UNIVERSITÀ DEGLI STUDI DI PADOVA
FACOLTÀ DI SCIENZE MATEMATICHE, FISICHE E NATURALI
LAUREA SPECIALISTICA IN BIOLOGIA MARINA

RELATING NUTRITIONAL CONDITION AND FOOD OF ANCHOVY LARVAE (*Engraulis encrasicolus*, LINNAEUS 1758) IN THE GULF OF LIONS (NORTH-WESTERN MEDITERRANEAN)

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ANNO ACCADEMICO 2007/2008

Index

Summary

Appare ormai accertato che lo stato attuale delle attività di pesca a livello globale, se mantenute ai livelli di sfruttamento odierni, non può essere sostenuto a tempo indefinito. Lo stato degli *stock* ittici richiede interventi a più livelli volti sia ad aumentare la conoscenza scientifica sull'ecologia delle specie e gli effetti della pesca, che ad ottenere una maggiore sensibilizzazione dell'opinione pubblica e, di conseguenza, un incremento della volontà politica di gestione delle risorse marine (Dulvy *et al*., 2003; Pauly *et al*., 2005).

L'attività di pesca porta una serie di effetti sulle popolazioni sfruttate quali la diretta riduzione del numero di individui, il cambio nella struttura di taglia ed età delle popolazioni, ed altri effetti indiretti come la modificazione della struttura della comunità nonché i conseguenti mutamenti nella rete trofica. Inoltre, in base al tipo di strategia riproduttiva, anche il reclutamento delle specie sfruttate (che determina l'abbondanza delle classi adulte) può essere più o meno influenzato dalla pesca (Botsford *et al*., 1997).

Le pesca dei "piccoli pelagici" rappresenta una parte preponderante dell'attività di pesca globale. Nel mondo esistono marinerie in grado di sostenersi esclusivamente sfruttando queste specie. Questo avviene non per un alto prezzo di vendita all'ingrosso ma soprattutto per le notevoli quantità sbarcate.

Gli *stock* di piccoli pelagici, in particolar modo *Engraulis encrasicolus* e *Sardina pilchardus*, sono di grande importanza nelle marinerie del Mar Mediterraneo e contribuiscono a circa il 50% dello sbarcato totale. Come per ogni altra specie pelagica a ciclo vitale breve, la biomassa degli *stock* mediterranei dipende principalmente dal reclutamento, il quale a sua volta è primariamente correlato ai regimi oceanografici e climatici locali.

Attualmente la conoscenza sugli stadi larvali e giovanili dei piccoli pelagici nel Mediterraneo è scarsa; in aggiunta, le fasi larvali tardive non sono ancora state prese in considerazione in studi scientifici. Appaiono quindi necessarie ricerche per determinare l'influenza delle condizioni ambientali su queste fasi di sviluppo e di conseguenza sul reclutamento. La determinazione della dieta di questi stadi dovrebbe perciò contribuire al miglioramento degli attuali modelli a singola specie ed ecosistemici che sono stati sviluppati in questi anni per il Mediterraneo nord-occidentale.

Nei pesci il contenuto lipidico gioca un ruolo fondamentale nello sviluppo, perché viene utilizzato come riserva energetica primaria. Se un evento di deficit della riserva lipidica avvenisse nelle fasi di sviluppo, il reclutamento potrebbe esserne di conseguenza danneggiato (Rainuzzo *et al*., 1997). Uno studio della condizione

nutrizionale di questi stadi vitali offrirebbe una migliore comprensione dei fattori esterni in grado di influenzare lo *stock*.
In questo quadro è inserito il presente lavoro di tesi, che si accosta agli obiettivi del progetto europeo SARDONE. Questo progetto, che coinvolge diversi istituti di ricerca del Mediterraneo (ISMAR Ancona, ICM Barcelona, HCMR Iraklio, IFREMER Sète, AZTI Pasaia) si pone lo scopo di sviluppare una serie di strumenti per migliorare la conoscenza (e di conseguenza, la gestione) degli *stock* di piccoli pelagici nel mare Mediterraneo. Il lavoro di internato è stato condotto presso l'Institut de Ciències del Mar di Barcelona, analizzando il contesto del Golfo del Leone e considerando la specie *E. encrasicolus* (Linnaeus 1758), che rappresenta la specie pelagica economicamente più importante della regione.
In questo lavoro, una prima analisi ha riguardato la valutazione della condizione nutrizionale delle larve e la loro comparazione per definire se vi fossero differenze rispetto a due periodi diversi di cattura (e quindi ad una diversa condizione trofica e oceanografica). A questo fine è stata compiuta un'analisi delle classi lipidiche delle larve, per ottenere indici di condizione basati sui lipidi. Sui medesimi campioni è stato effettuato anche uno studio del profilo frazionato degli acidi grassi dato che questi vengono assunti con la dieta e possono perciò essere impiegati come indicatori dei livelli trofici inferiori.

I dati dei due filoni d'analisi sono stati entrambi ottenuti sul medesimo gruppo di campioni. Il campionamento è stato effettuato nei mesi di agosto e dicembre 2007, a bordo della nave oceanografica *L'Europe* dell'IFREMER (Francia) nel Golfo del Leone. Il periodo riproduttivo conosciuto di questa specie nella regione presenta un picco durante l'estate, e si conclude tipicamente a ottobre. Durante queste due campagne sono stati prelevati campioni di zooplancton per mezzo di retini e sono state effettuate cale con una rete a strascico pelagica per la raccolta di larve tardive di acciuga.
In laboratorio, le larve sono state misurate e pesate, e quindi raggruppate in pool di 4-6 individui per ottenere una maggiore capacità di discriminazione da parte delle metodiche analitiche applicate. Ogni larva è stata pesata e misurata e, successivamente, è stato calcolato il peso secco utilizzando una relazione empirica peso umido-peso secco in precedenza stimata con altri pool di larve raccolti nel contesto della ricerca. Sono stati, inoltre, preparati e pesati tre campioni di zooplancton, ciascuno contenente tutto il materiale filtrato in ogni stazione.
I campioni sono stati sottoposti ad una procedura di estrazione dei lipidi totali, seguendo il metodo di Folch *et al*. (1957). La soluzione lipidica è stata quindi conservata in soluzione antiossidante.

Per determinare se la condizione nutrizionale delle larve tra i due periodi fosse la medesima, ne sono state quantificate le classi lipidiche principali (triacilgliceridi, colesterolo, acidi grassi liberi, lipidi polari) utilizzando una tecnica cromatografica su strato sottile a doppio eluente. I risultati hanno permesso di calcolare, per ciascun pool di larve, un indice comprendente i contenuti in triacilgliceridi e in colesterolo (TAG/CHOL); questo indice viene ritenuto un indicatore affidabile della condizione nutrizionale negli stadi di sviluppo dei pesci (Fraser, 1989). Inoltre, è stato possibile rilevare anche il contenuto in lipidi polari (PL), anch'essi componenti strutturali delle cellule; alti livelli di lipidi polari possono indicare un'aumentata proliferazione cellulare e, da questo, una migliore capacità di crescita. Utilizzando i dati morfometrici raccolti sull'insieme delle larve utilizzate è stato calcolato anche l'indice di condizione di Fulton, che integra i parametri lunghezza standard ed peso umido.
Per l'analisi della dieta, un'aliquota dei campioni è stata sottoposta a trans-esterificazione come procedura preparatoria; una cromatografia su gas accoppiata ad una spettrometria di massa ha quindi consentito di acquisire il profilo degli acidi grassi frazionati delle larve e dello zooplancton. La composizione in acidi grassi è stata ottenuta comparando i tempi di ritenzione di ciascun picco dello spettrometro di massa con quelli di una miscela standard di acidi grassi.

Dai dati ottenuti sulla composizione delle classi lipidiche, non sono emerse differenze significative tra larve di agosto e di dicembre; questo sia per ciascuna classe lipidica, sia per il rapporto TAG/CHOL calcolato (Tab. 2, pag. 26).
Per quanto riguarda la composizione in acidi grassi, l'analisi statistica multivariata ha evidenziato differenze tra larve dei due periodi e zooplancton. Osservando gli acidi grassi più abbondanti, alcuni mostravano marcate differenze mentre altri non sembravano variare in base al periodo dell'anno (Tab. 4, pag. 28). Per meglio interpretare questi risultati, sono stati calcolati alcuni rapporti tra acidi grassi, già utilizzati in precedenti studi: 16:1(ω-7) / 16:0, 18:1(ω-9) / 18:1 (ω-7), e EPA/DHA. Questi rapporti sono stati impiegati per valutare il contributo di ciascuna classe di zooplancton, sia nella comunità planctonica che nella dieta delle larve (Tab. 5, pag. 29).

Utilizzando i dati dei tassi di crescita già noti, è stato calcolato che le larve raccolte in dicembre erano nate a metà ottobre, cioè verso la fine del normale periodo riproduttivo nella regione. Nello stesso periodo iniziano ad essere presenti le larve di un'altra specie pelagica, *S. pilchardus*, la qual cosa porta ad una competizione interspecifica e quindi ad uno svantaggio ulteriore per le larve di *E.*

encrasicolus. Questa competizione potrebbe essere mantenuta a bassi livelli differenziando le preferenze alimentari. Tale ipotesi è già stata verificata su adulti delle due specie, in un'area dell'Africa del sud; in quel caso, le acciughe selezionavano prede planctoniche di dimensioni maggiori mentre le sardine includevano fitoplancton nella loro dieta (van der Lingen *et al*., 2006).

La temperatura potrebbe essere un ulteriore fattore che determina la condizione nutrizionale nelle larve studiate. Le maggiori temperature estive, rilevate nelle due campagne, causano negli individui un aumento del tasso metabolico. Per mantenere quindi la stessa condizione, nelle larve di agosto si è ipotizzata una dieta energeticamente più ricca oppure più abbondante. Durante il periodo di studio sono state compiute, anche, analisi preliminari sullo zooplancton nei due periodi ed è stato riscontrato che in agosto la biomassa zooplanctonica era quattro volte superiore a quella presente in dicembre. Questa differenza potrebbe favorire una possibile spiegazione della medesima condizione nutrizionale, anche se sono comunque necessari studi di alimentazione più approfonditi per poter dare una risposta definitiva in merito.

La campagna oceanografica condotta in dicembre 2007 è stata la prima occasione in cui larve tardive di acciuga venivano trovate nei mesi invernali, ma va precisato che è stata la prima campagna di campionamento condotta nella regione in quel periodo. Non è quindi stato chiarito se la presenza di larve tardive di acciuga fosse un evento isolato oppure abituale. Una possibile spiegazione, nel caso di ipotesi di evento isolato, sarebbe un'aumentata portata da parte del fiume Rodano; questa possibilità si è già verificata in passato, nell'anno 1983, quando fu riscontrata una biomassa planctonica maggiore degli anni precedenti e, di conseguenza, una riduzione significativa della mortalità larvale (García & Palomera, 1996).

L'analisi degli acidi grassi si è basata principalmente sui valori dei rapporti calcolati. In base ad essi, è stato rilevato che le larve tardive di acciuga della taglia considerata preferiscono un'alimentazione a base di zooplancton rispetto ad una di fitoplancton (sia esso costituito da diatomee o da dinoflagellati). Questo è in accordo con le ipotesi sollevate all'inizio dello studio. Un altro indice, utilizzato per valutare il contributo nella dieta di diatomee e flagellati, ha stimato una maggiore presenza di questi ultimi, sia nelle larve che nello zooplancton. Questo secondo risultato va ad ogni modo considerato con attenzione in quanto la traccia lipidica del fitoplancton può essere stata trasmessa in modo conservativo alle larve creando un *bias*.

Riunendo i dati dei due filoni d'analisi compiuti in questo studio, si può concludere che le larve in dicembre riescono a conservare una condizione nutrizionale accettabile, anche se in quel periodo la disponibilità di cibo è minore

rispetto alla stagione estiva. In entrambi i periodi, comunque, le larve sembrano preferire lo zooplancton al fitoplancton, questo probabilmente perché il primo è energeticamente più conveniente. In dicembre, inoltre, il ridotto tasso metabolico dovuto alle temperature più basse contribuisce probabilmente a mantenere la loro condizione a buoni livelli.

Introduction

It is now well established in the scientific community that most of the world fisheries, when managed without strict rules, tend to grow uncontrolled and will likely result in the collapse of the exploited stocks (Jennings *et al*., 2001). In fact, throughout the world, the uncontrolled fishing pressure is determining a dramatic reduction in the biodiversity of the oceans, causing the extirpation (i.e. the extinction of species at a local/regional scale) of several marine species (Dulvy *et al*., 2003). All this contribute to the process which has been defined by scientists as the "sixth mass extinction" that, unlike the previous five "mass extinctions", that occurred on a geological time-scale, it is mainly induced by human overexploitation of natural resources, habitat disruption, introduction of alien species, pollution and climate change (Pimm *et al*., 1995). It is widely accepted that the current situation of the world fisheries cannot be sustainably maintained, and that both an increase in public awareness and in scientific knowledge are required to reverse this trend (Pauly *et al*., 2005).
Fishing pressure causes a series of important effects on exploited populations, communities and ecosystems, which include both direct and indirect effects. In this framework the direct decrease in population biomass and the changes in its size- and age-structure is just one of the multi-level impacts which should be addressed. Other indirect effects such as the modification of the composition of fish and benthic communities and the consequent changes in trophic webs (reflected, for instance, on the worldwide reduction of the mean trophic level of the catches) have attracted the attention of researchers (Botsford *et al*., 1997; Pauly *et al*., 1998).

Life-history traits have been recognized to play a major role in determining species' vulnerability to fishing exploitation: typical traits are, for instance, age and size at sexual maturity, longevity and dispersal ability (Roberts & Hawkins, 1999). As a general rule, slow growth rates, relative low dispersion and aggregative spawning are characteristics that may prevent sustainable fishing of exploited species (Jennings *et al.*, 2001).

Among the many different fishing techniques adopted, those which target aggregative species are usually highly selective; in these cases, by-catch is limited to species of conservational interest like sharks, marine mammals, and sea turtles (Tudela, 2004). On the other hand, as nets are towed when large shoals of fish are detected with acoustic instruments, fishery may result in significant depletion of

target species, especially of adult spawners. All this does not only influence the subsequent catches, but also the spawning success of the stock and, therefore, the strength of its "recruitment", which can be described as the event that adds a new cohort of young individuals to a population (Sale, 1990).
Even in cases when some fishery management took place, for example by means of catch or fishing effort limitations, many fish stocks couldn't recover completely. This could be attributed, among other factors, to the *Allee effect* (Allee, 1931). Basically, this effect occurs at low population densities and causes an additional decrease in the reproduction capability of the stock (usually called *depensation*), due to its positive density-dependence (i.e., lower probability of finding a mate). This effect is typically underestimated by the use of models where the stock-recruit relationship is not well defined, and thus it is not taken into consideration that recruitment can reach zero even if the stock isn't completely depleted (Dulvy *et al*., 2003). In addition, environmental fluctuations can exert an even more negative impact on populations, especially on the first life stages. For this reason, study of these stages appears of great importance to include more information in the models currently in development.

In fact, larvae are by far the life stage of fishes that experiences the highest natural mortality rate: not only their escape and defence abilities to predation are scarce, but also their resistance to starvation is at its minimum. In addition, they have also low capacity of coping with environmental changes (Jennings *et al*., 2001). In species characterised by high fecundity, producing a high number of eggs per unit of weight with the least energy expenditure, only a small fraction of larvae from each spawn reaches the adult phase. In other words, in the so-called *r-type strategy*, "the average larva does not become a recruit" (Sale, 1990). These phenomenon and characteristics lead to the fact that even species that produce abundant and/or dispersive offspring may be vulnerable to the Allee effect (Roberts & Hawkins, 1999) and, thus, might be at risk of extirpation.

The case-study of small pelagics

Small pelagic fishes are exploited throughout the world by means of various trawling techniques. Although these species have not relatively high prices, the high catches and landings cause them to be of great economic interest and for this reason they experienced high fishing pressure in the past decades. All over the world, fishing fleets do exist that rely only on small pelagic species such as anchovies and sardines. Therefore the extent of the management problem of these

resources assume a global scale and the finding related to the study of small pelagics' vulnerabilities are probably applicable to other regions in the world.
Populations of small pelagic fishes around the world are known to undergo important fluctuations in abundance, even well before the industrial fishing was developed (Jennings *et al*., 2001). Larvae and late-larval stages of these fishes are deemed to be fundamental in determining subsequent year class-strength, and their growth (and thus their future condition) is firmly related to their diet and oceanographic features (Folkvord & Hunter, 1986).

The north-western Mediterranean is a large portion of the Western Mediterranean basin that extends from the Balearic sea to the Ligurian sea. Its water circulation is mainly characterised by the cyclonic current that come from the Corsica channel, following the Liguria coast up towards the Gulf of Lions, and then flows to the Iberian Mediterranean coast reaching the Atlantic ocean (Bas, 2002).
The most abundant clupeids in the north-western Mediterranean are European pilchard *Sardina pilchardus* (Walbaum 1792) and European anchovy *Engraulis encrasicolus* (L.). Although *S. pilchardus* is more abundant, fishing pressure is concentrated on the latter species due to its higher commercial value (García & Palomera, 1996). Fishing techniques nowadays include purse seines and bottom trawling in the Catalan Sea, carried out by the Spanish fleet, and mid-water pelagic trawling in the Gulf of Lions, by the French fleet (Barange *et al*., 2007). Other less important pelagic species targeted by the same fishing fleets are the Atlantic horse mackerel *Trachurus trachurus* (L.), Mediterranean horse mackerel *Trachurus mediterraneus* (Steindachner 1868), and mackerels *Scomber scombrus* (L.) and *Scomber japonicus* (Houttuyn 1782). The other small pelagic species that inhabit the area are Round sardinella *Sardinella aurita* (Valenciennes 1847) and European sprat *Sprattus sprattus sprattus* (L.), both of little commercial interest in this area (Palomera *et al.*, 2007).

The European anchovy *E. encrasicolus* (Teleostei, Engraulidae) is a pelagic fish distributed in the whole Mediterranean Sea, including the Black and the Azov Seas, and also along the eastern Atlantic coast, from Norway to South Africa (Whitehead *et al*., 1988). Anchovy can live up to four years, forms large schools, and is mainly a coastal marine species (Palomera, 2008; Pertierra & Lleonart, 1996). In the north-western Mediterranean region, *E. encrasicolus* forms a genetically panmictic stock which reproduces over the continental shelf areas associated with the runoff from the Ebre and Rhône rivers (Barange *et al*., 2007). Studies conducted on north-western Mediterranean anchovy stocks showed that

adults feed mainly on copepods, and to a lesser extent on cladocerans and other crustaceans (Plounevez & Champalbert, 2000). Feeding activity takes place mostly during daytime, with the stocks approaching the deep chlorophyll maximum to feed on the zooplankton situated there (Palomera *et al*., 2007). Regarding larvae, studies on stomach content showed that they feed on smaller fractions of zooplankton, namely copepodites, nauplii and copepod eggs (Tudela *et al*., 2002).

In the Gulf of Lions fishery, catches of anchovy increased from the mid-1980s to early 1990s and began to fluctuate thereafter from 1500 to 10000 tonnes (Barange *et al*., 2007). However, according to the most recent data acquired in 2007 by means of the daily egg production method (DEPM), mean biomass values are presently at around 20000 tonnes (Palomera, pers. comm.).

Lipids as nutritional biomarkers

The nutritional condition of a fish is an expression of both the biological and physical circumstances that acted on the individual, in the period prior to the assessment, and it reflects also the amount of energy available to that individual. In order to assess the nutritional condition of marine species various types of "indices of condition" have been defined and applyed. While morphometric indices take into account only the size and weight of the individuals, indices that consider biochemical content such as lipids or nucleic acids may be a more effective method of evaluating the conditions that led to that particular state (Lloret *et al*., 2002; Suthers *et al*., 1992).

Lipid contents play an important role during the larval and juvenile stages of fishes, since they are essential for cell and tissue membranes formation; moreover they are also heavily used as energy sources during development (Rainuzzo *et al.*, 1997). A series of experiments have demonstrated that during starvation, a general rapid decrease of lipids in fish larvae takes place, suggesting their use as indicators of the nutritional condition (for a review, see Rainuzzo *et al*., 1997).

A commonly used method of measuring fish condition is the assessment of the *total lipids*. This can be substantially achieved after extraction with either a gravimetric method, by which lipids are evaporated to dryness and weighted for difference, or a colorimetric method, which depends on the reaction of lipids with sulphuric acid, phosphoric acid and vanillin to give a red complex (Barnes & Blackstock, 1973). One advantage of the total lipids measurement is that the extraction procedure is done for every other lipid analysis and thus the quantification of total lipids is a obliged step. However, this measure does not discriminate between reserve lipids and structural lipids, so results are often

difficult to be interpreted in terms of condition. Besides, total lipid content maintains a strong correlation with body weight (Sabatés *et al*., 2003).

Tryacylglycerols (TAG) are used by fishes as primary forms of energy storage (Shul'man, 1960). Absolute expression of TAG content maintains a strong dependency on individual size and therefore should be standardized by any means. Use of dry weight would be an option but the analysis of lipids cannot be safely performed after freeze-drying of the sample. The most accredited option which is used at present is standardizing with another lipid type, cholesterol (CHOL), since it has been verified that they possess a high positive correlation with dry weight (Fraser, 1989; Fraser *et al*., 1987).

The resulting TAG/CHOL index has been thoroughly employed for a series of studies on developmental stages of fishes, starting from embryos (MacFarlane & Norton, 1999) up to larvae and juveniles. For example, Norton *et al.* (2001) applied this ratio to clarify the lipid metabolism in shortbelly rockfish *Sebastes jordani*. Pedersen *et al.* (1999) put in relation the TAG/CHOL ratio with diet in the juveniles of *Liparis spp.*. Another study verified a relationship between nutritional condition and growth rate in *Engraulis mordax* larvae, estimating the former variable by means of the aforementioned ratio (Håkanson, 1993). A similar study was conducted for *Oncorhynchus tshawytscha* juveniles (MacFarlane *et al*., 2005). This TAG/CHOL index appears therefore a well-known method for assessing the nutritional condition in various developmental stages of fishes.

Lipids as diet indicators

The first review that highlighted the dynamics of lipids in marine fishes was that of Shul'man (1960), who evidenced their high importance as metabolic energy sources, but also paved the way for a series of experiments focused on fish diet that started in the 80s'. Fish larvae, when they are in their phytoplanktivorous or zooplanktivorous phases, possess a fatty acid composition that can be related to their prey (St. John & Lund, 1996). This phenomenon was studied by Klungsøyr *et al*. (1989) to clarify the diet of larval cod (*Gadus morhua*), and was also used for feeding manipulation experiments such as in the case of the white sturgeon *Acipenser transmontanus* (Xu *et al*., 1993). The fatty acid compositions of phytoplankton (Dunstan *et al.*, 1994) and zooplankton (Rossi *et al*., 2006; Rossi *et al*., 2008) were also addressed in various studies.

Fatty acid composition was also used to readily distinguish cod adults fed with different preys, suggesting that fatty acids are conserved during transfer from prey to predator, and therefore could be used as effective diet indicators (Kirsch *et al*.,

1998). Starting from these examples, the analysis of fatty acids for diet has become a common practice.
In addition, several studies have been conducted to establish a series of ratios between fatty acids, which can describe some trophic relations in a more interpretable way (Auel *et al*., 2002; Cripps & Atkinson, 2000; Pedersen *et al*., 1999; Reuss & Poulsen, 2002; St. John & Lund, 1996).

Purposes of this study

The present study was carried out in the framework of the European Union's SARDONE research project, that is directed to improving assessment and management of small pelagic species in the Mediterranean. In detail, its work package 3 focuses on the study of trophic ecology of larval stages and juveniles in three regions: north-western Mediterranean, Adriatic Sea and Aegean Sea.
The present thesis work adhered to this research project and was carried out at the Institut de Ciències del Mar, in Barcelona, Spain. The two objectives of this work were:

- The evaluation of the nutritional condition of anchovy (*E. encrasicolus*) late-larvae, by using indicators based on lipid contents;
- The study of anchovy late-larvae diet, by comparing the accumulation of fatty acids in larval body with those in lower trophic levels.

Materials and methods

Samples were collected during two oceanographic/acoustic cruises carried out in the Gulf of Lions in 2007 on board the *R/V L'Europe* (IFREMER, France), in the framework of the SARDONE project. The first cruise, PELMED07, was conducted in summer from the 28th of July to the 9th of August 2007 and the second cruise, JUVALION07, was carried out in late Autumn from the 8th to the 21st of December 2007. The main objectives of these cruises were the stock assessment of small pelagic species by means of acoustic survey (mainly anchovy and sardine), the collection of samples of larvae and juveniles, along with the retrieval of relevant oceanographic parameters.

Sampling area

The sampling area was located on the continental shelf of the Gulf of Lions, in the north-western Mediterranean Sea. In this region the shelf extends widely into the oceanic zone and this, coupled with hydrographical features such as increased river runoff, allows for well-defined small pelagics stocks to live and spawn there (Palomera *et al*., 2007). The Gulf of Lions hosts one of the highest concentrations of anchovy in north-western Mediterranean, and for this reason it is the target of the French mid-water trawling fishery (Bas, 2002; Palomera *et al.*, 2007). Zooplankton standing stock in this area is dominated mainly by copepods; cladocerans can be abundant during seasonal swarming events; other groups such as the appendicularians and gelatinous plankton, while scarce in biomass, have to be mentioned for their considerable contribution to particle recycling (Champalbert, 1996).

During each cruise a set of oceanographic stations was distributed in the sampling area to collect data on environmental variables. Figure 1 shows the stations where anchovy late-larvae and zooplankton used in this work were collected.

Sampling methods

At each station, a CTD (Seabird model 19) probe was cast to obtain profiles of temperature and salinity. At the same stations, samples of phytoplankton and zooplankton (divided into microzooplankton, 53 – 200 μm, and mesozooplankton, 200 – 3000 μm) were collected. Mesozooplankton was captured using a standard WP2 cylindrical-conic net, with a mouth width of 58 cm, a total length of 2.6 m and a mesh size of 200 μm. Similarly, microzooplankton was collected by means of a scaled-down version of the WP2 net, with a mouth opening of 25 cm, a total length of 1.8 m and a 53 μm mesh size (calculated for a mesh porosity of 33 %).

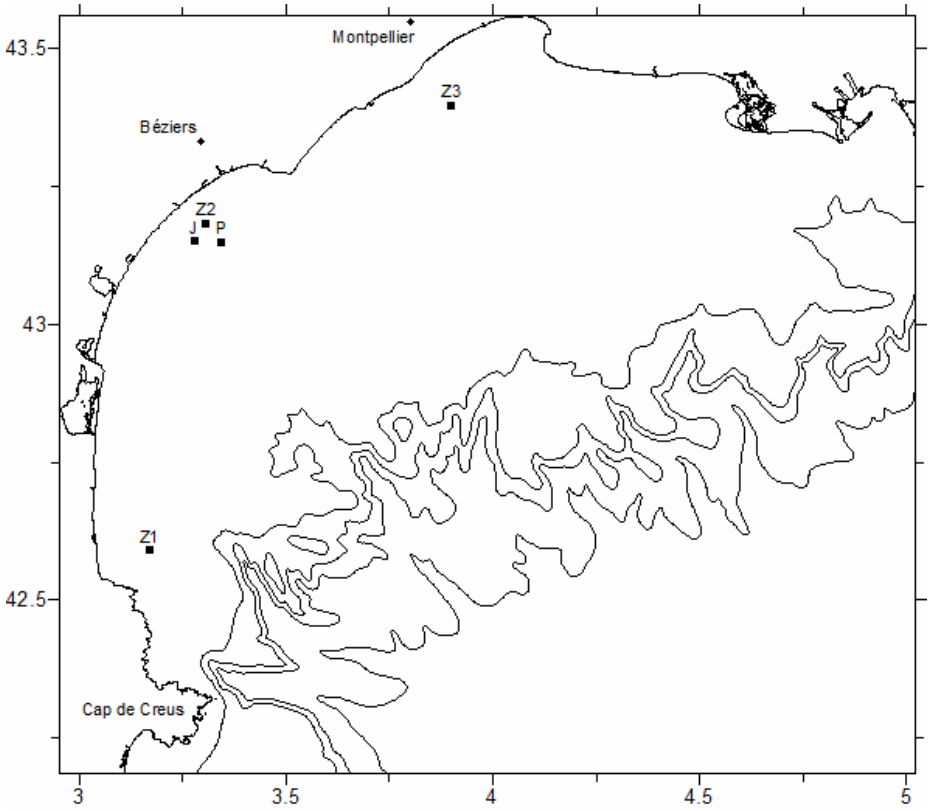


Figure 1 - Sampling map of the Gulf of Lions. Z1, Z2 and Z3 are the stations where zooplankton was collected; P and J mark the positions where hauls for anchovy larvae were made. P = PELMED07 cruise, J = JUVALION07 cruise.

Both nets were fitted with a General Oceanics 2030 flow-meter, in order to obtain an estimate of the volume of filtered water. Nets were towed vertically from bottom to surface, at a speed of 1 m/s.

Zooplankton samples were filtered on board, using pre-dried and pre-weighted Whatman® GF/C glass microfiber filters with 25 mm diameter for microzooplankton and 47 mm diameter for mesozooplankton. The samples were carefully folded, enveloped in aluminium foil, and frozen in liquid nitrogen. For this study, zooplankton samples were only available for the JUVALION07 cruise, as the possibility of lipid analysis during the previous cruise was not considered yet.

Late-larvae of *Engraulis encrasicolus*, defined as larvae in post-flexion stage from 15 mm length until metamorphosis, were captured by means of a pelagic trawling net fitted with two divergent doors. The net had a mouth height of 46.50 m and a mouth width of 26.00 m; the cod end mesh size was 12 mm. Tows were made when a school of small pelagic fishes was found with acoustic methods and were conducted at 4 knots (= 7.4 km/h), with durations comprised between 30-40 min.

At the PELMED07 cruise the larvae samples were stored at -20 °C. At the JUVALION07 cruise larvae were put in cryovials and frozen in liquid nitrogen immediately after sorting. This two different methods of conservation of samples

did not represent any problem for the following analyses, since Håkanson (1989) verified that the assessment of lipid composition is not influenced by differences in the samples freezing methods. Larvae and zooplankton samples previously frozen in liquid nitrogen at the JUVALION07 cruise were transferred into a -80 °C freezer after returning from sea.

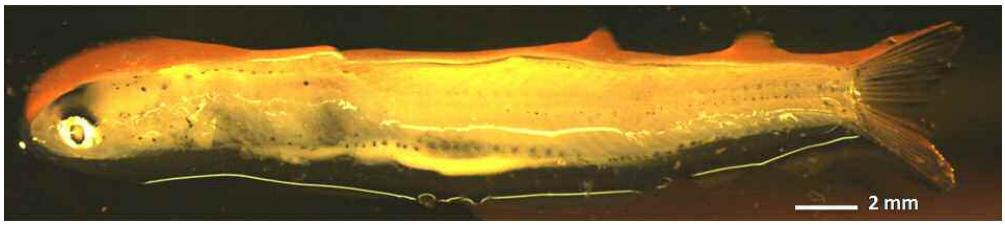


Figure 2 – Late-larva of *E. encrasicolus*.

Total lipids extraction

Pools of 4-6 anchovy larvae were prepared, and their wet weight measured by means of a digital scale (OHAUS Analytical, d = 10 μg), after removing excess water with an adsorbent paper. The standard length of each larva was also measured using a metal caliper (d = 0.1 mm). Data of larval length and weight were used to calculate the Fulton's condition factor (Ricker, 1975), using the formula:

$$K = wet\ weight * (standard\ length)^{3}$$

Each larva was carefully dissected under a stereomicroscope, removing the gut and the head that were stored for subsequent stomach content and otolith analyses. Lipid extraction was performed on the remaining muscular parts. Regarding zooplankton, all the material filtered at each station (microzooplankton and mesozooplankton together) was weighted and used for lipid analysis.

In order to obtain a purified extract of total lipids a modified procedure based on that of Folch *et al.* (1957) was applied. Samples were transferred into 25 ml glass tubes with Teflon-lined screw cap; 5.0 ml of chloroform:methanol (2:1) solution were then added. The sample was minced for one minute with a manual homogenizer, and then sonicated for 30 seconds (frequency 40%, pulse profile 0.8). After this procedure, another 5.0 ml of chloroform:methanol (2:1) solution were added, followed by 2.5 ml of potassium chloride (0.88%).

The essay tubes were agitated vigorously and centrifuged at 325 x g for 5 minutes in a refrigerated centrifuge, pre-cooled at 4 °C. This procedure accelerated the separation process, allowing the typical formation of an upper water layer, a central solid layer and a lower organic layer containing mostly chloroform and

lipids. The upper layer was carefully removed with a glass pipette. Samples were then filtrated with Whatman® paper filters into clean tubes.
These tubes were left at least 24 hours in a -20 °C freezer, in order to further separate the unwanted water from the lipid extract. Traces of remaining floating water were then removed with a glass pipette.
Samples were nearly completely evaporated in a Heto MAXI centrifugal-vacuum evaporator, redissolved in chloroform:methanol and transferred into 2 ml pre-weighted glass vials fitted with Teflon cap. After another cycle of total evaporation, vials were weighted again to obtain the total lipids weight. Samples were then stored at -20 °C until further analyses.
All reagents used for this and for subsequent analyses were of HPLC grade, and were acquired from Sigma Aldritch.

Lipid classes analysis

Two standard mixtures of lipids were used to calculate the concentrations of the various lipid classes. The neutral lipid mix (Matreya LLC) was composed of cholesteryl oleate, triolein, C18:1 methyl-ester, oleic acid and cholesterol, for a total of 25 mg/ml and dissolved in 1 ml chloroform. The phospholipid mix was obtained from Supelco and was composed of lysophosphatidylcholine, phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, dissolved in 2 ml chloroform at various known concentrations (factory-verified by TLC). Layers used were 20 cm long x 10 cm wide glass TLC plates coated with 0.25 mm thick Silica Gel 60, and were purchased from Albet Labscience.
Based on data of total lipid weight, dried samples were redissolved in chloroform:methanol 2:1 with 0.01% buthylated hydroxytoluene, up to a final lipid concentration of 10 mg/ml.
The protocol followed for the high-performance thin-layer chromatography (HPTLC) was that of Olsen & Henderson (1989). The eluent for polar lipids was prepared with 5.0 ml methylacetate, 5.0 ml isopropanol, 5.0 ml chloroform, 2.0 ml methanol and 1.8 ml KCl 0.25% (0.25 g KCl in 100 ml distilled water). The eluent for neutral lipids was 16.0 ml hexane, 4.0 ml diethylether and 0.4 ml glacial acetic acid.
Layers were first washed with hexane:diethylether 1:1, left drying under a stream of air, and then activated at 110 °C for 30 minutes in a ventilated oven. Aliquots of 1 µl of the samples were transferred on the layers, along with the reference standards, using a Hamilton Gastight® 10 µl glass syringe. The first run was carried over with the eluent for polar lipids, up to 5.5 cm height. The plates were then dried in a silica gel desiccator, in complete darkness, for 15 minutes. The

second run was made with the eluent for neutral lipids, up to an height of at least 12 cm; after that, plates were left drying once again for 15 minutes.
After development, layers were sprayed with a mixture of 3% copper acetate in 8% orthophosphoric acid, and charred at 160 °C for 20 minutes. This staining technique is preferable to the sulphuric acid – potassium dichromate reagent in that no darkening of the background occurs, and expensive glassware for spraying is not required (Fewster *et al.*, 1969).
Quantitative densitometry was performed in visible light with a Bio-Rad Gel Doc XR densitometer, using Quantity One 4.6.2 software. The concentration of each lipid class was obtained by calculating the proportion between spot density and concentration of the standard mixture. Each lipid class was expressed as weight per larva, which is the most used measure in literature. For completeness, data were calculated also referring to the larval weight; no differences were found with this change, so it has been decided to stick with the first unit of measurement.

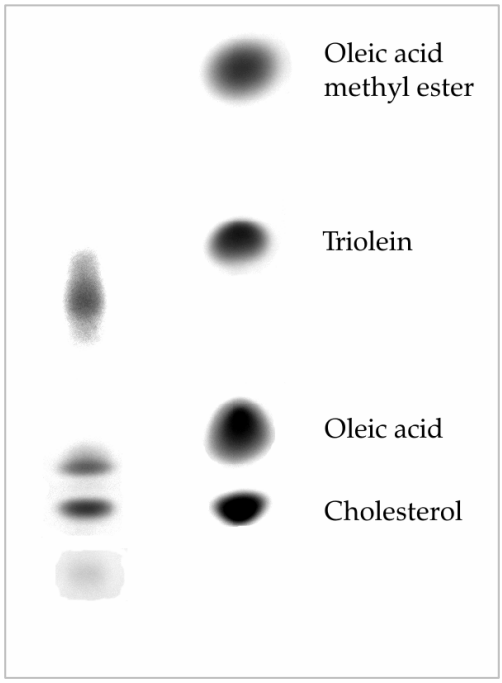


Figure 3 - Example of a TLC plate development for neutral lipids. Standard mixture is in right lane, with names of compounds as stated on the data sheet provided by the manufacturer; to the left, a preliminary sample from JUVALION07 cruise.

Fatty acid composition

The preparation of samples for the subsequent gas-chromatography analysis was carried over by methylation. This method was first described thoroughly by Christie (1989), and the protocol concretely followed was that of Li & Watkins (2001), modified as follows.

Samples were transferred into glass tubes with Teflon-lined screw caps, and dried completely with a centrifugal-vacuum evaporator, at 40 °C for 2 hours. 0.4 ml of 0.5 N sodium hydroxide in methanol were added, tubes were tightly capped and left in a 100 °C heating block for 5 minutes, carrying over the preliminary saponification. After cooling them down with tap water, 0.4 ml of 14 % boron trifluoride (BF_3) in methanol were added, and the tubes again placed at 100 °C for 5 minutes.

To extract the resulting fatty acid methyl esters (FAME), 0.5 ml of hexane and 8.5 ml of distilled water were added. Tubes were shaken for 1 minute in a vortex shaker (Bannon *et al*., 1982), and then centrifuged at 1000 x g for 5 minutes, at room temperature. This separated the sample in its organic and aqueous phases. The upper organic layer was recovered with a glass pipette and placed in 1.5 ml GC sample vials containing a 1 mm-deep layer of anhydrous sodium sulphate on the bottom, in order to remove residual water from the sample (Li & Watkins, 2001).

Gas-chromatographic analyses were performed with a Thermofisher Scientific GC8060 gas-chromatograph coupled with a MD800 mass-spectrometer. The apparatus was fitted with a BPX-70 capillary column (30 m x 0.25 mm i.d. x 0.25 μm). Helium was used as carrier gas, with a speed of 1 ml/min. The programmed oven temperature was 60 °C (1') to 260 °C (10') with an increment of 8 °C/min. Injector temperature was 270 °C and injector split was set at 35 ml/min.

Mass-spectrometry was conducted with an ion source temperature of 200 °C and an interphase temperature of 260 °C. Ionization was performed by electron impact at 70 eV. Weight range analyzed was 50 – 550 Da.

Fatty acid methyl esters were identified by comparing their retention times with those of the standard mixture, Supelco™ 37 Component FAME mix, which has a concentration of 10 mg/ml and is factory-verified by gas-chromatography – flame ionization detection. Data acquisition and peak identification were performed with Weightlab software.

Dry weight measurement

Data of lipid content were standardized by using the dry weight of the individuals. For this reason, as dried larvae cannot be used for analysis on biochemical components such as lipids, subsets of 15 larvae from each cruise were thawed and weighted while wet, removing only excess water by means of an absorbent paper, using a digital scale (OHAUS Analytical, d = 10 μg). They were then left drying in a static oven at 60 °C for 24 hours, and weighted again with the same scale to

obtain the dry weight. Larvae chosen for this measurement were discarded after weighting. These results were then used to obtain a function to estimate the dry weight of larvae analyzed for lipid content, on the basis of their wet weight.

Statistical analyses

The empirical relationship between wet and dry weight was calculated on the whole group of summer and late-autumn larvae by means of linear regression after the absence of significant differences in the slopes of wet and dry weight seasonal relationship was first tested (Underwood, 1997) and ANCOVA analyses showed that season does not significantly influence, as a fixed factor, the above mentioned relation.

Differences between water temperature, Fulton's condition factor, total lipid content, lipid classes and fatty acid ratios were tested using Mann-Whitney's non-parametric test for independent samples (Dytham, 2003). Fatty acids were pairwise compared using Mann-Whitney's test, between larvae from the two cruises and between December 2007 larvae and zooplankton.

Statistical analyses were performed with STATISTICA 6.0 software by Statsoft, Inc. Significance level for all tests was adopted at $p < 0.05$.

Figure 4 - Retrieving a Sardonet pelagic trawl net.

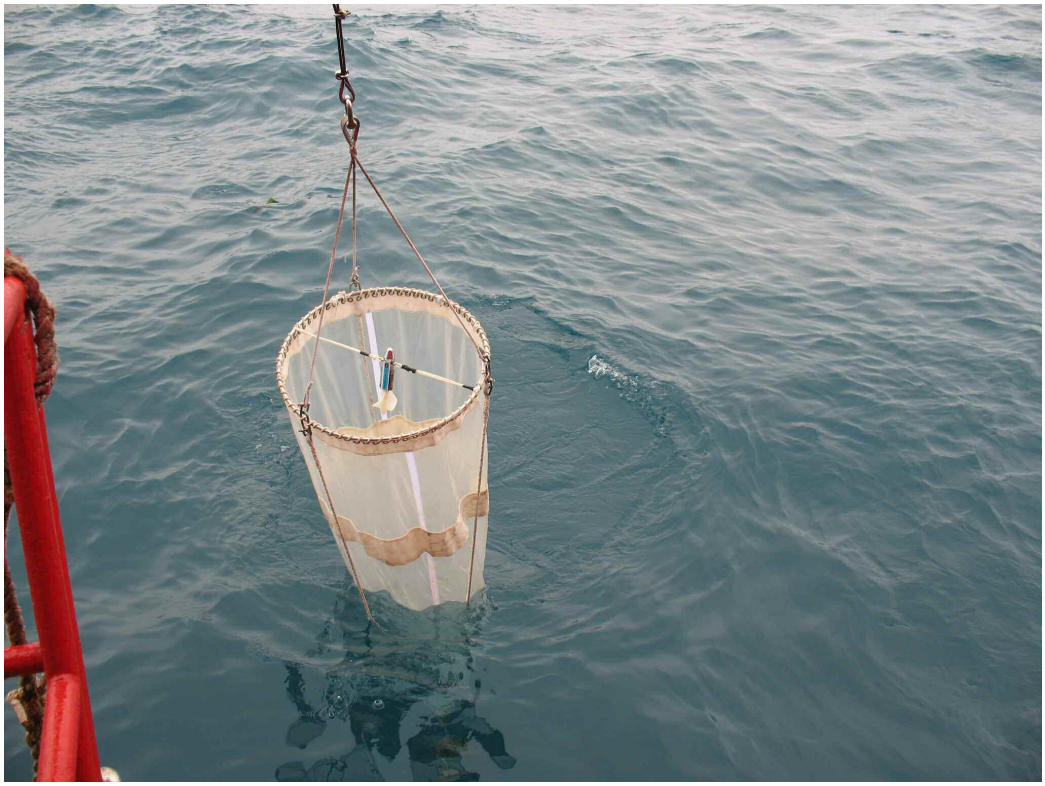


Figure 5 - A WP2 mesozooplankton net coming out of the surface.

Results

Oceanographic data

During August cruise, the mean temperature in the water column (0-50 m) was 16.6 ± 1.0 °C, and the salinity was 38.0 ± 0.1 PSU. In December, the temperature was 13.1 ± 0.5 °C and the salinity was 38.0 ± 0.3 PSU. Regarding the surface temperature (as measured in the first 5 meters), it was 18.8 ± 1.4 °C in August and 12.7 ± 0.8 °C during December cruise. Temperatures at the surface and over the water column were significantly higher during the August cruise compared with December cruise ($p < 0.05$). Salinity did not show differences between the two periods.

Larvae and nutritional condition

The analysis of the homogeneity of slopes showed the absence of significant differences in the slopes of linear relationship between dry weight and wet weight of larvae ($p > 0.05$). For this reason, the presence of differences between summer and late-autumn larvae was tested by means of one-way ANCOVA (considering wet weight as covariant). The test showed that no significant difference between the two regressions occurred ($p > 0.05$), thus a single linear relation between wet weight and dry weight of larvae was computed (shown in Fig. 6). A clear, significant ($p < 0.05$), linear correlation between the two parameters was observed ($R^2 = 0.9578$). The resulting empirical relationship was:

$$\textit{Dry weight} = 0.1692 * \textit{wet weight} + 1.8262$$

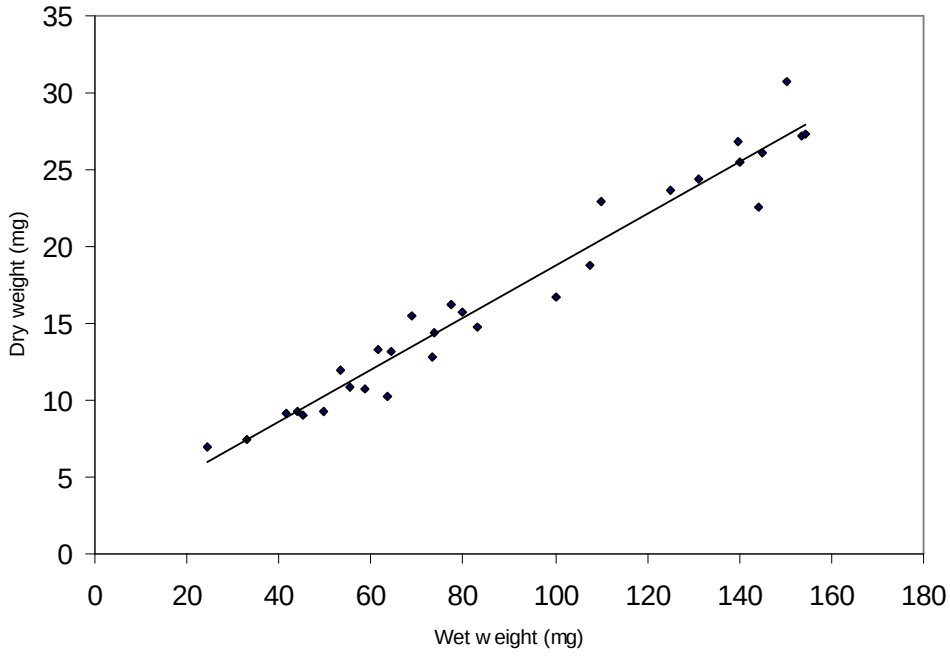


Figure 6 – Wet weight / dry weight relationship of anchovy late-larvae.

Subsequently this equation was applied to calculate the dry weight of samples analyzed for lipids.

A total of 7 pools of larvae (n = 4-6) from PELMED07 cruise and 5 pools (n = 4-6) from JUVALION07 cruise were processed for lipid extraction and used to assess the total lipid content. Anchovies larval mean standard length did not differ between the two seasons (Tab. 1). Larvae wet weight (considering both cruises) ranged from 28 mg to 208 mg. Samples prepared for lipid extraction ranged in weight between 314 mg and 543 mg, which is considered a sufficient amount for the quantification by gravimetric method. Dry weight was calculated using the relationship explained above. Values of Fulton's condition factor showed a significant difference between cruises ($U_{33,21} = 200$; $p < 0.05$), with values higher in August larvae than in December ones.

Table 1 – Characteristics of *E. encrasicolus* larvae used for lipid analysis. Standard length data is presented as Mean ± Standard deviation and as size range. Weights are presented as Mean ± Standard deviation.

	August 2007	**December 2007**
Total number of larvae	32	21
Number of samples	7	5
Standard length (mm)	27.3 ± 3.0 19 ÷ 31	27.0 ± 3.4 21 ÷ 35
Wet weight (mg / larva)	127.0 ± 44.0	97.0 ± 44.0
Estimated dry weight (mg / larva)	23.0 ± 7.0	18.0 ± 7.0
Fulton's condition factor	0.598 ± 0.131	0.489 ± 0.153

Lipids accounted for between 2.9 % and 5.8 % of total body dry weight, with average values of 4.2 % in August and 4.4 % in December (Tab. 2). Values for larvae of the two cruises showed no significant differences ($U_{7,5} = 8$; $p > 0.05$).

Usable samples for thin-layer chromatography were 7 for PELMED07 and 4 for JUVALION07, due to a problem with one sample of the latter cruise desiccating after chloroform:methanol:BHT was added. Data on lipid classes is presented on table 2. No significant differences were found between larvae of the two cruises regarding triacylglycerol ($U_{7,4} = 14$; $p > 0.05$), cholesterol ($U_{7,4} = 12$; $p > 0.05$), free fatty acids ($U_{7,4} = 11$; $p > 0.05$) and polar lipids ($U_{7,4} = 14$; $p > 0.05$).

The calculated TAG/CHOL condition index ranged 0.53 – 0.72 for August larvae, and 0.60 – 0.82 for December larvae (Fig. 7). Values also showed no statistical differences between cruises ($U_{7,4}$ = 7; p > 0.05).

Table 2 – Lipid content of *E. encrasicolus* larvae. Data are presented as Mean ± Standard deviation. Total lipid content and lipid class values are μg per larva. – indicates not detected.

	August 2007	**December 2007**
Total lipid content	770.4 ± 275.1	664.5 ± 92.7
% lipids / dry weight	4.2 ± 0.8	4.4 ± 0.2
Neutral lipids		
Triacylglycerol (TAG)	922.2 ± 379.4	873.8 ± 242.2
Cholesterol (CHOL)	1414.4 ± 476.6	1227.8 ± 249.9
Free fatty acid	1406.0 ± 425.6	1228.2 ± 221.7
Steryl esther	–	–
Polar lipids	230.6 ± 65.9	223.1 ± 37.8
TAG / CHOL index	0.64 ± 0.06	0.70 ± 0.09

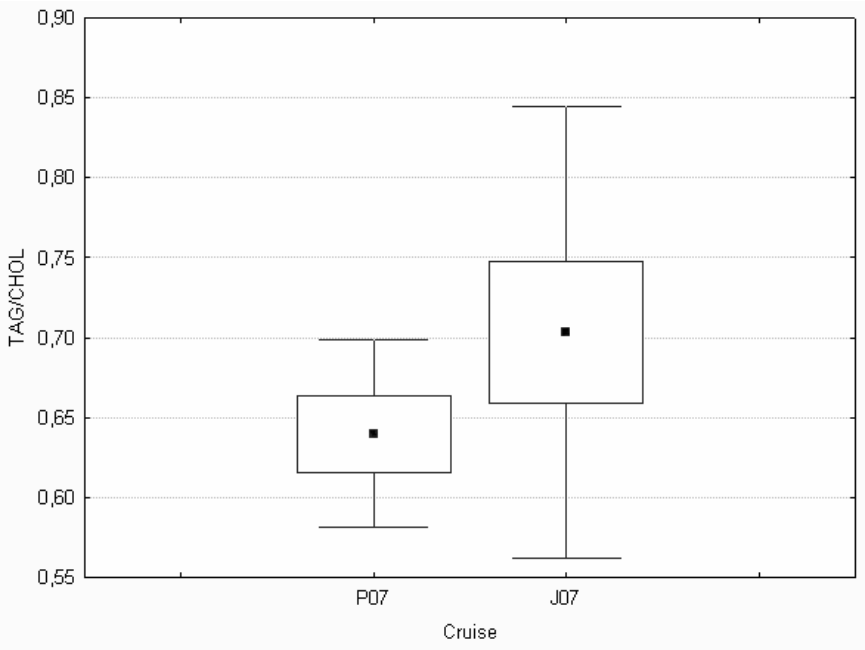


Figure 7 - TAG/CHOL index of *E. encrasicolus* larvae. Central dots indicate means, boxes indicate standard error and whiskers indicate 95% confidence interval. P07: PELMED07, August; J07: JUVALION07, December.

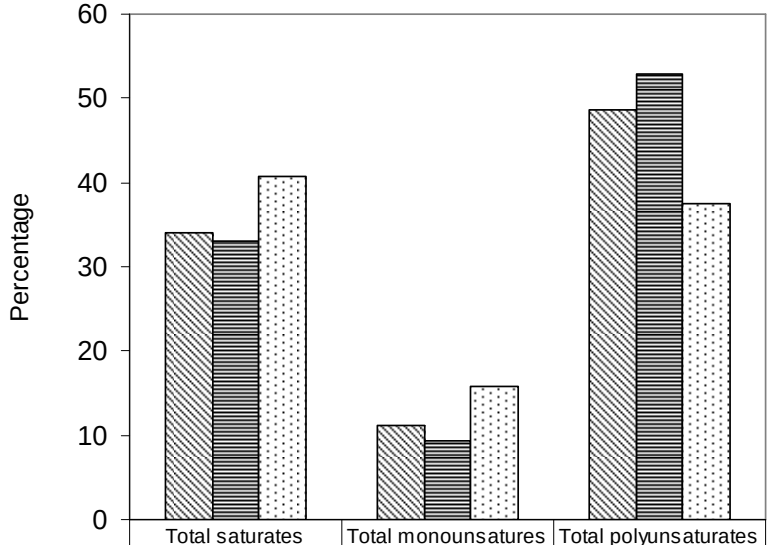
Fatty acid composition

A total of 9 pools of anchovy larvae (4 for PELMED07 and 5 for JUVALION07) and 3 zooplankton samples were correctly read by the gas chromatography – mass spectrometry apparatus.

Polyunsaturated fatty acids (PUFA) accounted for the majority of total fatty acid content in anchovy larvae, ranging between 49 % and 53 %, while in zooplankton the most important group in terms of percentage was that of the saturated fatty acids (SFA), with an abundance just below 41 % (Tab. 3). The most important fatty acids in anchovy larvae were 16:0, 20:5(ω-3) and 22:6(ω-3); these are respectively palmitic acid, timnodonic acid (EPA) and docosahexanoic acid (DHA). Zooplankton showed accumulation also of 14:0, 16:1(ω-7) and 18:1(ω-9); these are called myristic acid, palmitoleic acid and oleic acid, respectively.

Table 4 presents fatty acid composition for each group, each acid expressed as percentage of the total. Where significant differences were found by means of Mann-Whitney's test ($p < 0.05$), a letter is indicated. Regarding anchovy larvae, the abundant fatty acids that showed higher concentrations in August than in December were 14:0, 15:0, 20:0, 24:0, 16:1(ω-7) and 18:2(ω-6); the fatty acids that were higher in December were 17:0, 18:1(ω-9) and 18:3(ω-3). Moreover, differences were found between December larvae and zooplankton: the 14:0, 15:0, 18:0, 16:1(ω-7), 18:1(ω-9) and 18:3(ω-3) fatty acids showed higher concentrations in zooplankton, with only the 22:6(ω-3) being more abundant in anchovy larvae.

Table 3 - Comparison of fatty acid content by degree of saturation. Data are presented as percentage of the total fatty acid content.



	Total saturates	Total monounsatures	Total polyunsaturates
Anchovy - August 2007	34,0	11,1	48,7
Anchovy - December 2007	33,0	9,3	52,9
Zooplankton - December 2007	40,7	15,8	37,6

Table 4 – Fatty acid composition of *E. encrasicolus* late-larvae and zooplankton. Data are presented as Mean percentage ± Standard deviation. – indicates not detected.
A: Statistical difference between August and December larvae, $p < 0.05$.
B: Statistical difference between December larvae and zooplankton, $p < 0.05$.

	E. encrasicolus		**Zooplankton**	
Fatty acids	**August 2007 N = 4**	**December 2007 N = 5**	**December 2007 N = 3**	**Differences**
14:0	5.06 ± 0.39	1.71 ± 0.32	8.94 ± 2.10	A B
15:0	0.61 ± 0.02	0.49 ± 0.06	0.88 ± 0.37	A B
16:0	23.39 ± 1.47	25.63 ± 7.17	24.31 ± 4.83	
17:0	0.51 ± 0.02	0.69 ± 0.03	0.67 ± 0.19	A
18:0	4.22 ± 0.52	4.32 ± 0.12	5.73 ± 0.48	B
20:0	0.06 ± 0.01	0.04 ± 0.003	0.11 ± 0.02	A
22:0	0.05 ± 0.01	0.04 ± 0.01	–	
24:0	0.14 ± 0.03	0.08 ± 0.02	0.07 ± 0.00	A
Total saturates	*34.0*	*33.0*	*40.7*	
15:1	0.04 ± 0.01	0.04 ± 0.01	0.11 ± 0.00	
16:1 (ω-7)	3.66 ± 0.38	1.42 ± 0.09	6.47 ± 1.74	A B
18:1 (ω-9)	5.16 ± 0.33	5.57 ± 0.13	6.82 ± 1.48	A B
18:1 (ω-7)	1.91 ± 0.28	1.92 ± 0.16	1.84 ± 0.36	
20:1 (ω-9)	0.20 ± 0.15	0.13 ± 0.01	0.42 ± 0.10	
22:1 (ω-9)	–	–	0.08 ± 0.00	
24:1 (ω-9)	0.12 ± 0.03	0.27 ± 0.05	0.08 ± 0.00	A
Total monounsaturates	*11.1*	*9.3*	*15.8*	
18:2 (ω-6)	1.19 ± 0.11	0.62 ± 0.12	0.83 ± 0.29	A
18:3 (ω-6)	0.55 ± 0.09	0.40 ± 0.05	0.64 ± 0.08	
18:3 (ω-3)	0.85 ± 0.15	1.18 ± 0.15	1.89 ± 0.05	A B
20:3 (ω-6)	0.04 ± 0.00	0.03 ± 0.01	–	
20:3 (ω-3)	0.05 ± 0.01	0.07 ± 0.01	–	
20:4 (ω-6)	0.48 ± 0.06	0.55 ± 0.09	0.31 ± 0.05	
20:5 (ω-3)	11.29 ± 0.64	10.79 ± 0.85	16.57 ± 6.34	
22:6 (ω-3)	34.25 ± 2.96	39.28 ± 4.47	17.35 ± 6.01	B
Total polyunsaturates	*48.7*	*52.9*	*37.6*	
Others	6.2	4.8	5.9	
Total	100	100	100	

In order to have another set of indices that could improve evaluation, ratios between percentages of fatty acids were assessed for 16:1(ω-7) / 16:0, 18:1(ω-9) / 18:1 (ω-7), and EPA/DHA [20:5(ω-3) / 22:6(ω-3)]. These ratios are presented in Tab. 5, along with ratio between polyunsaturated and saturated fatty acids (PUFA / SFA). Additionally, as samples of zooplankton from the summer cruise (PELMED07) were not available, data of zooplankton fatty acid composition in the same area and season were retrieved from the work by Rossi *et al*. (2006), who applied the same methodology. Those data were used to calculate the same ratios and presented alongside our results for comparison.

Larvae from the two periods showed a significant difference ($p < 0.05$) in the 16:1(ω-7) / 16:0 and the 20:5(ω-3) / 22:6(ω-3) ratios; in both cases, values were higher in August larvae. The same two ratios were statistically different between December larvae and zooplankton ($p < 0.05$): in this case, the latter exhibited higher values for both indices.

Table 5 – **Ratios between relevant fatty acids in *E. encrasicolus* larvae.** Data of June 2000 was only available without variability.

	E. encrasicolus		**Zooplankton**	
	August 2007	**December 2007**	**June 2000 (from Rossi *et al*., 2006)**	**December 2007**
16:1(ω-7) / 16:0	0.16 ± 0.01	0.06 ± 0.01	0.96	0.27 ± 0.08
18:1(ω-9) / 18:1 (ω-7)	2.73 ± 0.30	2.91 ± 0.23	3.15	3.75 ± 0.62
20:5 (ω-3) / 22:6 (ω-3)	0.33 ± 0.01	0.28 ± 0.02	0.53	0.96 ± 0.42
PUFA / SFA	1.44 ± 0.21	1.68 ± 0.42	0.65	0.97 ± 0.42

Discussion

Recruitment influences the strength of the first year-classes, thus its success becomes of increased importance when considering species exploited especially in those classes (Jennings *et al.*, 2001) which is the case of the anchovy fishery, as well as the other small pelagics' fisheries.
The success in recruitment is related, apart from density-dependency, to several factors and is mainly driven by food availability and environmental processes. It is generally assumed that larvae exhibit a preference for specific planktonic organisms, so the zooplankton community composition may influence larval feeding and, thus, its energy reserves. Larval condition can then play a fundamental role in determining recruitment for this species, and is therefore an important factor to be considered in order to predict the strength of recruitment.

The Gulf of Lions represents somewhat an exception in the general oligotrophy of the north-western Mediterranean basin, since its primary production is high due to the presence of the Rhône river discharge plume (Cruzado & Velasquez, 1990). Food availability in the region is correlated with nutrients carried to the sea by river discharge and as it has been verified for a similar region – the southern Catalan Sea – this factor can strongly influence the strength of early age-classes (Lloret *et al.*, 2004). Another important environmental factor is wind stress, which is high in this region, and contributes to the mixing of the water column (Millot, 1990). Strong winds can also produce local upwellings, further increasing the nutrient availability and thus the primary production (Salat, 1996). Both the Gulf of Lions and the Catalan Sea are known as main spawning areas for anchovy, with a spawning period from April to October, and a peak in June-July (Palomera, 1992).
As expected, the water temperatures in August were higher than those recorded in December, while salinity was stable in the two periods.
During the study period, parallel analyses have been conducted on mesozooplankton from the same cruises, to obtain data on species composition and biomass (data courtesy of I. Álvarez and D. Costalago). Preliminary analyses showed a significant difference in mesozooplankton total biomass (dry weight / m^3) between the two periods, the December biomass being less than a fourth of that recorded in August.
In August both cladocerans and copepods were abundant, with the latter group dominated by calanoids and to a less extent by poecilostomatoids. By contrast, in December no cladocerans were present and copepods (both calanoids and

cyclopoids) accounted for the majority of the biomass. These data agree with those already described for mesozooplankton composition in another area of the north-western Mediterranean, the Bay of Blanes (Calbet *et al*., 2001).

The finding of this study that no differences in nutritional condition exist between *Engraulis encrasicolus* larvae of the two periods, considering both TAG/CHOL and the polar lipids content, may seem puzzling at start. The TAG/CHOL index includes two lipid classes with different meanings: triacylglycerol is considered an indicator of individual potential of resisting starvation conditions (Fraser *et al*., 1987), while cholesterol is mainly a structural membrane component so it is used to standardize for the fish size. The resulting ratio between the two represents therefore a set of conditions: energy reserves used for periods of starvation, environmental stress (which affects metabolic rate), and growth capacity. Polar lipids are also structural cell components and high levels of PL may indicate increased cell proliferation and thus improved growth capacity (Norton *et al*., 2001).

The TAG/CHOL index is considered an indicator of poor nutritional condition for anchovy when values are below 0.2 - 0.3 (Håkanson, 1989; Håkanson, 1993). Following this boundary, neither summer anchovy larvae nor late-autumn ones showed a deficiency in their energy reserves, although in December a higher variability was detected. For this reason, zooplankton biomasses and compositions of the two periods could be considered favourable environments for anchovy late-larvae growth.

A contrasting result comes from the Fulton's condition factor, which was statistically higher in August larvae. However, the absolute difference between the two periods was not very marked, and in addition this index has been demonstrated to suffer from various biases. Larval weight was very variable in both periods, and this could have partially influenced this result. Besides, this data contrast with all the other results obtained in this study, and should be considered with caution.

Based on known data on growth rates (obtained by otolith analyses, Palomera *et al*., 2007), it may be hypothesized that larvae collected in December hatched in mid-October, i.e. at the end of the known reproductive period in the region (Palomera, 1992). Spawning after October leads anchovy larvae in the Gulf of Lions to an interspecific competition for food with *Sardina pilchardus* larvae, which begin to be present in the same period (Palomera *et al*., 2007), so at first it could not be considered an advantage. However, one way for minimizing or avoiding this competition may be the differentiation of feeding preferences. For example, in the southern Benguela, it has been demonstrated that differences

between diet of anchovy and sardine late-larvae do exist, the former feeding on larger prey sizes while the latter including phytoplankton in its habitual diet (van der Lingen *et al*., 2006). This point should be carefully analysed also in the area addressed by this study.

Temperature is an important factor in determining metabolism in fishes. Warmer waters in summer increase metabolic rates, thus raising the animal energy expenditure (Randall *et al*., 1997); the contrary happens during winter. As already mentioned, the water temperatures in the two periods differed significantly, both at the sea surface and in the first 50 meters. In order to justify the same nutritional condition, in August larvae an energetically richer diet was therefore expected, and the above described differences in mesozooplankton composition could partially agree with this hypothesis.

It is also worth noting that JUVALION07 cruise was the first attempt to conduct a pelagic sampling in the region during winter months, and so it is unclear if presence of anchovy late-larvae in December is a punctual event or an usual occurrence. In the case of an isolated event, a higher-than-usual runoff from the Rhône river may have positively influenced primary production and, consequently, zooplankton abundance. Also, as the sampled area is described as hosting frequent upwelling events (Salat, 1996), heavy late-autumn rains could have caused the winds that produced the water mixing. These occurrences would not be unusual, as it has been already documented, e.g. for the year 1983, when increased plankton abundance and, consequently, reduced larval mortality for anchovy larvae was reported (García & Palomera, 1996). However, a detailed comparison between present and past data should be carried out to confirm or reject this hypothesis.

The interpretation of results of fatty acid analysis is not a straightforward one. First of all, the number of samples processed precluded some types of statistical analyses that could have better clarified the possible relations between larvae and zooplankton.

Saturated fatty acids, specifically 16:0, 20:0, 22:0 and 24:0, can all be synthesized by the organism and therefore do not constitute a significant trophic marker, unless in the case some *iso* or *anteiso* odd-chain acids – markers for North Atlantic copepods – were found (Ackman, 1980), but this was not the case. The two most abundant monoethylenic fatty acids in marine organisms, namely 16:1(ω-7) and 18:1(ω-9), showed differences between larvae of the two periods. Copepod typical 20:1(ω-9) and 22:1(ω-11) were not detected in anchovy larvae, but this was more or less expected because these two acids are utilized by fishes

for metabolic energy and not for biomembrane functioning, thus precluding their accumulation (Dalsgaard *et al*., 2003).

Considering previous knowledge from literature, 18:2(ω-6) and 18:3(ω-3) do not accumulate in fishes more than 1-2 % (Ackman, 1980); this was also our case. Although differences were found between groups for these two fatty acids, their trophic meaning has not been clarified yet.

Phytoplankton traces can be detected with 18:4(ω-3) and 22:6(ω-3) for flagellated algae such as Prymnesiophyceae or dinoflagellates, while 20:5(ω-3) is regarded as a marker for diatoms (Bell & Sargent, 1996; Dalsgaard *et al*., 2003). The first one, 18:4(ω-3), was not detected in our samples, neither in zooplankton nor in anchovy larvae. The other two fatty acids can be summarized in the EPA / DHA ratio, to evaluate preferences between diatoms and flagellates (Auel *et al*., 2002). Values calculated for zooplankton, both in December and in June (Rossi *et al*., 2006) are higher than values calculated for anchovy larvae of both periods. Applying the same ratio to anchovy larvae requires more attention, as traces of phytoplankton prey eaten by zooplankton may conservatively transfer to upper trophic levels such as larvae. It has been proposed that larvae could show accumulation of markers from two or more inferior trophic levels and not only from their prey. With this effect in mind, it does not surprise that anchovy larvae of both cruises present a ratio that indicates a higher contribution of flagellates over diatoms, even if the latter are thought to be energetically more convenient than flagellates (St. John & Lund, 1996). In fact, phytoplankton community of both periods was indeed dominated by dinoflagellates (data courtesy of I. Álvarez). However, further analyses are needed in order to test this hypothesis, so this remains only matter of speculation.

Another trophic ratio, 18:1(ω-9) / 18:1(ω-7), can be used as an index of carnivory (Auel *et al*., 2002) and data indicated that larvae preferred carnivorous feeding to diatoms. This is also supported by the 16:1(ω-7) / 16:0 ratio, which is used to evaluate diatom contribution in diet (St. John & Lund, 1996; Pedersen *et al*., 1999). In our case, its low values indicate just a small contribution of diatoms in anchovy larvae diet, which is consistent with the results of the EPA / DHA index. The same ratio was also slightly lower in December than in August, and this was expected since there were no reported phytoplankton blooms in December.

The last calculated ratio, PUFA / SFA, has been used only once, as an indicator of carnivory in the invertebrate *Euphausia superba* (Cripps & Atkinson, 2000). Results from this index seem to contradict the conclusions from the other indices, but some observations have to be done. In this study, mixed zooplankton was analyzed, while the other study distinguished between species; furthermore, the

work by Cripps & Atkinson (2000) is the only one in literature that addresses this index. The lack of other applications may thus make the interpretation of the PUFA / SFA ratio not univocal.

These results from fatty acids indices cannot be obviously conclusive without associating other analyses. For example, data from stomach contents may be helpful to confirm the interpretation of these ratios in anchovy larvae. In addition, following on with biochemical analyses would add more detail and provide other points of view. For instance, the adoption of stable isotopes analysis, as food web tracers able to discriminate the proportion of autotrophic and heterotrophic food eaten by anchovy larvae, might contribute to more robust understanding of late-larvae diet composition (Michener & Schell, 1994).

Summarizing, data from this study seem to indicate that in late-autumn, even if zooplankton and phytoplankton are very low in abundance, anchovy larvae can maintain an acceptable nutritional condition, aided by the reduced metabolic expenditure. During these months, they seem to disregard phytoplankton and it could be hypothesized this is due in order to invest their energies on selecting the more convenient zooplankton. In summer, when temperatures are higher and so are energetic costs, larvae still concentrate on zooplankton, even if both phytoplankton and zooplankton are abundant.

This ability of selective feeding could be achieved with a specialized differentiation of the gill-raker morphology, which is structured to retain only particles above a "desired" size. Smaller gaps between gill-rakers typically indicate lack of selectivity, while larger gaps allow for the exclusion of smaller particles, i.e. phytoplankton and detritus. A marked difference in gill-raker morphology has been already verified for anchovy and sardine adults in other regions (van der Lingen *et al.*, 2006), while the same analysis on anchovy developmental stages has not been accomplished yet. It is therefore not sure that late-larvae have already sufficiently developed their gill-rakers to functionally use them as food selectors.

Further improvements

During the definition of the analytical protocol, two different methods for lipid classes analysis were evaluated. Other than the high-performance thin-layer chromatography (HPTLC), another commonly used method utilizes the Iatroscan™, a commercial apparatus combining a thin-layer chromatograph with a flame ionization detector, using silica-coated quartz rods as stationary phase and allowing for rapid analysis of large numbers of samples (Fraser *et al.*, 1985; Shantha, 1992). While this second technique has been applied in many studies,

even for anchovy larvae (Håkanson, 1993), a rather complex calibration is required and it was decided to rely on the HPTLC due to the reduced number of samples processed and also because it requires a rather small sample load (Olsen & Henderson, 1989).

A possible way of increasing analysis detail for fatty acids would be transitioning to the gas chromatography – flame ionization detection technique, which would provide more quantitative results and would also be capable of discriminating fatty acids by stereochemistry and functional groups (Christie, 1989). With that analysis, though, the number of samples would have to be increased, but this methodology would allow also for advanced statistical analyses to be conducted.

Additional details may come from zooplankton samples of August, which as already said were not available at the time of this study, and from *S. pilchardus* larvae. In this latter case, comparative studies regarding accumulation of dietary fatty acids between the two small pelagic species would become possible. Finally, conducting the same analyses on juveniles and adults of the two species would permit a clarification of ontogenic shift in feeding habits.

Conclusions

The present work was aimed to address the issue of the nutritional condition in late-larvae of *E. encrasicolus*, a life stage that has not been extensively studied yet. The analysed pools of larvae showed the same nutritional condition based on lipid contents, and the preliminary study of the dietary accumulation of fatty acids did not lead to a clear discrimination. In addition, results from the Fulton's condition factor added another point of incertainty.

However, a series of possible relations between the anchovy larvae and the oceanographic conditions were also identified and discussed: the possible influence of temperature on metabolism and thus on energetic condition, the effect of fatty acid transfer from more trophic levels, and the probable differentiation of diet between late-larvae of anchovy and sardine. It is my personal opinion that this path should be followed in the subsequent studies, as this species has already been demonstrated of great importance in regulating the community dynamics.

Knowledge of dynamics at early stages of anchovy may also prove useful for inclusion in the current ecosystem models. Although different in many aspects, the Catalan Sea – where another anchovy spawning stock is present – has been studies with a mass-balance ecosystem model in recent times (Coll *et al.*, 2006). A more detailed description of the recruitment and early life-stages could lead to improvements of the management politics, also transposable to other ecosystem

models such as the one set up for the Northern and Central Adriatic Sea (Coll *et al.*, 2007) with very encouraging results.

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Acknowledgements

Un ringraziamento alla prof.ssa Maria Berica Rasotto e al dott. Saša Raicevich, per la pazienza e la disponibilità.

Quiero además agradecer
a la tripulación del B/O *García del Cid*, por su colaboración;
a las "chicas de l'IRTA" Noelia Gras y Mireia Andrés, por enseñarnos el protocolo de extracción de lípidos y soportar nuestras continuas preguntas sobre cromatografía;
a Pilar Teixidor y colaboradores de los Serveis Cientificotècnics de la Universitat de Barcelona, por llevar a cabo los análisis de cromatografía de gases;
y por último, pero no por ello menos importante, a David Costalago, compañero de trabajo, colega de mil cervezas, capitán del mejor equipo de voleibol del mundo mundial, y lo más importante, un amigo.

Isabel, en aquests mesos el teu somriure i la teva hospitalitat sempre m'han enllumenat el camí, des de la primera encaixada de mans fins als moments més difícils. Gràcies per la teva ajuda i els teus consells.

Questo lavoro è dedicato alle persone sulla cui spalla ho avuto la fortuna di piangere.
Rendete questo mondo un posto migliore.