Values of these metabolites reached the highest values from day 24 to 27 (Fig. 2.1.2.).

Survival

Larval density at first exogenous feeding was 60 larvae l^{-1} (SD 20). The data of larval survival in the three treatments is shown in Fig. 2.1.3. Survival decreased in all treatments until day 9 after hatch. Next followed a steady period of scarce or null mortality which, for the Fed larvae, continued until the end of the experiment. This phenomenon was also observed in the Starved group until day 14, although accumulated mortality was

higher. After day 17, the Starved group underwent a drastic drop in the number of larvae, all of which died in a week time. When larvae from the Delayed group were fed (day 13), their mortality rate continued dropping for three days (following a similar dynamics to the Starved treatment), until a steady-state similar to that of Fed treatment was attained.

Growth

Growth was described by two-cycle Gompertz curves in both Fed and Delayed treatments, while Starved treatment followed a one cycle Gompertz curve (Fig. 2.1.4.). Larvae from each treatment were considered as replicates both when they were sampled from a single cylinder (Delayed treatment) or from several cylinders (Starved and Fed treatments). For a same treatment, the homogeneity of the log-linearised growth within cylinders was confirmed by ANCOVA using age as a covariate (through GLM procedure, MINITAB Inc.). Significance was set at = 0.05.

It becomes apparent that all treatments underwent an initial high growth rate, up until day 4. After that day, growth rate decreased to a plateau that lasted differently depending on the treatment, but which was similar for all groups until day 14. Fed treatment showed a second increase in growth rate (GR) from this day (estimated GR on day 13 = 0.60). After urostyle flexion (days

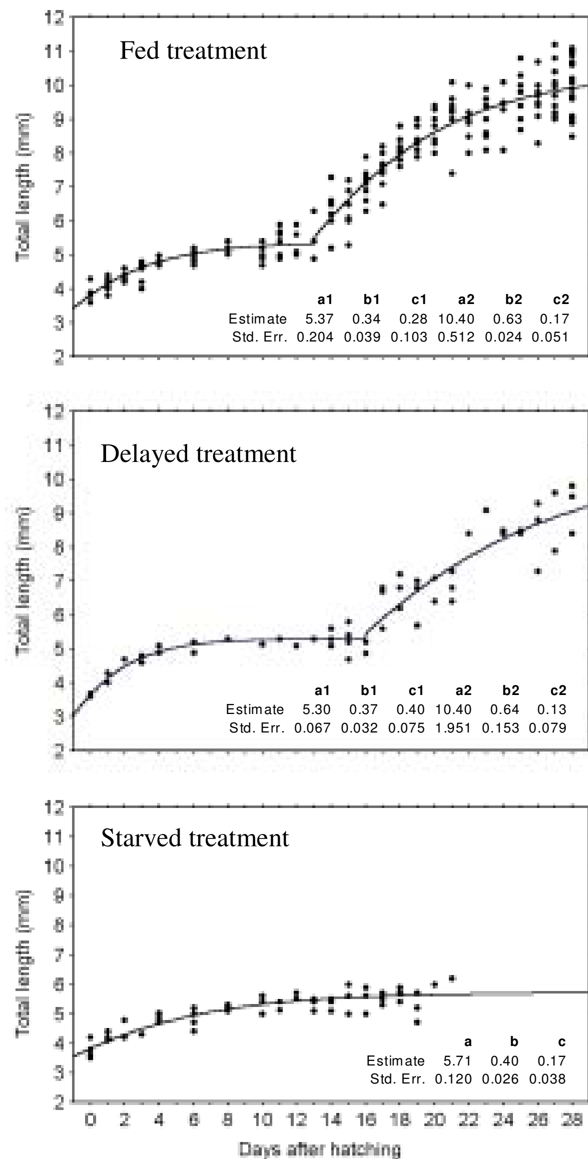

Figure 2.1.4. Gompertz fit of total length *vs* age of *Dicentrarchus labrax* larvae reared under different feeding conditions. Fed: larvae always fed. Delayed: Feeding was delayed until day 13 after hatching. Starved: larvae were deprived of food from hatching day. Estimated growth parameters and Standard Errors of the Gompertz curves inside each figure.

20-22) it was observed a conspicuous decrease in growth rate (estimated GR on day 22 = 0.22). Larvae to which first feeding was delayed until day 13 did not show the second increase in growth rate until day 16 (estimated GR on day 16 = 0.43). The estimated asymptotic size was similar for Fed and delay treatments. The second growth cycle of both Fed and Delayed treatments were compared and found to be significantly different (Hotelling's test: T2 (3814) > T02 (12)). This difference was due to the lower value of parameter *c* of the fitted curve in Delayed treatment.

DISCUSSION

The most dangerous metabolites for larval development (unionised ammonia and nitrite) are even more important in the first phases of ontogeny (Holt and Arnold 1983). However, in the case of nitrite, its toxicity in salt water is much lower than in fresh water systems. Concentrations of unionised ammonia and nitrite during the present experiment were much lower than those considered dangerous for survival (Brownell 1980; EPA 1989; Holt and Arnold 1983).

The system design permitted an easy adjustment of the most important physical and chemical parameters and allowed for the successful maintenance of low levels of ammonia, nitrite and nitrate. However, the system performance was not optimal, as an increase in nitrogen compounds was observed along with time (Fig. 2.1.2.). There are several explanations for this phenomenon. At the beginning of the exogenous feeding, fast accumulation of food waste could have exceeded the filter capacity (the filter was probably too "young" so bacteria could not cope with the input of organic matter). Also, feeding with *Artemia* implies the introduction of aerobic bacteria into the system, which can outcompete the nitrifying bacteria (Blancheton and Canaguier 1995). The increase in metabolites towards the end of the experiment could be related to the gradual accumulation of non captured prey into the mechanical filter.

The observed levels of unionised ammonia (0.013 mg l^{-1}) and nitrite (0.118 mg l^{-1}) in the present experiments, from the start of exogenous feeding and along the first month of live, were lower than those cited as responsible for mortality or decreased feeding ability in

larvae and juvenile marine fish. Brownell (1980) investigated the tolerance of marine fish larvae to ammonia by monitoring the decrease in first-feeding incidence following a 24-h exposure as the criterion of response. This author found concentrations of unionised ammonia N-NH_3 between 0.03 and 0.13 mg l^{-1} responsible for a 10% decrease in first-feeding success. Holt and Arnold (1983) observed that, after 14 days exposure to N-NH_3 concentrations of 0.3 mg l^{-1}, survival of *Sciaenops ocellatus* larvae decreased.

Percentage hatching and survival on the first week of live obtained herein lies within the range found in previous works (Barnabé et al. 1976; Johnson and Katavic 1986; Oyen et al. 1997). The maximum larval length also agrees with the literature (Chatain 1987; Johnson and Katavic 1986; Regner and Dulcic 1994). The two different growth cycles fitted in the present study show good parallelism with the growth in body length (Regner and Dulcic 1994) and mg of Carbon (Bergeron et al. 1997) of sea bass larvae reared in semi-enclosed and flow through systems respectively. In both studies it was observed a first phase of slow growth followed by a sharp increase from days 12-14, which again decreased by the time of urostyle flexion, as found in the present work. Survival of feeding larvae was of the same order or even higher than in previous studies (Barahona-Fernandes 1978; Coves 1985; Johnson and Katavic 1986). Therefore, it is unlikely that the obtained values for the main rearing parameters caused any stress on the larvae.

In the marine environment, variation of prey abundance is particularly important for first-feeding larvae (Cushing 1990). The experimental setting of different nutritional regimes could simulate an uncoupling between larvae and its prey in the wild. The obtained results on growth and survival, which we believed were not affected by other parameters than feeding ration, could be of value for further studies on trophic ecology in the field. Survival of Fed larvae showed higher values than those of Starved larvae from day 6 after hatching. Nevertheless, larvae which did not receive any food, showed little mortality all along the second week of life. Sea bass larvae are very resistant to food deprivation during the first stages (Bergeron et al. 1997; Johnson and Katavic 1986). This is due to the large yolk sac and oil globule, which are used as energy source during first development (Klaoudatos et al. 1990). This fact could explain the similarity in the growth curves, regardless feeding treatment, until day 13 after hatch. Initial high growth rate in the three treatments, up until day 4 (Fig. 2.1.4.), was probably due to the conversion of most yolk

reserves directly into skeletal growth, measurable as an increment in TL. From day 4 until day 13 it was observed a decrease in growth rate, which followed a plateau-shaped curve. During that time, Starved and Delayed treatments could have been using the internal reserves purely as maintenance energy (Klaoudatos et al. 1990). For the Fed larvae, this could be partly related to the inefficiency in the prey capture during the first days of feeding. However, the sudden increase in growth rate after day 13 suggests that the Fed animals have been investing the energy in building up body tissues or accumulating reserves in some organs (McGurk 1985 a).

The sharp decrease in larval survival of the Starved treatment observed between 15 and 17 DAH occured after total oil globule resorption. Delayed-feeding larvae did not attain a survival stabilisation until four days after food was supplied. From that moment on, mortality remained near zero as in the Fed treatment.

There were significant differences in the second growth cycle between Fed and Delayed treatments. The starting point for these second growth cycle was on day 13 in fed larvae and on day 16 in delayed feeding larvae. The initial growth rate was also smaller for delayed feeding larvae, indicating their poorer nutritional condition, which coincided with the observations for *Engraulis mordax* (Theilacker and Watanabe 1989). In our work, differences in size and growth rates were reduced after two weeks of normal feeding.

Summarising, in this work reference values for total and unionised ammonia and nitrite which permit suitable rearing conditions for sea bass larvae during the first month of life are given. To the light of the results presented throughout this section it is concluded that differences in growth and survival among treatments were exclusively due to feeding regime. The good values for survival and growth as well as the normal larval development prove the adequacy of the rearing system. We believe that future nutritional studies of field collected larvae will benefit from this kind of environmentally controlled experimental design.

2.2. MORPHOMETRIC INDICES

ABSTRACT

In this section, a study of condition based on the analysis of shape was conducted on reared sea bass larvae subjected to varying feeding rations (Fed, Starved and Delayed). Our results showed that the shape of pre-flexion *Dicentrarchus labrax* changes during development, and it does so at different rates, which depend on both the source of food (maternal or external), and the time from experimental feeding treatment. The effect of size in five morphometric variables was successfully removed by applying a normalisation method that enabled the incorporation of the effects of allometry.

Multivariate analysis (PCA) of such normalised data identified two extreme shape groups: yolk-sac type larvae and extremely emaciated larvae. A Stepwise Discriminant Analysis (SWDA) showed that the highest percentage of correct classification corresponded to the eldest emaciated category: between 76.0 and 61.5% of these larvae (depending on whether all data or a learning sample was used) was correctly identified. When all ages of the Starved treatment were pooled, between 80.0% and 69.2% of the 14-21 day old larvae were identified as Starved. Pre-anal length, body depth at anus level and head length were the variables that most contributed to the definition of the shape-groups. Also, the first canonical variable of a stepwise discriminant analysis using these 3 normalised variables was the measurement that best correlated with the survival of the Starved larvae. It is believed that this type of standardisation procedures can be of value for the study of condition in larvae exhibiting strong allometry. The potential use for field studies is discussed.

INTRODUCTION

Morphometrics applied to the study of starvation effects usually address the factor of shape variation between individuals. Therefore, the effect of size is usually undesired as it is a reflection of individual growth, and it causes variation in the variables that are potential indicators of a starving condition. The removal of size effects has been attempted through several mathematical operations. The use of ratios (the starvation-sensitive variable to the less-sensitive variable, e.g. body depth at anus level to eye diameter or length) is only valid in the case of isometric growth, which seldom occurs in fish larvae. The presence of allometry in several studies on larval development suggests that extreme caution should be observed in the use of ratios to remove size-effects (Reist 1985; Hare and Cowen 1995; Suthers et al. 1996). The use of residuals has been shown to still be affected by allometry, although the use of small size-ranges (Suthers 1989) can solve it. Performing a principal component analysis on a multivariate set and discarding the first component, which is usually correlated with size, is thought to effectively eliminate size effects. This approach was applied by McGurk (1985) and defended on the grounds that it was the only statistical method which satisfied the requisites for an ideal morphometric condition factor: size-independence, biological meaning and orthogonality. However, some studies observed that information on shape was also incorporated in the first PCA (e.g. Jolicoeur and Mosimann 1960). In this respect, the prior elimination of ontogenetic effect of size has been proved to yield a better performance than the use of PCA. A normalisation method for removing the effect of size accounting for allometric effects was empirically applied by Thorpe (1975) to the study of snakes. This method scaled all individuals to a same size and adjusted their shape to that they would exhibit in the new size. It was first applied to the study of fish by Lombarte and Lleonart (1993), and generalised by Lleonart et al. (2000).

The objectives of this section must be considered in the framework of a comparative study of condition indices in fish larvae reared in the laboratory. The first objective was to generate morphometric data from reared *D. labrax,* which contained only information on the individual shape of the larvae, whilst accounting for allometric effects. The second

objective was to select the morphometric variables that best discriminated the nutritional status of the reared larvae, and to measure their discriminating power. The relationship of the different measures with the observed survival in the laboratory was also assessed. The potential use of this shape information for field studies is discussed.

MATERIAL AND METHODS

Sea bass larvae obtained from the experimental culture described in Section 2.1 were used for the analyses. The larvae had been fixed in 10% phosphate-buffered formalin for 30 days before being measured. The sampling scheme for morphometric analyses is shown in Table 2.2.1. Only larvae from 6 DAH were considered. The study was extended until 21-day-old larvae, as this was the last day for which larvae of the three main treatments were available. We used larvae from treatments Fed, Starved and Delayed, described in the previous section. The total number of larvae used was 195.

Table 2.2.1. Number of Larvae utilised for morphometric analyses.

Feeding treatments	Days after hatch													
	6	8	10	11	12	13	14	15	16	17	18	19	20	21
Fed	12	6	7	8	6	4	7	8	9	10	9	8	9	15
Starved	6	4	4	4	4	4	3	3	4	4	5	3	2	1
Delayed	-	-	-	-	-	-	4	3	2	3	4	3	3	4

Several body measurements were taken using a binocular microscope with an eyepiece graticule, to an accuracy of 0.1 mm.

The morphometric measurements used for this study were selected from the literature and are known to relate to starvation at several degrees (Ehrlich et al. 1976; McGurk 1985). The following measurements were recorded (Figure 2.2.1.): total body length (TL), the distance along the midline of the body from the tip of the snout to the tip of the notochord in preflexion larvae, and to the end of the caudal fin in postflexion individuals; preanal length (PAL), the distance along the midline of the body from the tip of the snout to the anus; head length (HL), the distance from the tip of the snout to the posterior margin of the cleithrum; head depth (HD), the maximum depth of the head; body

depth at the level of the initiation of the pectoral fins (BDP); body depth at the anus level (BDA); and horizontal eye diameter (ED).

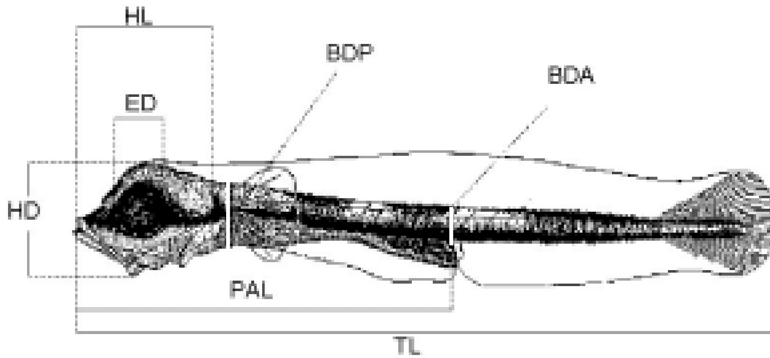

Figure 2.2.1. Morphometric measurements in *D. labrax*. **BDA**, body depth at anus level; **BDP**, body depth at pectoral fin buds; **ED**, eye diameter; **HD**, head depth; **HL**, head length; **PAL**, pre-anal length; **TL**, total length. Drawing from Barnabé et al. (1976). 7 mm (TL) larva, 17 DAH, 15°C.

Removing the effect of size.

The method for removing the effect of size was empirically applied by Thorpe (1975) and generalised by Lleonart et al. (2000). It basically standardises all measurements of a larva to a pre-determined size, taking into account the allometric relationships between the morphometric variables and body length. In other words, it calculates the new shape of an individual if it was brought to a pre-defined length, taking into account the variation in shape that it would undergo during growth. If this procedure is used, the experimental effect (food regime) should be the most likely factor to explain variations in shape.

The allometric relationships between the various body measurements and TL were calculated by using the equation $y = a\,x^{b}$, where x is TL, y is the other measurement being related, *b* the allometric factor, and *a* the expected value of y at x=1 (Gould, 1966).

The values a, b and the standard deviations (in ln-transformed units) were first calculated.

Once these values were obtained, morphometric variables were normalised according to

$$\mathbf{Z_{ij}=Y_{ij}[X_0/X_j]^{b}i} \qquad \textbf{(Eq.1.)}$$

where X_j is the TL of the individual j, X_0 is the reference length and b_i is the allometric parameter which relates the variable under study and the TL. If this procedure is followed, all measures of a particular individual are scaled up to a mean reference length (Fig.2.2.2.)

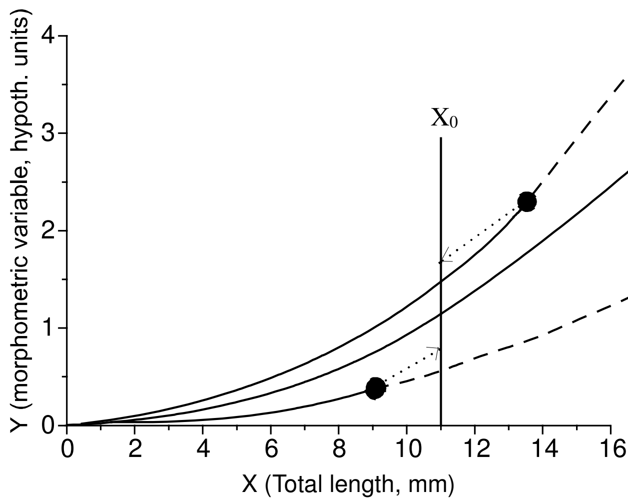

Figure 2.2.2. Illustration of the normalisation procedure. The central curve is an estimated regression on the variation of a particular morphometric measurement with respect to total length. The lower and upper curves represent the growth trajectories of two larvae, one of which has a positive shape factor (and has reached a TL of ca. 14 mm) and another of which shows a negative shape factor, reaching a TL of ca. 9 mm). The intersection with the vertical line show the particular value for the morphometric variable that they would have if they were normalised to a total length of 11 mm. The dashed parts of the curves represent the hypothetical growth trajectories of the two individuals in the future. Adapted from Lleonart et al., 2000.

One requirement for the application of the normalisation method is that the growth pattern must be the same throughout the period analysed. Larval growth pattern (Fig. 2.1.4.) showed that there was an inflexion point around 6 mm TL for Fed and Delayed larvae.

To test whether there was any difference in the morphometrical variables between the two size groups ($\leq$ 6 mm and > 6 mm), we compared the slopes of the ln-linearised morphometric variables of the Fed treatment of the two groups (the latter treatment would ideally reflect a size-effect, with no interference of food availability). The comparison of slopes was performed through ANCOVA (through GLM, MINITAB 12.1 Inc.) (Table 2.2.2.). We used each morphometric variable as a response. TL was used as a covariant.

Table 2.2.2. Comparison of slopes of each morphometric variable *vs* TL between the two size-groups ($\leq$ 6 mm and >6 mm) in the Fed larvae by ANCOVA through GLM.

	PAL		HL		ED		HD		BDA	
	F	p	F	p	F	p	F	p	F	p
Significance of slope of Interaction term (size-variable)	18.30	<0.001	10.79	<0.005	3.08	0.082	0.70	0.405	29.1	<0.001

Slopes of PAL, HL and BDA *vs* TL were significantly different between the two size-groups. This further justified the separate analysis for the two groups. In spite of these

preliminary results, normalisation had to be done using the coefficients for the pooled larvae (see results), normalised to 6 mm (median for the size distribution). The success of the normalisation method for each variable was assessed by analysing the significance of the correlations with size.

Bivariate analysis

Data was first explored in regard to age and length in non-normalised variables. The allometric patterns of each variable (ln-linearised) with respect to TL were analysed for each size-group and for each feeding treatment. The significant departure from isometry was checked by T-tests (H0: b=1).

Multivariate analyses.

The rationale for the use of multivariate analysis was two-fold. Firstly, the aim was to study the behaviour of the body shape as a whole and to discern the particular contribution of each variable to the total variance of shape. This was done by Principal Component Analysis (PCA, Rao 1964). Secondly, Stepwise Discriminant Analysis (SWDA) (Legendre and Legendre 1998) was used to ascertain the predictive power of the morphometric variables, and for the purpose of comparison with other condition indices performed on larvae obtained from the same experimental culture. Both analyses were performed using STATISTICA 5.0.

All multivariate analyses were conducted on variables normalised by the previously described method. Before proceeding with the multivariate study of the data, we considered the suitability of using separate coefficients for each feeding treatment or a single coefficient derived from all treatments. The suitability of applying different sets of coefficients to each size-range was also assessed.

The normalised variables for PCA needed no further standardisation as multivariate normality and homocedasticity held in all variables. Also, variables were measured in the same units, so the covariance matrix was used for the analyses.

The rationale behind the discriminant analysis is to determine whether groups differ with regard to the mean of a variable (in this case morphometric) and to then use that

variable to predict group membership. The discrimination was done using the Mahalanobis distances (D). By this procedure, each individual is allocated to the group for which D has the smallest value. The data need not be standardised as discriminant analysis is not affected by the scaling of individual variables (Manly 2001). Discriminant analysis was done in a forward stepwise fashion, so that variables are added to the model individually according to their discriminating power. The observations were plotted on canonical axes, which have the maximum discriminating power. The input data to the discriminant analysis was based both on the results of PCA analysis and on the biological meaning of the pre-defined groups.

Relationship with survival

The relationship between each normalised morphometric variable (or function of them) and the survival of the Starved larvae was examined through non-parametric (Spearman) correlations due to the often non-linearity of the relationships even after data transformation.

RESULTS

Bivariate analysis

The observation of the change in morphometric variables (non-normalised) in each treatment with age (Fig. 2.2.3.) suggested several ongoing processes. Firstly, growth patterns approximated that described for TL (Section 2.1.). The presence of a shift in growth was evident in the Fed treatment of most variables and occurred around day 13. On the other hand, variables measured in Starved larvae showed no apparent shift in growth, and either grew at a slower rate (Fig. 2.2.3.; ED, HL, PAL) or showed little or no apparent growth (Fig. 2.2.3.; BDA). Delayed larvae, to which food had been supplied from day 13, showed an apparent recovery in all variables 4-5 days after food was supplied.

For all variables, significant differences between Fed and Starved individuals were not detected until 10 days after food was introduced into the system. The time to noticeable recovery after food was supplied to Delayed larvae (day 13) was 4-5 days for BDA, HD and PAL. Eye diameter and HL never departed significantly from the Starved treatment during this period.

Correlations between TL and the linearised (non-normalised) variables were performed and relationships were plotted (Fig. 2.2.4.). Only BDA from Starved larvae showed a non significant correlation with TL. From this feeding group, only 2 individuals (from 51) had a TL of over 6 mm, and were excluded from the analyses. All significant

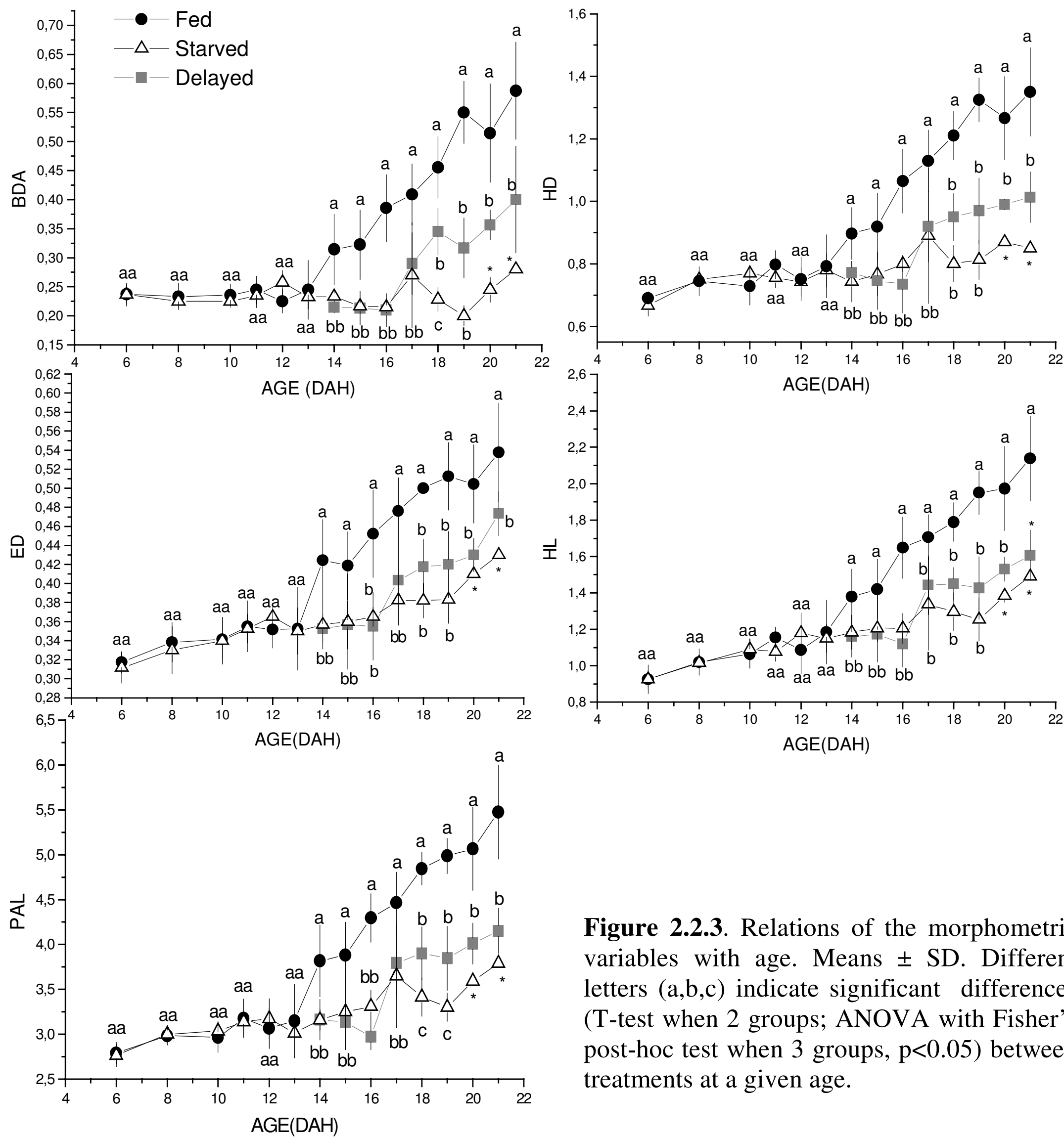

Figure 2.2.3. Relations of the morphometric variables with age. Means ± SD. Different letters (a,b,c) indicate significant differences (T-test when 2 groups; ANOVA with Fisher's post-hoc test when 3 groups, p<0.05) between treatments at a given age.

correlations had a $p<0.001$ except ln HL and ln BDA of the Delayed larvae < 6mm ($p<0.005$) and ln ED ($p<0.05$), also from the same treatment. According to this, allometric equations were calculated for all variables and size-groups except for BDA of Starved larvae (Table 2.2.3.).

In Table 2.2.3., significant values of $b<1$ indicate that the morphometric variable is growing at a slower rate than TL (negative allometric growth). Significant values of $b>1$ indicate that the morphometric variable is growing at a faster rate than TL (positive

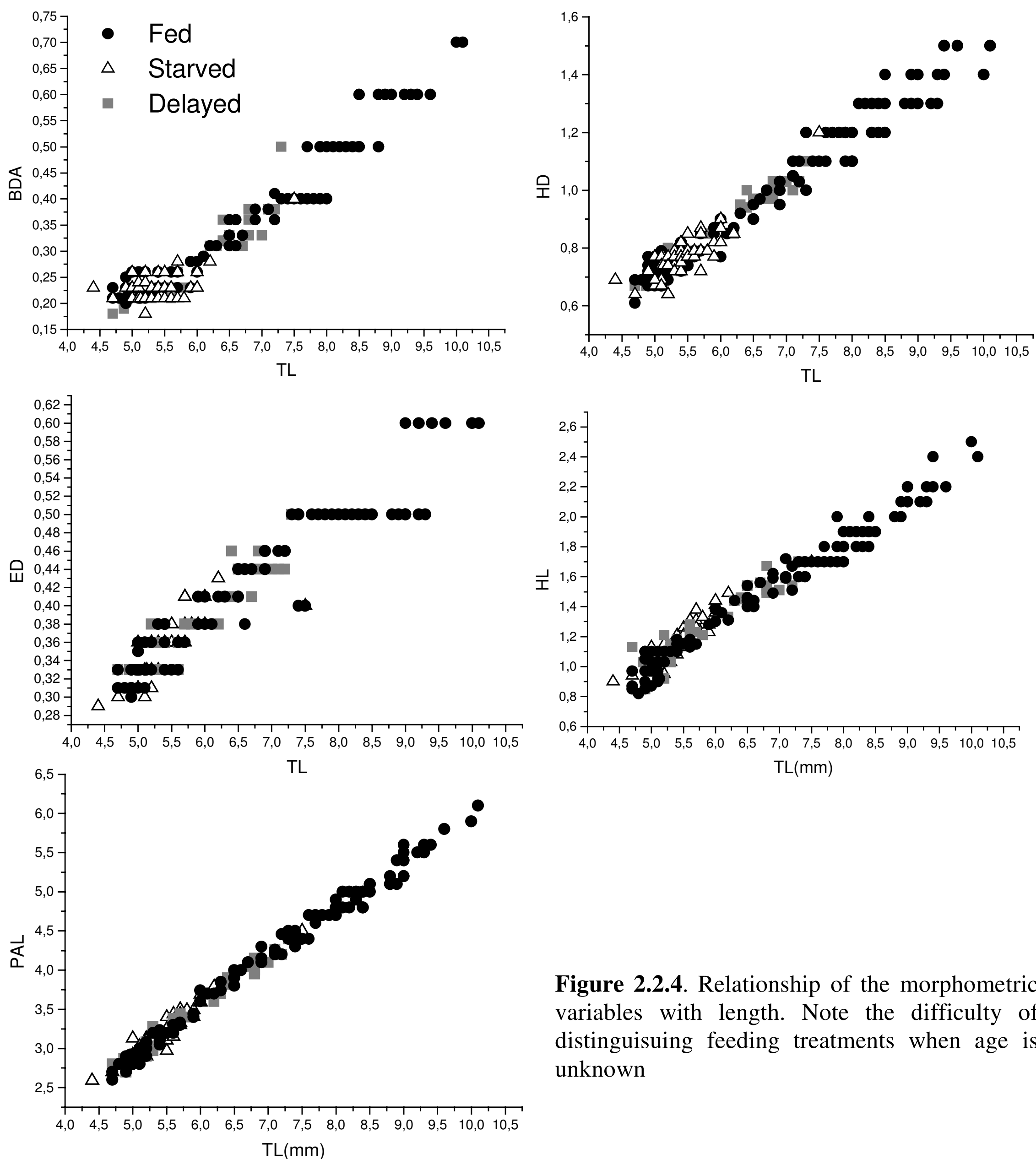

Figure 2.2.4. Relationship of the morphometric variables with length. Note the difficulty of distinguisuing feeding treatments when age is unknown

allometric growth). Slopes not significantly different from 1 mean that the morphometric variable is growing at a non-differentiable rate from TL (isometric growth).

Larvae ≤ 6mm.

This group contained all the Starved individuals, plus some Fed and Delayed larvae. Fed larvae showed positive allometric growth for PAL and HL. From the latter, HL grew at a faster rate. The remaining variables showed isometry. Fed larvae of this size-class were composed mainly of young individuals (under 13-14 DAH, see Fig. 2.1.4.). The positive allometry of PAL and HL indicates that the diggestive tract, and particularly the structures of the head are developing fast.

Starved individuals showed positive allometry for the same variables. However, these larvae comprised the whole age spectrum, until 21 DAH. The relatively fast growth of PAL and HL in comparison with TL in the Starved larvae may be indicating that these variables are particularly important for development.

Delayed larvae showed isometric growth in this size range, except for a negative allometry in eye diameter. However, the low number of observations for this group suggests that caution should be observed in the interpretation.

A word of caution should be addressed to the study of morphometric data using all treatment in this size-fractionated way (a case that can be found in field studies). As shown in the results of the pooled data (Table 2.2.3.) if the feeding treatments were unknown, some fictitious allometry would appear in this population of small larvae.

Larvae >6mm

Only Fed and Delayed larvae over 13 DAH composed this group. In the Fed larvae, BDA showed strong positive allometry. The HL of Fed larvae continued growing with a slight positive allometry, thus reflecting the importance of the development of the head part in the larval stages of this species. A slow-down in the growth (negative allometry) was observed in ED, and to a lesser extent in PAL, in comparison with the previous size-class.

Delayed larvae showed positive allometric growth in BDA, the rest of the variables growing isometrically.

.

Table 2.2.3. Parameters of regression lines which relate the morphometric variables to the total length according to (Ln(y)=Lna+bLn(TL)). Test for allometry with significance (*) p<0.05,(**) p<0.01, (***) p<0.001, ns, non-significant.

Group ≤ 6mm (age 6-20 DAH)

	Ln BDA			Ln PAL			Ln HD			Ln HL			Ln ED		
	Ln a	b	r^2	Ln a	b	r^2	Ln a	b	r^2	Ln a	b	r^2	Ln a	b	r^2
Fed (46)	-2.842	0.846 ns	0.364	-0.866	1.191 **	0.912	-1.914	0.979ns	0.596	-2.51	1.550**	0.691	-2.58	0.914 ns	0.602
Starved (49)	There is no correlation			-0.805	1.160*	0.850	-1.775	0.894 ns	0.514	-2.711	1.689***	0.784	-3.00	1.164 ns	0.739
Delayed (11)	-3.630	1.259ns	0.652	-0.645	1.07ns	0.916	-2.099	1.093 ns	0.747	-1.172	0.793 ns	0.453	-1.822	0.472*	0.258
Pooled (106)	-2.346	0.526***	0.145	-0.845	1.182***	0.888	-1.861	0.946 ns	0.590	-2.568	1.599***	0.720	-2.726	1.003ns	0.657

Group >6mm (age13-21 DAH)

	Ln BDA			Ln PAL			Ln HD			Ln HL			Ln ED		
	Ln a	b	r^2	Ln a	b	r^2	Ln a	b	r^2	Ln a	b	r^2	Ln a	b	r^2
Fed N=72	-4.379	1.740 ***	0.930	-0.437	0.962(*)	0.976	-2.071	1.079 ns	0.905	-1.74	1.127***	0.936	-2.119	0.677***	0.701
Delayed N=15	-4.260	1.687 *	0.683	-0.521	1.002ns	0.947	-2.147	1.120 ns	0.861	-1.439	0.976 ns	0.788	-2.218	0.727 ns	0.574
Pooled N=87	-4.33	1.716***	0.922	-0.464	0.975 ns	0.978	-2.078	1.078 *	0.916	-1.698	1.106 **	0.936	-2.134	0.684***	0.730

All larvae

	Ln BDA			Ln PAL			Ln HD			Ln HL			Ln ED		
	Ln a	b	r^2	Ln a	b	r^2	Ln a	b	r^2	Ln a	b	r^2	Ln a	b	r^2
Fed N=118	-4.0851	1.598***	0.962	-0.652	1.0651***	0.990	-2.101	1.093***	0.965	-2.0361	1.2678***	0.967	-2.428	0.825 ***	0.921
Starved N=51	There is no correlation			-0.722	1.141*	0.898	-2.016	1.037ns	0.681	-2.470	1.545 ***	0.814	-2.586	0.919ns	0.654
Delayed N=26	-4.732	1.930 ***	0.914	-0.575	1.030 ns	0.983	-2.133	1.113*	0.949	-1.641	1.078 ns	0.913	-2.360	0.798**	0.848
Pooled N=195	-4.222	1.657 ***	0.930	-0.64	1.059***	0.986	-2.116	1.100 ***	0.957	-0.1.961	1.235***	0.950	-2.423	0.823***	0.909

All larvae (pooling treatments and considering the whole size-range).

The absolute number of larvae from each size-class had relevance on this final result. This is particularly evident if the slopes for the pooled feeding treatments are observed. The large number of Fed larvae were responsible for the observed allometries in the final table.

Removal of size effects and multivariate analysis

A normalisation with specific coefficient sets for each feeding treatment was not appropriate, as the Starved treatment showed no correlation with length for BDA. For the same reason, a normalisation by separate size-groups was judged unwise as the Starved larvae were all $\leq$ 6mm (Table 2.2.3.). Indeed, the use of all larvae from this size-range would have involved the use of a strong negative allometric coefficient for BDA (b=0.526). The latter would not have been a reflection of the allometry for the whole population of small larvae, but a result of the non-significant correlation showed by Starved larvae. Therefore, data from all larvae (pooling all sizes) had to be used. Due to the comparatively large number of Fed larvae, in the pooled data, we analysed the relative influence of this treatment in a normalisation procedure using all data. The normalisation using only larvae from Fed larvae showed no significant differences from the normalisation using larvae from all feeding treatments, thus confirming the strong influence of this group in the final results. According to the arguments exposed above, all multivariate analyses were performed on normalised variables obtained from data from pooled treatments and sizes. Also, this procedure implies no previous knowledge on the feeding treatment, which is the analogous situation that would be encountered in the field.

The absence of correlation of the normalised variables with TL confirmed the total removal of size-effect by the normalisation procedure. The first two principal components of the PCA performed on these data explained 84.2% of total variance (Table 2.2.4.). PC1 explained 66.8% of the variance and was mainly a contrast between PAL/ HL and BDA. PC2 explained 17.4% of total variance and was a contrast between HL and PAL (Figure 2.2.5., Table 2.2.4.).

Table 2.2.4. Results from the PCA on all larvae, normalised to 6 mm.

	PAL	HL	ED	HD	BDA	Eigenvalue	%variance
PC1	0.805	0.575	0.078	0.086	-0.085	0.011559	66.8
PC2	-0.589	0.792	0.062	0.143	-0.025	0.003013	17.4

PC1 separates individuals that exhibit a slender body and large guts and heads. At the other extreme of PC1 one would find larvae with thick bodies and relatively short digestive tracts and head. PC2 would further distinguish between individuals having opposite trends in HL and PAL.

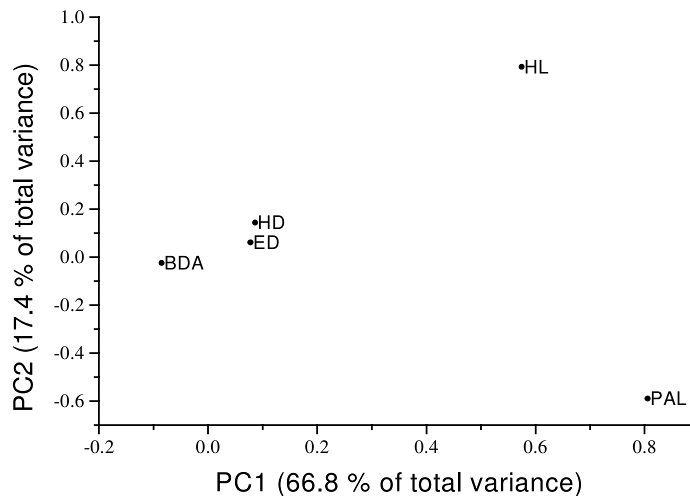

Figure 2.2.5. Plot of the loadings of each variable for the first two Principal Components. All larvae, normalised to 6 mm TL.

These results confirm the shape patterns suggested by the bivariate analyses. The scores for the first two principal components plus the information on the feeding treatments and age was represented (Figure 2.2.6.). Each feeding group was assigned a symbol. For the sake of clarity, larvae from each feeding treatment were divided into 4-day categories. Figure 2.2.6. showed the existence of two defined extreme groups, and a more numerous middle group. A first group would be composed of young larvae of 6-9 DAH, both Fed and Starved. This group would be composed of larvae with a relatively thick trunk and short

heads and guts. The second extreme group would be composed of Starved larvae (mainly over 14 DAH) and some Delayed larvae. This group would have a slim trunk and large heads and guts.

Finally, we identified a large group of larvae that had intermediate characteristics. This group would be composed of Fed and Starved larvae over 9 days-old, (but the latter not extremely starved), plus some Delayed larvae.

Figure 2.2.6. reflects the strong effect of age and development (see discussion) in the ordering of the results. The first group (6-9 days old) is composed by larvae which exhibited oil globule, and some of them remains of the yolk sac (see Section 2.1.). In these larvae, ontogeny influences shape independently of food regime. It thus seems unwise, for these species and under our experimental conditions, to study the effect of starvation on morphometric measurements in larvae under 9 DAH. As time progresses, the maternal reserves deplete, for Starved larvae the effect of starvation becomes dominant over purely ontogenetic processes and the body shape moves gradually to the top right-hand corner of Figure 2.2.6. These extremely emaciated larvae do not grow in length (Fig. 2.1.4.) but undergo a relative development of heads and gut. Well fed larvae show mean values for the different variables on both PCs.

Overall, the PCA analysis discriminates two extreme groups, and contains a mixed information of food regime and ontogenetic processes.

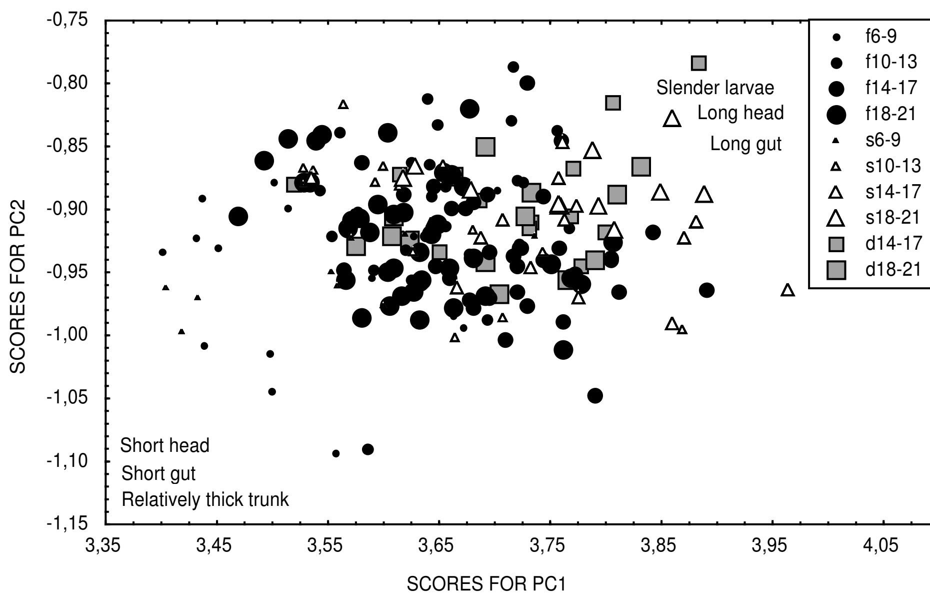

Figure 2.2.6. Plot of the scores for the first two PCs. Legend: f, s and d are Fed, Starved and Delayed treatments, respectively. Numbers refer to the age-interval. Size of symbols represents time (DAH). For example, f14-17 means Fed individuals between 14 and 17 DAH.

Discriminant analysis was performed on two age-groups: 6-13 and 14-21 DAH. The criterion for doing so was based on the fact that maternal reserves were detected in individuals up to 13 DAH.

Two kinds of SWDA were performed. First, SWDA was performed with all data. Secondly, the dataset was split in two, so that one half of the data was used to build the classification functions, whereas the other half was used to validate the model. All data conformed multinormality requirements.

Discriminant analysis with all data

The discriminant analysis selected the following variables in order of importance: BDA>PAL>HL>ED>HD. Only the first 3 variables contributed significantly to the analysis. A second SWDA was performed using these three variables. The classification results were practically identical, so only the results based on the 3 variables are shown.
Classification functions are shown in Table 2.2.5. (A). The percentage of correct classification varied greatly in each group. The best classification by age-food groups (76

Table 2.2.5. Results from the SWDA performed on 3 variables using normalised data of all larvae (n=195). A; classification functions for the different age-food groups. B; Classification matrix (equal *a priori* probabilities). The first column is the real treatment. The remaining columns are classification results. Correct classifications in bold. Values are percentages.

A

	Age-food groups				
	F6-13	**F14-21**	**S6-13**	**S14-21**	**D14-21**
BDA	699.466	679.84	692.103	617.15	666.05
PAL	510.097	516.62	508.938	519.58	515.42
HL	4.384	7.20	6.441	20.87	18.17
constant	-997.238	-1017.88	-993.587	-1029.51	-1024.04
%correct	39.53	37.33	34.61	76.00	38.46

B

		Fed			Starved			Delayed
		6-13	14-21	**Total**	6-13	14-21	**Total**	14-21
Fed	6-13 (n= 43)	**39.53**	16.28	55.81	20.93	2.32	23.25	20.93
	14-21(n=75)	14.66	**37.33**	52	21.33	5.33	26.66	21.33
	total		**53.38**			25.42		21.2
Starved	6-13 (n=26)	30.77	19.23	50	**34.61**	0	34.61	15.38
	14-21(n=25)	0	8.00	8.00	4.00	**76.00**	80.00	12.00
	total		29.41			**56.86**		13.73
Delayed	14-21 (n=26)	4.00	24.00	28.00	12.00	24.00	36.00	**40.00**

%) was obtained for the extremely emaciated larvae (s 14-21, Table 2.2.5. A). The remaining age-food groups correctly classified around 36% of larvae, which is over the random classification value for all groups (random value = 20%). Most misclassifications occurred in young-Starved larvae of 6-13 DAH (Table 2.2.5. B; altogether, 50 % were misclassified as Fed). The proportion of Fed larvae of any age-group classified broadly as Fed was 53%. This proportion was of 57% for the Starved treatment and 38% for the Delayed treatment.

If the classification into 3 broad categories (Fed, Starved ad Delayed) is considered by age groups, then the oldest Starved larvae (aged 14-21 DAH) were the best classified (80% classified broadly as Starved, Table 2.2.5. B).

The first two canonical functions explained ca. 98% of all the discrimination (Fig. 2.2.7.) Looking at the standardised coefficients for canonical variables, it was seen that individuals lying on the positive side of the first canonical variable have thick BDA and

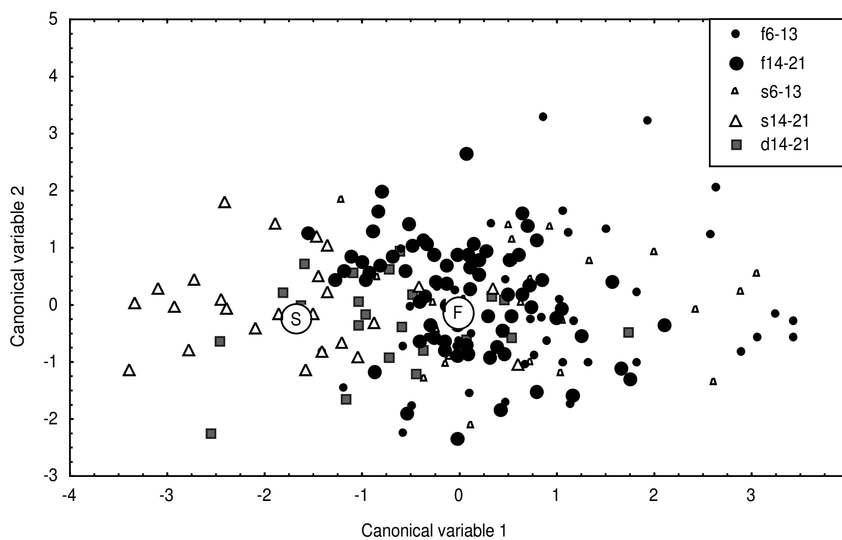

Figure 2.2.7. Values of each individual with respect to the first two canonical variables, using normalised values of 3 variables for all larvae. Centroids of Fed (F) and Starved (S) individuals of age 14-21 are indicated. Legend. f, s, d indicate Fed, Starved or Delayed treatments, respectively. Numbers refer to age-interval (DAH).

small HL and PAL. They correspond mainly to young individuals (Fed and Starved). On the other hand, larvae located at the negative side were characterised by a reduced BDA, and a proportionally larger PAL and HL. The second canonical variable accounts for 12% of the discrimination power and the variable with most weight is PAL.

Discriminant analysis with split data

The predictive value of this method was studied by splitting the dataset. One set was used to build the classification functions, and the remaining data were used to validate it. The SWDA chose, by order of importance, the following variables: BDA>PAL>HL>HD>ED. This result is similar to the previous one except that the order of second and third variables was inverted. Only the first two variables contributed significantly to the analysis. The third variable, HL, was only marginally significant. The result of the SWDA with 3 variables yielded a similar classification matrix to the SWDA with 5 variables. These results are presented.

The classification functions were obtained by analysing the data that had not been used for building up the model. These results were consistent with those using all data in that the extremely starved individuals were the group which was best classified (ca. 62%). Fed individuals of 14-21 DAH came in second place, and young Starved individuals came third. These results differ slightly from the previous classification. It is possible that, due to the smaller number of observations (approximately half of the data), the groupings are less reliable despite being formed from different data sets than the observational data. Nevertheless, the variables chosen as significantly contributing to the analysis remained the same.

Table 2.2.6. Results from the SWDA on 3 normalised variables (split data). A;, Classification functions (n=96). B; Classification matrix (n=98). Column ordination as in Table 2.2.5.

A

	Age-food groups				
	F6-13	**F14-21**	**S6-13**	**S14-21**	**D14-21**
BDA	522.94	509.85	521.66	420.95	484.52
PAL	572.65	580.29	567.58	591.44	578.60
HL	-11.28	-8.48	-8.12	8.18	1.42
Constant	-1068.55	-1095.05	-1054.57	-1133.00	-1094
%correct	28.57	44.73	38.46	61.53	30.77

B

		Fed			Starved			Delayed
		6-13	14-21	**Total**	6-13	14-21	**Total**	14-21
Fed	6-13 (n= 21)	**28.57**	14.29	42.86	38.10	0	38.10	19.05
	14-21(n=38)	5.26	**44.73**	49.99	21.05	7.89	28.94	21.05
	total		**47.46**			32.20		20.34
Starved	6-13 (n=13)	7.69	30.77	38.46	**38.46**	7.69	46.15	15.38
	14-21(n=13)	0	15.38	15.38	7.69	**61.53**	69.23	15.38
	total		26.92			**57.69**		15.39
Delayed	14-21 (n=13)	7.69	15.38	23.07	7.69	38.46	46.15	**30.77**

The first two canonical variables explained 99.2 % of the discriminating power. Looking at the standardised coefficients, it was seen that the first canonical variable discriminates individuals with thick BDA and small PAL and HL. The second canonical axis accounted for 5.6 % of total variance and the variables contributing most were PAL and HL.

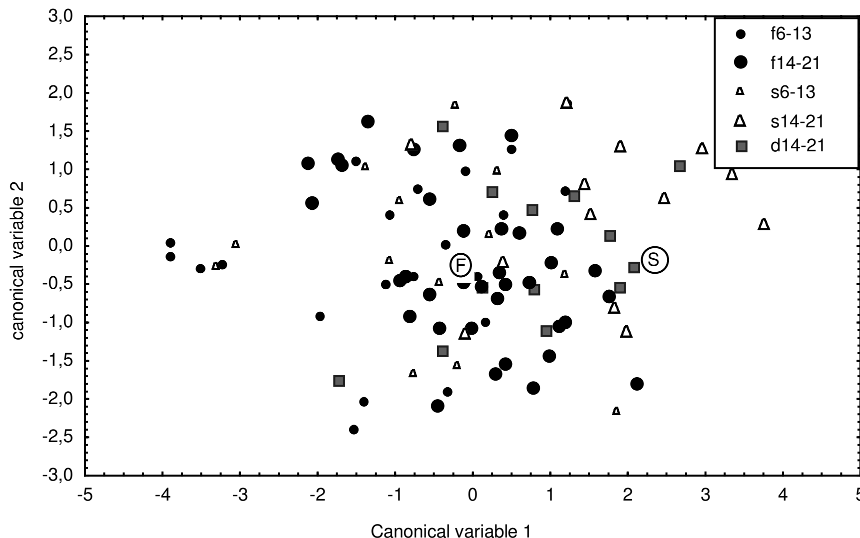

Figure 2.2.8. Values of each individual with respect to the first two canonical variables (normalised data of 3 variables, split data). Centroids of Fed (F) ad Starved (S) individuals of age-groups 14-21 are indicated. Centroids seem uncentered because they are derived from the classification function, obtained from different data to that represented in this figure. Legend. F, S, D indicate Fed, Starved or Delayed treatments, respectively. Numbers refer to age-interval (DAH).

Relationship with survival

The single normalised variables that best correlated with the survival of the Starved larvae were HL and BDA (Table 2.2.7.). Head depth was the only variable which did not correlate significantly with survival. From all correlations, canonical variable 1 obtained from the SWDA performed on 3 normalised variables previously chosen by a SWDA with 5 variables yielded the highest significant correlation (79.8%, Table 2.2.7., Fig. 2.2.9). Discriminant functions and single variables (BDA and HL) yielded better correlations than PC1 performed on all larvae.

Table 2.2.7. Spearman rank correlations between percentage survival of Starved larvae and the different variables or functions. S, significant. NS, not significant

Variables or functions	DF	r_s	t_s	P	Significance
PAL	49	-.547334	-4.57794	<0.001	S
HL	49	-.716459	-7.18893	<0.001	S
ED	49	-.549258	-4.60097	<0.001	S
HD	49	-.003819	-.02674	0.979	NS
BDA	49	.704518	6.94902	<0.001	S
Principal component 1 (all data)	49	-.663628	-6.20990	<0.001	S
Canonical variable 1(all data)	49	.798272	9.27765	<0.001	S
Canonical variable 1 (split data)	49	-.760043	-8.18667	<0.001	S

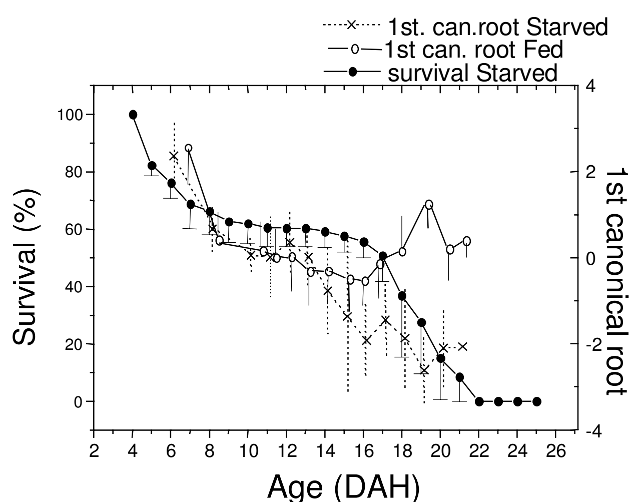

Figure 2.2.9. Relationship of the 1st canonical root with survival of the Starved larvae. Survival for the Fed larvae was constant for this period (not shown). Mean values represented. Error bars are ± SE for survival, and ± SD for scores.

For Starved larvae, the scores for the 1st canonical variable decreased continuously (Fig. 2.2.9.). Fed larvae underwent a decrease in this “shape factor” until day 16, and increased thereafter.

DISCUSSION

D. labrax proved to be relatively resistant to starvation in the absence of food. The Fed larvae reflected, through all morphometric variables, the shift in growth that takes place after the end of the oil-globule reserves (13 DAH, Figure 2.2.3.). It is known that the European sea bass is relatively insensitive to starvation during the early development (Johnson and Katavic 1986, Bergeron et al. 1997) , and it is likely that internal reserves are being used to line up feeding and digestive structures, as well as being used up as maintenance energy (McGurk 1985 a). Also, the specific characteristics of morphometric indices (short sensitivity) would mask any fast response to feeding regime (Ferron and Leggett, 1994).

In Starved larvae over 13 DAH, the time of food deprivation was not reflected in a worsening of morphometric variables but rather in the slowdown in their growth. This confirms the suggestions made by several authors that extremely starved larvae can seldom be separated from moderately starved larvae on a morphometric basis.

The strong influence of size on all morphometric variables (Fig. 2.4.4.) confirmed the need for the removal of size effects if the influence of food availability is to be detected.

Allometry in larvae ≤ 6mm

The positive allometric growth of PAL and particularly of HL of Fed and Starved larvae ≤ 6mm (Table 2.2.3.) is an indication of the development of structures that are of particular value at first feeding, like gut, jaws and gills. The number of Delayed larvae ≤ 6mm was low, so the interpretation of the growth for this treatment should be addressed with caution.

Similar observations to those recorded in the present study are registered in the literature for larvae of fish species including sea bass, and has been interpreted in terms of the survival advantage conferred by a fully functional visual, feeding and digestive apparatuses (Pedersen et al. 1990; Polo 1991; Hunt Von Herbing 2000; Koumondouros 2001).

Allometry in larvae > 6mm

Larvae > 6mm were composed of Fed and Delayed treatments only. Strong positive allometry was detected for BDA of both treatments, indicating a thickening of the trunk at the anus level. This was paralleled by a faster growth in length than in the previous growth phase (Fig. 2.1.4.), which is indicative of a successful transition to external feeding. In Fed larvae, there was a relative slowdown in the growth of HL and ED in comparison with smaller sizes, which is reflected in the weaker positive allometry for HL and in the negative allometry of ED (Table 2.2.3.). This reinforces the idea of a somatic growth period for this size-class, as opposed to a developmental growth period observed for smaller larvae. The relative slowdown of eye growth respect to the TL may be an indication of the importance of the development of the visual system in the first growth cycle. Delayed larvae show a similar pattern to Fed larvae in this size-class, the differences in significance probably being explained by the small sample size.

Removal of size-effects

The application of the method of Lleonart et al. (2000) retained information on the shape of the individuals and completely removed the effect of size from morphometric variables. This enabled the use of the data without the interference of size-effects, which are commonly present in the use of ratios or residuals (McGurk 1985 a; Suthers 1992). The initial planned comparison between a normalisation using a separate set of coefficients for each treatment was not possible, as BDA from the Starved treatment showed no significant correlation with TL. The coefficients used for the normalised data were drawn from the pooled feeding treatments, and were biased towards those of Fed larvae, which was the most abundant group (Table 2.2.3.). Infact, the comparisons of each morphometric variable between age-groups using two types of normalisation coefficients (those derived from the Fed treatment and those derived from all treatments) showed no significant differences. This implies that the absolute difference in shape between feeding treatments could not be

fully detected, but only as a relation to the mean allometry of each variable for the whole reared population.

Multivariate analyses

Through multivariate analysis on size-free variables, we found that BDA, PAL and HL were particularly sensitive to starvation. These variables have also been found to be useful for distinguishing underfed individuals in other species of marine fish larvae (Theilacker 1978; Martin and Wright 1987; Polo et al. 1991; Dou et al. 2002). Also, the finding that young larvae possess a particular shape irrespective of feeding condition is widely registered in the literature (Ferron and Leggett 1994).

SWDA was conducted on 2 age-intervals which were previously defined by the duration of maternal reserves. The aim of conducting an SWDA was not to get the maximum discrimination value but to get a quantitative value of the discrimination power of the method, which could be compared with posterior analyses of condition obtained by different indices performed on larvae from the same culture and sampling dates. The SWDA showed that BDA was the variable that most contributed to the discrimination of the age-feeding groups.

Our classification results are generally lower than those of other works that have used SWDA (e.g. Theilacker 1978). There are several plausible explanations for this. Firstly our results are drawn from a long-term starvation experiment. As a consequence, the shape of the individuals from the three treatments was a highly variable continuum as shown in Figure 2.2.6., which posed difficulties for the pre-definition of groups. This is inherent to all studies of condition through morphometry, in which samples are gathered over a considerable starvation time (e.g. Powell and Chester 1985). On the other hand, comparisons of larvae from treatments sampled on a single day, or restricted to very narrow size-ranges are prone to yield better percentages of correct classifications. Secondly, a great proportion of larvae which were still dependent to some extent on internal reserves were included in the analyses. This probably lowered the proportion of correct classifications in comparison with similar works which have used relatively size-free data (McGurk 1985 a). Lastly, most values of high classification scores in the literature are based on SWDA

derived from ratios or residuals. According to McGurk (1985 a), these values are unreliable because data are correlated with size.

The use of independent data sets for building a classification model and validating it yielded slight differences in the classification matrix (Table 2.2.6.). In these split data, sample size probably affected the classification results and hence their reliability, despite being derived from a learning sample.

Relationship with survival

Few data on the correspondence between starvation indices and survival are found in the literature. Most available information on survival and starvation is concerned with the determination of the time at which starved larvae can no longer feed (and hence recover) even if food becomes available. This is a way of defining the so called Point of no Return (PNR; Blaxter and Hempel 1963; Theilacker and Dorsey 1980; Yin and Blaxter 1987; Dou et al. 2002). Although the PNR must theoretically exist at some point during starvation in any species, it can be highly variable. The time to PNR in larvae deprived of food, once maternal reserves are exhausted, depends mainly on temperature and is highly species-specific. The lower the temperature, the longer the time larvae can withstand starvation without it being "irreversible". Data on PNR (calculated from yolk-resorption) show that for temperatures between 15 and 28°C, PNR ranges from 14 to 48 DAH for several species (Dou et al. 2002). Also, it is recognised that PNR is delayed in species with a long-lasting oil globule, like *D. labrax*. In our experiment the PNR was not determined, but a mass mortality of Starved larvae was noted around day 17 (Fig. 2.1.3.). It would be unwise, however, to equal this increased mortality to the existence of a PNR. Infact, data on several species including the Striped sea bass *Morone saxatilis* show that the PNR may be non existent (May 1971; Eldridge et al. 1981; Rogers and Westin 1981).

From the correlations with survival, it was clear that a high explanation of total variance in shape (PC 1) does not imply a relationship with survival. Head length itself may be an indirect measure of condition in older larvae, whilst being related to ontogenetic processes in early individuals, whereas BDA is probably a direct reflection of a deficient nutritional status in larvae over 9 DAH (muscle protein is known to be quickly degraded as

starvation progresses (Shelbourne 1957). The simultaneous plot of the first canonical root for Starved or Fed larvae and the survival over age showed that the change in shape pattern underwet a rapid decrease in the first days, then stabilised on days 8-12 and it either decreased sharply in Starved larvae or remained largely unchanged in Fed larvae until 16 DAH, increasing afterwards (Fig. 2.2.9).

Application in the field.

Morphometric analyses of condition of reared larvae have been repeatedly used with the aim of applying them in field studies. This is mainly because of the ease with which measures can be obtained from routine ichthyoplankton surveys and the low processing costs (Hunter 1981). The major obstacles to this general utilisation are: 1) Shrinkage problems arising from net treatment add to fixation effects (Theilacker 1980; Hay 1982; McGurk 1985 a) and are known to influence larval shape and 2) calibration experiments are rarely useful because experimental conditions (size of containers, feeding regime etc.) tend to drive larval shape away from that of wild populations. Other main drawbacks for the utilisation of morphometry to detect suboptimal condition are the strong influence of age and size, the large species-specificity and the high response time (Ferron and Leggett 1994). Indeed, strong morphological changes may occur as a response to the adaptation to first feeding (Theilacker 1978; Neilson et al. 1986) that do not depend on feeding condition as confirmed in the present study. Also, the ontogenetic uncoupling between growth in length and mass (i.e. an increase in mass or volume may be preceded by an increase in length) suggested by Farbridge and Leatherland (1987) may force misinterpretations of morphometrically-related condition in wild fish.

Nevertheless, the good points still seem to exceed the drawbacks and morphometric analyses are still used today, through multivariate statistical procedures to eliminate age and size effects and to increase the discriminating power between groups.

The normalised variables used in the present study of each individual were probably biased towards the characteristics of the Fed larvae, as these were the most abundant in the sampling. To some extent, this is the situation that would be encountered in the field, where larval populations are known to be dominated by individuals in a relatively good condition,

as factors like predation may exert a strong influence by removing poorly performing/slow-growing larvae (Hare and Cowen 1997, but see Leggett and Deblois 1994). The way in which the normalisation method could be applied to field studies could be as follows: firstly, meaningful morphometric variables should be selected. Secondly, the set of samples to be normalised should be delimited (eg. each haul, set of pooled hauls, etc.). In this respect, speciose waters (having low density of a given species) could also be included in the analyses, as long as there were enough larvae to draw the allometric curves. Thirdly, the set of allometric coefficients to be used for normalisation should be selected. One possibility would be to normalise the data from each haul with its own set of allometric coefficients. Alternatively, a single set of coefficients derived from a selected haul (eg. the one which best correlated with food abundance or a set of selected environmental parameters, hence assumed to be formed by a "well-fed" population) could be used. Finally, comparisons of normalised data (or its residuals) between sites/houls could be adopted in a frame of "apparent condition" (McGurk 1985 a).

The latter procedure can be justified for most species since variables which respond to food deprivation are usually similar between species, the latency and dynamics (*sensu* Ferron and Leggett 1994) being the main interspecific variation. Therefore, as long as the species follows a similar allometric growth pattern for the time-period studied, this method could be applied without prior laboratory calibration.

It should be emphasised that allometric changes are referred to as smooth changes in shape. As noted by Lleonart et al. (2000), processes like major structural changes, typical from metamorphosis, do not meet the assumptions of the general allometric model, so care should be taken in avoiding these major developmental changes in the study of wild populations.

2.3. HISTOLOGICAL INDICES

Part of this section has been published in: "**Catalán IA** and Olivar MP (2002) Quantification of muscle condition using digital image analysis in *Dicentrarchus labrax* larvae, and relationship with survival. J Mar Biol Ass UK. 82: 649-654."

ABSTRACT

The histological response of muscle, liver, pancreas, hindgut and cartilage to varying feeding regimes was recorded for reared *Dicentrarchus labrax*. Tissular response was recorded both qualitatively and quantitatively using digital image analysis, and the results were compared. In general, the tissues responding faster to food deprivation were gut-associated glands and muscle. The cartilage took the longest to respond.

Liver gave more consistent results than pancreas in the determination of feeding treatments. Liver scoring was better than hepatocyte maximum diameter for detecting emaciated larvae. Gut associated glands proved to be age dependent and showed high within-treatment variability. From all indices, the quantitative determination of muscle fibre separation (MFS) was the single index that correctly classified the highest proportion of larvae to their corresponding feeding treatments. It was not correlated with age or size and, in the Starved larvae, it underwent a significant worsening coinciding with the initiation of mass mortality. We regard the quantitative determination of MFS as a useful contribution to the determination of condition in fish larvae. It is a relatively fast quantitative method, and its use would reduce the bias caused by discrete grading (scores) and by differences in individual expertise. Finally, a method for weighting histological indices is proposed for use in field studies.

INTRODUCTION

The use of histological methods in the determination of larval nutritional condition has at least two unresolved problems. One regards the low objectivity of these methods, as the measures are mainly qualitative (O'Connell 1976; Sieg 1992; McFadzen et al. 1997). To date, quantitative data have been restricted to the measurement of cell heights of few tissues, mainly gut and liver, and have proved useful for early larval stages of some species (Ehrlich et al. 1976; Oozeki et al. 1989; Theilacker and Watanabe 1989, Theilacker and Porter 1995). However, some of these measurements are only obtainable from species with elongated digestive duct (Theilacker and Watanabe 1989), or have been restricted to particular larval stages.

Digital Image analysis offers versatile tools that permit the study of trends (areas, volumes, colour-related differences etc.) whose calculation was tedious some years ago. Johnston (1993) measured organelle areas in the muscle of *Clupea harengus* larvae using an image analyser and McFadzen et al. (1994) quantified starvation in *Scophtalmus maximus* using the ratio perimeter/area of the foregut. However, there are no studies in which multiple quantitative and qualitative measurements of several organs taken from the same individuals can be compared.

The second main problem of histological indices (extensive to any condition index) is the large dependence of condition on the experimental rearing parametres, with the subsequent poor applicability to field studies. Until further evidence is supplied, there is a need to establish a relationship between survival and each condition measurement in laboratory conditions.

Attending to the above, the goals of this section were a) to study the variation of several histological indices as a response to long and short-term food deprivation and delayed feeding, b) to compare classical qualitative measurements of tissue degeneration with quantitative measurements and c) to explore the relationship between histological measurements and the survival of larvae in the laboratory.

MATERIAL AND METHODS

Rearing design and sampling

The rearing design and feeding scheme were described in Section 2.1. Here, the histological study was divided into a long-term food deprivation experiment and a short-term one. The former aimed to study of the starvation effect from the day the food was first supplied (4 DAH) until 21 DAH, plus the effect of Delayed feeding (starting at 13 DAH). The short-term experiment aimed to test the effect of punctual fast on well developed larvae aged between 24 and 28 DAH. The sampling scheme and feeding treatments are shown in Table 2.3.1. A total of 124 larvae were used for the analyses.

Table 2.3.1. Number of larvae per treatment that yielded interpretable results for most tissues. A, long-term starvation experiment. B, short-term starvation experiment. Shaded cases indicate the day at which food was withdrawn.

A

Feeding Treatments	Age (DAH)					
	6	**10**	**14**	**17**	**19**	**21**
Fed	5	5	5	5	5	5
Starved	6	5	5	5	5	3
Delayed	-	-	5	5	4	5

B

Feeding treatments	Age (DAH)				
	24	**25**	**26**	**27**	**28**
Fed	4	5	7	3	4
1d of Fast		2			
2d of Fast			8		
3d of Fast				3	
2d of Fast, 1d re-fed				5	
2d of Fast, 2d re-fed					5

Histological analyses

Sampled larvae were placed in chilled sea water until torpid and immediately fixed in 10% phosphate-buffered formalin (pH 7.2). After one-month fixation, larvae were

measured to the nearest 0.1 mm and transferred to 70% ethanol until analysis. Prior to histological procedures, larvae were coded so that no bias related to the knowledge of the treatment was introduced. Larvae were dehydrated in an ethanol series up to 95%, embedded in historesin (Leica) and serially sectioned at 3 µm in an approximate sagittal plane. They were mounted and stained with Lee's Methylene Blue-Basic Fuchin (Benett et al. 1976). Sections were blow-dried and coverslipped with Eukitt mounting medium.

We studied the degree of starvation by looking at liver, pancreas, hindgut, mucosa, muscle and cartilage. The study of midgut was here limited to general cellular observations, as its quantification was not possible due to uniform staining and high folding of the tissue.

Before any tissue was measured, all sections from the larva under study were inspected and the best ones marked for further study. Both quantitative and qualitative measurements were taken.

Qualitative measurements consisted in the scoring of the tissues of several organs. Maximum differences between tissues of 19 and 21 day old Fed and Starved larvae were used to establish the extremes of the scores. Few modifications were introduced in the scoring methods in comparison with other authors, as the tissular characteristics and alterations were similar to those described for other species (O'Connell 1976; Yúfera et al. 1993; Theilacker 1978; Sieg 1992; McFadzen et al. 1997). Details of the scoring procedure for each character are given in Table 2.3.2.

Quantitative measurements were taken with an image analyser (OPTIMAS 6.0 CORP, USA) connected to a CCD SONY video camera. Two types of quantitative measurements were recorded.

A) Area-ratio measurements. These measurements consisted in the calculation of the percentage of muscle fibre separation (MFS) of a determined area of trunk muscle and the determination of the percentage zymogen (% zymogen) of a pancreas section. Muscle fibre separation was measured on the central part of the trunk, in sections where myotomes and notochord were clearly visible. For several specimens examined, variability of MFS was high for areas comprising less than three myotomes. Therefore, regions comprising a minimum of three myotomes were selected. For the determination

of % zymogen, a section of the pancreas close to the endocrine tissue was chosen when possible. To determine both MFS and % zymogen, a macro from Optimas was run on the selected area. This macro automatically detected the lighter areas (muscle gaps or zymogen granules) against a luminance-corrected background (Fig. 2.3.1. A, B)

B) Linear measurements. Linear measurements included hepatocyte diameters and enterocyte cell heights. Only individuals that had a minimum of 4 readable cells of each organ were included in the analyses. Enterocytes were measured from the basal membrane to the inner part of the microvilli brush. Hepatocyte cell heights were obtained by measuring the maximum cell diameters (Fig. 2.3.1. C).

Relationship of the indices with age and size

For the long-term starvation experiment, data was analysed by age and length. As shown in the growth studies, two size-categories were established (4.5-6 and over 6mm). Almost all Starved larvae were included in the small size category (27 out of 29). These few Starved larvae over 6 mm were not sufficient to provide statistically valuable information so they were excluded from the analyses. Feeding groups of each age class were compared through non-parametric tests (Mann-Whitney when 2 treatments, Kruskall-Wallis when 3 treatments, α=0.05) due to the strong non-normality of the data even after several transformations. The effect of size on each histological variable was tested by comparing Fed individuals smaller and larger than 6 mm of the Fed treatment, as this group would probably reflect a size effect independently of food regime. Groups were compared using Mann-Whitney tests, at α=0.05.

The short-term starvation experiment was only analysed by age.

Classification properties by histological variable

These analyses were only conducted for the long-time experiment, for which maximum histological differences were observed. Also, this age-range enabled the comparison with other studies of condition (see Sections 2.2. and 2.5.). In order to study the classification properties of the different histological variables, we defined 3 nutritional

Table 2.3.2. . Histological traits used to score *D. labrax* larvae

Organ/tissue	Observational criteria	Grade 4 (only for hepatocyte intracellular space)	Grade 3	Grade 2	Grade 1
Mucose	Degree of detachment of the body		Tight contact	Detached in some areas	Highly detached along the trunk
Muscle	Fibre separation		Densely packed	Slight separation, fibres often bent	Gaps as wide as fibres, fibres severely bent
	Intercellular substance		Abundant	Scarce	Absent or scarce
Pancreas	Zymogen /pancreas ratio		High, >30%	Mid, 20-30%	Small, < 20%
	Cell (acini) appearance		Cytoplasm appears bright	Cytoplasm darker	Cells darkly stained
	Cell (acini) boundaries		Well defined	Poorly defined.	Indistinguishable
Cartilage	Capsular cavities		Filled with cytoplasm	Cytoplasm partly retracted	Cytoplasm severely reduced and nuclei darkly stained
Hepatocyte intracellular space	Vacuolisation	Conspicuous.	Reduced	Only occasionally, small	Absent, cells compact
	Inclusions	Absent	Some acidophilic ones	Abundant	Inclusions filling cytoplasm
	Cell boundaries	Marked	Less distinguishable	Barely distinguishable	Indistinguishable
Hepatocyte nuclei	Size and placement		Large and marginal	Large, some not marginal	Pycnotic
	Stain		Lightly stained	Some stain darkly in blue.	Darkly stained.
Hepatocite cytoplasm	Presence of inclusions		None or few	Coarse	Large number, acidophilic
Hindgut	Cellular adhesion		Tight	Some detached	Most widely separated
	Acidophilic Inclusions		Small	Some large	Most larger than nucleus

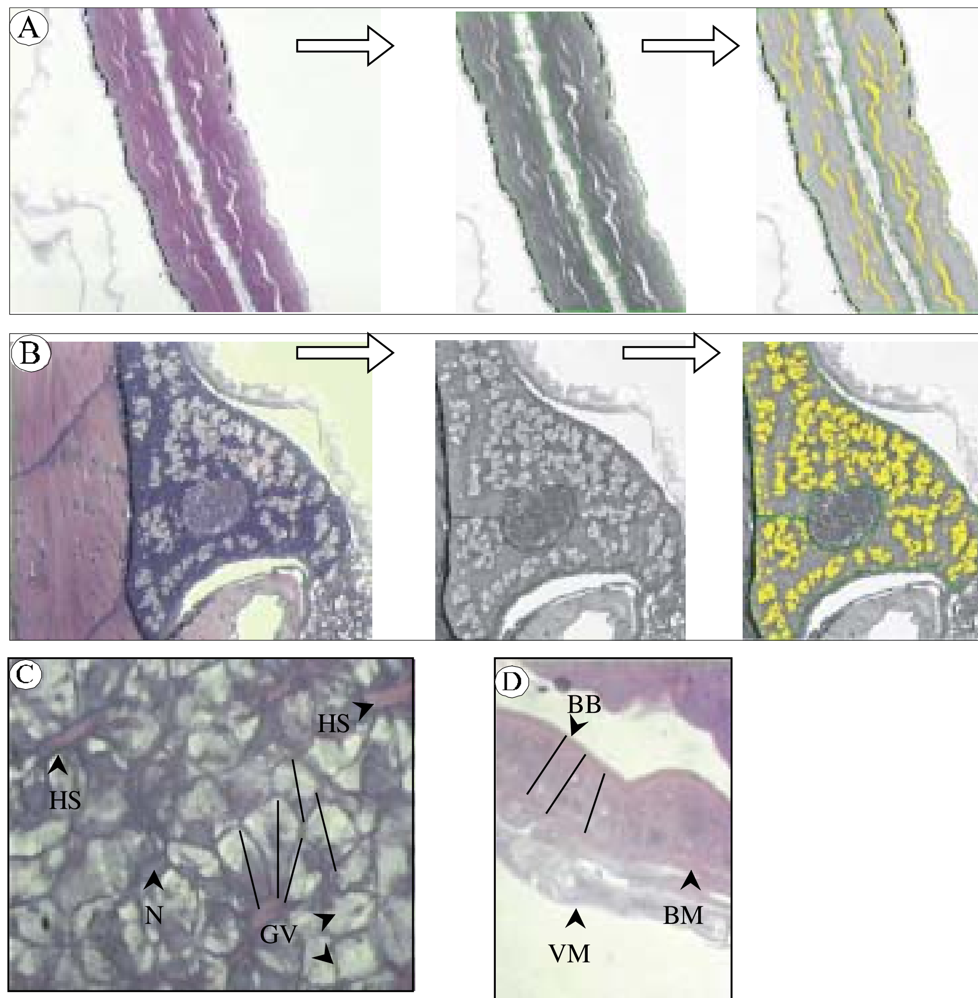

Figure 2.3.1. Quantitative measurements. A, and B, determination (in yellow) of MFS and % zymogen, respectively, using image analysis. C, measurement of maximum hepatocyte diameters in the liver. D, measurement of hindgut cell heights. **BB**, brush border; **BM**, basal membrane; **GV**, glycogen vacuole; **HS**, hepatic synus; **N**, nucleus; **VM**, ventral mucosa.

ranges for each type of measurement. Data from both qualitative and qualitative measurements were divided into Healthy, Moderately Healthy and Emaciated condition. These ranges were established in a different way for qualitative or quantitative variables.

For qualitative variables, equal ranges were built. As an example, a tissue with 3 possible qualitative categories, 1 to 3, would have the following ranges of nutritional condition: 2.34-3.00 (Healthy), 1.67-2.33 (Moderately Healthy) and 1-1.66 (Emaciated). For quantitative variables, ranges for each nutritional condition corresponded to these that yielded the best classification through several tentative trials (Table 2.3.3.). Medians and

95% confidence intervals were used when possible. The goodness of each histological variable to detect starvation was established as the percentage of larvae of a given treatment correctly assigned to its corresponding nutritional class (For example, % of Fed larvae classified as Healthy by the liver scores, etc.).

Table 2.3.3. Ranges used to define nutritional categories in quantitative indices for each larva.

	Nutritional category		
Quantitative index	**Healthy**	**Moderately Healthy**	**Emaciated**
Hepatocyte max. Diametre (µm)	>15.5	15.5-11.5	<11.5
Percentage zymogen (%)	> 20.0	20.0-16.0	< 16.0
Hindgut cell height (µm)	> 17.0	17.0-15.3	< 15.3
MFS (%)	< 1.3	1.3-3.9	> 3.9

In order to combine the information of the different indices, and to compare it with the previously described single classification, a Stepwise Discriminant Analysis (SWDA) was performed on selected variables (see Section 2.2. for a more extensive explanation of the method). This methodology has been successfully applied to discriminate fish larvae of diverse feeding condition from pre-defined groups, and to select the variables that most contribute to the discrimination between groups (O'Connell 1976; Theilacker 1978). Variables were represented onto the two first canonical axes to assess graphically the results of the SWDA.

Relationship of histological variables with survival

In order to analyse the degree of correspondence between the nutritional descriptors and survival, we compared the time-variation of each variable with the survival of the Starved larvae. This analysis was only performed in the long-term experiment. Spearman's Rank correlation was used due to the general lack of linear relationships even after data transformation.

RESULTS

Among the several histological variables analysed, the degree of mucosa detachment had to be excluded from the analyses for showing extreme within-treatment variability. The general histomorphology of *D. labrax* is shown in Figure 2.3.2.

General histological differences between Fed and Starved larvae

The following results describe maximum differences observed for several organs of Fed and Starved larvae of days 19-21 (Fig. 2.3.3.).

The hepatocytes of well fed larvae were polygonal and showed defined cell boundaries, exhibiting a high degree of vacuolisation that presumably corresponded to glycogen (O'Connell and Paloma 1981). Cell nuclei showed basophilic stain and tended to appear at the cell corners. In severely Starved larvae, glycogen vacuoles were almost absent, and a variable degree of intracellular inclusions (possibly degeneration bodies) was commonly observed. In these larvae, cell boundaries were difficult to distinguish and nuclei usually appeared darkly stained (Fig. 2.3.3. A, B).

The pancreas of well fed individuals tended to show high amounts of zymogen granules that showed a varying degree of affinity for basic Fuchin, ranging from intense red to whitish. Acinar structure was visible in most cases. Extremely emaciated larvae showed shrunk Acinar cells that stained intensely in blue, and zymogen granules were almost absent.

Enterocytes of the Fed larvae showed a thick microvilli layer and were closely adhered to the basal membrane, unlike those of Starved larvae. Intracellular lipid inclusions were observed in the midgut of Fed larvae, and tended to disappear in emaciated individuals. Small acidophilic inclusions were observed in the hindgut of Fed larvae. In Starved animals, these inclusions tended to increase in number and size (Fig. 2.3.3. C, D).

The trunk muscle of Fed larvae showed compact myomeres, with straight muscle fibres and abundant interfibrillar substance. Starved larvae had widely separated muscle

fibres, a phenomenon that was more conspicuous towards the rear end of the body (Fig. 2.3.3. G, H).

The procartilage of the Fed larvae was formed by a thick matrix enclosing the chondrocytes, which usually obliterated the capsular space. In extremely starved larvae, the chondrocytes appeared shrunk in the luminal space (Fig. 2.3.3. I, J).

Histological response to feeding conditions along age and size

Significantly different patterns were observed between feeding treatments for most histological variables. Despite this, high within-treatment variability occurred, particularly in the gut-associated glands. Below, the most relevant results are commented for each tissue and type of measurement for the long-term starvation experiment. The short-time starvation experiment is discussed at the end of this Section.

At the start of the experiment, the liver of the Fed and Starved larvae exhibited low scores and small cell diameters (Fig. 2.3.4. A). Starved larvae remained in poor condition throughout the experiment whilst Fed larvae underwent a gradual improvement in liver condition. Hepatocyte traits of Starved larvae differed significantly from those of Fed larvae at 17 DAH (scores) or 19 DAH (diameters) (Fig. 2.3.4. A, B). Delayed feeding larvae took 4 days to reach values similar to these of Fed larvae, showing significant differences from Starved larvae at 19 DAH. The reasons for the apparent improvement in condition are discussed further in this Section. A significant effect of size was observed in both hepatocyte measurements; the Fed larvae over 6 mm showed significantly higher scores and cell diameters (Fig. 2.3.5. A, B) thus suggesting a developmental effect on hepatocyte cellular properties.

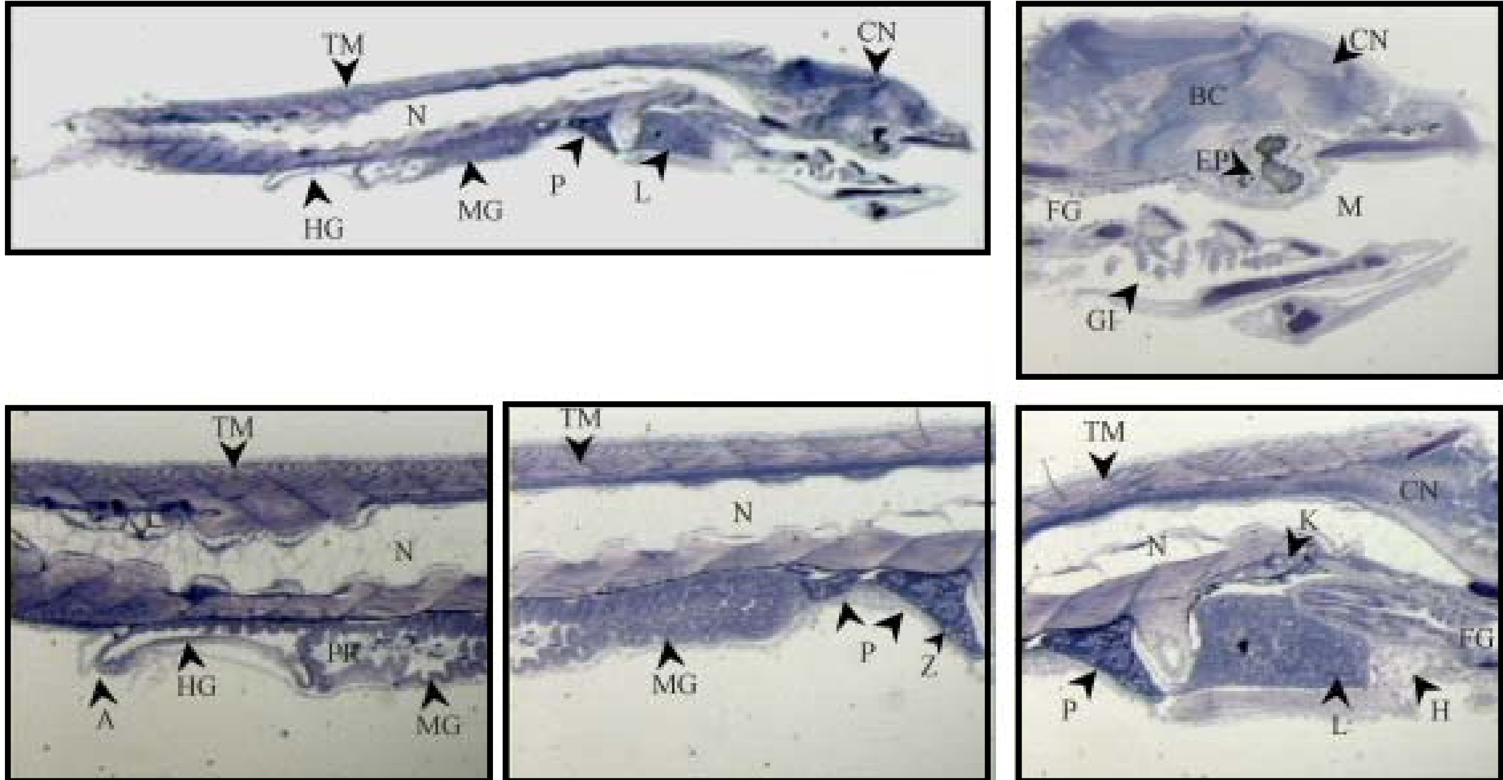

Figure 2.3.2. General histomorphology of a Fed 19 day-old *D. labrax* larva. **A**, anus; **BC**; brain cells; **CN**, central nervous system; **EP**; eye pigments; **FG**, foregut; **GI**, gills; **H**, heart; **HG**, hindgut; **K**, kidneys; **L**, liver; **M**, mouth; **MG**, midgut; **N**, nothochord; **P**, pancreas; **PR**, prey remains; **TM**, trunk muscle; **Z**, zymogen granules.

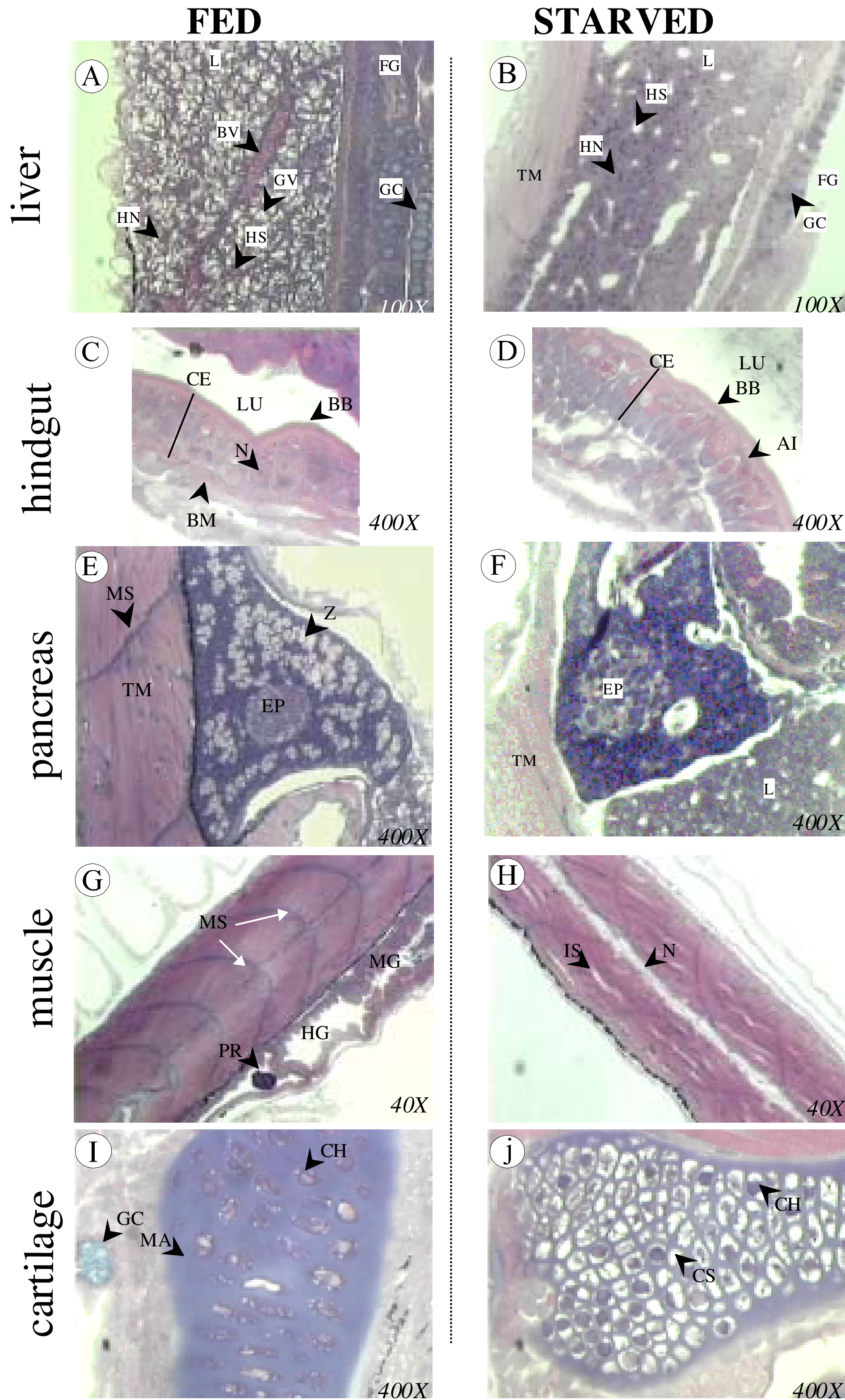

Figure 2.3.3. Examples of healthy and degraded tissues of *D.labrax* larvae. **AI**, acidophilic inclusions; **BB**, brush border; **BM**, basal membrane; **BV**, blood vessel; **CE**, cuboidal epithelium; **CH**, Chondrocite; **CS**, capsular space; **EP**, endochrine pancreas; **FG**, foregut; **GC**, goblet cell; **GV**, glycogen vacuole; **HG**; hindgut; **HN**, hepatocyte nucleus; **HS**, hepatocyte sinus; **IS**, interfibrillar space; **L**, liver; **LU**, luminal space; **MA**, matrix; **MG**, midgut; **MS**, miotome septa; **N**, nothochord; **PR**, prey; **TM**, trunk muscle; **Z**, zymogen.

The pancreas scores of the Fed larvae were significantly higher than those of Starved larvae at 14 DAH.The pancreas condition improved in Delayed larvae until 17 DAH. The % zymogen showed little variation in Fed larvae over time and followed a similar pattern to that of the classical scores. For both pancreas measurements, Starved larvae showed the highest median on day 6 and decreased until day 19. As before, on day 21 there was an improved condition for Starved larvae (Fig. 2.3.4. C, D). For % zymogen, the first significant differences between Fed and Starved larvae were detected on day 19. Delayed larvae had fully recovered to levels of Fed larvae by day 17 (significant K-W, $p<0.05$). No size-effects were detected (Fig. 2.3.5. C, D).

The medians for hindgut scores of Starved larvae were always below those of Fed larvae, although significant differences were only observed on days 14 and 19 (scores) or 19 (cell heights) (Fig. 2.3.4. E, F). This was probably due to the lack of interpretable measures for this tissue, and the high variability. There was no significant size-effect on this measurement (Fig. 2.3.5. E).

Muscle scores of Fed larvae showed a higher daily variation than MFS until day 21 (Fig. 2.3.4. G, H). Mean MFS of the Fed larvae was 1.2 (SD = 1.55, n=27) throughout all the experiment. Both muscle scores and MFS of Starved larvae had a worsened condition from day 10. The poorest condition was attained on day 17 according to both measurements. Both muscle measurements were significantly different between Fed and Starved treatments from 14 DAH. Muscle response of Delayed larvae appeared earlier in the MFS (17 DAH) than in the muscle scores (19 DAH). Delayed larvae fully recovered on day 21 according to the scores and on day 19 according to the MFS. There was no size effect on muscle measurements in larvae over or under 6 mm (Fig. 2.3.5. G, H).

Cartilage scores of Fed larvae varied around 2 and 3 during the experimental period (Fig. 2.3.4. A). Starved larvae showed significantly different scores for cartilage from day 14. After that day, there was never a sufficient number of larvae (generally around 3) to make reliable statistical comparisons. However, the trends in medians suggested a general poorer condition of Starved larvae, with a recovery of Delayed larvae detectable 3 days after food was supplied (17 DAH). No size effects were detected (Fig. 2.3.5. I).

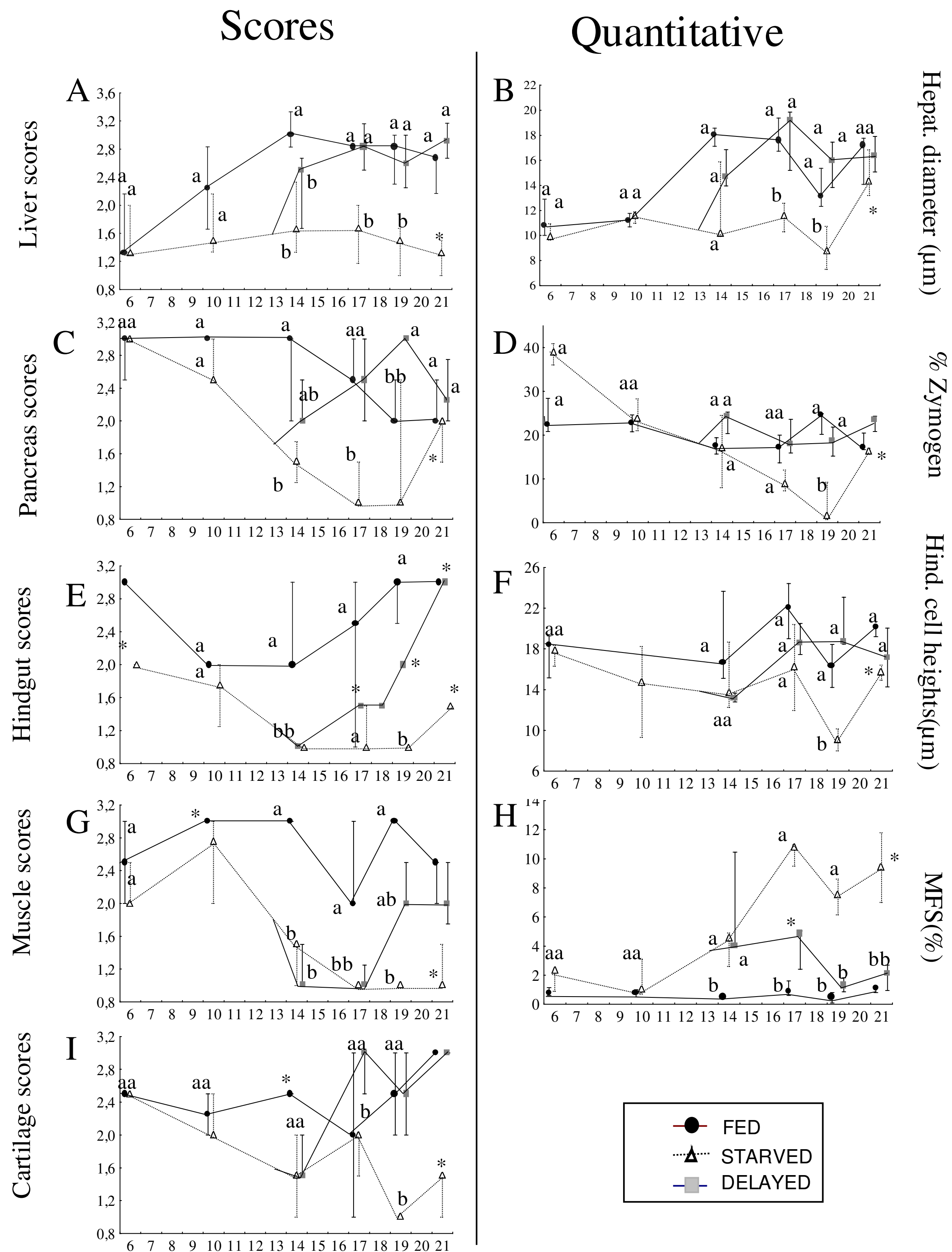

Figure 2.3.4. Relationship between each index (scores or quantitative) with age for each feeding treatment. Data are medians and 25 and 75% percentiles. Different letters are significant differences at $p<0.05$ (Mann-Whitney if 2 groups, Kruskal-Wallis if 3 groups). Asterisks indicate small sample($n<5$). When scores were coincident among treatments, no letter is shown.

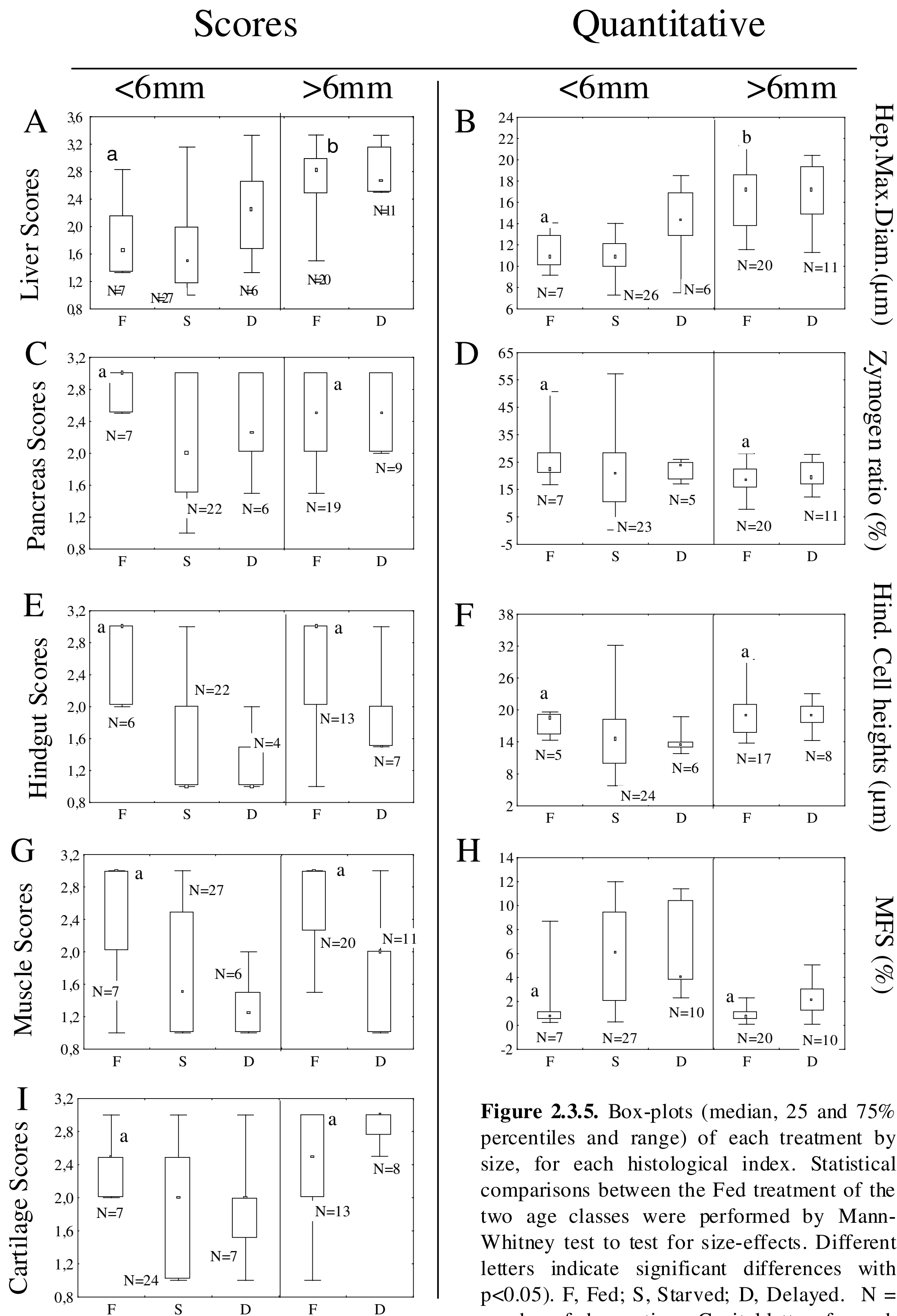

Figure 2.3.5. Box-plots (median, 25 and 75% percentiles and range) of each treatment by size, for each histological index. Statistical comparisons between the Fed treatment of the two age classes were performed by Mann-Whitney test to test for size-effects. Different letters indicate significant differences with p<0.05). F, Fed; S, Starved; D, Delayed. N = number of observations. Capital letters for each figure are drawn to help explanation in the text.

The short-term experiment revealed some trends in the tissular response to short-term fast, in larvae that can theoretically cope with starvation for longer periods. Though statistical differences were probably influenced by the low number of available larvae, a general responsiveness of most tissues was suggested by our results (Fig. 2.3.6.).

One day of starvation was not significantly reflected in a variation of the means in any tissue. Two days of starvation produced significant differences between Fed and Starved treatments in MFS, and the same pattern (though not significant) was shown by the rest of the measurements except for hepatocyte diameters. In fact, hepatic condition was found to be far from that expected in Fed larvae (see discussion). Larvae subjected to 3 days of fast showed either a similar or a lowered condition in all cases. After 2 days of starvation, larvae refed for 1 or 2 days showed a generalised improved condition, although with noticeable variability values.

Classification by each index into nutritional classes

Results from the classification into nutritional classes showed that Fed and Starved larvae between 14-21 DAH were better classified than their counterparts of 6-13 DAH by all indices (Table 2.3.4. A, B), reflecting the more pronounced differences caused by the longer starvation time. Except for MFS, qualitative variables of a given tissue offered better classification results than the quantitative measurements for either age-class.

Misclassifications patterns in 6-13 DAH larvae were different for several indices and deserve further explanation. Liver measurements, (both scores and hepatocite diameters) of young larvae were characterised by misclassifying the majority of Fed larvae into the Emaciated category (Table 2.3.4. A). On the other hand, pancreas classified the vast majority (between 82 and 100%) of young Starved larvae into the Healthy class. Also, Muscle scores, MFS and Cartilage scores classified over half of the larvae as Healthy. Hindgut measurements of this age-class misclassified between 46 and 56% of Starved larvae either as Healthy or Moderately Healthy. The best classification scores for larvae of

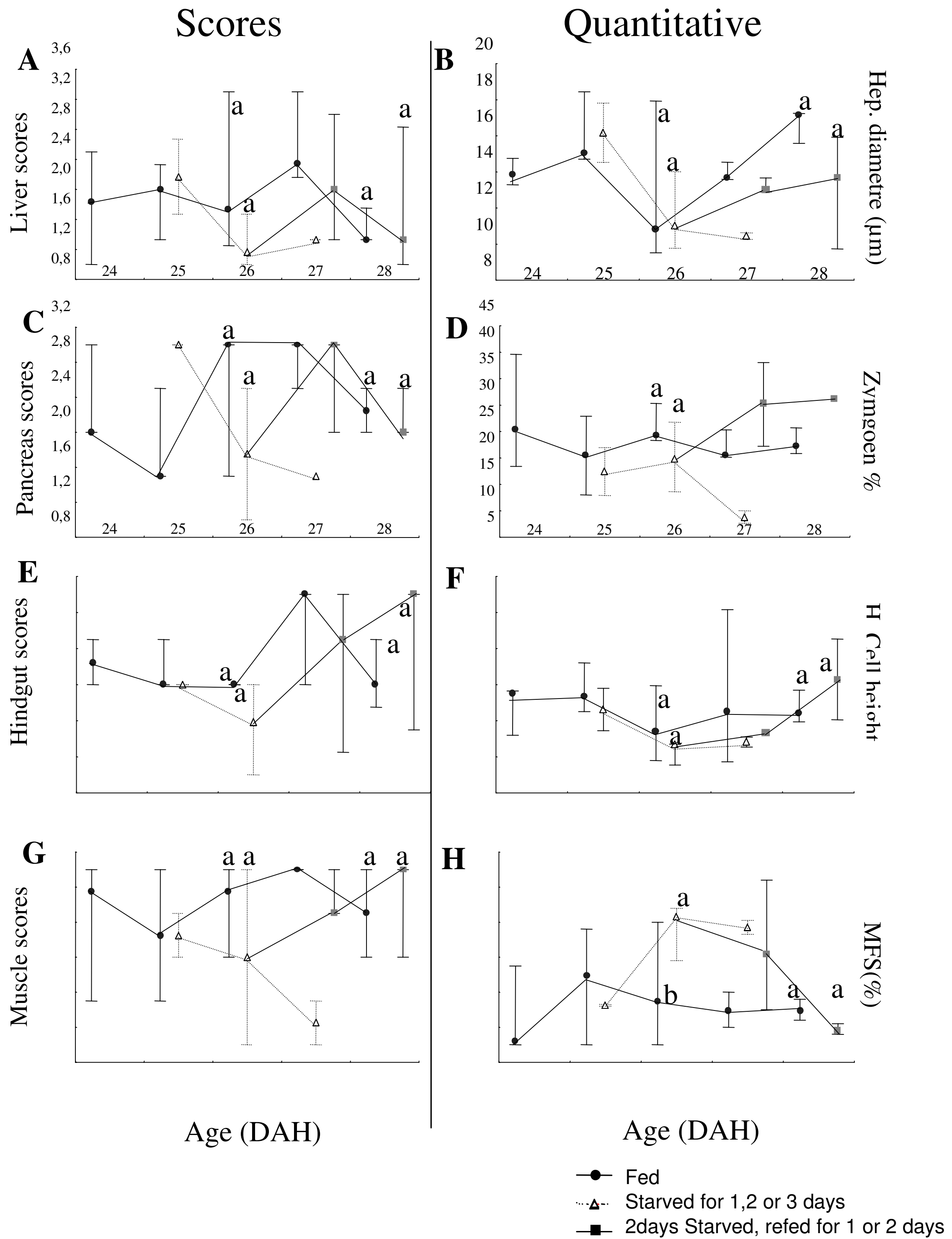

Figure 2.3.6. Response of both quantitative and qualitative histological indices to 1-3 days of starvation and 1-2 days of re-feeding. Plots are medians plus 25 and 75% percentiles. Different letters represent significant differences (K-W, $p<0.05$). The lack of letters indicates insufficient number of observations for safe comparison.

Table 2.3.4. Classification table of each histological variable for the two age-groups considered. Table is read as follows: "from Fed larvae of 6-13 DAH, liver scores classified 14% as "Healthy", 57% as "Emaciated" and 29% as "Moderately Healthy". H,E,M, Healthy, Emaciated and Moderately Healthy, respectively. F,S and D are number of larvae analysed for Fed, Starved and Delayed treatments, respectively. Values in bold are correct classifications. (*) percentage of total correct classifications for a given index and age-group.

A. Larvae of 6-13 DAH

	Liver scores F=7, S=11			Hepatocite diameter F=7, S=11			Pancreas scores F=7, S=11			% zymogen F=7, S=11			Hindgut scores F=6, S=9			Hindgut cell heights F=5, S=11			Muscle scores F=7, S=11			MFS F=7, S=11			Cartilage scores F=7, S=9		
Nutritional class →	H	E	M	H	E	M	H	E	M	H	E	M	H	E	M	H	E	M	H	E	M	H	E	M	H	E	M
Treatment ↓																											
Fed	**14**	57	29	**0**	57	43	**86**	14	0	**86**	0	14	**67**	0	33	**60**	20	20	**71**	14	14	**86**	14	0	71	0	29
Starved	9	**64**	27	0	**60**	40	82	**9**	9	100	**0**	0	11	**33**	56	46	**36**	18	55	**18**	27	54	**18**	27	56	0	44
%correct*	**39**			**30**			**49**			**43**			**50**			**48**			**44.5**			**52**			**35.5**		

B. Larvae of 14-21 DAH

	Liver scores F=20, S=18, D=17			Hepatocite diameter F=20, S=18, D=17			Pancreas scores F=19, S=13, D=15			% zymogen F=20, S=14, D=16			Hindgut Scores F=13, S=14, D=11			Hindgut cell heights F=17, S=14, D=14			Muscle scores F=20, S=18, D=17			MFS F=20, S=18, D=16			Cartilage scores F=13, S=18, D=14		
Nutritional class →	H	E	M	H	E	M	H	E	M	H	E	M	H	E	M	H	E	M	H	E	M	H	E	M	H	E	M
Treatment ↓																											
Fed	**74**	5	21	**65**	0	35	**53**	5	42	**40**	30	30	**69**	8	23	**65**	23	12	**75**	5	20	**90**	0	10	**69**	8	23
Starved	5	**72**	22	17	**56**	28	8	**69**	23	14	**71**	15	0	**100**	0	22	**71**	7	11	**83**	6	0	**83**	17	11	**72**	17
Delayed	59	12	**29**	59	**12**	29	53	**7**	40	50	**12,5**	37,5	9	64	**27**	57	43	**0**	12	59	**29**	25	31	**44**	64	21	**14**
%correct*	**58.3**			**50**			**54**			**49.5**			**65.3**			**45.3**			**62.3**			**72.3**			**51.7**		

6-13 DAH corresponded to Fed individuals; up to 86% were correctly classified as healthy by MFS and pancreas measurements.

For larvae aged 14-21 DAH, the best classification results corresponded to MFS, which correctly classified 72.3% of all larvae (Table 2.3.4. B). This was the index that best classified the Fed larvae (90% correct classification). Starved larvae were best classified by Hindgut scores (100%), and Delayed larvae were again best classified by MFS (44%).

The effect of maternal reserves was probably interacting with starvation effects in the first age-class (see discussion), so the results were ordered from best to worse only in the second age-class, i.e. MFS > hindgut cell heights > muscle scores > Liver scores > pancreas scores > Cartilage scores > Hepatocite diameter > %zymogen > Hindgut cell heights.

Multivariate analysis

In order to study the gain in classification power by combining several variables, a SWDA was performed. Larvae were assigned to 5 predefined groups of treatment-age. Variables that shared information were not included in the analysis in order to avoid the ill-conditioning of the matrix (Manly 2001). Also, variables that showed more than 20% of missing data were excluded from the analyses. As seen from Table 2.3.4., all quantitative indices (except muscle scores) yielded better classification results than the qualitative ones. Therefore, complying with the above-mentioned conditions, the variables finally included in the SWDA were MFS, liver scores, pancreas scores and cartilage scores. All data were ln-linearised except for MFS, which was root-transformed in order to avoid negative values. Multiple correlation between the selected indices showed that no single variable showed a significant correlation with more than half of the remaining variables.

SWDA chose, in order of importance for the discrimination of groups, MFS>liver scores>pancreas scores>cartilage scores. Only the three first variables contributed significantly to the results.

The group composed of 6-13 DAH larvae corresponded to Fed individuals; up to 86.0% of those larvae were correctly classified as Healthy by MFS and pancreas measurements.

Table 2.3.5. (A) shows that the groups that were best classified were Fed larvae of 14-21 DAH (83.3%) and Starved larvae of 14-21 DAH (91.7%). Overall, 88.9% of Fed larvae were classified as Fed (either in the 6-13 or 14-21 classes). Over ninety percent of all Starved larvae were correctly classified as Starved (of any age-category, see Table 2.3.5.B). All Starved larvae aged between 14 and 21 days were classified as Starved (Table 2.3.5. B).

The first two canonical variables explained 96.6% of all variation (Fig. 2.3.7.). Looking at the standardised coefficients of the first canonical variable it was seen that individuals lying on the positive side were characterised by having a high MFS and low scores for the remaining three variables. These individuals corresponded to Starved larvae of 14-21 DAH plus some Delayed individuals. The negative side of the first canonical axis comprised individuals with low MFS and high scores for Liver, pancreas and cartilage. Individuals falling into this area were young Fed and Starved larvae, most Delayed larvae and all Fed larvae over 14 DAH. The second canonical axis only explained 8% of the variation and was mainly a contrast between pancreas scores and liver scores.

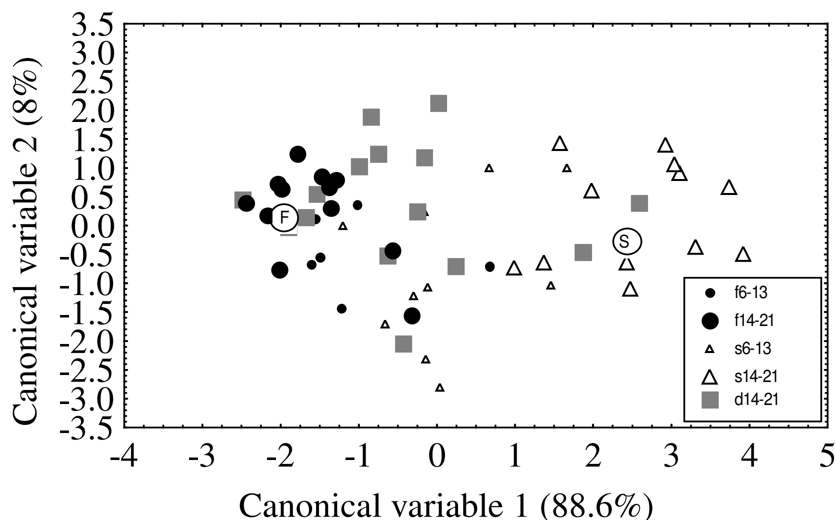

Figure 2.3.7. Values of each individual with respect to the first two canonical variables. Centroids of Fed (F) and Starved (S) individuals of age-group 14-21 DAH are indicated. Legend as in Fig. 2.2.7.

Table 2.3.5. Results of the SWDA on four histological variables. A; parameters of the classification functions. Age-food grups arecomposed by feeding treatment (Fed (F),Starved (S), or Delayed (D)) and the age period. B; classification mattrix. In bold, correct classifications. Values are percentages.

A

	Age.food groups				
	F6-13	**F14-21**	**S6-13**	**S14-21**	**D14-21**
MFS	3.3401	2.8874	3.9382	5.9651	4.5822
Liver scores	7.0211	9.9827	4.1275	3.0654	8.0759
Pancreas scores	12.7317	11.2856	10.9639	5.2217	10.9767
Cartilage scores	11.1233	11.7913	9.7643	6.1145	11.4749
Constant	-17.0505	-18.3481	-13.4293	-12.0931	-18.3060
%correct	33.3	83.3	60.0	91.7	46.7

B

		Fed			Starved			Delayed
		6-13	14-21	**Total**	6-13	14-21	**Total**	14-21
Fed	6-13 (n= 6)	**33**	50	**83**	17	0	17	0
	14-21(n=12)	8.3	**83.3**	**91.6**	8.3	0	8.4	0
	total		**88.9**			11.1		0
Starved	6-13 (n=10)	10	0	10	**60**	20	**80**	10
	14-21(n=12)	0	0	0	8.3	**91.7**	**100**	0
	total		45			**90.1**		
Delayed	14-21 (n=15)	6.7	20	26.7	13.3	13.3	26.6	**46.7**

Relationship of the histological variables with survival

Percentage of zymogen, the 2 muscle measurements and the calculated values for the first canonical variable showed the highest significant correlation's with survival of the Starved larvae (Table 2.3.6.). However, the contribution to the correlation meaning should be looked at for each variable before any interpretation on causality is made.

As seen in Fig. 2.1.4., mortality of Starved larvae showed the sharpest decline at 17 DAH. From Fig. 2.3.4., it is seen that muscle measurements for Starved larvae do vary reflecting the mass mortality, showing the lowest scores (or highest MFS) on day 17. On the other hand, % zymogen (which yielded the highest correlation) descended constantly from day 6 until day 19, thus failing to encompass the survival trends. Regarding the correlation of the first canonical root, the Spearman correlation coefficient was similar to those yielded by muscle measurements. Therefore, it is concluded that the highest correlation with survival held by pancreas scores did not encompass the peak mortality

occurring at ca. 17 DAH, whereas muscle scores did. Also, the use of a combination of several histological variables (1st canonical root) did not significantly improve the correlation of survival with muscle measurements, so the latter are preferred as single indicators of starvation mortality in the laboratory, for this species and under the present experimental conditions.

Table 2.3.6. Results of the Spearman rank correlation analyses between the different histological variables and the survival of the Starved treatment. NS, non significant. S, significant.

Treatments	r_s	T	DF	Significance
Liver scores	0.204	1.062	26	NS
Hepatocyte diameter	-0.333	1.670	23	NS
Pancreas scores	0.256	1.242	22	NS
% zymogen	0.819	6.846	25	S (p<0.001)
Hindgut scores	0.586	3.318	23	S (p<0.01)
Hindgut cell heights	0.404	2.117	25	S S (p<0.05)
Muscle scores	0.697	4.950	26	S (p<0.001)
MFS	-0.668	4.660	27	S (p<0.001)
Cartilage scores	0.384	2.037	24	NS
First canonical root	-0.686	-4.526	25	S (p<0.001)

DISCUSSION

D. labrax larvae proved to be histologically sensitive to food deprivation in all the examined tissues. However, response-times and within-treatment variability differed among histological measurements. The comparison of qualitative and quantitative measurements showed that, except for muscle measurements, qualitative scores correctly classified a larger proportion of individuals than quantitative measurements at any age-class. This is partly to be expected as the qualitative indices take into consideration more cell or tissular characteristics, than quantitative ones. The latter notwithstanding, the search for quantitative histological indicators of larval emaciation is well documented in the literature (e.g. Oozeki et al. 1989) as it is generally thought that tissular scoring is an exceedingly

expertise-dependent procedure, as was proved in fish larvae by O'Connell and Paloma (1981).

A general observation that is common to other works on fish larvae of several species (O'Connell, 1976; Martin and Wright; 1987) is that at all ages, but particularly during the first week of life, there are some Fed individuals that show symptoms of emaciation. This has been interpreted in terms of genetic conditioning and competition for food (O'Connell 1976; Sclafani et al. 2000), and explains why in the literature, and in this work, the percentage of Fed larvae categorised as Healthy rarely shows values over 90%. In fact, most of the variations and misclassifications occurred during the period in which larvae were still dependent on maternal reserves.

Relationship of the histological variables with age and size

Before commenting separately on the different results, it should be said that the response-times obtained in this work are only of use for the purpose of comparison among variables. Indeed, an increased sampling frequency or/and sample size might detect earlier responses to starvation or recovery.

It is generally accepted that liver is one of the fastest tissues to respond to food deprivation. Its vacuoles store glycogen and lipids and are the first substances to be mobilised in starvation episodes (O'Connell and Paloma 1981; Margulies 1993). In Starved larvae a constant poor condition in hepatocite scores and vacuolation through time were observed. On the other hand, Fed larvae reflected an increase in both liver scores and cell diameters during the first days of development. This increase was obvious by age (day 6 until day 14) and size (Fig. 2.3.4. A, B, and Fig, 2.3.5. A, B). This can be partially explained by the fact that liver in *D. labrax* is not fully formed until 17 DAH (Barnabé 1976). In fact, increased cellular size and tissular complexity is common in the tissues of the digestive canal during early development, even if there is no change in tissular organisation (Govoni 1986). Therefore, the lack of response in the liver of the Starved larvae could be due to the initial inhibition of liver development and subsequent degradation of hepatic cells. It is also noteworthy the relatively high variation in the

medians of both quantitative and qualitative liver measurements, in all the feeding treatments.

Great variability has been observed in the condition of gut-associated glands of many species subjected to starvation in the laboratory (Yúfera et al. 1993). The time-response of glycogen stores in larval fish may vary from hours (Watanabe 1985; Oozeki et al. 1989) to days (Kashuba and Mathews 1984). In the present study, long-term starvation was translated into a significantly reduced condition of both hepatocyte measurements (Fig. 2.3.4.), but in well developed larvae subjected to 1, 2 or 3 days of starvation, variability in hepatocyte condition prevented from the detection of significant differences between treatments. Although in the present work larvae were sampled right before food was supplied, individual variability in the ingestion rate and time at ingestion might explain the within treatment differences in hepatocyte vacuolation. Therefore, liver measurements should be regarded with caution except for cases of severe starvation, as they may be reflecting immediate digestive status.

The pancreas scores were high and the % zymogen abundant in early Starved larvae of day 6. From then on they underwent a continuous decrease until day 19. Fed larvae maintained a high level of pancreas condition during the experiment, although the variation was considerable. Zymogen is protein based and is formed by enzyme precursors (mainly trypsinogen) that will be activated in the gut once food intake elicits its release. Zymogen is known to be synthesised in non-feeding larvae (O'Connell 1976), which probably explains the high zymogen levels found in young Starved larvae of the long-term experiment (Fig. 2.3.4. D). As shown by Pedersen et al. (1990), starved larvae also accumulate zymogen, which might account for some of the high values in the pancreas scores found in larvae subjected to one day of fast (Fig. 2.3.6.). The fact that these larvae did not show a corresponding increase in the quantitative zymogen determination could be partly explained by varying zymogen/pancreas area ratios. With starvation time, %zymogen and pancreas scores decreased, in both long and short-term starvation episodes (Figs. 2.3.4. and 2.3.6.). The high variation in the medians of Fed larvae might be due to short-term processes, as in the liver, that are not adequately explained by our data due to the low sampling frequency for this type of analysis. However, there are other sources of variability: the amount of

trypsinogen secreted by the pancreas is proportional to the amount of ingested food (Pedersen et al. 1990), which in turn is higher in larger larvae. Therefore, age could have influenced the results of the pancreas scores in the Fed treatment. For *D. Labrax* of 16 to 38 DAH, Zambonino-Infante et al. (1996) proved an age-dependency in the pancreatic secretion patterns. Thus, it is not unlikely that some part of the variability found in the experiments could be attributed to the latter findings. An observation which is crucial for the interpretation of the results of gut-associated glands is that when larvae were fed, it was common to find remains of live food in the rearing cylinders. Therefore, ingestion, although less likely, could have occurred during a wide time-interval in the Fed treatment, adding variability to the digestive processes. Delayed larvae showed a consistent recovery that was significant for pancreas scores already 24h after food was supplied (day 14). This was observed also in 27 day-old larvae subjected to 2 days of fast and refed for one day (Figs 2.3.4. I, F and 2.3.6. C, D). This was due to the general recovery of the integrity of Acinar cells and zymogen contents. From the above results, it is concluded that liver and pancreas, though responsive to food deprivation in *D.labrax*, should be interpreted with caution if they are to be used in field studies. Their use should ideally be accompanied by other starvation measurements obtained from the same individual.

In this work, we recorded the presence of presumptive acidophilic inclusions in the hindgut and lipid droplets in the midgut (Fig. 2.3.8.). These observations agree with the regional differences in assimilation thought to occur in the gut of fish larvae (Govoni 1986; Boulhic and Gabaudan 1992; Yúfera et al 1993.). The acidophilic inclusions of the hindgut are probably indicative of protein digestion. The massive presence of lipid droplets in the midgut has been previously described in sea bass larvae (Deplano et al. 1991; García-Hernández et al. 2001) and has been related to an excess fatty acid absorption in relation to the exporting capacity of the enterocytes (Crespo et al. 2001).

Both quantitative and qualitative hindgut measurements reflected the general trends found for the previous variables. The enterocytes of Starved larvae, although generally smaller in size, tended to have more abundant acidophilic droplets. These pinocytotic inclusions have been observed to occur in starved individuals of other species (e.g. Kjørsvik et al. 1991). The latter observation was accompanied, in severely starved

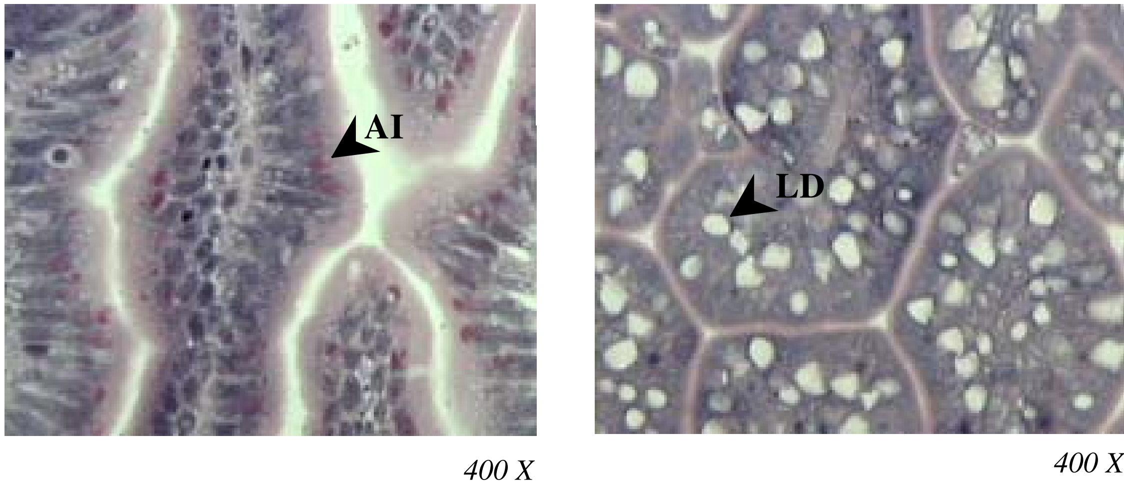

Figure 2.3.8. Light micrographs of the hindgut (left) and the midgut (right) in 22 DAH *D. labrax* larvae . **AI**, acidophilic inclusions. **LD**, lipid droplets.

individuals, by a loss of cellular integrity that caused a large variation in the cell size, making Starved individuals of 14 DAH show either extremely large or small enterocytes (Table 2.3.4. B). The number of valid measurements was lower for this tissue as sections through hindgut were often biased. This partly accounted for the high variability observed. Hindgut cell heights were less useful to distinguish feeding treatments than other quantitative measurements and than hindgut scores.

The use of digital image analysis enabled a relatively rapid determination of MFS, which showed an improved resolving power in comparison with the classical muscle scoring (see Table 2.3.4.). Our results agree with most of the literature on larval condition which, for most of the species studied, report a gradual separation of muscle fibres and loss of interfibre substance in starving individuals (Gas 1972; O'Connell 1976; Theilacker 1978; Cousin et al. 1986; Martin and Wright 1987; Green and McCormick 1999). This has been explained by the ability of fish larvae to quickly mobilise protein by degrading muscle when lipid and glycogen reserves have been exhausted (Love 1970; Pedersen 1990). Also, muscle degradation was most severe coinciding with the maximum observed mortality rate, which is important when it comes to the interpretation of the different indices for survival (see below). In the short-term starvation experiment, muscle responded as fast as liver to food deprivation.

The cartilage is known to respond only to prolonged periods of food deprivation in several species (Theilacker 1978; McFadzen et al. 1994). In the present experiment significant differences between treatments could only be established on days 19 and 21, confirming the aforementioned works. In the analysed larvae of 6 and 10 DAH, the majority of Starved individuals were misclassified as Healthy or Moderately Healthy (Table 2.3.4. A). The recovery of Delayed larvae was relatively fast, and 78% of the larvae from the Delayed treatment were classified either as Healthy or as Moderately healthy (Table 2.3.4. B). Variability in the cartilage scores have probably a large experimental error, as the chondrocyte reduction in the capsular space was seldom uniform across different parts of the cartilage of the same individual. In view of these results, the inclusion of this tissue in the determination of starvation in *D.labrax* is only of use to discriminate extremely emaciated individuals.

Multivariate Classification

The results of the SWDA agreed with the individual classification procedure. MFS was the variable that contributed most to the classification of single larvae. The SWDA improved the classification of yolk-sac and oil-globule larvae. However, there was no gain in the classification power of larvae of the 14-21 DAH period in comparison with the single use of MFS. For that period, SWDA correctly classified 83.3% of Fed larvae (*vs* 90.0% using MFS), 91.7% of Starved larvae (*vs* 83.0% using MFS) and 46.7% of Delayed larvae (vs 44.0% using MFS). Therefore, the effort of measuring several variables in that period is not justified in this species. Our classification values are of the same order as those obtained by Theilacker (1978) for jack mackerel. However, great caution should be taken in referring to classifications for comparative purposes, as experimental design (days of starvation, food type, temperature) and species-specificity are likely to make comparisons inadvisable.

It should be said that SWDA assumes multinormality requirements and homogeneity of within-groups covariance matrices (Manly 2001). Although this was not the case for our data, violations of one or more of these assumptions do not compromise the

results as long as significance tests are not the objective (Manly 2001). The agreement of the results of univariate and multivariate classifications support this view.

Relationship with survival and importance for field studies

The exact importance of starvation-mortality in the field is unclear. Results are often contradictory and highly species-specific. Ferron and Leggett (1994) concluded that most studies fail to establish any clear relationship between field-determined starvation and larval survival. For this reason, the study of this coupling in the laboratory must be udnertaken when possible.

As explained in the results section, we chose MFS as the best descriptor of survival for its quantitative nature, its good classifying performance, and its parallelism with the initiation of mass mortality.

The susceptibility to mortality could not be studied following the “necrotic criteria” used to define a point of irreversible starvation established for other species (Blaxter and Hempel 1963; McGurk 1984). Instead, we defined a critical value of MFS that might severely handicap survival. Based on a “mass mortality” criterion, this value was used to delimit larvae with higher or lower probabilities of survival. We chose a critical (approximate) value of MFS of 6% (Mmax) that might be associated with the mass mortality period observed around day 17 (Fig. 2.3.9.) We then re-classified the larvae into pre and post-Mmax. According to this, ca. 96 % of all Fed larvae were classified as pre-Mmax. Of Starved larvae, ca. 69% of the larvae under 14 DAH would be classified as pre-Mmax (therefore having higher survival probabilities), and ca. 87% of Starved larvae of 14-21 DAH would be classified as post-Mmax (reflecting a worsened condition). Of all Delayed larvae, 87.5% would be classified as post-Mmax (would presumably survive accounting only for starvation data). This result corresponds well with the null mortality observed for Fed and Delayed larvae over 16 DAH (see Figure 2.1.3.).

Our results on susceptibility to mortality based on this mass mortality criterion should not be interpreted according to the existence of a true Point of no Return (PNR) *sensu* Lasker (1970). As explained in Sections 2.1. and 2.2., it is likely that European sea

bass has an extremely delayed PNR (Barnabé et al. 1976). We believe that in cases of such extended periods before PNR is reached, the experimental correlation with survival in the laboratory must be interpreted as an increased susceptibility to starvation mortality.

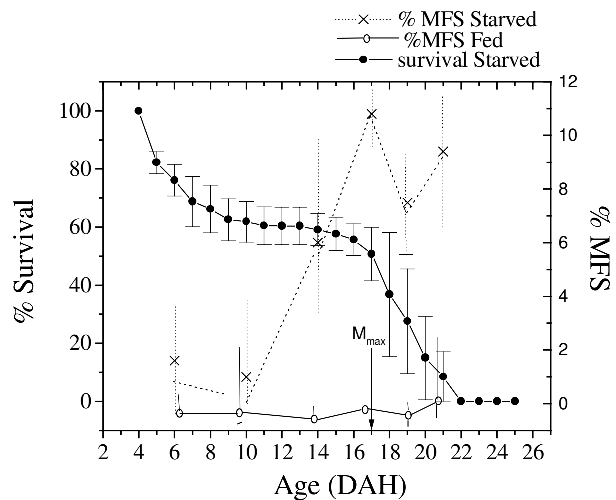

Figure 2.3.9. Relationship of MFS with survival. Double plot of MFS (Fed and Starved larvae) and mean percentage survival (Starved larvae) *vs* age. Mmax is the value of MFS over which survival is supposed to be handicapped. Survival is represented by means ± SE. MFS are medians and 25 and 75% percentiles.

If one considers the role of the body muscle in a larva, it comes clear that a severe degradation of the muscle fibres could be particularly important for survival for at least the following reasons: a) a reduction in the feeding success, b) a reduced capacity of avoiding the predators and c) a decrease in growth rate. The "growth-mortality" hypothesis, which includes several of the aforementioned mechanisms that are possibly involved, has been the object of numerous studies (see Hare and Cowen 1997 and references cited therein). The major role of muscle for these processes has been pointed out by Green and McCormick (1999). These authors also proved empirically that muscle degradation was directly related to a decreased striking success. Furthermore, according to the works on buoyancy (Neilson et al. 1986; Sclafani et al., 1993, 1997, 2000), muscle condition may be involved in the positioning of the larvae in the water column and hence in their interaction with prey and predators. The relationship of other nutritional indices (particularly those which have a short-time response to food deprivation) with survival in the wild seems less obvious.

However, it is difficult to assign a particular mortality rate to each of these mechanisms. In the field, collected larvae are usually found at different degrees of emaciation and few seem to be near an irreversible point of starvation (O'Connell 1980; Theilacker 1986). In the realm of nutritional indices, a single index is often used to determine the physiological status of a sampled larva (e.g. RNA/DNA, lipid content, etc.). If several variables are

considered (e.g. several histological variables), it is common to simply average the obtained values to describe different larval groups. As a consequence, the particular relationship between the different starvation indices and their probable relation to survival in the wild is seldom considered. We propose that indices that can be obtained from a single larva (e.g. several histological measurements) should be weighed to account for their theoretical (and empirical when available) relationship with survival. Therefore, indices that suggest that larvae will have a lowered performance or that have been experimentally correlated with high mortality rates (e.g. muscle measurements) or that imply that larval growth-rate or size will be severely handicapped for a long time (e.g. morphological indices) should be given more importance than indices that are just indicators of immediate feeding status. Figure 2.3.10. illustrates how a methodology based on this idea could be used for field-collected larvae.

As a conclusion, we propose the use of MFS as a main factor (within the variables studied herein) to discriminate nutritional condition in *D.labrax* over the oil-globule period.

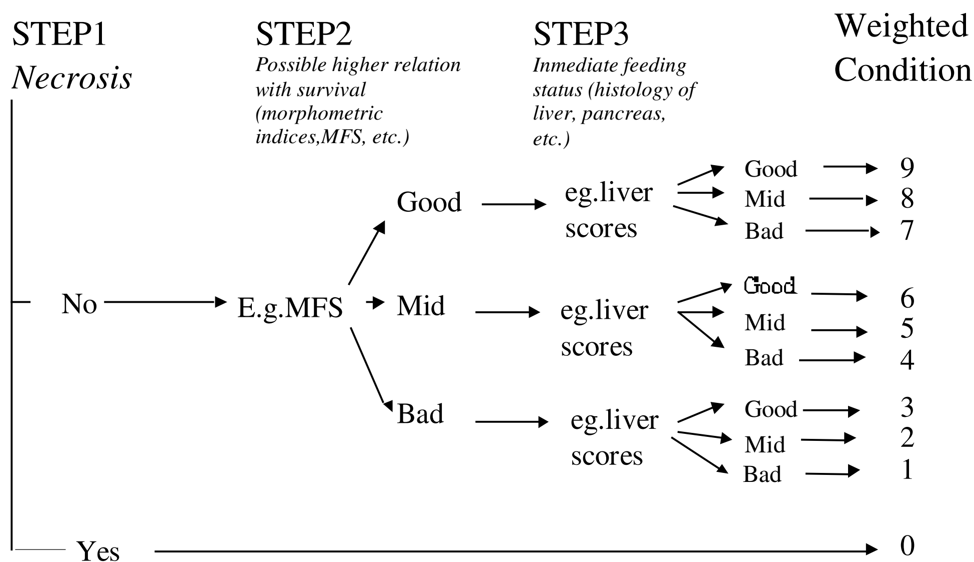

Figure 2.3.10. Proposed method for weighting measurements of nutritional condition according to their potential relationship with survival. Necrotic tissues (Step 1) are assumed to have the highest associated probability of death. Maximum weight (9) would be given to larvae that exhibit optimum values for indices that are highly related to survival (Step 2) plus high values of indices that are indicators of immediate nutritional status (Step 3).

It is a single quantitative index that is relatively easy to obtain, and it yielded one of the best classifications by feeding treatments. The combined information of survival and this index could be used to classify larvae with high or low survival probabilities. From our results, we believe that indices that are experimentally (or theoretically) more related to survival should be given more weight than others for determining the condition of field-collected larvae.

2.4. BIOCHEMICAL INDICES

ABSTRACT

The aim of this section was to compare several biochemical condition indices, i.e. RNA/DNA, protein/DNA, and the activities of 2 metabolic enzymes, Lactate dehydrogenase (LDH) and Citrate Synthase (CS) in homogenates of single *Dicentrarchus labrax* larvae subjected to varying feeding regimes. Also, the cell proliferation rates from well developed larvae was assessed in Fed larvae *vs* larvae subjected to 2 days of fast.
Two developmental events, i.e. the exhaustion of maternal reserves and notochord flexion were found to affect drastically some biochemical parameters independently of the food regime.

In pre-flexion larvae, only RNA/DNA could be tested, and revealed to be of use only in extremely emaciated larvae, well after the exhaustion of maternal reserves. An RNA/DNA value of 1.7 (± 0.62 SD) was associated with the initiation of maximum mortality rates and was considered to indicate starvation. This value is similar to that indicative of extreme starvation in larvae of other fishes. In postflexion larvae, RNA/DNA and LDH activity best encompassed nutritional condition.

Response time to food deprivation and re-feeding was longer in pre-flexion than in post-flexion larvae. For the latter, recovery time decreased with increasing starvation time. The use of metabolic enzymes revealed that LDH was a better descriptor of short-term food deprivation than CS, as confirmed for other species. In postflexion larvae, LDH/DNA could be a good way of detecting nutritional condition if applied to narrow size ranges.

Cell proliferation rates enabled the differentiation of larvae subjected to short-time fast in muscle tissue but not in brain tissue. Further methodological refinements are needed in order to use this index extensively, however our results seem promising.

The relationship between indices and their usefulness for future studies is discussed.

INTRODUCTION

Biochemical condition indices have a tradition in determining fish larval nutritional stress (Ferron and Leggett 1994, see Section 1). Within these, nucleic acid-based indices (RNA/DNA, protein /DNA etc) belong to the most widely used, whereas metabolic enzyme activities and cell proliferation rates are seldom utilised.

The objectives of this section were to test and compare several biochemical parameters, all with extensive background (Section 1) in *D. labrax* subjected to both long-term and short-term food deprivation and refeeding episodes. Also, the effect of initial delayed feeding was explored. To our knowledge, no comparative analysis of the variables measured herein has been done hitherto for this species. The tested biochemical indicators of condition were RNA/DNA, protein/DNA, the activity of 2 metabolic enzymes (lactate dehydrogenase and citrate Synthase) and cell proliferation rates. A brief explanation of each index is given below, but further references can be found in Section 1.

The RNA/DNA ratio gives a measure of the synthetic capacity of the cell and it has been used extensively in fish larvae to detect nutritional status and changes in growth rates (Ferron and Leggett 1994; Bergeron 1997; Buckley et al. 1999). Theoretically, a well nourished larva grows fast by increasing the total muscle mass. This is linked to increased RNA synthesis. While RNA contents show high variability as a response of food availability, the DNA contents per cell have a much reduced variability and is therefore a good proxy for body size. It is assumed that, during starvation, DNA contents per cell is maintained but RNA contents decrease.

Protein content is a particularly useful variable to detect emaciation in fish larvae because, unlike in other vertebrates, fish are known to quickly mobilise protein from body muscle to meet metabolic demands (Buckley 1979; Pedersen et al. 1990). Therefore, it is expected that a deficient nutrition would be reflected in a decreased protein content. Obviously, this fact is closely related to the interpretation of lowered RNA in starved larvae. Further, a reduction in protein content has important effects in the behaviour,

locomotory capacity and floatability of larvae and consequently survival rates can be severely affected by reduced protein contents (Neilson et al. 1986; Sclafani 1993). It is necessary, however, to correct the total soluble protein contents for size. Standardisation by DNA or length are two of the possible ways of accounting for size-effects.

The activities of the two metabolic enzymes selected, lactate dehydrogenase (LDH) and citrate Synthase (CS), have shown to be sensitive to emaciation in fish larvae (Clarke et al. 1992; Fiedler et al. 1998), and to be correlated with growth rates in juvenile fish (Couture et al. 1998). Nevertheless, species-specific data are still scarce. LDH is the terminal enzyme of anaerobic glycolisis in vertebrates. It is assumed that LDH activity increases proportionally to feeding rate and is correlated to anaerobic potential and locomotory performance. Citrate synthase is an important enzyme of the Krebs cycle and its activity is believed to increase as a response of feeding intensity. Increased CS activity indicates enhanced aerobic metabolism. When standardised (classically to protein contents), both enzymes can serve as an indicator of general condition of fish larvae.

The use of cell proliferation rates to study nutritional condition relies on the hypothesis that optimum feeding status promotes growth, which is in turn related to cell division rates. Studies of *in vitro* cell cultures have shown that extracellular factors such as nutrient deprivation determine that normal proliferating cells in G1 stage of the cell cycle revert to quiescence (Pardee 1989; Murray and Kirschner 1989; Alberts et al. 1994). Despite the promising results obtained using this technique to detect starvation in fish larvae (Bromhead et al. 2000; Theilacker and Shen 2001), numerous methodological problems have been found, and further research has been recommended. In this section, the cell proliferation rates were studied in brain and muscle tissue. It was anticipated that these two tissues could respond differently to short term nutrient deprivation, due to their particular developmental and physiological characteristics.

MATERIAL AND METHODS

All sampled larvae were frozen alive in liquid-nitrogen and stored at –80°C until analysed. Detailed sampling schemes and analytical methods will be described further in this section.

Marked developmental transitions, for example the shift from maternal to exogenous feeding, or urostyle flexion are known to be a threshold influencing biochemical dynamics in *D. labrax* (Bergeron and Pearson-Le Ruyet 1997). We therefore explored these possible effects before further analysis of the results. The type of feeding treatments was different, as explained in other sections, for early stage or well developed larvae.

Feeding treatments

A first group of treatments was called “long-term food deprivation experiment” and comprised larvae from 6 to 18 days after hatching (DAH). The feeding groups were as follows: “Fed” larvae that had been continuously feeding from 4 DAH, “Starved” larvae which were deprived of food since hatching, or “Delayed” larvae that were starved until 13 DAH and then fed (Table 2.4.1. A). Due to the small size of the youngest larvae, it was not possible to measure all the biochemical parameters during this period. It was decided to measure only RNA and DNA. The condition indices explored was the RNA/DNA ratio.

A second group of treatments was set for postflexion larvae from days 22 to 28 and is referred to as “short-term food deprivation experiment”. One treatment “Fed” was formed by larvae that had been continuously fed since day 4. Another set of treatments was formed by a combination of food deprivation and re-feeding days, according to the sampling scheme in Table 2.4.1. (B). During the analysed period, each larvae yielded sufficient material to quantify RNA, DNA, total soluble protein and LDH and CS enzymatic activities. The tested potential indicators of nutritional condition were

RNA/DNA, protein/DNA, protein-specific LDH and CS activity and DNA-specific LDH and CS activities.

Also, the relationship between daily growth rate and nutritional condition was explored. This was done using Fed larvae from 14 to 28 DAH. RNA/DNA was the selected index as it was the only one available for that age-span. Daily growth rate was expressed in mm/day, calculated from:

Growth rate (mm/day)=(SL(t2)-SL(t1))/t2-t1

where SL equals standard length (mm), and t1 and t2 are time in days.

For both long and short-term starvation experiments, the possible effect of size and age in condition indices was only assessed in continuously fed larvae, as this was the treatment for which the effect could not be attributed to a food deficiency.

Table 2.4.1. Number of larvae examined for biochemical measurements (except for cell proliferation rates). For the long-term starvation experiment (A), nucleic acids were analysed. For the short-term starvation experiment (B), variables measured were nucleic acids, LDH and CS activities and protein contents. In B, shaded cases indicate the day of food withdraw. (*) For protein-based measurements, only 10 points were available

A

Treatment	Days after hatching (DAH)									
	6	**8**	**10**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Fed	5	6	5	4	5	4	6	3	8	6
Starved	7	5	6	2	4	4	2	1	3	6
Delayed	-	-	-	-	-	4	3	4	4	4

B

Treatment	Days after hatching (DAH)						
	22	**23**	**24**	**25**	**26**	**27**	**28**
Fed	5	14	5	2	12	10	11
Delayed			8				
1d of fast				2			
2d of fast					16*		
2d of fast 1d refed						11	
2d of fast 2d refed							7
3d of fast						5	
3d of fast 1d refed							5

Finally, the cell proliferation rates of 26 DAH continuously fed larvae were compared to larvae of the same age subjected to 2 days of fast. Muscle and brain tissue were analysed separately in larvae sampled every 6 hours (ca. 20 larvae were sampled each hour-treatment) during a 24 hour period to analyse the short-time response of cell division. Following, the protocols used for each type of analyses are detailed.

Sample dissociation

The determination of nucleic acid, protein and enzyme activities was conducted on the same homogenates obtained from single larvae. Flow citometry analyses were obtained from separate larval batches due to methodological constraints.

The sample dissociation procedure used for all analyses (except cell proliferation rates) is schematised in Figure 2.4.1. Larvae were thaw, the SL measured and the head and gut regions excised from the rest of the body, in order to avoid interference of gut contents or tissue-specific interference in the interpretation of results. Thus, only muscle tissues were used for the analyses. The muscle portion of a single larva was homogenised using a borosilicate tissue grinder and a Teflon pestle, rotating at middle speed for 1 min, at 0-4ºC. The volume (125 to 250 µl) of grinding buffer (GB, 10 mM Tris-HCl, pH 7.2) was adapted depending upon the size of the larva. The homogenates were transferred to an eppendorf tube, well mixed and split into two aliquots for the analyses of protein and enzyme activities (200 µl) and nucleic acids (25 to 40 µl). In the case of the smallest samples ground on 125 µl GB, most homogenate (110 µl) was used for nucleic acids only (as indicated earlier). An additional chemical extraction using EDTA and sarcosyl was performed for the nucleic acid estimation. The corresponding aliquots were diluted 1:1 with 1% TSEB buffer (Tris buffer containing 1% sarcosyl and 0.5 mM EDTA). The mixture (now containing 0.5% sarcosyl) was kept at RT for 1 hour (well mixed after the first 30 min). The homogenates were kept frozen at –80ºC until the corresponding analyses were performed.

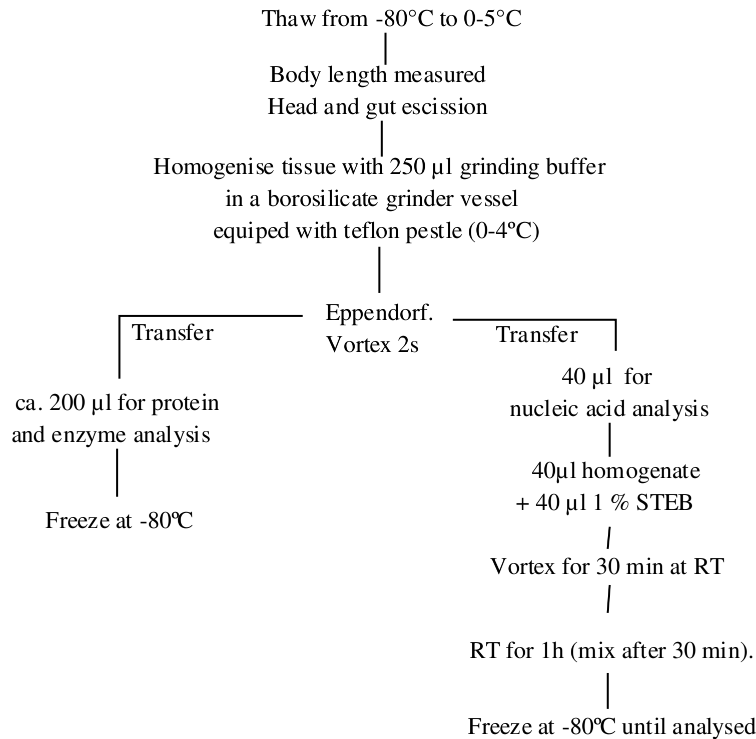

Figure 2.4.1. Procedure for the obtention of final homogenates from a single larva in order to analyse protein, enzyme and nucleic acid analyses.

The individual larvae used for cell proliferation analyses were processed on the same day as they were thawed. The specific protocol is detailed further in the text.

Quantification of nucleic acid contents

The methodology followed the procedure described by Berdalet (2002). Basically, the method uses SYBR Green II fluorochrome to estimate DNA and RNA in combination with Rnase and Dnase digestion on separate aliquots.

Briefly, the homogenate (containing 0.5% sarcosyl) was thawed and diluted down to 0.05% sarcosyl using 100 mM Tris buffer (pH 8.0, containing 100 mM NaCl, 0.9 mM

$CaCl_2$ and 0.9 mM $MgCl_2$). After centrifugation (10000 rpm, 2 min, 4ºC) three aliquots (125 to 250 µl, depending on the size of the samples) were transferred to three test tubes. An aliquot was incubated in the presence of RNase, to allow for the estimation of the DNA. A second aliquot was incubated with DNase, for the estimation of RNA. The third aliquot accounts for the blank correction of the samples. The highly sensitive fluorochrome SYBR Green II (Molecular Probes) allows the quantification of the nucleic acids. DNA Type I from calf thymus (D-3664) and RNA Type III from baker's yeast (R-7125), both from Sigma, were used as standards. The analyses were run in 1 ml assays on 100 mM Tris buffer. It is essential that the concentration of the sarcosyl in the assay was below 0.0125%. A Shimadzu RF-570 spectrofluorometer was used for the readings at 497 and 520 nm of excitation and emission wavelengths, respectively.

Quantification of total soluble protein and metabolic enzymes

Total soluble protein was analysed using the colorimetric method of the Bicinchoninic acid (BCA, Smith et al. 1985). This method was selected for its highest tolerance to common buffers and for its highest simplicity (permits a one-step analysis) and flexibility compared to other methods such as that of Lowry et al. (1951). A daily standard curve was made using bovine serum albumin (BSA, SIGMA P-0915). Standards included a blank (just buffer) and increasing concentrations of BSA, from 0.1 up to 8 mg BSA/ml. Absorbance read at 562 nm was measured using a UVIKON 923 (Bio-Tek Kontron Instruments) spectrophotometer in a total of 1200 l of assay (total Working Reagent). Due to the small amount of protein yielded by each larva, we studied the possibility of making a single reading using the whole sample rather than several replicates using smaller amounts. The test of replicability yielded coefficients of variation between 2 and 7, which were considered small. Therefore, it was decided to use a single reading of each larva using 35 l of sample supernatant. Prior to reading, homogenates were vortexed and centrifuged at 14000 r.p.m. for 1 min.

The determination of enzyme activity is based on the premise that, for a given temperature and in the presence of saturating substrate concentration, the activity (rate of

conversion of the substrate into a product) is proportional to the total amount of enzyme present in the sample. The protocol for LDH and CS determination was basically that of Childress and Somero (1979), with certain modifications (described below) to optimise the usage of reagents.

Frozen homogenates (-80°C) did not loose activity respect to fresh homogenates for at least 2 months. This was calculated by comparing a set of 15 samples. Prior to the analyses, homogenates were vortexed and centrifuged at 14000 r.p.m. for 1 min in a refrigerated (4°C) centrifuge. A refrigerated multi-cuvette carrier adapted to the spectrophotometer enabled the simultaneous reading of 3 samples at a time. Both LDH and CS activities were measured at 15°C.

For the LDH activity, the decrease in Absorbance due to the NADH oxidation was recorded at 340 nm in a medium containing 80 mM Tris/HCl (pH 7.2 at 20°C), 5 mM Na-Pyruvate, 0.15 mM NADH and 100 mM KCl. The assay reagents were prepared daily. The assay amounts were optimised for the use of pre-weighed 2 mg NADH vials (SIGMA-4730) which permitted the analysis of 16 samples (or 8 duplicates) per vial. The volume of assay per sample was 1 ml. The amount of homogenate (supernatant) varied between 25 and 40 l depending on the size of the larva. Total reading time was of 8 min per larva.

The CS assay contained 50 mM Imidazole/HCL buffer (pH 7.8 at 20 °C), 0.5 mM oxaloacetate, 0.1 mM Acetyl-CoA, 0.1 mM 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and 1.5 mM $MgCl_2$. Total assay volume was 1 ml. The AcCoA was prepared in a 1mg/ml solution to reduce weight error and kept at –80°C in 1 ml eppendorfs. Handled in this way, solutions can be used for at least 6 months (SIGMA-ALDRICH). For the CS reaction to start, it is needed that the sample (supernatant) is introduced first as it produces a background activity that must be recorded. This activity is subtracted from the enzymatic reaction that takes place once the substrate (oxaloacetate 25 l) is added, 2 min after sample addition. The CS activity was recorded for 20 min at 412 nm as the increase in Absorbance produced by the reaction of reduced Acetyl-CoA liberated from the enzymatic reaction with DTNB. The amount of sample varied between 25 and 50 l depending on the size of the larva.

The extinction coefficients utilised for calculations were 6.22 for NADH and 13.6 for DTNB. Enzymatic activity was calculated as Units (U), where 1U= 1 mol substrate

converted to product per min. Final results were presented as specific enzyme activity (Units/mg of protein or Units/µg DNA).

Flow cytometry

Samples were prepared following the general procedures for flow cytometry analyses as described by Shapiro (1988) and Vindeløv and Christensen (1994). Thawed larvae were measured (SL) and the head and gut regions were separated from the rest of the body. The tissue to be analysed, i.e. brain or muscle, was placed in 0.5 ml of a hypotonic breaking solution kept on ice (Fig. 2.4.2.). Tissues were mechanically dissociated with a 1-ml syringe and a 23-gauge needle in the breaking solution to obtain single-cell suspensions. One litre of breaking solution contained: 1 g sodium citrate, 8 g NaCl, 0.560 g PIPES, 0.372 g EDTA, 1 ml Triton X-100, 40 mg RNase (heated in boiling water for 5 minutes) and distilled water up to 1000 ml.

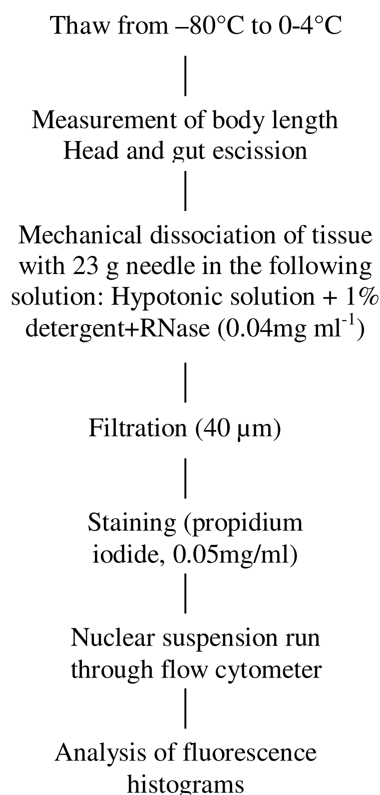

Figure 2.4.2. Schematic of the steps conducted to analyse the muscle and brain cell proliferation rates.

The cell (nuclei) suspension obtained as previously described was filtered throughout a 40 µm mesh and subsequently incubated at 37°C for 30 minutes. Nuclei staining was performed by adding Propidium Iodide solution to the cell suspension (final concentration of 50 µg/ml). Samples were kept on ice until the flow cytometric analysis, which was performed in the subsequent 2 hours. The suspension containing the stained nuclei was run throughout an Epics XL flow cytometer (Coulter Corporation, Hialeah, Florida). The instrument was set up with the standard configuration: excitation of the sample was done using a 488 nm air-cooled argon-ion laser at 15 mW power. The complex DNA-fluorochrome emits a fluorescence, which depends on the amount of DNA of each nucleus. Light emission is higher in DNA synthesising nuclei prior to division. DNA analysis on

single fluorescence histograms was done using ModFit LT software (Verity Software House, 1995). The program was used to calculate the amount of proliferating cells (stages S, G2 and M) in relation to those in stages prior to DNA synthesis (G0 and G1 stages of the cell cycle). Only those samples with coefficients of variation of the G0+G1 peak below 6 were considered useful for interpretation of the results.

Statistical analyses

The existence of significant relationships between a given variable and age or length was tested by linear regression (variables were ln-linearised when appropriate). Differences between slopes and means of the different treatments were tested by ANCOVA and ANOVA using GLM procedure of MINITAB Inc., USA, (version 12.1). Differences between biochemical parameters for given days were tested by Student's-T-test (2 treatments) or one way ANOVA (3 treatments) with post-hoc Tukey's comparison when the number of observations was over 4 for each treatment-day. The relationship with survival of the starved larvae with the RNA/DNA ratio was explored by Spearman's rank-correlation.

RESULTS

D. labrax larvae underwent two shifts in growth associated probably with the total oil globule exhaustion (around day 13) and with the notochord flexion (between days 20 and 22). This was evident in the RNA and DNA changes in continuously fed larvae (Fig. 2.4.3., Table 2.4.2.). Both RNA and DNA slopes were significantly higher in the second growth period (days 14 to 18) than in the third growth period (days 22 to 28).

The RNA/DNA ratio was fairly constant with time (no significant correlation) in both periods. This is of extreme importance as it allows for its direct use to estimate condition without further size or age standardisation.

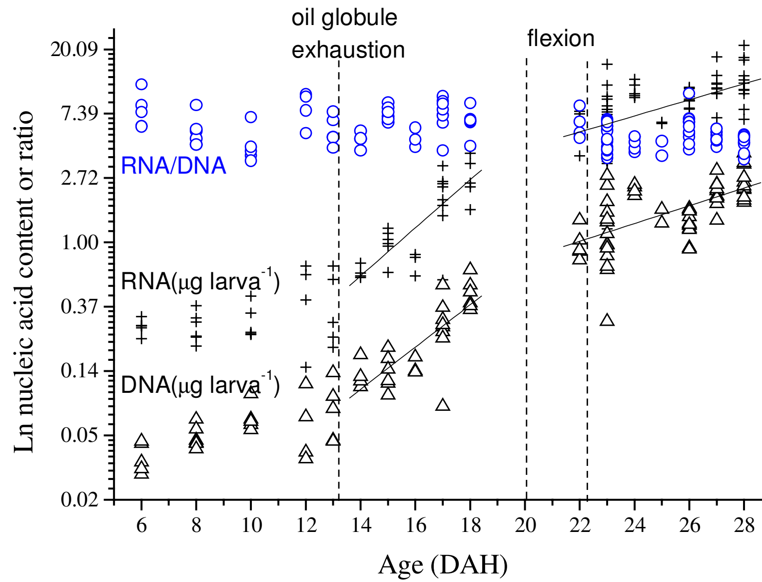

Figure 2.4.3. Variations in RNA and DNA contents per larva, and RNA/DNA ratio (all ln-transformed) in continuously fed *D.labrax* larvae along the experiment. Regression lines were only fitted if statistically significant (see Table 2.4.3.) and only over 14 DAH (when maternal reserves are exhausted).

Table 2.4.2. Regressions and comparisons of slopes of RNA, DNA and the RNA/DNA ratio with time (days) between pre and post-flexion continuously fed larvae. Only larvae over 14 DAH are considered to avoid the effects of maternal reserves. Fitted regressions lines can be found in figure 2.4.3. NS= not significant.

Dependent variable (Y)		Regression equations	r^2	N	P	Test of slope
RNA(µg larva^{-1})	Preflexion	LnY=-5.75 + 0.374 X	0.64	27	<0.0001	P<0.001
	Postflexion	Ln Y= -1.03 + 0.125 X	0.28	60	<0.0001	
DNA(µg larva^{-1})	Preflexion	LnY= -6.88 + 0.328 X	0.60	27	<0.0001	
	Postflexion	Ln Y= -3.02 + 0.138 X	0.36	60	<0.0001	P<0.001
RNA/DNA	Preflexion	Ln Y= 1.129 + 0.045 X	0.080	27	NS	
	Postflexion	LnY= 1.99 – 0.013 X	0.020	60	NS	-

"Long-term food deprivation experiment" (preflexion period)

Fed larvae exhibited a significant and exponential increase of the RNA and DNA content with age (Fig. 2.4.4., Table 2.4.3.). This increase was negligible or slow until day 12-13 and faster afterwards. Mean RNA/DNA values of Fed larvae fluctuated between 4 and 8. For these larvae RNA/DNA ratios decreased slightly from days 6 to 10. After that, the ratio did not vary significantly.

The length (SL) of Fed larvae increased significantly and linearly from day 6. Length was not significantly correlated to RNA/DNA in Fed larvae in any of the 3 developmental periods (correlation, p>0.05). This is an important result as it is the basis for its use as indicator of nutritional status in larvae of unknown age, which is often the case encountered in the field.

In Starved larvae, RNA and DNA remained fairly low through time compared to Fed larvae (Fig. 2.4.4.). RNA/DNA decreased from near 6 to ca. 2 from days 6 to days 17-18. However, its variability was considerable and significant differences respect to Fed larvae may only be real on day 12 and after day 15. The minimum RNA/DNA value of Starved larvae was observed around days 16-18 coinciding with the initiation of mass mortality (Fig. 2.4.5). The value for day 17 was of 1.7 ± 0.62 (SD). There was a significant and positive relationship between survival and RNA/DNA ratio between days 6 and 18 (Spearman's r=0.58, DF=42, p<0.0001).

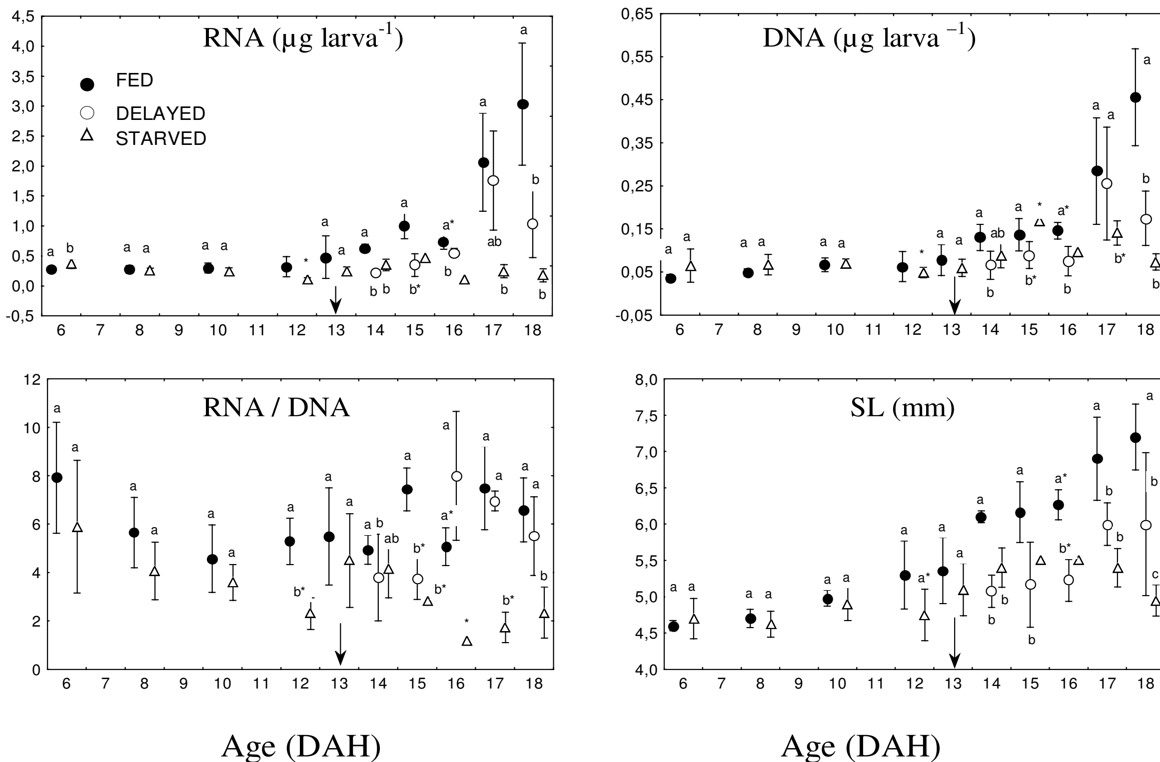

Figure 2.4.4. Variation of the mean RNA and DNA contents per larva, RNA/DNA ratio and SL (± SD) along age in larval *D. labrax* for each feeding treatment in the long-term starvation experiment. Arrow indicates the time of oil globule exhaustion in fed larvae. Different letters are significant differences at =0.05. Asteriscs indicate less than 4 observations. The relationship with length is commented in the text.

Table 2.4.3. Regression parametres for each variable *vs* age (x), for each treatment during the long-term starvation study. Only days 14 to 18 are considered (preflexion period, no dependence on maternal reserves). Only equations of significant regressions are shown. Different letters indicate significant differences in slopes or means among treatments, at = 0.05. NS= not significant.

Dependent variable (Y)	Treatments	Regression equations	R^2	P	N	Slope	Intercept/ mean
RNA(g larva^{-1})	Fed	LnY=-5.75 + 0.374 X	0.64	<0.0001	27	A	A
	Delayed	LnY= -7.67 + 0.443 X	0.58	<0.0001	17	A	B
	Starved	Y= 1.01-0.046X	0.35	<0.05	15	B	C
DNA(g larva^{-1})	Fed	LnY= -6.88 + 0.328 X	0.60	<0.0001	27	A	A
	Delayed	LnY=-6.96 + 0.299 X	0.49	<0.001	17	A	B
	Starved	NS				-	-
RNA/DNA	Fed	NS				-	-
	Delayed	Y= -4.64 + 0.620 X	0.21	<0.05	17	A	A
	Starved	Y= 10.02-0.45X	0.34	<0.05	15	B	B
SL (mm)	Fed	Y= 1.652 + 0.106 X	0.52	<0.0001	27	A	A
	Delayed	Y= 1.294 + 0.267 X	0.38	<0.01	17	A	B
	Starved	Y=7.007 – 0.106 X	0.32	<0.05	15	B	B

In the Delayed larvae, RNA and DNA contents per larva increased significantly and exponentially with time (Fig. 2.4.4.). Mean values of RNA contents, DNA contents, and SL were always significantly lower in delayed than in fed larvae. However, the rate of increase (comparison of slopes) was not significantly different between Fed and Delayed larvae (Table 2.4.3.). The RNA/DNA of Delayed larvae increased abruptly from day 15 to 16, reaching similar values to those of fed larvae (Fig. 2.4.4.). An increase in length occurred on day 17, which is one day later than the observed increase in the RNA/DNA values. Delayed larvae had similar values to starved larvae until day 17 for DNA and SL. RNA increased for Delayed larvae on day 16, which was responsible for the high increase in RNA/DNA. Therefore, for this period it took 2-3 days (latency) for

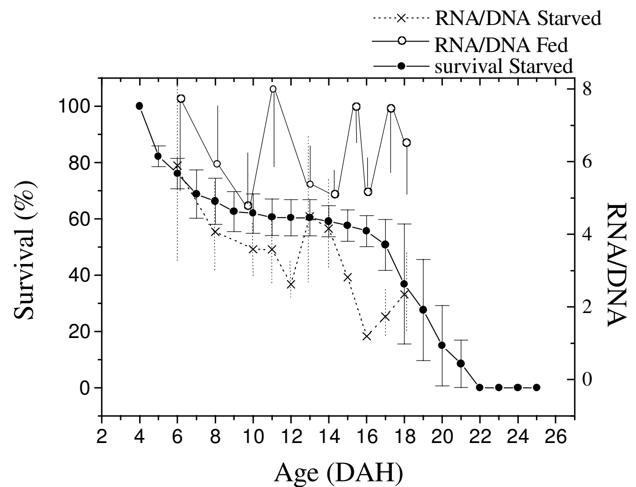

Figure 2.4.5. Relationship between mean survival of the Starved larvae and the mean RNA/DNA ratio. Fed treatment is shown for comparison. Error bars are SE for survival and 1SD for RNA/DNA ratios.

RNA/DNA to respond to a food addition, and the response was quite abrupt (dynamics of just one day).

Data of Delayed larvae after flexion (not shown) are commented in this subsection due to the relationship with the past feeding history described in this paragraph. These larvae were significantly smaller and had lower RNA and DNA contents than continuously fed larvae on day 24 (all t-tests showed $p<0.05$). However, they showed significantly higher RNA/DNA contents than Fed larvae of that age.

"Short-term starvation experiment" (post-flexion larvae)

For this section, only data of day 25 were not statistically considered as only 2 larvae from each treatment were available, and they showed high variance in length, therefore greatly influencing all the biochemical results. The size of the analysed larvae is shown in Fig. 2.4.6.

The length of continuously fed larvae (Fig. 2.4.6.) showed a slight (but significant) positive correlation with age, RNA, DNA and protein contents and total LDH and CS activities (Pearson r between 0.33 and 0.67, all $p<0.05$). The condition indices (RNA/DNA and both protein-specific or DNA-specific LDH and CS activities) showed no significant correlation with body length.

The age of Fed larvae was positively related to SL, RNA, DNA and protein contents (Pearson r between 0.33 and 0.59, $p<0.05$) but not with total LDH and CS activity. Regarding the condition indices, there was a negative correlation between age and protein specific CS activity, Prot/DNA, DNA-specific CS activity, and DNA-specific LDH activity (Pearson's r values between –0.28 and –0.45, all $p<0.05$).

Larvae subjected to 1, 2, or 3 days of fast exhibited a general decrease (except for day 25 in some cases, for the reasons explained above) in all biochemical contents (raw variables, Fig 2.4.7. A-C; Fig. 2.4.8. A, B) and condition indices (ratios, Fig. 2.4.7. D, E; Fig. 2.4.8. C-F). The decrease in the raw biochemical values can be explained by a growth-

inhibition, which was evidenced by a lowered mean body length of short-term starved and refed animals from days 26 to 28 (Figure 2.4.6.). This was probably accompanied by a loss of body mass, as suggested by the decrease in total soluble protein (Fig. 2.4.7. B).

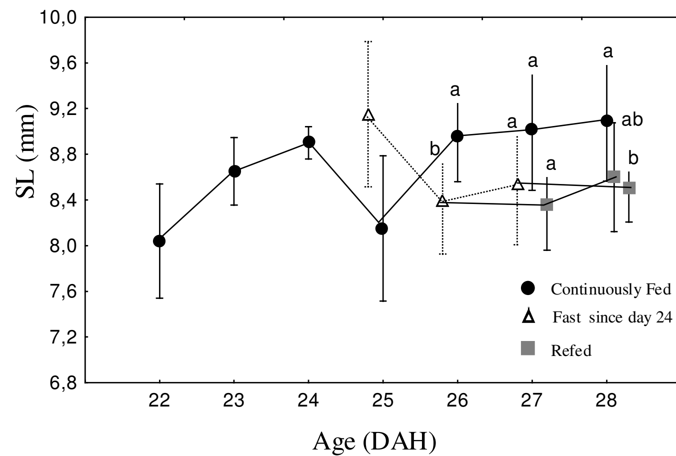

Figure 2.4.6. Mean size variation (SL in mm ± SD) with age of the larvae continuously Fed, subjected to 1 to 3 days of fast since day 24 or refed after 2 or 3 days of fast, corresponding to the larvae of the short-term starvation experiment. For each day, different letters indicate significant differences between treatments.

From all condition indices, RNA/DNA was the one that best described the starvation process (Fig. 2.4.7. E). Short-term starved larvae experienced a continuous decline in the RNA/DNA ratios until day 27 and significant differences with regard to Fed larvae were detected 2 days after food deprivation. Other indices also reflected a significant lowered condition after 2 starvation days (DNA-specific LDH and DNA-specific CS activities, Fig. 2.4.8. E, F). It was therefore proved that these measurements were also a reflection of condition, although the response was less uniform than that of RNA/DNA. For example, DNA-specific CS activity underwent an unexpected increase after 3 starvation days. Regarding the protein-based condition index, it showed a delayed response (significant differences detected only on day 27, after 3 starvation days, Fig. 2.4.7. D). Protein-specific metabolic enzyme activities (Fig. 2.4.8. C, D) did not yield significant differences between Fed and Starved larvae. However, mean values tended to be below those of Fed larvae in the case of protein-specific LDH activity, which indicates that it could be a potential index of condition if sample size was increased.

The re-introduction of food to larvae starved for 2 or 3 days caused a response in biochemical contents and condition indices. In general, length was not responsive to the refeeding scheme (Fig. 2.4.6.). In the case of biochemical contents (not indices), food that was supplied to larvae that had been starved for 2 or 3 days caused a recovery in total RNA,

DNA, total soluble protein and LDH activity. However, it is difficult to make statements about the rate of increase due to the data variability. From crossed t-test comparisons between days, RNA was the variable that responded faster to refeeding. After just one day, RNA contents of refed larvae were significantly higher than those of starved larvae from the previous day (T-test, $p<0.05$) (Fig. 2.4.7.A).

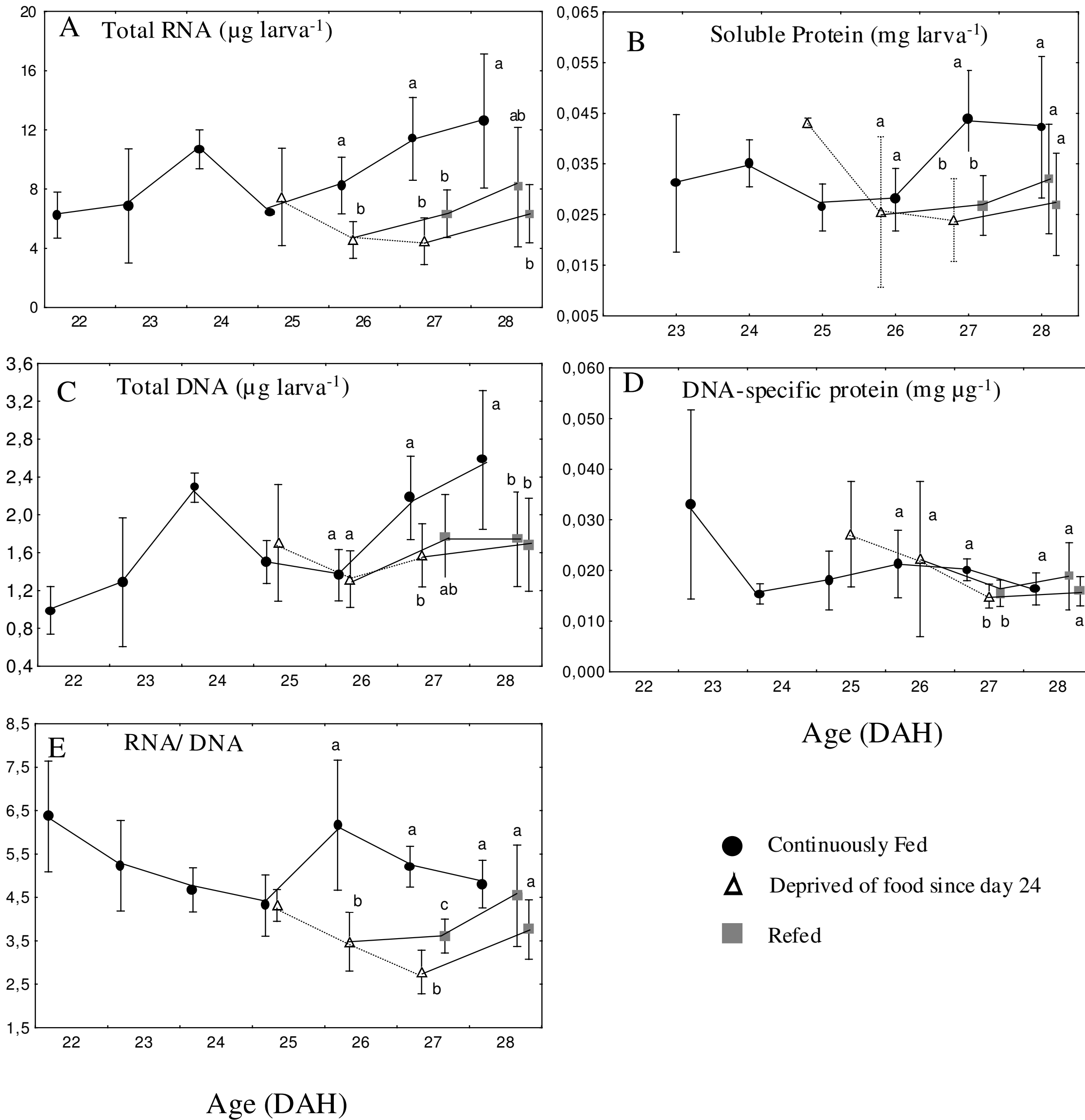

Figure 2.4.7. Time-variation of mean RNA contents (A), total soluble protein (B), DNA contents (C), DNA.specific protein contents (D) and RNA/DNA ratio (E) along the short-term starvation period. Error bars are 1 SD. Significant differences of within-day comparison between individuals continuosly Fed,

Regarding the condition indices, RNA/DNA offered the clearest recovery response (Fig. 2.4.7. E). One day of refeeding provoked a faster response in 3d starved larvae than in 2d starved larvae. Other condition indices either responded slowly to refeeding (DNA-specific protein or protein-specific LDH activity) or did not respond at all .

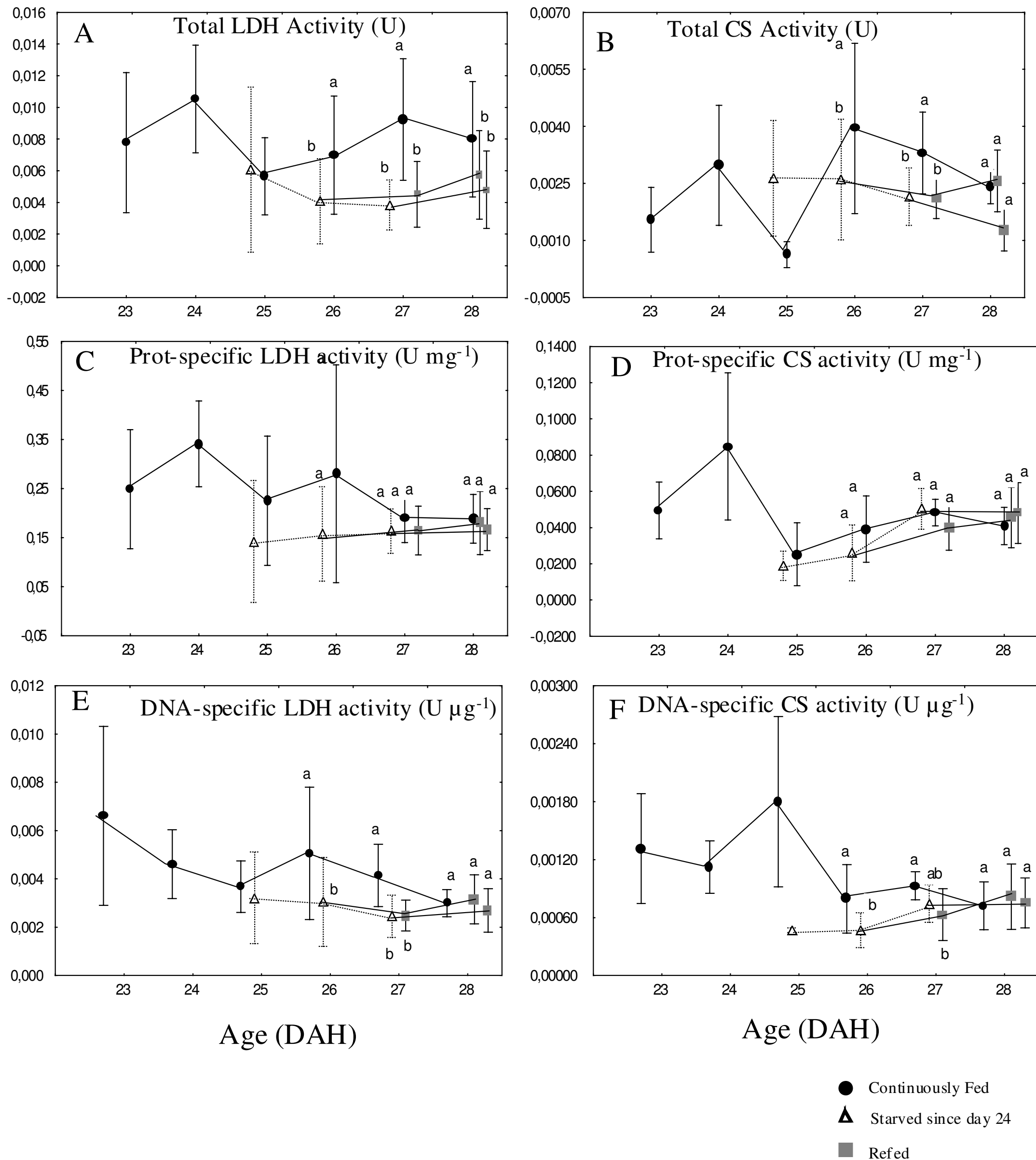

Figure 2.4.8. Time-variation of mean total (A,B) or specific metabolic enzyme activities (C-F) in the short-term starvation experiment. Error bars are 1 SD. Significant differences of within-day comparison between individuals continuosly Fed, deprived of food since day 24 and refed are indicated by different letters (a,b) at = 0.05. Comparisons were made only when n >5 for each treatment. Student's T-test was used when there were 2 groups, or ANOVA when there were 3 groups.

There was no significant correlation between RNA/DNA ratios and daily growth in length (mm/day) in Fed larvae from days 14 to 28.

Flow cytometry analysis was used to quantify the percentage of growing cells in twenty six day old Fed larvae compared to larvae of the same age subjected to two days of fast. The body length of the analysed larvae measured from 8.5 to 10.6 mm (data not shown). The histograms obtained using brain cells had smaller coefficients of variation (CV) than those of muscle tissues, which presented a higher proportion of debris. Of all data, only a small fraction (around 30%) of the analysed larvae yielded interpretable results.

The proliferation rates estimated in muscle cells of Fed larvae ranged from 12.3 to 23.0% (Fig. 2.4.9.). For this treatment, there were no significant differences among the values obtained at the different hours sampled. In larvae subjected to two days of fast, the proliferation rates of muscle cells ranged from 7.8 to 10.1%.

Pooled data of the different hours showed that larvae subjected to two days of fast, exhibited mean cell proliferation rates in muscle tissues significantly lower than that obtained on Fed larvae (Table 2.4.4.).

In the case of brain, the cell proliferation rates ranged from 8.9 to 19.1% on Fed larvae and from 6.2 to 17.8% on larvae subjected to two days of fast (Fig. 2.4.9.). The Brain tissue showed no significant differences within treatments related to the time of sampling.

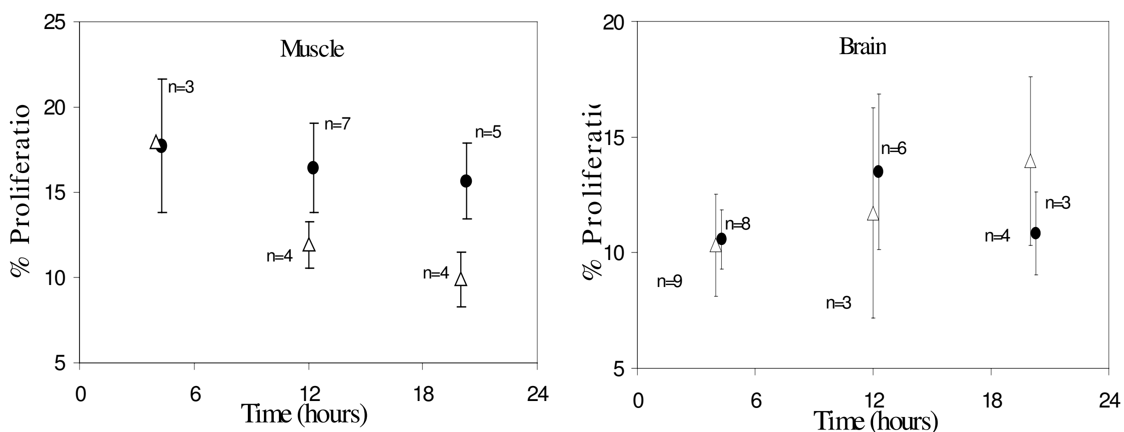

Figure 2.4.9. Mean cell proliferation rates of muscle and brain of *Dicentrarchus labrax* larvae along sampled at 8 hours intervals during day 26 after hatching. Bars indicate 95% confidence intervals.

Considering the whole data set, the mean proliferation rates of brain cells in the continuously fed larvae were not significantly different from that of the larvae subjected to two days of fast (Table 2.4.4.).

Table 2.4.4. Percentage proliferation rates of muscle and brain in larvae of the Fed and two days of Fast treatments.

		Mean	SD	N	T-value	df	P
Muscle	Fed	16.4	3.1	15	3.71	17	<0.01
	Starved	11.7	3.0	9			
Brain	Fed	11.7	2.9	17	0.14	28	0.89
	Starved	11.5	3.7	16			

DISCUSSION

The main goal of this section was to compare several biochemical indices of condition in sea bass larvae obtained from a single culture. In the coming section 2.5., these results will be compared to morphological and histological indices developed in previous sections. Also, the results presented herein serve as reference values of the response to food deprivation of several biochemical indices which had not been previously tested in a comparative framework, it is, in larvae obtained from a single controlled rearing system.

For sea bass larvae, some work on this direction was conducted by Bergeron and Person-Le Ruyet (1997). These authors successfully used DNA/C ratios to detect short-time starvation episodes. They found, as in the present study, that three developmental periods were reflected in larval DNA contents. Further, we found that these changes are reflected on the RNA, but not on the RNA/DNA contents. These changes in growth dynamics of fed larvae are defined by the exhaustion of the maternal reserves (around day 13) and by the notochord flexion (around days 20-22 in the present study). The observed changes were abrupt confirming the theory of saltatory ontogeny of Balon (1984). Other authors have confirmed these findings in sea bass through several methodologies, including otolith microstructure readings (Gutierrez and Morales-Nin 1986). It is therefore of extreme importance to have information on species-specific developmental characteristics in the

study of condition indices (although only RNA and DNA were studied during the two crucial shifts in growth, it is likely that any condition index will be altered, see Ferron and Leggett (1994)), before any extrapolation to field studies is made.

Long-term food deprivation experiment

The condition index analysed in this period was RNA/DNA measured in muscle tissue. The methodology utilised based in the method of Berdalet (2002) permitted the separate determination of RNA and DNA in individual larvae of sizes down to 4.5 mm using a highly sensitive fluorometric technique. Fluorimetric assays are very sensitive to procedural details and to the choice of standards (Caldarone and Buckley 1991; Clemmensen 1993). Nevertheless, these techniques are preferred as they are more sensitive, more rapid and equally precise than older UV-based methods. However, the particular variability encountered with highly sensitive fluorochromes claims for intercalibration studies among methods (Buckley et al. 1999). Extensive discussion of pros and cons of different methodologies used to determine nucleic acid contents in fish are available in Ferron and Leggett (1994), Bergeron (1997) and Buckley et al. (1999). The method used here improves the methods used by other authors because of its high sensitivity (about 100 times over Ethidium Bromide) and the independence of RNA from DNA estimations. The latter aspect is essential when dealing with small biomasses. In the previous methods, RNA was estimated from the subtraction of the DNA fluorescence from the Total (RNA+DNA) fluorescence. This caused mathematical artefacts when the RNA and DNA concentrations were close to the detection limit.

During the long-term food deprivation experiment (days 6 to 18), RNA/DNA was dependent on developmental stage and treatment. Size was not found to be a problem for the use of RNA/DNA as a condition index. During the period of maternal-reserves dependence (here until day 13), RNA/DNA was insensitive to feeding regime as noticed by other authors in several species (Clemmensen 1989; Bergeron ad Boulhic 1994; Clemmensen 1996; Houlihan et al. 1995). Fed and Starved larvae increased in length but showed smaller RNA/DNA values (Fig. 2.4.4.).

The initial drop in RNA/DNA has also been observed in larvae of several species regardless feeding treatment (Clemmensen 1987; Clemmensen and Doan 1996) and it is probably a reflection of a lose in protein and body mass, as confirmed by several works (eg. Brightman et al. 1997). Reasons for this may be related to allometric organ development. The fact that only muscle was analysed could account for this effect, as RNA could be synthesised from maternal reserves and used either as maintenance energy or to launch fully functional digestive apparatus (McGurk a 1985). It is therefore confirmed for sea bass that the utilisation of RNA/DNA to detect feeding condition should be avoided in the presence of maternal reserves.

After the oil globule resorption, RNA, DNA, and length of Fed larvae increased and were highly intercorrelated, following the usual pattern observed for several species (Folkvord 1996).

During days 14 to 18, RNA/DNA met the requirements to be used as an indicator of feeding status, as it was independent of size and age. Despite the relative low number of observations, Starved larvae could probably be distinguished from Fed larvae from day 15 (Fig. 2.4.4.). The long time required to observe such difference is well explained by the long-time dependence of sea bass from maternal reserves and their high resistance to starvation (see discussion in Sections 2.1. and 2.4). However, the increase in RNA/DNA of Starved larvae observed on days 13 and 14 is of difficult explanation. We hypothesise that right after oil globule resorption, a mobilisation of energy from muscle tissue to meet metabolic demands could be translated into a punctual increased RNA content, which would cease in only 2 days.

The values indicative of extreme starvation were of 1.7 ± 0.62 and were detected around the day of the initiation of mass mortality (see Fig. 2.4.1.). We believe that this minimum RNA/DNA value can be associated with the “point of irreversible starvation” or “point of no return” (PNR), which has an inherent maximum associated probability of death. The positive correlation with survival further confirms this hypothesis. However, this correlation should be interpreted with caution as, until day 13, values of the RNA/DNA ratio also decreased for Fed larvae, which then increased. Our value of 1.7 is slightly higher than the value found by Clemmensen et al. (1994) (minimum RNA/DNA=1) as indicative of PNR for other fish species. Reasons for this can be due to methodological differences or

may be species-specific. The decrease in length of extremely emaciated larvae of day 18 (Fig. 2.4.4.) is a further indication of the poor condition of these larvae. These length reduction could have been caused by a loss of osmoregulation observed in moribund larvae (Sclafani 1993).

It should be noted, however, that the RNA/DNA value of 1.7 is not the one that best classifies the different feeding treatments. If we consider only larvae after oil globule resorption (from days 14 to 18), the RNA/DNA value assigning the highest number of larvae to their correct treatment was of 4. This value classified correctly 100% of Fed larvae and 80% of Starved larvae. Of Delayed larvae, 71% would be missclassified as Fed, and 29% as Starved. These values will be used for comparison with other condition measurements in Section 2.5., for which criteria of maximum classification scores are also used.

Delayed larvae took between 2 and 3 days to recover to “normal” RNA/DNA values. This recovery was abrupt and took place in just one day. We think that some latent adaptation to feeding status was necessary due to the previous prolonged starvation time.

Overall, RNA/DNA was a useful condition index to distinguish Fed from Starved larvae 3 days after oil globule resorption. It was independent of size and its minimum values corresponded well with the initiation of mass mortality. Delayed larvae took 3 days to respond to food supply and responded in only one day. The relationship of RNA/DNA with survival was clear, as the lowest RNA/DNA values were reached coinciding with the mass mortality event.

Short-term food deprivation experiment

In general, the RNA/DNA ratio was the biochemical index that best responded to a short-time starvation and refeeding treatment (Fig. 2.4.7. E). The time response to food deprivation was 2 days, and continued dropping sharply after 3 days of starvation. This time response is one day faster than in the long-term starvation experiment. Several authors have proved that older larvae with well developed musculature respond faster to food

deprivation in terms of RNA decrease (Bisbal and Bengston 1995; Rooker and Holt 1996). This is so because at advanced developmental stages, the dynamics of RNA in muscle is directly dependent on feeding status, and the variation of ribosomal RNA (the bulk of RNA) increases with higher total muscle biomass. In particular, sea bass suffers a strong development of musculature after flexion (see Barnabé et al. 1976 for a picture of this phenomenon). The time to recovery of the RNA/DNA ratio was faster if larvae had been starved for 3 days than if they had been starved for 2 days. Three day starved larvae refed for 1 day recovered 1 RNA/DNA unit, whereas, 2 day starved larvae refed for 1 day recovered only 0,1 RNA/DNA units (Figure 2.4.7. E). This agrees well with the scheme of latency and dynamics proposed by Ferron and Leggett (1994) for biochemical indices, by which the longer the starvation time, the faster the recovery time. The minimum RNA/DNA values observed for these period (around 2.5) do not necessarily imply an increased starvation mortality. It is crucial to bare in mind that the relationship of minimum RNA/DNA values (like the one obtained for the long-time starvation experiment) and survival are dependent on larval developmental stage. It is therefore unlikely that a well developed larvae over the flexion stage with RNA/DNA values even close to 1.7 were close to a critical period. Due to this developmental effects on RNA/DNA ratios, several authors strongly recommend that only narrow size ranges of the same species collected at similar temperatures should be compared in field studies (Wang et al. 1993).

The lack of correlation between daily growth rates (in length units/time) and RNA/DNA ratios in Fed larvae was probably caused by individual RNA/DNA variability and by the low number of data points.

The protein-based condition indices analysed in this work showed lower resolution than the RNA/DNA ratios. Total soluble protein varied consistently with size and feeding treatment, but short-term Starved larvae showed high variability on day 26 (Fig. 2.4.6. B). High variability in protein as a descriptor of condition has been previously described (e.g. Bisbal and Bengston 1995). In the present work an standardisation by DNA was attempted (Fig. 2.4.7. D). Protein standardisation by DNA should give an idea of increased muscle cell size in well fed larvae if hypertrophic growth occurred. However, only for day 27 differences between fed and short-term starved larvae were evident. It is plausible that

growth was hyperplastic still at that age. In fact, Veggetti et al. (1990) showed that hyperplasic growth occurred in sea bass until 28 DAH. Standardisation by length (not shown) was of little use because when length is placed in the denominator total numbers varied little (length changes were small between days). However, if differences in length were large and could be accurately determined in field samples, protein/length ratios could be useful to detect starvation. Several authors have shown that protein is quickly mobilised in the absence of food or at low food rations, which can be used to detect starvation (Pedersen et al. 1990). It is anticipated, though, that protein variation will be of more use at early stages than in larvae with well developed musculature. This is so because early larvae tend to use protein quickly in the absence of food (up to 45% was lost in cod larvae of 2-11 days old, Buckley 1979). Older larvae may use up liver glycogen and lipids for longer before degrading muscle protein. Also, protein utilisation in early larvae is accompanied by increased water content and lose of buoyancy (Neilson et al. 1986; Sclafani 1993). This has been shown to vary the position of starved larvae in the water column and may greatly affect survival. Histological observations and field-experiments further confirm this hypothesis and will be further discussed.

To our knowledge, there are no studies on the response of metabolic enzymes to food deprivation in larval *D. labrax*. LDH activity is thought to vary with the capacity for anaerobic catabolism, whereas CS is thought to be indicative of feeding-induced capacity of aerobic catabolism (Sullivan and Somero 1980). The activity of both enzymes is strongly dependent on experimental and developmental characteristics. Within the former, temperature will decisively determine the enzyme kinetics. The strict control of the assay temperature suggests that this source of variability did not affect significantly our results. Developmental variability, however, is a major concern in the use of metabolic enzymes as indicators of condition. On one hand, there is the size-scaling phenomenon. Size-scaling has been observed in metabolic enzymes repeatedly and implies that, for a unit mass (eg. protein) the enzymatic activity increases (LDH) or decreases (CS) as a function of body size independently of feeding regime (Somero and Childress 1980; Torres and Somero 1988; Overnell and Batty 2000, but see Somero and Childress (1980) for a violation of this general law in fish). Size scaling in metabolic enzyme activity is related to oxygen

consumption rates. Younger larvae are thought to be more aerobic dependent, absorbing oxygen through all the body surface (Batty 1984) whereas older individuals have the glycolitic (anaerobic) pathways potentiated. These changes may have an adaptive value, as larger individuals tend to be "sprinters" whereas young larvae are "stayers" in their feeding behaviour. Another developmental cause of variability is related to changes in muscle growth patterns and types along development (Stoiber et al. 1999). Indeed, the changes in red to white muscle fibre types have associated changes in enzyme contents and isoforms. As a consequence of this, direct comparison of metabolic enzyme activities between larvae must be restricted to narrow size-ranges (Brightman et al. 1997).

The latter notwithstanding, LDH and CS have been successfully used to detect starvation in fish larvae, both in the laboratory and in the field. As an example, LDH has been found to be positively correlated with food quantity and quality. In fish larvae of *Sciaenops ocellatus* and *Lutjanus synagris* (Clarke et al. 1992) and negatively associated to swimming activity under suboptimal feeding conditions (Fiedler et al. 1998).

Our results showed that these metabolic enzymes are indicative of poorer feeding status in sea bass larvae. LDH activity (both total and standardised by protein or DNA) showed a better response to short-time food deprivation and refeeding than CS (Fig. 2.4.8. C-F). Standardisation by DNA gave higher discriminatory results than classical standardisation by total soluble protein (reduced within-treatment variance). Only DNA-specific LDH activity of short-term starved larvae decreased with starvation time and enabled a significant separation of Fed larvae from larvae starved for 2 or 3 days. Reasons for the higher between-treatment discrimination if standardised by DNA as opposed to protein may be due to the smaller variability of DNA. Also, the use of DNA in the denominator may reduce the effects of the size scaling phenomenon. These hypothesis should guarantee further research.

In view of the evidence presented herein, we propose the use of the ratio LDH/DNA as a potentially useful indicator of condition.

CS activity showed high variability and only when standardised by DNA some differences between treatments became significant. It is known that CS may not respond to food regime as well as LDH in fish larvae (Clarke et al. 1992). This lower response of CS

has been explained on the grounds of the more conservative nature of CS, which is needed for basal metabolic processes in the cell.

The negative correlation between specific enzyme activities and age of the Fed larvae could be explained by size-scaling for CS. For LDH, this relationship is difficult to explain. However, size-scaling studies usually include a higher length range.

To the light of the results presented, LDH activity is regarded as a potentially useful indicator of condition in sea bass. However, its dynamics should be further explored along a wider age span and feeding regimes. Respect to the other indices tested, it was the most reliable after RNA/DNA and showed a comparable response time.

The last part of this section showed that it is possible to detect short term food deprivation on sea bass larvae using the flow cytometric estimation of the muscle cells proliferation rates. In contrast, this methodology revealed that brain cells remained insensitive to the applied experimental treatment. The obtained trend should be related to the physiological role that each tissue plays on the whole organism rather than to methodological aspects.

As it was noted above, brain cells samples had a smaller presence of debris, which resulted in smaller coefficients of variation than the muscle samples. The finding that brain cells proliferation rates of larvae subjected to two-days of fast did not differ from that of Fed larvae, suggests that brain cells may obtain nutrients from body reserves, probably muscle, at least during a short period of (nutritional) stress. This hypothesis agrees with several histological observations that indicate that gut-associated glands and muscle reflect the food deprivation earlier than the brain (O'Connell 1976; Theilacker 1978; Martin and Malloy 1980). Also, there is evidence that biochemical parameters are least affected in the brain tissue (Yang and Somero 1993). This statement, however, does not discard that long term food deprivation could also affect brain cells proliferation rates as observed in other studies (Theilacker and Shen 1993 a, b; 2001). However, the usefulness of brain indicators to detect suboptimally fed larvae in the field is under question, particularly because few extremely starved larvae are usually identified in plankton samples (Theilacker 1986).

Fish muscle growth patterns are a combination of processes of fibre recruitment (hyperplasia) and fibre enlargement (hypertrophy) (Weatherley 1990; Johnston 1999). The

usefulness of cell proliferation rates as a measure of growth and condition requires a coupling between tissue growth and cell proliferation. However, it must be considered that muscle-generating (myosatellite) cells become more and more diluted over an increasing number of amitotic muscle-fibres nuclei with age (Koumans et al. 1991). Therefore, the use of the cell proliferation rate in muscle must be restricted to individuals of the same developmental stage. Considering that the time period of our experiment was of only two days, and that the growth in sea bass larvae is hyperplasic during the first 28 days (Veggetti et al. 1990), we think that the observed differences in cell proliferation rates between treatments can be explained by differences in nutrient availability.

The present results on cell proliferation rates add to the recent studies, using different methodologies, that regard larval muscle as a suitable tissue to asses the growth response to environmental factors as food shortage (Akster et al. 1995; Theilacker and Shen 2001) or temperature (Johnston et al. 1995; Temple et al. 2000).

2.5. COMPARISON OF INDICES AND DISCUSSION

The aim of this section is to compare the best performing condition indices in the two main experiments (called long and short-term starvation experiments) measured in *D. labrax* larvae at three organisation levels: morphometric, histological and biochemical. Whereas information on the response of some indices is available for this species, there is no comparative study on larvae obtained from a single culture. Studies of this type are valuable because they allow one to safely compare indices whilst reducing the variability due to different rearing systems, conditions or larval origin. It is also a goal of this section to briefly discuss the main results obtained in this experimental part.

Rearing system

We are confident that the small-scale closed recirculation rearing system, which served as an incubator and hatchery (Section 2.1.), provided quality data for the analysis of condition. Its design permitted the accurate regulation of rearing parameters and produced values that were equal to or higher than those found in other studies on sea bass in terms of hatching and survival (Barnabé 1976; Barahona-Fernandes 1978; Johnson and Katavic 1986; Oyen 1997), growth rates (Regner and Dulcic 1994; Bergeron et al. 1997) and timing of major developmental events (Gutiérrez and Morales-Nin 1986; Klaoudatos et al. 1987; Bergeron and Person-Le Ruyet 1997; Koumondouros et al. 2001). Also, the recirculation design allowed for the maintenance of levels of toxic metabolites under the threshold cited as being responsible for mortality or decreased feeding ability in fish larvae (EPA 1989).

Selection of condition indices to be compared

All condition measurements reflected the feeding regimes in the two main experiments. Although to different extents. For simplicity, we attempted to select a single index from each category (morphological, histological or biochemical) for making between-categories comparisons. The main points explored in order to make this selections

were their independence of age, size and development, which are a major problem as they tend to confuse the differences due to feeding (Ferron and Leggett 1994). These relationships were explored in continuously fed larvae in order to select the indices that best described condition independently of developmental processes. Most indices were affected at some point by pre-determined growth phases associated with the yolk absorption (7 DAH) and end of mixed feeding period (13 DAH). Other criteria used within each category in order to choose a particular index were its relationship with survival and its classification properties.

Morphometric analyses: Morphological characteristics are easy to obtain, inexpensive and have low processing time. The main drawback in the study of nutritional condition through morphometry in fish larvae are the extreme effect of size (more than age) on all body measurements. Standardisation is difficult due to allometric processes and has been attempted through the use of ratios and residuals, which are still correlated with size (McGurk 1985 a; Suthers 1996). We showed in Section 2.2. that is was possible to totally remove the effects of size by standardising each variable to a common size whilst conserving the allometric information (Lleonart et al. 2000), which is crucial for the definition of individual shape. Here, we used the scores of the first canonical root performed on standardised (size-free) BDA (body depth at anus level), HL (head length) and PAL (preanal length) as the best indicator of condition because it was not correlated with size, it showed the highest correlation with survival of all morphometric variables and it permitted the highest percentage of correct classification into feeding treatments.

Despite the lack of correlation with size, the shape was correlated with developmental stage and followed a U-shape in Fed larvae (Fig. 2.5.1.A). Shape of Fed larvae under 14 DAH was based on relatively large heads, long guts and thin muscle, and was associated with a slow growth in length (Section 2.1.). This period of steady TL and developing structures for maximising prey capture and digestion may be an adaptation to the mixed feeding period (Paine and Balon 1985, Klaoudatos et al. 1987; Koumoundouros 2001) and supports the hypothesis that success at exogeonus feeding is a developmental priority, which also fits the Critical Period hypothesis (Hjort 1914). After complete oil globule resorption and until 21 DAH, the shape of Fed larvae became relatively deep-

muscled and short-intestine, and was associated with increased growth rates, thus reinforcing growth in length, which would agree with a stage-duration hypothesis (Cushing 1975). Therefore, though size effects can successfully be removed, shape is strongly influenced by developmental stanzas and effects of feeding regimes are only likely to be detected in the absence of maternal reserves.

Hystological analyses: Age, size and development affected some of the histological condition indices. During the long-term starvation experiments, continuously Fed larvae had developing hepatocytes until 14 DAH. Also, pancreas scores (but not % zymogen) reflected a developmental effect, as it was at its maximum until 14 DAH, decreasing thereafter. Hindgut presented high acidophilic activity at yolk-sac stage, decreasing until exogenous feeding.

These ontogenetic effects on the digestive tract and associated organs have previously been described in species including sea bass (Barnabé 1976, Oozeki et al. 1989, García Hernández et al. 2001) and are usually related to the increase in both cell size and complexity during early development (Govoni et al. 1986). The latter agrees well with the little growth in length observed until 14 DAH and further suggests that energy during this period (obtained from both internal and external sources) was primarily invested in the development of the digestive apparatus. During this period, cartilage and muscle (the "structural indices") of Fed larvae showed no variation with age or size. In the growth phase from complete exogenous feeding until near flexion (14 to 21 DAH), evident developmental changes (the aforementioned increased growth rates) in Fed larvae exerted an effect on gut and gut-associated glands (high variability was detected), probably reflecting variability in individual feeding rates. On the other hand, the percentage muscle fibre separation (MFS) reflected an almost invariant "healthy" state.

The short-term starvation experiment was characterised by a high histological variability in all treatments including Fed larvae. From all indices, only MFS permitted a significant differentiation between Fed and short-term starved larvae.

We proved that not all histological quantitative indices behave better than scores (eg. liver measurements) but that some (MFS vs muscle scores) do. Regarding the time responses of the different measures, it is difficult to make comparisons between qualitative

and quantitative variables, as the statistical significance is affected by the nature of the measurement. Martin and Wright (1987) showed that the last tissues to be degraded were the first to recover. However, this statement, generalised by Ferron and Leggett (1994), is severely influenced by the developmental stage, as less developed larvae are more prone to starvation effects than more developed larvae (Sieg 1992), so for young larvae the ability to test this hypothesis needs high sampling resolution (greater than our 2-4 days sampling interval).

For the two experimental periods (long and short-term starvation experiments), we selected the MFS as the best histological descriptor of suboptimal feeding. It was unrelated to size or age for Fed larvae, responded as quickly as or faster than traditional short-term condition indices like liver (probably due to its quantitative nature, which reduces the inherent higher variability of discrete scores) and needed little expertise in histological interpretation.

Moreover, it offered the best classification success among the histological variables measured when used singly, and around the same when several variables were grouped through discriminant analysis. Further, it showed one of the best correlations with survival among the variables examined. From a physiological point of view, muscle degradation may be a better indicator of deficient condition than other histological indices as it tends to be degraded only when glycogen or lipids have been depleted (Love 1970; Pedersen 1990) thus potentially indicating a severe emaciation process. The later may have particularly important ecological consequences that will be discussed at the end of this section.

Biochemical analyses: the RNA/DNA was selected as the single best biochemical descriptor of condition from both short and long-term starvation experiments. The method utilised for nucleic acid determination (Berdalet 2002) improved other existing methods as it was based on a highly sensitive fluorimetric technique (around 100 times more sensitive than Ethidium Bromide-based methods), which is particularly useful when dealing with small biomasses. The RNA/DNA ratio of continuously fed larvae was uncorrelated with size throughout the experimental period, and a slight negative correlation with age was observed only for days 6 to 10. The lack of correlation with size for this species is a valuable property of this index if it is to be applied for field studies.

The initial decrease that we observed in RNA/DNA during the first days of the long-term starvation experiment (preflexion period) has been previously described in fish larvae (Clemmensen 1987; Clemmensen and Doan 1996) and is independent of feeding treatment. As in our study the RNA/DNA was measured just in muscle, its initial decline in Fed and Starved larvae was a biased picture of development, which was preferentially occurring in gut and associated glands. Therefore, for this species the RNA/DNA should not be used at ages in which development is not primarily based on muscle growth.

From the short-term starvation experiment RNA/DNA yielded similar results to the use of LDH/DNA (the numerator being lactate dehydrogenase, an anaerobic metabolic enzyme) except for the dynamics of degradation through starvation, which was steeper for RNA/DNA. However, the potential for LDH/DNA was clear and deserves further research. Other biochemical condition indices like specific CS activity (terminal enzyme of the Krebs cycle) or protein/DNA, although reflecting condition (Clarke et al. 1992; García et al. 1997; Fiedler et al. 1998; present study), were not selected for comparison because they showed less clear results than RNA/DNA. The use of muscle cell proliferation rates, studied through flow cytometry, enabled the differentiation of Fed from 2-day food-deprived larvae, but no refeeding treatments were available for comparison. The results showed that whereas muscle tissue reflected changes in the proliferation rates after 2 days of food deprivation, the brain was unaltered by food withdrawal during this period. This follows logically the adaptation to protect the most "essential" organs in the absence of a sufficient food supply (Uriarte and Balbontin 1987).

Time responses

Under *time responses* we will discuss the reaction time of the selected condition indices to different feeding regimes. In the following paragraphs we will use concepts like *latency* (time to a detectable change in condition) or *dynamics* (behaviour of this reaction, e.g. exponential, or simply faster or slower in relative terms, Ferron and Leggett 1994).

From an ecological perspective, the comparative study of time responses is of interest as it enables one to establish hypothesis on physiological responses to food deprivation that can be interpreted in terms of susceptibility to mortality, both directly by

starvation (e.g. by establishing concepts of "Point of no return" or PNR, which are thresholds over which larvae are unable to recover even if food is offered, e.g. Blaxter and Hempel 1963, Owen et al. 1989) or through increased predation in less fit individuals (e.g. stage-duration hypothesis, Cushing 1975). The literature shows that the *sensitivity* (defined as amount of environmental variation, i.e. food, needed to cause a change in a condition index) of the condition indices falls as the organisation level rises. Thus, in general, morphometrics take longer to show the effects of food deprivation than histology, and this in turn takes longer to respond than biochemical methods.

Long-term starvation analysis

The morphometric condition index was the one that took longer to show significant differences between Fed and Starved preflexion larvae (Table 2.5.1.). The percentage of muscle fibre separation showed significant differences between Fed and Starved larvae on day 14, whereas RNA/DNA underwent an apparent decrease on day 12, a later increase on days 13 and 14 and a decline on day 15 (Fig. 2.4.4.). The observation that at 14 DAH the MFS had responded significantly, whereas the RNA/DNA ratio had not, could be misleading due to the low number of observations on day 12 for RNA/DNA of Starved larvae. If infact that was a real difference, then the later RNA/DNA increase in Starved larvae (days 13 and 14) should be explained on a physiological basis. A plausible explanation would be that for Starved larvae the protein contained in the muscle pack was being used before day 14 as shown in Fig. 2.3.4. (H). This could enhance catabolic processes in the muscle that would be paralleled by an increase in RNA/DNA, which would explain the sudden increase in RNA/DNA ratio detected in the muscle of Starved larvae of days 13 and 14.

For delayed feeding larvae, the latency of recovery increased following the patterns of increasing organisation level, being shortest for RNA/DNA and longest for the morphometric index (Table 2.5.1.). Also, the time to full recovery (to levels of Fed larvae) followed a similar pattern: morphometry had not recovered on day 21. MFS took 5-6 days and RNA/DNA increased abruptly on the 3rd day after food was offered. This is logical for muscle tissue (the one used to measure RNA/DNA, MFS and and BDA, the latter being the

most discriminating variable within the morphometric ones) as the natural cascade of events will imply firstly a modification in RNA synthesis that will lead to an increased (or decreased) muscle fibre development, and this will eventually be detectable through morphometry some time later.

Table 2.5.1. Response times of the different indices in the long and short-time starvation experiments.

	Morphometric	Histological	Biochemical
Long-term starvation experiment (days 6 to 21)	1st canonical root on size-free BDA, PAL and HL	%MFS	RNA/DNA
Days from hatching until detection of differences between Fed and Starved larvae	17-18	11-14	15
Days to detection of response of delayed larvae after food supply on day 13	5	4	3
Days to full recover of delay larvae after food supply (on day 13)	Not fully recovered	5-6	3
Short-term starvation experiment (days 22 to 28)			
Days to detection of difference between Fed and Starved larvae	No data	2	2
Continuous worsening over 3 starvation days	No data	No	Yes
Time to significant recovery after 2 d of food deprivation 3 d of food deprivation	 No data No data	 2 no data	 2 1

Short-time starvation experiment

The time for food-deprived post-flexion larvae to exhibit a significantly worsened condition was similar for MFS and RNA/DNA ratios (Table 2.5.1.). The time of 2 days is within the range of values observed in several species for histological (Martin and Wright 1985; Green and McCormick 1999) and RNA/DNA ratios (Buckley 1984; Suneetha et al. 1999).

The RNA/DNA ratio showed a longer-lasting dynamics than MFS in the sense that it showed a continuous decrease during the 2nd and 3rd day of fast, whereas MFS did not. The time to significant recovery after 2 days of fast was similar for the two indices, again showing that MFS is a relatively sensitive indicator of condition. This is in agreement with the model of shorter latency and faster dynamics for quantitative histological and biochemical indices suggested by Ferron and Leggett (1994) for other species.

If food was offered after 3 days of fast, RNA/DNA ratio recovered faster than if food was offered after just 2 days of fast, suggesting a compensatory growth (table 2.5.1.). This agrees with the work of Martin and Wright (1985) for *Morone saxatilis*, which shows a faster recovery dynamics than decaying dynamics. However, further research into this aspect should be conducted for sea bass in order to establish a pattern of latency and dynamics for this species that can be useful for making interpretations in future field studies.

Classification properties

We define the classification properties or "power" of a condition index in the laboratory as its ability to correctly ascribe a particular larva to a predefined group. Classification results are only valid for the pre-defined groups, species and rearing conditions.

The three condition indices were compared only in the long-term starvation experiment (pre-flexion stage) and during the exogenous feeding period (days 14 to 21) in order to avoid differential developmental effects. The values utilised for comparison were those yielding the best discrimination for the 3 pre-defined feeding categories, using either multivariate or univariate measurements (Table 2.5.2.).

Fed larvae were best classified by MFS or RNA/DNA. Morphometry was not a very good predictor of condition in Fed larvae. This is so because the shape of Fed larvae keeps changing with age (even after removal of size effects, see below).

Starved larvae were equally well classified (around 80%) by any condition index, which is a good result considering that the identification of Starved larvae is the main goal in condition analyses.

Delayed larvae had a higher than chance (that would be 33%) percentage of correct classification using either morphometry or histology. However, RNA/DNA could not be used to define a "delayed class" as response of Delayed larvae was abrupt, with no continuous gradient. We used an arbitrary value to separate Fed from Starved larvae (it was the value that offered best discrimination through several trials). The latter perhaps increased the correct classification of Starved and Fed larvae as classified by RNA/DNA

(the existence of 2 categories instead of 3 increases the chance of spurious correct classification). The RNA/DNA was confirmed as an excellent index for distinguishing growing individuals for this phase. However, we found it to be a poor predictor of intermediate condition.

Multivariate analyses performed on several biochemical measures obtained from a single larva (traditionally not applied due to small biomass) could help the discrimination of intermediate nutritional classes. Multivariate approaches, widely applied to morphometry (Theilacker 1978, 86; McGurk 1985 a, Polo 1991) and histology (Theilacker 1978; O'Connell 1980) are currently being explored for biochemical indices in fish larvae (Høie et al. 2000) and would benefit from refined sensitive fluorimetric methods like the one used herein.

Table 2.5.2. Classification results for the three selected condition indices.

Organisation level	Measure	Treatment	N	%correct classification
Morphometric	Discriminant function on size-free BDA, PAL and HL	Fed Starved Delayed	67 25 26	52.0 80.0 40.0
Histological	MFS (categories derived from table 2.3.3.)	Fed Starved Delayed	20 18 16	90.0 83.0 44.0
Biochemical	RNA/DNA Reference value=4	Fed Starved Delayed	27 15 18	100.0 80.0 0 (no own class)

Relationship with survival

The value of a nutritional indicator of condition is higher if it can be directly related to decreased survival. The extreme case of this is the establishment of PNR values, which can tell us for certain that a single larva will die even if food is supplied. If the PNR is not established, or if it is assumed to be long (present study), then we must consider aspects such "how a lowered condition index may decrease the susceptibility to total mortality", and integrate these speculations into the known theories of starvation-predation mortality (reviewed by Leggett and Deblois 1994).

We explored the relationship of the three selected indices with survival in the Starved larvae, as they were the only ones evidencing a mass mortality episode that could be related to a critical condition for each index (Fig. 2.5.1.).

Although we could not calculate a PNR based on decreased feeding activity (e.g. Yin and Blaxter 1987) or the presence of necrotic tissues (McFadzen et al. 1997), it was observed that a mass mortality occurred in Starved larvae at around 17 DAH. We therefore observed how the different indices behaved on this day, and assumed that if they showed a particular worsening their value would be highly related to starvation mortality. The three selected condition indices were significantly related to survival. The lower the survival, the lower the shape factor and RNA/DNA, and the higher the MFS. However, there are some important differences regarding how each index related to the observed mortality.

The initial sharp drop in survival (days 4-6) was not related to food change, as it was also experienced in Fed larvae (see Section 2.1.), and must be related to genetic failures in the transition to the larval phase, which demands

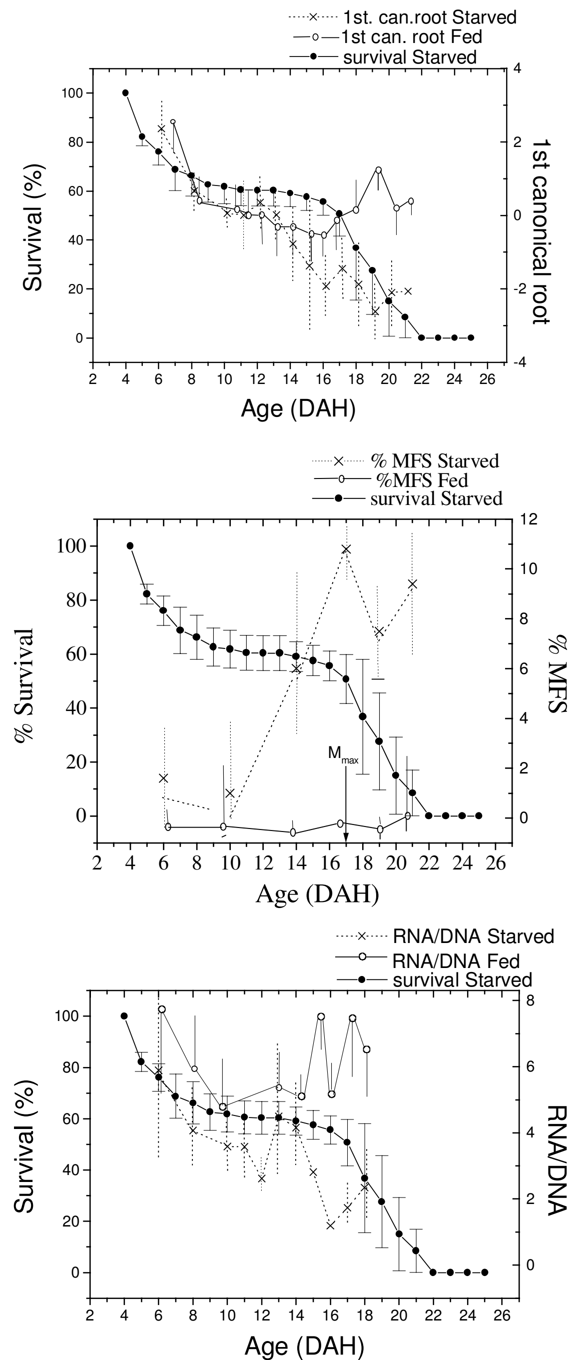

Figure 2.5.1. Relationship between selected morphometric (A), histological (B) and biochemical (C) indices and survival. The represented survival curve is that for Starved larvae. Fed larvae are represented for comparative purposes. Means represented for survival, morphometric and biochemical indices. Medians are used for the histological index. Error bars are SE for survival, SD for morphometric and biochemical indices and 25 and 75% percentiles for the histological index.

strong physiological and behavioural adaptations (Fuiman 1997). It is clear from Fig. 2.5.1. that at the initiation of mass mortality on day 17, the three indices are in their lower range of values for Starved larvae, whereas Fed larvae are in significantly better condition. It is also clear from Fig. 2.5.1. that morphometric and biochemical indices are strongly related to developmental events and may be reflecting both growth and condition (see the u-shape in the Fed treatment) whereas MFS could behave mainly as a condition index, as it varied little in Fed larvae.

The two types of information are of value for they have different implications if extrapolated to the wild. On the one hand, it could be argued that body shape and RNA/DNA have an added value because they reflect growth and thus can e associated to potential predation mortality (Cushing 1975). Counter to these argument, these indices will be age and size-dependent, a situation which is not easily solvable in the field unless routine otolith readings are conducted. Another interesting observation that relates the RNA/DNA to complex physiological processes is that RNA/DNA increased at the time that muscle initiated a massive degradation (day 14) and confirms that the use of a single index may lead to missinterpretations of nutritional condition.

Processing time, costs and requirements

One must not lose sight of the fact that the ultimate reason for the study of condition indices (to relate them to fluctuations in juvenile-adult populations) can only be achieved in routine studies, for which aspects of costs, requirements and processing time must be considered (Ferron and Leggett 1994). The processing time once the larvae have been collected was higher in histological measurements (around 3 days for a series of 15 larvae), and lowest in morphometric measurements (tens were measured daily for several variables), leaving biochemical indices in an intermediate position (a few tens measured on a day).

Costs were minimal for morphometric analyses and higher in histological indices (due to time requirements) and biochemical indices (due to cost of reagents). Given that the maximum level of information must be extracted from each larva to overcome the high individual variability (Chambers 1993), there should be a maximisation of information

revenue vs effort in the analysis. Morphometrics could be routinely included in a multivariate analysis based on either biochemical measurements or histology. In small larvae, the maximum information regarding condition is undoubtedly achieved through histology, as several organs can be explored for selected characteristics. However, some of this information could be sacrificed (taking only subsamples for histological measures as noted by Theilacker 1986) in favour of an increased sample size, by applying several biochemical indices which are sensitive enough to single larvae, like the ones used in the present study.

Usefulness for field studies

The different results obtained in this section have the value of serving as a reference for future field studies on this species, allowing for the widely commented differential effects between laboratory and field studies. However, following our initial goal for this part of the thesis, we consider that an effort must be made towards drawing conclusions that can be useful in a broader than species-specific sense.

Taking into account the peculiarities of each index in terms of time response, classification properties, relationship with survival and effort requirements, it is clear that each index offers a set of advantages and disadvantages (gathered in Table 2 of Ferron and Leggett (1994), and confirmed with the analysed indices in this study). As noted by those authors, a combination of various indices is probably the only way of approaching the relationship between nutritional condition and susceptibility to total mortality (a combination of direct starvation mortality and induced starvation mortality through predation, loss of buoyancy etc).

In the realm of the nutritional indices, a single index is often used to determine the “status” of the sampled individual larvae (e.g. RNA/DNA, prot/DNA, etc.). When several variables are considered (usually in histological or morphometric measurements), it is common to average the obtained values to describe the different larval groups (O’Connell 1976). When multivariate approaches are adopted, the real correspondence between the highest discriminating variables and survival is seldom considered. Indeed, though useful, experimental correlations with survival do not necessarily imply causality in mortality

rates. This is even more obvious when for wild populations, where multiple non-controlled factors interact with a weakened larval condition to cause the aforementioned increased susceptibility to mortality.

To account for this probable - yet difficult to measure - link between larval condition and susceptibility to total mortality, we propose here that different indices of condition should have different weights in the overall classification of field samples. These weights could be experimentally or theoretically based. Figure 2.5.2. illustrates how this idea could be developed. The concept of this method is extensive to any species and assumes that an effort for measuring several indices in each specimen is a better solution than the use of a single index (Ferron and Leggett 1994, Suthers 1998).

Necrosis should have the minimum associated score (score 0 in Fig. 2.5.2.), as it is the only true indicator of direct starvation mortality (it can be assumed to be a PNR, McFadzen et al. 1997). Maximum scores (step 2, scores 7-9), should be given to indices that may have higher associated survival probabilities. In this case, the muscle degradation could be chosen as it is indicative of a particular degraded condition (note that a degraded

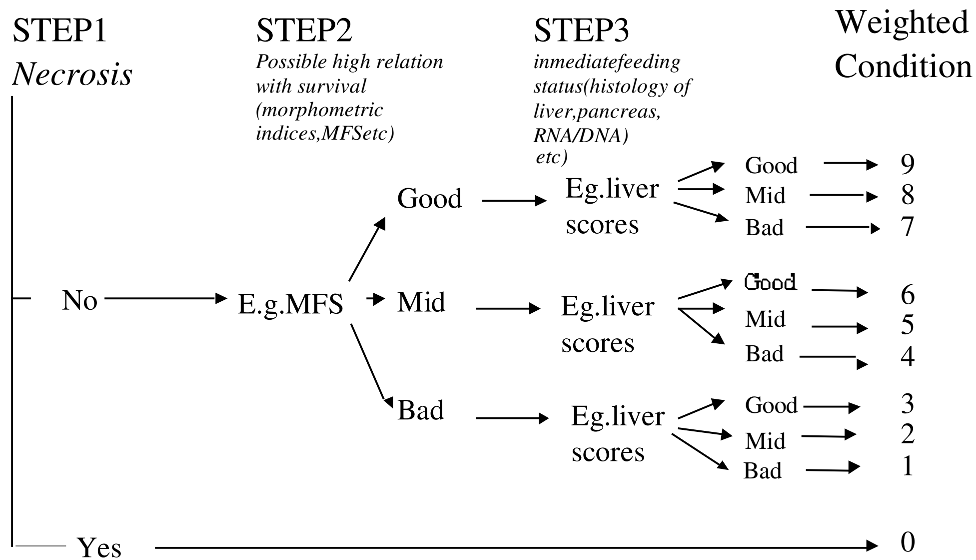

Figure 2.5.2. Proposed method for weighting measurements of nutritional condition according to their potential relationship with survival for field-collected samples.

muscle means that fast-burning reserves like triacylglycerols and glycogen are exhausted) Also, it is particularly related to swimming performance (e.g. Green and McCormick 1999),

which is in turn negatively related to predation mortality (Hunter 1972, Frank and Leggett 1982) and directly related to feeding ability (Laurence 1972). Moreover, a degraded muscle causes a change in density that has been shown to affect the relative position in the water column of emaciated larvae which may influence the interaction with predators or food patches (Neilson et al. 1986, Sclafani et al. 1993, 1997, 2000). Also, morphometric characters could be used (as an alternative or in conjunction) at this level. They are the last ones to recover after refeeding (e.g. present study). Given that morphometry, function and performance are correlated (Fuiman et al. 1997, Osse and Boogaart 1999), a prolonged time in a "suboptimal shape" may be indicative of severely handicapped survival. Lastly, "storage" indices (e.g. liver) or other short-time response indices could be used to refine the scores (Step 3). Undoubtedly, the degree of subjectivity in weighting condition indices can be high, but on the other hand this method can be used in parallel and confronted with non-weighted condition predictions that could be introduced into modelling schemes.

3. FIELD STUDIES

3.1. EARLY STAGES OF *Sardina pilchardus* AND ENVIRONMENTAL ANOMALIES IN THE CATALAN SEA

Most of this section has been published in: "Olivar MP, **Catalán IA**, Emelianov M, Fernandez de Puelles ML (in press) Early stages of *Sardina pilchardus* and environmental anomalies in the North Western Mediteranean. Est Coast Shelf Sci".

ABSTRACT

The influence of environmental conditions on the spatial distribution patterns of the early stages of *Sardina pilchardus* off the Catalan coast (NW Mediterranean) during November 1998, February 1999 and November 1999 was investigated. As a general rule, eggs were collected on the continental shelf, while larvae displayed a wider cross-shelf distribution. The local circulation during autumn-winter 1998-1999 was dominated by an anticyclonic eddy of new Atlantic Water that remained stationary at the centre of the Catalan Sea from September 1998 to the end of February 1999. Such a situation is very infrequent and the influence of these waters extended to the continental shelf zone, modifying the slope current by diverting the flow and even reversing the direction of the flow. On the third cruise, carried out at the beginning of the spawning season of 1999 (November), the circulation and water mass distribution in the region was typical, with the old Atlantic Water occupying the whole region. Abundance of pilchard eggs and larvae during this last cruise was higher than in the previous surveys. In November 1998, the abundance of pilchard eggs and larvae was relatively low in the zones affected by the new Atlantic Water. The most interesting result of these surveys was the very low concentrations of eggs and larvae found in February 1999, possibly due to the anomalous situation created by the eddy, which had remained almost stationary during the 1998-99 spawning season.

INTRODUCTION

The European pilchard, *Sardina pilchardus*, is a coastal pelagic species found in the Western Mediterranean and Adriatic Sea (Whitehead 1984). It is the most abundant small pelagic species in the Northwestern Mediterranean (Martín 1991), though the biomass of the population has decreased since 1993 (Anonim 2000). In spite of the fishery importance of the species, there is little information on the spatial distribution patterns of adults and recruits in this region.

Pilchard have a protracted spawning season, with some differences in the peak of spawning depending on the areas and the temperature regimes (Ré et al. 1990; Solá et al. 1992). In the NW Mediterranean the spawning season extends from October to May and the peak of spawning occurs in January-February (Gómez Larrañeta, 1960, Palomera and Olivar, 1996). This period coincides with that reported for other areas of the Mediterranean, like the Adriatic Sea (Dulcic and Grbec, 2000).

The major aspects of the dynamic conditions and the typical water mass structure in the study area are well known and have been described by several authors (*e.g.* Salat and Cruzado 1981; Font et al. 1988; Pinot et al. 1995; López-Jurado et al. 1996*;* Millot 1999; Salat 2002). The cyclonic circulation in the surface layer around the basin is controlled by two permanent density fronts: the Catalan front located over the Iberian Peninsula slope and the Balearic front located over the insular slope (Fig. 3.1.1). The Catalan front separates the saltier old Atlantic Water (AW)[1] in the central part from fresher shelf waters near the Catalan coast. The Balearic front separates the old AW from the less saltier and warmer new AW incoming into the basin through the Balearic Island channels (Pinot et al. 2002). The circulation is subjected to mesoscale variability, which has been attributed to the action of open sea eddies (Tintoré et al. 1990; García et al. 1998). Off the Catalan

[1] Water mass acronyms in this paper follow the recent recommendations of the Round table on Mediterranean Water Mass Acronyms, 36th CIESM Congress, Monaco, 2001.
(http://ciesm.org/events/RT5-WaterMassAcronyms.pdf).

coast, fertilisation of the shelf waters during the autumn-winter season is caused by local vertical mixing and shelf intrusions of slope water (Salat et al., 2002).

There have been many studies of the influence of oceanographic processes on temporal and spatial patterns of distribution of species reproducing during the spring/summer period off the Catalan coast (Sabatés 1990 a,b; Palomera 1992; Sabatés and Olivar 1996). However, few studies have addressed the environmental effects on species that reproduce during autumn-winter, the most unstable period of the year (Olivar et al. 2001).

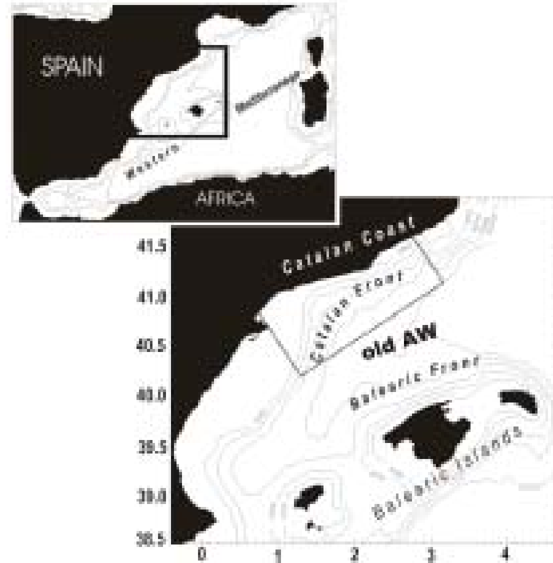

Figure. 3.1.1. Western Mediterranean basin with indication of the sampling area along the Catalan coast shelf and slope.

The aim of this work was to investigate the influence of environmental conditions on the spatial distribution patterns of *Sardina pilchardus* eggs and larvae in the NW Mediterranean.

MATERIAL AND METHODS

The study area corresponded to the southern sector of the Catalan coast, and extended from near Barcelona to the north of the Ebro river Delta (Fig. 3.1.1. and Table 3.1.1.). Three oceanographic cruises were conducted: November 1998, February 1999 and November 1999. According to previous information, these months fall within the main spawning period for this species (Gómez Larrañeta 1960; Palomera and Olivar 1996; Olivar et al. 2001). The total number of stations was not identical for the different cruises (Table 3.1.1.), although all of them were mainly designed to sample the area inhabited by spawning adults (the continental shelf) (Gómez Larrañeta, 1960).

Ichthyoplankton hauls and CTD casts were done on a grid of stations placed on cross-shelf transects almost perpendicular to the coastline and 5 to 10 nmiles apart. Oblique ichthyoplankton hauls covering from the surface to 200 m depth were made using a 60 cm Bongo equipped with nets of 300 µm mesh size. The ship speed during the haul was 2 knots. The volume of filtered water was estimated by means of a flow meter placed in the centre of the Bongo mouth, and the maximum depth of the haul was determined by means of a depth sensor (Minilog). CTD casts were carried out from surface to a maximum of 500 m depth using a SeaBird 25 profiler system. On the first two surveys (November 1998 and February 1999), microzooplankton hauls were made to assess the amount of potential food available to pilchard larvae. Microzooplankton samples were collected by vertical hauls from 70 m to the surface using a Juday-Bogorov net (40 cm mouth and mesh size of 53 µm). Posteriorly, samples were fractioned into copepod nauplii (N), formed by nauplii < 45 m, and the rest of the microzooplankton (T-N)

Table 3.1.1. Sampling details

Vessel	Sampling dates	Nº. of stations	Sampling location
R.V. García del Cid	4-9 November 1998	77	41° 36'-40° 31' N
R.V. García del Cid	12-15 February 1999	50	41° 28'-40° 40' N
R.V. García del Cid	20-25 November 1999	86	41° 20'-40° 35' N

The number of pilchard eggs and larvae collected at each station was standardised to number under 10 m^2 of sea surface using the volume of water filtered by the net and the maximum depth sampled. Larvae were measured to the nearest 0.1 mm, and abundance by size class was also standardised. Size frequency distributions were presented as mean abundance of positive stations. Abundance of copepod nauplii and the remaining microzooplankton organisms was standardised to number of specimens per volume of filtered water.

RESULTS

November 1998

The vertical structure of the water column was unusual for the autumn season, with a relatively shallow and narrow pycnocline (40-60 m) (Fig. 3.1.2.). Temperature profiles showed great changes from surface to 50 m ranging from 20 to 15°C, and 13.5 °C was reached at 125 m, below which the water column was almost homogeneous. Salinity was homogeneous in the first 40 m (37.85), but increased thereafter down to 300 m, where it reached 38.55. Fluorescence profiles still showed the importance of the deep chlorophyll maximum between 30 and 60 m, and very low values at surface (Fig. 3.1.2.).

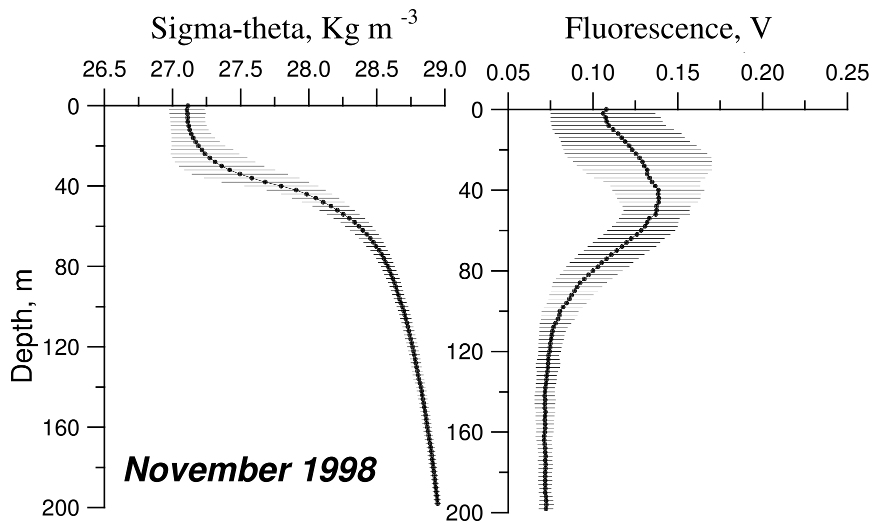

Figure 3.1.2. Mean vertical sigma-t (kg m^{-3}) and fluorescence (V) profiles during the November 1998 cruise. Horizontal lines indicate 1.96 x standard deviations

The horizontal distribution of temperature and salinity above the thermocline did not show cross-shelf gradients (Fig. 3.1.3). Surface temperatures in the area ranged from 18 to 20°C, which is unusually high for this period of the year. Two colder patches of water were detected in the central area and north of Barcelona. Surface salinity was lower than usual for this area, not reaching the 38 characteristic of old AW. Surface fluorescence

was very low in the study area. Values were higher at 50 m than at the surface, showing the importance of the Deep Chlorophyll Maximum (Fig. 3.1.3.). Main concentrations of copepod nauplii as well as other species of microzooplankton were distributed along the coastal band, and extended offshore in the zones where water temperatures were lower than 19°C (Fig. 3.1.3).

Pilchard eggs were predominantly found on the continental shelf, although in the central zone their distribution extended to the shelf break. Larvae also showed peak abundance on the shelf, but displayed a wider cross-shelf distribution, frequently extending to the slope. Higher concentrations of both eggs and larvae occurred in the zones of lower water temperatures (Fig. 3.1.3). Main larval densities were also associated with high microzooplankton concentrations (above 4500 nauplii/m^3 or above 5000 individuals/m^3 of the rest of microzooplankton).

The size of the collected larvae ranged from 3 to 16.5 mm. The modal class was found to be between 4 and 5 mm and the abundance per size class decreased thereafter. Larvae smaller than 5 mm were more abundant on the shelf and those larger than 9 mm appeared mainly offshore (>200m isobath) (Fig. 3.1.4).

Size frequency distributions were also analysed after the grouping of stations of three different sectors: the southern sector (near the Ebro Delta); the central zone occupied by colder waters, and the northern sector (north of Barcelona). In the northern and southern sectors an exponential decrease in the abundance of larvae larger than 5 mm was observed. In the central zone the decrease in larval abundance from the modal size class to 9 mm was less pronounced. In the northern sector the majority of larvae belonged to small sizes (more than 70% were <5 mm), and larvae larger than 9 mm were almost absent (Fig. 3.1.4).

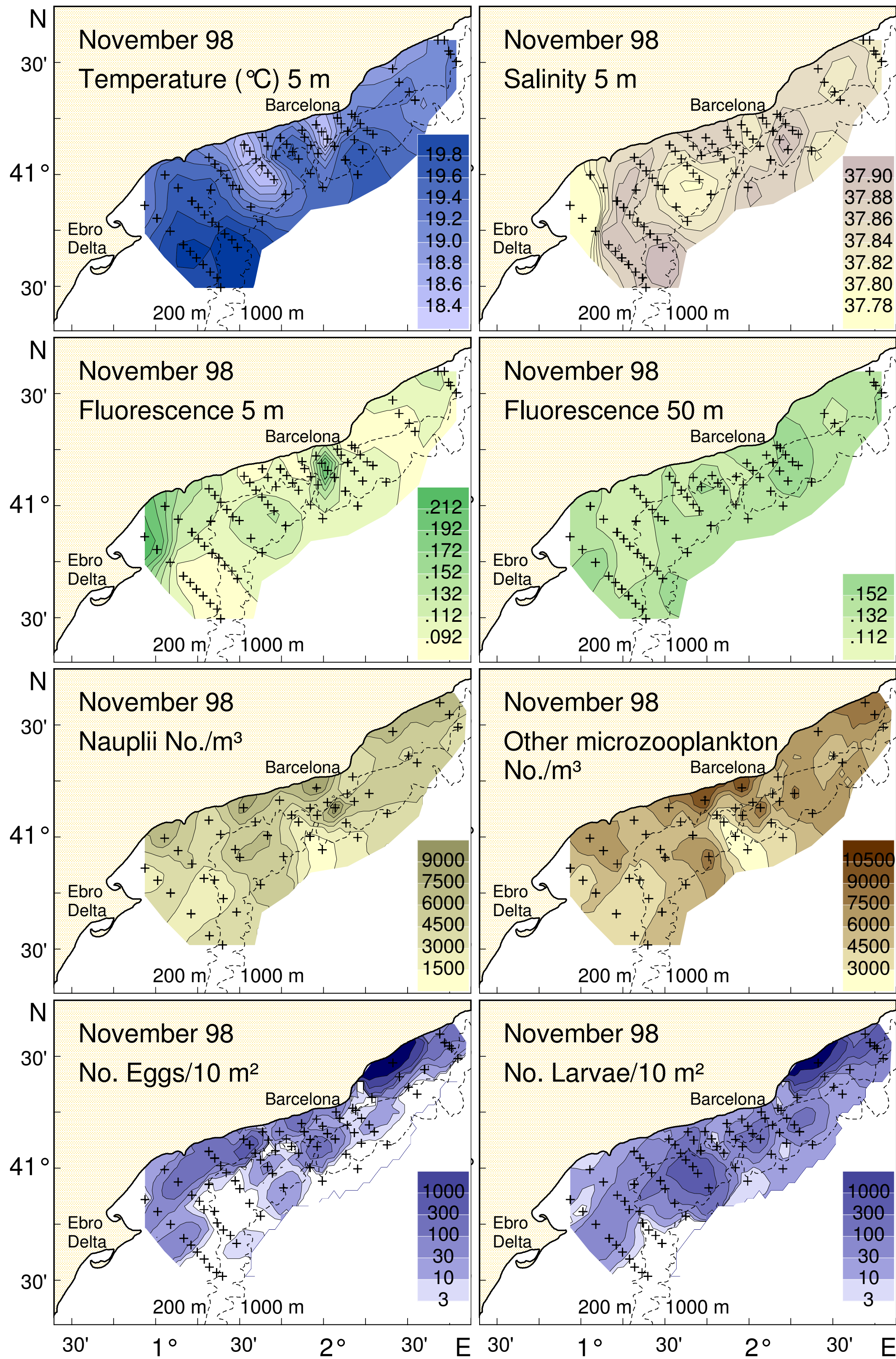

Figure 3.1.3. Horizontal distribution of surface temperature (°C), surface salinity, surface and 50 m fluorescence, copepod nauplii and other microzooplankton organisms and *Sardina pilchardus* eggs and larvae during the November 1998 cruise. Crosses indicated the sampling location.

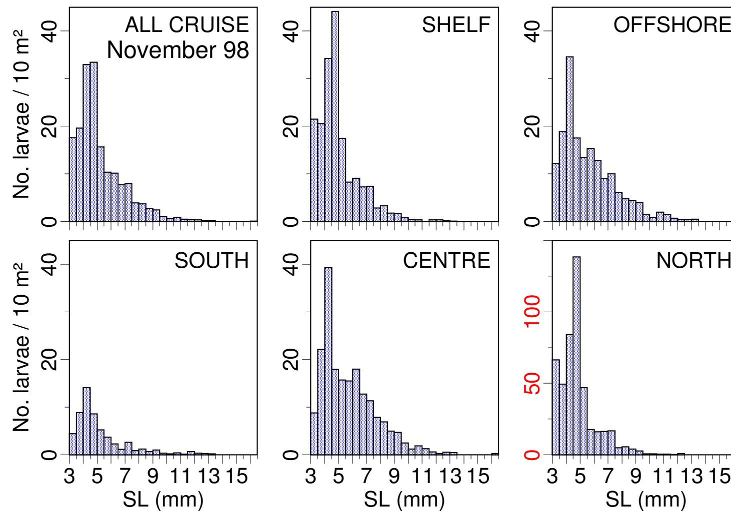

Figure 3.1.4. Size frequency distribution of *Sardina pilchardus* larvae during the November 1998 cruise.

February 1999

The water column was homogeneous during the period, as expected, due to the winter mixing. Mean temperature was *ca.* 13°C throughout the water column. Salinity was homogeneous in the first 20 m (38) with a slight increase at greater depths. The vertical density profiles showed a homogeneous water column with values of *ca.* 28.7 kg/m^3. Peak fluorescence was detected in the upper 20 m, although high fluorescence was found down to 40 m (Fig. 3.1.5).

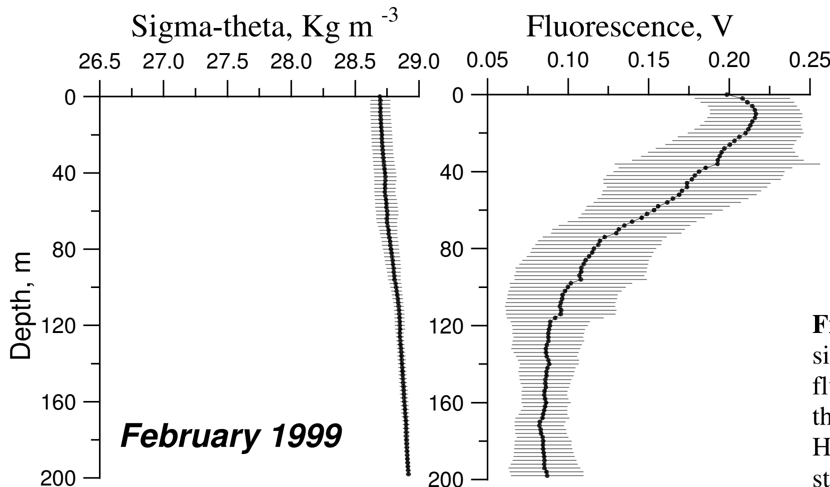

Figure 3.1.5. Mean vertical sigma t (kg m^{-3}) and fluorescence profiles during the February 1999 cruise. Horizontal lines indicate standard deviations

Horizontal distributions of temperature showed almost no differences in the study area. A reversed salinity pattern was observed, with higher values restricted to a narrow coastal band and lower values at the slope (Fig. 3.1.6). Fluorescence both at surface and 50 m was higher than in the autumn cruise. Lower fluorescence values were found in the coastal region near Barcelona (Fig. 3.1.6). Abundance of nauplii and other microzooplankton was higher than in the autumn cruise, with maximum values of *ca*. 13000 nauplii/m^3. Maximum concentrations were located from the centre to the Ebro Delta area and lower concentrations appeared near Barcelona (Fig. 3.1.6).

Pilchard eggs and larval concentrations during this survey were very low. However, spatial distribution patterns were similar to those of the autumn cruise, with the highest concentrations on the continental shelf (Fig. 3.1.6). Both eggs and larvae presented the highest abundance in the area close to the Ebro Delta, coinciding with the highest concentrations of microzooplankton.

Larvae collected during this survey ranged from 3 to 16.5 mm, although more than 80% were small (3 to 5 mm). At stations beyond the 200 m isobath (offshore), the abundance of larvae of the smallest sizes (3-4 mm) was very low. The size structure after separating by north, south and central sectors showed the presence of small larvae both in the south and northern sectors. No larvae larger than 5.5 mm were present in the northern sector (Fig. 3.1.7).

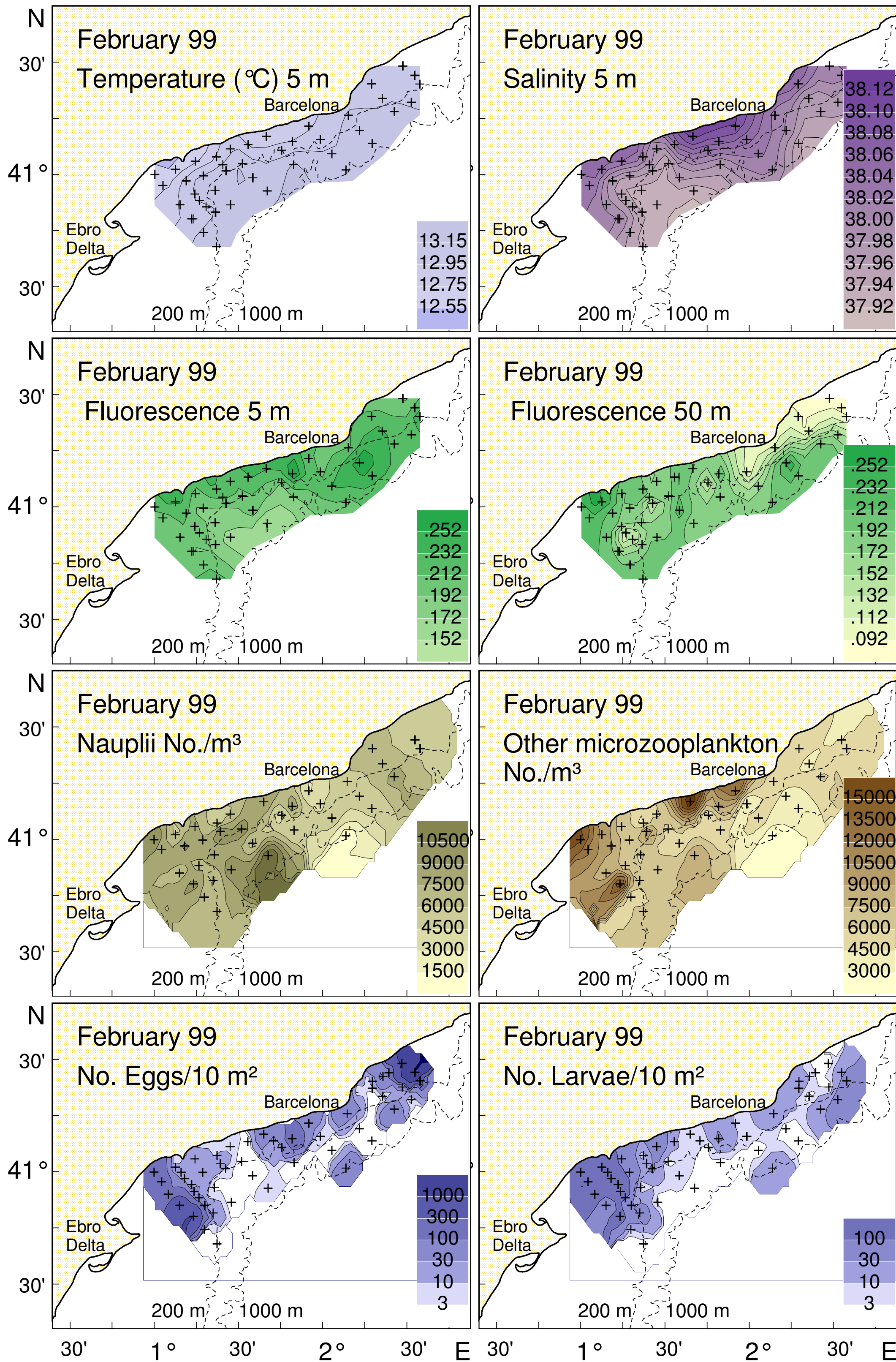

Figure 3.1.6. Horizontal distribution of surface temperature (°C), surface salinity, surface and 50 m fluorescence, copepod nauplii and other microzooplankton organisms and *Sardina pilchardus* eggs and larvae during the February 1999 cruise. Crosses indicate the sampling location.

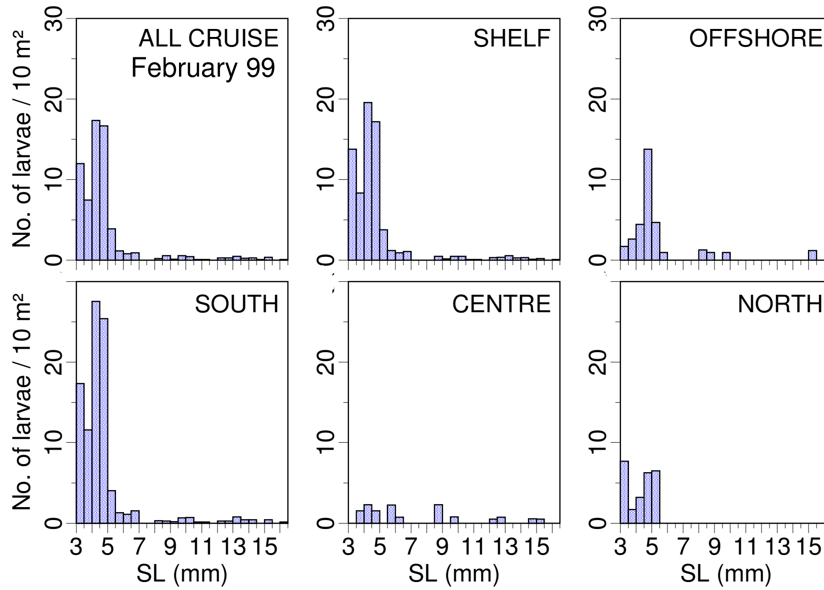

Figure 3.1.7. Size frequency distribution of *Sardina pilchardus* larvae during the February 1999 cruise.

November 1999

Oceanographic conditions during this cruise were relatively typical for this period of the year, with a deep and wide pycnocline (100-200 m) (Fig. 3.1.8). The water column between the surface and 100 m was well mixed at all the stations, as shown by the temperature, salinity and density profiles. Mean temperature in the first 100 m was 16.4°C, and 13.5°C was reached at 175 m. Mean salinity was 38.1. Fluorescence values were

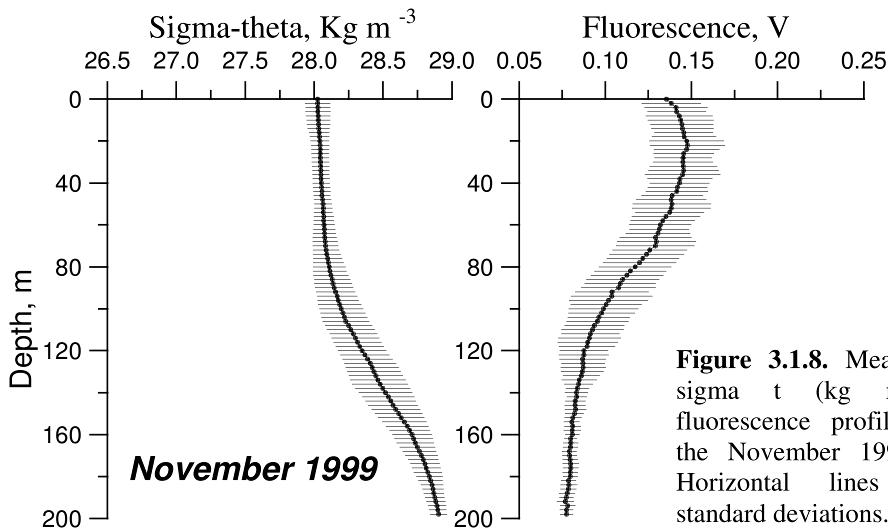

Figure 3.1.8. Mean vertical sigma t (kg m^{-3}) and fluorescence profiles during the November 1999 cruise. Horizontal lines indicate standard deviations.

higher in the upper 40 m of the water column (Fig. 3.1.8).

Sea surface temperature and salinity distributions were relatively homogenous throughout the area (around 16.5°C and 38) (Fig. 3.1.9). Salinity was higher than in the previous cruises. Fluorescence values were higher than in November 1998. Horizontal fluorescence distributions were similar at surface and at 50 m, with maximum values near the coast (Fig. 3.1.9).

Pilchard eggs and larvae showed a fairly typical distribution pattern, with eggs mainly located on the shelf and larvae spreading offshore (Fig. 3.1.9). Maximum concentrations were located in the Southwestern part, where the continental shelf is wider.

The size range of pilchard larvae during this cruise was from 3 to 20 mm with a modal class between 4 and 5 mm. Both shelf and offshore stations were mainly (90%) composed by larvae smaller than 5 mm, with no latitudinal or cross-shelf differences (Fig. 3.1.10).

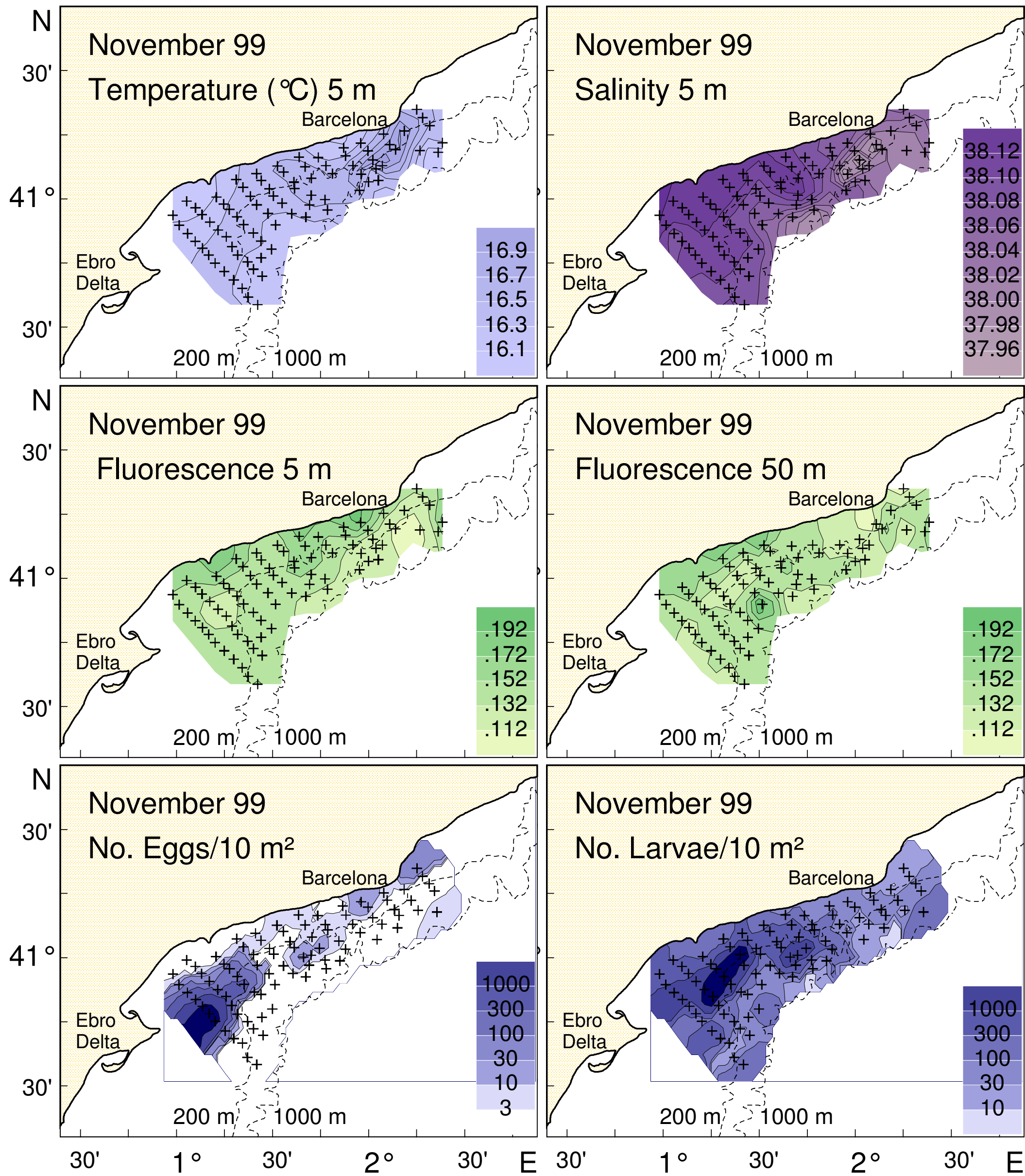

Fig. 3.1.9. Horizontal distribution of surface temperature (°C), surface salinity, surface and 50 m fluorescence and *Sardina pilchardus* eggs and larvae during the November 1999 cruise. Crosses indicate the sampling location.

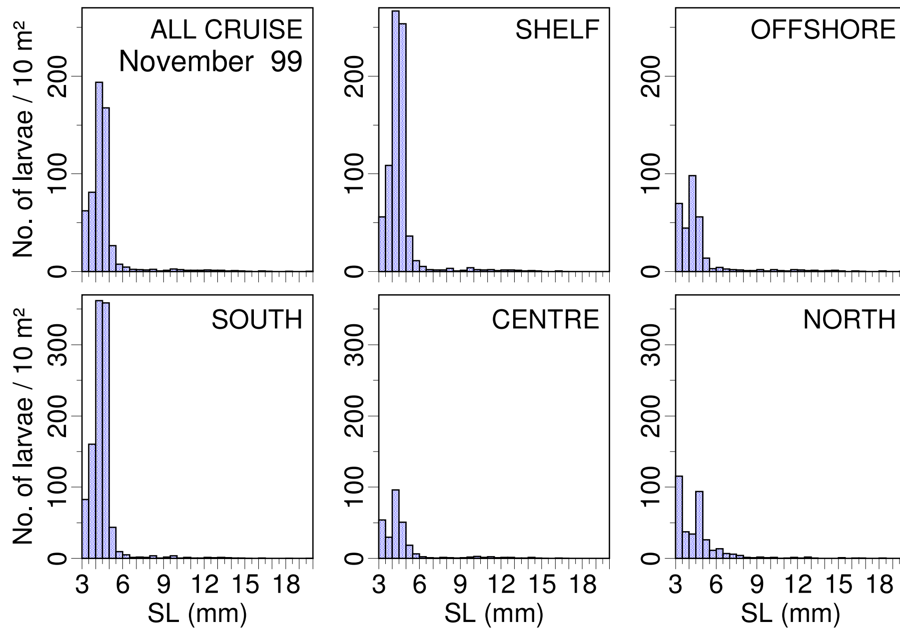

Figure 3.1.10. Size frequency distribution of *Sardina pilchardus* larvae during the November 1999 cruise.

DISCUSSION

Hydrography

Hydrographic characteristics during autumn-winter 1998-99 showed an anomalous situation. In contrast, in November 1999 the oceanographic features were more typical for the region. The unusual conditions encountered in autumn-winter 1998-99 were characterised by the anomalous strong intrusion of new AW into the basin that was reflected in the appearance of warmer and less saltier waters near the Catalan shelf. During November 1998 the surface salinities observed in the study area were particularly low (below 37.8), contrasting with previous data for that area and period (above 37.8) (Salat et al. 2002). The stratified water column, together with the high temperatures and low salinities in the upper layers during November 1998 are further indications of the influence of new AW. The effect of these lighter waters is more important at surface levels, and contributed to strengthen the vertical gradients, restricting the vertical mixing processes

that should have taken place at that time of the year (Salat 1996; Salat et al. 2002). In February 1999 the influence of new AW was weaker, as shown by the slightly increased salinity, but the hydrographical situation was still anomalous, as evidenced by the reversed salinity pattern (with higher salinities near the coast than at the slope).

We relate these anomalies to the presence, from October 1998 to February 1999, of an intense mesoscale anticyclonic eddy of new AW near the area of study. The presence of this eddy was revealed by Sea surface temperature images (NOAA AVHRR data) (Fig. 3.1.11). The influence of these warm low saline waters extended to the continental shelf zone, restricting the SW flow of the Catalan slope current and even inverting the flow direction (Pascual et al. 2000).

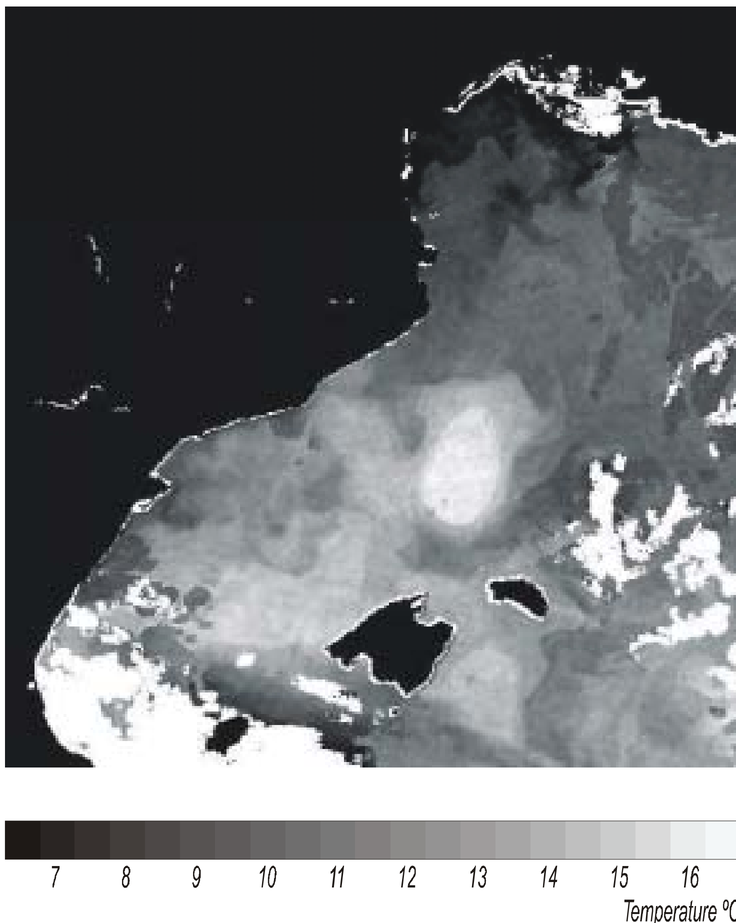

Figure 3.1.11. AVHRR image of Balearic Sea from 27/02/1999 showing the anticyclonic eddy of new Atlantic Water.

The data obtained during the present cruises agreed with the satellite observations indicating that new AW almost reached the continental shelf. In contrast, hydrographic conditions in November 1999 resemble previously reported data for that time of the year (Salat 1996; Salat et al. 2002): mixing of the upper 60-100 m of the water column, lower surface temperature (below 16-18°C) and higher surface salinity (38).

Effects on the organisms

The prolonged (6 months) hydrographical imbalance due to the eddy had an effect on the organisms living in the water column. The relation between environment and organisms was evident in the distribution and abundance patterns of pilchard eggs and larvae during autumn-winter 1998-99. However, the effect of the altered environment could have been direct or indirect (*i.e.* through the spawning stock, the planktonic phases or both). The development of *Sardina pilchardus* eggs takes 67 hours at 15°C (Miranda et al. 1990), while larvae remain in the plankton for more than one month (Ré 1984). Therefore, egg distributions reflect the adult spawning population, while those of larvae are more dependent on environmental influences. The egg distributions found during the three studied surveys indicated that the spawning of this species took place on the continental shelf.

Coastal oceanography and distribution of spawning adults have been judged as the main determinant of the spatial distribution patterns of *Sardina pilchardus* eggs and larvae off the Atlantic Iberian coast (Chesney and Alonso-Noval 1989; García et al. 1992). The anomalous situation during November 1998 could have affected spawners and early stages. Although pilchard spawning in the NW Mediterranean starts at temperatures around 20°C, it does not reach a high intensity until the temperature falls below 18°C (Oliver 1957). In November 1998 the temperature was closely associated with the distribution pattern of eggs and larvae, the highest eggs and larval concentrations being found in areas with temperature values below 19°C. The intense stratification in the water column, together with the depletion of nutrients in the upper layers after the summer period, could have been responsible for the low surface fluorescence levels and the consequent low concentrations of microzooplankton during that period. The highest concentrations of pilchard larvae and copepod nauplii, their main food item (Conway et al. 1994), corresponded to areas with

temperature values below 19°C. In this zone, larval abundance from 5 to 9 mm size classes showed a slow decline, indicating either a lower mortality or a retention of larvae in this zone.

The spawning peak for pilchard in the NW Mediterranean occurs in January-February (Gómez Larrañeta 1960; Palomera and Olivar, 1996). Previous data on egg and larval abundance for that period reported mean densities of ca. 500 individuals/10 m^2 (with areas where abundance was *ca*. 3000/10 m^2) (Olivar et al. 2001). The low egg densities found in February 1999 (mean value of 152 eggs/10 m^2) suggest that spawning during that period was severely reduced. Furthermore, the low larval abundance (mean density of 44 larvae/10 m^2) and the scarcity of larvae larger than 5.5 mm indicated that spawning during the previous weeks was very low and/or that larval survival was adversely affected. The vertical structure of the water column evidenced the winter mixing processes that might have caused a nutrient enrichment in the upper layers, reflected by the high fluorescence values that resulted in high microzooplankton concentrations throughout the study area. In the light of the high levels of potential food, it is likely that the scarcity of larvae was due to a negative effect of the unusual hydrographical conditions on the spawning stock.

During this winter cruise the horizontal distribution of sigma t, with denser waters in the coastal zone, was the opposite of the usual distribution in the NW Mediterranean. This was reflected by the reversed current in a NE direction (Pascual et al., 2001). In the northern part of the Catalan coast, during spring, it has been observed that a reversal in the density patterns has an adverse effect on the distribution of larvae of shelf-dwelling species (Sabatés and Masó, 1992). In the present study it is likely that the adverse effect on the organisms was caused not only by the anomalous hydrographic structure found during the days of the study, but also by the long persistence (since October 1998) of new AW near the shelf. The marked difference in the water characteristics could have led to the development of prey populations less suitable for pilchard feeding and/or might have modified the distributions patterns of the spawning stock.

Finally, in November 1999 the typical hydrographic situation paralleled the usual pilchard egg and larval distribution patterns. The most conspicuous feature during this

cruise was the high number of early larval stages, which was indicative of a high spawning intensity during the study period and a few weeks before.

Our observations indicate that both spatial distribution and abundance of early stages of pilchard are associated with oceanographic variability caused by the influence of the eddy. An unanswered question is: why was new AW displaced towards the coast in the winter of 1998-99?. Studies carried out from 1996 to 1998 in this region showed an inter-annual evolution, with increasingly warm winters. Due to this increase in temperature, the formation of Winter Intermediate Water decreases. This weakened the Catalan current at the end of spring, which could have facilitated the entrance of new AW to the north (Pinot et al. 2002). We think that it would be of interest to investigate the influence that large-scale oscillations affecting the NE Central Atlantic (*e.g*. North Atlantic Oscillation, NAO) may have on the Western Mediterranean.

3.2. RELATIONSHIPS BETWEEN ENVIRONMENT, GROWTH, AND CONDITION OF PILCHARD *Sardina pilchardus* IN THE CATALAN SEA

ABSTRACT

In this chapter we show several relationships between the environmental characteristics in two contrasting periods (November 1998 and February 1999), the long-term growth (derived from otolith and muscle growth analyses), and the nutritional condition (assessed through histological and biochemical indices) of pilchard *Sardina pilchardus* (Walbaum) in the Catalan Sea. These measurements were compared between larvae grouped by selected environmental variables through PCA. Further, non-parametric correlation was performed between 12 environmental variables and larval condition. The study of long-term mortality was also attempted.

November 1998 was characterised by unusual hydrographic conditions, probably caused by the influence of a mesoscale eddy of Atlantic Waters, that provoked higher than usual temperatures and stratification (Olivar et al. in press). Pilchard larvae collected from stations with lower stratification, lower temperatures and higher levels of potential food showed better histological condition and higher RNA/DNA ratios than larvae from highly stratified areas and lower food levels. Correlation analyses further supported this view. We found no differences in long-term growth between these two main groups, but higher larval abundance was found in the areas where larval condition was better.

In February 1999, the effect of the eddy was weaker but still persistent, and defined an inverted coastal-offshore salinity pattern, which is not usual for this area. However, several hydrographical features were typical for this time of the year, like the strong vertical mixing and the low temperature of the water column (ca. 6ºC less than in November). Larvae grouped in stations attending to the prevailing environmental conditions (differing trends in temperature and salinity) showed no between-group differences in growth, mortality or condition. However, the lower larval availability for this cruise advises caution in the interpretation of results.

Comparison of both cruises, pooling all larvae, showed that November 1998 was characterised by higher growth rates and better nutritional condition than February 1999. Whereas it seems clear that temperature had an effect on growth rates, the differences in nutritional condition between sites and years might have also influenced growth and hence potential survival. The overall percentage of starving larvae was low in both cruises (ca. 2-7%), according to necrotic criteria. However, the amount of larvae in suboptimal condition was relatively large in both cruises. Possible relationships between all the studied variables are discussed.

INTRODUCTION

The European pilchard *Sardina pilchardus* (Walbaum) is within the most commercially important species in the NW Mediterranean and supports the largest fishery in terms of biomass on the Catalan coast.

Whereas some literature exists on the spatial and vertical distribution of eggs and larvae in the area of study (Sabatés and Olivar 1996; Olivar et al. 2001; Olivar et al. in press, see Section 3.1.), there is no data on the condition or growth in relationship with the environment.

The survival of pilchard larvae, as in most pelagic species, is thought to be determined at early stages. It is believed that small variations in egg and larval mortality may explain a large portion of the sharp fluctuations in the year-class strength (Houde 1989). Starvation and predation, or their interaction, are probably the main cause of the observed mortality (Leggett and Deblois 1994). Growth is probably a function of successful feeding, and mortality due to predation is thought to be greater in slower-growing individuals (Hare and Cowen 1997). Given the fact that predation is difficult to study in the wild (Bailey and Houde 1989), starvation and growth are usually the variables gathered to infere survival potential in fish larvae.

It was shown by Olivar et al. (in press) (see Section 3.1.) that the horizontal distribution and abundance of pilchard eggs and larvae was influenced by environmental factors in the spawning season 1998-1999. However, it was not shown whether this distribution responded to an effect on the parental stock or could be attributed to a certain

degree to the effect of environmental variables on the larvae themselves. In order to shed some light on this aspect, it was the aim of this section to study the effect of several environmental characteristics on the long-term growth and nutritional condition of pilchard larvae collected from two cruises, November 1998 (early season) and February 1999 (peak of spawning season).

Long-term growth was here estimated through otolith and muscle growth analyses. Otolith microstructure has long been proved to serve as an indicator of larval age and growth (e.g. Brothers et al. 1976; Campana and Neilson 1985). For pilchard larvae, the daily nature of increments was demonstrated by Ré (1984), and the coincidence between hatching and the first readable ring was shown by Alemany and Álvarez (1994). Some field studies exist in SW Mediterranean pilchard in regard to otolith-derived growth parameters (Ramírez et al. 2001). It is known that environmental changes can be reflected in the width of otolith rings (Neilson and Geen 1985), and therefore, assuming a series of premises, the total otolith width can be thought of as a measure of long-term growth.

Also, long-term growth can be assessed by the variation of certain muscle characteristics, like the number of superficial (presumptive slow muscle fibres) or inner (presumptive fast muscle fibres) fibres over larval age or length. Several works on the changes in larval muscle cellularity in response to external factors have shown that ambient temperature can affect the number and width of muscle fibres. These modifications can occur already at the egg stage and imprint the larvae through older stages (Johnston et al. 1998; Galloway et al. 1998). Once hatched, larvae experiencing diverse thermal conditions can have their muscle growth patterns modified by either hypertrophy (increase in fibre size) or hyperplasia (increase in number of fibres) (Stickland et al. 1988; Vieira ad Johnston 1992; Johnston et al. 1995). For clupeiform larvae, this change in muscle cellularity has been associated with changes in swimming capacity that are directly related to survival potential (Morly and Batty 1996; Johnston 2001a). Until present, most studies have been laboratory-based. However, the study of Temple et al. (2000) on herring *Clupea harengus*, suggested that food concentration might influence muscle growth patterns. Those authors recommended further research on the topic. No data on this issue are available for pilchard.

It has been suggested by several authors (Ferron and Leggett 1994; Suthers 1998) that no single condition index is “the best index”, as all offer different information on the physiological status of an individual. We consider that there is a need to test different methodologies in samples taken from the same sites (provided the same individual cannot be simultaneously tested for some measurements) in order to compare the information offered.

Histological indices are thought to be the most reliable indicators of condition (Ferron and Leggett 1994; Suthers 1998). These indices imply that different tissues can be graded and reveal large amounts of information about a single individual. Histological data on the response of pilchard larvae to suboptimal food levels under controlled conditions are scarce (Silva and Miranda 1992), and studies in the wild have been based upon general patterns of other species (McFadzen et al. 1997). Indeed, the general histopathological alterations observed in the absence of food are reasonably maintained across species, which enables the use of literature information (O’Connell 1976; Cousin et al 1986; Theilacker and Watanabe 1989; Uriarte and Balbontin 1987; Margulies 1993; McFadzen et al. 1997).

Within the biochemical condition indices, those based on nucleic acids are the most widely used (Ferron and Legget 1994). The amount of DNA is considered to be relatively constant in a cell, and is often used as a measure of number of cells or larval size. On the other hand, RNA is known to vary greatly within a cell when protein synthesis occurs. Protein is rapidly laid down in growing larvae, which are thought to rely on the fast increase in size to reduce their susceptibility to mortality. RNA/DNA or protein/DNA ratios have been successfully used to detect growth or condition in several species including European pilchard (Chícharo 1998 a b; Ramírez et al. 2001).

The aim of this study was to relate several measurements of growth and condition to environmental variables in pilchard collected in the Catalan Sea. An approach to the study of mortality was also attempted.

MATERIAL AND METHODS

Sample collection and preservation

The general collection techniques and sampling stations were described in Section 3.1. Hauls were between 15 and 20 min. As soon as the haul was up onboard, contents of the cod-end of one of the bongo nets was poured into a tray and the pilchard larvae were sorted into 2 groups. One group was preserved in 10% phosphate-buffered formalin and later transferred to 70% ethanol for histological analyses. The second group was deep-frozen individually in liquid nitrogen for biochemical and otolith analyses. The onboard sorting was performed so that larvae from a sufficient size-range were represented. The larvae from the other bongo net were fixed in standard 5% borax-buffered formalin for later study.

The whole sorting process was usually finished between 5 and 10 minutes after haul recovery to minimise post-mortem cellular degradation. Depending on larval abundance, between 5 and 15 larvae were preserved from each station for each analysis. The final available number of larvae differed for each analysis and will be specified in the corresponding section.

We next comment on how the different analyses were related to environmental characteristics. The two types of growth analyses and the two types of condition measurements were derived from different individuals. This often implied differences in the number of larvae or stations analysed. For the sake of clarity, we first explain the common methods for relating the results to environmental characteristics. The specific methodology for each type of analysis is detailed below.

Relationships with environmental data

The relationship of growth, mortality and condition *vs* environmental data was approached in several ways.

Firstly an *a priori* grouping of stations into "environmental groups" (A and B) was conducted within each cruise using PCA. This enabled the comparison of, for example,

growth with nutritional condition of larvae collected from stations with similar environmental characteristics. For the PCA analysis, we only used the variables that we considered representative of the factors that might be affecting the biological parametres measured (e.g. condition). The selected variables are represented in bold in Table 3.2.1.

Within the variables selected, we decided to include the Brunt-Väissälä (B-V) frequency as water stability index. The rationale behind this decision is that the degree of strength of the pycnocline may interact with the production dynamics, food distribution and larval vertical migration, and therefore might be related to larval nutritional condition. We calculated the B-V frequency for each station, according to

$$B - V = \sqrt{\frac{g}{\rho}\frac{d\rho}{dz}}$$

where g is acceleration due to gravity, ρ is water density and z is depth (Mann and Lazier 1996). This value was calculated for dz= 2 meters, and the maximum value for each station was selected. The value used was B-V in cycles hour^{-1}, and was calculated using the program “Buoyancy” from SEASOFT (v.4.244, SEASOFT ELECTRONICS, Inc., USA). For February 1999, the variables used for PCA were the same except for B-V, which was not included due to highly uniform mixing of the water column, as seen in Section 3.1. Regarding the potential food, we used two categories. Copepod nauplii < 45 µm (which we called N) and other copepod stages, of any size, except eggs (which we called T-N). These are the main food items found in the guts of pilchard larvae ranging from 4 to 24 mm, as described by Conway et al. (1994).

Variables showing high dispersion in values were appropriately transformed (depth *(ln)* and potential prey abundance *(sqrt)*). All variables were then normalised to zero mean and unit SD and PCA was conducted on the correlation matrix.

Also, correlation analyses between individual larval condition and the values of each environmental variable associated with the station of collection were performed (Table 3.2.1.). This made it possible to explore the importance of individual environmental variables on condition. Long-term growth and mortality could not be analysed in this way as data were derived from pooled larvae. We used non-parametric Spearman-rank

correlations due to the often non-linear relationships observed in the plots, even after appropriate data transformation.

Table 3.2.1. Environmental variables analysed on both cruises. In bold, variables used for station grouping through PCA. (*) The Brunt-Väissälä (B-V) frequency as water stability index was used only for November 1998, as stratification was almost homogeneous in February 1999.
All variables were used for Spearman's rank-correlation analyses with condition.

Environmental variables
Station Depth (m)
Copepod nauplii m^{-3} (<45 m)
Other microzooplancton m^{-3}
Temperature at 5 m (˚C)
Salinity at 5m (psu)
Fluorescence at 5m (volts)
Integrated Fluorescence (0-70m) (volts)
Temperature at 50 m (ºC)
Salinity at 50 m (psu)
Fluorescence at 50 m (volts)
Water stability (max. B-V index at 2 m intervals, cycles h^{-1})*
Depth of maximum B-V index (m)

For November 1998, The first 2 PCs explained ca. 74% of total variance (Table 3.2.2., Fig. 3.2.1., top-left). Basically, stations with lower stratification (B-V) were paralleled by higher food levels (N and T-N). Stations showing negative values for PC1 were called "A", and those showing positive values were called "B". In Fig. 3.2.1. (mid-left, bottom-left) we represent the stations classified as A or B for this cruise, with

Table 3.2.2. Results of the PCA performed on several environmental variables (7 for November 1998, 6 for February 1999) in order to select those that most contributed to the definition of station groups.

November 1998

PC	Depth(ln)	Nauplii( )	Other Microzooplankton ( )	Temp (5m)	Sal (5m)	Fluorescence (integrated over 70m)	Stratification index (B-V)	Eigenvalue	% explained variance
1	0.385	-0.511	-0.498	0.099	0.001	0.297	0.494	2.06	52.5
2	-0.611	-0.270	-0.133	0.098	0.003	-0.602	0.404	0.84	21.4
3	-0.160	0.373	0.428	0.006	0.001	0.380	0.713	0.59	15.2

February 1999

PC	Depth(ln)	Nauplii( )	Other Microzooplankton ( )	Temp (5m)	Sal (5m)	Fluorescence (integrated over 70m)	Eigenvalue	% explained variance
1	0.436	0.064	-0.170	0.643	-0.549	0.250	2.29	49.0
2	-0.096	-0.504	-0.727	0.187	0.130	-0.395	1.25	26.8
3	-0.294	0.366	0.152	0.137	-0.419	-0.749	0.63	13.5

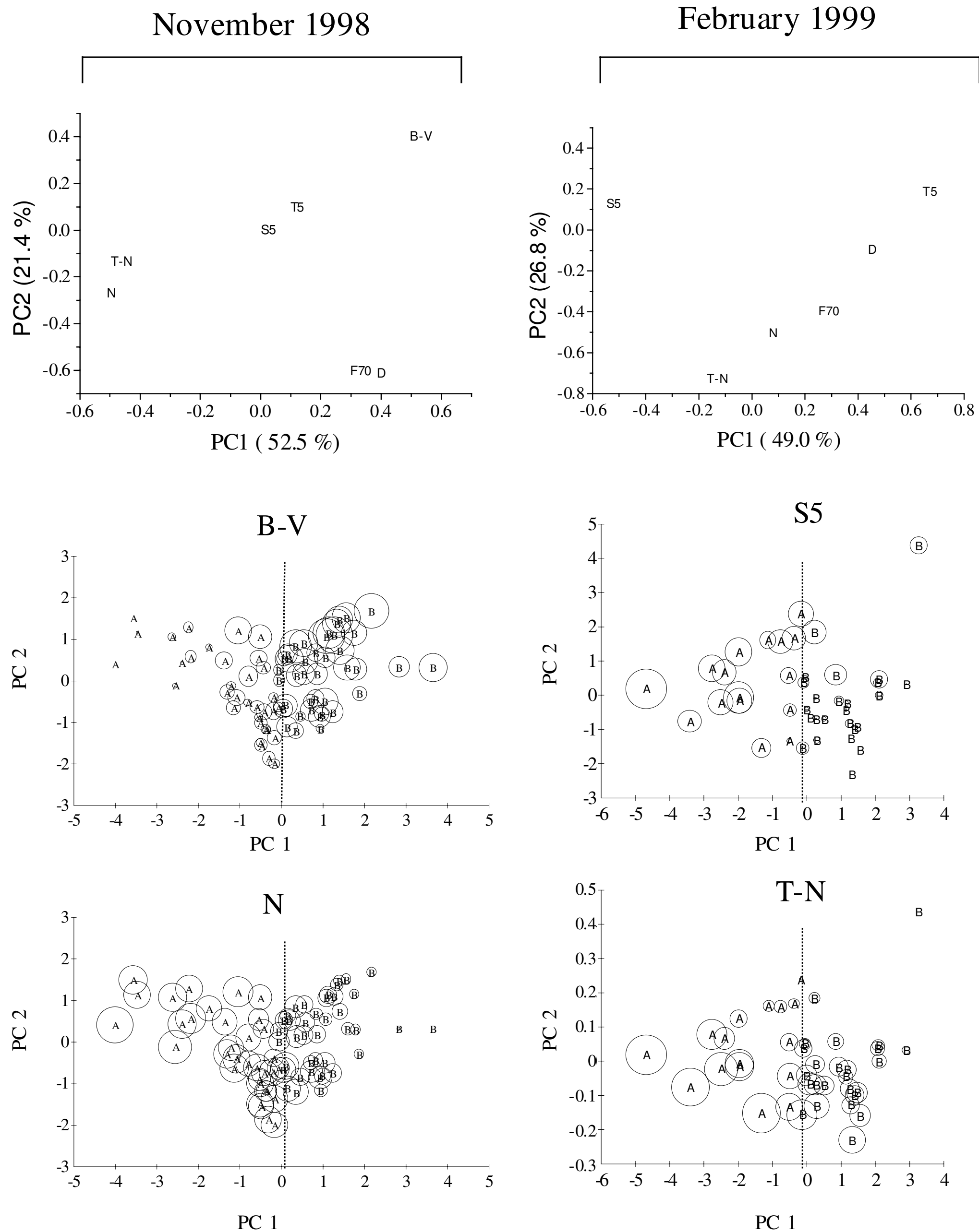

Figure 3.2.1. Results of the PCA analyses and classification into environmental groups A and B for both cruises. Top figures, loadings of the first 2 PCs. Middle and bottom figures, classification of stations into A and B according to the sign of the PC1, and superimposition of the values of selected variables. The diameter of the circles is proportional to the value of the variable for a given station (Primer-E Ltd.). **B-V**, Brunt-Väissälä frequency; **D**, station depth; **F70**, fluorescence integrated over 70m; **N**, copepod Naupli m^{-3} (< 45 m); **S5**, salinity at 5 m; **T5**, temperature at 5 m; **T-N**, other microzooplankton.

superimposed values of the variables explaining the highest amount of variance in PC1. In Fig. 3.2.2. (left column), stations A and B are plotted over the sampling sites, with superimposed values of selected environmental variables.

For February 1999, the first two PCs explained almost 77% of the total variance (Table 3.2.2., Fig. 3.2.1. top-right). The first PC was a contrast between low salinity and higher temperature and depth, which distinguised inshore from offshore stations. The second PC was mainly a contrast of abundance of potential food *vs* temperature and salinity. As for the previous cruise, stations A and B were defined by their positive or negative values with respect to PC1. In Fig. 3.2.1. (mid-right and bottom-right), S5 and T-N are superimposed. Surface temperature, despite explaining a high amount of variance in PC1, was not shown in Fig. 3.2.1. as it showed similar values to surface salinity in the second PC, and thus its representation would be redundant. As for the previous cruise, In Fig. 3.2.2. (right column), stations A and B are plotted over the sampling sites, with selected superimposed environmental variables. For each cruise, growth, mortality and histological and biochemical condition were analysed between areas A and B. Finally the two cruises were compared for the aforementioned parameters by pooling all larvae.

For all the studies, larval length was measured under a binocular microscope at the nearest 0.1 mm. In order to standardise comparisons, all length measurements are given as formalin-preserved standard length (SL). When animals were frozen (larvae used for biochemical analyses), length was corrected by applying a 5% reduction due to preservation in formalin (Watanabe and Kuroki 1997). No remains of yolk-sac or oil globule were observed in larvae under 5 mm (preserved length) in any of the cruises. Larvae under this length were not used for condition analyses, but were included in the growth and mortality studies.

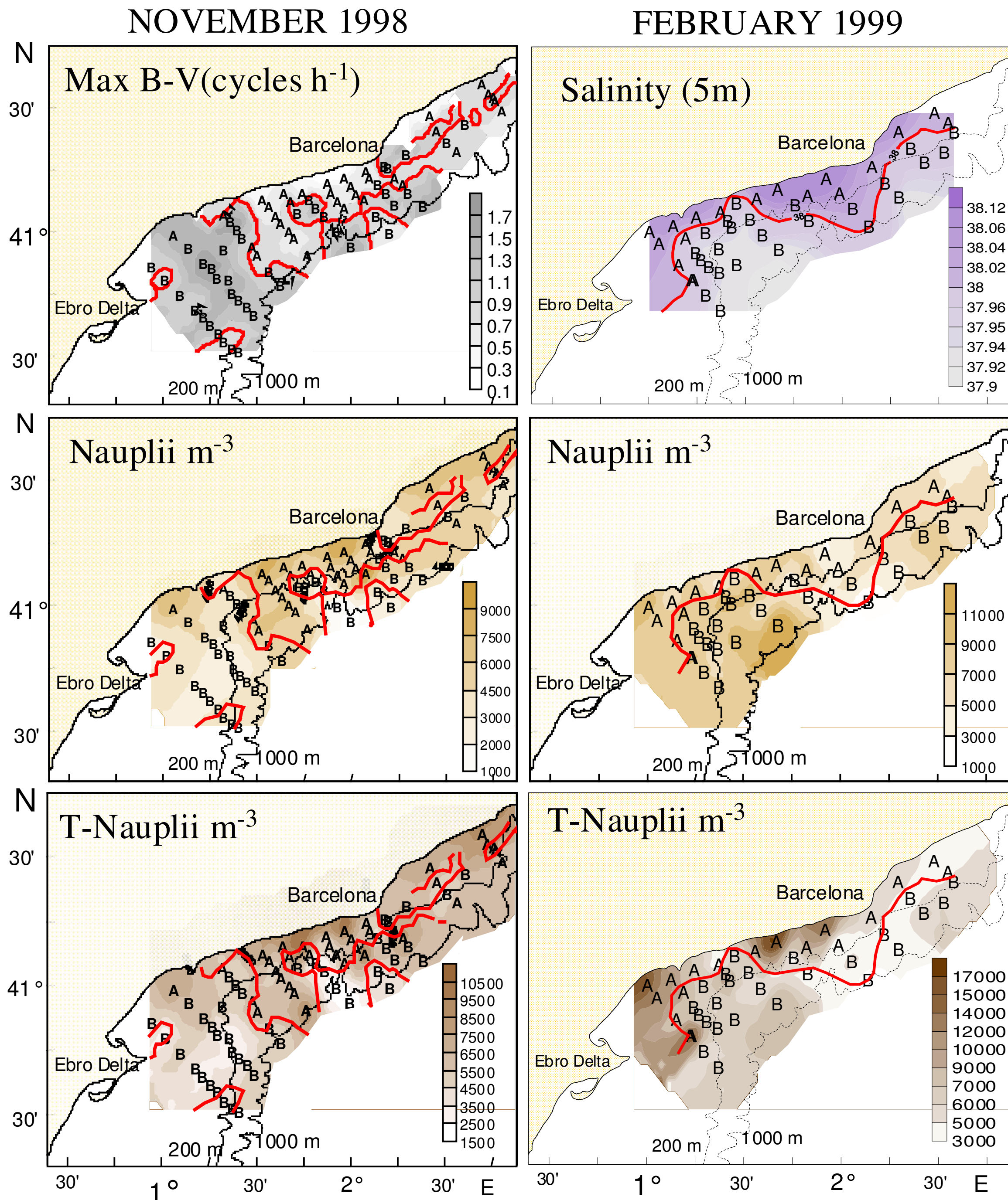

Figure 3.2.2. Classification of stations into environmental groups A or B, according to PCA.
For **November 98,** B-V frequency and the 2 types of food fractions are represented. Superimposed red line represents intermediate values of Maximum B-V.
For **February 1999**, salinity and the two fractions of potential prey are represented. Here, superimposed red line represent intermediate salinity values.
Lines do not necessarily embrace station groups.

Growth

Long-term growth was explored by 1) assessing the relationships between length, estimated age through otolith daily increment counts, and otolith radius, and 2) by studying the muscle growth cellularity along length. The hypothesis tested was that there were no significant differences between environmental groups (within-cruises) or between cruises in either type of growth.

Otolith analyses

Long-term growth in length of the grouped larvae from each environmental group or from each cruise was analysed by comparing standard body length to estimated age obtained through otolith readings. These data are part of a study which is currently being conducted by Palomera et al. (unpublished data). From each larva, both right and left sagittae were located and extracted under a dissecting microscope equipped with polarised light. Otoliths were rinsed in distilled water, allowed to dry and mounted with DPX onto slides. The number of daily growth rings and the maximum otolith diameter along the longest otolith axis was determined using an image analyser (OPTIMAS 6.0) connected to a PC through a CCD video camera. When possible, final estimation of age was made by averaging results from both sagittae. If one sagittae was difficult to read, data was obtained from only one sagitta, either left or right. The number of otoliths analysed is shown in Table 3.2.3. According to Alemany and Álvarez (1994), the number of presumptive daily rings was assumed to indicate age from hatching. The relationship between the examined variables and length was adjusted by either linear ($y=a+bx$), allometric ($y=ax^b$) or exponential ($y=ae^{bx}$) functions, whichever yielded the highest determination coefficient. Instantaneous growth in length (G_L, mm day^{-1}) or in otolith diameter (G_{OD}, µm day^{-1}) was calculated by taking the derivative for each equation for a given age.

Significance of each regression was tested through ANOVA, and differences in slopes and intercepts through ANCOVA by way of GLM (Minitab Inc.). With a view to standardisation, comparisons within each cruise were made between larvae ranging from 3

to 15 mm SL for November 1998, and from 3 to 16 mm SL for February 1999. Comparisons between cruises were made on larvae ranging from 3 to 15 mm SL.

Table 3.2.3. Otoliths analysed for each cruise and environmental group

Cruise	Environmental group	Number of larvae analysed	Mean larval SL (mm) ± SD	Number of stations used
November 1998	A	53	8.6 ± 2.57	13
	B	41	7.4 ± 2.78	7
	Total	94	8.1 ± 2.71	20
February 1999	A	45	10.03 ± 3.96	10
	B	20	11.3 ± 4.59	8
	Total	65	10.4 ± 4.17	18

Muscle growth variability

The data for the study of muscle growth variability was treated in a slightly different way due to the low number of stations available. In this case, for each cruise, we grouped the larvae into two categories defined only by a range of temperature and potential prey abundance (see further below). Temperature is known to be a major factor influencing muscle growth patterns (Johnston 1993; Galloway et al. 1999), and food has been suggested as a potentially important variable in this respect (Temple et al. 2000).

The hypothesis tested was that muscle cellularity, tested by several measurements, was not significantly different between environmental groups or between cruises. Histological analyses were conducted at the Gatty Marine Laboratory (St Andrews, Scotland), and at the Instituto de Ciencias del Mar (Barcelona). Larvae fixed in 10% phosphate-buffered formalin and preserved in 70% ethanol were processed for wax histology. Seven micrometer transverse sections were made from the whole larvae and mounted in poly-L-lysine coated slides. Analyses were performed on sections located at ca. 0.5 SL from the head region. Sections were de-waxed with xylene, rehydrated and stained with haematoxylin-eosin.

Data on 1) number of inner fibres, 2) number of superficial fibres and 3) the area of each inner fibre was taken using an image analyser (OPTIMAS 6.0) (Figure 3.2.3.). Larvae

were assumed to be bilaterally symmetrical, so measurements were taken from one half of the section and final values were multiplied by two.

Two groups of larvae were analysed for each cruise according to the amount of potential prey and the mean surface (5 m) temperature. We chose copepod nauplii to represent potential prey, as they are thought to make the bulk of ingestion items in larvae of the range considered herein (Conway et al. 1994). The two environmental factors could not be tested separately, so individuals were grouped as belonging to either a "Higher Food-Lower Temperature" group or a "Lower Food-Higher Temperature" group (Table 3.2.4.). For each cruise, food and temperature patterns were the opposite, so stations at lower temperatures always had higher food concentrations and *vice versa*. This made it possible to hypothesise that if a significant difference in muscle characteristics was observed between environmental zones and for the two years, and was always associated to a particular environmental group, then one could infer which variable (potential food or temperature) was probably exerting a greater effect on muscle growth. For both November 1998 and February 1999, the food abundance differed by about 100% between each zone. The temperature difference between zones was ca 1°C for November 1998 and ca. 0.5°C for February 1999. The temperature difference between the two cruises was around 6°C.

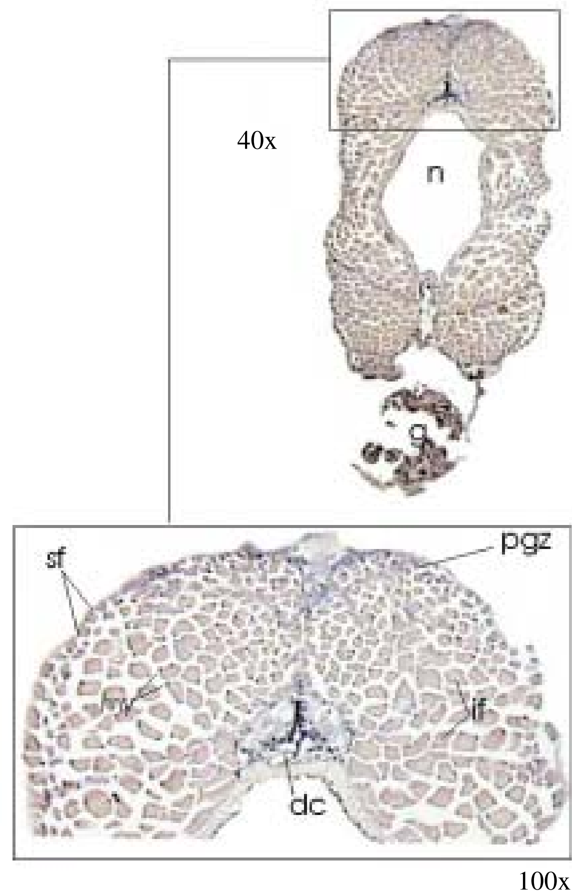

Figure 3.2.3. Transverse section of *Sardina pilchardus* stained with haematoxylin-eosin. Standard length = 10.26 mm. **dc**, dorsal chord; **g**, gut; **if**, inner fibres; **my**, myonuclei; **n**, notochord; **pgz**, presumptive germinal zone; **sf**, superficial

Table 3.2.4. Characteristics of the environmental features utilised for within-cruise comparisons (A) and between-cruise comparisons (B) in muscle growth parameters. Mean values for each environmental variable were calculated by assigning to each analysed larvae the temperature and number of potential prey of the station from which it was collected. N= number of larvae analysed

A	November 1998 (N=57)		February 1999 (N=51)	
	Environmental group		Environmental group	
	Higher food Lower T	Lower food, Higher T	Higher food Lower T	Lower Food Higher T
Nauplii m^{-3} (± SD)	5164 (1262)	2782 (828)	9300 (1380)	4397 (1040)
Mean surface Temperature in °C (± SD)	18.9 (0.34)	19.8 (0.25)	12.7(0.12)	13.1 (0.079)
Number of larvae analysed	40	17	21	30
Number of stations	6	10	5	4

B

Cruise	Mean T (°C) (SD)	Mean Naupli m^{-3} (SD)	Number of larvae	Number of Stations
November 1998	19.2 (0.47)	4439 (1500)	57	16
February 1999	12.9 (0.21)	6577 (2641)	51	9

Larvae between 3.5 and 13.5 mm SL were selected from each cruise and compared between zones (Table 3.2.4. A). Between-cruise comparisons were conducted only for the number of inner and superficial fibres as there was indication of higher muscle fibre shrinkage in February 1999, which might affect the fibre diameter for a given length.

Regressions between muscle parameters and length were adjusted by either linear, potential or exponential equations, and significance was tested by ANOVA on linearised (when appropriate) equations. Comparisons of slopes and means between areas or years was made by an ANCOVA by way of GLM (MINITAB Inc.).

The frequency distributions of the inner fibre areas was compared within each cruise in the two environmental areas characterised in Table 3.2.5. Two length groups (called “small” and “mid”) were made for each area and cruise. These groups were not comparable between cruises (Table 3.2.5.) but enabled a size comparison within each group, which was performed by the non-parametric Kolmogorof-Smirnoff test.

Table 3.2.5. Larvae used for inner fibre distribution analysis in the two cruises

		November 1998		February 1999	
		Environmental group		Environmental group	
		Higher food Lower T	**Lower food Higher T**	**Higher food Lower T**	**Lower Food Higher T**
	Mean surface Temperature in °C (± SD)	18.7	19.8 (0.20)	12.6 (0.05)	13.1 (0.10)
	Naupli m^{-3} (± SD)	6200	2515 (231)	9195 (1135)	5035 (606)
	Number of stations	1	3	3	3
Size group	**Small**				
	SL (mm) ± SD	4.71 ± 0.18	4.82 ± 0.27	4.09 ± 0.021	4.17 ± 0.099
	Nº larvae measured	3	3	5	3
	Nºfibres measured per larva (± SD)	37 (4)	26 (4)	28 (10)	32 (2)
	Mid				
	SL (mm) ± SD	6.52 ± 0.36	6.15 ± 0.44	5.26 ± 0.22	5.28 ± 0.26
	Nº larvae measured	3	3	4	4
	Nºfibres measured per larva (± SD)	36 (3)	33 (13)	36 (6)	31 (8)

Mortality

In order to make reasonable interpretations on mortality curves, the critical assumption of equilibrium spawning must be met. This means that the abundance of the oldest age-classes must be related to the previous age-structure. There was evidence from the previous cruise that November 1998 corresponded to the beginning of the spawning season, as shown by the large number of eggs and small larvae at some stations. In February 1999, the spawning season was around its theoretical maximum, and the structure of the population might be closer to equilibrium spawning during the weeks prior to collection.

Despite the latter considerations, to obtain a graphical visualisation of the data, we built age-frequency data from the length-frequency data and the otolith-derived age-length relationships for both environmental groups and cruises. Larvae between 4 and 16 mm SL were used. Smaller larvae were excluded from the analyses due to the possible extrusion phenomenon observed in Section 3.1. We ascribed a mean age to each size category, calculated every 0.5 mm. Total frequencies at age were given as mean number of larvae 10 m^{-2} per positive stations. Station 70 was excluded from group A in November 1998 because it exhibited anomalous amount of eggs and recently hatched larvae, indicative of recent spawning. Mortality coefficients were not calculated for November 1998 due to the lack of

reasonable evidence for constant spawning. For February 1999, the abrupt drop in apparent mortality prevented us from fitting negative exponential fits, and presumptive mortality was calculated by difference at two ages.

Histological indices

The condition of larvae from the predefined environmental groups A and B was analysed for both November 1998 and February 1999. Also, correlations between the 12 environmental variables and condition of the larvae was assessed, separated by two size-classes using a 8 mm cutpoint. The separation into size classes was conducted in order to allow for possible differences in tissular organisation levels.

The tissues analysed were divided into short and long-term indices. This grouping responds to the different response time displayed by each tissue in several species reared in the laboratory (Kashuba and Mathews 1984, Theilacker 1986). Also, a weighted index was implemented, following the idea of assigning differential susceptibility to mortality to different indices or tissues, as explained in Section 2.3. (Fig. 2.3.10.).

Larvae older than the yolk-sac stage were processed for Historesin (Leica) embedding as described in Section 2.3. They were sectioned at 3 m in an approximate sagittal plane and stained with Lee's methylene blue – basic fuchin (Bennet 1976). Larvae showing strong bents were sectioned in two steps, rotating the resin block to the appropriate position.

Of all the larvae analysed, a large portion could not be fully interpreted histologically. These larvae had missing guts that were probably lost during net collection. This explains why the amount of larvae analysed for short-term indices was lower than that for long-term indices (Table 3.2.6.). The number of larvae analysed for February 1999 was lower than that of November 1998. This was due partly to the lower larval abundance, as shown in Section 3.1. Some considerations about histological problems encountered in February 1999 ought to be further developed. These samples were characterised by pronounced shrinkage of muscle and general overstaining of the tissues. We disregard the trawling speed or onboard sorting time as possible causes of tissue degradation as they were

similar to those of November 1998, for which no alterations or unusual shrinkage were evident. However, preservation time could have played a determinant role in the fixation product. Larvae from February 1999 had been transferred to ethanol almost two months after collection, about twice the time that elapsed for November 1998 larvae. It is possible that a degradation of the fixing medium prior to the alcohol transfer occurred. Although the utilisation of 10% buffered formalin has several advantages (it produces low shrinkage, allows for relatively long preservation before transference to alcohol, reduces sample washing before processing etc.), it has some drawbacks. One problem of formaldehyde is the slowly increasing concentration of methanol (an unwanted by-product of ageing formaldehyde). Methanol promotes clumping of proteins, which could have accounted for the anomalously dark coloration of the samples, particularly the muscle.

Table 3.2.6. Larvae used for histological analyses, classified by type of index, size-class and cruise

CRUISE	Nº OF ST. USED	SHORT-TERM INDICES Larval size-class (SL, in mm)						LONG-TERM INDICES Larval size-class (SL, in mm)						WEIGHTED INDICES Larval size-class (SL, in mm)					
		8mm			> 8mm			8mm			> 8mm			8mm			> 8mm		
		N	Mean SL	SD	N	Mean SL	SD	N	Mean SL	SD	N	Mean SL	SD	N	Mean SL	SD	N	Mean SL	SD
Nov. 98	**36**	42	6.6	1.05	47	10.1	2.02	78	6.5	0.93	100	10.5	2.28	49	6.8	0.74	92	10.5	2.27
Feb. 99	**25**	16	6.3	0.92	42	14.2	2.94	28	6.1	1.16	51	13.4	2.95	16	6.5	0.74	42	14.2	3.00

Selection of indices and histological grading

We used both qualitative and quantitative histological measurements. Qualitative histological criteria used in this study were derived from both the available information in the literature on both pilchard larvae (Uriarte and Balbontin 1987; Silva and Miranda 1992; McFadzen et al. 1997) and other clupeids (O'Connell 1976; Sieg 1998). Qualitative measurements were made on cartilage, pancreas, liver and midgut. These measurements were classified into nutritional grades according to various cell and tissular characteristics, from 1 (poor condition) to 3 or 4 (good condition) (Table 3.2.7., Fig. 3.2.4., Fig. 3.2.5.). Also, two quantitative measurements were incorporated. One was the percentage muscle fibre separation (MFS). It was chosen over the classical muscle scoring for its best performance in laboratory rearing (Catalán and Olivar 2002, see Section 2.3.).

Table 3.2.7. . Histological traits selected to analyse *S. pilchardus* larvae.

LONG-TERM INDICES

Organ/tissue and nature of the measurement	Feature measured	Division into nutritional grades		
		Grade 3	**Grade 2**	**Grade 1**
Cartilage (qualitative)	Capsular cavities and matrix	Large nuclei. Cytoplasm occupying most of the capsular space	Some nuclei condensed at several degrees. Cytoplasm frequently reduced, occupying only a small portion of the capsular space.	Condensed nuclei. Cytoplasm often absent or severely reduced.
MFS (only Nov 98) (quantitative)	Fibre separation	0-9 %	9.1-17%	Over 17.1%

SHORT-TERM INDICES

Organ/tissue and nature of the measurement	Feature measured	Division into nutritional grades			
		Grade 4 (only for hepatocyte intracellular space)	**Grade 3**	**Grade 2**	**Grade 1**
Pancreas (qualitative)	Zymogen /pancreas ratio Pancreatic sinus Cell (acini) boundaries		High, >30% Always full and rel. large Well defined	Mid, 20-30% Partly filled and/or coarse appearance Poorly defined.	Small, < 20% Sinus almost empty or extr. coarse Indistinguishable
Hepatocyte intracellular space (qualitative)	Vacuolisation Inclusions Cell boundaries	Conspicuous. Absent Marked	Reduced Some acidophilic ones Less distinguishable	Only occasionally, small Abundant Barely distinguishable	Absent, cells compact Inclusions filling cytoplasm Indistinguishable
Hepatocyte nuclei (qualitative)	Size and placement Stain		Large and marginal Lightly stained	Large, some not marginal Some stain darkly in blue.	Pycnotic Darkly stained.
Midgut integrity (qualitative)	Midgut epithelium		No separation between cells. Light cytoplasm. Supranuclear inclusions	Gaps between basal membrane and enterocytes. Cytoplasm often dark	Cells frequently dark, small and detached from each other and from the basal membrane
Residuals of the foregut specific surface against SL (quantitative) (only Nov 98)	Specific cell surface		-0.3 a –0.06	-0.059 to –0.08	-0.081 to 0.3

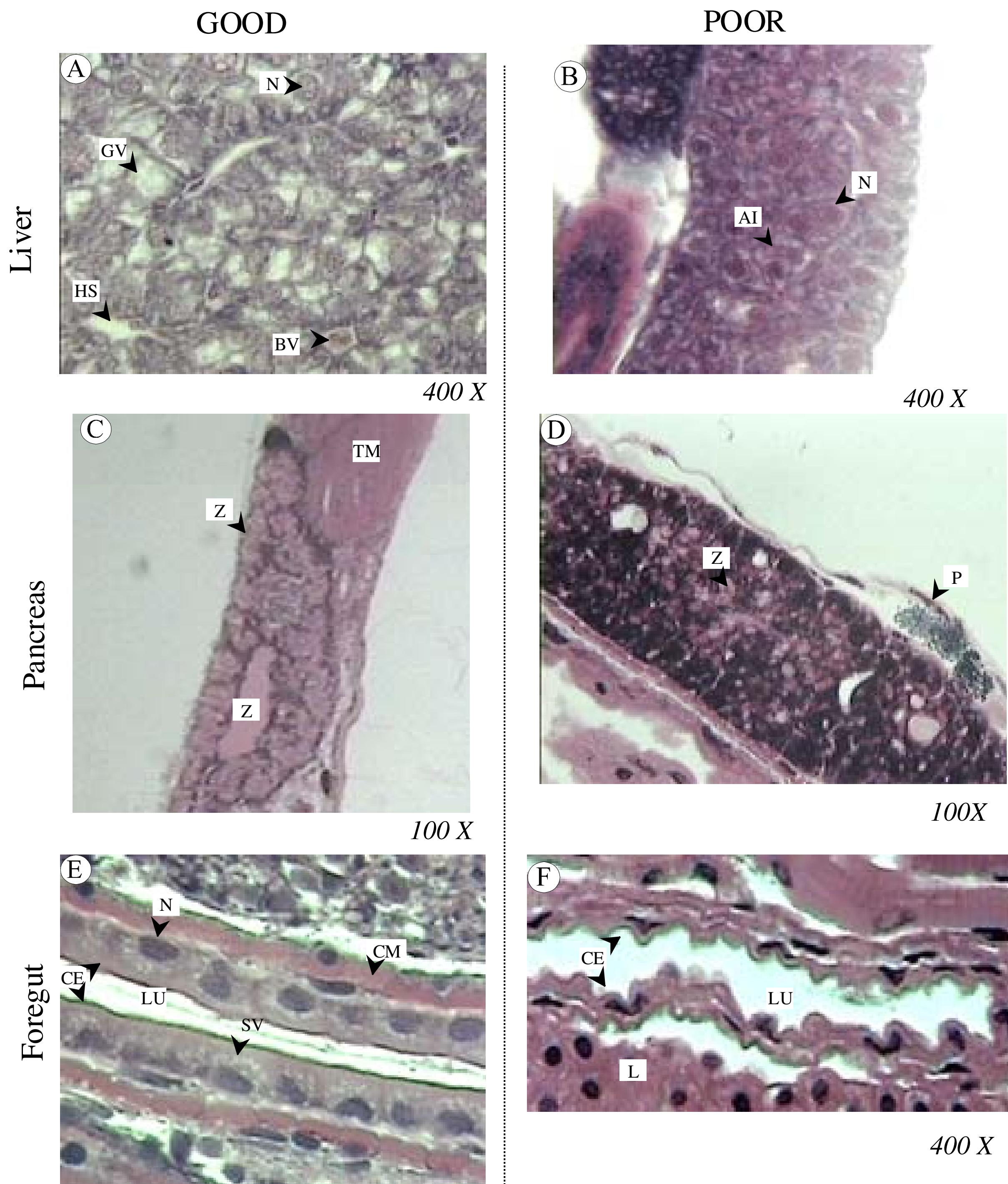

Figure 3.2.4. Examples of healthy and degraded tissues (short-term response) of *S.pilchardus* larvae stained with methylene blue-basic fuchsin 3 Φm sections in the sagital plane. Grades for the "good" tissues are all= 3. Grades for the "poor" tissues are all = 1 except liver, which is = 2 (see Table 3.2.2). A and B, liver: note the difference in vacuolation. C and D, pancreas: note the difference in zymogen abundance. E and F, posterior part of the foregut: note the loss of cell integrity and increase in folding of the degraded tissue. **AI**, acidophilic inclusions; **BB**, brush border; **BM**, basal membrane; **BV**, blood vessel; **CE**, cuboidal epithelium; **CM**, circular musculature; **CS**, capsular space; **FG**, foregut; **GC**, goblet cell; **GV**, glycogen vacuole; **HS**, hepatic synus; **IS**, interfibrillar space; **L**, liver; **LU**, luminal space; **MA**, matrix; **MG**, midgut; **N**, nuclei; **P**, pigment; **SV**, supranuclear vacuoles; **TM**, trunk muscle; **Z**, zymogen. Magnification is indicated under each image.

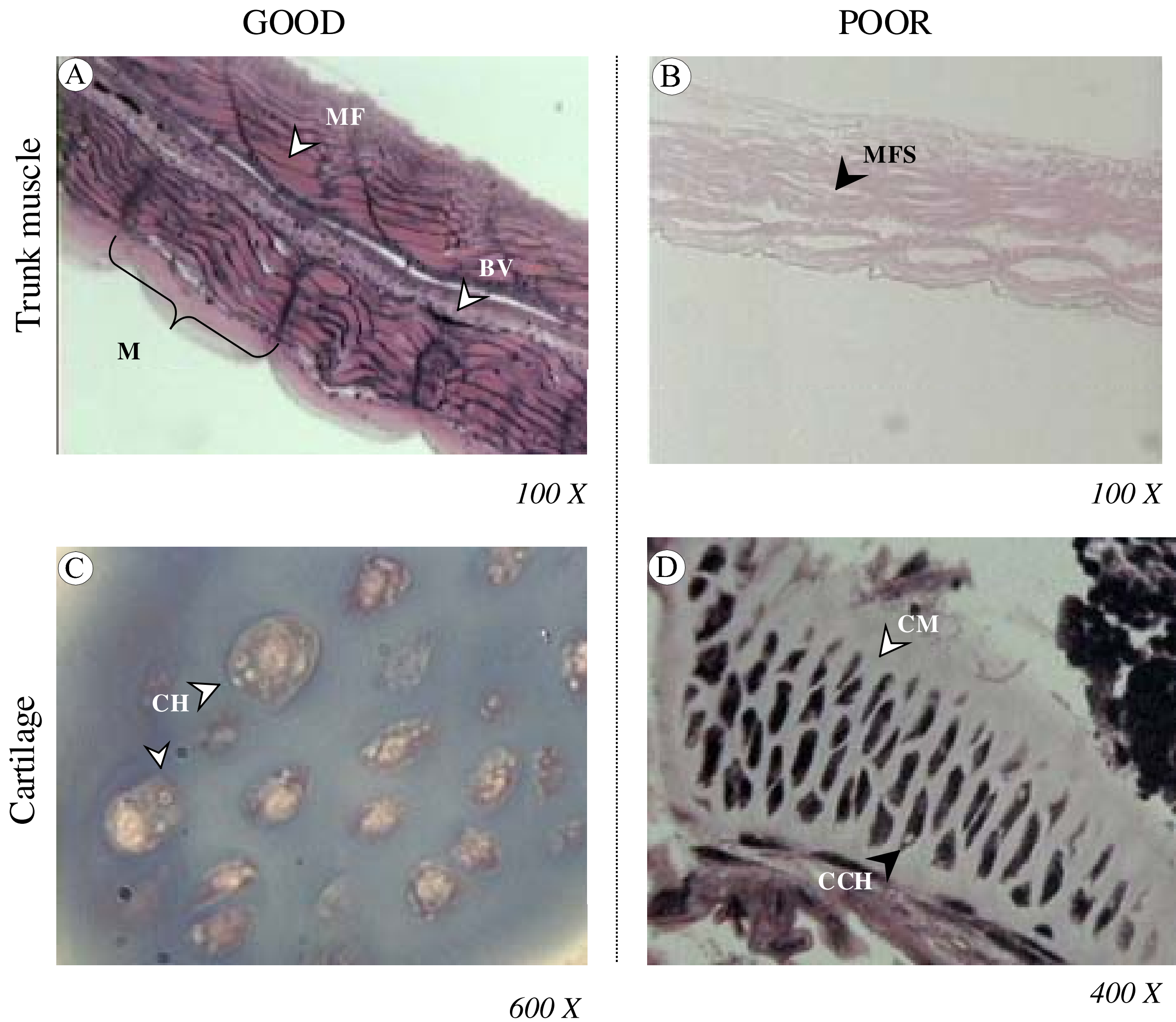

Figure 3.2.5. Examples of healthy and degraded tissues (long-term response) of *S. pilchardus* larvae stained with methylene blue-basic fuchsin 3 Φm sections in the sagital plane. Grades for the “good” tissued are all 3. Grades for the “poor” tissues are all (see Table 3.2.2). A and B, trunk muscle: note the difference in muscle fibre separation. C and D, cartilage of the buccal cavity: note collapsed matrix of the degraded cartilage. **BV**, blood vessel; **CCH**, condensed chondrocytes; **CH**, chondrocytes; **CM**, collapsed mattrix; **M**, myotome; **MF**, muscle fibre; **MFS**, muscle fibre separation. Magnification is indicated under each image.

The second quantitative measurement was the ratio between the perimeter of a section of the foregut epithelium and its contained cellular area (foregut specific surface) (Fig. 3.2.6.). This measurement was utilised by McFadzen et al. (1994) in larvae of *Scophtalmus maximus*. Its use was advantageous for our study as it enabled the study of specimens at an advanced developmental stage that usually showed a folded midgut.

Moreover, the foregut was usually present in specimens that had damaged mid and hindguts.

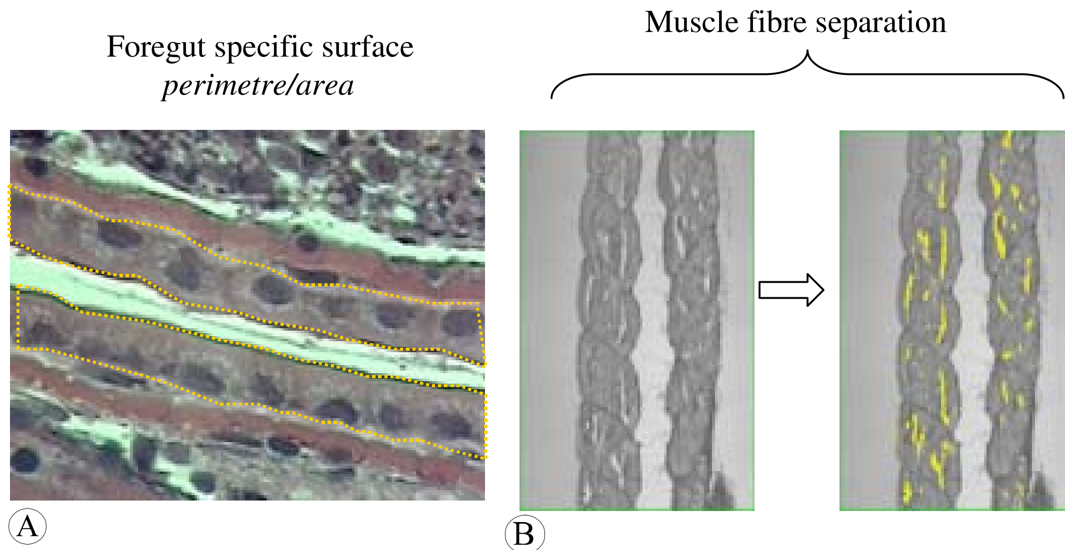

Figure 3.2.6. Determination of the quantitative measurements. A, foregut specific surface. In yellow dotted line, example of area that would be selected. B, quantification of MFS (%) after applying a Macro from Optimas.

For the classification into grades of MFS and foregut specific surface, for which no literature information was available, we used data of several pilchard larvae kept without food that were hatched onboard during the February cruise. Although hatching was around 90%, mortality was extremely high and only 2 larvae were recovered on day 8 after hatching. They showed severe signs of emaciation and no yolk was present. The values of MFS and foregut specific surface of these starved larvae were used as reference values. MFS was 19 and 23% in these larvae. Specific surface was 0.41 (only one larvae readable).

MFS was classified into nutritional classes taking into consideration both the laboratory data and images taken from the literature (Figures from Silva and Miranda 1992 and McFadzen et al. 1997). Intervals are shown in Table 3.2.7. The grading of the Foregut specific surface was made at three equal intervals. Foregut specific surface was the only measurement that significantly correlated with size even within each of the two larval size-groups (r_p^2 was between –0.41 and –0.29, $p<0.001$). Therefore, residuals of the foregut perimeter/area against ln-SL were used to overcome this problem (Table 3.2.7.).

In the final decision step of what measurements should be used in the short, long-time and weighted indices, we optimised for the maximum number of larvae analysed. For the determination of the pancreas and midgut integrity, there was a lower number of readable larvae than for the readings of liver scores and foregut specific surface. Therefore, we chose the latter. MFS and cartilage were easily readable in most larvae so we included the two for the analyses.

For November 1998, we used ((MFS+Cartilage scores)/2) as long-term indices, and ((foregut perimeter/area + liver scores)/2) as short-term measures. The weighted index was made using only MFS and liver scores (corresponding to steps 2 and 3 respectively in Fig. 2.3.10). The results were compared with the short and long-term indices.

Measurements for February 1999 were restricted to Cartilage (long-term) and Liver scores (short-term), which retained recognisable characters of emaciation in their cell structure despite the differential fixation effects. The weighted index was therefore made of these two measurements.

A last criterion of condition was necrosis. A separate record of any tissue showing necrotic areas was kept. We interpret the existence of necrosis as a sign comparable to the concept of “point of no return” (PNR) (Blaxter and Hempel 1963). It was assumed that if a larva exhibiting necrotic tissues was to encounter sufficient prey, it would not be able to conduct efficient digestion and assimilation and would probably die (McGurk 1984). All tissues and sections from each larva were looked at in order to detect necrosis. The criterion of necrosis was incorporated in the weighted index as the lowest possible outcome of the analysis (Fig. 2.3.10., step 1).

Biochemical indices

Larvae frozen in liquid nitrogen were transported to the laboratory for protein and nucleic acid analyses. The methods used herein are those described in Section 2.4. Nucleic acid analyses was conducted for both cruises. Protein analyses were available only for February 1999 (Table 3.2.8.).

The condition indices explored were RNA/DNA for November 1998 and RNA/DNA plus protein/DNA for February 1999. The relationship between size and the condition indices was explored through correlation (Pearson) analysis. If no significant correlation was found, larvae were compared between areas A and B defined at the beginning of Materials and Methods. These within-group studies were made through T-test, after checking for normality (Anderson-Darling) and homogeneity of Variance (F-test). Indices correlating significantly with size were compared through the aforementioned ANCOVA procedure. Also, relationships between each environmental variable and condition indices were compared through non-parametric correlations (Spearman). The level of significance was set at <0.05.

Comparisons between cruises were performed on RNA/DNA ratios in larvae from a common size-range (4.8-16.0 mm).

Table 3.2.8. Number of larvae used for the biochemical indices of condition. A; within-cruise comparisons. B; between-cruise comparisons (common size-range).

A

Cruise	Environmental group	N° of larvae used for RNA/DNA	SL ± SD	N° of larvae used for Protein/DNA	SL ± SD
	A	76	8.9 ± 2.26	-	-
November 98	B	40	8.2 ± 2.24	-	-
	Total	116	8.7 ± 2.27	-	-
	A	43	12.7 ± 4.65	26	13.9 ± 2.63
February 99	B	36	12.9 ± 2.49	29	13.5 ± 2.13
	Total	79	12.8 ± 3.79	55	13.6 ± 2.37

B

Cruise	N° of larvae used for RNA/DNA	SL ± SD
November 1998	116	8.7 ± 2.27
February 1999	66	11.6 ± 2.73

RESULTS

Growth

Otolith analyses

November 1998

All performed regressions were significant (ANOVA; $p<0.0001$). For both groups A and B growth in length was best described by a linear function (Fig. 3.2.7., top-left). G_L (mm day^{-1}) was 0.77 for group A and 0.63 for group B but there were no significant differences in slopes or intercepts between both areas.

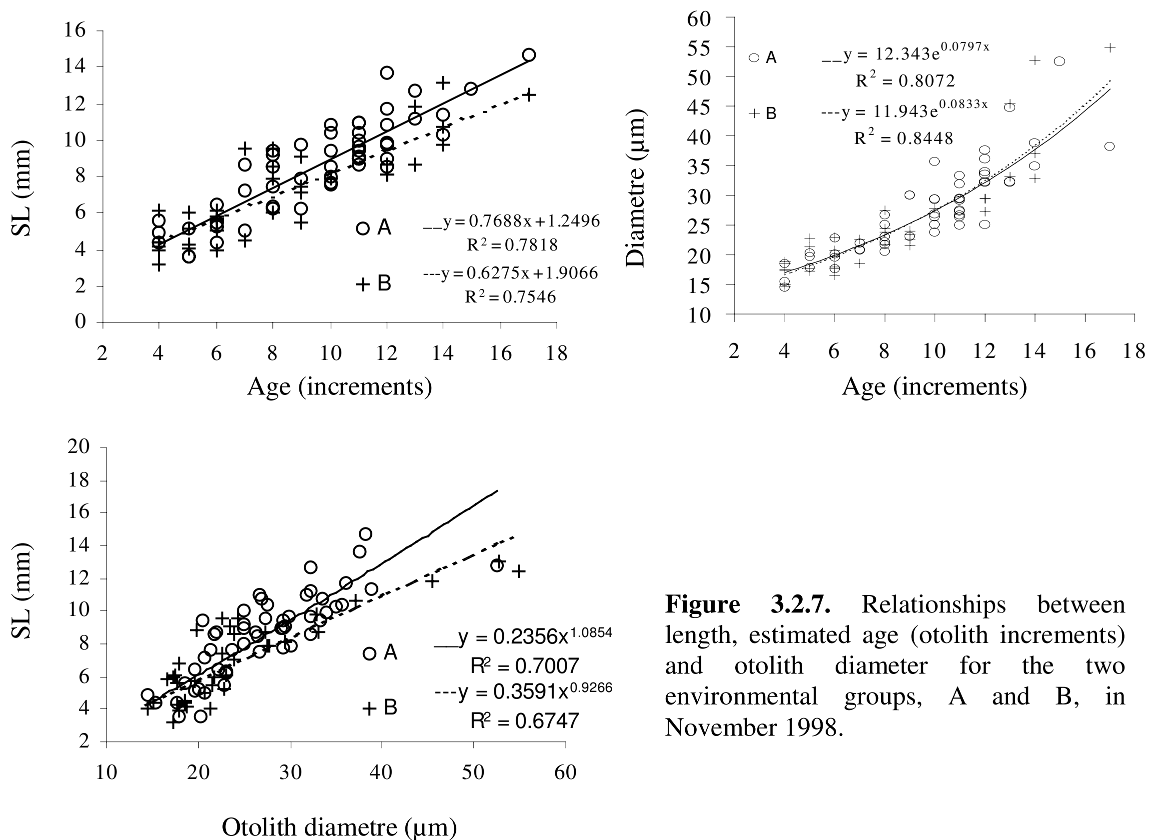

Figure 3.2.7. Relationships between length, estimated age (otolith increments) and otolith diameter for the two environmental groups, A and B, in November 1998.

The otolith diameter increased following an exponential equation (Fig. 3.2.7., top right). There were no significant differences between the two environmental groups. The length-otolith diameter relationship adjusted well to a potential equation for both

environmental groups (Fig. 3.2.7., bottom-left). The significant regressions between SL and otolith diameter indicate that otolith size encompassed somatic growth. Although there were no significant differences between slopes in both groups, the mean length at a given otolith diameter was significantly higher for group A (ANCOVA; F= 4.33, $p<0.05$). The interpretation of these results must be approached with caution (see discussion).

February 1999

All regressions were significant (ANOVA, $p<0.0001$). We found no significant differences between environmental groups in any of the comparisons performed. Potential functions were best adjusted to the length/age relationships (Fig. 3.2.8., top left). For both

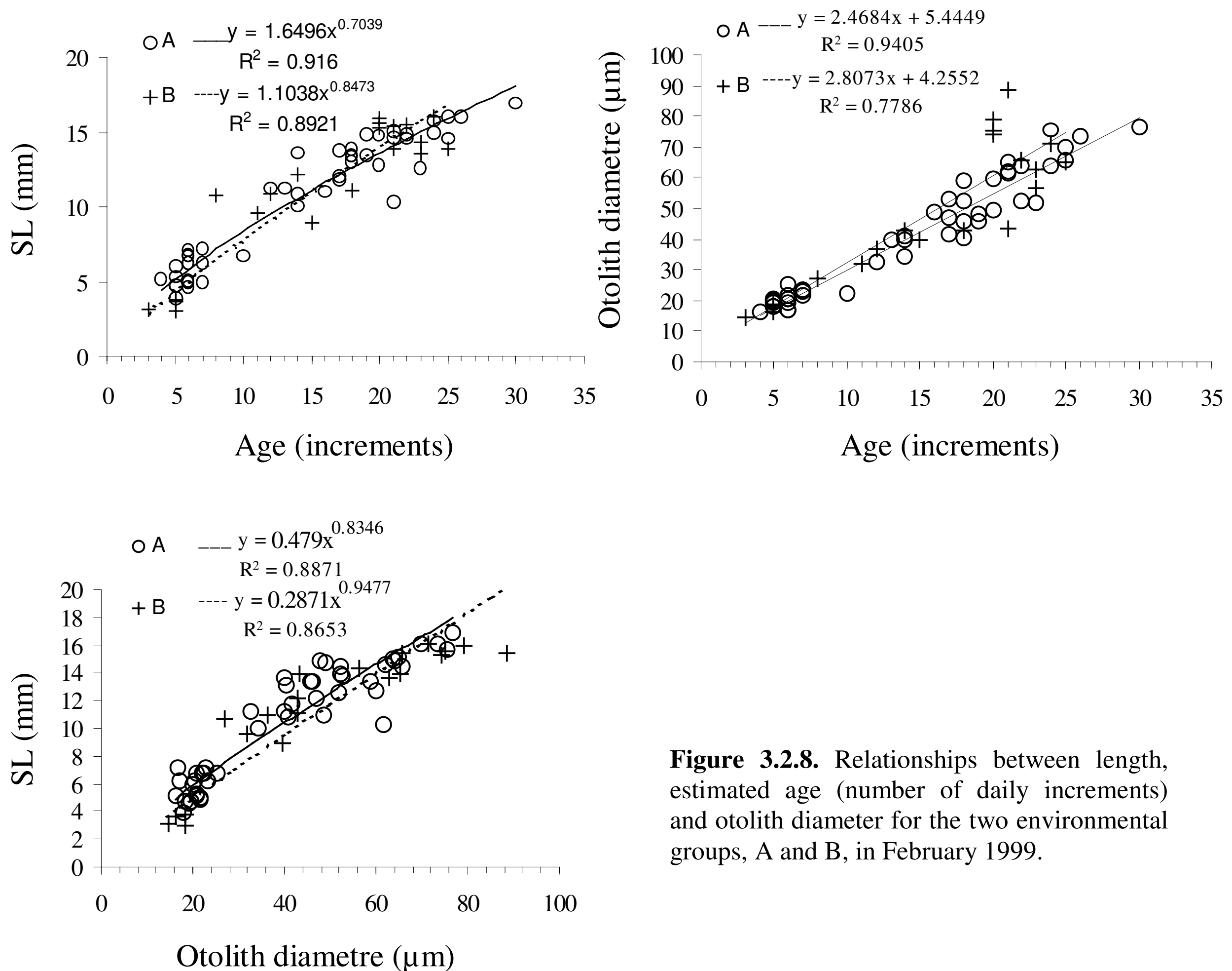

Figure 3.2.8. Relationships between length, estimated age (number of daily increments) and otolith diameter for the two environmental groups, A and B, in February 1999.

groups, otolith diameter increased linearly with age (Fig 3.2.8., top right), whereas body length related to otolith diameter through a potential function (Fig. 3.2.8., bottom).

Comparison between cruises

The two cruises were compared by pooling all larvae, taking the same length range in each cruise. All regressions were significant (ANOVA, $p<0.0001$).

Length related significantly to age by a potential fit (Fig. 3.2.9., top left). G_L was higher at young ages (Day 5: G_L for November 1998=0.79 , G_L for February 1999=0.64), than at older ages (Day 15: G_L for November 1998=0.71 , G_L for February 1999= 0.53). Although statistical difference could not be established, our data suggests a possible older age for a given length in larvae between 8 and 15 mm for February 1999, suggesting a slower growth.

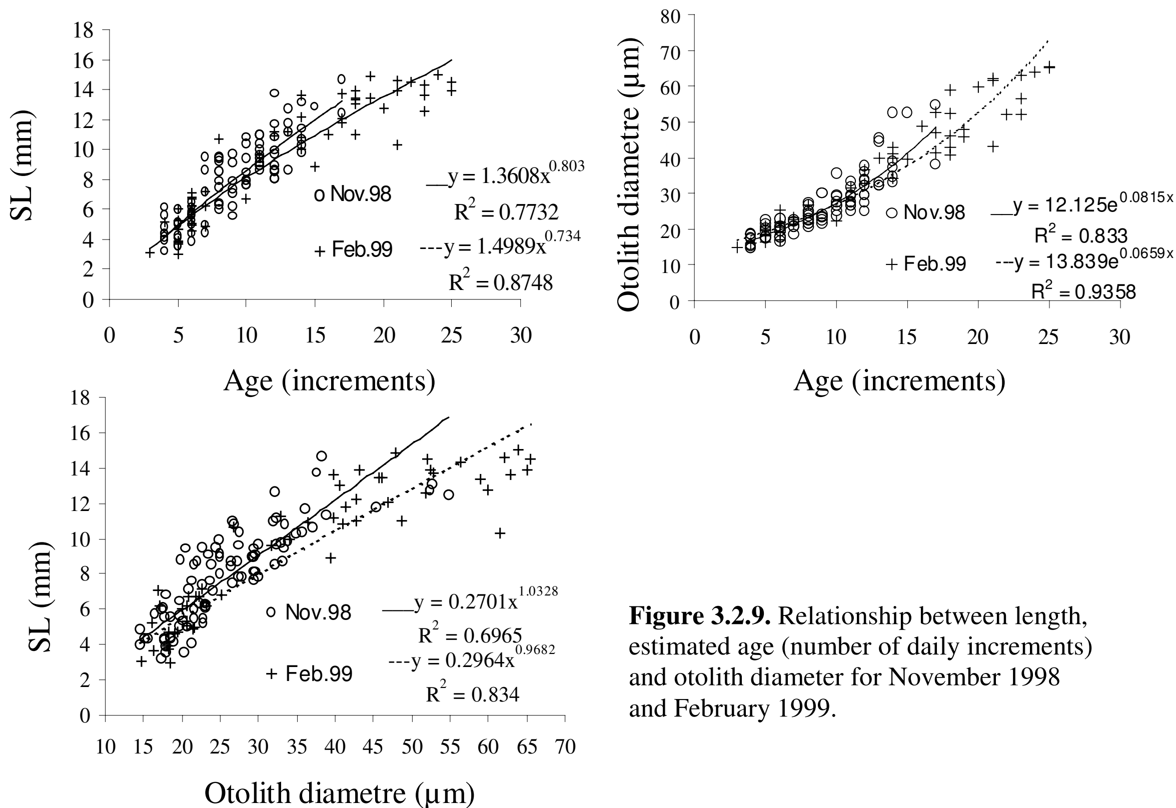

Figure 3.2.9. Relationship between length, estimated age (number of daily increments) and otolith diameter for November 1998 and February 1999.

For the length-range considered, the otolith diameter increased exponentially with age in both cruises (Fig. 3.2.9., top-right). This long-term otolith growth was lower for

younger ages (Day 5: G_{OD} for November 1998 = 1.02, G_{OD} for February 1999 = 0.87) than for older ages (Day 15: G_{OD} for November 1998 =3.05, G_{OD} for February 1999=2.60). We were unable to find statistically significant differences between slopes or means between cruises. However, Fig. 3.2.9. suggests that, for the length-range considered, larvae from February 1999 tend to be older, and have larger otoliths than their November 1998 counterparts.

Somatic growth was reflected in otolith diameter. Figure 3.2.9. (bottom) shows that changes in length, rapid between 3.5 and 10 mm, were accompanied by fast increases in otolith diameter.

Muscle growth variability

For both cruises, changes in number of inner fibres and maximum inner fibre area increased exponentially with larval length, whereas the number of superficial fibres fitted a potential equation with $b < 1$, hence reducing its rate of increase at larger lengths, consistently with surface-length associated growth properties.

November 1998

For both environmental groups, regressions between the studied variables and length were all significant (ANOVA, $p < 0.005$).

For a length ranging from 3.5 to 13.5 mm SL, the number of inner fibres increased from 78 to 750 fibres per section, and the number of superficial fibres from 40 to 100. Maximum inner fibre area increased from 27 to 250 m^2 (Fig. 3.2.10.).

No significant differences were found in either slopes or intercepts between the two environmental groups.

The histogram analysis of inner fibre area distributions (Fig. 3.2.11.) showed that, for the small larvae (ca. 4.7 mm SL) and mid larvae (ca 6.3 mm SL), increased environmental temperature was associated with higher mean inner fibre cross-sectional areas (distributions shifted to the right). The small larvae had a unimodal fibre distribution peaking between 20-25 m^2 (Higher food-Lower T group,) and 25-30 m^2 (Lower food, Higher T group).

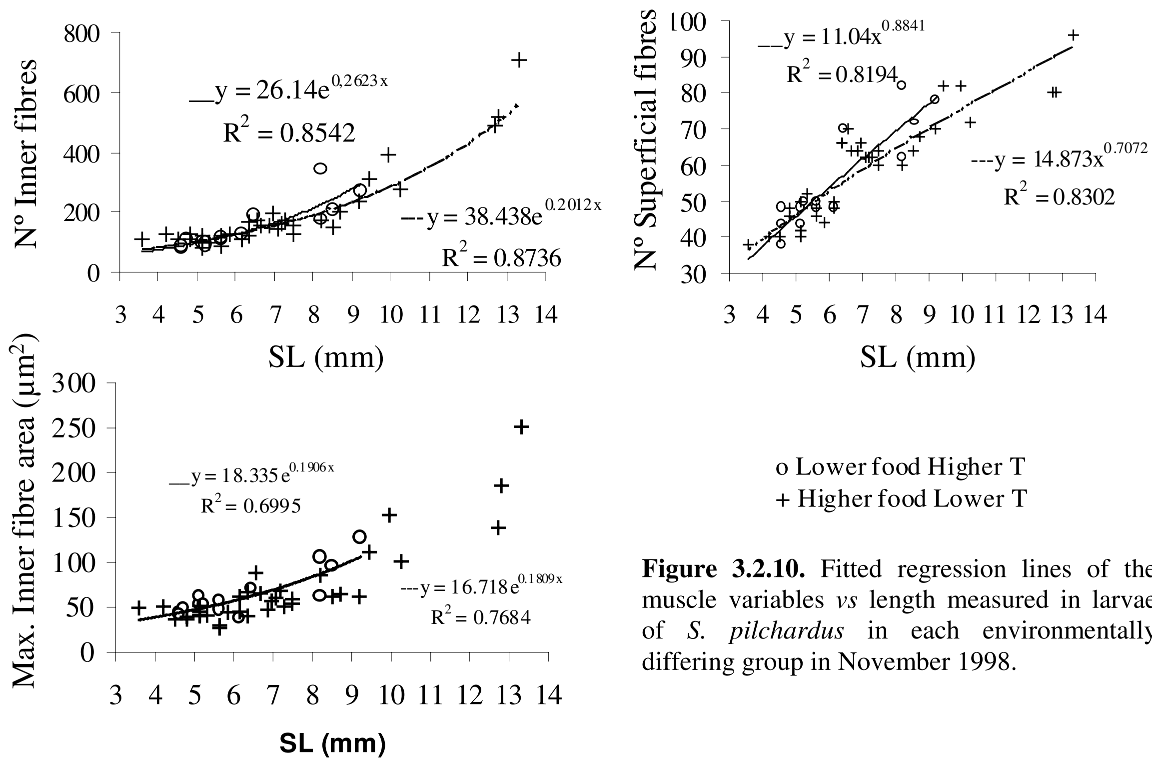

Figure 3.2.10. Fitted regression lines of the muscle variables *vs* length measured in larvae of *S. pilchardus* in each environmentally differing group in November 1998.

Larvae of ca. 6.3 mm showed increased within-environmental group variability although medians showed similar values to those of small larvae (Fig. 3.2.11. B). The Kolmogorof-Smirnoff test was not significant between environmental groups in the small larvae (p=0.069), and it could not be performed in the mid group due to the high variability between individuals of the same environmental group. However, we believe that the graphical results enable one to suspect that a 1°C difference in temperature has a greater effect on inner muscle fibre areas (hypertrophic growth) in larvae of these size ranges than food abundance.

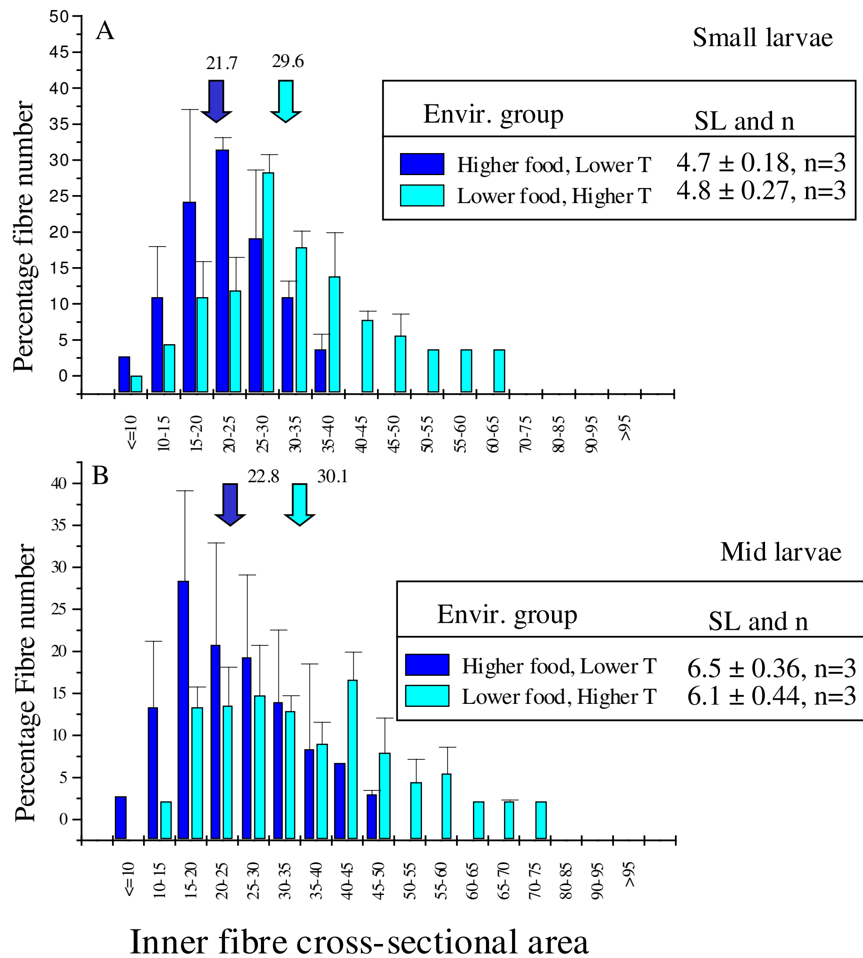

Figure 3.2.11. *S. pilchardus* larvae frequency distribution of inner fibre cross-sectional areas of two size-classes (small and mid) collected from two groups of stations differing in surface temperature and potential prey abundance in November 1998. Arrows indicate medians. Error bars are SD. Bars without error bar indicate that they belong to only one specimen.

February 1999

The mean surface temperature on this cruise was around 6°C lower than in November 1998. Temperature differences between environmental groups were lower than in 1998 (ca. 0.5°C in February, *vs* 1°C in November), but the abundance of potential food within groups was around twice that in 1998 (Table 3.2.5.).

All regressions of the analysed variables *vs* length were significant (ANOVA, $p<0.001$).

The number of inner fibres increased from ca. 70 to 450 in larvae measuring between 3.5 and 13.5 mm SL (Fig. 3.2.12.). The number of superficial fibres increased from 25 to 100, and the maximum inner fibre area from 30 to 350 µm^2. Larvae collected from stations with higher potential food and slightly lower temperature had a significantly higher number of inner fibres (ANCOVA (length as a covariate) F=14.42, P<0.001). Slopes were not significantly different. No significant differences in either slopes or intercepts was found in the other relationships. This suggests a possible effect of potential food abundance in the number of inner fibres.

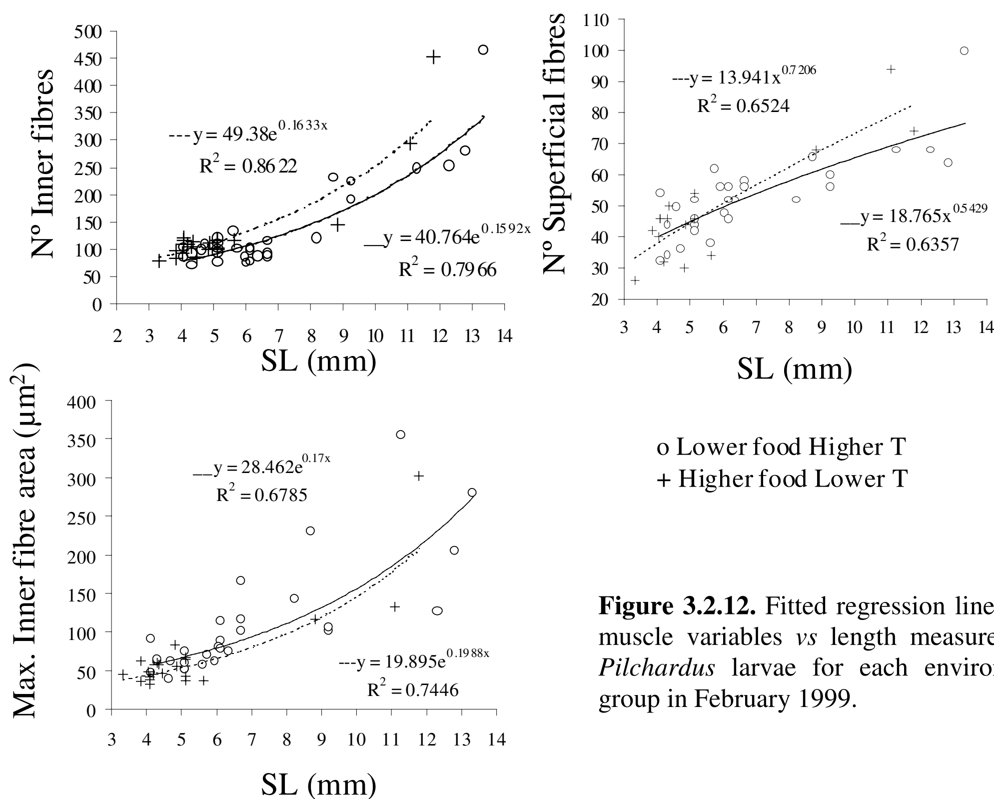

Figure 3.2.12. Fitted regression lines of the muscle variables *vs* length measured in *S. Pilchardus* larvae for each environmental group in February 1999.

The histogram analysis of fibre area distribution showed a pattern similar to that for November 1998 (Fig. 3.2.13.). Significant differences were not found. However, differences between environmental groups seemed smaller than in November 1998. The Medians of the inner fibre areas tended to be higher in larvae collected from lower food-higher T stations. Small larvae of ca. 4.15 mm showed a distribution peak between 20 and 30 m^2. From these small-size group, larvae from warmer-higher food areas had a greater

proportion of fibres over 45 m^2 (Fig. 3.2.13. A). These differences were not observed in the mid group (Fig. 3.2.13. B). No significant differences between environmental groups were detected through the K-S test.

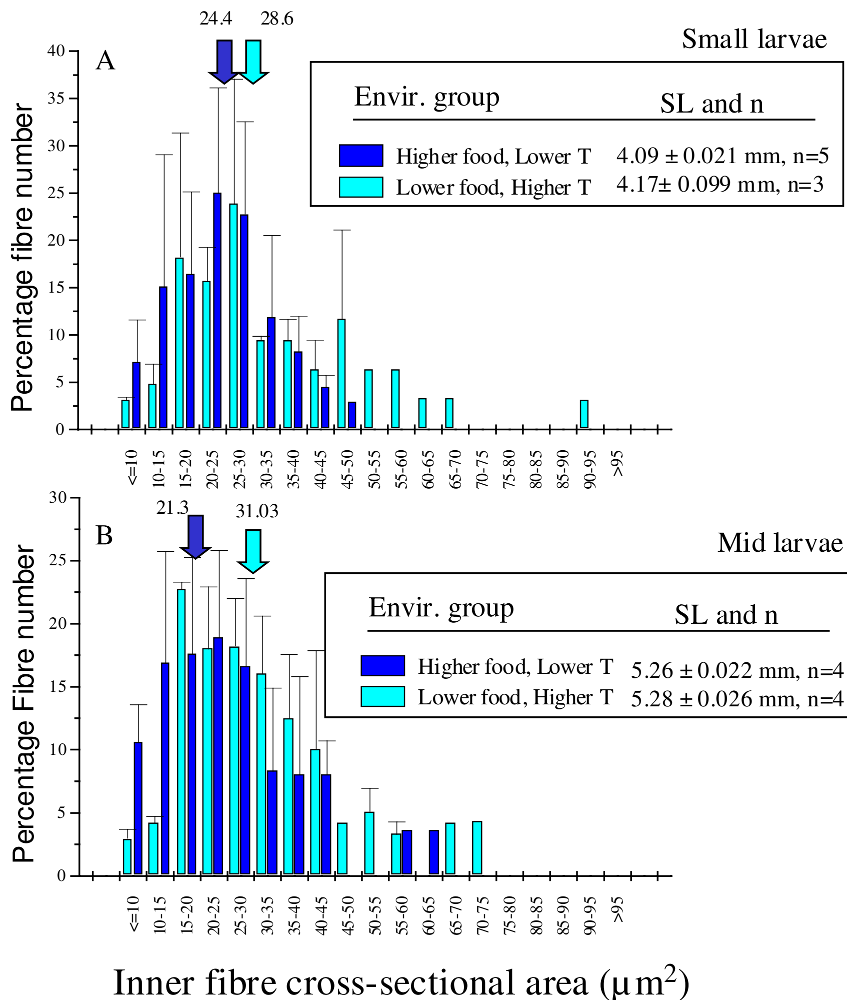

Figure 3.2.13. *S. pilchardus* larvae frequency distribution of inner fibre cross-sectional areas of two size-classes (small and mid) collected from stations differing in surface temperature and potential prey abundance in February 1999. Arrows are Medians. Error bars are SD. Bars without error bar indicate that they belong to only one specimen.

Comparison between cruises

Between-cruise comparisons (Fig. 3.2.14.) were performed in shrinkage-independent variables (number of inner and superficial fibres). Larvae from November 1998 (mean T=19°C) showed a significantly higher rate of increase of inner fibre number

(ANCOVA, F=13.43, p<0.005). Also, the mean number of superficial fibres was significantly higher for November 1998 (ANOVA, F=4.74, p<0.05), although the slopes did not differ. These results suggest that larvae from November 1998 had an overall faster muscle growth than larvae from February 1999. From Table 3.2.5., it is clear that the analysed larvae from November 1998 had been collected in an environment with less food than larvae from February 1999. On the other hand, surface temperature was 6ºC higher in November 1998. This suggests that temperature might be the prevailing factor in determining muscle growth under the studied conditions. Considerations about the type of muscle growth can also be drawn and will be addressed at the discussion section.

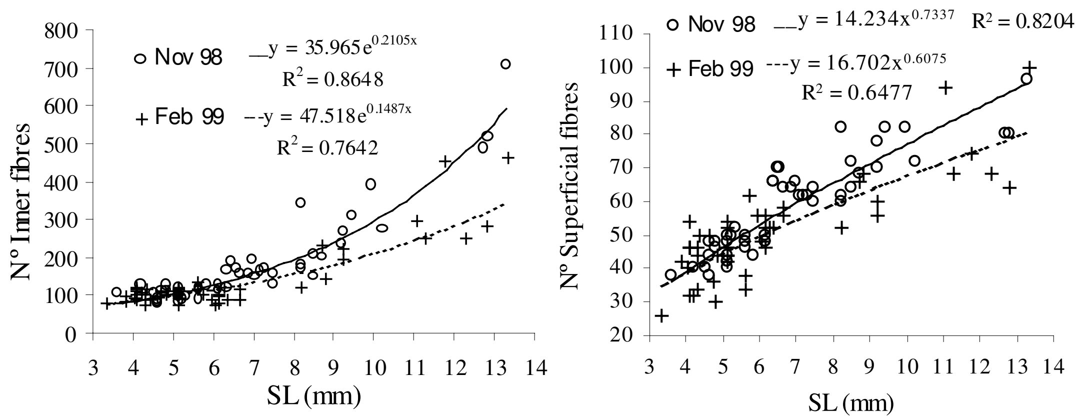

Figure 3.2.14. Comparison of the fitted regression lines on the number of inner and superficial fibres vs length measured in larvae of *Sardina pilchardus* in cruises of November 1998 and February 1999.

Mortality

Age-frequency distributions for November 1998 showed that mean larval abundance was around a higher order of magnitude in group A than in group B (not shown). This contradicts one of the assumptions for interpreting mortality from this kind of data, a reasonably constant spawning through time and space (Pepin 2002). However, it suggests that a positive environmental selection by spawners in environmental group A might have occurred.

In February 1999, the relative decline in larvae around 5 days old was abrupt (Fig. 3.2.15) and similar between groups A and B (not shown). The number of larvae over 6 days old was extremely low, which is unexpected for the peak of the spawning season. The reasons for both the sharp decrease in abundances at young ages and the low number of older larvae will be discussed.

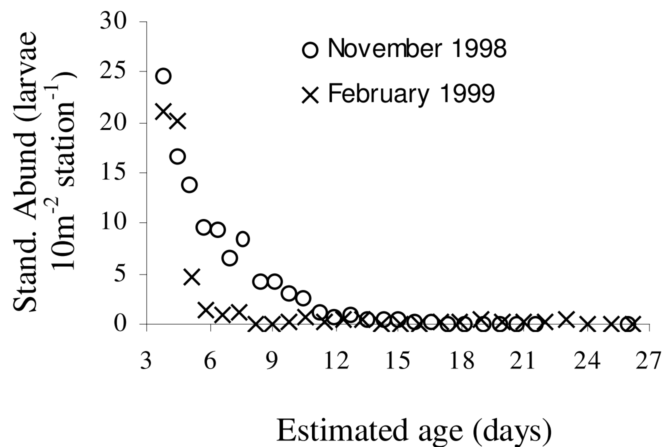

Figure 3.2.15. Age-frequency distributions for the pooled larvae for each cruise. Abundances are shown as mean number of larvae at each age-class.

Comparison of cruises reflects a higher apparent mortality in February than in November. In February 1999 a ca. 96% decrease in larval population between 3.8 and 6.5 days old was observed, *vs* 61% in November 1998 for the same age-range. Though these data are not comparable for the reasons mentioned above, our data suggests that February 1999 might be a worse period for larval production. The possibility that the environment might have affected individual larvae was later explored through the condition analyses in order to shed some light on the interpretation of the observed mortality.

Histological indices

In general, short-term histological measurements of condition showed intermediate scores for both cruises, with few larvae exhibiting extreme degeneration. In these cases, larvae showed almost null coloration of zymogen in the pancreas, had reduced liver vacuoles and the foregut specific surface was increased (Fig. 3.2.4.). In the long-term histological indices, cartilage showed a generally healthy appearance, whereas variability in muscle histomorphology was higher, suggesting that it was more affected by environmental conditions (Fig. 3.2.5.). In severely degraded larvae, other tissues appeared altered. The

brain cells exhibited condensed nuclei with conspicuous gaps between them and retinal cells, particularly ganglion cells, appeared disaggregated.
The particular observations regarding each cruise are detailed below.

November 1998

The nutritional condition of larvae differed between environmental groups A and B. Short-term indices showed a similar pattern between the two groups, having the modal class in the intermediate condition (Fig. 3.2.16.). The two size categories of larvae (8 mm and > 8 mm) showed no differences in the distribution of condition as assessed by the short-term index.

The frequency distributions of the long-term index was clearly different between groups A and B. Around 80% of larvae from group A were in good condition, irrespective of size-class. In group B, 80% of larvae under 8 mm were either in Poor or Mid condition. According to this, around 75% of larvae over 8 mm were in good condition and around 24% showed apparent degradation of muscle and cartilage.

Weighted indices (which put more weight on the long-term indices of condition and on the existence of necrosis, as a proxy for survival potential) showed that the percentage of larvae scoring under 7 was 4.4% for group A, *vs* 25% for group B (Fig. 3.2.16.).

Correlations between the 12 environmental variables and both short and long-term condition indices were performed. Short-term indices showed no significant correlations with any of the environmental variables studied, in either the small or larger larvae.

In contrast, the long-term histological condition indices showed a significant correlation with 8 out of 12 variables in larvae 8 mm (Table 3.2.9.). Larvae >8 mm did not correlate significantly with any variable. From the small larvae, the highest positive correlations were held with food abundance (both N and T-N). The highest negative correlations were held with temperature (both at surface and at 50 m) and with the water stability index (B-V). Therefore, larvae with higher condition indices were found in stations with higher food abundance and lower stratification and temperature. The correlation analysis confirms the patterns observed through the initial divisions in groups A and B, and the adequacy of the variables chosen a priori through PCA to study condition.

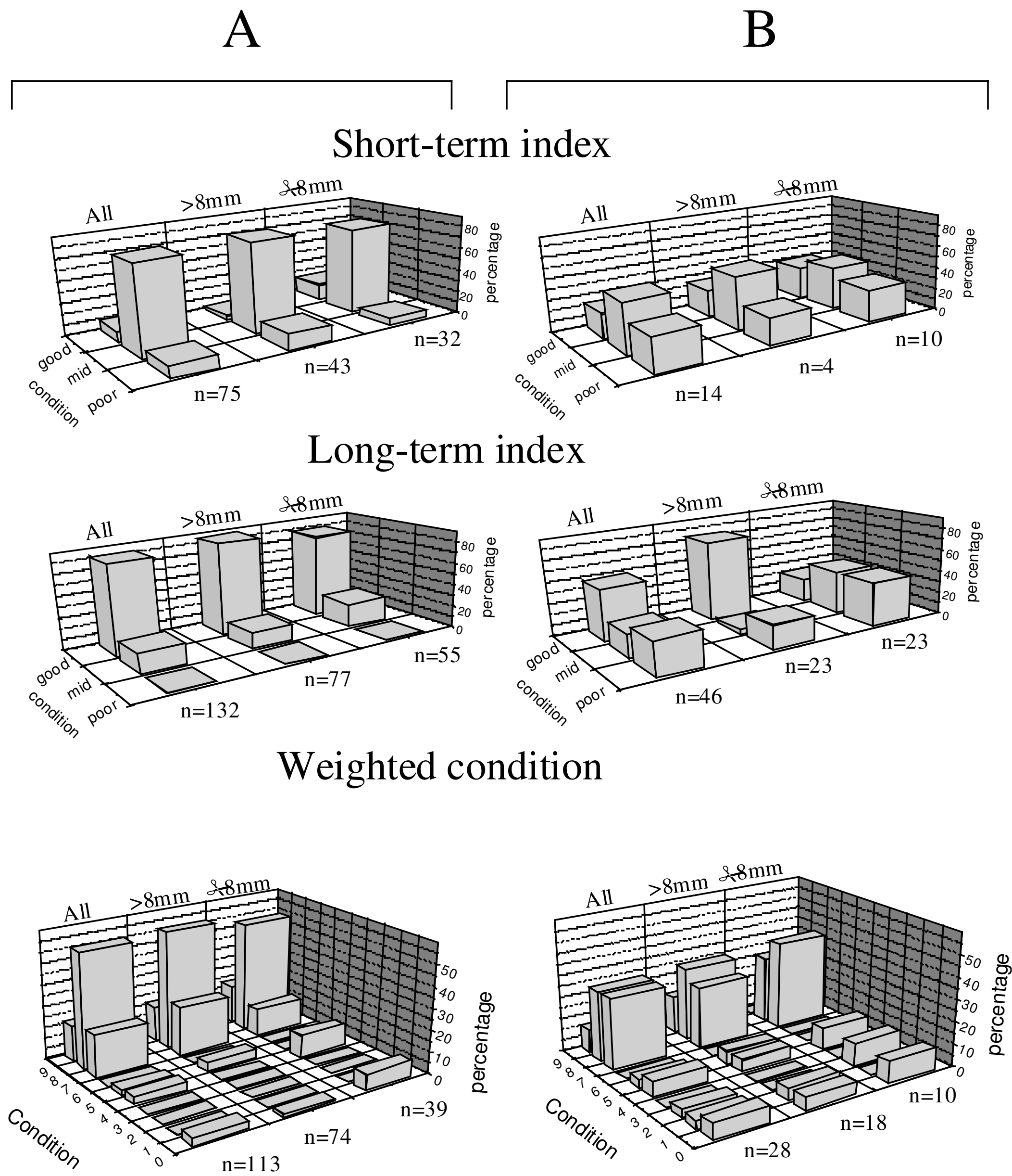

Figure 3.2.16. Frequency distributions of histological tissue grades for *S.pilchardus* in November 1998. A and B, environmental groups. Short-term indices, based upon liver scores and foregut specific surface. Long-term indices, based on MFS and Cartilage. Weighted indices, based on liver scores, MFS and necrosis.

Table 3.2.9. Non-parametric correlations between environmental variables and the long-term histological condition index for larvae 8 mm in November 1998. Valid N=78. NS, not significant.

Environmental variables	Spearman R	T (N-2)	P value
Station Depth (m)	-0.07	-0.639	NS
Nauplii/m^3	0.49	4.891	<0.00001
T-Nauplii	0.50	5.050	<0.00001
Temperature at 5 m (°C)	-0.49	-4.827	<0.00001
Salinity at 5m (psu)	-0.37	-3.453	<0.001
Fluorescence at 5m (Volts)	0.17	1.512	NS
Integrated Fluorescence (0-70m) (Volts)	0.18	1.540	NS
Temperature at 50 m (°C)	-0.45	-4.068	<0.001
Salinity at 50 m (psu)	0.28	2.371	<0.05
Fluorescence at 50 m (volts)	0.28	2.354	<0.05
Water stability (max. B-V index at 2m intervals, cycles h^{-1})	-0.44	-4.207	<0.0001
Depth of maximum B-V index (m)	-0.17	-1.477	NS

February 1999

For this cruise, the environmental groups A and B were not fully comparable as there were no available larvae under 8 mm for group B for the short-term index.

The short-term indices showed that in environmental group A, larvae 8 mm were in poorer condition than larvae > 8 mm (Fig. 3.2.17.). Both A and B groups presented between 40 and 50% of larvae in good condition (less than in November 1998).

The long-term condition index could only be based on the cartilage scores, which is known to be one of the last tissues to respond to starvation. The results showed no differences between size-categories or between environmental groups, and ca. 80% were in good condition.

The weighted index showed that larvae from group A were in somewhat better condition than larvae from group B, although the smallest size fraction could not be compared.

No single environmental variable correlated significantly with the short-term condition index in larvae 8 mm. This could be partly due to the low number of larvae available for the analyses. Five out of the 12 environmental variables showed a significant correlation with the short-term index in larvae > 8 mm (Table 3.2.10.). From this group of

larger larvae, those in better condition would have been collected from relatively deep stations, where the amount of large zooplankters, salinity and fluorescence was high.

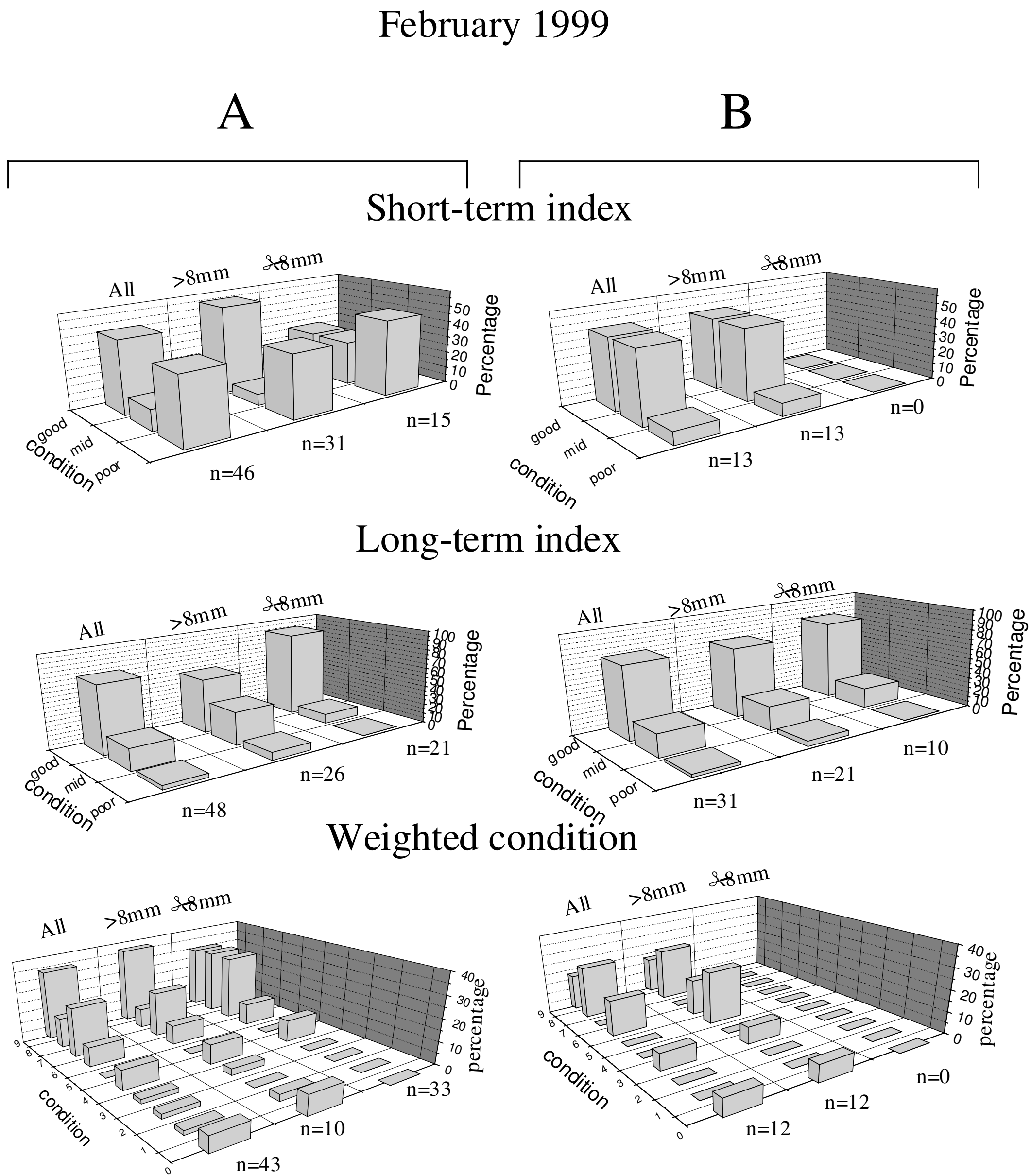

Figure 3.2.17. Frequency distributions of histological tissue grades for *S. pilchardus* in February 1999. A and B, environmental groups (see Fig. 3.2.1.). Short-term indices, based on liver scores. Long-term indices, based on Cartilage. Weighted indices, based on cartilage score, liver scores and necrosis.

From the long-term indices, only 2 variables were related to condition in larvae 8 mm. From these, a positive correlation was held with salinity at 50 m (r_s=0.50, N=29, T=2.983, $p<0.05$) and a negative correlation was held with T-N (r_s=-0.37, N=29, T=-2.105, $p<0.05$). In larvae > 8 mm a positive correlation was observed between condition and temperature at 5 m (r_s=0.34, T=2.390, $p<0.05$), temperature at 50 m (r_s=0.409, N=45, T=2.833, $p<0.01$) and salinity at 50 m (r_s=-0.34, N=45, T=-2.314, $p<0.01$). A negative correlation was held with fluorescence at 50 m (r_s=-0.34, N=45, T=-2.314, $p<0.05$). These results suggest that good condition assessed through the long-term index was associated with higher salinity values in both size classes. These were mostly coastal stations (Fig. 3.2.2.) and held the highest number of collected larvae (see Section 3.1.)

Table 3.2.10. Non parametric correlations between environmental variables and the short-term histological condition index of larvae > 8 mm in February 1999. Valid N=42. NS, not significant.

Environmental variables	Spearman R	T (N-2)	P value
Station Depth (m)	-0.39	-2.618	<0.05
Nauplii/m^3	0.16	1.043	NS
T-Nauplii	0.34	2.245	<0.05
Temperature at 5 m (°C)	-0.07	-0.457	NS
Salinity at 5m (psu)	0.32	2.105	<0.05
Fluorescence at 5m (Volts)	0.36	2.397	<0.05
Integrated Fluorescence (0-70m) (Volts)	-0.14	-0.873	NS
Temperature at 50 m (°C)	-0.30	-1.925	NS
Salinity at 50 m (psu)	-0.03	-0.157	NS
Fluorescence at 50 m (volts)	0.37	2.442	<0.05
Water stability (max. B-V index at 2m intervals, cyclesh^{-1})	0.05	0.302	NS
Depth of maximum B-V index (m)	0.26	1.702	NS

Comparison between cruises

Pooled larvae from groups A and B from each cruise are compared in Fig. 3.2.18. Short-term indices are the ones which can be compared more safely, as the two measurements utilised for November 1998 (liver scores and foregut specific surface) and the one utilised in February 1999 (liver scores) tend to covary in the same individual (significant correlation between liver scores and foregut specific surface for larvae in November 1998, r_p=-0.30, N=91 $p<0.05$), which does not happen between the long-term indices (cartilage and muscle).

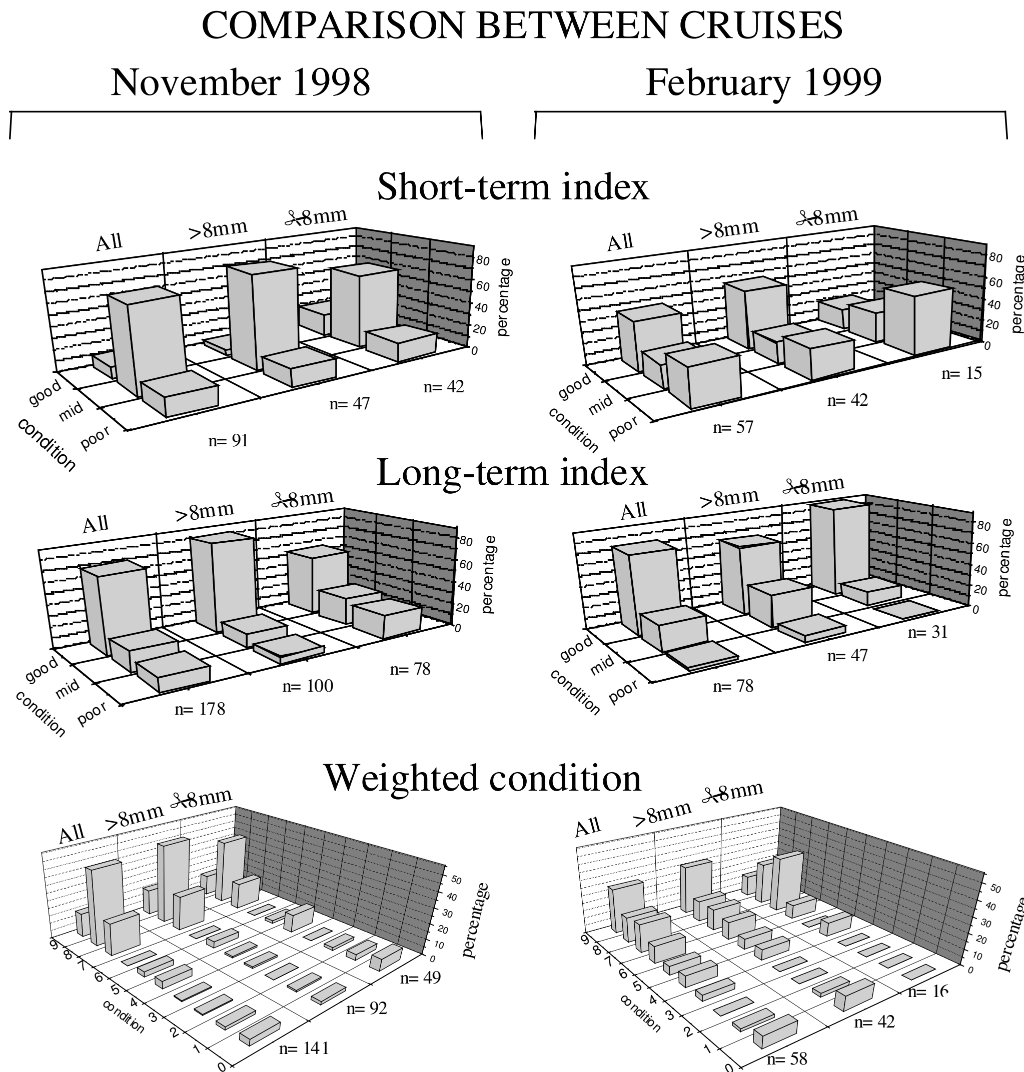

Figure 3.2.18. Comparison between global histological results (groups A and B pooled) for both cruises, by type of index and size-class.

The short-term indices showed that November 1998 had the vast majority of larvae in intermediate condition for all size classes. In contrast, larvae from February 1999 exhibited a poorer mean condition, with over 40% of larvae classified as “poor” (Fig. 3.2.18.). For this cruise, the larvae 8 mm were in obviously poorer condition than the larger larvae.

The long-term indices of November 1998 showed that 50% of larvae 8mm were in intermediate or poor condition. Most of this information was contained in the MFS index, the cartilage being affected only in cases of extreme degradation. For the same size category, larvae from February 1999 had only 15% of larvae in intermediate condition and none in poor condition. The lowered condition in larvae 8mm in November may be a combination of both the higher sensitivity of the measures used for November 1998 (only cartilage was used for February), and the higher environmental within-cruise differences in November 1998. The interpretations of the long-term index comparison in the February 1999 data, as said before, should be interpreted with caution, as only cartilage could be used for scoring.

Weighted indices reflected the previous patterns. The percentage of necrotic larvae was low in both cruises. Only 4 larvae from each cruise were graded as necrotic.

Biochemical indices

November 1998

No correlation between RNA/DNA and size was found. The RNA/DNA value for environmental group A was 7.0 ± 1.72 (SD) (n=76), whereas for group B was 6.2 ± 1.45 (n=40). The RNA/DNA in each area followed Normality, and the variance was homogeneous. Larvae from area A had significantly higher RNA/DNA contents than larvae from area B (T-test, T=2.40, DF=113, $p<0.05$) (Fig. 3.2.19.)

When all sizes of larvae were pooled, we found significant correlations between individual RNA/DNA and several environmental variables (Table 3.2.11.). In general, larvae in better condition (higher RNA/DNA values) were located in shallower stations, with a higher abundance of total microzooplankton except nauplii and lower salinity and fluorescence values (both integrated and at surface). Figure 3.2.19. suggests that larger larvae (over ca. 8 mm) were the groups that differed most between areas A and B. A separate correlation analysis using these larger larvae showed that RNA/DNA correlated with a larger number of environmental variables, including negative correlations with the B-V index (r_s =-0.30, N=71 T=-2.571, $p<0.05$) and surface temperature (r_s =-0.28, N=71,

T=-2.363, p<0,05) and positive correlations with the nauplii abundance (r_s =0.30, N=71, T=2.599, p<0,05). These data match the horizontal pattern for these variables (Fig. 3.2.2.), which showed that the areas characterised by low B-V had lower mean temperature and higher abundance of both N and T-N. On the other hand, when the RNA/DNA of larvae

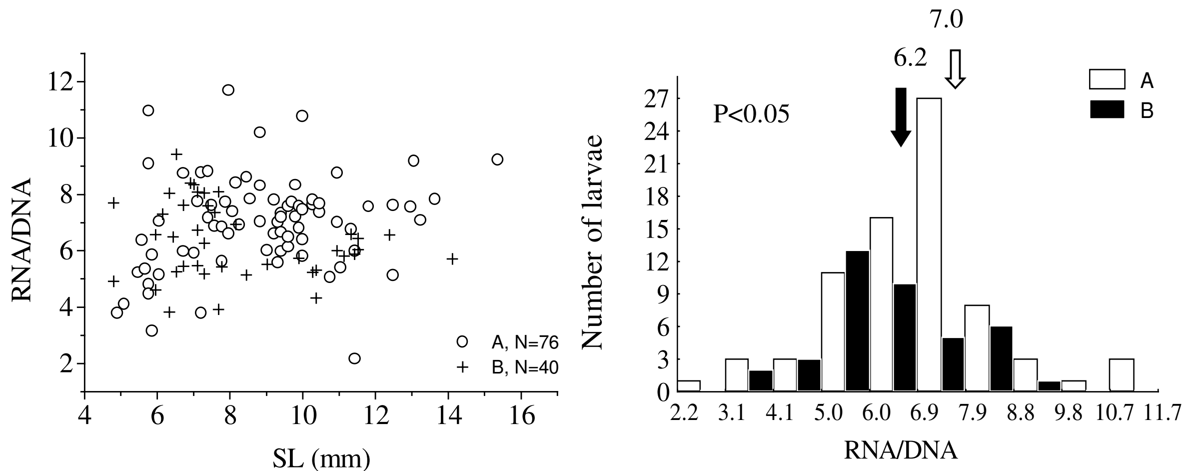

Figure 3.2.19. RNA/DNA results for November 1998. Left; RNA/DNA values *vs* standard length (SL). Right; frequency distributions of RNA/DNA in the two environmental areas. A and B, different environmental groups. Arrows are means. P value obtained after T-test is shown.

under 8 mm was confronted with environmental variables, they only correlated significantly, and negatively, with depth (r_s=-2.72, N=45 T=-2.012, p<0,05), suggesting that coastal stations offered better possibilities for larval development. The RNA/DNA values of these smaller larvae, however, showed higher variability than those of larger larvae. RNA/DNA values were similar between groups A and B at these larval sizes (Fig. 3.2.19.).

Table 3.2.11. Results from Spearman rank-correlations between environmental variables and RNA/DNA ratios for all larvae in November 1998. N=116.

Environmental variables	Spearman R	T (N-2)	P value
Station Depth (m)	-0.24	-2.686	<0.05
Nauplii/m^3 (sqrt)	0.13	1.438	NS
T-Nauplii	0.31	3.511	<0.0001
Temperature at 5 m (C)	-0.04	-0.422	NS
Salinity at 5m (psu)	-0.10	-1.119	NS
Fluorescence at 5m (Volts)	-0.25	-2.751	<0.01
Integrated Fluorescence (0-70m) (Volts)	-0.34	-3.816	<0.001
Temperature at 50 m (C)	-0.13	-1.437	NS
Salinity at 50 m (psu)	-0.25	-2.75	<0.01
Fluorescence at 50 m (volts)	-0.03	-0.281	NS
Water stability (max. B-V index at 2m intervals, cycles/h)	-0.10	-1.101	NS
Depth of maximum B-V index (m)	-0.12	-1.288	NS

February 1999

The RNA/DNA ratio showed no correlation with length in either group A or B. The dispersion of RNA/DNA values was similar for the two environmental groups, although there was an under representation of larvae under 9 mm in group B (Fig. 3.2.20).

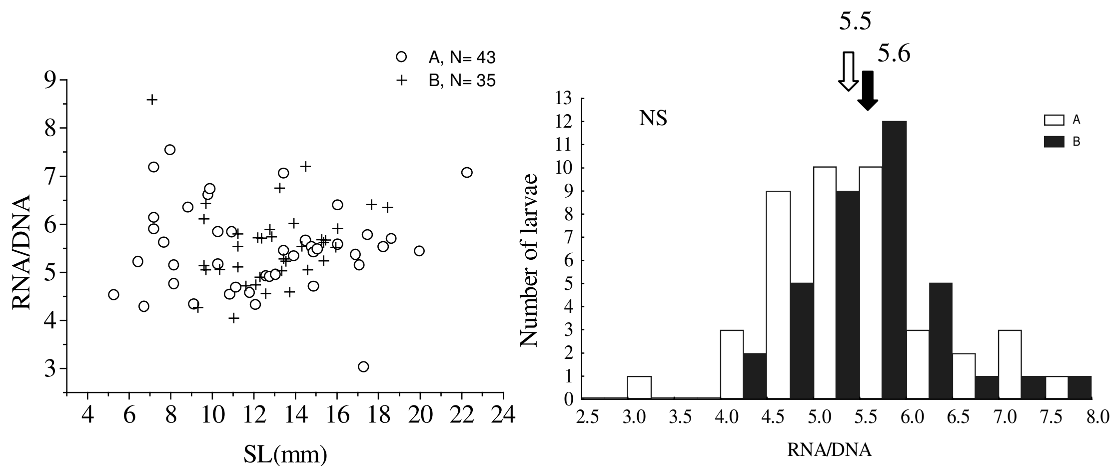

Figure 3.2.20. RNA/DNA results for February 1999. Left; RNA/DNA values *vs* standard length (SL). Right; frequency distributions of RNA/DNA in the two environmental areas. A and B, different environmental groups. Arrows are means. NS, non significant differences between the two groups after T-test.

The distribution of the RNA/DNA values from both areas followed normality and showed homogeneous variances. No significant differences between the two areas were found (Fig. 3.2.20.) and no single environmental variable correlated significantly with the RNA/DNA ratio.

Protein/DNA ratios showed a significant correlation with length in both A (r_p= 0.62, N=26, p<0.001) and B (r= 0.44, N=27, p<0.05) groups. Larvae between the two areas were compared by taking the length as a covariate in an ANCOVA analysis on linearised data. There were no significant differences between areas A and B for this condition index (Fig. 3.2.21.). A significant correlation with environmental variables (all data used) was only held with salinity at 50 m (r_s=0.33, N=38, p<0.05). Therefore, larvae assumed to be in better condition (higher protein/DNA ratio) were found in areas affected by higher salinity.

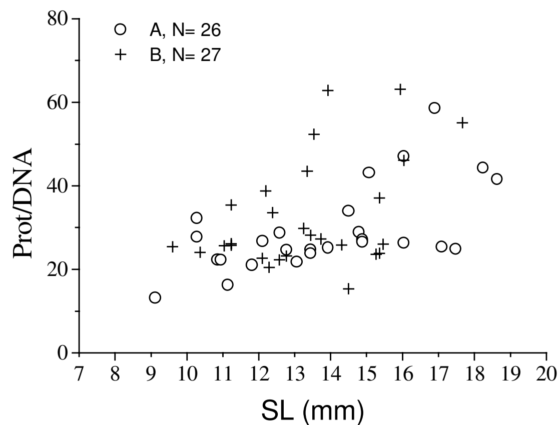

Figure 3.2.21. Relationship between prot/DNA *vs* length in the two environmental groups.

Comparison between cruises

The pooled larvae from November 1998 and February 1999 followed Normality, but the variances were not homogeneous. The two cruises were compared through the non-parametric Mann-Whitney test. The median of the RNA/DNA ratio in November 1998 was significantly higher than in February 1999 (W=4174, p<0.0001) (Fig. 3.2.22.). It seems apparent from this figure that for both cruises the smaller larvae (under ca. 9 mm) showed the highest dispersion in RNA/DNA values. Thus, most of the detected difference between cruises must be attributed to larvae over that size.

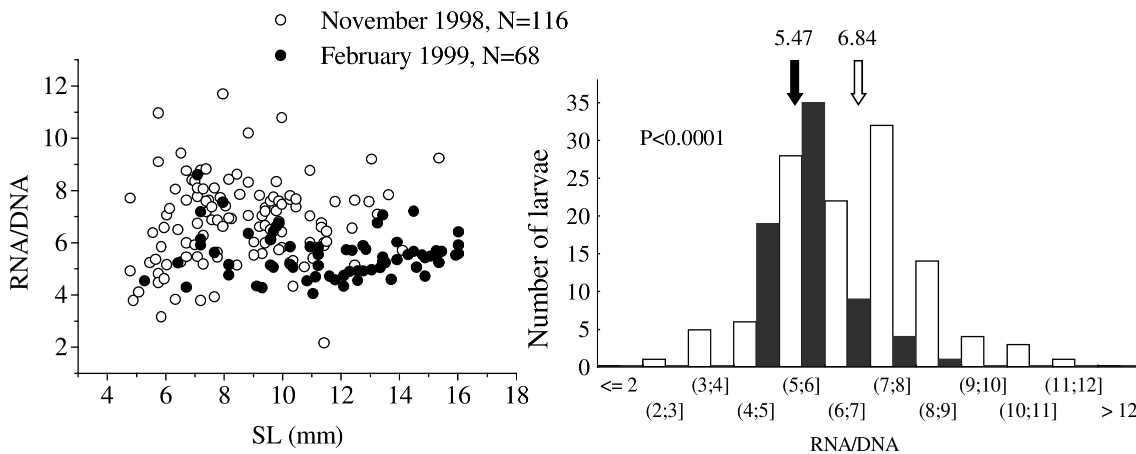

Figure 3.2.22. Left: RNA/DNA values *vs* length in the two cruises. Right; frequency distributions. Arrows indicate medians. P value after K-W test is shown.

DISCUSSION

General comments

According to the available literature, not only is there no study on the condition of pilchard larvae in the Catalan sea, but there is no single study on any species that uses histological, biochemical, muscle growth characteristics and age/length relationships to characterise the overall fitness of larval populations in relationship with biotic and abiotic factors. The results obtained in this section strongly suggest the existence of non-spurious relationships between environment, growth and condition in pilchard larvae during the period of analysis, which will be described throughout this discussion.

Abundance and adequacy of potential food

The two fractions of microzooplankton utilised in this study are adequate descriptors of potential prey for pilchard larvae in the studied size-range. As no remains of yolk were observed in the analysed specimens, it is assumed that all individuals could potentially be affected by the prey availability. We acknowledge, however, that in the

growth analyses some of the smallest specimens might be still dependant on oil globule reserves. The fraction N (nauplii under 45 µm) is known to build up to 60% of the total gut contents of larvae under 10 mm, and up to 40% in the larvae between 10 and 15 mm (Conway et al. 1994). The second main prey item found in the gut by these authors corresponds to copepodites. In the category T-N, we included most copepodite stages plus other microzooplankters. It is known that whereas naupli decrease slightly in the diet as larvae grow, copepodites are increasingly represented in the gut contents, from 10% in larvae under 10 mm up to 35% in larvae between 10 and 15 mm (Conway et al. 1994). We therefore believe that in the present study naupli may be an important food source for larvae under 8-10 mm, whereas the copepodites will have a larger influence in the larvae over this size.

Regarding the mean concentration of potential prey found on the two cruises, the values cannot be considered particularly high in either of them. For November 1998, the median of nauplii abundance was ca. 4.4 nauplii l^{-1}, and dispersion was high between stations (1.5 to 9.5 nauplii l^{-1}). The values for T-N were around 20% higher than those of N and also showed high dispersion values. In February 1999, the median nauplii concentration was ca. 6.3 nauplii l^{-1}, ranging from 1.5 to 10.5 nauplii l^{-1}. For this cruise, the fraction T-N was around 10% less abundant than that of N. These figures are generally lower than those found by other authors in the NW Mediterranean. Calbet et al. (2001) found mean values of nauplii of around 10 l^{-1} both in November and February. Also in the NW Med, during the summer, mean values of 10-20 nauplii l^{-1} were reported by Sabatés et al. (2001). In North Atlantic Iberian waters, mean nauplii abundances of between 2.4 and 17.3 have been found in several months (Conway et al. 1994).

The wide between-station variability in microzooplankton abundance, also observed by Calbet et al. (2001), is an advantage when it comes to incorporating potential food as a variable in the study of nutritional condition. Indeed, several studies have found correlations between food abundance and nutritional condition in pilchard (e.g. Chícharo et al. 1998 a,b).

It cannot be ignored, however, that food availability might have been higher than expected from our values on abundance, due to patchiness of prey and larvae at the vertical scale. Active larval vertical migration (Neilson and Perry 1990; Olivar et al. 2001) or turbulence,

which within certain values can favour the larval-prey encounter rates (Rothchild and Osborn 1988; McKenzie et al. 1994; see Franks (2001) for another implication of turbulence in enhancing feeding success) might have also influenced feeding success.

Growth

Otolith analyses

In this thesis we have used the relationship between body length, otolith diameter and age to estimate the growth of pilchard larval populations from either differing environmental groups or cruises. The daily deposition of rings in pilchard was demonstrated by Ré (1984), and the fact that the first detectable ring is formed at hatching was shown by Alemany and Álvarez (1994). We have assumed these two results in order to conduct our analyses and interpretations in pilchard larvae.

If environmental conditions were relatively persistent during the age-range considered, then strong environmental differences between areas, if they occurred, might have been reflected in a variation of the length-age relationship in the population sampled. We also studied the relationship between otolith diameter, age and size. The general correspondence between mean larval growth (e.g. in body length) and mean otolith diameter is well established. Several works have shown that changes in environmental factors affecting larval growth are also reflected in the width of daily deposition bands. However, other authors have reported an uncoupling between somatic growth and otolith growth. This uncoupling has been associated with changes in food availability and temperature, and may explain, for example, the fact that the slowest-growing larvae (e.g. older for a given size) sometimes exhibit the largest otoliths in relation to body length (Secor and Dean 1989; Barber and Jenkins 2001; Fey 2001).

The only significant difference between environmental group comparisons (within each cruise) was found in the body length to otolith diameter relationship in November 1998. Slopes did not differ between groups A and B, but mean otolith diameter was higher

in larvae from group B. The latter result, however, must be taken with caution as the number of data points corresponding to large otoliths is limited.

It is not surprising that there is a general lack of between-group differences within each cruise. It must be considered that, though the relationship between daily increments and length at the time of capture has been successfully used to estimate larval growth (Brothers et al. 1976; Methot and Kramer 1979; Palomera et al. 1988), this technique only enables a gross view of the growth parameters from the sampled population, as the length at age of a given collected specimen is the integral of the past growth history. Therefore, the past variation in parameters like food and temperature, which may have had an effect on growth during larval life, may have been obscured by subsequent environmental fluctuations. Indeed, the observed variability associated with a given age or length was relatively large. A closer study of the recent otolith growth would be required to relate the recorded environmental variables to larval growth. This study is currently being conducted by Palomera et al. (unpublished).

The length-at age relationships of NW Mediterranean pilchard was comparable to that reported for the same species in other areas. In November 1998 (mean surface T=ca. 19°C), the G_L value was 0.64 mm day^{-1} for day 15, whereas in February 1999 (mean surface T= 13°C) the G_L was 0.53 for the same day.

In SW Mediterranean pilchard collected from the Alboran Sea, Ramírez et al. (2001) showed a G_L= 0.43 mm day^{-1} for day 15 (mean surface T= 15°C). Ré (1984), for North Atlantic pilchard, showed mean growth values in body length of 0.49 mm day^{-1} for temperatures of 15.6ºC. The generally higher growth rates observed on our two cruises in comparison with the results of the aforementioned authors could be explained by differences in the length-range considered. They used larvae from 11.4 to 29 mm (Ramírez et al. 2001) or 8-26 mm (Ré 1984) *vs* our 3-15.5 mm size range.

In the present study, higher G_L was observed at younger ages, and decreased with age. This was also observed by Ramírez et al. (2001). Therefore, our use of smaller larvae could explain our higher growth rates. Of course, other factors might account for the observed differences, such as food availability, food quality or stock-related growth characteristics. Also, if growth-selective mortality (Hovenkamp 1992) was higher in our

sampled population than in the aforementioned works, we could have had a bias in the growth estimates, as our curves would be derived mostly from the faster growing individuals. However, in the absence of data on back-calculated length at age, it is not possible to speculate on this point.

Our otolith-based between-cruise comparison suggests, though not conclusively, that larvae from November 1998 might be experiencing higher growth than larvae from February 1999. In spite of the difficulties in making between-cruise comparisons, due to unequal representation of sizes at each age, Fig. 3.2.9. suggests that larvae from November 1998 might be growing faster. For sizes of 10-15.5 mm, larvae from February 1999 seemed to be older than those from November 1998 (Fig. 3.2.9., top left). Also, these older larvae had wider otoliths for the same length (Fig. 3.2.9., top right). This suggests that the otolith diameters of February collected larvae were not larger because they had experienced better conditions but because they belonged to older larvae. The latter might be indicative of uncoupling between otolith growth and somatic growth. This uncoupling, defined as an otolith growth in periods when somatic growth ceases or is reduced has been observed in larvae of other species (Brothers 1981; McGurk 1984; Secor and Dean 1989; Molony and Choat 1990, Secor and Dean 1992; Barber and Jenkins 2001).

Summarising, our results suggests that larvae from February 1999 might have a decreased growth, as they were older and had wider otoliths than larvae from November 1998 for a same length-range. In further research, it is expected that work on recent otolith growth can elucidate a possible relationship with condition or temperature that gives a closer time-scale view of the relationship of our data with the environment.

Muscle growth variability

There are no data on the variability of muscle cellularity in pilchard. Therefore, our results are not only valuable for the comparison of growth between areas and cruises but as

reference values for this species. However, until laboratory studies are conducted, our results must be considered as preliminary.

Literature on the effect of temperature on muscle fibre recruitment (hyperplasic growth) and fibre size (hypertrophic growth) has been mainly conducted under laboratory conditions, and shows that even small temperature differences during egg or larval development cause severe changes in muscle cellularity, which are often species-specific (Vieira and Johnston 1992; Usher et al. 1994; Johnston et al. 2001b). In herring *Clupea harengus*, the studied species which is phylogenetically closest to pilchard, higher temperatures experienced by eggs and larvae are known to increase the numbers of muscle fibres and their diameters (Vieira and Johnston 1992; Johnston 1993). The variation in muscle growth patterns can be of extreme importance to survival, as it may be associated with striking ability and hence with feeding success (Morley and Batty 1996; Johnston 2001 a).

Within cruises, larvae were compared between two zones, characterised by opposite trends in temperature and nauplii abundance (Table 3.2.4.). We were unable to show significant differences in muscle fibre recruitment or maximum inner fibre area between groups except for February 1999. The groups compared within this cruise were characterised by differing little in mean surface temperature (0.5ºC). However, food concentration in one of the groups was the highest observed in all the study (ca. 9 nauplii l^{-1}). Larvae from this group had a significantly higher amount of inner fibres than larvae collected from the stations with ca. 4 Nauplii l^{-1}. This result might be attributable to the difference in food concentration between the studied areas, as temperature differences were low. Nevertheless, we consider it unwise to extract solid conclusions from this particular comparison due to the low number of datapoints for larger larvae. The fact that in November 1998 the within-group comparison performed was unrevealing could be explained by the lack of larger larvae in the group collected from warmer stations and lower mean food items (Fig. 3.2.10.).

Recruitment of inner muscle fibres in pilchard larvae was low until 6-7 mm, increasing thereafter until 14 mm SL (Fig. 3.2.14.). This was true for both cruises. The low inner fibre recruitment at small larval sizes was also observed in herring by Johnston et al. (1998), who found that hypertrophic growth of embryonic myotomal muscle fibres occurred up to sizes of 12 mm. Our graphical analysis of inner fibre cross-sectional area distributions, performed on two size-groups within each cruise (Figs. 3.2.11. and 3.2.13.), suggests that hypertrophic growth might occur in small pilchard larvae, and might be more affected by small differences in temperature rather than food abundance (larvae from areas with lower food abundance but slightly higher temperature tended to have a higher mean inner fibre area). These suggested differences between areas were evident in November 1998, which was the cruise that displayed the largest mean surface temperature differences between the groups considered (1ºC). The number of superficial fibres in pilchard seemed to increase more constantly than that of inner fibres at the length-rage studied (Fig. 3.2.14.). This was also observed in herring larvae (Johnston et al. 1998; Temple et al. 2000).

Our data on number of fibres is difficult to compare to the range of values obtained for the herring. In the field study by Temple et al. (2000), the mean number of inner fibres of 16-18 mm SL (live) herring (their lowest size values), collected from areas with temperature ranging from ca. 10 to 15°C was around 700 and 1000 per cross-section. Our largest analysed pilchard larvae (ca. 14 mm SL (preserved)), collected in areas with water temperature around 13°C, showed ca. 400 inner fibres per cross section. This indicates that the number of inner fibres may lie within a comparable range.

The most revealing result of our analysis is that larvae from November 1998 had significantly higher inner fibre recruitment rates and a significantly higher number of superficial fibres than larvae from February 1999. Our results partly agree with the study on herring by Temple et al. (2000), which showed that, when larvae were compared by age (determined through otolith analysis), higher temperatures experienced at hatching were translated into higher number and size of superficial and inner myotomal muscle cells. These authors suggested that, though temperature might have been the main factor provoking the observed differences, the amount and suitability of prey should be further

studied in relation to muscle developmental patterns. In our study, the higher concentrations of potential food in February 1999 than in November 1998 induce us to believe that food concentration is not likely to be a factor explaining the lower fibre recruitment observed in February.

According to our results and the literature data on herring, we propose that muscle growth properties could be used as a way to determine the past influence of temperature on the young of the year recruits in pilchard. Firstly, small temperature differences experienced by eggs and early larvae are recognisable in the number of muscle fibres until advanced juvenile stages (Johnston et al. 1998). Secondly, there are data on different adult herring stocks showing large differences in the number of fast (white, usually derived from inner fibres) muscle fibres, which was suggested as a stock identification factor (Greer-Walker et al. 1972). If appropriate temperature-muscle growth patterns were available from laboratory studies, it should be feasible and cheap to study young of the year pilchard (from market sampling), and to have data on the probable thermal regime at hatching and during early larval development, according to the muscle growth characteristics and to surface temperature maps (obtained through ordinary satellite imagery). Then, some interesting conclusions like “the years of higher stock recruitment correlate with high success of larvae spawned during the coldest months” could be drawn. This kind of study might be of value for fisheries ecology and might complement the studies on otolith back-calculated age. Of course, several aspects such as the correspondence between recruits and spawning site, genetic differentiation of stocks, etc. should be taken into consideration.

The joint evidence of otolith analyses and muscle growth patterns strongly suggests that larvae in February 1999 had a reduced growth in length, lower inner fibre recruitment, and lower mean number of superficial fibres. These observation partly agree with the field study of Temple et al. (2000) on herring, who also related a lowered growth in length with a reduction in muscle fibre recruitment. It must be said that, though our otolith-based and muscle-based growth data are not fully comparable (we did not use “age” in the muscle growth analyses), the agreement of the two results strengthens the validity of the conclusions.

According to the growth-mortality hypothesis, larvae from February 1999 would remain longer at a less developed stage and/or smaller sizes in the plankton, which might involve a higher mortality due to predation (Houde 1989), and a higher probability of starvation due to the smaller storage capacity of less developed larvae.

We believe that muscle growth patterns should be further investigated in the laboratory with regard to food abundance and temperature, as they could be used as a measure of condition (and not only of growth) under a wide range of temporal scales. For example, differences in the properties of cross-sectional areas of the muscle pack could reflect starvation-induced condition at a short time-scale (variation of myonuclei, as suggested by Temple et al. 2000), whereas differences in fibre numbers could reflect long-time exposure to suboptimal food levels.

Mortality

Interpretation of mortality from age-frequency abundances cannot be done safely in this study. Some of the critical requirements for studying mortality from age-length derived data (see Pepin 2002) were not met. Our data on November 1998 suggest that spawning was not in equilibrium, as shown by the high number of eggs and larvae of the smallest size-classes (Olivar et al. in press, Section 3.1.). Also, spatial heterogeneity was high in the mean age-class abundances, being an order of magnitude higher in Environmental group A than in B. In spite of the aforementioned cautionary issues, the higher recent spawning detected in area A points towards a positive selection of this area by the spawning stock.

In February 1999, the abrupt apparent mortality might be explained by a number of reasons. As discussed in Olivar et al. (in press, see Section 3.1.), the low number of eggs and larvae observed might indicate a reduced spawning period during the weeks prior to collection. Unfavourable conditions during this month (reversed coast-shelf brake salinity pattern, inversion in the direction of the predominant current) could account for a reduced spawning.

In the above sections of the discussion devoted to the analysis of growth, we presented some elements that support the hypothesis that growth might have been reduced

in February 1999. If this was the case, the longer time during which larvae would remain at small sizes could have accounted, at least partially, for the sharp decrease in survival, due to growth-selective or size-selective mortality. In the next sections of the discussion, we will add the information obtained through condition analyses in order to build up a more comprehensive picture of how environmental factors might be operating at individual level.

Histological indices

Histological assessment of condition is probably the most accurate indicator of nutritional status (Ferron and Leggett 1994; Suthers 1998). Histological observations can be made from several tissues from a single specimen, which respond at different rates to food deprivation enabling a more precise description of the nutritional state of an individual. At present, histological criteria have not been successfully calibrated with regard to organ reaction of starving European pilchard larvae. In this respect, the existing laboratory data on this species (Silva and Miranda 1992) are difficult to interpret due to the high mortality observed in the control group (fed larvae). The general histological observations made in this work were adopted from several works on clupeids (O'Connell 1976; Theilacker and Watanabe 1989; Uriarte and Balbontin 1987; Silva and Miranda 1992). In general, the variation in the tissues observed herein was common to that described for other species, including turbot (Cousin et al 1986), stone flounder (Oozeki et al 1989), mackerel (Theilacker 1978; Margulies 1993) and Mediterranean Sea bass (Catalán and Olivar 2002; see Section 2.3.). We therefore believe that our interpretations on starvation-induced tissular degeneration have a sound base.

We decided to separate indices that may respond more quickly to suboptimal feeding from those that, allowing for the larval developmental stage, would take longer to be affected by low food concentrations. This approach had been previously adopted in *S. pilchardus* by McFadzen et al (1997).

Although increased tissue complexity was not observed throughout the size range analysed, organ growth during larval development is known to affect the sequence of energy usage as well as the duration of a particular energy reserve (Ferron and Leggett 1994). Therefore, the division into developmental stages, or at least size classes, as done in

the present work, is justified for condition studies. According to various works in European pilchard larvae (Blaxter 1969; Miranda et al. 1990; Silva and Miranda 1992), complete yolk-sac resorption occurs between 6 and 7 days old at temperatures of between 13 and 18ºC respectively. Corresponding sizes range from 4.5 to 5.5 mm (4% formalin-preserved SL). Therefore, our 8 mm SL cutpoint clearly separates larvae that are developing digestive and feeding abilities from larvae with well established external feeding.

We believe there is sufficient evidence in November 1998 to establish a relationship between environment and condition according to histological indices. Larvae from environmental group A were generally in better condition than larvae from group B. This was not, however, reflected in the short-term condition index. On this cruise, the modal class of the short-term index was in an intermediate condition for both A and B groups. However, groups A and B could not be compared safely as the number of positive readings for both tissues in area B was low. This was probably caused by the generally lower number of larvae collected for this area, coupled to the high number of larvae with broken or absent guts, as a result of net damage during collection.

It is known that short-term indices, and particularly liver and gut, can respond to food intake in a short time period that varies from hours to a few days (Watanabe 1985; Oozeki et al. 1989; Yúfera et al. 1993, see Section 2.3.). Therefore, the intermediate state of the short-term index for both sizes might indicate that larvae had not been subjected to severe starvation prior to collection.

Differences between environmental groups were conspicuously shown by the long-term index of condition and were more evident in larvae under 8 mm (Fig. 3.2.6.), which is in accordance with the general lower resistance to starvation of the more undeveloped larvae. This index was composed of the quantitative evaluation of muscle fibre separation (MFS) through image analysis developed in Section 2.3, and of the scoring of cartilage. Our microscope observations showed that MFS was mostly responsible for the differences in condition between the environmental groups, whereas cartilage only responded in cases of severe degeneration of muscle tissue. In environmental group B, long-term condition of larvae under 8 mm (mostly due to a poorer muscle state) had a clear inverted distribution shape in comparison with their counterparts from group A.

The fact that muscle tissue responds better and faster than cartilage to lowered food concentration is well documented in the literature. Fish are known to mobilise muscle protein during suboptimal feeding to supply for maintenance energy (Love 1970; Pedersen 1990). In the youngest phases, energy reserves in the form of lipids or glycogen are minimal, and muscle degradation will be almost immediate in the absence of the required external energy input (O'Connell 1976; Theilacker 1978; Cousin et al. 1986; Martin and Wright 1987; Green and McCormick 1999; Catalán and Olivar 2002). Furthermore, cartilage is known to respond only to prolonged periods of food deprivation (Theilacker 1978; McFadzen et al. 1994). Therefore, in group B, the better condition of larvae over 8 mm than of those under 8 mm could be explained by an increased buffer capacity due to larger storage reserves. The lowered condition of the smaller larvae according to the long-term index does not contradict the intermediate gradation by the short-term index, in view of the aforementioned sensitivity of the latter to food ingestion.

There was a good agreement in the correlation analysis of long-term condition *vs* environmental variables, and the divisions into A and B groups as potentially influencing condition. The histological condition of larvae under 8 mm was better (showed higher significant correlations) at stations that were less stratified, colder, and with higher values of N and T-N. These results correspond well with the differentiated horizontal patches shown in Fig. 3.2.2. Larvae over 8 mm showed no correlation with environmental variables, which follows from most of them being in good condition (Fig. 3.2.16.).

The use of the weighted index developed in Section 2.3. was applied based on the hypothesis that indices that could reflect an immediate feeding status would have a lower influence on final survival to juvenile stage than indices related to feeding success, escape reactions etc. as is the case of muscle. Our results of the weighted index, placing more weight in the long-term index, showed that the percentage of larvae with necrosis was extremely low (1.8% for group A and 7.1% for group B). We were unable, however, to apply the weighted index appropriately in area B because the number of larvae with positive readings for both short and long-term indices was low. As explained in Section 2.3., we believe that the results of the weighted index give a better picture of larval susceptibility to mortality than the separate use of each index, or the simple average of the obtained measurements.

The results from February 1999 require caution in their interpretation. The number of available larvae for analysis was lower than for the previous cruise, and there was no representation for the smallest size class in the short-term condition analyses for environmental group B. Moreover, preservation problems prevented the correct scoring of muscle and foregut specific surface.

Allowing for the latter observations, the short-term index suggested a poorer condition in larvae 8 mm than in their November 1998 counterparts, which could be an indication of food inadequacy (food abundance was not lower on this cruise). The lack of significant correlations between larvae 8 mm and environmental variables was not unexpected, due to the lower number of larvae compared to the previous cruise for the same size range, and the lower number of stations analysed. Also, for the short-term index, correlation results showed that larvae > 8 mm had a better liver condition in areas with a higher amount of larger potential prey (T-N), higher salinity and fluorescence values, and higher salinity values. These stations were also located in shallower waters. For this cruise, the long-term indices showed that larvae were in a generally good condition for both size-classes, though percentages of larvae in mid-condition were higher than in November 1998. For these larger larvae, the correlations with environmental variables were weaker than for the previous cruise, probably due to the fact that muscle, the most responsive tissue within the long-term indices, could not be used.

Overall, according to the most comparable histological index between cruises, larvae from February 1999 were in poorer condition than larvae from November 1998. Whereas long-term indices clearly showed differences between environmental groups in November 1998, this cannot be safely established for 1999. According to the histological indices, smaller larvae seemed to be more prone to starvation than larger larvae in both cruises.

Relationships between a poorer histological condition and lowered food concentration in field-collected clupeoid larvae has been previously reported (Lasker 1985; Sieg 1992), although data showing no relationships are also available (O'Connell 1980; McFadzen 1997). The finding that smaller larvae were generally in worse condition than larger larvae on both cruises agrees with the Critical Period hypothesis (Hjort 1914) which

implies that larvae are highly sensitive to suboptimal food concentrations during a short period after first feeding. In addition, evidence of an improved condition with age is abundant, which agrees with our data, and has been found for both wild and reared larvae (O'Connell 1980; Buckley 1984; Suthers 1993; Theilacker and Porter 1994; Grioche 1998). The latter has been explained in terms of an increased amount of body reserves, but other factors could also play a role, like the increased potential prey size and species range (Conway et al. 1994; Gonzalez –Quirós and Anadon 2001; Tudela et al. 2002).

The geographically closest study on pilchard condition assessed through histology was conducted by McFadzen et al. (1997) in Atlantic Iberian waters. They found that the vast majority of the larvae analysed were in good condition as assessed by short-term and long-term indices. They reported, however, a high percentage of starving larvae (up to 30%) identified by necrotic tissues. Our results showed far lower values (up to 7.1%). The mean length of larvae examined by McFadzen et al. (1997) was consistently lower than ours, which could explain their higher proportion of necrotic larvae. The mean nauplii concentration in our cruises was of the same order than theirs. Our results are more in accordance with those of Balbontin (unpubl.), who found values of ca. of 11-13% of necrotic *Sardinops sagax* larvae, calculated from histological grading. However, the large proportion of larvae in suboptimal condition detected in some areas (25% in group B *vs* 4% in group A in November, according to the weighted index) shows that susceptibility to mortality might be higher.

Summarising, our histological study provides evidence of an influence of potential food concentration in the histological condition of pilchard in the Catalan sea. The clearest results were obtained for November 1998, for which larvae from stations with lower B-V index, lower temperature and higher N and T-N were in better condition than larvae collected from areas showing opposite environmental trends. These differences were more evident in larvae under 8 mm. The use of MFS showed to be highly valuable for these analyses. The results for February 1999 were less clear, but a general pattern suggests that they were in poorer condition than those of November 1998.

Biochemical indices

The lack of correlation between body length and RNA/DNA ratios in all cruises and environmental groups enabled a comparison of the pooled larvae. This lack of correlation has been observed elsewhere in wild-collected clupeoids (Kimura et al. 2000), and when positive correlations have been reported, these are typically weak (Chícharo et al. 1998a).

For both cruises, variability in individual RNA/DNA was high. Our observed RNA/DNA ranges and modal classes were higher than those reported by Chícharo et al. (1998 a, b), fact which could be attributed to methodological differences. The aforementioned authors used Ethidium Bromide as a dye. Here, the use of SYBR Green II plus a detergent increased the sensitivity up to 100 fold in comparison with Ethidium Bromide (Berdalet 2002). We did not establish critical starvation values, due to the absence of laboratory calibration for this species using the current methodology. Our lowest estimated RNA/DNA values were between 2 and 4 (3.3% of all larvae), but the vast majority of larvae had values over 4. The critical RNA/DNA values, using dyes different to the one utilised in the present study, have been found to vary between 1.0 and 1.3 for some clupeoids including *S. pilchardus* (Clemmensen 1994, Chícharo 1997; Kimura et al. 2000), depending on temperature, size and species. However, these values cannot be taken for comparison purposes either, due to the differences in methodology for determining RNA and DNA. In order to clarify the differences between current methodologies, an intercalibration study is currently being conducted among several laboratories (Berdalet pers. com.). A laboratory calibration using the methodology utilised herein should be conducted in order to determine starvation percentages in this species.

For November 1998 significant differences between environmental groups were found (Fig. 3.2.19., right). This result agrees with our histological analyses and with the idea that environmental conditions were better for larval survival in group A. The correlation analyses also supported this view, and showed that the amount of potential food was an important variable explaining condition. It was evident from Fig. 3.2.19. and from subsequent separate correlation analyses that larvae over 8 mm differed most between groups A and B. The unsuitability of RNA/DNA for detecting starvation in the earliest developmental stages, particularly during transition to exogenous feeding, has been

repeatedly demonstrated and pointed out as drawback for the use of RNA/DNA even in larvae that are already ingesting prey items (Clemmensen 1989; Bergeron and Boulhic 1994; Clemmensen 1996, Section 2.4.). The fact that none of our analysed larvae showed signs of yolk, plus the fact that we used only one type of tissue for the analyses (muscle), reduced the possibility of ontogenetic artefacts in the interpretations of RNA/DNA as a condition measurement. Therefore, at least for November 1998, our results reinforce this criticism.

A factor also influencing RNA/DNA is temperature. On this cruise, within-group differences in surface temperature was ca. 1ºC. We do not believe that this temperature difference can account for the observed differences in the between-group RNA/DNA values. Several works indicate that temperature differences <2ºC enable direct comparison of RNA/DNA values (Buckley 1984, Buckley et al. 1999). On the other hand, the effect of food abundance has repeatedly shown to have a significant effect on RNA/DNA ratios in European pilchard (Chícharo 1998 a, b). These authors found that between 17 and 20% of their RNA/DNA variability was explained by zooplankton biomass, values that are comparable to our results.

The lack of significant differences between groups A and B in February 1999 is in agreement with the similar growth rates between the two groups as determined by otolith and muscle cellularity analyses. Our histological results are difficult to compare due to the low number of larvae available for analysis in this cruise. Also, few larvae under 10 mm were available for analysis for group B.

The protein/DNA analyses showed no significant differences between environmental groups either, but correlation analyses with all larvae showed that larvae in better condition were located in more saline areas. These results agree with those of histology in larvae > 8 mm and suggest that coastal areas, where the abundance of T-N was higher (Fig.3.2.2.), were better suited for larval survival.

Higher protein/DNA values were observed at larger sizes in both A and B groups. The latter agrees with the results of Ramírez et al. (2001) in SW Mediterranean pilchard. These authors hypothesised that if selective mortality existed upon slower growing individuals (in worse condition), the survivors would be characterised by having higher

protein/DNA ratios, which would explain its increase with length. However, the latter should also apply to RNA/DNA values. For both cruises and groups, we observed a bell-shaped form of the RNA/DNA frequencies. A truncation at the left-hand side would be indicative of growth-selective mortality, and this was not observed herein. Therefore, we suspect that increasing values of protein/DNA with length may be influenced by changes in physiological properties associated with muscle growth. Laboratory investigation on the latter could help in the future application of this index.

Larvae collected in November 1998 had significantly higher RNA/DNA values than those from February 1999, when compared using a common length-range and pooling all larvae. This is in agreement with our data suggesting higher growth in length and in muscle fibre numbers for that cruise. Also, the histological indices, particularly the short-term one (the most comparable measure), showed that larvae from February 1999 were in poorer condition. The major concern in comparing RNA/DNA values between cruises was temperature, which was around 6ºC higher in November 1998. Although growth rates of fish larvae are known to increase with increasing temperature, this does not necessarily imply increased RNA/DNA values. An initial reason for this is that, for a given RNA/DNA value, growth is higher at higher temperatures (Buckley 1984). In addition, larvae from colder environments, given other factors are not limiting, can grow at similar growth rates to larvae inhabiting warmer areas by increasing the amount of RNA, therefore compensating for temperature effects (Goolish et al. 1984). In the present study, we observed that the RNA/DNA ratio in individuals under 8 mm was highly variable and not distinguishable between the two cruises (Fig. 3.2.22.). According to Goolish et al. (1984), the similar RNA/DNA values between the November and February cruises for larvae under 8 mm, coupled with the lower RNA/DNA values of larvae over 8 mm in February 1999 than in November, would further reinforce the view of a general lowered growth or condition in the larvae collected in February.

Summary

European pilchard is a multiple spawning species with a protracted spawing season, which is well characterised both in the North Atlantic Iberian waters (García et al. 1992) and in the Mediterranean (Palomera and Olivar 1996). This wide period of spawning is an adaptation to overcome stochastic fluctuations in the numerous factors that can account for the high mortality at early larval ages, as a result of the low stability of the environment in which eggs and larvae develop.

In the Catalan Sea, *S. pilchardus* spawns from October to May within the continental shelf. During most of the spawning season, winter vertical mixing throughout the water column enriches the euphotic zone with nutrients, enhancing productivity in the areas in which larvae are found (Salat et al. 2002).

The area where larvae usually develop is also hydrographically delimited in its offshore boundary by the Catalan shelf-slope front. The Catalan front has a permanent SW associated current, the Liguro-Provençal-Catalan current. During the spawning season, variations to this general hydrographical and production pattern are diverse, including interactions with open sea eddies (see Introduction). The way in which environmental variables affect survival of pilchard larvae is difficult to grasp, as they probably act in concert, in an overlapped fashion, provoking high mortality rates. Nevertheless, the key role of food availability for larval survival in the wild is revealed by the close link that exists between the spawning time in temperate marine fish and the time of greatest abundance of planktonic food (Cushing 1977).

Acknowledging the difficulty of establishing causal links in this type of field study, we feel that an effort must be made to maximise the information extracted from the available environmental and individual or population-derived data.

In the light of the results presented in the chapter devoted to field studies (Sections 3.1. and 3.2., several relationships between environment and possible survival of larval pilchard in the Catalan sea can be established.

In November 1998 the pilchard population was at the beginning of the spawning season, as evidenced by the patches with high abundance of eggs and recently hatched

larvae. Certain areas could be characterised in which condition as assessed by histological and RNA/DNA ratios was particularly high. These areas may be described by ranges of environmental values that form a favourable “environmental window” (Cury and Roy 1989) for potential larval success. Such a suite of favourable conditions would include surface temperatures under 19ºC, B-Vvalues of 0.1-0.8 cycles h^{-1}, and mean potential food abundance over 4.5 nauplii l^{-1} or over 5.5 other microzooplankters l^{-1}. The areas characterising an unfavourable environment, where spawning was reduced and nutritional condition was lower, would exhibit surface temperatures over 19.0ºC, higher B-V values (0.81-1.8 cycles h^{-1}) and potential food abundance under the aforementioned values. We showed that the existence of these environmentally differentiated areas was probably caused by the influence of an oceanic mesoscale eddy of new AW that introduced warmer and less saline water into the shelf area and enhanced stratification at some sites. This oceanic eddy was very persistent (October 1998 to February 1999) and probably affected the larval production during the whole spawning season.

In February 1999 spawning and larval abundance was extremely low compared to other works conducted on the same month of the year (Olivar et al. 2001). The low number of larvae over 6 day old or 5 mm SL could be explained by a lower spawning during the weeks prior to collection and/or to increased mortality. The whole hydrographical situation on this cruise could be described as anomalous, as indicated by the reversed salinity pattern and the inversion in the direction of the dominant current. This observation was widely supported by calculations from our survey (Emelianov pers comm), by analyses derived from satellite data (Pascual et al. 2001), and by the ADCP results of a survey conducted a week after our cruise (Salat pers. comm). We found evidence to support the view that reduced spawning was paralleled by lower growth and condition in comparison to the previous cruise. The analyses of individual larvae, fewer in number than in November 1998, showed weak correlations with environmental conditions. Only larvae over 8 mm SL seemed to be in better condition at stations where the total amount of microzooplankters and surface salinity was high. On this cruise, the mean abundance of potential prey was not particularly low, and the poorer condition and lower growth observed could be attributed to a mixture of reduced temperature (6ºC lower than in November) and unsuitability of prey

composition. In this respect, we speculate that the long persistence of the new AW eddy might have contributed to a shift in the composition of microzooplankton species.

We believe that general environmental conditions on this cruise affected larval production negatively, probably due to a combination of reduced spawning intensity, reduced growth and poorer condition.

The joint interpretation of condition and growth through several methods confirmed that the type of information offered by measures that are commonly classified into a same category (e.g. “short-term condition indices” or “long-term growth”) is different. Whereas a comprehensive picture of the larval “fitness” with regard to the surrounding environment is undoubtedly enriched by the addition of methods, the interpretation of each one must be specific for the time-scale of the processes they integrate.

Within the growth measures, our results are valuable for several reasons. Firstly, we showed that there is an agreement between long-term growth estimated through otolith analyses and through the study of muscle cellularity. The clearest differences were detected between cruises and were probably due to temperature. These measures have an inherent great variability as they integrate the whole past history of the larva, but the general agreement between the two reinforces the validity of the results. Further research on both otolith microstructure and muscle cellular properties should be explored in *S. pilchardus* larvae with regard to their capability to detect recent growth as a response to varying environmental conditions. Secondly, muscle cellularity has never been used before in pilchard, and the results obtained herein seem promising. A future line of work could be devoted to the backward inference of environmentally important factors affecting larval survival, through the study of muscle cellularity of young of the year recruits. If appropriate temperature-muscle growth patterns were available from laboratory studies, it should be feasible and cheap to study young of the year pilchard (from market sampling), and to have data on their probable spawning times, according to the muscle growth characteristics and water surface temperature maps (obtained through ordinary satellite imagery). This kind of study might be of value for fisheries ecology and might complement the studies on otolith back-calculated age (e.g. Alvarez and Morales-Nin 1992). Of course, several aspects such as the correspondence between recruits and spawning site, genetic differentiation of stocks,

etc. should be taken into consideration. Our results on muscle growth suggest that pilchard larvae exhibit an initial hypertrophic growth that shifts into hyperplasic growth at around 6-7 mm SL. This implies that, if unexploited techniques like the cell proliferation rates (see section 2.4.) come to be of easy routine use, they should be applied only over these length-ranges.

Among the condition measurements, the information offered by histological and biochemical indices was complementary.

Within the histological methods, the use of the muscle fibre separation yielded the highest information on condition, which agrees well with the literature and with the experiments conducted in the first part of this thesis. Whereas the histological indices were effective in the larvae of all size-ranges, including the smallest size fraction, the results of RNA/DNA best differentiated larvae over 8-10 mm. This also conforms the information in the literature and our results from laboratory studies.

The adequacy of both histological and biochemical indices as indicators of condition or growth was further evidenced as they covaried with environmental conditions, particularly in November 1998 and in their between-cruise pattern. Comparative results within areas in February 1999 were less clear, which was not unexpected due to the lower number of available larvae and the weaker environmental differences between stations. Future lines of work regarding histological indices should focus on the standardisation of histological grading like the one used for MFS determination. Also, automatic quantification of key tissular traits is desired. The continuous, rather than discrete, obtained measures could clarify future correlation analyses with environmental variables.

Regarding biochemical indicators, further effort is needed to ascertain the physiological meaning of the variation in RNA/DNA ratios in tissues like muscle with growth. This would of course require species-specific validation. We believe that, as the muscle tissue is the most abundant in fish larvae, and is responsible for most growth, further research should focus on the link between ontogeny, environmental factors and muscle properties and its influence on behaviour and survival. Also, common methodological grounds should be aimed for among researchers, and efforts are currently being made in this direction.

The challenge of estimating survival from nutritional studies, given the usual lack of reliable data on the larval-recruitment correspondence, must ascribe theoretical chances of survival to particular indices. We believe that the muscle tissue should be given particular value for its importance in potential survival. Weighting schemes like the one presented in Section 2.3. and used in this chapter might be valuable for this purpose.

The data from this part of the thesis suggests, firstly, that in the Catalan sea environmental variations can influence future pilchard recruitment by modulating spawning intensity through hydrographic events at scales of several months and tens of kilometres. In addition, potential food abundance as a biological factor, and water column stability and temperature as physical factors, appear to be strongly associated with larval condition and growth, probably conditioning larval success at an individual level.

4. CONCLUSIONS

CONCLUSIONS

LABORATORY STUDIES

1) Morphometric indices proved useful in distinguishing the shape of larvae subjected to differing feeding conditions. This was done through multivariate analysis on variables from which the effect of size had been completely removed, but that conserved the information on allometry.

2) Within the histological variables tested, the quantitative determination of muscle fibre separation (MFS) showed to be the best method to detect a suboptimal nutritional state. This best performance was reflected in the percentage of correct classification of individuals to their age-feeding group, and in the speed of response to food deprivation or recovery from fast.

3) The RNA/DNA ratio is more appropriate in detecting a nutritional deficiency in post-flexion than in pre-flexion larvae.

4) Within the biochemical indices studied in post-flexion larvae subjected to a short-term food deprivation and refeeding, the RNA/DNA and the LDH/DNA ratios are considered particularly useful.

5) In post-flexion larvae subjected to 2 days of fast, the cell proliferation rate measured in the muscle was significantly lower than in Fed larvae. This was not observed in the brain, which is more conservative to the food withdrawal.

6) The comparison of morphometric, histological and biochemical indices shows that, in pre-flexion larvae, the quantitative determination of muscle fibre separation is the index that soonest detects the effect of food deprivation. For the same age-range, and excluding the period of yolk and oil-globule dependence, the RNA/DNA yields the

maximum correct discrimination percentage (100%). On the contrary, the maximum correct classification of starved and delayed-feeding larvae is obtained through the use of the MFS (83% and 44% respectively). In this study, the MFS was the condition index that held the best correspondence with larval mortality.

7) The larval susceptibility to mortality at sea may be more related to those deficiencies that involve organs or tissues that are of crucial importance for feeding behaviour, escape response or maintenance of floatability, like the muscle. It is here proposed that indices should be weighted in order to relate them with survival potential.

FIELD STUDIES

8) In November 1998, certain environmental characteristics were found to be positively associated with nutritional condition of *S.pilchardus*, measured through histological indices and RNA/DNA ratios. These areas can be characterised by an optimum "environmental window" defined by temperature values under 19ºC, values of the Brunt-Väissälä (B-V) water stability index under 0.8 cycles h^{-1} and values of potential food abundance over 4.5 nauplii l^{-1} and 5.5 individuals l^{-1} of the rest of the microzooplankton. The variables that best related to larval condition were the B-V index and the amount of potential food. Moreover, the larval abundance was higher in those areas. Therefore, it is considered that larval survival would be enhanced in these areas.

9) In February 1999 the eggs and larval abundance was lower than in November 1998.

10) The larval long-term growth studied through otolith analyses suggests that growth was enhanced in November 1998 with respect to February 1999. This result was confirmed by the analysis of the muscle fibre growth patterns. Condition was also better in November 1998.

11) The measures of growth and condition showed a general coincident pattern. The analysis of muscle growth patterns suggests that in pilchard larvae growth is hypertrophic until 6-7 mm SL, becoming hyperplastic at least until 13.5 mm SL. Within the histological measures studied, the MFS was the most informative. The RNA/DNA appeared more useful in larvae over 8-10 mm SL.

12) The amount of larvae in the November 1998 and February 1999 cruises was anomalously low for the time of the year. Hydrographic conditions during both cruises were highly unusual, exhibiting high stratification in November and an inverted salinity pattern in February. The presence of a mesoscale anticyclonic eddy of new AW, that remained in the area from October 1998 to February 1999, is thought to account for the unusual hydrographic conditions and the low numbers of eggs and larvae in some areas.

13) Overall, the data on larval abundance, nutritional condition and environmental characteristics suggest that the spawning season autumn 1998-winter 1999 was characterised by a low larval production of this species. It is concluded, from the joint interpretation of all data, that putative larval survival would be the result of a reduced spawning and nutritional condition in some areas and periods, and a reduced growth in the winter cruise.

LITERATURE CITED

LITERATURE CITED

Ahlstrom EH, Moser HG (1976) Eggs and larvae of fishes and their role in systematic investigations and in fisheries. Rev Trav Inst Pêch Mar 40: 379-398.

Akster HA, Verreth JA, Spierts ILY, Berbner T, Schmidbauer M, Osse JWM (1995) Muscle growth and swimming in larvae of *Clarias gariepinus* (Burchell). ICES Mar Sci Symp 201: 45-50.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1994) Molecular biology of the cell. Garland Publishing Inc. NY.

Alemany F, Alvarez F (1994) Formation of initial daily increments in saggital otoliths of reared and wild *Sardina pilchardus* yolk-sac larvae Mar Biol 121: 35-39.

Alvarez F, Morales-Nin B (1992) An attempt to determine growth and birthdate of juvenile sardine *Sardina pilchardus* Walb. in the Western Mediterranean Sea. Mar Biol 114: 199-203.

Amat F, Hontoria F, Navarro JC, Gonzalbo A, Varó Y (1991) Bioecología de *Artemia* (Crustácea, Branchiopoda) en la laguna de la mata, Torrevieja, Alicante. Instituto de Cultura “Juan Gil-Albert”. Diputación de Alicante. pp 7-173.

Anonim (2000) Provisional standard forms reporting information on stock assessment in the GFCM areas. GFCM-SAC. Stock Assessment Sub Committee. 12 pp.

Bailey KM, Houde ED (1989) Predation on eggs and larvae of marine fishes and the recruitment problem. Adv Mar Biol 25: 1-83.

Bailey KM, Canino MF, Napp JM, Spring SM, Brown AL (1995) Two contrasting years of prey levels, feeding condition and mortality of larval walleye pollock (*Theragra chalcogramma*) in the western Gulf of Alaska. Mar Ecol Prog Ser 119:11-23.

Bakun A (1998) Ocean triads and radical interdecadal stock variability: bane and boon for fishery management science. In: Pitcher TJ, Hart PJB Pauly D (eds) Reinventing Fisheries management. Chapman and Hall, London, pp 331-358.

Balon EK (1984) Reflections on some decisive events in the early life of fishes. Trans Am Fish Soc 113: 178-185.

Barahona-Fernandes ME (1978) Effect of aeration on the survival and growth of sea bass (*Dicentrarchus labrax* L.) larvae: a preliminary study. Aquaculture 14: 67-74.

Barber MC, Jenkins GP (2001) Differential effects of food and temperature lead to decoupling of short-term otolith and somatic growth rates in juvenile King George whiting. J Fish Biol: 1320-1330.

Barnabé G (1985) L'élévage du loup et de la daurade. In: Barnabé G, Silbard Y (eds) Aquaculture, Téchnique et Documentation , Lavoisier, Paris, pp. 627-666.

Barnabé G (1991) La cría de lubina y de dorada. In: Barnabé G (ed) Acuicultura. Omega, Barcelona pp 573-612.

Barnabé G, Boulineau-Coatanea F, Rene F, Martin V (1976) Chronologie de la morphogenese chez le loup ou bar *Dicentrarchus labrax* (L.) (Pisces Serranidae) obtenue par reproduction artificielle. Aquaculture 8: 351-363.

Batty RS (1984) Development of swimming movements and musculature of larval herring (*Clupea harengus*). J Exp Biol 83: 287-291.

Bennett HS, Wyrick AD, Lee SW, McNeil JW (1976) Science and art in preparing tissues embedded in plastic for light microscopy, with specific reference to glycol metacrylate, glass knives and simple stains. Stain Technol 51:71-97

Berdalet E (2002) Quantization of nucleic acids in marine microplankton samples. In: Pelagic Ecology Methodology. Subba Rao DV (ed). AA Balkema Publishers, Lisse, Abingdon, Exton (PA), Tokyo, pp 271-289.

Bergeron JP (1997) Nucleic acids in ichthyoplancton ecology: a review, with emphasis on recent advances for new perspectives J Fish Biol 51: 284-302.

Bergeron JP, Boulhic M (1994) Rapport ARN/ADN et évaluation de l'état nutritionel et de la croissance des larves de poissons marons: un essai de mise au point espérimentale chez la sole (*Solea solea* L.) ICES J Mar Sci 51: 181-190.

Bergeron JP, Person-Le Ruyet J (1997) Teneur en ADN de la larve de *Dicentrarchus labrax*: évolution ontogénétique et effect de la privation de nourriture. Aquat Living Resour 10: 247-250.

Bernard DR (1981) Multivariate analysis as a means of comparing growth in fish. Can J Fish Aquat Sci 38: 233-236.

Bierse SC, Leonard RB, Coggeshall RE (1980) Neuronal increase in various areas of the nervous system of the guppy, *Lebistes.* J Comp Neurol 194: 291-301.

Bisbal GA, Bengtson DA (1995) Description of the starving condition in summer flounder, *Paralichthys dentatus*, early life history stages. Fish Bull 93: 217-230.

Blancheton JP, Canaguier B (1995) Bacteria and particulate materials in recirculating seabass (*Dicentrarchus labrax*) production system. Aquaculture 133: 215-224.

Blaxter JHS, Hempel G (1963) The influence of egg size on herring larvae. J Cons, Cons Int Explor Mer 28: 211-240.

Blaxter JHS, Ehrlich KF (1974) Changes in behaviour during starvation of herring and plaice larvae. In: Blaxter JHS (ed) The early life history of fish. Springer-Verlag, Berlin, pp 575-588.

Boulhic M, Gabaudan J (1992) Histological study of the organogenesis of the digestive system and swim bladder of the Dover sole, *Solea solea* (Linnaeus 1758). Aquaculture 102: 373-396.

Brickman D, Frank KT (2000) Modelling the dispersal and mortality of Browns Bank egg and larval haddock (*Melanogrammus aeglefinus*) Can J Fish Aquat Sci 57: 2519-2535.

Brightman RI, Torres JJ, Donnelly J, Clarke ME (1997) Energetics of larval red drum, Sciaenops ocellatus. Part II: Growth and biochemical indicators. Fish Bull 95: 431-444.

Bromhead D, Kalish J, Waring P (2000) Application of flow cytometric cell cycle analysis to the assessment of condition and growth in larvae of a freswater teleost *Galaxias olidus.* Can J Fish Aquat Sci 57: 732-741.

Brothers EB, Mathews CP, Lasker R (1976) Daily growth increments in otoliths from larval and adult fishes. Fish Bull 74: 1-8.

Brothers EB (1981) What can otolith microstructure say about daily and subdaily events in the early life history of fish Rapp P-v Réun Cons Perm Int Explor Mer 178: 393-394.

Brownell CL (1980) Water quality requirements for first-feeding in marine fish larvae, I. Ammonia, nitrite and nitrate. J Exp Mar Biol Ecol 44: 269-283.

Buckley LJ (1979) Relationship between RNA-DNA ratio, prey density, and growth rate in Atlantic cod *Gadus morhua*. J Fish Res B Can 36: 1497-1502.

Buckley LJ (1984) RNA/DNA ratio: an index of larval fish growth in the sea. Mar Biol 80: 291-298.

Buckley LJ, Lough RG (1987) Recent growth, biochemical composition, and prey field of larval haddock (*Melanogrammus aeglefinus*) and Atlantic cod (*Gadus morhua*) on Georges Bank. Can J Fish Aquat Sci 44: 14-25.

Buckley LJ, Caldarone EM, Ong TL (1999) RNA-DNA ratio and other nucleic acid-based indicators for growth and condition of marine fishes. Hydrobiologia 401: 265-277.

Calbet A, Garrido S, Saiz E, Alcaraz M, Duarte C (2001) Annual zooplankton succession in coastal NW Mediterranean waters: the importance of the smaller size fractions. J Plankton Res 2001 23: 319-331.

Caldarone EM, Buckley LJ (1991) Quantitation of DNA and RNA in crude tissue extracts by flow injection analysis. Anal Biochem 199: 137-141.

Campana SE, Neilson JD (1985) Microstructure of fish otoliths. Can J Fish Aquat Sci 42: 1014-1032.

Canino MF, Bailey KM, Incze LS (1991) Temporal and geographical differences in feeding and nutritional condition of walleye pollock larvae *Theragra chalcogramma* in Shelikof Strait, Gulf of Alaska. Mar Ecol Prog Ser 79: 27-35.

Catalán IA, Olivar MP (2002). Quantification of muscle condition using digital image analysis in *Dicentrarchus labrax* larvae, and relationship with survival. J Mar Biol Ass UK 82: 649-654.

Chambers RC (1993) Phenotypic variability in fish populations and its representation in individual-based models. Trans Am Fish Soc 122: 404-414.

Chatain B (1987) La vessie natatoire chez *Dicentrarchus labrax* et *Sparus auratus*. II. Influence des anomalies de développement sur la croissance de la larve. Aquaculture 65: 175-181.

Chesney EJ, Alonso-Noval M (1989) Coastal upwelling and the early life history of sardines (*Sardin*a pilchardus) along the Galician coast of Spain Rapp P-v Réun Cons Perm Int Explor Mer 191: 63-69.

Chícharo MA (1997) Starvation percentages in field caught Sardina pilchardus larvae off southern Portugal. Sci Mar. 61 :507-516.

Chícharo MA, Chícharo L, Valdés L, López-Jamar E, Ré P (1998a) Does nutritional condition limit survival potential of sardine *Sardina pilchardus* (Walbaum, 1792) larvae off the north coast of Spain? RNA/DNA ratios and their variability. Fish Res 39: 43-54.

Chícharo MA, Chícharo L, Valdés L, López-Jamar E, Ré P (1998b) Estimation of starvation and diel variation of the RNA/DNA ratios in field-caught Sardina pilchardus larvae off the north of Spain. Mar Ecol Prog Ser 164: 273-283.

Childress JJ, Somero GN (1979) Depth-related enzymatic activities in muscle, brain, and heart of deep-living pelagic marine teleosts. Mar Biol 52: 273-283.

Clarke ME, Calvi C, Domeier M, Edmonds M, Walsh PJ (1992) Effects of nutrition and temperature on metabolic enzyme activities in larval and juvenile red drum, Sciaenops ocellatus, and lane snapper, *Lutjanus synagris*. Mar Biol 112: 31-36.

Clemmensen CM (1987) Laboratory studies on RNA-DNA ratios of starved and fed herring *Clupea harengus* and turbot *Scophtalmus maximus* larvae. J Cons Int Exp Mer. 43: 122-128.

Clemmensen CM (1989) RNA/DNA ratios of laboratory-reared and wild herring larvae determined with a highly sensitive fluorescence method. J Fish Biol 35: 331-333.

Clemmensen CM (1993) Improvements in the fluorometric determination of the RNA and DNA content in individual marine fish larvae. Mar Ecol Prog Ser 100: 177-183.

Clemmensen CM (1994) The effect of food availability, age or size on the RNA/DNA ratio of individually measured herring larvae: laboratory calibration. Mar Biol 118: 377-382.

Clemmensen CM (1996) Importance and limits of RNA/DNA ratios as a measure of nutritional condition in fish larvae. In: Watanabe Y, Yamashita Y, Ooseki Y (eds) Survival Strategies in Early Life Stages of Marine Resources. A.A. Balkema, Rotterdam, pp 67-82.

Clemmensen CM, Doan T (1996). Does otolith structure reflect the nutritional condition of a fish larvae?. Comparison of otolith structure and biochemical index (RNA/DNA ratio) determined on cod larvae. Mar Ecol Prog Ser 138: 33- 39.

Conway DVP, Coombs SH, Fernández de Puelles ML, Tranter, PRG (1994) Feeding of larval sardine, *Sardina pilchardus* (Walbaum), off the north coast of Spain. Bol Ins Esp Oceanogr 10: 165-175.

Coombs SH, Conway DVP, Morley SA, Halliday NC (1999) Carbon content and nutritional condition of sardine larvae (*Sardina pilchardus)* off the Atlantic coast of Spain. Mar Biol 134: 367-373.

Corneillie S, Noterdaeme L, Ollevier F (1989) Culture of sea bass larvae (*Dicentrarchus labrax*) in completely closed recirculation systems with artificial seawater In: De Pauw N, Jaspers E, Ackefors H, Wilkins N (eds) Aquaculture a biotechnology in progress. European Aquaculture Society, Bredene, Belgium pp 125-131.

Couture P, Dutil JD, Guderley H (1998) Biochemical correlates of growth and condition in juvenile Atlantic cad (Gadus morhua) from Newfoundland Can J Fish Aquat Sci 55: 1591-1598.

Coves D (1985) État actuel de l'élevage du loup, *Dicentrarchus labrax* en écloserie. Aqua Revue 3: 26-31.

Crespo S, Marín de Mateo M, Santamaría CA, Sala R, Grau A, Pastor E (2001) Histopathological observations during larval rearing of common dentex *Dentex dentex* L. (Sparidae). Aquaculture 192: 121-132.

Cury P, Roy C (1989) Optimal environmental window and pelagic fish recruitment in upwelling areas. Can J Fish Aquat Sci 46:670-680.

Cushing DH (1975) Marine ecology and fisheries. University press, Cambridge 277 pp.

Cushing DH (1977) The problems of stock and recruitment. In Gulland JA (ed) Fish population dynamics, Wiley J, London pp 116-133.

Cushing DH (1990) Plankton production and year class strength in fish populations: an update of the match/mismatch hypothesis. Adv Mar Biol 26 (1990) 249-293.

Deplano M, Diaz JP, Connes R, Kentouri-Divanach M, Cavalier F (1991) Appearance of lipid-absorption capacities in larvae of the sea bass *Dicentrarchus labrax* during transition to the exotrophic phase. Mar Biol 108:361–371.

Dou S, Masuda R,Tanaka M,Tsukamoto K (2002) Feeding resumption, morphological changes and mortality during starvation in japanese flounder larvae J Fish Biol 60: 1363-1380.

Dulcic J, Grbec B (2000) Composition and temporal fluctuations of ichthyoplankton community in the Konati Archipelago and Murter Sea, Eastern Adriatic. Vieu et Milieu 50: 163-170.

Ehrlich KF (1974) Chemical changes during growth and starvation of larval *Pleuronectes platessa*. Mar Biol 24: 39-48.

Ehrlich KF, Blaxter JHS, Pemberton R (1976) Morphological and histological changes during the growth and starvation of herring and plaice larvae. Mar Biol 35:105-118

Eldridge MB, Whipple JA, Eng G, Bowers MJ, Jarvis BM (1981) Effects of food and feeding factors on laboratory-reared stripped bass larvae. Trans Am Fish Soc 110:111-120

Elliott JK, Leggett WC (1997) The effect of temperature on size-dependent predation by a *fish (Gasterosteus aculeatus*) and a jellyfish (*Aurelia aurita*) on larval capelin (*Mallotus villosus*). Can J Fish Aquat Sci 54: 2759-2766.

Elliott JK, Legget WC (1998) Larval condition and vulnerability to predation: an analysis based on mixed-prey experiments. Can J Fish Aquat Sci 55: 626-630.

EPA (United States Environmental Protection Agency) (1989) Ambient water quality criteria for ammonia (salt water), EPA 440/5-88-004, Office of water regulations and standards criteria and standards division, Washington DC 1989.

Felip A, Zanuy S, Carrillo M, Martínez G, Ramos J, Piferrer F (1997) Optimal conditions for the induction of triploidy in the sea bass (*Dicentrarchus labrax* L.), Aquaculture 152: 287-298.

Ferron A, Leggett WC (1994) An appraisal of condition measures for marine fish larvae. Adv Mar Biol 30:217-303.

Fey DP (2001) Differences in temperature conditions and somatic growth rate of larval and early juvenile spring-spawned herring from the Vistula Lagoon, Baltic Sea manifested in the otolith to fish size relationship. J Fish Biol 58(5): 1257-1273.

Fiedler TJ, Clarke ME, Walsh PJ (1998) Condition of laboratory-reared and wild-caught larval Atlantic menhaden *Brevoortia tyrannus* as indicated by metabolic enzyme activities. Mar Ecol Prog Ser 175: 51-66.

Font J, Salat J, Tintoré J (1988) Permanent features of the circulation in the Catalan sea. Oceanol Acta 9: 51-57.

Frank KT, Leggett WC (1981) Wind regulation of emergence times and early larval survival in Capelin (*Mallotus villosus*) Can. J Fish Aquat Sci 38: 215-223.

Frank KT, Leggett WC (1982) Environmental regulation of growth rate, efficiency, and swimming performance in larval capelin (*Mallotus villosus*), and its application to the Match/Mismatch hypothesis. Can J Fish Aquat Sci 39: 691-699.

Franks PJS (2001) Turbulence avoidance: An alternate explanation of turbulence-enhanced ingestion rates in the field Limnol Oceanogr 46: 959-963.

Fuiman LA (1994) The interplay of ontogeny and scaling in the interactions of fish larvae and their predators. J Fish Biol 45:55-79.

Fuiman LA (1997) What can flatfish ontogenies tell us about pelagic and benthic lifestyles? J Sea Res 37:257-257.

Fuiman LA, Higgs DM (1997) Ontogeny, growth, and the recruitment process. In: Chambers RC, Trippel EA (eds). Early Life History and Recruitment in Fish Populations. Chapman and Hall pp 225-249.

Gallego A, Heath MR, McKenzie E, Cargill LH (1996) Environmentally induced short-term variability in the growth rates of larval herring Mar Ecol Prog Ser 137: 11-23.

Galloway TF, Kjørsvik E, Kryvi H (1999) Muscle growth and development in Atlantic cod larvae (*Gadus morhua* L.) related to different somatic growth rates J Exp Biol. 202: 2111-2120.

García A, Franco C, Solá A (1992) Sardine, *Sardina pilchardus* (Walb.), egg and larval distribution off the Spanish North Atlantic coast (Galician and Cantabrian areas) in April 1987. Bol Ins Esp Oceanogr 8: 87-96.

García MA, Arnau P, Sánchez-Arcilla A (1998) Advection of anticyclonic eddies across the Blanes canyon, NW Mediterranean. Rap Pro-Verb Réun Comm Int Exp Sci Mer Méd 35: 142-143.

García Hernández MP, Lozano MT, Elbal MT, Agulleiro B (2001) Development of the digestive tract of sea bass (*Dicentrarchus labrax* L). Light and electron microscopic studies. Anat Embryol (2001) 204:39–57.

Gas N (1972) Structural alterations in the white muscle of carp (*Cyprinus carpio* L.) during prolonged starvation. Cr hebd Séances Acad Sci 275:1403-1406.

Gaughan DJ, Mitchell RW, Blight SJ (2000) Impact of mortality, possibly due to *Herpesvirus* sp., on pilchard, *Sardinops sagax*, stocks along the south coast of Western Australia 1998-1999. Mar Fresh Res 51: 601-612.

Gómez- Larrañeta M (1960) Synopsis of biological data on *Sardina pilchardus* of the Mediterranean and adjacent seas. FAO Fish Biol Syn 9: 137-173.

González-Quirós R, Anadón R (2001) Diet breadth variability in larval blue whiting as a response to plankton size-structure J Fish Biol 59: 111-1125.

Goolish EM, Barron MG, Adelman IR (1984) Thermoclimatory response of nucleic acid and protein content of carp muscle tissue: influence of growth rate and relationship to glycine uptake by scales. Can J Zool 62: 2164-2170.

Gould SJ (1966) Allometry and size in ontogeny and phylogeny. Biol Rev 41: 587-640.

Govoni JJ (1980) Morphological, histological and functional aspects of alimentary canal and associated organ development in larval [*Leiostomus xanthurus*] Rev Can Biol 39: 69-80.

Govoni JJ, Boehlert GW, Watanabe Y (1986). The physiology of digestion in fish larvae. Env Biol Fish 16: 59-77.

Govoni JJ, Pietrafesa LJ (1994) Eulerian views of layered water currents, vertical distribution of some larval fishes, and inferred advective transport over the continental shelf off North Carolina, USA, in winter. Fish Oceanogr 3: 120-132.

Green BS, McCormick MI (1999) Influence of larval feeding history on the body condition of *Amphiprion melanopus.* J Fish Biol 55: 1273-1289.

Greer-Walker MG (1970) Growth and development of the skeletal muscle fibres of the cod (*Gadus morhua* L.). J Cons Perm Int Explor Mer 33:228-244.

Greer-Walker MG, Burd AC, Pull GA (1972) The total number of white skeletal muscle muscle fibres in cross section as a character for stock separation in North Sea herring (*Clupea harengus* L.) J Cons Int Explor Mer 34: 238-243.

Grioche A (1998) Dynamique de l'écophase ichtyoplanctonique en Manche orientale et sud Mer du Nord. Approche multispécifique et description de deux espèces cibles: *Solea solea* (L.) et *Pleuronectes flesus* (L.). PhD thesis, 286 pp.

Gutiérrez E, Morales-Nin B (1986) Time series analysis of daily growth cycles in *Dicentrarchus labrax* (Pisces: Serranidae) J Exp Mar Biol Ecol 103: 163-179.

Hakanson JL (1989) Analysis of lipid components for determining the condition of anchovy, *Engraulis mordax* Mar Biol 102: 143-151.

Hare J, Cowen R (1997) Size, growth, development, and survival of the planktonic larvae of *Pomatomus saltatrix* (Pisces: pomatomidae) Ecology 78: 2415-2431.

Hay DE (1982) Fixation shrinkage of herring larvae: effects of salinity, formalin concentration, and other factors. Can J Fish Aquat Sci 39: 1138-1143.

Hinrichsen H, John M, Aro E, Grønkjær P,Voss R (2001) Testing the larval drift hypothesis in the Baltic Sea: retention versus dispersion caused by wind-driven circulation. ICES J Mar Sci 58: 973-984.

Hjort J (1914) Fluctuations in the great fisheries of northern Europe viewed in the light of biological research. Rapp P-v Réun Cons Perm Int Explor Mer 20: 1-228.

Høie H, Folkvord A, Johannessen A (2000) A multivariate analysis of herring larvae from different environmental conditions. ICES CM R:04.

Holt GJ, Arnold CR (1983) Effects of ammonia and nitrite on growth and survival of red drum eggs and larvae. Trans Am Fish Soc 112: 314-318.

Houde ED (1987) Fish early life dynamics and recruitment variability. Am Fish Soc Symp 2: 17-29.

Houde ED (1989) Subtleties and episodes in the early life of fishes. J Fish Biol 35: 29-38.

Houde ED (1997) Patterns and trends in larval-stage growth and mortality in teleost fish. J Fish Biol 51: 52-83.

Houlihan DF, Pedersen BH, Steffensen JF, Brechin J (1995) Protein synthesis, growth and energetics in larval herring (*Clupea harengus*) at different feeding regimes. Fish Phys Biochem 14: 195-208.

Hovenkamp F (1992) Growth-dependent mortality of larval plaice *Pleuronectes platessa* in the North Sea. Mar Ecol Prog Ser 82: 95-101.

Hunt von Herbing I (2001) Development of feeding structures in larval fish with different life histories: winter flounder and Atlantic cod J. Fish Biol 59: 767-782.

Hunter JR (1972) Swimming and feeding behavior of larval anchovy *Engraulis mordax*. Fish Bull US 70: 821-838.

Hunter JR (1981) Feeding ecology and predation of marine larvae. In: Lasker R (ed) Marine fish larvae: morphology, ecology and relation to fisheries, University of Washington Press, Seattle pp 34-77.

Hunter JR, Lo NCH, Leong RJH (1985) Batch fecundity in multiple spawning fish. In: Lasker R (ed) An egg production method for estimating spawning biomass of pelagic fish: application to the Northern Anchovy, *Engraulis mordax.* NOAA Tech Rep NMFS 36: 67-68.

Jennings S (1991) The effects of capture, net retention and preservation upon lengths of larval and juvenile bass *Dicentrarchus labrax* (L.) J Fish Biol 38: 349-357.

Johnson DW, Katavic Y (1986) Survival and growth of sea bass (*Dicentrarchus labrax*) larvae as influenced by temperature, salinity and delayed initial feeding. Aquaculture 52: 11-19.

Johnston IA (1993) Temperature influences muscle differentiation and the relative timing of organogenesis in herring (*Clupea harengus)* larvae. Mar Biol 116:363-379.

Johnston IA (1999) Muscle development and growth: potential implications for flesh quality in fish. Aquaculture 177: 99-115.

Johnston IA, Vieira VLA, Abercromby M (1995) Temperature and myogenesis in embryos of the Atlantic herring *Clupea harengus*. J Exp Biol 198: 1389-1403.

Johnston IA, Cole NJ, Abercromby M, Vieira VLA (1998) Embryonic temperature modulates muscle growth characteristics in larval and juvenile herring. J Exp Biol 201: 623-646.

Johnston IA, Vieira VLA, Temple GK (2001a) Functional consequences and population differences in the developmental plasticity of muscle to temperature in Atlantic herring *Clupea harengus* Mar Ecol Prog Ser 213: 285-300.

Johnston IA, Temple GK, Vieira VLA (2001b) Impact of temperature on the growth and differentiation of muscle in herring larvae. In: Atkinson D, Thorndyke M (eds) Environment and Animal Development:Genes, Life Histories and Plasticity. BIOS Scientific Publishers Ltd, Oxford, pp 99-120.

Jolicoeur P, Mosimann JE (1960) Size and shape variation in the painted turtle. A principal component analysis. Growth 24: 339-354.

Kashuba SA, Matthews WJ (1984) Physical condition of larval shad during spring-summer in a south-western reservoir. Trans Am Fish Soc 113:199-204.

Kendall AW, Ahlstrom EH, and Moser HG (1984) Early life history stages of fishes and their characters. Ontogeny and Systematics of Fishes: Am Soc Icht Herpet (spec pub) 1: 11-22.

Kimura R, Watanabe Y, Zenitani H (2000) Nutritional condition of first-feeding larvae of Japanese sardine in the coastal and oceanic waters along the Kuroshio Current. ICES J Mar Sci 57: 240-248.

Kingsford MJ (1990) Linear oceanographic features.: a focus for research on recruitment processes. Aust J Ecol 15: 391-401.

Kiørboe T, Munk P, Richardson K, Christensen V, Paulsen H (1988) Plankton dynamics and larval herring growth, drift and survival in a frontal area. Mar Ecol Prog Ser 44.: 205-219.

Kjørsvik E, Van der Meeren T, Kryvi H, Arnfinnson J, Kvenseth PGK (1991) Early development of the digestive tract of cod larvae, *Gadus morhua* L., during start-feeding and starvation. J Fish Biol 38: 1-15.

Klaoudatos S, Tsevis N, Conides A (1990) Energy sources during the early larval development of the European sea bass, *Dicentrarchus labrax* (L.). Aquaculture 87: 361-372.

Kloppmann M, Hillgruber N, von Westernhagen H (2002) Wind-mixing effects on feeding success and condition of blue whiting larvae in the Porcupine Bank area. Mar Ecol Prog Ser 235: 263-277.

Koslow JA (1992) Fecundity and the stock-recruitment relationship. Can J Fish Aquat Sci 49: 210-217.

Koumans JTM, Akster HA, Booms GHR, Lemmens CJJ, Osse JWM (1991) Numbers of myosatellite cells in white axial muscle of growing fish: *Cyprinus carpio* L. (Teleostei). Am J Anat 192: 418-424.

Koumoundouros G, Divanach P, Anezaki L, Kentouri M (2001) Temperature-induced ontogenetic plasticity in sea bass (*Dicentrarchus labrax*) Mar Biol 139: 817-830.

Lasker R, Feder HM, Theilacker GH, May C (1970) Feeding, growth and survival of Engraulis mordax larvae reared in the laboratory. Mar Biol 5:345-353.

Lasker R (1981) Marine fish larvae. Morphology, ecology, and relation to fisheries. University of Washington Press, Seattle, 131 pp.

Lasker R (1985) What limits clupeoid production? Can J Fish Aquat Sci 42: 31-37.

Laurel BJ, Brown JA, Anderson R (2001) Behaviour, growth and survival of redfish larvae in relation to prey availability. J Fish Biol 59: 884-901.

Laurence GC (1972) Comparative swimming abilities of fed and starved larval largemouth bass (*Micropterus salmoides*). J Fish Biol 38:1–15.

Legendre P, Legendre L (1998) Numerical Ecology. Elsevier Science BV, 853 pp.

Leggett WC, Deblois E (1994) Recruitment in marine fishes: Is it regulated by starvation and predation in the egg and larval stages? Neth J Sea Res 32: 119-134

Litvak MK, Leggett WC (1992) Age and size-selective predation on larval fishes: the bigger is better paradigm revisited. Mar Ecol Prog Ser 81:1-12.

Lleonart J, Salat J, Torres GJ (2000) Removing allometroc effects of body size in morphological analysis. J Theor Biol 205: 85-93.

López-Jurado JL, García-Lafuente J, Pinot JM, Álvarez A (1996) Water exchanges in the Balearic Islands. Bull Inst océanogr, Monaco 17: 41-63.

Love RM (1970) The chemical biology of fishes, Vol.1. Academic press, New York.

Lowery MS, Roberts SJ, Somero GN (1987) Effects of starvation on the activities and localization of glycolytic enzymes in the white muscle of the barred sand bass *Paralabrax nebulifer.* Physiol Zool 60: 538-549.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) J Bioch Chem 193: 265-275.

MacKenzie BR, Leggett WC (1991) Quantifying the contribution of small-scale turbulence to the encounter rates between larval fish and their zooplankton prey: effects of wind and tide. Mar Ecol Prog Ser 73:149-160.

MacKenzie BR, Miller TJ, Cyr S, Leggett WC (1994) Evidence for a dome-shaped relationship between turbulence, and larval fish ingestion rates. Limnol Oceanogr 39: 1790-1799.

Manly BFJ (2001) Multivariate Statistical Methods. Chapman and Hall, London. 159 pp.

Mann KH, lazier, JRN (1996) Dyamics of Marine Ecosystems. Biological-physical interactions in the oceans, 2nd ed. Blackwell Science 394 pp.

Margulies D (1993) Assessment of the nutritional condition of larval and early juvenile tuna and spanish mackerel (Pisces: Scombridae) in the Panamá Bight. Mar Biol 115:317-330.

Martín P (1991) La pesca en Cataluña y Valencia (NO Mediterráneo): análisis de las series históricas de captura y esfuerzo. Inf Téc Sci Mar 162: 1-43.

Martin FD, Malloy R (1980) Histological and morphometric criteria for assessing nutritional state of larval striped bass, *Morone saxatilis* US Fish and Wildl Serv Biol Serv Prog FWS/OBS- 80/43: 157-166.

Martin FD, Wright DA, Means JC, Setzler-Hamilton EM (1985) Importance of food supply to nutritional state of larval stripped bass in the Potomac River estuary. Trans Am Fish Soc 114: 137-145.

Martin FD, Wright DA (1987) Nutritional state analysis and its use in predicting striped bass recruitment: laboratory calibration. Am Fish Soc Symp 2: 109-114.

Mathers EM, Houlihan DF, Cunningham MJ (1992) Nucleic acid concentrations and enzyme activities as correlates of growth rate of the saith *Pollachius virens*: growth-rate estimates of open-sea fish. Mar Biol 112: 363-369.

May RC (1971) Effects of delayed initial feeding on larvae of the grunion, *Leuresthes tenuis* (Ayres). US Nat Mar Fish Ser Fish Bull 69:411-425.

May RC (1974) Larval mortality in marine fishes and the critical period concept. In: JHS Blaxter (ed) The early life history of fish. Springer-Verlag, Berlin pp 3-19.

McFadzen IRB, Lowe DM, Coombs SH (1994) Histological changes in starved turbot larvae (*Scophtalmus maximus*) quantified by digital image analysis. J Fish Biol 44:255-262.

McFadzen IRB, Coombs SH, Halliday NC (1997) Histological indices of the nutritional condition of sardine *Sardina pilchardus* (Walbaum) larvae off the north coast of Spain. J Exp Mar Biol Ecol. 212: 239-258.

McGurk MD (1984) Ring deposition in the otoliths of larval pacific herring, *Clupea harengus pallasii*. Fish Bull US 82: 113-120.

McGurk MD (1985a) Multivariate analysis of morphometry and dry weight of Pacific herring larvae. Mar Biol 86: 1-11

McGurk MD (1985b) Effect of net capture on the postpreservation morphometry, dry weight, and condition factor of Pacific herring larvae Trans. Am. Fish. Soc. 114: 348-355.

McGurk MD (2000) Comparison of fecundity-length-latitude relationships between nonanadromous (Kokanee) and anadromous sockeye salmon (*Oncorhynchus nerka*). Can J Zool 78: 1791-1805.

Methot RD, Kramer D (1979) Growth of northern anchovy, Engraulis mordax, larvae in the sea. Fish Bull US 77: 413-423.

Michaeltz PH (1997) Factors affecting abundance, growth, and survival of age-0 gizzard shad. Trans Am Fish Soc 126: 84-100.

Mihelakakis A, Yoshimatsu T, Tsolkas C (2001) Spawning in captivity and early life history of cultured red porgy, *Pagrus pagrus.* Aquaculture 199: 333-352.

Millot C (1999) Circulation in the Western Mediterranean Sea. J Mar Syst 20: 423-442.

Miranda A, Cal RM, Iglesias J (1990) Effect of temperature on the development of eggs and larvae of sardine *Sardina pilchardus* Walbaum in captivity. J Exp Mar Biol Ecol 140: 69-77.

Molony BW, Choat JH (1990) Otolith increment widths and somatic growth rate: the presence of a time-lag. J Fish Biol 37:541-551.

Morales-Nin B, Pertierra JP (1990) Growth rates of anchovy (*Engraulis encrasicolus*) and sardine (*Sardina pilchardus*) in the Northwestern Mediterranean Sea. Mar Biol 107: 349-356.

Morales-Nin B (2000) Review of the growth regulation processes of otolith daily increment formation. Fish Res 1006: 1-15.

Morley SA, Batty RS (1996) The effects of temperature on “S-strike” feeding of larval herring, *Clupea harengus* L. Mar Fresh Behav Physiol 28: 123-136.

Murray AW, Kirschner MW (1989) Dominoes and clocks: The union of two views of the cell cycle. Science 246: 614-621.

Nakata H, Zenitani H (1996) Did offshore shift of the main spawning ground of Japanese sardine affect the availability of food for the larvae? In: Watanabe Y, Yamashita Y, Ooseki Y (eds) Survival strategies in Early Life Stages of Marine Resources. A.A. Balkema, Rotterdam, pp 115-123.

Neilson JD, Geen GH (1985) Effects of feeding regimes and diel temperature cycles on otolith increment formation in juvenile chinook salmon, *Oncorhynchus tshawytscha*. Fish Bull 83: 91–101.

Neilson JD, Perry RI, Valerio P, Waiwood KG (1986) Condition of Atlantic cod (*Gadus morhua*) larvae after the transition to exogenous feeding: morphometrics, buoyancy and predator avoidance. Mar Ecol Prog Ser 32:229-235.

Neilson JD, Perry RI (1990) Diel vertical migrations of juvenile fish: an obligate or facultative process?. Adv Mar Biol 26: 115-168.

O'Connell CP (1976) Histological criteria for diagnosing the starving condition in early post yolk sac larvae of the northern anchovy, *Engraulis mordax Girard.* J Exp Mar Biol Ecol 25: 285-312.

O'Connell CP (1980) Percentage of starving northern anchovy *Engraulis mordax* larvae in the sea as estimated by histological methods. Fish Bull US 78:475-489.

O'Connell CP, Paloma PA (1981) Histochemical indications of liver glycogen in samples of emaciated and robust larvae of the northern anchovy *Engraulis mordax.* Fish Bull US 79: 806-812.

Olivar MP, Shelton PA (1993) Larvae fish assemblages of the Benguela Current. Bull Mar Sci 53: 450-474.

Olivar MP, Sabatés A (1997) Vertical distribution of fish larvae in the north-west Mediterranean Sea in spring. Mar Biol 129: 289-300.

Olivar MP, Sabatés A, Abelló P, García M (1998) Transitory hydrographic structures and distribution of fish larvae and neustonic crustaceans in the north-western Mediterranean Oceanol Acta 21: 95-104.

Olivar MP, Ambrosio PP, Catalán IA (2000) A closed recirculation system for ecological studies in marine fish larvae: growth and survival of sea bass larvae fed with live prey. Aquat Living Resour 13:1-7.

Olivar MP, Salat J, Palomera I (2001) A comparative study of the spatial distribution patterns of the early stages of anchovy and pilchard in the NW Mediterranean Sea. Mar Ecol Prog Ser 217: 111-120.

Olivar MP, Catalán IA, Emelianov M, Fernández de Puelles ML (in press) Early stages of Sardina pilchardus and environmental anomalies in the North Western Mediteranean. Est Coast Shelf Sci.

Oliver M (1957) Biology of the sardine of the Balearic Islands. Con génér pêch Med, Déb doc tech FAO, Rome 4: 51-62.

Oozeki Y, Ishii T, Hirano R (1989) Histological study of the effects of starvation on reared and wild-caught larval stone flounder, *Kareius bicoloratus.* Mar Biol 100:269-275.

Overnell J, Batty RS (2000) Scaling of enzyme activity in larval herring and plaice: effects of temperature and individual growth rate on aerobic and anaerobic capacity J Fish Biol 56: 577-589.

Owen R W, Lo NCH, Butler JL,Theilacker,GH, Alvariño A, Hunter JR, Watanabe Y (1989) Spawning and survival patterns of larval northern anchovy, *Engraulis mordax*, in contrasting environments- a site-intensive study Fish Bull 87: 673-688.

Oyen FGF, Bruce MP, Asturiano JF, Ramos J, Carrillo M, Zanuy S (1997) An improve method for monitoring eggs and larvae of sea bass (*Dicentrarchus labrax* L.), Actas del VI Congreso Nacional de Acuicultura, Cartagena. Spain. Mimeo: 1-5.

Paine MD, Balon EK (1985) Early development of the northern logperch, *Percina caprodes semifasciata* according to the theory of saltatory ontogeny. In: EK Balon (ed) Early life history of fishes Dr. W. Junk Publishers, Dordrech. pp 132-149.

Palomera I (1992) Spawning of anchovy *Engraulis encrasicolus* in the northwestern Mediterranean relative to hydrographic features in the region. Mar Ecol Prog Ser 79: 215-223.

Palomera I, Rubíes P (1979) Ichthyoplankton de la mer Catalane. Larves de poissons recoltées sur deux stations fixes devant Barcelone au cours d'un cycle annuel. Rapp Comm Int Expl Mer Medit 25/26, 10: 201-206.

Palomera I, Morales-Nin B, Lleonart J (1988) Larval growth of anchovy, *Engraulis encrasicolus*, in the Western Mediterranean. Mar Biol 99: 283-291.

Palomera I, Olivar MP (1996) Nearshore ichthyoplankton off the Costa Brava (Northwest Mediterranean). Bol Inst Esp Oceanogr 22: 71-76.

Pardee AB (1989) G1 events and regulation of cell proliferation. Science 246: 603-608.

Parrish RC, Nelson CS, Bakun A (1981) Transport mechanisms and reproductive success of fishes in the Californian Current Biolog Oceanogr 1: 175-203.

Pascual A, Emelianov M, Larnicol G, Gomis D, Salat J, Buongiorno Nardelli B, D'Acunzo E (2000) Detection by altimetry and AVHRR images of an intense anticyclonic eddy in the Balearic Sea (Western Mediterranean). Proc Int Soc Opt Eng 4172: 31-36.

Pedersen BH, Ugelstad I, Hjelmeland K (1990) Effects of a transitory, low food supply in the early life of larval herring (*Clupea harengus*) on mortality, growth and digestive capacity. Mar Biol 107: 61-66.

Pepin P, Shears TH, Lafontaine Y (1992) Significance of body size to the interaction between larval fish (*Mallotus villosus*) and a vertebrate predator (*Gasterosteus acu*leatus) Mar Ecol Prog Ser 81: 1-12.

Pepin P (2002) Population analysis. In: Fuiman LA; Werner RG (eds) Fishery science: the unique contrubutions of early life stagesBlackwell Science, Iowa State press, pp 112-142.

Pinot JM, Tintoré J, Gomis D (1995) Multivariate analysis of the surface circulation in the Balearic Sea. Progr Oceanogr 36: 343-376.

Pinot JM , López-Jurado JL, Riera M (2002) The CANALES experiment (1996-1998). Interannual, seasonal, and mesoscale variability of the circulation in the Balearic Channels. Progr Oceanogr 55: 335-370.

Polo A (1991). Crecimiento y alimentación durante el desarrollo larvario de la dorada, *Sparus aurata* L., en cultivo. PhD Thesis, Universidad de Cádiz, 179 pp.

Polo A, Yúfera M, Pascual E (1991) Effects of temperature on egg and larval development of *Sparus aurata* L., Aquaculture 92: 367-375.

Powell AB, Chester AJ (1985) Morphometric indices of nutritional condition and sensitivity to starvation of Spot larvae. Trans Am Fish. Soc 114:338-347.

Power JH, Walsh PJ (1992) Metabolic scaling, buoyancy, and growth in larval Atlantic menhaden, *Brevoortia tyrannus*. Mar Biol 112: 17-22.

Puvanendran V, Leader LL, Brown JA (2002) Foraging behaviour of Atlantic cod (*Gadus morhua*) larvae in relation to prey concentration. Can J Zool/Rev Can Zool 80: 689-699.

Ramírez T, Cortés D, García A (2001) Growth of north Alboran Sea sardine larvae estimated by otolith microstructure, nucleic acids and protein content. J Fish Biol 59: 403-415.

Rao CR (1964) The use and interpretation of principal component analyis in applied research. Sankyhyaá Ser. A 26: 329-358.

Ré P (1984) Evidence of daily and hourly growth in pilchard larvae based on otolith growth increments, *Sardina pilchardus* (Walbaum, 1792). Cybium 8: 33-38.

Ré P, Farinha A, Meneses I (1988) Diel spawning time of sardine, *Sardina pilchardus* (Walbaum, 1792) (Teleostei, Clupeidae), off Portugal. Inv Pesq 52: 207-213.

Ré P, Silva C, Cunha E, Farinha A, Meneses I, Moita T (1990) Sardine spawning off Portugal. Bol Inst Nac Inv Pesc 15: 31-44.

Regner S, Dulcic J (1994) Growth of sea bass, *Dicentrarchus labrax*, larval and juvenile stages and their otoliths under quasi-steady temperature conditions. Mar Biol 119: 169-177.

Riveiro I, Guisande C, Lloves M, Maneiro I, Cabanas JM (2000) Importance of parental effects in larval survival in *Sardina pilchardus*. Mar Ecol Prog Ser 205: 249-258.

Rogers BA, Westin DT (1981) Laboratory studies on effects of temperature and delayed initial feeding on development of striped bass larvae. Trans Am Fish Soc 110:100-110.

Rooker JR, Holt GJ (1996) Application of RNA/DNA ratios to evaluate the condition and growth of larval and juvenile red drum (*Sciaenops ocellatus*) Mar Freshwat Res 47: 283-290.

Rothchild BJ, Osborn TR (1988) The effects of turbulence on planktonik contact rates. J Plankton Res 10: 465-475.

Sabatés A (1990a). Changes in the heterogeneity of mesoscale distribution patterns of larval fish associated with a shallow coastal haline front. Est Coast Shelf Sci 30: 131-140.

Sabatés A (1990 b) Distribution pattern of larval fish populations in the Northwestern Mediterranean. Mar Ecol Prog Ser 59: 75-82.

Sabatés A, Masó M (1992) Unusual larval fish distribution pattern in a coastal zone of the western Mediterranean. Limnol Oceanogr 37: 1252-1260.

Sabatés A, Olivar MP (1996) Variation of larval fish distributions associated with the variability in the location of a shelf-slope front. Mar Ecol Prog Ser 135: 11-20.

Sabatés A, Salat J, Olivar MP (2001) Advection of continental water as an export mechanism for anchovy, *Engraulis encrasicolus*, larvae. Scient Mar 65: 77-87.

Salat J (1996) Review of hydrographic environmental factors that may influence anchovy habitats in the northwestern Mediterranean. Scient Mar 60: 21-32.

Salat J, Cruzado A (1981) Masses d'eau dans la Méditerranée occidentale: Mer Catalane et eaux adjacents. Rapp et P-V des Réun Com Int Exp Scient Mer Méd 27: 201-209.

Salat J, García M, Cruzado A, Palanques A, Arín L, Gomis D, Guillén J, de León A, Puigdefàbregas J, Sospedra J, Velásquez ZR (2002) Seasonal changes of water mass structure and shelf slope exchanges at the Ebro shelf (NW Mediterranean). Cont Shelf Res 22: 327-346.

Sánchez-Velascoa L, Valdez-Holguína JE, Shirasagoa B, Cisneros-Matac MA, Zaratea A (2002) Changes in the Spawning Environment of Sardinops caeruleus in the Gulf of California during El Niño 1997-1998. Est Coast Shelf Sci 54.

Sawant MS, Zhang S, Li L (2001) Effect of salinity on development of zebrafish, *Brachydanio rerio*. 81: 1347-1350.

Sclafani M, Taggart CT, Thompson KR (1993) Condition, buoyancy and the distribution of larval fish: implications for vertical migration and retention. J Plankton Res 15: 413-435.

Sclafani M, Stirling G, Leggett WC (1997) Osmoregulation, nutritional effects and buoyancy of marine larval fish: a bioassay for assessing density changes during the earliest life-history stages. Mar Biol 129:1-9.

Sclafani M, Stirling G, Leggett WC (2000) Osmotic condition, buoyancy change and mortality in larval cod Gadus morhua. A bioassay for assessing near-term mortality Mar Ecol Prog Ser 193:157-166.

Secor DH, Dean JM (1989) Somatic growth effects on the otolith-fish size relationship in young pond-reared stripped bass, *Morone saxatilis*. Can J Fish Aquat Sci 46: 113-121.

Secor DH, Dean JM (1992) Comparison of otolith-based back-calculation methods to determine individual growth histories of larval stripped bass *Morone saxatilis*. Can J Fish Aquat Sci 49: 1439-1454.

Shapiro HM (1988) Practical flow cytometry. Alan R. Liss, Inc. New York.

Shelbourne JE (1957) The feeding and condition of plaice larvae in good and bad plankton patches. J Mar Biol Ass UK 36:539-552

Shelton PA, Hutchings L (1989) Ocean stability and Anchovy spawning in the outhern benguela current region. Fish Bull 88: 323-338.

Sheperd JG, Cushing DH (1980) A mechanism for density-dependent survival of larval fish as the basis of a stock-recruitment relatioship J Cons int Explor Mer 39: 160-167.

Shepherd TD, Costain KE, Litvak MK (2000) Effect of development rate on the swimming, escape responses, and morphology of yolk-sac stage larval American plaice, *Hippoglossoides platessoides*. Mar Biol 137: 737-745.

Shimizu H, Nakata K, Nakano H (1989) Comparison of nutritional condition of sardine larvae, Sardinops melanostictus (T and S) taken from the coastal and offshore region of the Kuroshio Current. Nippon-Suisan Gakkaishi Bull-Jap Soc Sci Fish 55: 179.

Sieg A (1992) A histological study on the nutritional condition of larval and metamorphosing fishes of the genus *Vinciguerria* (Photichtidae) sampled in two contrasting environments. J Appl Ichtyol 8:154-163.

Sieg A (1998) A study on the histological classification of the in situ-nutritional condition of larval South-west Atlantic anchovy, *Engraulis anchoitia* Hubbs and Marini, 1935. Arch Fish Mar Res 46:19-35.

Silva A, Miranda A (1992) Laboratory rearing of sardine larvae, Sardina pilchardus (Walb.), and early effects of starvation: a preliminary experiment. Bol Inst Esp Oceanogr 8: 163-174.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 159: 76-85.

Solá A, Franco C, Lago de Lanzós A and Motos L (1992) Temporal evolution of *Sardina pilchardus* (Walb.) spawning on the N-NW coast of the Iberian peninsula. Bol Inst Esp Oceanogr 8: 97-114.

Somero GN, Childress JJ (1980) A violation of the metabolism-size scaling paradigm: activities of glycolytic enzymes in muscle increase in larger-size fish. Physiol Zool 53: 322-337.

Steele MA, Forrester GE (2002) Early postsettlement predation on three reef fishes: effects on spatial patterns of recruitment. Ecology 83: 1076-1091.

Stickland NC, White RN, Mescall PE, Crook AR, Thorpe JE (1988) The effect of temperature on myogenesis in embrionic development of the Atlantic salmon (*Salmo salar* L.) Anat Embryol 178: 253:257.

Stoiber W, Haslett JR, Sänger AM (1999) Myogenic patterns in teleosts: what does the present evidence really suggest?. J Fish Biol 55: 84-99.

Sullivan KM, Somero GN (1980) Enzyme activities of fish skeletal muscle and brain as influenced by depth of occurrence and habits of feeding and locomotion. Mar Biol 60: 91-99.

Suneetha K-B, Folkvord A, Johannessen, A (1999) Responsiveness of selected condition measures of herring, *Clupea harengus*, larvae to starvation in relation to ontogeny and temperature. Envi Biol Fish 54: 191-204.

Suthers IM (1998) Bigger? Fatter? Or is faster growth better? Considerations on condition in larval and juvenile coral-reef fish. Aust J Ecol 23: 265-273.

Suthers, IM, Fraser A, Frank KT (1992) Comparison of lipid, otolith and morphometric condition indices of pelagic juvenile cod *Gadus morhua* from the Canadian Atlantic. Mar Ecol Prog Ser 84: 31-40.

Suthers IM, Cleary J, Battaglene SC, Evans R (1996) Relative RNA Contents as a measure of condition in larval and juvenile fish. Mar Freshwat Res 47: 301-307.

Suthers IM, van der Meeren T, Jorstad KE (1999) Growth histories derived from otolith microstructure of three Norwegian cod stocks co-reared in mesocosms; effect of initial size and prey size changes. ICES J Mar Sci 56: 658-672.

Temple GK, Fox CJ, Stewart R, Johnston IA (2000) Variability in muscle growth characteristics during the spawning season in a natural population of Atlantic herring *Clupea harengus*. Mar Ecol Prog Ser 205: 271-281.

Theilacker GH (1978) Effect of starvation on the histological and morphological characteristis of jack mackerel, (*Trachurus symmetricus*), larvae. Fish Bull 76: 403-414.

Theilacker GH (1980) Changes in body measurments of larval northern anchovy , Engraulis mordax, and other fishes due to handling and preservation. Fish Bull US 78: 685-692.

Theilacker GH, Dorsey D (1980) Larval fish diversity, a summary of laboratory and field research. UNESCO Int Oceanogr Comm Workshop Rep 28:105-142.

Theilacker GH (1986) Starvation-induced mortality of young sea-caught jack mackerel, *Trachurus symmetricus*, determined with hystological and morphological methods. Fish Bull 84: 1-17.

Theilacker GH, Watanabe Y (1989) Midgut cell hight defines nutritional status of laboratory raised larval northern anchovy, *Engraulis mordax*, Fish Bull 87: 457-469.

Theilacker GH, Shen W (1993a) Fish larval condition analyzed using flow cytometry. In: Walther BT, Fyhn HJ (eds) Physiological and biochemical aspects of fish development University of Bergen, Norway, pp 346-355.

Theilacker GH, Shen W (1993b) Calibrating starvation-induced stress in larval fish using flow cytometry. Am Fish Soc Symp 14: 85-94.

Theilacker GH, Porter SM (1995) Condition of larval walleye pollock, *Theragra chalcogramma*, assessed with histological and shrinkage indices. Fish Bull 93: 333-344.

Theilacker GH, Bailey KM, Canino MF, Porter SM (1996) Variations in larval walleye pollock: a synthesis. Fish Oceanogr 5: 112-123.

Theilacker GH, Shen W (2001) Evaluating growth of larval walleye pollock, *Theragra Chalcogramma*, using cell cycle analysis. Mar Biol 138: 897-907.

Thorpe RS (1975) Quantitative handling of characters useful in snake systematics with particular reference to intraspecific variation in the Ringed snake *Natrix natrix* (L.). Biol J Linn Soc 7: 27-43.

Tilseth S, Stromme T (1976) Changes in buoyancy and activity during starvation of cod larvae (Gadus morhua L.) ICES. CM f:33: 11

Tintoré J, Wang DP, La Violette PE (1990) Eddies and thermohaline intrusions of the shelf/slope front off northeast Spanish coast. J Geoph Res 95: 1627-1633.

Torres JJ, Somero GN (1988) Metabolism, enzymic activities and cold adaptation in Antarctic mesopelagic fishes. Mar Biol 98: 169-180.

Tudela S, Palomera I, Quílez G (2002) Feeding of anchovy *Engraulis encrasicolus* larvae in the north-west Mediterranean. J Mar Biol Ass UK 82. 349-350.

Uriarte I, Balbontín F (1987) Caracterización del estado de hambruna en las larvas de sardina *Sardinops sagax musica* (Pisces, clupeiformes), mediante criterios morfométricos e histológicos Rev Biol Mar Valparaíso 23: 77-106.

Usher ML, Stickland NC, Thorpe JE (1994) Muscle development in the Atlantic salmon (*Salmo salar*) embryos and the effect of temperature on muscle cellularity. J Fish Biol 44: 953-964.

Veggetti A, Mascarello F, Scapolo PA, Rowlerson A (1990) Hyperplastic and hypertrophic growth of lateral muscle in *Dicentrarchus labrax* (L.). Anat Embryol 182: 1-10.

Vieira VLA and Johnston IA (1992) Influence of temperature on muscle-fibre development in larvae of the herring *Clupea harengus* Mar Biol 112: 333-341.

Vindeløv LL, Christensen IJ (1994) Detergent and proteolitic enzyme-based techniques for nuclear isolation and DNA content analysis. In: Darzynkiewicz Z, Robinson JP, Crissman HA (eds) Flow cytometry, Academic Press, Inc., New York, 41: 219-230.

Wang SY, Lum JL, Carls MG, Rice SD (1993) Relationship between growth and total nucleic acids in juvenile pink salmon, *Oncorhynchus gorbuscha*, fed crude oil contaminated food. Can J Fish Aquat Sci 50: 996-1001.

Ward T, Hoedt F, Mcleay L, Dimmlich W, Kinloch M, Jackson G, Mcgarvey R, Rogers P, Jones K (2001) Effects of the 1995 and 1998 mass mortality events on the spawning biomass of sardine, *Sardinops sagax*, in South Australian waters. ICES J Mar Sci 58: 865-875.

Watanabe Y (1985) Histological changes in the liver and intestine of freshwater goby larvae during short-term starvation. Bull Jap Soc Sci Fish 51:707-710.

Watanabe Y, Kuroki T (1997) Asymptotic growth trajectories of larval sardine Sardinops melanostictus in the coastal waters off weatern Japan. Mar Biol 127: 369-378.

Weatherley AH (1990) Approaches to understanding fish growth. Trans Am Fish Soc 119: 662-672.

Werner FE, Quinlan JA, Lough RG, Lynch DR (2001) Spatially-explicit individual based modeling of marine populations: a review of the advances in the 1990s. Sarsia 86:411-421.

Westerman M, Holt GJ (1994) RNA/DNA ratio during the critical period and early larval growth of the red drum *Sciaenops ocellatus.* Mar Biol 121: 1-9.

Whitehead PJP (1984) Clupeidae. In: Whitehead PJP, Bauchot M-L, Hureau J-C, Nielsen J, Tortonese E (eds) *Fishes of the North-eastern Atlantic and the Mediterranean*, UNESCO 1: 268-281.

Yang T-H, Somero GN (1993) Effects of feeding and food deprivation on oxygen consumption, muscle protein concentration and activities of energy metabolism enzymes in muscle and brain of shallow-living (*Scorpaena guttata)* and deep- living *(Sebastobolus alascanus)* scorpaenid fishes. J Exp Biol 181: 213-232.

Yin MC, Blaxter JHS (1987) Feeding ability and survival during starvation of marine fish larvae reared in the laboratory. J Exp Mar Biol Ecol 105:73-83.

Yúfera M, Pascual E, Polo A, Sarasquete MC (1993) Effect of starvation on the feeding ability of gilthead seabream (*Sparus aurata* L.) larvae at first feeding. J Exp Mar Biol Ecol 169:259-272.

Zambonino-Infante JL, Cahu CL, Perés A, Quazuguel P, Le Gall MM (1996) Sea bass (*Dicentrarchus labrax*) larvae fed different Artemia rations: growth, pancreas enzymatic response and development of digestive functions. Aquaculture 139: 129-138.

Zweifel JR, Lasker R (1976) Prehatch and posthatch growth of fishes a general model. Fish Bull 74: 609-621.

RESUMEN

RESUMEN

1. INTRODUCCIÓN Y OBJETIVOS GENERALES

Las poblaciones de peces están sometidas a fuertes fluctuaciones en el número de individuos que se incorporan al stock pescable, proceso que se conoce como reclutamiento. La difícil predicción de estas fluctuaciones supone uno de los mayores problemas en la gestión de las pesquerías (Koslow 1992). Dado que en muchas especies sólo un 0.01% acaba reclutando al stock, mucha de la variabilidad observada se suele atribuir a las pequeñas variaciones en la mortalidad de huevos y larvas.

También es sabido que los factores que afectan a la mortalidad en las primeras fases del desarrollo son diversos e interactúan, probablemente, de forma solapada. En una primera división, estos factores pueden clasificarse en bióticos (principalmente éxito alimentario, depredación y enfermedad) y abióticos (hidrografía e hidrodinámica). Los estudios sobre la importancia relativa de cada uno de ellos presenta un grado de dificultad variable, ya sea en términos de la tecnología necesaria, el tiempo requerido o los costes económicos que implican.

La comprensión de la relación entre procesos hidrodinámicos y supervivencia larvaria requiere la utilización de modelos complejos (ej. Brickman y Frank 2000; Hinrichsen et al. 2001), y el estudio *in situ* del efecto de la depredación es difícil (Bailey 1989). Por el contrario, el estudio del estado nutricional o “condición” (tras Shelbourne 1957) de las larvas, puede ser realizado sobre cada especimen y, como aproximación al estado de crecimiento, goza todavía de gran popularidad en la estimación de la susceptibilidad de muerte del individuo (Ferron y Leggett 1994; Theilacker 1996; Suthers 1998). Aunque la muerte directa por inanición cabe en lo posible, se observa raramente en estudios de campo. Más probablemente, y de acuerdo con las hipótesis actuales, las larvas en un estado nutricional deficiente experimentarán una mayor mortalidad debida a predación, transporte desfavorable, etc. (Houde 1987; Leggett y Deblois 1994; Hare y Cowen 1997).

Los trabajos sobre el uso de índices de estado nutricional en larvas de peces son abundantes (Ferron y Leggett 1994; Suthers 1998). La mayoría de estos trabajos pueden

incluírse en dos grupos principales: aquellos que estudian las propiedades del índice en cuestión bajo condiciones controladas, y los que aplican índices existentes (previa calibración en laboratorio o sin ella, en cuyo caso la utilidad aparece en términos relativos a los grupos considerados) para estudiar la relación entre ambiente y condición e inferir así la posible supervivencia de las larvas.

La presente tesis pretende contribuir al conocimiento de estas dos grandes divisiones centrándose en los dos siguientes objetivos generales.

- ***Profundizar en el estudio de diversos índices de condición en una especie (lubina* Dicentrarchus labrax*) que cría con facilidad en el laboratorio.***

Para poder comparar las propiedades de cada índice de condición, es deseable estudiar un gran número de ellos en individuos de historia ambiental conocida y similar. La mayoría de estudios de laboratorio centran su esfuerzo sobre un sólo índice de condición, con contadas excepciones (ej. Martin et al. 1985; Bisbal y Bengston 1995). La obtención de resultados fiables implica un conocimiento de los parámetros de cría de las especies, de forma que el efecto de los factores estudiados (ej: cantidad de comida) no varíen de forma impredecible. Por esa razón, se eligió la lubina (*Dicentrarchus labrax*) para esta parte de la tesis.

Los índices de condición se han dividido clásicamente con relación al nivel de organización biológica que integran. Así, los índices pueden clasificarse atendiendo a un nivel de organismo, tisular o celular. Estos índices operan a diferentes escalas temporales. Típicamente, cuanto mayor es el nivel de organización, más tiempo tarda el índice en reflejar un cambio ambiental para un determinado estado de desarrollo, especie, conjunto de condiciones ambientales o estado nutricional específico en el momento de la captura. Tal como concluyen Ferron y Leggett (1994), no existe un "índice único" óptimo, y normalmente debe elegirse uno o la combinación de ellos que mejor responda al objetivo concreto del estudio.

En la primera parte de esta tesis se abordó el estudio de índices en los tres niveles de organización mencionados. Se estudiaron índices morfométricos (integran el nivel de organismo), histológicos y bioquímicos (integran el nivel celular). Además, se describe de

forma detallada el sistema de cultivo y se justifica la idoneidad del mismo para el ulterior análisis de índices de condición. Teniendo en cuenta que los valores absolutos de los índices son específicos para cada especie, la orientación de esta parte, experimental, se dirigió hacia la comparación de las propiedades de los índices, la mejora de algunos de ellos, el ensayo de métodos poco utilizados y la relación con la supervivencia en el laboratorio. A continuación se ofrecen algunos datos sobre los índices estudiados.

Los índices morfométricos de condición se han utilizado desde antiguo (Shelbourne 1957; Theilacker 1978) y se basan en el estudio de la forma, o el cambio de forma, en respuesta a la variación de alimento. La hipótesis común a estos índices es que, en respuesta a un déficit alimenticio, algunas partes del cuerpo se modifican respecto a otras menos sensibles a una deficiencia nutricional. Los principales problemas en la utilización de índices morfométricos para detectar un deficiente estado nutricional estriban en los efectos de la talla y de la alometría (tipo de crecimiento en el que una parte del cuerpo crece de forma diferencial respecto a otra) sobre las variables corporales, cuestiones que a menudo pueden confundir los efectos de la cantidad/calidad del alimento en la forma del individuo. En estudios de campo, la edad de la larva suele ser desconocida y la estandarización por talla es común. En la sección 2.2. de la tesis se aplica un método para la eliminación del efecto de la talla, que incorpora la información sobre alometría.

Los índices histológicos consisten en el examen de células y órganos, y su variación, en respuesta al tratamiento alimenticio. La gradación de los resultados obtenidos sobre distintas variables histológicas permite extraer una gran información de cada individuo. Otra ventaja de este tipo de índices es que el patrón de degradación, tisular o celular, suele mantenerse entre especies y es poco dependiente de la talla (Ferron y Leggett 1994; Grioche 1998). Por el contrario, uno de los problemas que plantean es la difícil objetivación de los métodos de valoración de las características de los tejidos o células, ya que las medidas suelen basarse en apreciaciones cualitativas o semi-cuantitativas (O'Connell 1980; Sieg 1992; McFadzen 1997). En la sección 2.3. se aborda este problema, comparándose las ventajas e inconvenientes de las medidas cualitativas respecto a las cuantitativas.

En cuanto a los índices que caracterizan el nivel de organización celular, o índices bioquímicos, se caracterizan por tener bajos tiempos de respuesta respecto a los anteriores.

Este tipo de medidas han tenido un enorme éxito (Ferron y Leggett 1994; Suthers 1998). Al igual que la mayoría de los índices, se basan en establecer una relación entre variables sensibles a la alimentación y aquellas menos sensibles. Uno de los índices más populares es el cociente ARN/ADN (Buckley 1979; Bergeron 1997; Buckley et al. 1999). Se basa en la asunción de que la cantidad de ARN celular es proporcional al crecimiento proteico. Ante una alimentación inadecuada, el crecimiento y la cantidad de ARN se reduce mientras que el ADN permanece relativamente inalterado. Esta medida se ha utilizado con éxito para calcular crecimiento y condición (Buckley 1979; Westerman y Holt 1994; Clemmensen 1996).

Otro de los índices bioquímicos utilizados, aunque con menor frecuencia, se basa en la actividad de ciertas enzimas metabólicas (Lowery et al. 1987; Yang and Somero 1993; Mathers et al. 1993). Típicamente se estudia la actividad de la lactato deshidrogenasa (LDH) y de la citrato sintetasa (CS). La LDH es el enzima terminal de la glicólisis anaeróbica y transforma piruvato en lactato. Este enzima es abundante en el tejido muscular, y su actividad se ve potenciada en condiciones anaeróbicas como las que se dan en natación rápida para acometer presas. En varios estudios realizados en larvas de peces, la actividad LDH se ha relacionado positivamente con la cantidad y calidad de alimento (Clarke et al. 1992) y negativamente con la actividad natatoria en larvas debilitadas (Fiedler et al. 1998). La actividad CS tiene lugar en el ciclo de Krebs y refleja la capacidad aeróbica del tejido estudiado. Se ha observado que la actividad CS es menor en larvas en hambruna respecto a larvas alimentadas (Mathers et al. 1993). La actividad de ambas enzimas suele normalizarse por unidad de proteína. Una mayor actividad se equipara a una mayor cantidad de enzima, en condiciones de saturación de sustrato.

Otro de los métodos, todavía más infrecuente en los análisis de condición, consiste en el cálculo de las tasas de proliferación celular medidas por citometría de flujo. Se basa en observaciones *in vitro* que muestran una parada en el ciclo de crecimiento celular en ausencia de fuente de energía (Pardee 1989; Murray y Kirschner 1989). Los escasos estudios donde se ha aplicado esta técnica al crecimiento larvario utilizaron tejido muscular y cerebral (Theilacker y Shen 1993 a,b, Bromhead et al. 2000; Theilacker and Shen 2001), sobre la base empírica de que las células de estos dos tejidos se dividen durante la mayor parte del ciclo vital en los peces (Greer-Walker 1970; Bierse et al. 1980).

- *Explorar las relaciones entre factores ambientales, estado nutricional y crecimiento de la sardina* ***Sardina pilchardus*** *(Walbaum 1792) en el Mar Catalán.*

La sardina Sardina pilchardus es un clupeido que habita en el Atlántico noroccidental, Mar Mediterráneo, Mar de Mármara y Mar Negro. En el Mediterráneo es más frecuente en su parte Oeste y Adriático que en la parte Este (Whitehead 1984). Como la mayoría de los clupeidos, se trata de una especie iterópara (una misma hembra desova de forma intermitente a lo largo del período de puesta). La época reproductiva es estacional y, en el Mediterráneo, tiene lugar entre octubre y mayo, con un pico en enero-febrero (Gómez-Larrañeta 1960; Palomera y Rubíes 1979; Palomera y Olivar 1996). Es la especie pelágica más abundante en el Mediterráneo nor-occidental (Martín 1991) y soporta la mayor pesquería (en términos de biomasa) de la costa catalana. A pesar de su importancia económica y del conocido declive del stock de adultos (Anónim 2000), los estudios sobre las relaciones entre el ambiente y sus primeros estadíos de vida no están desarrollados en esta zona.

La oceanografía del área es bien conocida (Salat y Cruzado 1981; Font et al. 1988; López-Jurado et al. 1996; Millot 1999; Pinot et al. 2002; Salat et al. 2002). Se caracteriza por una circulación ciclónica controlada por dos frentes de densidad permanentes: el frente catalán plataforma-talud, y el frente balear. El primero separa las Aguas Atlánticas (AW) antiguas del centro de la cuenca, más saladas, de las aguas de plataforma, menos saladas. El frente balear separa las AW antiguas de la menos salina AW nueva, que se introduce en la cuenca a través de los canales de las Baleares (Pinot 2002). La circulación está sujeta a fuertes variaciones de mesoescala, que se han atribuído a la acción de remolinos de aguas abiertas (Tintoré 1990; García et al. 1998). Han sido descritos algunos de los efectos de la hidrografía sobre poblaciones de larvas de anchoa (en verano y primavera) y marginalmente sobre sardina (Palomera 1992; Sabatés y Masó 1992; Sabatés y Olivar 1996; Salat 1996; Olivar y Sabatés 1997; Olivar et al. 1998), y se asocian al cambio de posición del frente catalán plataforma-talud, la circulación anticiclónica en plataforma y el efecto de advección superficial de aguas dulces en primavera.

Los únicos trabajos llevados a cabo en larvas de sardina durante la época de puesta, abordan sus patrones de distribución vertical y horizontal (Olivar et al. 2001). No existen otros estudios sobre la ecología larvaria de sardina en la costa catalana durante la época de puesta, ni sobre la relación de variables ambientales con su estado nutricional. En este contexto, la segunda parte de la tesis ofrece una serie de elementos en respuesta a esta problemática. En una primera aproximación (sección 3.1.) se estudian las distribuciones de huevos y larvas con relación a diversas variables ambientales, en tres campañas efectuadas en la época de puesta (noviembre 1998, febrero 1999, noviembre 1999). En una segunda fase (sección 3.2.) se exploran las relaciones entre ambiente y estado nutricional, medido con índices histológicos y bioquímicos. Además, se aplican análisis de crecimiento a largo plazo, por medio de análisis de otolitos y determinación de la variación en el patrón de crecimiento de fibras musculares (ver introducción).

2. ESTUDIOS DE LABORATORIO

2.1. Sistema de cría, crecimiento y supervivencia

La mayor parte de esta sección ha sido publicada en: "Olivar MP, Ambrosio PP, **Catalán IA** (2000) A closed recirculation system for ecological studies in marine fish larvae: growth and survival of sea bass larvae fed with live prey. Aquat Living Resour 13: 1-7."

Esta sección describe la validez de un sistema de cultivo de circulación cerrada, a pequeña escala, para el estudio de larvas de peces en respuesta a diferentes tratamientos alimenticios. El sistema sirvió a la vez como incubadora y "hatchery". Se estudió la variación temporal de parámetros físico-químicos, la supervivencia y el crecimiento longitudinal de larvas de lubina durante el primer mes de vida, prestándose especial atención a la variación de compuestos nitrogenados. Los resultados que se presentan en este capítulo corresponden sólo a los tres de mayor duración temporal: tratamiento Alimentado (A), Hambruna (H) y Alimentación Retrasada (AR). El diseño del sistema permitió mantener unos niveles de amonio, nitritos y nitratos por debajo de los valores considerados perjudiciales para la supervivencia o la capacidad alimenticia de varias especies de peces marinos.

La mortalidad del tratamiento A fue prácticamente nula a partir del noveno día después de la eclosión (DDE). El tratamiento H indujo una mortalidad masiva tras 17 días en ausencia de alimento. Las larvas del tratamiento AR, que permanecieron sin comida hasta 13 DDE, sufrieron una mortalidad similar a la del tratamiento H hasta dia 13, recuperándose hasta mortalidad cero cuatro días después. El crecimiento en longitud fue similar entre los tratamientos alimenticios durante las dos primeras semanas de vida larvaria, y fue descrito por una curva Gompertz con asíntota entre 5,3 y 5,7 mm de longitud total (LT). En los tratamientos A y AR, se observó un segundo ciclo de crecimiento Gompertz algún tiempo después del inicio de la alimentación. Las diferencias en el crecimiento (en tasa puntual y en longitud total) entre los tratamientos A y AR fueron especialmente evidentes durante la tercera semana de vida larvaria. Sin embargo, tras dos semanas de alimentación continuada estas diferencias se redujeron. Los patrones de crecimiento bajo el tratamiento A fueron similares a los descritos para sistemas de cría de circulación abierta.

El presente estudio ofrece suficiente evidencia para afirmar que los tratamientos alimenticios fueron los causantes de las diferencias observadas en el crecimiento y la supervivencia de las larvas. Se considera que este sistema de cría puede ser de utilidad para futuros ensayos de laboratorio.

2.2. Índices morfométricos

El primer objetivo de esta sección fue de generar datos morfométricos que contuviesen sólo información sobre la forma de las larvas, eliminando el efecto de la talla sobre la alometría sin despreciar la información sobre ésta. En segundo lugar, se quiso averiguar qué variables morfométricas, de entre las medidas, ofrecen una mejor discriminación entre grupos de larvas sometidas a los tratamientos alimenticios A, AR y H anteriormente descritos.

La variación de cinco variables morfométricas con relación a la edad, confirma que la forma de las larvas en estadio de pre-flexión cambia en el curso del desarrollo y a diferentes velocidades, dependiendo, sobre todo, de la fuente de energía (interna o externa) y del tiempo transcurrido desde el inicio de un tratamiento alimenticio. Esto es, sin

embargo, escasamente evidente con relación a la talla. El método aplicado para la eliminación del efecto de la talla normaliza las variables corporales de las larvas a una talla común, incorporando el crecimiento alométrico de las variables corporales (Lleonart et al. 2000). De esta forma, los efectos de la falta de alimento pueden ser observados con menos interferencias.

Se realizó un análisis multivariante de los datos (Análisis de Componentes Principales y Análisis Discriminante) así normalizados. Las variables normalizadas que más discriminaron fueron la longitud preanal, la altura del cuerpo a nivel del ano y la longitud de la cabeza. El análisis multivariante mostró dos grupos de forma diferenciados: Las larvas dependientes de las reservas vitelinas, y las larvas del tratamiento H que llevaban más tiempo sin alimentarse (14-21 DDE). El mayor porcentaje de clasificación correcta (la que adscribe un individuo a su grupo de edad-alimentación) se obtuvo para las larvas de más edad (14-21 DDE) del grupo H. Entre el 76 y 62% de estas larvas (dependiendo de la utilización de todas las larvas o de una submuestra sin reposición) fueron clasificadas correctamente. Cuando el grupo de clasificación se amplió a todas las larvas H, el porcentaje de las mismas (de entre 14-21 DDE) correctamente clasificado osciló entre el 80.0 y el 69.2%. La medida que ofreció mayor correlación con la mortalidad en las larvas H fue la resultante de calcular la primera variable canónica obtenida de un análisis discriminante utilizando las anteriores 3 medidas normalizadas. En larvas con fuerte crecimiento alométrico, este tipo de análisis puede ser de utilidad en análisis de condición.

2.3. Índices histológicos

Una parte de esta sección ha sido publicada en: **Catalán IA**, Olivar MP (2002) Quantification of muscle condition using digital image analysis in *Dicentrarchus labrax* larvae, and relationship with survival. J Mar Biol Ass UK 82: 649-654.

Los objetivos específicos de esta sección fueron tres: a) el estudio de la variación de diferentes tejidos en respuesta a diversos tratamientos alimenticios, que incluyeron los tratamientos A, AR y H en larvas en estadio de pre-flexión, y un ayuno puntual y realimentación durante varios días en larvas en estadio de post-flexión; b) comparación de la degeneración tisular producida por los anteriores tratamientos mediante dos tipos de

medidas histológicas: unas basadas en la gradación clásica de características celulares y tisulares, y otras basadas en la cuantificación de ciertas características mediante análisis de imagen; c) la exploración del grado de relación existente entre la supervivencia larvaria y las diferentes medidas histológicas.

Los tejidos u órganos estudiados fueron músculo, hígado, páncreas, intestino posterior y cartílago. De entre las glándulas asociadas al tubo digestivo, el hígado mostró resultados más consistentes que el páncreas en la determinación de tratamientos alimenticios. La gradación clásica del hígado (determinación de la cantidad potencial de glicógeno, estado de condensación de los núcleos, estructuración del citoplasma) se mostró más efectiva en la detección de larvas sometidas a un ayuno prolongado que la determinación cuantitativa (diámetro máximo de los hepatocitos). Tanto el hígado como el páncreas presentaron una gran dependencia del estado de desarrollo y exhibieron una alta variabilidad intra-tratamiento.

De todos los índices analizados, la determinación cuantitativa del grado de separación de las fibras musculares (SFM) fue el que, individualmente, adscribió correctamente un mayor porcentaje de larvas a su correspondiente tratamiento alimenticio. En larvas del tratamiento A, este índice no se correlacionó significativamente con talla ni edad y, en las larvas del tratamiento H, sus valores experimentaron una brusca y significativa variación coincidiendo con la mortalidad masiva de las larvas. Por todo lo anterior, se considera que la determinación cuantitativa de la SFM es una contribución útil a los métodos de detección de un deficiente estado nutricional en larvas de peces. Al tratarse de un método cuantitativo, su uso reduce el sesgo atribuíble a las medidas cualitativas y a diferencias en la experiencia del observador.

Finalmente, en esta sección se propone un esquema para ponderar los métodos histológicos, extensible a otros métodos o a una combinación de ellos, para futuros estudios de campo. Este esquema implica la atribución de más peso a las variables que muestren una mayor relación con la posible supervivencia.

2.4. Índices bioquímicos

El objetivo de esta sección fue analizar y comparar varios índices bioquímicos de condición, a saber, el cociente ARN/ADN, el cociente proteína/ADN, la actividad lactato deshidrogenasa (LDH) y citrato sintetasa (CS), en larvas de lubina con diverso estado de desarrollo y sometidas a varios tratamientos alimenticios. Además, se estudió la utilidad de la tasa de proliferación celular como medida de estado nutricional en larvas en estadio de post-flexión sometidas a un ayuno puntual.

Las medidas de ácidos nucléicos, proteínas y actividad enzimática se obtuvieron utilizando sólo tejido muscular, el más apropiado según la literatura revisada. Las tasas de proliferación celular se midieron tanto en músculo como en cerebro. Dos importantes eventos en el desarrollo larvario, la total absorción del saco vitelino y la flexión de la notocorda, fueron los causantes de importantes variaciones en algunos índices bioquímicos, con independencia del tratamiento alimenticio de las larvas.

En larvas en estadio de pre-flexión, se pudo estudiar únicamente el cociente ARN/ADN. Éste sólo fue de utilidad para distinguir las larvas en hambruna cuando éstas hubieron agotado las reservas de orígen materno. El valor de ARN/ADN asociado al inicio de la mortalidad masiva en estas larvas (H) fue de 1,7 (± 0,62 desv.est.). Tras la flexión de la notocorda, las medidas más útiles para discriminar un pobre estado nutricional fueron el cociente ARN/ADN y la actividad LDH.

El tiempo de respuesta a la falta de comida y realimentación fue mayor en larvas en estadio de pre-flexión que en larvas en estadio de post-flexión. Para estas últimas, el tiempo necesario para la recuperación disminuyó de forma inversa al tiempo de ayuno.

De entre las dos actividades enzimáticas estudiadas, la de LDH describió mejor los efectos de un ayuno puntual que la actividad CS. Esta observación ha sido confirmada para otras especies. En larvas en estadio de post-flexión de *D. labrax*, la actividad LDH/ADN podría ser un buen método para detectar un deficiente estado nutricional si se aplicase a rangos de talla pequeños.

La tasa de proliferación celular medida en músculo, permitió diferenciar, para una misma edad, larvas sometidas a un ayuno corto de aquellas otras alimentadas. En tejido cerebral no se observaron diferencias significativas. Aunque se necesita mayor

investigación en el aspecto metodológico de este índice, el uso de las tasas de proliferación celular parece prometedor. En la discusión de esta sección se abordan los pros y contras de una posible aplicación de estos índices en larvas recolectadas en el mar.

2.5. Comparación de índices y discusión

El objetivo de esta sección fue comparar los diferentes índices de condición durante los dos tramos principales del experimento y de acuerdo a los tres niveles de organización en los que se enmarcan la mayoría de estudios de la literatura: organismo, histológico y celular. Los índices se compararon de acuerdo al tiempo de respuesta, la bondad de clasificación, la relación con la supervivencia y los tiempos de procesado, costes y requerimientos específicos. En aras de la simplicidad, en esta sección se eligieron sólo los índices que mejor se comportaron dentro de cada categoría. Si bien en el primer tramo del cultivo (larvas en estadio de pre-flexión, 6-21 DDE) se compararon los tres tipos de índices, en el segundo (larvas en estadio de post-flexión, 22-28 DDE) sólo se utilizaron los histológicos y bioquímicos.

Los criterios para la elección de cada medida se basaron en la independencia de la edad, talla o estadío de desarrollo. Estas relaciones se exploraron en larvas sometidas al tratamiento A que son las que, teóricamente, mejor podrían mostrar la dependencia de uno o varios de los factores citados sin interferencia del regimen alimenticio.

Selección de índices

Todos los índices reflejaron el estado nutricional, aunque de forma diversa.

De entre las medidas morfométricas, se eligió la primera variable canónica resultante de un análisis discriminante realizado sobre tres variables normalizadas ya citadas con anterioridad (altura del cuerpo a nivel del ano, longitud preanal y longitud de la cabeza). Se tomó esta variable porque no está correlacionada con la talla, mostró la mejor correlación con la supervivencia y ofreció los mayores porcentajes de clasificación correcta entre los grupos de edad-tratamiento alimenticio estudiados.

Durante el primer tramo del cultivo en larvas alimentadas, el hígado, intestino y páncreas sufrieron variaciones atribuíbles a la ontogénesis y no al tratamiento alimenticio. Ni el músculo ni el cartílago experimentaron cambios apreciables respecto a la edad o la talla. De todas las variables histológicas medidas en larvas A, la SFM fue la que presentó menor variabilidad. En la segunda fase del experimento (larvas en estadio de post-flexión), la variabilidad fue grande en todos los tejidos, incluso en las larvas alimentadas. En este tramo, la SFM fue la única que permitió distinguir entre tratamientos. Por todo lo anterior, se seleccionó la SFM como el mejor descriptor de una alimentación no óptima para los dos tramos del experimento. Además de las ventajas anteriormente mencionadas, la SFM fue la medida individual que clasificó correctamente el mayor porcentaje de larvas y que mejor se correlacionó con la supervivencia.

Entre los índices bioquímicos, se seleccionó el ARN/ADN para el primer tramo del experimento, ya que era la única medida disponible para ese período. Este cociente no se correlacionó con la talla pero mostró una correlación negativa con la edad en el período 6-10 DDE. El descenso en los valores del cociente en los primeros días de edad ha sido observado por otros autores y es independiente del regimen alimenticio (Clemmensen 1987; Clemmensen y Doan 1996). El ARN/ADN sí sirvió para distinguir las larvas del tratamiento A de las del tratamiento H a partir de 17 DDE. En larvas en estadio de post-flexión, los resultados del ARN/ADN y LDH/ADN fueron similares excepto por la pendiente de descenso ante 3 días de ayuno, que fue continuada para el ARN/ADN. En consecuencia, se eligió también este cociente para comparar con los otros índices.

Tiempos de respuesta

Se compararon los tiempos de respuesta de los diferentes índices ante las variaciones en los tratamientos alimenticios. El estudio de los tiempos de respuesta (ver introducción) es útil para la interpretación ecológica de los índices de condición. Por ejemplo, si se conoce que un determinado índice exhibe una dinámica de respuesta lenta ante la adición de alimento, puede inferirse que ese individuo permanecerá en un estado “subóptimo” durante más tiempo en el medio, lo cual permite suponer que tendrá más

probabilidades de ser depredado, transportado hacia lugares menos favorables para la alimentación etc.

En larvas preflexión, el índice morfométrico fue el menos sensible a las variaciones de los tratamientos alimenticios. En general, el índice histológico mostró tiempos de respuesta a la alimentación -o degradación ante la falta de ella-, superiores a los del ARN/ADN. Dado que el tejido muscular fue el estudiado en esta comparación (incluso en el índice morfométrico, la altura del paquete muscular fue una de las variables con más peso), los resultados obtenidos son coherentes desde un punto de vista fisiológico, ya que la cascada natural de acontecimientos ante una falta de alimento seguirá un orden creciente molecular-histológico-morfométrico. En el experimento de ayuno y recuperación a corto plazo, el índice histológico y el ARN/ADN mostraron unas propiedades similares, siendo los tiempos de degradación y de recuperación, comparables. Estudios utilizando ARN/ADN en otras especies muestran tiempos de respuesta a la falta de comida y tiempos de recuperación tras un ayuno corto (2 dias) similares a los aquí obtenidos. Esta similaridad en los tiempos de respuesta ante la falta de comida y una realimentación a corto plazo es sorprendente, teniendo en cuenta la naturaleza de los dos índices comparados.

Propiedades de clasificación

En este apartado se eligieron grupos de la misma edad y se dividieron por tipo de tratamiento alimenticio. Sólo se utilizaron las larvas de alimentación exógena. Mediante la comparación del número de larvas adscritas correctamente a su grupo, se dio una medida de cuán “útil” es cada índice para detectar un estado de alimentación deficiente. La máxima discriminación del tratamiento A correspondió al ARN/ADN con un 100% de acierto. Por el contrario, la máxima discriminación del tratamiento de inanición y recuperación lo ofreció el índice de separación muscular, con un 83% y 44% respectivamente. Mientras que la SFM se perfila como más adecuado para distinguir larvas en estado nutricional intermedio, se interpreta que el RNA/DNA es más útil para la detección del crecimiento, y no del estado nutricional.

Relación con la supervivencia

El valor teórico de un índice nutricional es mayor cuanto mayor sea su directa relación con la supervivencia. El caso extremo vendría dado por el establecimiento de un "punto de no retorno" (PNR, Blaxter y Hempel 1963), o valor del índice tras el cual la mortalidad fuera segura. Criterios tales como la existencia de tejidos necróticos suelen utilizarse para determinar el PNR. Si no puede establecerse con certeza el PNR por existir una alta tolerancia a la falta de alimento (caso del presente estudio), cabe considerar otros aspectos: la relación entre el valor de un determinado índice y la posible supervivencia, integrando estas especulaciones en las teorías generales de mortalidad por predación-hambruna (revisadas por Leggett y Deblois 1994).

En este apartado, se comparó la variación de la curva de mortalidad de las larvas del tratamiento H con relación a los valores de los diferentes índices a lo largo del tiempo. Dado que la mortalidad masiva ocurrió el día 17, se asumió que un índice reflejaría la mortalidad con más precisión cuanto mejor contemplase su variación este evento de mortalidad masiva.

Las correlaciones no paramétricas entre mortalidad y cada uno de los tres índices fueron significativas. Cuanta menor supervivencia, menor factor de forma, menor el cociente ARN/ADN y mayor SFM. La figura 2.5.1. muestra que, aparte del inicial decremento en todos los índices y la supervivencia durante los días 6-10 DDE, los tres índices exhibieron los valores indicativos de mayor degradación a partir del dia 17 DDE.

En el mar, se supone que la mortalidad actúa preferentemente sobre las larvas que crecen menos. En cultivo, el crecimiento debido al tratamiento alimenticio se detectó alrededor de los 13 DDE. A los 14 DDE, sólo la separación de las fibras musculares reflejó significativamente este crecimiento diferencial en las larvas del tratamiento A. Por tanto, desde un punto de vista teórico, puede suponerse una mejor relación de este índice con las probabilidades de supervivencia de las larvas, en comparación con los índices morfométricos o bioquímicos analizados.

Perspectivas para futuros estudios de campo.

Uno de los objetivos de esta serie de análisis fue la de extraer conclusiones de carácter general a partir de la comparación de los índices, que pudiera ser de utilidad en un marco que trascendiese el de la especie en estudio. Dada la diversidad de información que proporciona cada índice y sus características diferenciadas en cuanto a tiempo de respuesta, requerimientos, relación con la supervivencia etc., se conviene con otros autores que una combinación de índices es siempre mejor solución que la utilización de uno sólo de ellos. Consideraciones económicas y de tiempo requerido para los análisis, aconsejan la utilización del ARN/ADN o de los índices morfométricos. Sin embargo, la cantidad de información obtenida mediante los índices histológicos y su mejor comportamiento como descriptor de supervivencia y estado nutricional, sugieren la utilización de estos últimos.

A tenor de la bibliografía sobre el tema, la cantidad de larvas que muestran un estado fisiológico comprometido en el mar es bajo. Se ha argüído que la mortalidad probablemente opera sobre larvas que tienen alguna desventaja en cuanto a capacidad locomotora, de alimentación etc. Por tanto, es útil abordar el problema de la posible mortalidad en el campo valorando el estado fisiológico de las larvas en una escala que puede ser asimilada a una alta o baja probabilidad de morir por factores diversos (predación, enfermedad, etc.). En estudios de campo, por problemas de tiempo y costes, es frecuente utilizar un solo índice para el estudio de una población determinada. En los casos en que se consideran varias variables (ej: varias medidas histológicas), no es infrecuente que éstas se traten de forma similar para conseguir un valor promedio por larva. Por otra parte, en estudios donde se utilizan análisis multivariantes, pocas veces se adscribe un peso específico a cada variable concreta.

Para dar cuenta del hecho de que, en el campo, muchos fenómenos inciden en la mortalidad larvaria, se considera adecuado dar diferente peso a los índices de condición, de acuerdo a los conocimientos teóricos o experimentales de que se disponga. En esta tesis se propone un modelo de ponderación en el que los índices que tengan mayor tiempo de respuesta o se revelen (o supongan) particularmente importantes para la supervivencia, sean los de más peso. Así, por ejemplo, el criterio de necrosis debería inmediatamente diferenciar las larvas con una probabilidad máxima de muerte. Seguidamente, índices como

la SFM deberían tener más peso que el estado del hígado o los índices bioquímicos. Esto es así porque un tejido muscular degradado sugiere de forma evidente, y así se ha comprobado en algunos estudios, una posible desventaja en términos de capacidad natatoria, lo que a su vez está relacionado con una mayor probabilidad de muerte por predación (Hunter 1972; Frank y Leggett 1982), aumentada por el mayor porcentaje de fracaso de captura de presas (Laurence 1972). Además, un músculo degradado provoca pérdidas de flotabilidad, lo cual conduce a posicionamientos en la columna de agua que pueden alejar a las larvas de los patches óptimos de presas, temperatura, corrientes, etc. (Neilson et al. 1986; Sclafani et al. 1993, 1997, 2000). Por el contrario, los índices que son más sensibles a la alimentación inmediata y que muestran un rápido tiempo de respuesta, caso de los índices bioquímicos (ARN/ADN, enzimas metabólicas) podrían utilizarse para refinar este modelo en el supuesto de que varias medidas pudieran obtenerse simultáneamente de un mismo individuo (medidas morfométricas e histológicas, por ejemplo).

3. ESTUDIOS DE CAMPO

3.1. Primeros estadíos de *Sardina pilchardus* y anomalías ambientales en el Mar Catalán

La mayor parte de esta sección ha sido publicada en: Olivar MP, **Catalán IA**, Emelianov M, Fernández de Puelles ML (in press) Early stages of *Sardina pilchardus* and environmental anomalies in the North-Western Mediterranean. Est Coast Shelf Sci.

En esta sección se investigó la influencia de las condiciones ambientales en los patrones de distribución de los primeros estadíos de *Sardina pilchardus* en un área del Mar Catalán, durante los meses de Noviembre de 1998, Febrero de 1999 y Noviembre de 1999. Por regla general, los huevos fueron hallados en la plataforma continental mientras que las larvas mostraron una distribución más amplia, ocupando buena parte de la plataforma. La circulación local durante el otoño-invierno de 1998-1999 estuvo dominada por un remolino anticiclónico de Agua Atlántica (AW) nueva, que permaneció estacionario en el centro del Mar Catalán desde octubre de 1998 hasta el final de febrero de 1999. Esta situación es muy infrecuente. La influencia de las aguas del remolino se extendió a la plataforma continental, desviando la corriente del talud e incluso invirtiendo su dirección.

En la tercera campaña de muestreo, realizada al comienzo de la época de puesta de 1999 (noviembre), la circulación y distribución de la masa de agua fue la habitual, con el Agua Atlántica antigua ocupando toda la región. La abundancia de huevos y larvas de sardina durante esta última campaña fue superior a la de las dos campañas anteriores. En noviembre de 1998, la abundancia de huevos y larvas de sardina fue relativamente baja en las zonas afectadas por Agua Atlántica nueva. El resultado más interesante de esta serie de campañas fue la baja cantidad de huevos y larvas halladas en febrero de 1999, probablemente debida a la situación anómala creada por el remolino.

3.2. Relaciones entre ambiente, crecimiento y estado nutricional de *Sardina pilchardus* en el Mar Catalán

Esta sección tuvo como objetivo el establecer relaciones entre las características ambientales observadas en dos de las campañas (noviembre de 1998 y febrero de 1999) y su posible efecto sobre las larvas en términos de crecimiento y estado nutricional.

El estado nutricional se estudió mediante índices histológicos y el cociente ARN/ADN. El crecimiento se estudió mediante análisis de otolitos (talla-edad y diámetro máximo) y crecimiento de las fibras musculares (número de fibras internas y superficiales, y diámetro de las fibras internas) respecto a la variación en talla.

En noviembre de 1998 la sardina estaba al principio del período de puesta, lo que resultó evidente por el alto número de huevos y larvas de las primeras clases de edad. Se pudieron caracterizar ciertas áreas donde el estado nutricional, de acuerdo a los dos tipos de índices utilizados, fue particularmente favorable. Estas áreas pueden ser definidas por unos rangos de valores, en algunas variables ambientales, que conformarían una "ventana ambiental óptima" para la supervivencia larvaria. Este elenco de condiciones favorables incluiría temperaturas superficiales menores de 19°C, bajos valores del índice de estabilidad de la columna de agua (Brunt-Väissälä = 0.1-0.8 ciclos h^{-1}) y concentraciones de alimento potencial superiores a 4.5 nauplii l^{-1} o a 5.5 individuos l^{-1} del resto de microzooplankton. Las zonas que caracterizaron un ambiente desfavorable, donde la puesta fue reducida y el estado nutricional deficiente respecto al área anterior, se distinguieron por temperaturas superiores a los 19.0 °C, mayores valores del índice de estabilidad de la

columna de agua (Brunt-Väissälä = 0.81-1.8 ciclos h^{-1}) y concentraciones de alimento potencial menores a las anteriormente indicadas. La diferenciación de estas áreas coincide con la atribuída a la influencia del remolino de AW nueva mencionado en el anterior apartado.

En febrero de 1999 la puesta fue reducida, y la cantidad de larvas de más de 5 mm (LS) o 6 días de edad escasa en relación a datos de otros trabajos para el mismo período (Olivar et al. 2001). Las relaciones entre las variables ambientales y la condición fueron débiles. Tan sólo la condición de las larvas mayores de 8 mm (deducida de los dos tipos de índices utilizados) se correlacionó positivamente con la salinidad y la cantidad de microzooplankton superior a los 45 m. Las tasas de crecimiento de la población de larvas fueron similares entre las áreas consideradas.

Las tasas de crecimiento muscular fueron significativamente más bajas en febrero que en noviembre, observación que concuerda con los resultados obtenidos mediante los análisis talla-edad a partir de otolitos. A su vez, los índices de estado nutricional muestran una peor condición o crecimiento a corto plazo en febrero respecto a noviembre. La distinta temperatura media superficial entre ambos meses (6ºC menor en febrero) podría explicar las diferencias de crecimiento a largo plazo y los valores inferiores de ARN/ADN. No obstante, no se descarta que la importante influencia de las aguas del remolino en el área de estudio pudiera haber modificado la composición específica del alimento potencial, afectando a las tasas de crecimiento y a los índices de estado nutricional. En cualquier caso, hay suficiente evidencia para concluir que, en febrero, las condiciones ambientales afectaron negativamente la producción larvaria y la posible supervivencia de la población, probablemente por una combinación de efectos sobre la intensidad de puesta, crecimiento reducido y deficiente estado nutricional.

Los resultados obtenidos sobre medidas de crecimiento tienen interés por varias razones: aparte de que este tipo de datos son inexistentes para larvas de sardina en el área de estudio, se ha aplicado por primera vez en esta especie un índice de crecimiento a largo plazo basado en el reclutamiento de las fibras musculares. Los resultados se corresponden bien con los obtenidos por análisis de otolitos. Ambas estimas de crecimiento sólo permitieron obtener una idea general sobre el crecimiento poblacional de las larvas, el cual

puede no estar ligado a las condiciones observadas en el momento. Es necesario por tanto, para esta especie, profundizar en el estudio de estas medidas en respuesta a variaciones ambientales como descriptores de crecimiento o estado nutricional a corto plazo. Para los rangos de talla estudiados, los resultados de los otolitos sugieren, aunque no de forma concluyente, que el crecimiento del otolito en sardina se produce incluso en casos de lento crecimiento somático. Los análisis de fibras musculares apuntan a que el crecimiento muscular en larvas de sardina es hipertrófico hasta los 6-7 mm LS, tornándose entonces hiperplásico. El efecto de la temperatura en este tipo de crecimiento parece claro.

De entre las medidas histológicas de estado nutricional, el análisis de la separación de las fibras musculares fue el más informativo, variando de forma máxima en noviembre de 1998 para larvas recolectadas en zonas diferenciadas por la cantidad de alimento potencial. La utilidad de este índice respecto a otras medidas histológicas está de acuerdo con la literatura revisada y los resultados de la primera parte de la tesis.

El ARN/ADN se mostró efectivo para diferenciar larvas de las mismas zonas en noviembre de 1998. No obstante, las diferencias entre áreas son más claras en individuos de una cierta talla, mayores de 8-10 mm LS, lo que está de acuerdo con datos de otros autores. Ambas medidas covariaron en sus patrones entre las dos campañas y entre dos áreas diferenciadas en noviembre de 1998, lo que añade validez a la interpretación de los resultados. Los resultados fueron menos claros en febrero. El porcentaje de larvas sobrepasando el "PNR" (presencia de tejidos necróticos) fue bajo de acuerdo a los índices histológicos, variando entre un 2 y un 7%. Los valores de ARN/ADN no tienen calibración de laboratorio para esta especie con el protocolo utilizado, y se interpretaron sólo en términos de comparación entre áreas o campañas.

De los estudios sobre estado nutricional se desprende que, si bien la mortalidad debida únicamente a inanición es dudosa, la falta de alimento adecuado o en cantidad suficiente en el momento de muestreo afectó a las poblaciones de larvas, pudiendo influir decisivamente en el crecimiento y aumentando la probabilidad de muerte.

CONCLUSIONES

ESTUDIOS DE LABORATORIO

1) Se ha comprobado la utilidad de los índices morfométricos para diferenciar la forma de larvas sometidas a diferentes regímenes de alimentación. Esto se consigue utilizando métodos multivariantes sobre variables en las que se elimina completamente el efecto de la talla , a la vez que se conserva la información sobre la alometría.

2) Entre las variables histológicas estudiadas, la determinación cuantitativa del grado de separación de las fibras musculares se demuestra el mejor método para detectar un deficiente estado nutricional. Este mejor comportamiento se manifiesta tanto en los porcentajes de correcta clasificación a grupos de alimento y edad, como en la rapidez con que refleja el ayuno o la recuperación.

3) El cociente ARN/ADN es más apropiado para la detección de una alimentación deficiente en larvas en estadio de post-flexión que en larvas en estadio de pre-flexión.

4) Entre los índices bioquímicos estudiados en larvas en estadio post-flexión, sometidas a un ayuno y realimentación de corto plazo, el cociente ARN/ADN y la actividad LDH/ADN se revelan como los indicadores más útiles.

5) En larvas en estadio de post-flexión sometidas a un ayuno puntual de 2 días, la tasa de proliferación celular en músculo es significativamente menor que en larvas alimentadas. En el cerebro no ocurre lo mismo al tratarse de un órgano más conservativo ante la falta de alimento.

6) La comparación de los índices morfométricos, histológicos y bioquímicos estudiados, muestra que en larvas en estadio de pre-flexión, el índice que más rápidamente detecta el efecto de la inanición es la cuantificación de la separación de fibras musculares. Para un mismo rango de edades, excluyendo el período de dependencia de reservas de

orígen materno, la máxima discriminación del tratamiento alimentado corresponde al ARN/ADN, con un 100%. Por el contrario, la máxima discriminación del tratamiento de inanición y recuperación lo ofrece el índice de separación muscular, con un 83% y 44% respectivamente. En este estudio, el grado de separación de las fibras musculares determinado de forma cuantitativa es el índice de condición que tiene una mejor correspondencia con la mortalidad larvaria.

7) La posible vulnerabilidad en el mar puede estar más relacionada con aquellas deficiencias que impliquen órganos o tejidos cruciales para el comportamiento alimentario, de huída o del mantenimiento de la flotablidad, como es el caso del músculo. Se propone un método de ponderación de índices para estudiar la condición de las larvas y relacionarlo con su viabilidad potencial.

ESTUDIOS DE CAMPO

8) En noviembre de 1998, las larvas analizadas en ciertas áreas mostraron una mejor condición nutricional, reflejada en un mayor valor de los grados histológicos y del cociente ARN/ADN. Estas zonas se caracterizaron por una “ventana ambiental” definida por temperaturas inferiores a los 19ºC, valores del índice de Brunt Väissälä inferiores a 0.8 ciclos h^{-1} y una abundancia de alimento potencial superior a los 4.5 nauplii l^{-1} y 5.5 individuos l^{-1} del resto de microzooplankton. Las variables que mejor se relacionaron con la condición larvaria fueron la cantidad de alimento potencial y el índice de estabilidad de la columna de agua. Además, estas áreas coinciden con una mayor cantidad de larvas. Se considera, pues, que en estas zonas la probabilidad de supervivencia larvaria sería mayor.

9) En febrero de 1999, la cantidad de huevos y larvas fue inferior a la de noviembre de 1998.

10) Los estudios de crecimiento larvario a largo plazo mediante análisis de otolitos sugieren que el crecimiento fue mayor en noviembre de 1998 que en febrero de 1999.

Este resultado fue confirmado por los análisis de reclutamiento de fibras musculares. La condición también fue mayor en noviembre, aunque en este caso los datos de campo de Febrero fueron menos concluyentes.

11) Las medidas de crecimiento y condición utilizadas, muestran un patrón general coincidente. El análisis del crecimiento muscular en larvas de sardina sugiere que éste es hipertrófico hasta los 6-7 mm, tornándose después hiperplásico hasta como mínimo los 13.5 mm LS. De entre las medidas histológicas utilizadas, la determinación de la separación muscular fue la más informativa. El ARN/ADN es de mayor utilidad en larvas mayores de 8-10 mm LS.

12) La cantidad de larvas en las campañas de noviembre de 1998 y febrero de 1999 fue anormalmente baja para esas épocas. Las condiciones hidrográficas durante las dos campañas fueron anómalas para esos períodos, presentando elevados grados de estratificación en noviembre y un patrón de salinidad invertido en febrero. La presencia de un remolino anticiclónico de mesoescala constituído por AW nueva, que persistió durante los meses de octubre de 1998 a febrero 1999, se perfila como probable causante de las anomalías hidrográficas observadas y las bajas cantidades de huevos y larvas en ciertas zonas.

13) En conjunto, los datos sobre cantidades de larvas, condición nutricional y diferencias ambientales, sugieren que la estación reproductiva otoño de 1998 - invierno de 1999 fue de una baja producción larvaria para esta especie. Se infiere, de la interpretación conjunta de los datos, que la posible supervivencia larvaria será una suma de una puesta reducida, un estado nutricional deficiente en ciertas áreas y períodos y un crecimiento menor durante el muestreo de invierno.