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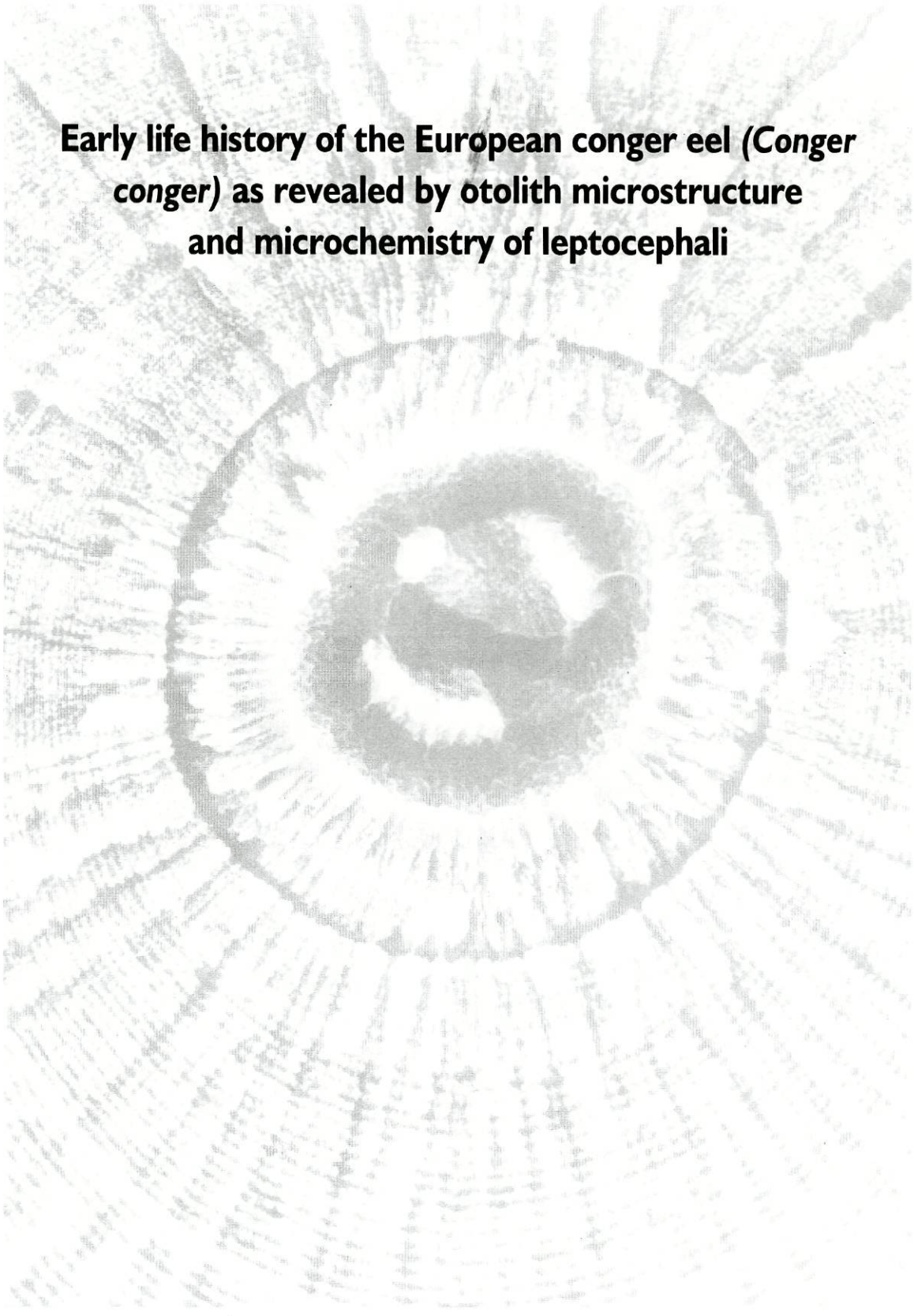

Instituto de Ciências Biomédicas de Abel Salazar
Universidade do Porto
Porto, 2004

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Early life history of the European conger eel (*Conger conger*) as revealed by otolith microstructure and microchemistry of leptocephali

Dissertação de candidatura ao grau de Doutor em Ciências do Meio Aquático, submetida ao Instituto de Ciências Biomédicas de Abel Salazar

Orientador: Prof. Doutor João Coimbra
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Porto, 3 de Fevereiro de 2004

Humberto Tomaz Pinheiro Nunes Lima

I dedicate this PhD thesis to my mother and wife, Eulália and Sandra, the two most special people in my life. They, not only have given me strength and support, but have also fill my life with all the love and affection one could wish for.

Acknowledgments

First of all, I want to thank my supervisors, Prof. João Coimbra and Dr. Carlos Antunes, for their support and guidance, without which this adventure would not have been possible. I am special grateful to Carlos Antunes for giving me his time, effort, advice, friendship and above all, for opening for me the doors to this fascinating (although sometimes hard) world of otolith research.

I want to express my special thanks to Dr. Eduardo Isidro, from the Department of Fisheries and Oceanography of the University of Azores, co-author on papers II and III, for giving me the opportunity to work in his lab and for the stimulating and creative discussions. My thanks goes also to all the crew and support staff of the R.V. "Arquipélago" for their help and assistance during the "Arictio 99" cruise.

In particular I want to thank Dr. Kenneth Able from the Rutgers University Marine Field Station for allowing me to work with him on the American conger eel early life history and for giving me helpful comments on the early draft of the paper V. Thanks also to Angie Muzeni for help with the collection of the *Conger oceanicus* specimens.

A special thanks to all the anonymous reviewers who provided constructive comments and suggestions on earlier drafts of the conger eel papers (I, II, III, VI) already published.

I want to thank Dr. Farinha Ramos and Dr. Paula Piedade for helping me in preparing and analysing the otolith microchemistry. My special thanks also to Dr. Carlos Sá and Daniela Silva for their help and useful comments on the SEM observations.

I am most grateful to the fisherman Eduardo Martins, for his help with the fishing of the conger eel leptocephali (when I had the chance of fishing something!) on the many cold nights that I passed on the Minho River.

I include a special thank to Dr. Jonathan Wilson for his assistance with the English language and for many valuable comments on my early manuscripts. I thank also to my sister Genoveva by help me on the drafting of the abstract in French.

A special thanks to all my colleagues at the ICBAS and CIIMAR institutions who have helped me during my thesis work.

And finally, the Portuguese Foundation for Science and Technology is gratefully acknowledged for the fellowship during my PhD work.

Resumo

O congro europeu, *Conger conger*, é um peixe comum nos ecossistemas marinhos do Atlântico oriental e Mediterrâneo. Contudo, a informação biológica acerca da sua fase larvar leptocéfala é escassa. A morfologia, pigmentação e características merísticas de larvas capturadas entre 1998 e 2000, no norte de Portugal, sobretudo no estuário do Rio Minho, e a sul do grupo central das ilhas dos Açores foram analisadas. O estudo da microestrutura e da relação Sr:Ca nos otólitos durante as várias etapas do desenvolvimento larvar, foi realizado com recurso à microscopia electrónica de varrimento (SEM) e à espectrometria por dispersão de comprimento de onda (WDS), respectivamente. O retro-cálculo das datas de eclosão larvar realizado a partir da microestrutura dos otólitos de larvas premetamórficas indica que a época de desova é longa, de Dezembro a Julho, com um pico observável no início do Verão. A idade das larvas capturadas a sul do Arquipélago dos Açores e o conhecimento actual da circulação do Atlântico NE, sugere que o congro tem um local de postura, algures perto dos Açores. O estudo preliminar do ADN mitocondrial de larvas capturadas na zona oriental e central do Atlântico, fornece alguma evidência para a existência de uma diferenciação genética significativa entre populações locais, sugerindo que o congro não compreende apenas uma única população panmíctica. Nos otólitos, a espessura dos incrementos, relativamente constante e fina na fase larvar pré-metamórfica, aumenta rapidamente por volta dos 170 a 280 dias. A elevada relação Sr:Ca observada nos otólitos durante a fase pré-metamórfica, mostra um súbita queda, coincidente com o aumento da espessura dos incrementos. Estas mudanças simultâneas são apontadas como o início da metamorfose nesta espécie. Os dados obtidos indicam que o congro leva cerca de 6 a 9 meses desde a eclosão larvar até atingir a metamorfose. Por sua vez, as larvas de crescimento rápido, metamorfoseiam e são recrutadas mais cedo para as águas costeiras do norte de Portugal, com um tamanho maior e num estádio de desenvolvimento larvar mais avançado. A presença nos otólitos das larvas metamórficas de uma zona difusa (DZ) periférica, sem incrementos diários observáveis, e de alguns centros acessórios de crescimento (AGCs) impossibilita a correcta estimativa da duração de vida larvar desta espécie. A microestrutura e composição química elementar dos otólitos de larvas metamórficas de *C. oceanicus* revelou uma variação semelhante na espessura dos incrementos e na relação Sr:Ca, incluindo também a presença de uma ZD e alguns AGCs. O uso da tetraciclina no estudo da taxa de deposição da ZD em larvas metamórficas mantidas em cativeiro, mostrou a existência de um crescimento anormalmente exagerado do otólito, provavelmente causado pelo stress da captura, manuseamento e marcação.

Résumé

Le congre européen, *Conger Conger*, est un poisson commun aux écosystèmes marins de l'Atlantique oriental et Méditerranée. Cependant, sa vie larvaire leptocéphale demeure mal connue. La morphologie, pigmentation et caractères méristiques de larves capturées entre 1998 et 2000 au Nord du Portugal, surtout dans l'estuaire du Minho, et aux Açores, au sud des îles du groupe central, ont été analysées. L'étude de la microstructure et du ratio Sr:Ca dans les otolithes pendant les étapes du développement larvaire a été réalisée à l'aide de microscopie électronique à balayage (SEM) et de spectrométrie à dispersion de longueur d'onde (WDS), respectivement. Le rétro-calcul des dates d'éclosion larvaire réalisé à partir de la microstructure des otolithes des larves pré-métamorphiques indique que la saison de ponte est longue, de décembre jusqu'en juillet avec un maximum remarquable au début d'été. L'âge des larves capturées au sud de l'archipel des Açores et la connaissance actuelle de la circulation de l'Atlantique NE, suggère que le congre ait une frayère, quelque part, près des Açores. L'étude préliminaire de l'ADN mitochondrial de larves capturées dans l'Atlantique oriental et central, fournit quelque évidence pour l'existence d'une différenciation génétique significative parmi les populations locales, suggérant que le conger comprenne plus qu'une population panmictique. Dans les otolithes, l'épaisseur des stries, relativement constante et faible dans la phase larvaire pré-métamorphique, augmente rapidement vers les 170 à 280 jours. Le ratio élevé Sr:Ca observé dans les otolithes pendant la phase pré-métamorphique montre une brusque chute coïncidente avec l'augmentation de l'épaisseur des stries. Ces changements simultanés sont indiqués comme le début de la métamorphose pour cette espèce. Les données obtenues indiquent que le congre prend environ 6 à 9 mois depuis l'éclosion larvaire jusqu'au début de la métamorphose. De son côté, les larves de croissance rapide, métamorphosent et sont recrutées plus tôt pour les eaux côtières du Nord de Portugal avec une taille plus grande et dans un stade de développement larvaire plus avancé. La présence dans les otolithes de larves métamorphiques d'une zone diffuse (DZ) périphérique, sans stries journalières observables, et de quelques primordiums accessoires (AGCs), ne permet pas l'estimation précise de la durée de vie larvaire de cette espèce. La microstructure et microchimie des otolithes de larves métamorphiques de *C. oceanicus* a révélé une variation pareille dans l'épaisseur des stries et dans le ratio Sr:Ca, incluant aussi la présence d'une ZD et des quelques AGCs. L'usage de tétracycline dans l'étude du taux de déposition de la ZD en larves métamorphiques gardées en captivité, a révélé une croissance anormalement élevée de l'otolithe, probablement causée par le stress de la capture, manipulation et marquage.

Abstract

The European conger eel, *Conger conger*, is a common fish of the NE Atlantic and Mediterranean marine ecosystems; however, information on several biological aspects of this species is scarce, especially concerning its larval leptocephalus phase. The morphology, pigmentation and meristic characteristics of leptocephali collected between 1988 and 2000, in northern Portugal, mainly from the Minho River estuary, and in the waters south of the central group of the Azores Islands are analysed. Otolith microstructure and Sr:Ca ratios are also examined during the larval developmental stages by scanning electron microscopy (SEM) and wavelength dispersive spectrometer (WDS), respectively. Back-calculated hatching dates from the otolith microstructure of developing leptocephali indicate a long spawning season, from December to July, with one annual peak, occurring in the beginning of summer. The early age of the larvae captured south of the Azores Archipelago and the current oceanographic knowledge of the NE Atlantic circulation suggests that the conger eel has a spawning area somewhere near the Azores. A preliminary mitochondrial DNA study using leptocephali from different locations around the central and eastern Atlantic Ocean, provides some evidence for the existence of a significant genetic differentiation among local populations, suggesting that the conger eel does not comprise a single panmictic population. Otolith increment width, which was relatively constant and narrow in the developing leptocephalus stage, increased sharply at age 170 to 280 days. Sr:Ca ratios in the otolith, which increased during the developing leptocephalus stage, showed a rapid drop coinciding with the increase in increment width. These coincidental changes are regarded as the onset of metamorphosis for this species. Our results indicate that the conger eel takes about 6 to 9 months from hatching to reach the onset of metamorphosis. Our data shows the faster-growing larvae metamorphose and recruited earlier to the northern Portuguese coastal waters, with a larger size and in an advanced developmental stage. The existence of a peripheral diffuse zone (DZ), where the daily increments are unclear, and some accessory growth centres (AGCs) in the otoliths of the metamorphosing leptocephali prevents an accurate estimation of the larval stage duration of this species. The otolith microstructure and microchemistry of metamorphosing leptocephali of *C. oceanicus* revealed a similar increment width and Sr:Ca ratios profiles along the otolith radius, including also the presence of a DZ and some AGCs. The use of tetracycline as an otolith time marker to study the DZ growth rate in reared metamorphosing specimens, showed an anomalous high otolith growth, probably caused by capture, handling and marking stress.

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AC: Azores Current

AGC: Accessory Growth Centre

% AGCA: % Area occupied by the AGC

BD: Body Depth

BSD: Back-Scattered Detector

C: Core

CC: Canary Current

CZW: Countable Zone Width

D: Diameter

DLGZ: Developing Leptocephalus Growth Zone

DOM: Dissolved Organic Matter

DZ: Diffuse Zone

DZW: Diffuse Zone Width

E: Edge

ED: Eye Diameter

FFC: First Feeding Check

GAG: GlycosAminoGlycans

HC: Hatch Check

HL: Head Length

ICTZ: Increment Countable Terminal Zone

(I)CZ: (Increment) Countable Zone

I_G: Gonadosomatic Index

I_O: Ocular Index

k: Growth constant

K: Condition factor

LM: Light Microscope

L_T: = TL

LVBV: Last Vertical Blood Vessel

$L\infty$: Asymptotic Length

M: Metamorphosis

MA/TNM: = PAM/TNM

MOD: Maximum Oocyte Diameter

MT: Metric Tonnes

MW: Mediterranean Water outflow

n: Sample size

N: Nucleus

OGR: Otolith Growth Rate

OL: Otolith Length

PAL: PreAnal Length

PAM: PreAnal Myomeres

PC: Portuguese Current

PCCC: Portuguese Coastal Counter Current

PDL: PreDorsal Length

PDM: PreDorsal Myomeres

POM: Particulate Organic Matter

R: Radius

SD: Standard Deviation

SED: Secondary Electron Detector

SEM: Scanning Electron Microscope

SGR: Somatic Growth Rate

TGL: = SGR

TGS: = OGR

TL: Total Length

TNM: Total Number of Myomeres

To: Length at birth

TZ: Transition Zone (=WIZ)

VtG: Vitellogenin

W: Weight

WDS: Wavelength Dispersive Spectrometer

WIZ: Wide Increment Zone

1. GENERAL INTRODUCTION

1.1 Biology of the European conger eel

Geographic distribution and habitat

The European conger eel, *Conger conger* (Linnaeus, 1758), is a fish widely distributed in the north-eastern Atlantic (Fig. 1), being found from Norway to Senegal, including the Canary Islands, Azores and Madeira, as well as in the Mediterranean and western Black Sea (Bauchot and Saldanha 1986). Conger eels are strictly marine benthic fishes that live on the continental shelf on rocky and sandy bottoms, from the shoreline to depths of 500 m (Smith 1981; Bauchot and Saldanha 1986; Bauchot 1987). On rocky grounds they are commonly found associated with piers, rocks, crevices and old wrecks below the low-tide mark (Lythgoe and Lythgoe 1971; Bagenal and Kenney 1973; Wheeler 1985; Hayward and Ryland 1995).

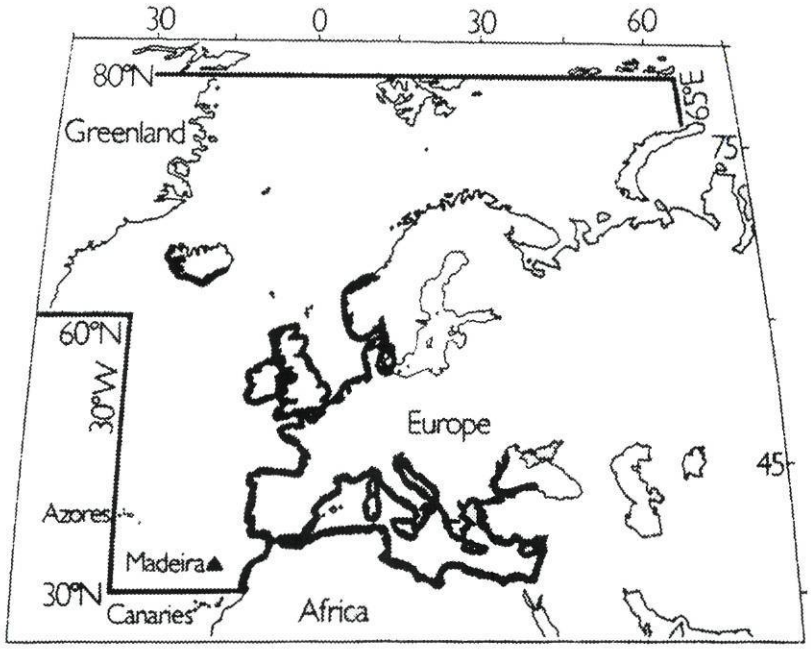

Fig. 1 *Conger conger*. Map of the geographical distribution of the European conger eel (from Bauchot and Saldanha 1986).

Feeding ecology

Little is known about the nutrition of eel leptocephali in general (Smith 1989), and no recognizable food organisms have ever been found in the gut of a premetamorphic leptocephalus (Hulet and Robins 1989). However, the gut content and ultrastructure of midgut

mucosal cells of several leptocephali, including the Japanese conger eel (*C. myriaster)*, suggested that both POM (Particulate Organic Matter) and DOM (Dissolved Organic Matter) are food sources for eel leptocephali during the premetamorphic stage (Otake et al. 1993). During metamorphosis, the relatively large amounts of sulphated glycosaminoglycans (GAG) accumulated during the premetamorphic stage are broken down and used for nutrition (Pfeiler 1986). When metamorphosis is complete, the animal begins feeding normally (Smith 1989).

Concerning the juveniles, congers eels are carnivorous and feed chiefly at night, their diet consisting mainly of bottom-living fishes, crustacea and cephalopods (Lythgoe and Lythgoe 1971; Bagenal and Kenney 1973; Smith 1981; Cau and Manconi 1984; Wheeler 1985; Bauchot and Saldanha 1986; Bauchot 1987; Saldanha et al. 1995). Being nocturnal fishes, olfaction should play an important role in feeding behaviour (Goh et al. 1979). Although no significant seasonal variations were found in the alimentary pattern of *C. conger* in the central-eastern Mediterranean, alimentation is modified according to the depth. The neritic (20-200 m) population fed on a higher number of species that the epimesobathyal individuals (200-800 m), and a high percentage of empty stomachs are found in the bathyal population, suggesting that the neritic females, at the end of the trophic phase, cease to feed and set out on a reproductive migration towards the bathyal level (Cau and Manconi 1984). Studies based on the stomach content composition of *C. myriaster* showed that although conger eels are significantly piscivorous (Matsumiya et al. 1980), their food differs depending on their habitat, season and year (Matsumiya and Imai 1987). Levy et al. (1988) showed that the American conger eel (*C. oceanicus*) within the same size range, collected in the Mid-Atlantic Bight, fed also mainly at night, on the same major prey types (decapod crustaceans, cephalopods and fish), and suggested that the differences observed between the diet of offshore and inshore individuals probably reflects the differences in prey avaibility.

Systematic and identification

Conger eels are primitive teleosts, belonging to the Elopomorpha, Anguilliformes, which includes *Anguilla* (De Pinna, 1996). Fishes of the superorder Elopomorpha share one important feature to the exclusion of all other fishes, the presence of a leptocephalus larva (Hulet and Robins 1989). Congrid leptocephali (family Congridae) vary widely in form and consequently are difficult to characterize. The larvae are mainly identified by the relative position of the dorsal and anal fins, pigmentation, shape of the head and eyes, number of myomeres (total, predorsal and preanal myomeres), position of the posterior end of the kidney

and posterior renal artery, among others characters (Smith 1989). Metamorphosis in fishes with a leptocephalus larva is a profound and non-instantaneous process, but the changes occur in a relatively short time. This process includes morphological, behavioural and physiological changes (Smith 1989; Yamano et al. 1991). Unfortunately, the only character consistently carried through metamorphosis to the adult is the number of vertebrae (Smith 1989). Figure 2 shows a typical anguilliform leptocephalus with some of the principal characters and commonly used counts and measurements.

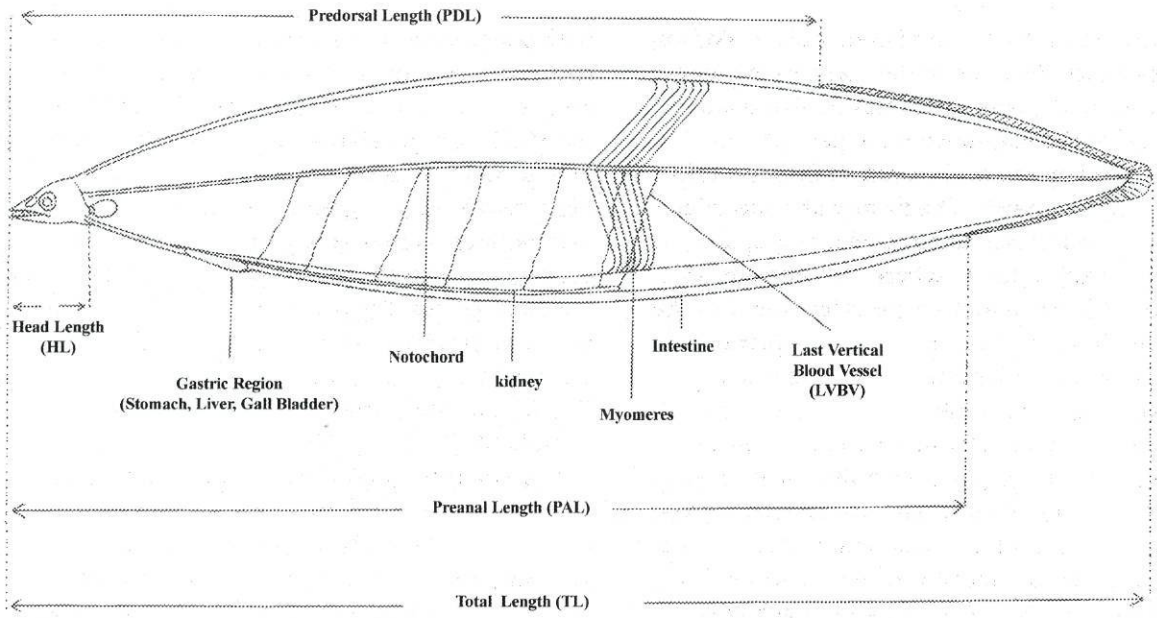

Fig. 2 A leptocephalus larva showing morphological features and principal measurements (modified from Smith 1989).

D'Ancona (1931) described in detail the morphology, dentition and pigmentation pattern of the European conger eel, through the leptocephalus life stage. The summary, which follows, is based on his work. Conger eel eggs are not known. The smallest larva of *C. conger* as been captured in Messina, on August 1912, being 8 mm long and 1 mm in depth. This prelarva, had oval eyes, an upper jaw shorter than lower, both with three teeth on each side, and with some pigmentation along the intestine, behind the anus at the base of the anal fin and also on the caudal fin. Larvae exceeding 50 mm in length already have the appearance of a fully developed leptocephalus (Fig. 3A). The snout is relatively blunt, the upper jaw projects slightly more than the lower, the angle of mandible is sharp, the eye is oval, 2-3 dots are evident around the heart and some pigmentation appears along the lateral line. During metamorphosis the body becomes shorter (reaching a length of 80 mm), lower and thicker, the

head becomes longer and the eyes rounded, losing the irido-chorioid process (Fig. 3B). The dorsal fin shifts forward and the larval punctuation becomes denser. Upon transition to the definitive form, the final pigmentation appears along the spinal cord, extending from the tail forward. Superficial pigment also appears at the caudal apex. The body becomes coloured, the bile yellowish and the swim bladder silvery. The silvery coloration of the belly appears and the definitive pigmentation becomes denser when the specimens reached the juvenile definitive form (Fig. 3C).

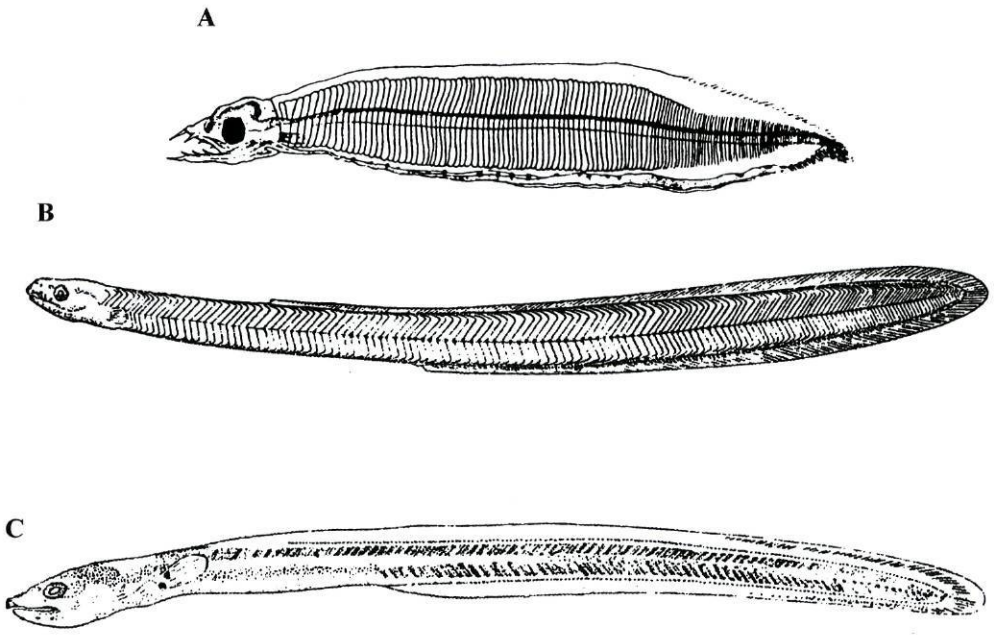

Fig. 3A-C *Conger conger*. **A** premetamorphic leptocephalus; **B** Metamorphic leptocephalus; **C** young conger eel (from D'Ancona 1931).

The juvenile conger eels are scaleless elongate fish with a snake-like body, cylindrical anteriorly and laterally compressed posteriorly (Smith 1981; Bauchot and Saldanha 1986; Bauchot 1987) (Fig. 4A). The dorsal outline of the head is convex with depressions over eyes, which are elliptical with a broad and flat inter-orbital space. Anterior nostril openings on the snout extremity have a short tube directed forward; while the posterior nostrils consist of an oval pore at mideye level. The mouth rictus is at level of the posterior edge of eye; snout slightly prominent; well-developed flange on upper lip (Fig. 4B). The lower jaw is shorter that the upper jaw. In both jaws two rows of teeth: an outer row of close set large incisiform teeth form a cutting edge, and an inner row of small conical and sharp teeth; larger conical teeth on premaxillary plate and vomer (Fig. 4C). Gill openings consist of sublateral, crescentiform long slits. The bilateral line has 44-47 preanal, six prepectoral and one supra-temporal pores.

Vertebrae: total 148-153; abdominal 53-57. The dorsal, caudal and anal fins are joined. Pectoral fins present, but pelvic fins absent. The dorsal fin origin is slightly behind the pectoral fin tip. Dorsal and anal fins have a black margin. The body is more or less dark grey, although pale ventrally, with white marked pores on the lateral line.

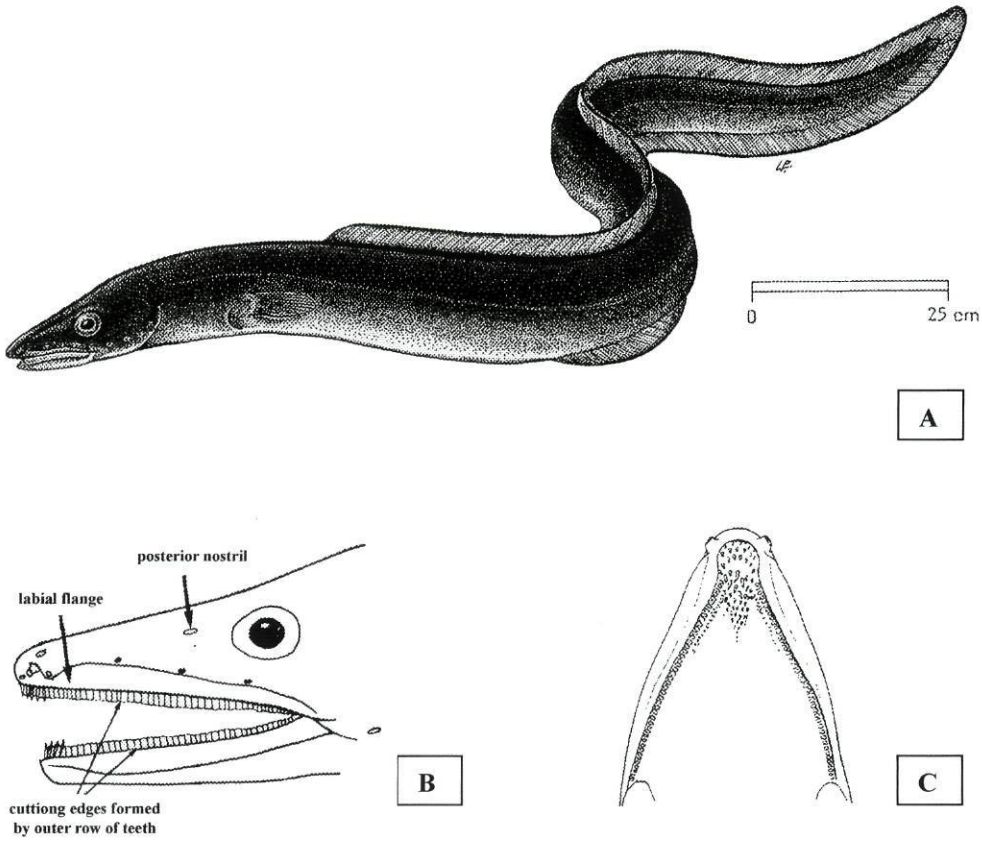

Fig. 4A-C *Conger conger*. **A** whole view of a conger eel; **B** detail of the head; **C** Teeth of the upper jaw (from Bauchot 1987).

Growth and age

According to Bigelow and Schroeder (1953), *C. conger* is the largest of the family Congridae with records existing of specimens of over 2.7 m and weighing 65 kg (Wheeler 1985), although such large fishes are uncommon. *C. oceanicus*, for example, reaches a maximum size of 2 m length and a weight of 8 kg (Bigelow and Schroeder 1953). In the Mediterranean, male conger eels are reported to be smaller than females, with males rarely exceeding 1 m in length and females reaching over 2 m (Cau and Manconi 1983). It has also been reported that the male of *C. myriaster* grows slower that the female, and as a result

males appear more smaller in size (Takai 1959). Females were found in the inshore waters but males were only found at much greater depths (>550 m) in offshore waters in the study of Cau and Manconi (1983). In the case of the American conger eel, *C. oceanicus*, both males and females were captured by longline and trawl in inshore waters (Hood et al. 1988). A recent study on Japanese conger eel found no males in samples from the net pot fisheries at sea water depths from 11 to 145 m off (Katayama et al. 2002). In a sampling survey using pots carried out in Shijiki Bay (Nagasaki Prefecture) from 1980 to 1983, 1741 conger eels (25-84 cm TL) were caught, showing an overwhelming dominance of the females (sex ratio 857/9) (Matsumiya and Imai 1987). The female dominance tends to be more significant when the conger eels become older (Takai 1959; Kubota 1961). Recently, a sexually mature female weighing 54 kg and measuring 2 m in length was trawled off Co. Wexford (southeast coast of Ireland). Von Bertalanffy growth parameters have been estimated for this single conger, indicating $L\infty$=2.65 m, k= 0.0633 and t_o of –0.3861 (Fannon et al. 1990). Both vertebrae and otoliths were successfully used to construct growth curves of conger eels captured between August 1998 and June 1999, from the inshore south coastal waters of Ireland (Sullivan et al. 2003). The estimates of the asymptotic lengths ($L\infty$) of 214 and 271 cm, appear realistic values as conger can grow to such large sizes. These values are similar to those reported by Fannon et al. (1990). Another study (Sbaihi et al. 2001) showed that the post-metamorphic age of conger eels collected in the south coast of Brittany, France, from November 1996 to March 1997, ranged between 2 and 11 years. A linear relationship was also observed between age and length, indicating a body growth increment of 10 cm.year^{-1}, although the grow rate was seen to slow down from 10 years upwards.

Spawning area(s), larval stage duration and migratory routes

Schmidt (1931) caught small conger eel larvae (9-20 mm) in the Sargasso Sea, Mediterranean and North-East Atlantic, suggesting a similar migratory behaviour to the European eel (*Anguilla anguilla* L.), i.e. spawning in the Sargasso Sea, following a larval transoceanic migration to the European and North Africa coasts. This author reported that the conger eel leptocephali collected in the Sargasso Sea belonged to two species, the American conger, *C. oceanicus,* and the European conger, *C. conger*. However, Kanazawa (1958) described a new species *C. triporiceps* as an adult form distinct from other conger species in the North Atlantic, with a range from Bermuda to Brazil. Earlier confusion was caused by a

substantial overlap of vertebral counts and myomeres counts between *C. triporiceps* and *C. conger* (McCleave and Miller 1994). In the Mediterranean Sea, Cau and Manconi (1983) reported, for the first time, the capture of sexually mature males and females *C. conger*, in deep water south-east of Sardinia, and concluded that a spawning ground exists in the Sardinian channel at depths between 600 and, at least, 800 m. This assumption is supported by the length and otolith analyses of leptocephali collected in the North and Central Atlantic Ocean (Strehlow et al. 1998). Another spawning place, in the eastern Atlantic Ocean, such as the area between Gibraltar and the Azores, in mid-water over depths of 3000-4000 m, is frequently suggested for *C. conger* (Lythgoe and Lythgoe 1971; Bagenal and Kenney 1973; Hayward and Ryland 1995). This spawning site probably serves the northern European conger population (Lythgoe and Lythgoe 1971). Even though sexually mature animals have not yet been caught is this area, in 1988, a sexually mature female was trawled off in the southeast coast of Ireland. When the ovaries were examined, individual eggs, measuring 0.57±0.15 mm, were visible to the naked eye, and their total number ranged from 12.4 to 17.3×10^6 (Fannon et al. 1990). In short, and on the basis of present knowledge, it is believed that the European conger does not spawn in the Sargasso Sea, but in the Mediterranean and possibly in the eastern North Atlantic, around the Azores.

It is assumed that the leptocephali take one to two years to drift inshore to the feeding grounds (Lythgoe and Lythgoe 1971; Bagenal and Kenney 1973; Strehlow 1992). A long larval life could explain the large dispersal range of the leptocephali until the beginning of metamorphosis (Strehlow et al. 1992). Antunes (1994) estimated 300 days as the duration time of the conger eel leptocephalus stage, based on the otolith microstructure of premetamorphic larvae (with 100 mm mean length) captured in May 1989. Recently, based on the capture of premetamorphic leptocephali in the eastern and central Atlantic, Strehlow et al. (1998) suggested a scenario for the growth and larval migratory route of the conger eel. After a short growth period, the larvae (> 30 mm) start, around November, a migration in a NW direction toward southern Portugal and Spain, extending to all the East and Central zone of the Atlantic. The conger eel has a second growth period lasting until the beginning of the next summer (reaching 130-150 mm and maximally of 165 mm length), after which they start a migration in the direction of the coastal waters of the continental slope, with a possible return to the Mediterranean. It is thought that this coastal migration induces metamorphosis. However, the location and timing of metamorphosis is unknown.

Reproductive biology

It is believed that when the European conger eel reaches sexual maturity (at 5-15 years old), it goes to spawn terminally in deep waters, in summer, with the females producing 3-8×10^6 eggs (Lythgoe and Lythgoe 1971; Wheeler 1985). The absence of males and of ripe or spent females in the coastal inshore waters, suggests that sexual maturation in conger eels occurs during their migration towards their deep-sea spawning areas (Cau and Manconi 1983, 1984; Sbaihi et al. 2001; Sullivan et al. 2003). Also the Japanese conger eels are generally assumed to spawn at offshore regions during spring and summer seasons (Matsumiya and Imai 1987). A blue-sensitive type rod opsin genes is expressed in the retina and pineal complex of conger eels, with a maximal light absorption of 487 nm, that could adapt their visual sensory system to the deep water light (Archer and Hirano 1996; Zhang et al. 2002).

Recently, Sbaihi et al. (2001) made the first study on the reproductive biology and endocrinology of *C. conger*. Examination of the gonads showed that all *C. conger* caught were females, aged between 2 and 11 years, with gonadosomatic indices (I_G) ranging between 0.04 and 4.78. Two main stages of ovarian development were described: 1) a pre-vitellogenic stage, observed in fish with $I_G<1$ showing small oocytes (diameter 20-70 μm) with a dense cytoplasm and a large nucleus; and 2) an early vitellogenic stage, observed in fish with $I_G>1$, showing enlarged oocytes (70-125 μm) containing lipid vesicles in the cytoplasm. In this latter stage, there was also a large deposition of adipose tissue surrounding the oocytes in the conger eel. The early vitellogenic stage was reached at TL>120 cm and at age≥5 years, with large individual variations. A very similar range in I_G was found for females of *C. oceanicus* collected in the Mid-Atlantic Bight (Hood et al. 1988). These authors also found pre-vitellogenic oocytes, with 20-80 μm of diameter, in females with low I_G, and vitellogenic oocytes, with a diameter superior to 80 μm, for $I_G>1$. The pre-vitellogenic stage in conger is equivalent to the pre-vitellogenic stage observed in sexually differentiated females *A. anguilla*, at the yellow stage ($I_G<1$) (Colombo et al. 1984). The early vitellogenic stage, characterized by lipid inclusions in the cytoplasm, is equivalent to the oil droplet stage observed at the silver stage in *A. anguilla* (Colombo and Grandi 1996) and in *A. japonica* (Ohta et al. 1997). However, even in female *C. conger* having the highest I_G value (4.78) yolk globules were not observed (Sbaihi et al. 2001).

Radioimmunoassays of sex steroids revealed low serum levels of estradiol and of 11-ketotestosterone, but higher levels of testosterone correlated with an increase in I_G. An immunoenzymatic assay indicated low serum levels of vitellogenin (VtG), which were

significant correlated with I_G (Sbaihi et al. 2001). The range of circulating VtG levels in these conger eels (up to 250 $ng.ml^{-1}$ for the highest I_G) was similar to that found in the majority of female European eels at the silver stage (Burzawa-Gérard and Dumas-Vidal 1991). However, presently, significant amounts of circulating estradiol and estrone in male of *C. conger* have only been reported by Cedard and Nomura (1961).

The pre-vitellogenic stages and early vitellogenic stages observed in these coastal conger eels were similar to the oocyte stages found in the European eel, *A. anguilla*, at the yellow and silver phases of its life cycle, respectively. However, other morpho-functional changes associated with silvering in *A. anguilla* (Fontaine 1994), such as the increase in ocular index (Io) and regression of the digestive tract, did not occur at the early vitellogenic stage in conger eels (Sbaihi et al. 2001). Nevertheless, a few observations suggest that some of these morphophysiological changes, such as the increase in Io, regression of the digestive tract, calcium teeth and bone resorption, body coloration and migratory behaviour, may also be observed in conger eels at advanced stages of sexual maturation (Lythgoe and Lythgoe 1971; Cau and Manconi 1983, 1984; Hood et al. 1988).

These data indicated that in *C. conger*, the increase in I_G values between 1 and 5, is related to the development of numerous adipose cells surrounding the oocytes, and do not correspond to a very advanced stage of vitellogenesis, as observed in European eels at the silver stage (I_G 1-2) (Sbaihi et al. 2001). The transition from the pre-vitellogenic stage to the early vitellogenic stage, as observed in conger eel with $I_G>1$, corresponds to the early stage of sexual maturation, also defined as the initiation of puberty in female teleosts (Goos and Schulz 1997; Dufour et al. 2000). Conger eels may reach advanced stages of sexual maturation under aquarium conditions (Hermes 1879; Buckland 1891; Tuzet and Harant 1933). Studies on female *C. conger* undergoing sexual maturation in captivity indicate that they attain a much higher I_G (15.6; Tuzet and Harant 1933).

Recently a study on age, growth and reproductive status of the European conger eel was made in the Irish coastal waters (Sullivan et al. 2003). All the conger eels examined in this work were females, either immature or developing, and no males or sexually mature specimens were collected. The ovaries of immature fish were transparent or pink ribbon-like structures. Maturing ovaries were larger in size, white in colour and possesed a frill on the distal surface with large amounts of adipose tissue. All ovaries were smooth with no granular texture. Ovaries contained oocytes in various stages of development from pre-vitellogenic oocytes to vitellogenic oocytes. Maximum oocyte diameter (MOD) ranged from 180 to 450 μm and the largest oocytes were still undergoing vitellogenesis. The smaller specimens

contained pre-vitellogenic oocytes and the larger had vitellogenic oocytes with yolk vesicles present. Oocytes in the cortical alveoli stage were observed in specimens of intermediate stage. No fully hydrated oocytes or postovulatory follicles were observed, and there was no evidence of oocyte atresia. These results are similar of the observations made by Sbaihi et al. (2001). In these species, the condition factor (K) was highest in autumn and lowest in winter. The low winter K value may result from lower food availability and the mobilization of somatic energy reserves for reproductive development. I_G was at its lowest in autumn and increased and was high in winter, when the ovaries were at their largest size (Sullivan et al. 1993). Similar findings were observed with *C. oceanicus*, where I_G was also highest in late winter and spring and lowest in autumn (Hood et al. 1988). Sbaihi et al. (2001) obtained a K value of 0.23±0.01 and there was no significant variation with mass or body length.

Fisheries

Conger eels are an important commercial and recreational fishing species of the central and eastern Atlantic, and are caught primarily with bottom-trawl, hook and line gear (Figueiredo et al. 1996). The world total catch reported for this species to FAO for 2001 was 14 238 metric tonnes (MT) (Fig. 5). The countries with the largest catches were France (5 225 MT) and Spain (3 311 MT). In Portugal this species has a significant commercial value, and the catch in 2001 was 1 896 MT. In Portugal, the conger eel is sometimes captured as by-catch of the crustacean trawl fishery, and is marketed mainly fresh or frozen (Moura 1995). Although there is a complete lack of fisheries management studies for the European conger eel, data collected by Sullivan et al. (2003) suggests that the younger fish are not over exploited by commercial longline and trawl fisheries.

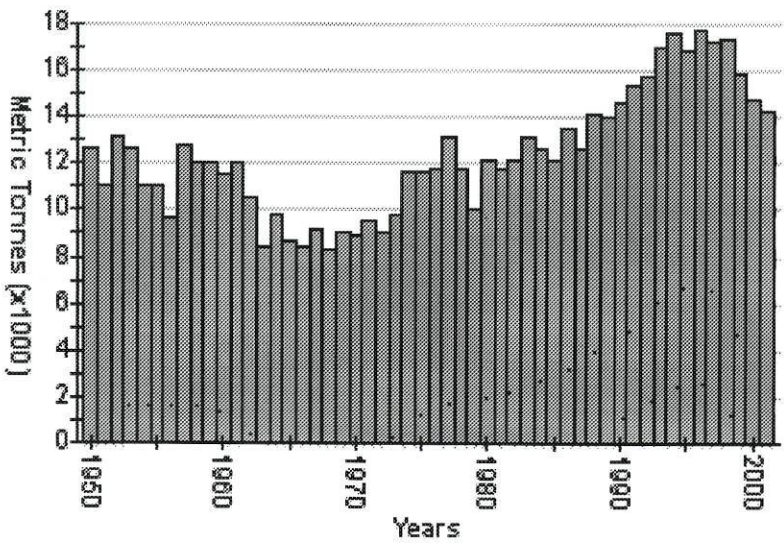

Fig. 5 *Conger conger*. World total catch reported for this species to FAO between 1950 and 2001.

1.2 Otoliths as a tool for studying fishes

Otoliths, what are they?

Fishes have a variety of different sensory receptors that enable them to glean a good deal of information about their environment (Atema et al. 1988). Otoliths are the inner ear bones of fishes used to detect sound and to aid in balance and orientation in the water column (Popper and Fay 1993). The other mechano-receptive system of fishes, the lateral line, is mainly involved with the detection of hydrodynamic signals and changes in water pressure as the fish swims (Montgomery et al. 1995).

The inner ear in fishes is typical of that of other vertebrates, having three semicircular canals and three otolithic organs (Fig. 6A). The semicircular canals and associated ampullae detect angular accelerations, whereas the otolithic organs appear to have a dual function, vestibular and auditory (Popper and Lu 20000). In the labyrinth of teleosts there are generally three otoliths (sagitta, lapillus and asteriscus), each having an irregular unsymmetrical shape, which is characteristic of the species. They are located in the otolithic organs, respectively in the sacculus, utriculus and lagena (Fig. 6B) (Carlström 1963). From the three pairs of otoliths, the sagittae are usually the largest and most common otoliths used for microstructure studies, however the two other pairs, lapilli and asterisci, are also useful for studying species of fish where the sagittae are small and relatively delicate (Campana and Neilson 1985).

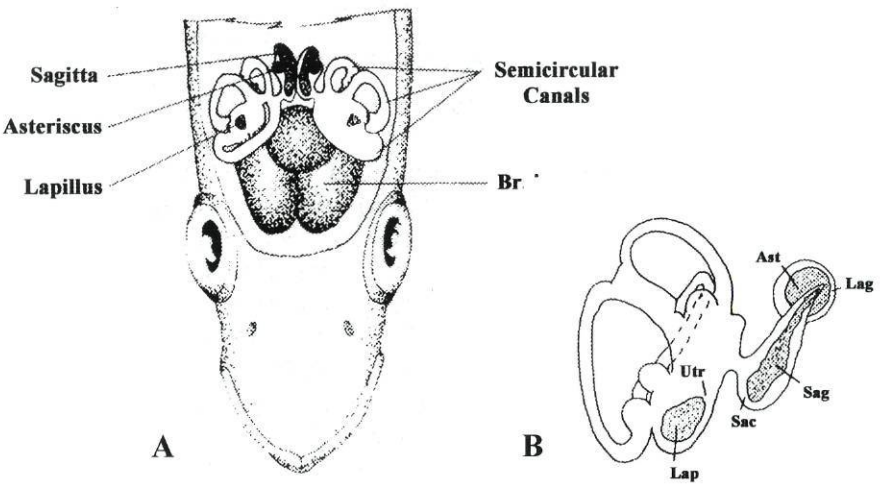

Fig. 6A, B Anatomy of the vestibular apparatus in a typical teleost fish. **A** Dorsal view (top of head is cut away). **B** Otoliths within the right side labyrinth system. Legends: *Ast* asteriscus; *Lag* lagena; *Lap* lapillus; *Utr* utriculus; *Sag* sagitta; *Sac* sacculus (modified from Stevenson and Campana 1992).

Otoliths are calcium carbonate concretions, mainly composed of polycrystalline aragonite (Carlström 1963) that contains a protein organic matrix, characterized by a high abundance of acidic amino acids (Degens et al. 1969). About half of the otolith proteins, named otolin, are water-insoluble (Asano and Mugiya 1993). Other crystal forms, such as calcite and vaterite occur, although less frequently, as partial or total replacements of aragonite in some otoliths (Gauldie 1999).

Based on the ability of the otoliths to record the life history of the fish, they are extensively used in several types of study of fish biology and fisheries science, namely for ageing fishes (Secor et al. 1995a). Tree xylem rings are the archetypal ageing structures and have been used not only to determine age and height-growth rates, but also to document interannual growth variations in terrestrial systems and to develop biochronologies extending over thousands of years (Schweingruber 1989). Others living organisms exist, however, that presents external or internal structures available for age purposes. Growth bands in calcified structures corresponding to annual, seasonal or daily patterns are common in a number of aquatic phyla, and include coral skeletons, bivalve shells, mammal teeth, as well as fish otoliths (Campana and Thorrold 2001). In fishes, interpretation of age and growth from any bony structure (scales, opercular bones, fin rays, vertebrae and otoliths) is based on the assumptions that periodic features are formed at a constant frequency, and that the width of these features is proportional to fish growth (Casselman 1987). As both fish and tree chronologies are assemblages of unitless measures of relative growth, these data could offer, for example, a rare opportunity for the comparisons of annual growth variations, between diverse life forms, from neighbouring aquatic and terrestrial ecosystems (LeBreton and Beamish 2000).

Fish ageing has an important role in fish stock assessment and fisheries biology. Age determinations in fish can occur in one of two scales. Reibisch (1899) was the first to observe and use the translucent and opaque rings to determine the age of fish. Since Reibisch´s observations of the annular ring formation otolith (annuli) in *Pleuronectes platessa*, there has been a growing interest in the use of the otolith as an indicator of annual age. Annual ageing is often used in support of harvest calculations and populations studies, and can be based on any bony structure in the fish, although scales and otoliths are the structures most frequently used (Campana and Thorrold 2001). In contrast, daily ageing based on the otolith microstructure tends to be targeted more at recruitment questions and studies of young fish (Campana and Neilson 1985). Although the use of otolith to age fish is one of the most common and spreading uses of these crystalline stones, today the applications of the otoliths go far beyond the simplest classical age calculations (Campana et al. 2000).

Daily growth increments in calcified structures are restricted to species in which the depositional environment of the structure can be controlled by the organism without subsequent resorption (Campana and Thorrold 2001), and includes bivalve shells (Richardson 1988), squid statoliths (Jackson 1990) and fish otoliths (Campana and Neilson 1985). Through diel variations in calcium and protein deposition, bipartite structures, consisting of an incremental and discontinuous zone (Mugiya et al. 1981), known as daily growth increments often form at the microstructural level of the otolith (Pannella 1971). The discontinuous matrix consists primarily of a proteinaceous matrix, while the incremental zone has a higher content of calcium (Dunkelberger et al. 1980; Mugiya et al. 1981; Gauldie and Xhie 1995). Daily growth increments in otoliths of teleost fish are now known to be a widespread phenomenon, present in taxa in both freshwater and marine habitats, and in species distributed from the polar regions to the tropics (Campana and Neilson 1985). Individual otoliths can be analysed to provide a daily record of age and growth rate with high accuracy and precision (Campana 1984). The otolith daily growth increments result from an endogenous circadian rhythm of increment formation, entrained by photoperiod (Campana and Neilson 1985), but susceptible to modification by other cyclic environmental variables, like the temperature and feeding regime (Campana and Neilson 1982; Neilson and Geen 1982; Alhossaini and Pitcher 1988).

Microstructural examination of otoliths is a three-stage process consisting of otolith mounting, preparation and observation. The application of specific techniques varies with the age and size of otolith, increment width and clarity, degree of resolution required and available equipment (Secor et al. 1991; Morales-Nin 1992, Stevenson and Campana 1992). The advantages and disadvantages of the various otolith examination methodologies (namely the use of a light microscope - LM or scanning electron microscope - SEM) are linked to the size, morphology and features of the otolith to be examined (Campana and Neilson 1985). The increments forming at irregular, non-daily intervals, reported for example in some studies could be caused by limitations in the resolution of the LM (Feet et al. 2002). With SEM, resolution exceeds that needed for observation of the narrowest increments known and visual artifacts usually do not occur. In addition, counts and measurements are usually made from photographs, which generally reduces observer error. Restrictions on SEM use include the need for expensive specialized equipment and more involved sample preparation (Campana and Nielsen 1985). The rate of formation of otolith growth increments theoretically permits

age determination with high precision, often to the daily level. Although the apparent rate of increment formation may vary, thus causing difficulty in the interpretation of age from otolith microstructure, only a few studies have provided validation of the frequency of formation of increments (Beamish and McFarlane 1983). Indeed, the results of many studies carry the explicit assumption that the otoliths increments were formed daily (Campana and Neilson 1985). Despite the differences in time scale, application and mode of formation, both annual and daily age date are governed by similar rules of analysis and are susceptible to similar sources of error. Age determination is invariably accompanied by various sources of error, some of which can have a serious effect on age-structure calculations. Campana (2001) has recently reviewed the best available methods for insuring ageing accuracy and precision, whether in support of a large-scale ageing program or a small-scale research project, through the use of quality control monitoring in age determination.

Otolith formation starts with the primordium, which is generally the first calcified tissue in the embryo (Morales-Nin 2000). The nucleus is formed when the first discontinuous unit is laid down (Dunkelberger et al. 1980). The age at which the first increment is produced is normally assumed to be at the onset of an event of biological significance in the fish´s life (Campana et Neilson 1985), such as the hatch, first feeding or start of activity (Taubert and Coble 1977; Brothers and McFarland 1981; Moksness 1992), although some species with long embryonic periods may start forming increments before hatching (Morales-Nin 2000). The periodicity of the increment formation is under an endogenous circadian rhythm entrained by photoperiod, although other environmental factors such as, temperature fluctuations and feeding frequency could have the potential to influence otolith deposition (Campana and Neilson 1985). Environmental conditions affect the otolith growth rate (increment width) but increment periodicity may be disrupted in extreme cases of physiological stress (Morales-Nin 2000). In fact, some works showed that the daily rhythm of the increment formation continued in fish held under constant light (Campana 1984), darkness (Radtke and Dean 1982) or in absence of cyclical variations in other major environmental factors (Wright et al. 1992), although the effects of environmental conditions on increment formation vary among species (Jones 1986). On the other hand, once deposited, the calcium carbonate of the otolith is reabsorbed only in extreme stress (Mugiya and Uchimura 1989).

Aside from the importance of counting daily increments for age and growth rate calculations, otolith microstructure examination may be also used to determine life history transitions, ambient temperature, trophic status, stock identification and taxonomic identification (Campana and Neilson 1985). Checks, or discontinuities (i.e. growth

interruptions), which are characteristic of most otolith growth sequences, may record periods of perturbation or stress to the fish (Campana and Neilson 1985). Non-periodic check formation is generally associated with periods of stress or sexual maturity (Campana 1983), while periodic series may be linked to the lunar cycle (Pannella 1980). However, little is known of the physiological processes responsible for check formation despite substantial variance in their visual prominence. They may be an anomalous incorporation of the otolith components (Campana and Neilson 1985) or regions of otolith resorption (Pannella 1980). The transition of width and appearance of the daily increments could indicate important physiological and behavioural events, such as metamorphosis and/or settlement (Lee and Byun 1996; Wilson and McCormick 1997). Other microstructural features, like the accessory growth centres are frequent in species that undergo a marked habitat change at the transition from the larval to juvenile stage (Secor et al. 1995a), as for example in the Dover sole *Microstomus pacificus* (Toole et al. 1993), the sandeel *Ammodytes marinus* (Wright 1993) and the European hake *Merluccius merluccius* (Morales-Nin and Aldebert 1997).

Otolith microchemistry (Sr:Ca ratios) as a tool for study environmental and physiological changes

The current interest in the chemistry of otoliths is driven by the environmental chronological capabilities of these structures rather that any unique chemical properties. Nonetheless, otoliths do have several properties (e.g. otolith growth is continuous; otolith growth occurs in an isolated compartment from the environment; the otolith is generally not subject to resorption and the crystalline structure of the otolith is aragonite) that set them apart from all other skeletal structures and without which many of the current applications would be impossible (Campana and Thorrold 2001). The same two properties of the otolith that allow it to form and retain daily growth increments (i.e. the otolith is metabolically inert, therefore newly deposited material is neither resorbed nor reworked after deposition; and the otolith grows throughout the lifetime of fish) are also responsible for its ability to record aspects of the environment to which the fish is exposed (Campana et al. 1997). The fact that the otolith is acellular and metabolically inert means that any elements or compounds accreted onto its growing surface are permanently retained, whereas the continued growth of the otolith from before the time of hatch to the time of death implies that the entire lifetime of the fish has been recorded (Campana and Neilson 1985). The otolith may contain a complete record of exposure to both the temperature and composition of the ambient water, which coupled with

age or date-structured examination of otolith growth increments (either annual or daily) allows a detailed chronological record of the environment to which the fish was exposed (Campana 1999).

Biomineralization of otoliths differs somewhat from that of vertebrate bone, molluscan shell and coral skeleton in that the otolith epithelium is not in direct contact with the region of calcification. As a result, the calcification process is heavily dependent upon the composition of the endolymphatic fluid surrounding the otolith, and to a certain extent, can be described on the basis of purely physical principles (Campana 1999). The key physical regulating factor appears to be the pH of the endolymph, which is determined by the concentration of bicarbonate ions in this fluid (Payan et al. 1997). However, a solely inorganic process cannot account for many of the features of biomineralization, and the otolith protein matrix, namely the recently characterized water soluble proteins (calcium-binding glycoproteins), should plays a pivotal role in otolith calcification (Asano and Mugiya 1993), perhaps through inhibition of crystal nucleation (Wright 1991). Although several authors have analysed the otolith constituents (Carlström 1963; Degens et al. 1969; Dunkelberger et al. 1980; Morales-Nin 1986; Gauldie and Xhie 1995), the complex physiology of otolith growth and formation has been less studied. Romanek and Gauldie (1996) recently proposed an otolith growth model based on the chemistry of the endolymph, where pH and temperature are the main factors controlling the fishes otolith growth.

The basic pathway of the bulk of inorganic elements into the otoliths is from the water into the plasma via gills or intestine, then into the endolymph and finally into the crystallizing otolith (Fig. 7) (Campana 1999). The primary source of inorganic elements into the otoliths is from the water, via branchial uptake (freshwater fishes) or intestine assimilation (seawater fishes) (Olsson et al. 1998), although a small proportion could be assimilated from food sources (Limburg 1995; Farrel and Campana 1996; Gallahar and Kingsford 1996). Salinity, pH, dissolved oxygen concentration and other factors can also influence elemental uptake into the fish (Mayer et al. 1994). While the factors influencing endolymph composition remain poorly understood (Campana 1999), the transfer of calcium and other ions into the endolymph may occur via a transcelullar route, thus ensuring significant regulation of both the selection of elements and their concentrations in the endolymph (Mugiya and Yoshida 1995). A final stage of the elemental pathway from environment to otolith occurs during the otolith crystallization process (Campana 1999).

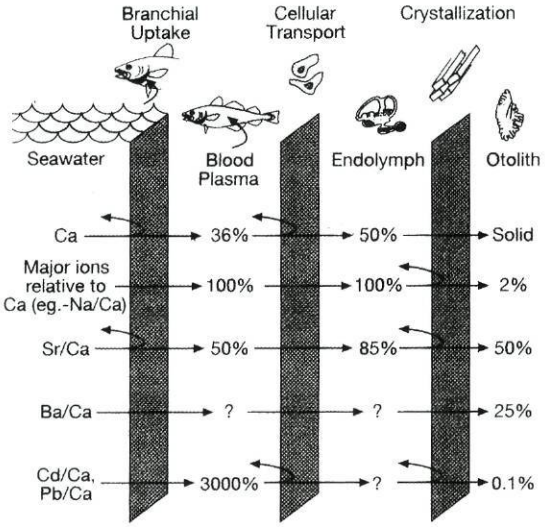

Fig. 7 Overview of elemental pathways and barriers between seawater and the otolith, with coarse estimates of transfer rates for selected elements at each physiological barrier. Elemental discrimination is greatest for major and physiologically regulated ions and least for trace elements, but the site of maximum discrimination is often unpredictable (from Campana 1999).

The potential application of trace element concentrations as paleoindicators of oceanographic conditions and for reconstructing the temperature and salinity exposure history of individual organisms, as mainly focused of the Sr:Ca ratios (Campana 1999). Calcium (Ca) can be partially substituted for strontium (Sr) during deposition in otolith, because Sr has the same valence as Ca, as well as a similar ionic radius (Amiel et al. 1973). Changes in otolith Sr:Ca ratios have been considered related to environmental factors such as water temperature (Radtke 1989; Townsend et al. 1992, 1995) and salinity (Secor 1992; Secor et al. 1995b; Radtke et al. 1996). It appears, for example that most of the discrimination against Sr occurs during otolith crystallization, rather that during Sr uptake into the plasma from water (Kalish 1989).

The underlying basis of the Sr:Ca temperature dependence is presumed to be kinetic effect, since the rate of Sr:Ca incorporation into inorganic aragonite varies inversely with temperature (Kinsman and Holland 1969). Examples of other otolith elements influenced by temperature include Mg, K, Na, Mn, Zn and Fe (Arai et al. 1995; Hoff and Fuiman 1995). However, particular attention has been focused on Sr:Ca, in which positive (Fowler et al. 1995; Arai et al. 1996; Limburg 1996), negative (Townsend et al. 1989; Radtke et al. 1990; Sadovy and Severin 1992) and non-existent (Gallahar and Kingsford 1996; Tzeng 1996) correlations with temperature have been reported. It appears that variations in the rate of otolith matrix formation are at least partially responsible for the observed range of Sr:Ca ratios across species within a given salinity regime, with temperature and/or growth being occasional, and often frequent, correlates (Campana 1999).

The concentrations of many of the most common elements (Ca, Na, K, Mg, Cl) differ substantially between fresh water and salt water, although those differences do not appear to be reflected in the plasma and in the otolith. The molar ratio of Sr to Ca in fresh water

(1.8×10^{-3}) is 4.8 times less that of salt water (8.6×10^{-3}), for example. However, the relative concentration of some trace elements, whose uptake is more likely to be unregulated, compared to those of the common salts, such as Sr, Zn, Pb, Mn, Ba and Fe in freshwater and marine otoliths is consistent with an environmental effect. These findings suggest that the elements under strict physiological regulation are unsuitable for use as environmental indicator (Campana 1999). WDS analysis of Sr to Ca ratios in otoliths has recently been developed as a method for distinguishing between freshwater and marine migratory phases in several diadromous fishes, such as brown trout, *Salmo trutta*, Atlantic salmon, *Salmo salar*, rainbow trout, *Oncorhynchus mykiss* (Kalish 1990), striped bass, *Morone saxatillis* (Secor 1992), sockeye salmon, *Oncorhynchus nerka* (Rieman et al. 1994), and masu salmon, *Oncorhynchus masou* (Arai and Tsukamoto 1998). Many reports have also shown relationships between otolith characteristics, such as growth pattern and Sr:Ca ratios and the timing of metamorphosis in *Anguilla* sp (Otake et al. 1994; Cheng and Tzeng 1996; Arai et al. 1997). In the conger eel *C. myriaster* leptocephali, it has been reported that otolith increment widths increased at the beginning of metamorphosis (Lee and Byun 1996), which coincided with dramatic decreases in otolith Sr:Ca ratios (Otake et al. 1997). The increase in otolith Sr:Ca ratios during the leptocephalus stage is the result of some endogenous factors rather than environmental factors (Arai et al. 1997). Variations in otolith Sr:Ca ratios in the leptocephali possibly reflected the synthesis and accumulation of body glycosaminoglycan (GAG) during its ontogeny. Further, Otake et al. (1994, 1997) ascertained that drastic changes in otolith Sr:Ca ratios in metamorphosing leptocephali were associated with decreasing body Sr content, caused by catabolism of GAG in the body during metamorphosis.

There are others examples of successful reconstructions of the fish's life history using otolith chemistry, ranging from temperature and salinity history (Iacumin et al. 1992; Patterson et al. 1993; Tzeng 1994), detection of anadromy (Secor 1992; Limburg 1996; Tzeng et al. 1997), determination of migration pathways (Thresher et al. 1994; Proctor et al. 1995; Thorrold et al. 1997), age determination and validation (Kalish 1993; Milton et al. 1995; Campana and Jones 1998), stock identification (Edmonds et al. 1989, 1991, 1992), use as a natural tag (Campana et al. 1995; Gillanders and Kingsford 1996; Kennedy et al. 1997) or physiological indicator (Kalish 1992; Gauldie 1996; Schwarcz et al. 1998), and for a rapid inexpensive mass marking (Tsukamoto 1985; Ennevor and Beames 1993; Brown and Harris 1995). A comprehensive review of this field and the main applications involving otolith composition and chemistry has been addressed by Campana (1999).

1.3 Framework, strategies and objectives

The starting point of this work was a three year national project, started in October 1997 and supported by the Portuguese Foundation for Science and Technology, concerning the study of the life cycle of two anguilliform species of the north-eastern Atlantic Ocean, the European eel (*Anguilla anguilla*) and the European conger eel (*Conger conger*). Even though conger eels are strictly marine fishes, while eel species may colonize fresh water during their growth phase, many similarities are found between *Conger* and *Anguilla* in their reproductive life cycles, e.g. spawning in the deep-sea, a leptocephalus oceanic larval stage, long juvenile growth phase before sexual maturation and extensive oceanic migrations between conclusion of growth and spawning. The significant economic importance of the conger eel and the lack of scientific knowledge about this species, namely concerning its early life stage (only a few papers published), led us to decide to study this species.

The existence of some scientific background of the early life history of *A. anguilla* was decisive in choosing the use of otoliths microstructure to study the early life stage of the conger eel. The existence of some logistical material (a small fishing boat, glass eel nets, and the help of fishermen) make the Minho River, in northern Portugal, the main site for catching metamorphosing conger eel leptocephali. During three years (from 1999 to 2001) an intensive fishing effort (one to two times of fishing per month) has been made, although the majority the samples collected were obtained by local fishermen during the official glass eel fishery (November to April). The participation on two research cruises in the central group of the Azores Archipelago, during October 1999 and June 2000, allowed us to catch some premetamorphic leptocephali. Unfortunately, the Azores summer cruise was interrupted due to a serious problem with the fishing net.

The examination of the otolith microstructure and microchemistry of the conger eel leptocephali, jointly with the biometric information was used to elucidate some aspects of the early life history of this species, such as the spawning area(s) and season, duration of the leptocephalus phase, age, growth, larval migratory routes and coastal recruitment mechanism. We also started some preliminary work on conger eel genetics, regarded as the first step to elucidate the structure of the Atlantic conger eel population, which makes the connection with one of the proposed lines for future conger eel research. The resulting improvement of the state of the art of conger eel biology could be important in the future in providing new insights for fisheries management purposes, like as the preservation of the reproductive stages and migratory routes.

1.4 Structure of the thesis

This PhD thesis is organized into seven chapters. In chapter one, we present the state of the art, i.e. the accumulated knowledge of the European conger eel biology and some introductory scientific background about the use of otolith microstructure and microchemistry (Sr:Ca ratios) - the main strategy tool used - in studying the early life history of fishes. The main objectives of this work and the thesis outline are also included. The middle chapters (2 to 5) are composed of journal articles already published (4), submitted (2) and in preparation (1), in which I am the principal author (excluding chapter 4.2) and represent the main body of this doctoral thesis. Since in these sections each sub-chapter represents a paper, they include an introduction, material and methods, results, discussion, acknowledgments and references. Although, this thesis structure has the disadvantage of some sub-chapters (scientific journal articles) having common material in the "Introduction", "Materials and Methods" and "References" sections, the degree of experimental detail, the number and quality of the figures and tables, and the data present in each paper is enough for the reader to understand the main conclusions of the work. Consequently, from my point of view, the classic format of individual sections like the "Materials and Methods" and "Results" are not justified as independent thesis chapters and were not included. In the main body of the thesis, the journal articles have been grouped based on the main subject addressed in each paper, although some subjects will inevitably overlap. Chapter two basically concerns the age, growth, leptocephalus duration stage, recruitment mechanisms and routes of the conger eel larvae migration. Chapter three focuses on the otolith microstructure and Sr:Ca ratios of the conger eel leptocephali, suggesting some ecological, behavioural and physiological hypotheses to explain the observed otolith morphology pattern. In chapter four, I analysed the otolith microstructure and microchemistry, of two closely related anguilliform species, in an attempt to identify some similarities with the European conger eel. Finally, chapter five presents some preliminary work on conger eel population genetics, in an attempt to provide further knowledge in order to better understand the spawning ground(s), ecology and migration of conger eel leptocephali in the ocean. Chapter six presents the final discussion and conclusions of the thesis, including some new questions left open and giving suggestions for future work on conger eel biology. Finally the references cited in chapters one and seven are presented.

2. AGE, GROWTH, LARVAL DURATION AND MIGRATORY PATTERN OF THE CONGER EEL

2.1 Aspects of the early life history of the European conger eel (*Conger conger*) inferred from the otolith microstructure of metamorphic larvae

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Abstract

The life cycle of the conger eel (*Conger conger* L.) is still fairly unknown, especially its larval leptocephalus phase. The morphology, biometry and meristic characteristics of 184 metamorphosing conger eel larvae collected from the Minho River, northern Portugal, between December 1998 and April 1999 were analysed. The total number of myomeres, the general body morphology and the pigmentation pattern of leptocephali are in agreement with the corresponding data found by other authors for this species. The sagittae microstructure of 90 specimens was viewed by scanning electron microscopy. The numbers of daily increments obtained in the otolith's countable zone were between 205 and 324. The final part of the countable zone is characterised by a sharp increase in width of the daily increments (transition zone), followed by a peripheral diffuse zone. In the diffuse zone no narrow circumscribed rings are visible, which prevents an accurate estimate of the duration of metamorphosis. The data indicates that the size variation of metamorphosing leptocephali is large, suggesting that their hatching time must be variable. Our data also show that the largest larvae, in later stages of metamorphosis, arrive first to the northern Portuguese coastal waters.

Introduction

The European conger eel (*Conger conger* L.) is a marine benthic fish, commonly found along sandy and rocky shores of the Atlantic coast of Europe from Norway to the Mediterranean, and extending into the western Black Sea (Bauchot and Saldanha 1986). In spite of its relatively wide distribution, its life cycle is poorly known, especially during its leptocephalus phase.

Schmidt (1931) caught small conger eel larvae in the Sargasso Sea, Mediterranean and North-East Atlantic, suggesting a similar migratory behaviour to the European eel (*Anguilla anguilla* L.), i.e. spawning in the Sargasso Sea, following a larval transoceanic migration to the European coast. However, the larvae caught in the Sargasso Sea and wrongly identified as *C. conger*, were indeed *C. triporiceps* (McCleave and Miller 1994). Nowadays the larvae of both species are distinguished by the catch location, being separated by a line that goes through the Canary Islands and the western zone of the Azores Islands, in a NW direction. The European conger eel leptocephali are restricted to the central and eastern zones of the Atlantic Ocean (Strehlow et al. 1998).

Different spawning places have been suggested for *C. conger*. Lythgoe and Lythgoe (1971), Bagenal and Kenney (1973) and Wheeler (1985) found that conger eel spawn only once at great depths (3000-4000 m), during the summer, in the North-East Atlantic between Gibraltar and the Azores. There are also spawning areas in the Mediterranean (Wheeler 1985). However, until now, the only spawning area well known for this species is in the central-east basin of the Mediterranean (Cau and Manconi 1983).

It has been suggested that the leptocephali has a long larval life (Bauchot and Saldanha 1986), taking about 1 or 2 years to drift inshore and to reach the juvenile elver form (Lythgoe and Lythgoe 1971; Wheeler 1985; Strehlow 1992). Recent studies, based on morphometric and sagitta analysis of premetamorphic larvae, showed that the spawning occurs in the Mediterranean Sea, between July and September. After a short growth period, the larvae (> 30 mm) start migration around November, in a NW direction toward southern Portugal and Spain, extending throughout the eastern and central zones of the Atlantic. The conger eel has a second growth period, lasting until the beginning of the next summer (normally reaching 130-150 mm, with a maximum of 165 mm length), after which they start migration in the direction of the coastal waters of the continental slope, with a possible return to the Mediterranean. It is supposed that this coastal migration induces metamorphosis (Strehlow et al. 1998). However, the location and timing of metamorphosis is unknown.

The microstructure of the otoliths provides us with valuable information about the early life cycle of fishes. Strehlow et al. (1998) obtained a mean value of 280 days by analysis of the sagittae of premetamorphic leptocephali (between 80 and 120 mm long), using the clear daily increments from the nucleus to the otolith margin (Antunes 1994; Antunes and Tesch 1997). However, until now the sagitta microstructure of metamorphic larvae has not been described.

The present paper examines the relationships between the biometry/meristic data and the sagitta microstructural growth in *C. conger* leptocephali during metamorphosis, in an attempt to elucidate some aspects of its larval life history.

Materials and methods

The conger eel larvae (*Conger conger* L.) were obtained as by-catch from the glass eel fishery at the mouth of the Minho River in northern Portugal (Fig. 1), monthly between December 1998 and April 1999. Fishing, using a stow net, took place in the estuarine area during the night, in the period of the new moon during the flood-tide current (Antunes 1994).

After collection, the leptocephali (n = 184) were preserved in commercial ethanol (95%) and the general body morphology, pigmentation, morphometric and meristic characters (number of myomeres) were analysed according to the methodology described by Smith (1989). Because the preservation method induces shrinkage of the body (approximately 7.5 %), the length measurements were corrected.

Both sagittae were removed from 90 specimens (randomly selected), cleaned, mounted on cylindrical stubs, and polished with 2400 silicon carbide abrasive paper and aluminium paste until the core was revealed. After that, they were etched for 8 s with a 0.5 % solution of HCl, sputter-coated with gold under vacuum and viewed with scanning electron microscope (SEM; Jeol JSM 630-1F) at 15 kV.

Following SEM analysis, core diameter (C), maximum otolith diameter (D), maximum otolith radius (R), maximum width of the countable incremental zone (CZW) and maximum width of the diffuse zone (DZW) were measured from the SEM photographs (Fig. 2).

We considered that the growth increment in the larval otolith of conger to be daily, although daily deposition has not been validated in this species. We base this assumption on the results of several related species, e.g. *Conger myriaster* (Mochioka et al. 1989), *Anguilla japonica* (Umezawa et al. 1989; Umezawa and Tsukamoto 1991) and *A. rostrata* (Martin 1995), which have been shown to have daily depositions.

Fig. 1 *Conger conger*. Sampling location of the metamorphosing conger eel leptocephali.

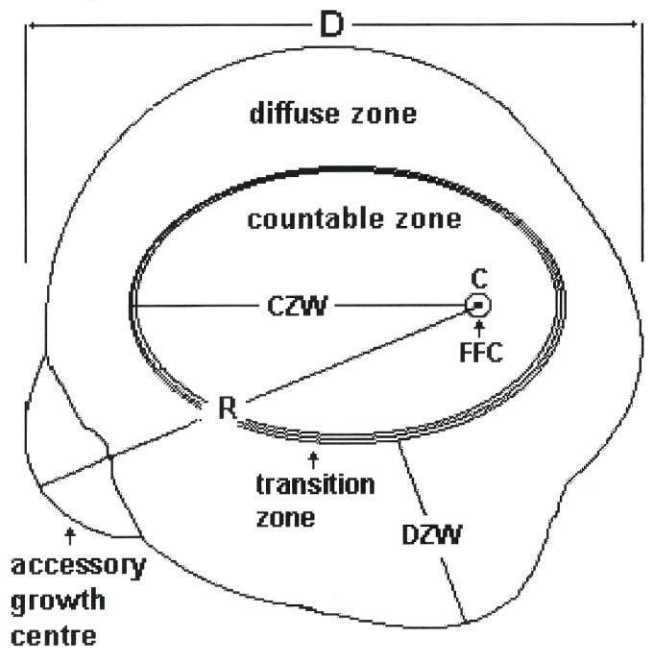

Fig. 2 *Conger conger*. Schematic diagram of the otolithometric measurements in the sagitta (*D* maximum diameter; *R* maximum radius; *CZW* maximum width of the countable zone; *DZW* maximum width of the diffuse zone; *C* core; *FFC* first feeding check).

In the countable zone (CZ), the total number and width of daily increments were registered. The average of every ten increment widths from the first feeding check to the edge of the CZ was used to measure the otolith growth rate. The maximum axis of the CZ was regarded as the otolith radius, along which increment widths were measured.

The percentage of the area occupied by the accessory growth centres (% AGCA) in the total otolith surface was also calculated.

Statistica 5.0 was used to perform all statistical analyses. After testing for normality and homogeneity of variances, a one-way analyses of variance (ANOVA) was used to examine the differences in the relationships between morphometric and otolitometric measures among sampling periods. ANOVA was followed by a Tukey HSD-test for unequal n (Spjotvoll/Stoline test). Data are presented as means ± standard deviations (SD).

Results

Morphometric and meristic data

The conger eel (*Conger conger*) larvae were identified mainly by counting myomeres. Unfortunately, because some specimens were damaged from the preservation method and/or the catch procedure, only the myomeres of a part of the collected specimens were counted. However, as the general body morphology, pigmentation and length were similar, the damaged specimens were clearly the same species. The meristic and morphometric values obtained are in Table 1.

The mean body length obtained for each month indicates a gradual decrease in larval size along the study period (Table 2; Fig. 3), although between neighbouring months the means are not significantly different ($P > 0.05$). The largest larvae (153 mm) and the smallest (103 mm) were caught, respectively, in January and April 1999.

The ratio of the number of preanal myomeres to total myomeres (PAM/TNM) and the ratio of the preanal length to total length (PAL/TL) were not significantly related to the total length (TL) ($r^2=0.0056$, n=81, $P>0.05$ and $r^2=0.0003$, n=153, $P>0.05$, respectively).

The PAL/TL index was positively and significantly correlated with the PAM/TNM ratio ($r^2=0.58$, n=81, $P<0.05$) (Fig. 4).

Table 1 *Conger conger*. Morphometric and meristic counts of metamorphic conger eel larvae (*SD* standard deviation; *n* sample size; *TNM* total number of myomeres; *PDM* predorsal myomeres; *PAM* preanal myomeres; *PDL* predorsal length; *PAL* preanal length; *BD* body depth; *HL* head length).

Parameter	Range	Mean ± SD	n
TNM	154-163	-	89
PDM	25-51	-	82
PDM/TNM	-	0.23 ± 0.04	
PAM	47-65	-	81
PAM/TNM	-	0.36 ± 0.03	
PDL (mm)	-	37.35 ± 5.97	146
PDL/TL	-	0.29 ± 0.04	
PAL (mm)	-	55.33 ± 5.97	153
PAL/TL	-	0.43 ± 0.04	
BD (mm)	-	7.48 ± 1.35	142
HL (mm)	-	9.50 ± 0.55	184

Table 2 *Conger conger*. Body length (mm) of the conger eel leptocephali collected from December 1998 to April 1999 (*SD* standard deviation; *n* sample size; the values marked with the same *superscripted letters* are not significantly different, P>0.05).

Month	Range	Mean ± SD	n
Dec 1998	122-149	134.5 ± 6.6^{a}	23
Jan 1999	118-153	$131.6 \pm 8.8^{a,b}$	19
Feb 1999	104-148	$128.5 \pm 8.0^{b,c}$	75
Mar 1999	115-135	$123.9 \pm 5.0^{c,d}$	15
Apr 1999	103-130	119.4 ± 5.8^{d}	52

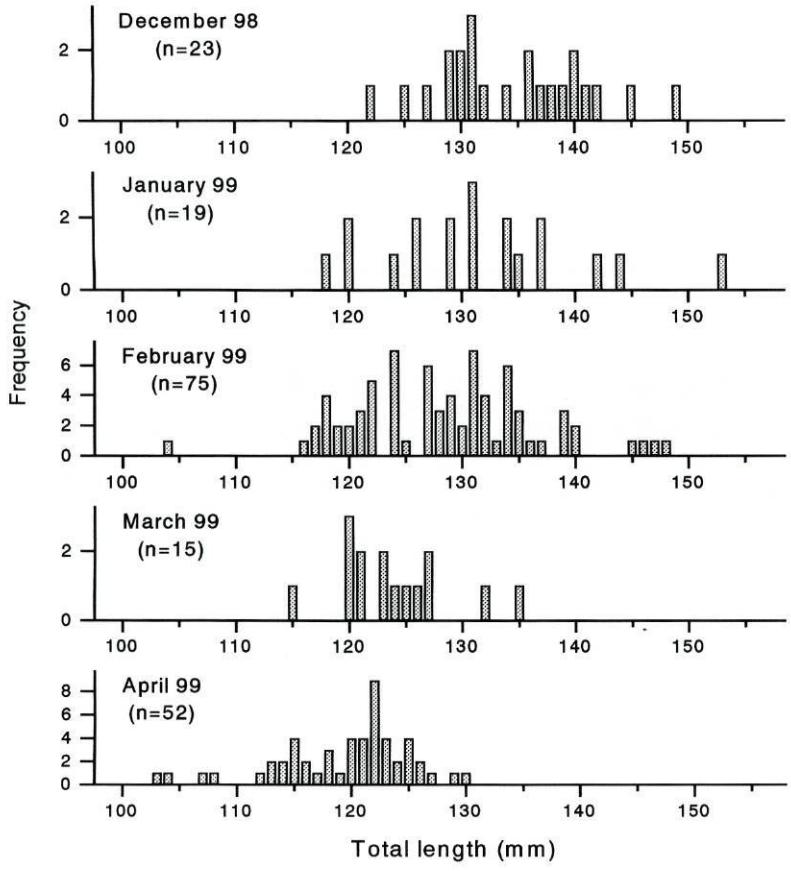

Fig. 3 *Conger conger*. Length-frequency distribution of metamorphosing conger eel leptocephali (collected from mouth of Minho River) between December 1998 and April 1999.

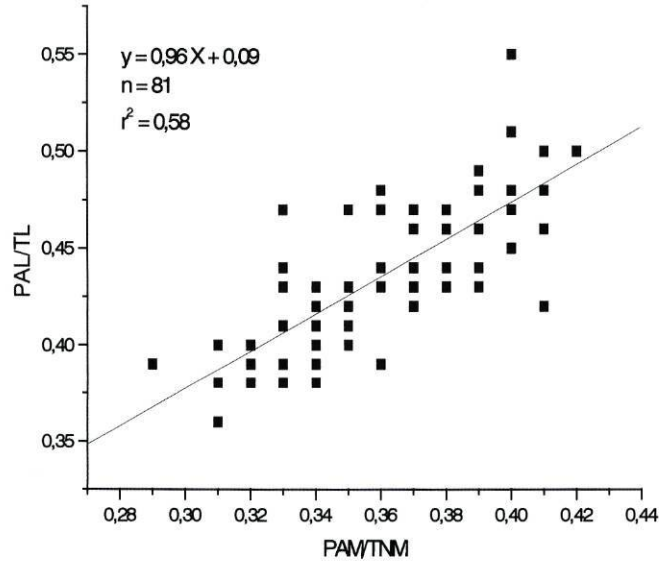

Fig. 4 *Conger conger*. Scatter diagram of PAL/TL index versus PAM/TNM ratio (the regression line represents a least squares fit of the linear equation). Abbreviations, see Table 1.

General body morphology and pigmentation

There is little information about the body morphology changes and the development of the pigmentation pattern in the conger eel larvae, and the existent data is restricted to that of D'Ancona's (1931) study.

The metamorphic larvae had the body shape associated with the general and identifying features of this species (Fig. 5). They had a laterally compressed, transparent body, with W-shaped myomeres and a simple tubular gut along the ventral margin of the body. The head was of medium size and had rounded eyes. Long dorsal and anal fins were confluent with a rounded caudal fin. Larvae had small pectoral fins, and pelvic fins were absent.

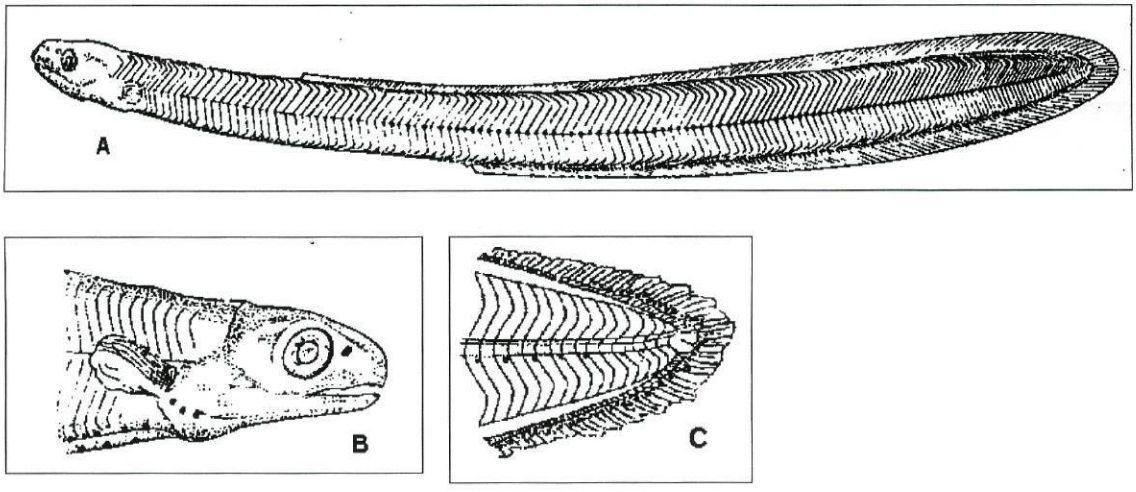

Fig. 5A-C *Conger conger*. Metamorphic lepthocephalus (modified from D'Ancona 1931): **A** whole view, **B** head, **C** tail.

Concerning the pigmentation, they possessed large dots along the lateral line, which became sparser or disappeared anteriorly. We also observed some melanophores at the bases of the caudal and anal fin rays, and limited to posterior region of the dorsal fin. They also exhibited a double ventral pigmentation on the sides of the intestine, sometimes extending slightly beyond the anus. In some specimens several spots were visible around the anus and at the mandibular angle. The crescent pigment patch under the eye (sometimes called irido-coroid process), characteristic of the premetamorphic stage, was absent.

Concerning the dentition, they had lost their larval teeth, or, as in several cases, the presence of vestigial teeth were found in both maxillas, yet with no calcification signs. One specimen captured in February, in a more advanced stage, exhibited dorso-lateral spots in the tail zone and had well developed calcified teeth.

Sagitta microstructure

The microstructure of 90 metamorphic larval sagittae were studied by SEM. A representative scanning electron micrograph is show in Fig. 6C. From the nucleus to the periphery, the sagittae showed permanent structures like the central core (C), the increment countable zone (CZ), the diffuse zone (DZ) and the existence of one or more accessory growth centres (AGC) (Fig. 6A, C).

The core, located at the posterior side of the countable zone (usually with an amorphous primordium in the centre), was surrounded by a thick ring, presumed to be the hatch check (HC). The core had a mean diameter value of 22±3 μm, and no significant differences were observed between the sagittae of conger larvae captured in different months ($P>0.05$).

During the yolk-sac stage, i.e. between the HC (when visible) and the first feeding check (FFC), no distinct daily growth was discernible. Beyond the FFC, clear daily growth increments marked the beginning of the countable zone. This check was postulated to be the FFC, since its morphology was similiar to other anguilliform fishes (Tabeta et al. 1987; Lecomte-Finiger and Yahyaoui 1989; Wang and Tzeng 1998, 2000).

In the countable zone, the otolith increment width showed a characteristic curve along the sagitta radius (Fig. 7). From hatching to approximately 30 days afterwards there was a pronounced increase of the daily ring width (until a maximum of 0.80 μm). Then, the increments became progressively narrow, until they reached a constant minimum value (0.35-0.45 μm) at about 160-180 days. This period of narrow rings can be quite extensive (between 170 and 280 days) depending of the specimen. After that, they abruptly widened (to a maximum of 0.65-0.90 μm) and became less clear (transition zone), until they disappeared (Fig. 6B), which corresponds to the beginning of the diffuse zone, at about 205-324 days.

The mean number of increments of the total countable zone for each month of capture was not significantly different ($P>0.05$). We obtained an overall mean of 260±28 days. The exclusion of the transition zone from the total countable zone in this analysis also gave non-significant results. The overall mean was 228±30 days.

As the diffuse zone (DZ) grew, one or more accessory growth centres (AGC) were formed (Fig. 6C). These structures appeared in the majority of the sagittae analysed (97 %). With a different spatial arrangement of the aragonite crystals compared to the diffuse zone (Fig. 6D), the AGC are responsible for the secondary growth layers which will generate the elliptical otolith shape in adults (Fig. 6E).

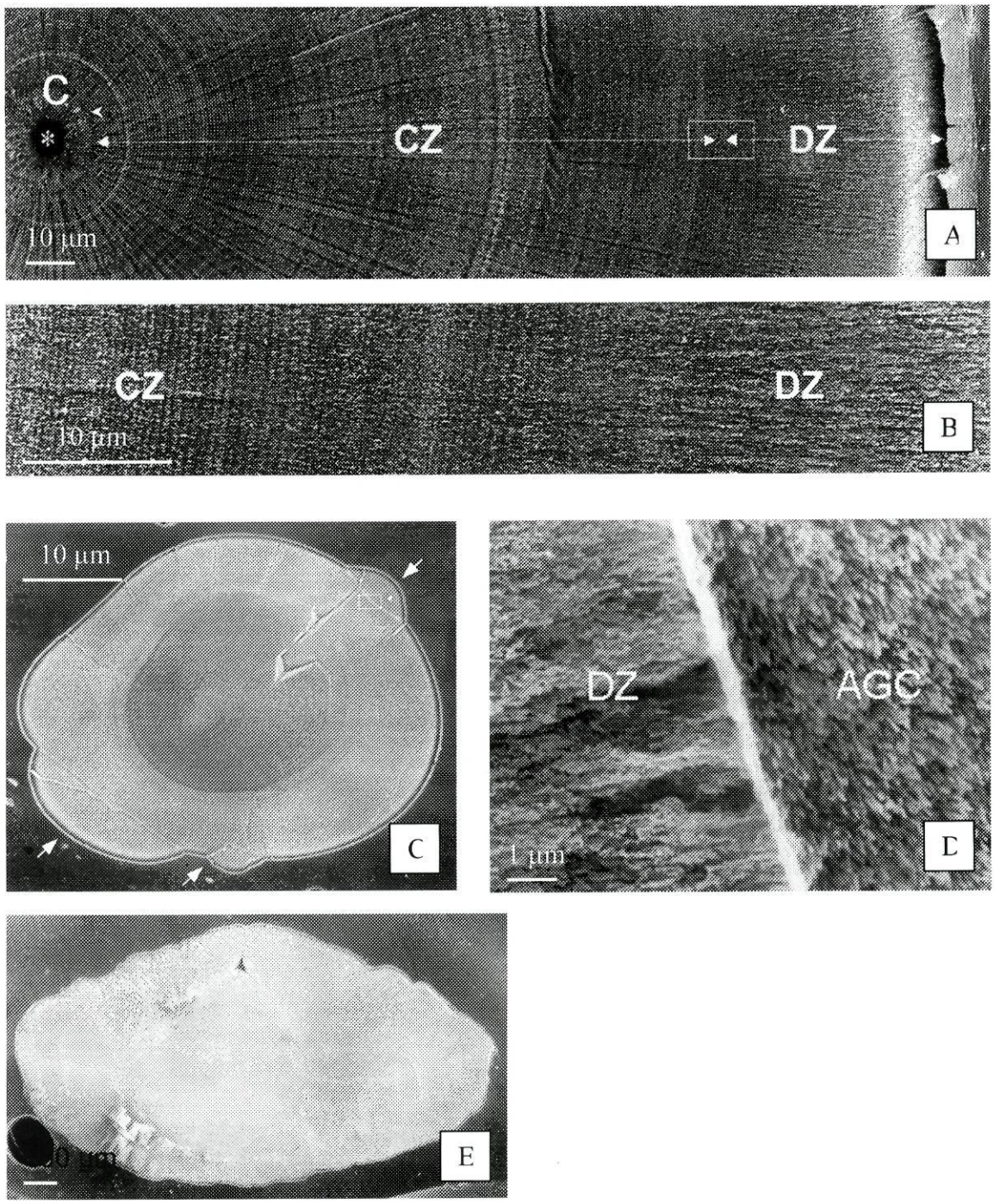

Fig. 6A-E *Conger conger*. SEM micrographs showing the sagittae microstructure pattern of metamorphic conger eel leptocephali. **A** Sequence of the different otolith zones (*C* core; *CZ* countable zone; *DZ* diffuse zone; *asterisk* primordium; *arrowhead* first feeding check). **B** Detail of the transition zone indicated by a box in A. **C** Whole view of the otolith, with the accessory growth centres indicated by *arrows*. **D** Spatial arrangement of the aragonite crystals between the sagittal plane of the diffuse zone (*DZ*) and the accessory growth centre (*AGC*) (enlargement of the region indicated by a box in C). **E** Whole view of the otolith of an elver conger eel (which had successfully completed metamorphosis in an aquarium).

Otolithometric measures

There is a very good relationship between the diameter (D) and the radius (R) of the sagitta (r^2=0.88, n=90, P<0.05), so either of these otolithometric measures are helpful in describing the otolith growth rate.

The sagitta size (D and R) is not significantly related to the total body length (r^2=0.040, n=90, P>0.05 and r^2=0.033, n=90, P>0.05, respectively), but is significantly correlated with the developmental stage. The diameter and the radius of the sagittae exhibited a moderate negative correlation with the PAL/TL index (r^2=0.42, n=81, P<0.05 and r^2=0.32, n=81, P<0.05, respectively). Also the DZW and the % AGCA are negatively correlated with the PAL/TL index (r^2=0.27, n=81, P<0.05 and r^2 =0.36, n=76, P<0.05, respectively).

The maximum otolith diameter and radius ranged between 328 to 589 μm and from 188 to 326 μm, respectively. The width of the countable zone (CZW) was approximately constant with a mean value of 148 μm (±14 SD) and no significant differences between months were observed (P>0.05). The width of the diffuse zone (DZW) presented a minimum value of 59 μm and a maximum of 141 μm. The otolith area occupied by the accessory growth centres (% AGCA) ranged between 0.3 % and 35.0 %.

So, through the study period, the majority of the otolith length differences are apparently due to the contribution of the DZW and %AGCA. Indeed, there is a good correlation between sagitta length expressed as a radius (r^2=0.59, n=87, P<0.05) or diameter (r^2=0.54, n=87, P<0.05) and the % AGCA of the otolith surface. In the same manner the otolith diameter (D) and radius (R) are positively correlated with the DZW (r^2=0.30, n=90, P<0.05 and r^2=0.49, n=90, P<0.05).

In fact the R, DZW and %AGCA in the first two months (December/January) were significantly higher that those in the last three months (P<0.05). The inverse situation was observed for the PAL/TL index (Fig. 8).

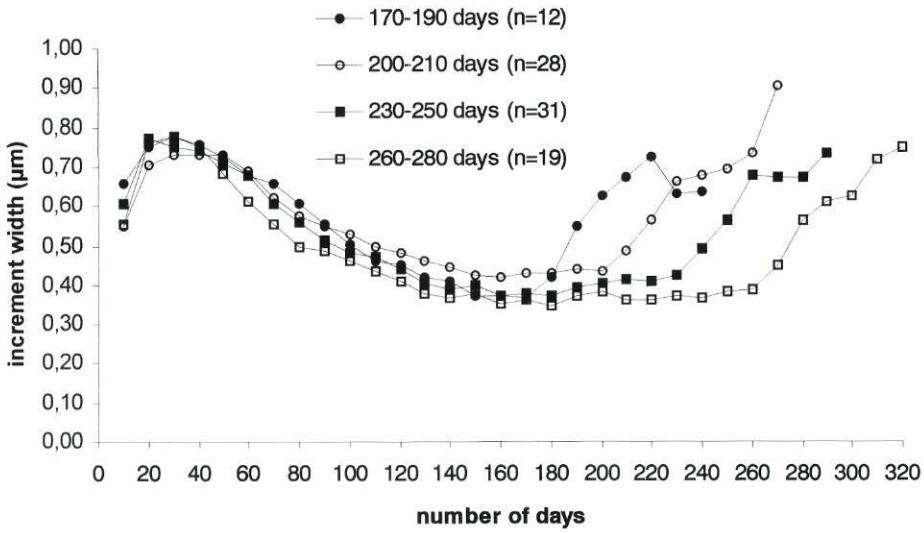

Fig. 7 *Conger conger.* Variations in mean increment width throughout the countable zone of sagittae. *Arrows* represent the points where the daily rings abruptly increase, i.e. the beginning of the transition zone (the specimens have been grouped according to the time when this happened).

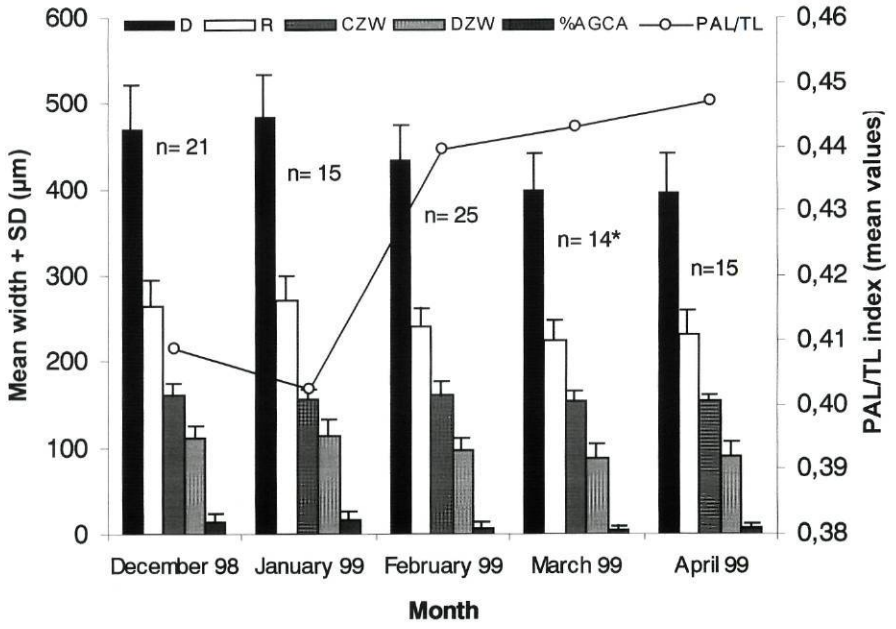

Fig. 8 *Conger conger.* Monthly variation of the otolithometric data (*, for AGC only 12 specimens presented this structure) and the PAL/TL index of the larvae captured during the study period. For the R, DZW and %AGCA, the values for the first two months are significantly higher than the values for other months ($P<0.05$). The inverse situation is observed for the PAL/TL index (*D* maximum diameter; *R* maximum radius; *CZW* maximum width of the countable zone; *DZW* maximum width of the diffuse zone; *%AGCA* percent area occupied by accessory growth centres; *PAL/TL* preanal length/total length).

Discussion and conclusions

Occasionally, metamorphic conger eel larvae are caught in the Portuguese glass eel fishery (from November to April), when conducted in the mouth of the Minho River. Indeed, the entry of metamorphic conger eel larvae into the northern coastal waters of Portugal has been observed from late October until June (Correia, unpublished data). For *Conger myriaster* leptocephali this coastal water presence has been recorded between November and July, during the last developmental and metamorphic stage (Tanaka et al. 1987).

The total number of myomeres (TNM) of *Conger conger* leptocephali collected varied between 154 and 163, which is in agreement with the corresponding data found by other authors (Schmidt 1931; Strehlow et al. 1998). Nevertheless, the range of values reported can be slightly different (D'Ancona 1931; Castle 1970), probably due to errors in counting (for instance, it is extremely difficult to count the most posterior myomeres near the end of the tail), explaining, in part, some of the intraspecific variation found in the literature.

The ranges of the predorsal (PDM) and preanal (PAM) myomeres of the metamorphic larvae, 25-51 and 47-65, respectively, were, as expected, smaller than the counts obtained by Strehlow et al. (1998) for the premetamorphic stage. Indeed, during the transition to metamorphosis the anus and also the origin of the dorsal fin begin to move to a distinctly more anterior position in Congridae leptocephali (Otake et al. 1997; Strehlow et al. 1998). Thus, the PAM/TNM obtained for this stage (0.36) is less than half the reported value (0.77) for premetamorphic larvae (Strehlow 1992).

The PAM/TNM ratio have been successfully used as a criterion to describe the developmental stage of conger eel leptocephali, as stage is difficult to determine on the basis of other morphometric characteristics (Tanaka et al. 1987). However, since the PAL/TL index is correlated with the PAM/TNM ratio, it was decided to preferentially use this index as a developmental stage indicator, as it is easier to measure, especially in poorly preserved specimens. Indeed the PAL/TL index was successfully used in classifying three different metamorphosing stages in *C. myriaster* (Yamano et al. 1991).

The lack of a relationship between the PAM/TNM ratio (or the PAL/TL index) and the total length indicates that the size variation in the metamorphosing leptocephali (and possibly the full-grown leptocephali) is large, suggesting that they belong to different hatching populations. Data also show (TL and PAL/TL index) that early migrating leptocephali are large in size and of advanced metamorphic stage.

There is no relationship between body size and otolith size, although an inverse correlation between them would be expected for a population in which somatic growth is correlated with otolithic growth. However, the sagitta size is significantly related with the PAL/TL index, a useful indicator of developmental stage.

The monthly variation in otolith size is supported by the PAL/TL index. The largest larvae in late stages of metamorphosis that were caugth during the first 2 months (December 1998 and January 1999) have the largest R, DZW and %AGCA, suggesting that they are older in spite of the total age remaining unknown. Furthermore, if early migrating leptocephali are older than late migrating ones, their hatching times must be quite different, as previously suggested by the variation in body size.

All morphological features and pigmentation patterns of the collected leptocephali are in agreement with the descriptions of D'Ancona (1931). Our specimens appear to belong to a single developmental stage. In the future, it would be useful to establish a criterion, based on pigmentation, for differentiation of the developmental stages occuring during metamorphosis.

The otolith morphology of the European conger eel is similar to that observed in the Japanese conger eel (Lee and Byun 1996); however, the opaque zone is replaced by a diffuse zone in this species.

In the countable zone, the width of the increments presented the same pattern when compared with the sagittae of premetamorphic stages (Antunes 1994), with the exception of the relatively wide increments at the periphery of the countable incremental zone (transition zone). These wide rings appears to be a metamorphosis check, since this particular increment pattern is associated with a drastic change in the otolith strontium:calcium ratios in *C. myriaster* (Mochioka et al. 1989; Lee and Byun 1996; Otake et al. 1997), *Anguilla japonica* (Otake et al. 1994; Arai et al. 1997), *A. rostrata* (Wang and Tzeng 1998, 2000) and *A. anguilla* (Wang and Tzeng 2000).

The different thicknesses of the increments along the sagitta radius are probably associated with both endogenous and exogenous factors. The seasonal variations in seawater temperature as well as photoperiod should be two of the most important environmental factors. In fact, previous studies on growth of herring larvae have shown the major influence of the environment (temperature, photoperiod and food abundance) on the deposition of increments in otoliths (Lough et al. 1982; Pavlov et al. 2000).

If we consider that the countable zone without the transition zone of the sagittae corresponds to the premetamorphic larval stage, as has been described in other anguilliform species, our results suggest that the larvae of the European conger eel take about 6-9 months

from hatching to onset of metamorphosis. These estimates shorten the hypothesised 15 months (1¼ years) proposed by Strehlow et al. (1998). Furthermore, since the mean increment number in the otolith countable zone is almost constant among the recruitment seasons, it suggests that the early migrating individuals metamorphose at the same age as late migrating ones.

However, the transition zone and also a part of the diffuse zone can be present in the late premetamorphic larvae, representing additional time for this larval phase. For instance, Otake et al. (1997) considered that the onset of the metamorphosis for *C. myriaster* occurs early, approximately in the fourth month.

At this point, assuming that: (1) the peak of the hatching season occurs in summer (Schmidt 1931; Strehlow et al. 1998), (2) the leptocepahalus stage lasts about 6-9 months and (3) the latest larvae captured (in June 1999) were not yet at the end of the metamorphic process, we can estimate that the duration of metamorphosis is at least 1 year, and probably occurs on the continental shelf and in coastal waters. This estimate gives a total larval phase of about 2 years before the juvenile elver stage is reached in the European conger eel.

Asano et al. (1978) reported that, in the laboratory, the metamorphosis of *C. myriaster* leptocephali takes about 22 days, at temperatures of 18-22 °C. Lee and Byun (1997), based on sagitta analysis of specimens collected from the wild, estimated the duration of metamorphosis to be 53-75 days (10-16 °C). However, the temperature effect is not the only parameter to be taken into account. The difficulty and ambiguity in identifying the beginning and completion of metamorphosis of reared leptocephali may also result in inaccurate estimation of the duration of the metamorphic stage (Otake et al. 1997). It is also plausible that captive specimens may accelerate metamorphosis (Butler et al. 1996). In fact, metamorphic conger eels captured in the Minho River in June 1999 and 2000 and reared in aquaria at two different temperatures (26 and 16 °C, respectively) completed metamorphosis at a final length of 70-80 mm, in about 1 ½ months (Correia, unpublished data).

Whether metamorphosis is triggered by some environmental stimulus or occurs spontaneously at a certain age or size is unknown (Smith 1989). For some species we know that metamorphosis is initiated when pelagic leptocephali migrate to inshore waters, suggesting that the shallow, near-shore environment is somehow involved in the triggering mechanism (Pfeiler et al. 1990). However, metamorphosis in some species takes place in the open ocean, indicating that factors other than proximity to shore are involved (Pfeiler 1999).

In contrast to the sagitta rings of premetamorphic conger eel larvae, which are easily countable (Antunes 1994), the existence of a diffuse zone and several accessory growth

centres in the metamorphic specimens hinders an accurate estimate of the duration of metamorphosis.

Although larvae did not feed and body length diminished during metamorphosis, otoliths continued to grow, since a diffuse zone was formed outside the countable zone, which was associated with a clear change in the otolith growth direction.

This diffuse zone, non-existent in the premetamorphic larvae up to 133 mm of total length (Strehlow et al. 1998), has also been described in European eel larvae; however, in this species, the diffuse zone is already present before the onset of metamorphosis (Antunes and Tesch 1997). Since the growth rate of otoliths often changes during transitive periods of fishes, in glass eel otoliths, this diffuse zone has been suggested to mark a period of very slow growth and to be made up of many daily growth increments that are so narrow they cannot be distinguished (Antunes and Tesch 1997). Despite the favourable external factors, namely the high temperatures of the end of summer and early autumn, the diffuse zone could represent a decrease in increment width and may be due to the nearly-completed growth of the larvae before metamorphosis starts. According to a new theory, this zone is formed during a period when the vertical rhythmic movements (i.e. diurnal vertical migration) of the leptocephali cease (Williamson et al. 1999).

The fact that the Japanese conger eel *C. myriaster* has a continuous series of daily growth rings and no "diffuse zone" (Tanaka et al. 1987; Mochioka et al. 1989; Lee and Byun 1996), as do the Japanese eel, *A. japonica* (Arai et al. 1997) and the American eel *A. rostrata* (Wang and Tzeng 1998), suggests a different manner of larval development from European eels (*Conger*, *Anguilla*). Although American and Japanese eels occur in the Atlantic and Pacific Oceans, respectively, their spawning grounds and larval migratory routes are symmetrical, and larvae drift to estuaries by similiar currents (Wang and Tzeng, 2000).

The high incidence of AGCs indicates that they are universal structures in metamorphic conger eel sagittae, appearing in a late stage of metamorphosis. The same morphological features have been observed in *C. myriaster* (Lee and Byun 1996). The cause behind the formation of these structures is not yet completely understood. However, in other species their formation is often associated with life-history transitions such as metamorphosis (Secor et al. 1995). They represent an extra period of growth, which is difficult to estimate, and are probably produced during a significant habitat change, for example, the entry into less saline coastal waters.

Otolith growth appears to progress in three stages: stage 1 corresponds to the countable zone; stage 2 to the diffuse zone and stage 3 corresponds to the formation of the accessory

growth centres. The transition between these zones is probably triggered by internal signals, but the rate of deposition of aragonite in the sagitta's distinctive parts could be modulated by external factors.

This study indicates that the growth pattern of otoliths of conger eel leptocephali may be similar between eel species, although some differences exist between European and Japanese/American species, probably due to differences in their life cycles, namely in the distance of their larval migratory routes.

Acknowledgements

This work was supported by "Fundação para a Ciência e Tecnologia" (PRAXIS XXI: 2/2.1/MAR/1728/95). A.T. Correia has a grant for PhD studies (BD18082/98) from the same sponsor. We thank Dr. J. Wilson for critically reading the manuscript.

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2.2 Age, growth, distribution and ecological aspects of *Conger conger* leptocephali collected in Azores, based on otolith analysis of premetamorphic specimens

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Abstract

A total of 29 specimens of the anguillid leptocephali *Conger conger* were collected by the R.V. "Arquipélago", Department of Fisheries and Oceanography, University of Azores, in the waters south of the central group of the Azores Islands in October 1999. The meristic counts, morphologic features and pigmentation patterns of the leptocephali agree with the descriptions made by other authors for this species. Leptocephali were found to ranged from 51.5 to 126.5 mm length and show a positively skewed distribution. The somatic growth rate (0.31 $mm.day^{-1}$), estimated from the linear regression of length on age, falls within the ranges reported by several authors for other anguilliform species. Back-calculated hatching dates from the otolith microstructure suggests a long spawning season, from January to July, with one visible annual peak, occurring in summer. The analysis of our collection data in light of the current physical oceanographic knowledge of the NE Atlantic suggests that it is unlikely that the Azorean specimens came from the Mediterranean, unless the larvae were capable of very active and oriented swimming; thus the existence of another spawning place for this species somewhere near the Azores Archipelago is probable.

Introduction

The European conger eel (*Conger conger* Linnaeus, 1758) is a marine fish widely distributed in the NE Atlantic, Mediterranean and western Black Sea (Bauchot and Saldanha 1986). However, knowledge about its early life history, such as spawning area(s) and season(s), duration of the leptocephalus phase and larval migratory route(s), is very limited, since few studies on this species have been reported.

Schmidt (1931) caught small *C. conger* leptocephali in the Sargasso Sea, Mediterranean and NE Atlantic, and proposed a similar migratory behaviour to the European eel (*Anguilla anguilla* L.), i.e. spawning in the Sargasso Sea, following a larval transoceanic migration to the European and North African coasts. However, Schmidt's claim that *C. conger* spawn in the Sargasso Sea is contested by McCleave and Miller (1994). These authors state that the small conger eel larvae caught in the Sargasso Sea were *C. triporiceps*, a species with an overlapping number of myomeres, which was described later by Kanazawa (1958). Nowadays, the larvae of both species are distinguished by the catch location, whereby the *C. conger* leptocephali are restricted to the central and eastern zones of the Atlantic Ocean (Strehlow et al. 1998).

The length and otolith analyses of *C. conger* leptocephali collected in the North and Central Atlantic Ocean showed that spawning occurs in the Mediterranean Sea, between July and September (Strehlow et al. 1998), supporting the existence of a spawning area in the Mediterranean for the European conger eel (Cau and Manconi, 1983).

Otolith microstructure analysis provides important information about age and growth of fishes. Daily increment studies contribute to the knowledge of important events in the early life history of individual fish, such as hatching time, duration of the larval phase, growth rate and transition to another mode of life (Campana 1985).

The present study examined the otolith record of age, growth and ontogeny in *C. conger* leptocephali, to provide a better understanding of the recruitment process of this species in the coastal habitat, and to provide further knowledge about its early life history.

Materials and Methods

The leptocephali used in this study were collected during a research cruise (R.V. "Arquipélago") conducted by the Department of Oceanography and Fisheries of the

Azores University in mid-October 1999, in the Central North Atlantic Ocean, near the central group of the Azores Islands. Figure 1 shows the sites where sampling took place.

Three transects (23 hauls, 18 at night and 5 during daytime) across the southern area of the Pico and Faial Isles provided 29 leptocephali of *Conger conger*. These transects were accompanied by CTD (conductivity-temperature-depth) and XBT (expendable bathythermograph) casts, which provided temperature profiles for each sampling site. The vertical profiles of temperature presented abrupt changes between 70 and 80 m deep, forming a sharp thermocline. Surface water temperature and salinity of the sampling area ranged from 20.9 to 21.7 °C and 35.9 to 36.1 psu, respectively. In Table 1 the sampling data, location, environmental oceanographic values and lengths of the collected leptocephali are presented.

All leptocephali were taken with a rectangular midwater trawl with a mouth opening of 8 m^2 (RMT8) and a 4.5 mm mesh net, mainly in the surface layers of water (0-200 m) during the night. After capture, the leptocephali were preserved in 4% seawater formalin and transferred in the laboratory to 70 % ethanol. We identified the specimens using the criteria of D'Ancona (1931). Measurements were made to the nearest 0.1 mm, and meristic counts were done using a dissecting microscope following the method adopted by Smith (1989). Two readers made the myomere counts. The counts were repeated until a consistent value was obtained (no more than two units of difference). The shrinkage caused by fixation and preservation method was not corrected.

Left side sagittae were removed from 16 specimens, cleaned, mounted on cylindrical stubs, and polished with 2400 silicon carbide abrasive paper and alumina suspension (1:20) until the core was revealed. After that, they were etched for 10 s with 0.05 M HCI, sputter-coated with gold under vacuum and viewed with a scanning electron microscope (SEM; Jeol JSM 630-1 F) at 15 kV.

Following SEM analysis, core diameter (C), maximum otolith diameter (D) and maximum otolith radius (R) were measured from the SEM photographs (at magnifications between 300× and 2500×). The otolith radius was measured along the longest axis of the otolith. On the same axis, the total number of increments and their widths were also registered. Enumeration of otolith micro-increments was straightforward. All the increment counts were performed three times, by the same reader, between the first visible increment to the last increment near the sagitta's edge. The coefficient of variation (CV) between counts was < 3.0 %.

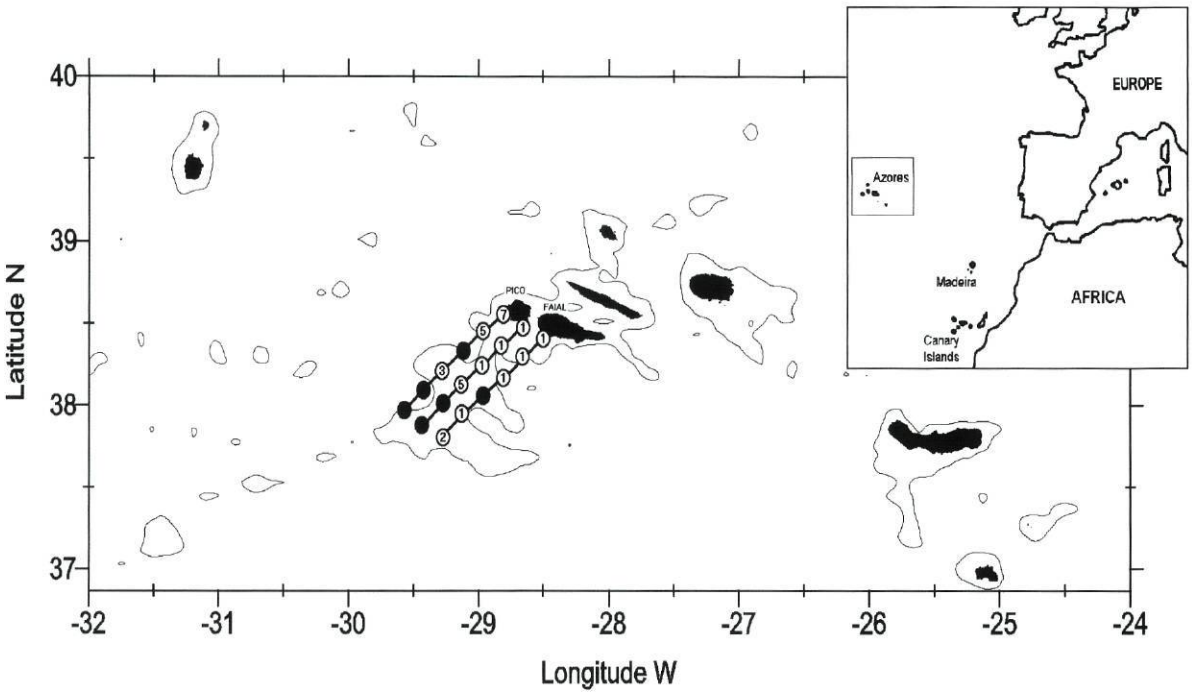

Fig. 1 *Conger conger*. Location of the Azores Archipelago in the middle of the North Atlantic Ocean (*inset*). Station locations of the R.V. "Arquipélago" (*main map*) (*lines* represent transects; *circles* indicate the stations; *solid circles* are the stations without larvae; *open circles with number* indicate the stations where the conger eel larvae were collected and the respective number of specimens).

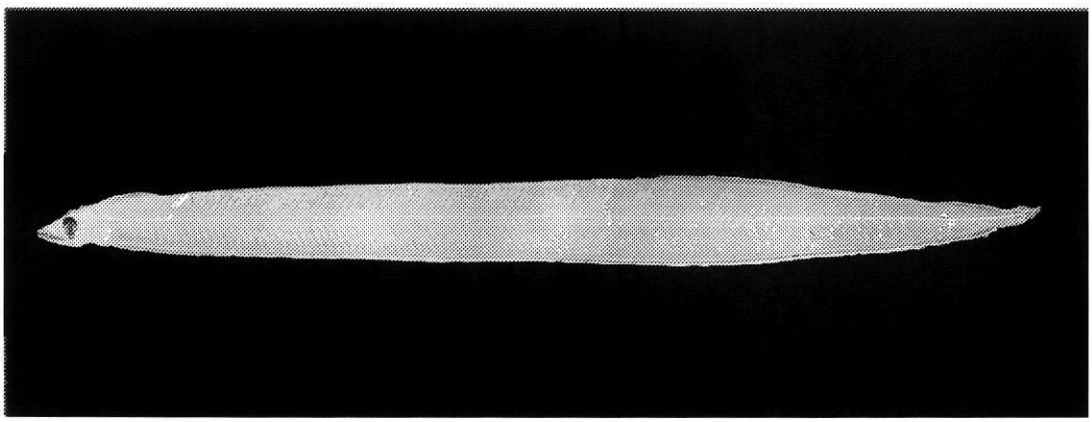

Fig. 2 *Conger conger*. Leptocephalus in a premetamorphic stage (80.5 mm in total length).

Table 1 *Conger conger*. Sampling location, date (in 1999), environmental parameters, length, age and hatching date (in 1999) of the collected leptocephali [[a]Estimated from the linear regression of length on age (Y=32.9 + 0.31X)].

Sampling location	Sampling date	Salinity (PSU) (Surface/Depth)	Temperature (°C) (Surface/Depth)	Depth (m)	Total length (mm)	Age (days)	Hatching date
38°33'N; 28°52'W	8 Oct	-/-	-/-	200	51.5	76	25 Jul
					68.0	114[a]	17 Jun
					80.5	122	9 Jun
	12 Oct	-/-	-/-	200	63.5	98[a]	6 Jul
					65.0	103[a]	1 Jul
					71.5	126[a]	9 Jun
					75.5	140[a]	26 May
38°26'N; 28°59'W	8 Oct	36.00/35.88	21.11/19.99	45	55.5	71[a]	29 Jul
					58.0	79[a]	22 Jul
					59.0	83[a]	18 Jul
					68.0	114[a]	17 Jun
					78.5	150[a]	12 May
38°12'N; 29°18'W	9 Oct	35.97/36.00	21.04/18.24	40	56.0	71	30 Jun
					64.5	102[a]	30 Jun
					70.5	103	29 Jun
38°28'N; 28°40'W	10 Oct	36.03/35.91	21.50/15.16	140	67.5	114	19 Jun
38°21'N; 28°50'W	10 Oct	35.99/35.99	21.18/19.83	58	57.0	76[a]	27 Jul
38°15'N; 28°58'W	10 Oct	35.89/36.01	21.06/15.66	90	75.0	115	18 Jun
38°08'N; 29°07'W	10 Oct	35.98/35.96	20.92/16.13	65	63.0	122	11 Jun
					81.0	159[a]	5 May
					82.0	182	12 Apr
					83.5	154	10 May
					104.0	224	28 Feb
38°25'N; 28°31'W	13 Oct	36.05/35.85	21.77/14.18	200	114.0	275	2 Jan
38°18'N; 28°40'W	13 Oct	36.11/35.87	21.69/14.44	200	58.5	90	16 Jul
38°11'N; 28°49'W	12/10/99	36.03/35.93	21.37/16.84	50	80.5	205	22 Mar
37°57'N; 29°07'W	10 Oct	36.06/35.83	21.22/14.22	200	126.5	246	26 Jan
37°50'N; 29°16'W	11 Oct	36.07/36.02	21.20/16.02	80	65.5	105	29 Jun
					88.5	185	10 Apr

Table 2 *Conger conger*. Morphometric and meristic characters (lengths expressed in mm).

Parameter	Abbrev.	Range	Mean±SD	Sample Size
Total length	TL	51.5-126.5	73.5±17.5	29
Predorsal length	PDL	33.5-78.5	48.0±10.2	24
Preanal length	PAL	46.0-110.0	64.3±14.9	29
Head length	HL	4.2-6.6	5.1±0.6	29
Body depth	BD	4.2-11.9	6.3±1.7	29
Eye diameter	ED	1.0-1.7	1.2±0.2	24
Total no. myomeres	TNM	155-161	158±2	28
Last vertical blood vessel	LVBV	57-62	60±1	27
Predorsal myomeres	PDM	76-102	86±5	24
Preanal myomeres	PAM	120-127	124±2	28

We assumed the growth increment in the larval otolith of *C. conger* to be daily, although daily deposition has not been validated in this species. We base this assumption on the results of several related anguilliform species, e.g. *Conger myriaster* (Mochioka et al. 1989), *Anguilla japonica* (Tsukamoto 1989; Umezawa et al. 1989), *A. rostrata* (Martin 1995), *A. celebesensis* (Arai et al. 2000) and *A. marmorata* (Sugeha et al. 2001), which have been shown to have daily depositions.

In most marine fishes, the deposition of the first daily increment occurs during their first exogenous feeding, when larvae have completed yolk-sac absorption (Lough et al. 1982; McGurk 1984; Tzeng and Yu 1988). In the Japanese eel the yolk sac was completely absorbed 4-6 days post-hatching (Umezawa et al. 1989). Like other similar studies on the Japanese eel (Tzeng 1990; Cheng and Tzeng 1996; Wang and Tzeng 1998), we regarded the first distinct increment as a first feeding check (FFC) and have also added 5 days to the number of the increments on the assumption that *C. conger* begin exogenous feeding 5 days after hatching like *A. japonica* larvae.

Daily growth rates of the body (SGR, somatic growth rate) and otolith (OGR, otolith growth rate) were calculated by the ratios of total length and maximum radius of otolith to estimated age, respectively. Furthermore, the correlations between body and otolith growth rates and age were also calculated.

Data are presented as mean values with standard deviations (±SD).

Results

External body morphology and pigmentation

A whole body view of a premetamorphic *Conger conger* leptocephalus is presented in Fig. 2. The leptocephali had a very elongate body, compressed laterally, with "W"-shaped myomeres and a very long simple tubular gut along the ventral margin of the body. The larvae had small heads and oval eyes, slightly elongated in a dorsoventral direction. Dorsal, caudal and anal fins were joined. The dorsal fin extends anteriorly, but does not reach half of the total length. Larvae had small pectoral fins; pelvic fins were absent.

Preserved specimens were translucent with some pigmentation. They had a few branched dots along mediolateral line, which became sparser or disappeared altogether anteriorly. We also observed some punctuate melanophores at the bases of the caudal

and anal fin rays, but limited to posterior region of the dorsal fin. Leptocephali also exhibited a paired row of punctuate melanophores along the ventral sides of the intestine, extending slightly beyond the anus. A crescentic patch of pigment (irido-chorioid process) was present under the eye. All the leptocephali had larval teeth in both maxillas.

Biometric and meristic data

The morphometric and meristic values obtained are in Table 2. The total number of myomeres (TNM) of *C. conger* leptocephali varied between 155 and 161. The predorsal (PDM) and preanal (PAM) myomere numbers of the premetamorphic leptocephali ranged from 76 to 102, and 120 to 127, respectively. The position of the last vertical blood vessel (LVBL) ranged between 57 and 62 myomeres.

The total length (TL) of leptocephali ranged from 51.5 to 126.5 mm, with a mean of 73.5 (±17.5) mm. The length-frequency of *C. conger* leptocephali (Fig. 3) also suggests a positively skewed distribution, with a peak around 70 mm.

The head length (HL) (r^2=0.85, n=29, P<0.001) and body depth (BD) (r^2=0.91, n=29, P<0.001) were significantly correlated with the TL of larvae. The HL/TL is negatively related to the TL (r^2=0.76, n=29, P<0.001).

The preanal length/total length (PAL/TL) ratio was significantly correlated with the preanal myomeres/total number of myomeres (PAM/TNM) ratio (r^2=0.48, n=28, P<0.001) (Fig. 4). The mean values obtained for the PAM/TNM and PAL/TL ratios, were 0.78±0.01 and 0.88±0.01, respectively.

There is a weak though statistically significant relationship between the PAL/TL and the TL of the larvae (r^2=0.15, n=29, P<0.05). However, no relationship exists between the PAL/TL index and the larval age (r^2=0.06, n=16, P=0.36).

Sagitta microstructure and growth increment

The sagitta, characterized by a rounded shape, viewed under SEM, exhibited clear daily increments from the first feeding check (FFC) to the otolith's edge (Fig. 5A, B). However, the core, which extends from the center to the FFC, is composed of a central amorphous primordium, surrounded by a thick ring, presumed to be the hatch check (HC), and a zone with no distinct increments between the HC and the FFC (i.e. during

the yolk-sac stage) (Fig. 5B). These checks were postulated to be the HC and the FFC, since their morphologies were similar to those in other anguilliform fishes (Tabeta et al. 1987; Lecomte-Finiger and Yahyaoui 1989; Wang and Tzeng 1998, 2000).

The core presented an average diameter value of 24±2 μm. The otolith radius (and diameter) ranged from 61 to 164 μm (101-209 μm), with a mean of 101±32 μm (146±38 μm).

The mean increment widths along the radius of sagittae showed a characteristic curve (Fig. 6). From the FFC to approximately 30 days afterwards, there was a pronounced increase in the increment widths (until a maximum of 0.82 μm). Then, the increment widths became progressively narrow, until they reached a constant minimum value (0.38-0.42 μm) at about 160 days. After that, and only for the two largest individuals, they abruptly widened to a maximum of 0.65-0.72 μm after about 200-230 days (Fig. 7). The average width of each increment was 0.61±0.17 μm (range 0.28-1.05 μm).

Increment counts ranged from 71 to 275 (Table 1), with a mean value of 149±63.

The OGR was 0.71±0.11 $\mu m.day^{-1}$. The otolith radius is significantly correlated with the total length (r^2=0.93, n=16, P<0.001) and age of the leptocephali (r^2=0.96, n=16, P<0.001).

A very good relationship was found between the total length and the age of the leptocephali (r^2=0.86, n=16, P<0.001). The linear regression obtained between size and larval age (estimated from otolith daily ring increments) is displayed in Fig. 8. The slope of this regression indicates a SGR of 0.31 $mm.day^{-1}$, and the Y-intercept indicates a predicted length at hatching of 32.9 mm.

Estimates of maximum SGR and OGR were 0.79 $mm.day^{-1}$ and 0.96 $\mu m.day^{-1}$, respectively. The SGR (r^2=0.77, n=16, P<0.001) and the OGR (r^2=0.65, n=16, P<0.001) were negatively correlated with the age.

Age and hatching season

Hatching dates were back-calculated for 16 leptocephali. The hatching time for the remaining 13 specimens, since their otoliths had been damaged during the formalin fixation process, were estimated from the equation obtained between the size and age of the larvae (TL=32.9+0.31AGE) (Table 1). Although, hatching occurred from January to July, one hatching peak is observed during the summer season (June and July) (Fig 9).

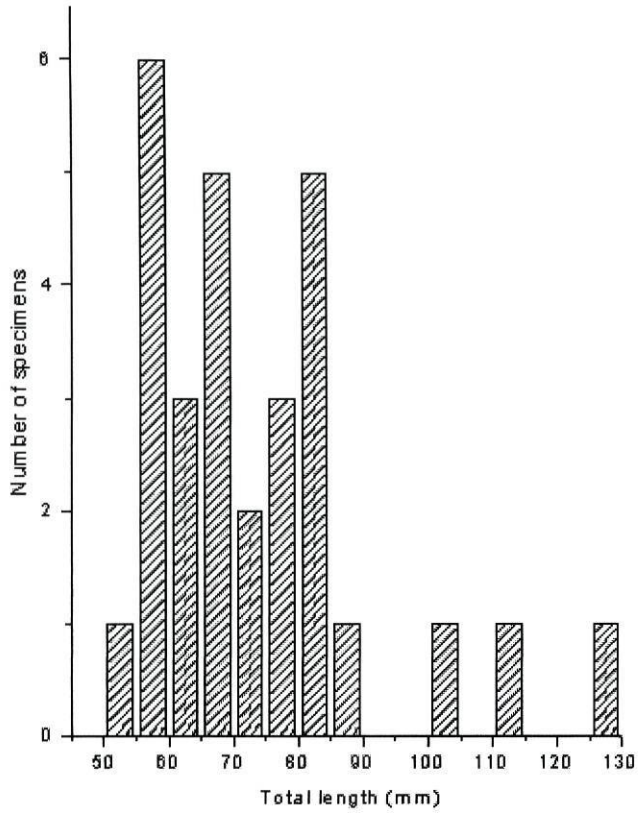

Fig. 3 *Conger conger*. Length-frequencies of the conger eel leptocephali collected during the R.V. "Arquipélago" in October 1999.

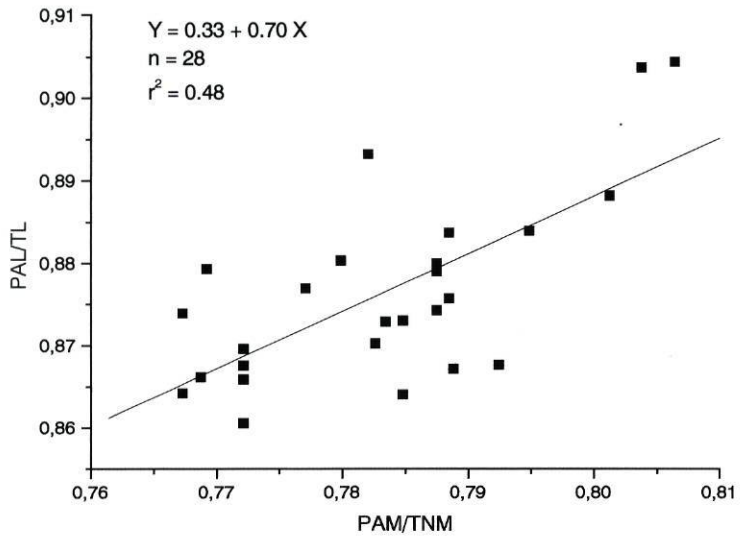

Fig. 4 *Conger conger*. Relationship between PAL/TL and PAM/TNM ratios (*line* represents a least squares fit of the linear regression, $P<0.001$).

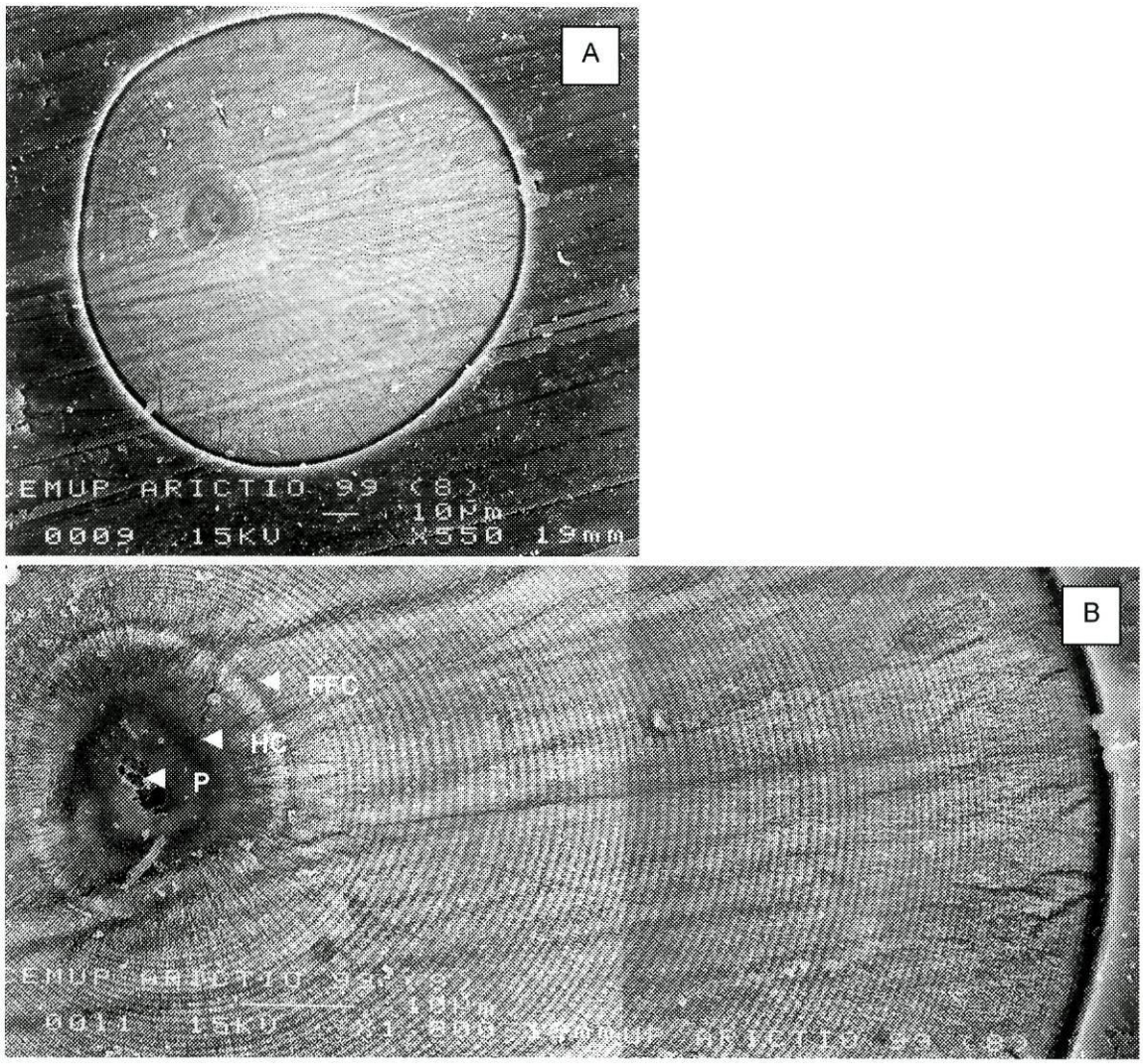

Fig. 5A, B *Conger conger*. SEM microphotographs of a sagitta of a premetamorphic leptocephalus (65.5 mm total length): **A** whole sagitta; **B** radius (*P* primordium; *HC* hatch check; *FFC* first feeding check).

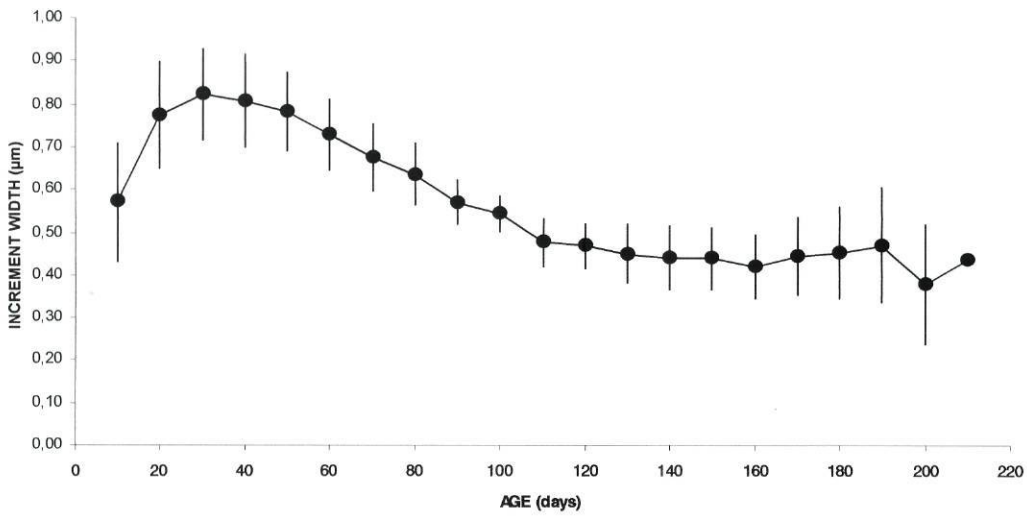

Fig. 6 *Conger conger*. Profile of otolith increment widths from ages 10-210 d. *Each data point* shows a mean value (±SD) for every ten successive increments. Number of samples for each average: 10-70 days (n=14); 80 days (n=12); 90-100 days (n=10); 110 days (n=8); 120 days (n=6); 130-150 days (n=5); 160-180 days (n=3); 190-200 days (n=2); 210 days (n=1).

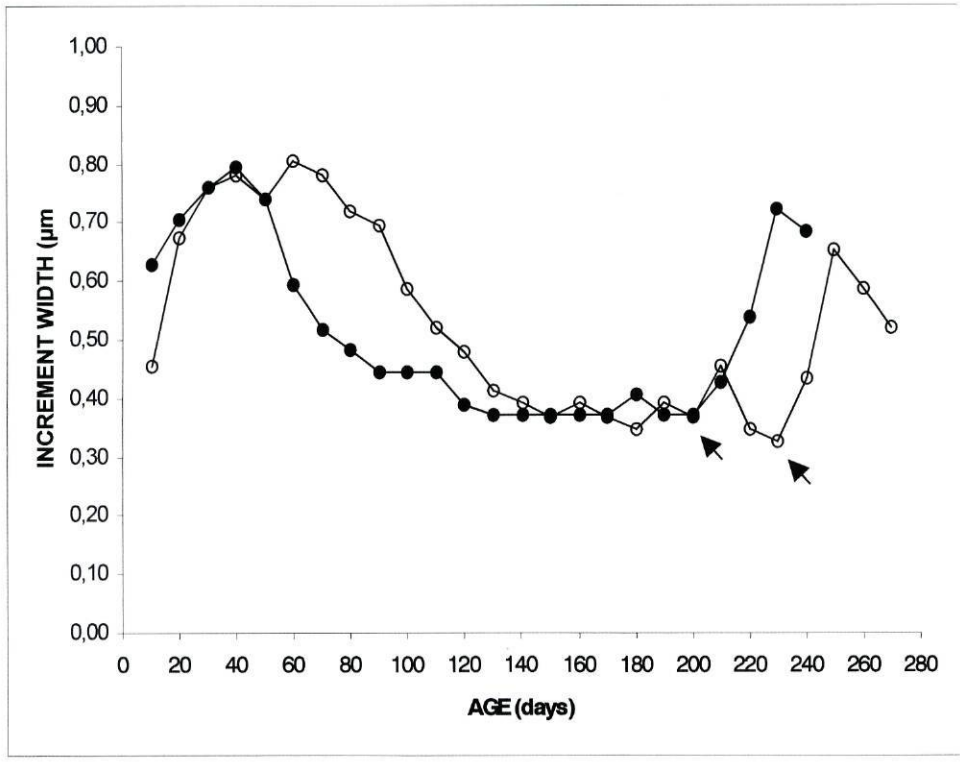

Fig. 7 *Conger conger*. Otolith increment width versus age of two individuals: 126.5 mm (•) and 114.0 mm (o) long (*arrows* sharp increase in the increment width).

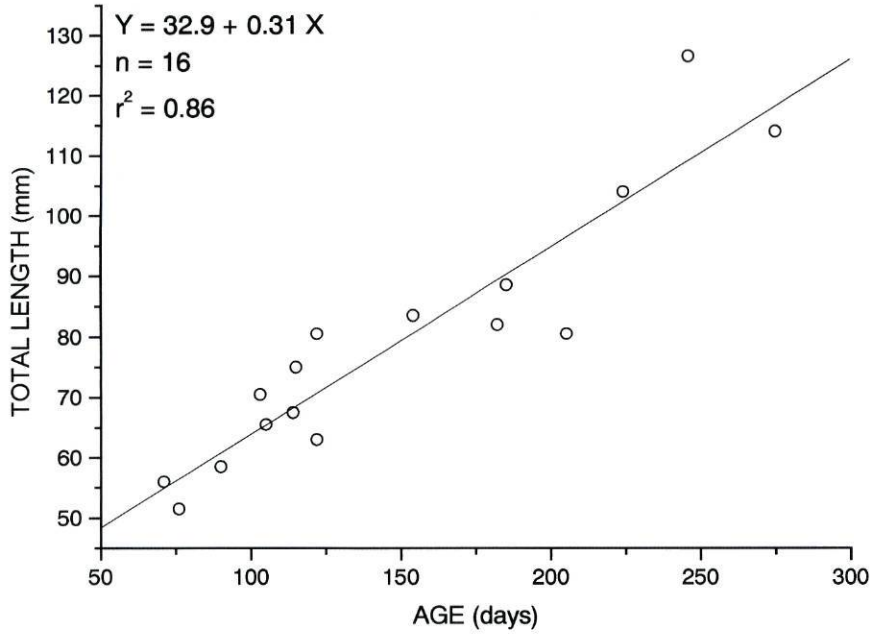

Fig. 8 *Conger conger*. Relationship between the larval length and the age (*line* represents a least squares fit of the linear equation, $P<0.001$).

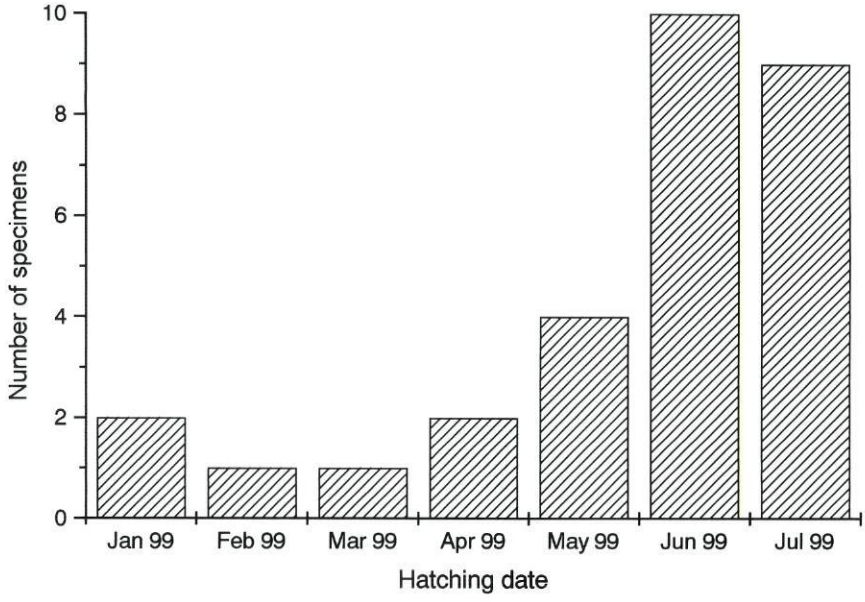

Fig. 9 *Conger conger*. Frequency distribution for eels on the estimated hatching dates.

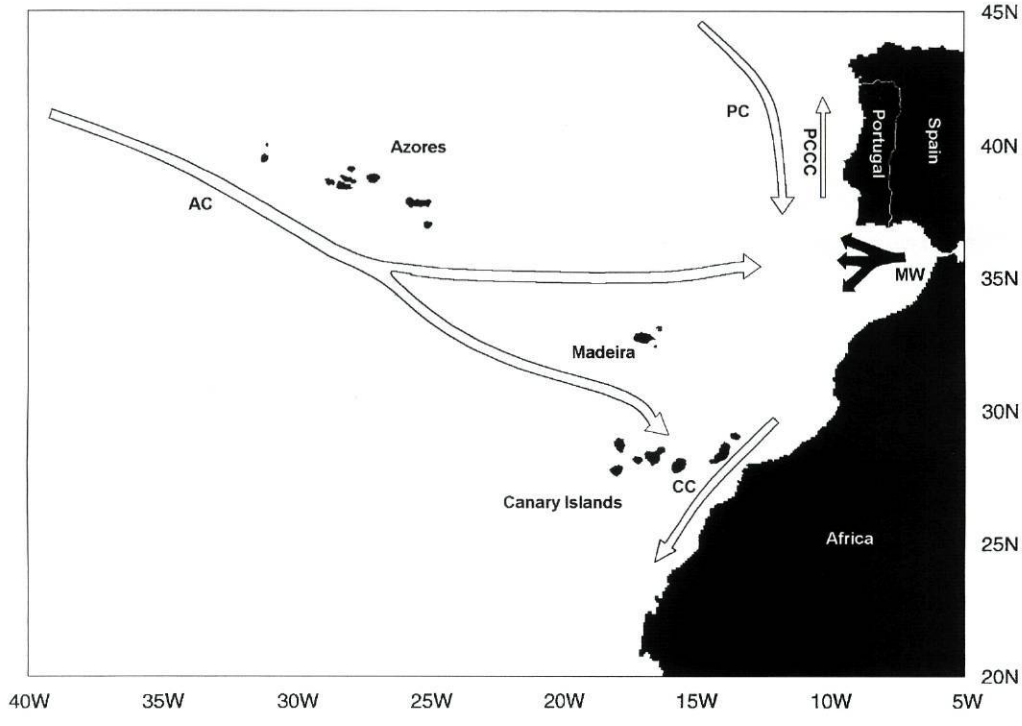

Fig. 10 *Conger conger*. Schematic diagram of the eastern North Atlantic Circulation between the Azores, the Canary Islands and the Strait of Gibraltar (*open* and *solids arrows* represent the surface and deep currents, respectively; *AC* Azores current; *CC* canary current; *MW* Mediterranean water outflow; *PC* portuguese current - summer season; *PCCC* Portuguese coastal counter current - winter season).

Discussion

Conger conger leptocephali were identified by the external body morphology, pigmentation and mainly by counting myomeres. The descriptions of the morphological features and pigmentation patterns of the leptocephali agree well with those made by D'Ancona (1931).

The number of myomeres (TNM, PDM, PAM and LVBV) of the leptocephali is in agreement with the data reported by several authors (D'Ancona 1931; Schmidt 1931; Castle 1970; Strehlow et al. 1998; Correia et al. 2002). However, our values appear to be more consistent than the existent data, probably because of less error in myomere counting. Differences in mean myomere counts are common in the literature. According to Vladykov and March (1975), variations between myomere frequencies could be attributed to several causes: counting technique, different numbers of specimens, variation in size of specimens and difference in collecting localities. Recent studies on American and European eels (Kleckner and McCleave 1985) and also on the European conger eel (Strehlow et al. 1998) show that there is no correlation between the myomere counts and the length of the leptocephali. The most probable cause of the literature discrepancies in myomere frequency distributions of leptocephali is the result of faulty counting techniques (Kleckner and McCleave 1985; Correia et al. 2002).

The position of the LVBV according to Smith (1979) is also a characteristic to be looked at, when identifying leptocephali. Our LVBV values are highly consistent, suggesting that this parameter, like the TNM, can be used for species identification.

The PAM/TNM ratio has been used as a criterion for the metamorphic stage (Tanaka et al. 1987; Correia et al. 2002), since it diminished drastically in the course of metamorphosis as a result of the anus beginning to move to a more anterior position in Congridae leptocephali (Otake et al. 1997; Strehlow et al. 1998). The PAM/TNM ratio obtained for this stage (0.78) is similar to the value (0.77) reported by Strehlow (1992) and nearly double the observed value (0.36) for the metamorphic stage (Correia et al. 2002). The PAM/TNM (and also the PAL/TL) ratio in *C. conger* appears to be almost constant throughout the leptocephalus (or premetamorphic) phase, but diminishes during the metamorphic stage (Correia et al. 2002). Since the PAL/TL ratio is correlated with the PAM/TNM, it has been successfully used in classifying metamorphosing stages in *C. myriaster* (Yamano et al. 1991) and in *C. conger* (Correia et al. 2002). The

PAL/TL has also the advantage of being more easily obtained, namely in specimens poorly preserved (Correia et al. 2002).

The smallest PAM/TNM (PAL/TL) value for the premetamorphic larvae was 0.77 (0.86), and, since the largest PAM/TNM (PAL/TL) observed value for metamorphic larvae was 0.42 (0.55) (Correia, unpublished data), these results suggest that *C. conger* leptocephali began to metamorphose at PAM/TNM (or PAL/TL) values between 0.77 and 0.42 (or 0.86 and 0.55). Lee and Byun (1996) reported, for instance, that *C. myriaster* leptocephali began to metamorphose at PAM/TNM values between 0.82 and 0.74.

The length at which the *C. conger* leptocephali undergo metamorphosis appears to be between 155 and 165 mm, based on the largest reported premetamorphic (Strehlow et al. 1998) and metamorphic leptocephali (Correia et al. 2002), with 165 and 153 mm length, respectively. About 90 % of the larvae had a length between 50 and 90 mm, with an overall mean value of 73.5 mm long, suggesting that the majority of the leptocephali in our collection are in the middle of the premetamorphic leptocephalus stage.

The length-frequency of the conger eel larvae shows one peak around 70 mm, and is positively skewed as a result of the three largest individuals (104, 114, 126.5 mm). These older specimens (224, 275 and 246 days old, respectively) may have a different migration route and environmental history.

The negative correlation between the HL/TL and HL indicates that the head grows more slowly that the whole body, leading at the end of this larval stage to a typically large leptocephalus with a short head.

The relationship between larval/otolith size and age indicates that older specimens are larger and have bigger otoliths, as expected if the somatic growth is correlated with the otolith growth.

The otolith morphology pattern of *C. conger* leptocephali is similar to that observed by Antunes (1994) and described in other anguilliform species (Tabeta et al. 1987; Lecomte-Finiger and Yahyaoui 1989; Wang and Tzeng 1998, 2000). The majority of the specimens presented a width-increment profile identical to the reported by Antunes (1994). However, the two largest specimens presented a peripheral zone in the otoliths with wide rings, i.e. a similar pattern recently described in the countable zone of the metamorphic conger eel sagittae (Correia et al. 2002). These wide increments are unexpected since they have often been associated with the onset of metamorphosis in several anguilliform fishes, namely in *C. myriaster* (Mochioka et al. 1989; Lee and

Byun 1996; Otake et al. 1997), *Anguilla japonica* (Otake et al. 1994; Arai et al. 1997), *A. rostrata* (Wang and Tzeng 1998, 2000) and *A. anguilla* (Wang and Tzeng 2000). Since these large specimens (114.0 and 126.5 mm long), based on their morphology (i.e. on the PAL/TL and PAM/TNM ratios of 0.86-0.87 and 0.77-0.78, respectively), are without any doubt in the premetamorphic larval stage, these results could indicate that the internal signal, marked on the otolith surface, for the beginning of metamorphosis occurs prior to the body morphological changes. However, further studies, namely on otolith Sr:Ca ratios, are needed to validate or refute this hypothesis.

The existing growth curves for anguilliform leptocephali are based on regressions of the length on the estimated age or date of capture. However, they are usually based on few data and much speculation. The somatic growth rate estimated from the linear regression (0.31 mm.day^{-1}) falls within the ranges reported by several authors for other anguilliform species: *A. rostrata*, 0.24 (Kleckner and McCleave 1985), 0.22 (Tesch 1998) and 0.19 mm.day^{-1} (Boëtius and Harding 1985); *A. Anguilla*, 0.15 (Tesch 1998) and 0.18 mm.day^{-1} (Boëtius and Harding 1985); *Anguilla* sp., 0.38 mm.day^{-1} (Castonguay 1987). The growth rate of 0.31 mm.day^{-1}, however, appears somewhat low for the following reasons. (1) The specimen sizes of the leptocephali are underestimates, since they have not been adjusted to account for shrinkage due to the fixation and preservation method, i.e. specimen shrinkage would underestimate the slope and intercept of the linear regression equation. (2) *C. conger* leptocephali undergo metamorphosis between 150 and 160 mm (Strehlow et al. 1998). A leptocephalus growing 0.31 mm.day^{-1} would reach metamorphosis length in about 13 months. However, the conger eel leptocephalus time duration was recently estimated to be between 6 and 9 months (Correia et al. 2002). (3) The predicted size at hatching, 32.9 mm long (Y-intercept), makes no sense when compared with the reported values for related species. Observed size at hatching of experimentally reared larvae of *A. anguilla* (Bezdenezhnykh et al. 1983) and *A. japonica* (Yamamoto et al. 1975) are 2.7 and 2.9 mm, respectively. (4) Finally, the growth of the conger leptocephali probably is not linear through the entire premetamorphic phase. The growth rates of the young leptocephali are comparatively higher than those of older ones, as indicated by the negative correlations between the SGR (and OGR) and age. To assume a simple linear regression is probably not suitable for a leptocephali growth curve.

The larvae of *C. conger* and *C. triporiceps* are very similar in morphology and number of myomeres, in contrast to the adults of both species which differ distinctly

with regard to the number of their sensory pores and in their dentition (Strehlow et al. 1998). The existing distinction of the American conger eel leptocephalus, *C. triporiceps*, from the European conger eel leptocephalus, *C. conger,* based on the catch locations, in the West versus the East-Atlantic (McCleave and Miller 1994; Strehlow et al. 1998) is from our point of view scientifically poor. The aforementioned authors used the Smith's (1989) criterion to identify both larval species; however, Smith's (1989) work only describes the western North-Atlantic conger eel leptocephalus species, i.e. *C. oceanicus*, *C. esculentus*, *C. triporiceps* and leptocephali of Congridae genus A species A, without any reference to its European congener, *C. conger*. In fact, the only study, we know of that describes the larval development of the *C. conger* in detail, and which agrees well with our descriptions of the leptocephalus (present study) and metamorphic stage (Correia et al. 2002) is the work of D'Ancona (1931). From the bibliographic descriptions available, these larvae were very similar, with one important difference: *C. triporiceps* do not have any lateral pigment (Smith, 1989), in contrast to a fully developed leptocephalus of *C. conger*, which presents a series of large dots along the lateral line, from the caudal extremity to a fairy considerable distance from the head (D'Ancona, 1931). According to these two descriptions the western North Atlantic species, *C. triporiceps*, can be easily distinguished from the eastern North Atlantic species, *C. conger*, based on the absence or presence of lateral pigmentation, respectively. To overcome the unequivocal systematic status of the larvae of *C. triporiceps* and *C. conger* with similar TNM, molecular biological investigations should be applied.

The interpretation of the daily increments of the leptocephali is extremely important, because the biological oceanographic study of the early life cycle of these fishes is extremely difficult and expensive. Assuming that the micro-increments are deposited on a daily basis, although the deposition rate of the rings has not been validated directly in this species, age of the conger eel ranged between 71 and 275 days (including the additional 5 days yolk-sac period). These data indicate that hatching dates for the European conger eel varied greatly between early January and late July, with a visible peak in the summer season. This result agrees with observations made from the capture of small leptocephali in the Mediterranean Sea (Schmidt 1931) and from the temporal and spatial distribution of the conger eel larvae in the NE Atlantic (Strehlow et al. 1998).

Naturally spawning conger eels have not yet been observed, and reports about the capture of maturing conger eels are restricted to the Mediterranean (Cau and Manconi, 1983) and to a single female specimen in the Irish Sea (Fannon et al. 1990). It is commonly suggested, in some textbooks, that the European conger eel, *C. conger*, has several different spawning places. Lythgoe and Lythgoe (1971), Bagenal and Kenney (1973) and Wheeler (1985) found that conger eel spawn once during the summer, at great depths (3000-4000 m) in the NE Atlantic, between Gibraltar and the Azores. Spawning areas in the Mediterranean have also been reported by Wheeler (1985). However, these authors did not mention any references to sustain their assumptions. Nowadays, the only spawning area well known for this species is in the central-east basin of the Mediterranean (Cau and Manconi 1983).

It has been suggested that the leptocephalus has a long larval life (Bauchot and Saldanha 1986), taking about 1 or 2 years to drift inshore and to reach the juvenile elver form (Lythgoe and Lythgoe 1971; Wheeler 1985; Strehlow 1992). Recently, Strehlow et al (1998), based on spatial and temporal distribution of conger eel leptocephali identified in oceanographic collections, showed that spawning occurs in the Mediterranean Sea, between July and September. After a short growth period, the larvae (> 30 mm) start migration, around November, in a NW direction, namely to southern Portugal and Spain, extending into the east and central zones of the Atlantic. The conger eel has a second growth period, lasting until the beginning of summer (130-150 mm to a maximum length of 165 mm), after which when they start migration in the direction of the coastal waters of the continental slope, with a possible return to the Mediterranean. It is supposed that this coastal migration induces metamorphosis (Strehlow et al. 1998). The exact timing of metamorphosis, however, remains unknown, since age determination of conger eels by otoliths is difficult due to an uncountable area of the sagittae presumably structured during metamorphosis (Correia et al. 2002). In short, the premetamorphic leptocephali of *C. conger* appear to be restricted to the continental slope (Strehlow et al. 1998; present study), suggesting that the leptocephali do not enter the continental shelf and coastal waters until they have attained the metamorphosing stage, as previously suggested by Correia et al. (2002).

The leptocephali occupy a discrete depth stratum, which changes daily and ontogenetically (Schoth and Tesch 1984; Castonguay and McCleave 1987). Fishing was largely carried out in the upper 200 m of the water column, a depth range typically inhabited by leptocephali (Tesch 1980; Kracht 1982; Schoth and Tesch 1982, 1984;

Kleckner and McCleave 1985; Kajihara et al. 1988; Strehlow et al. 1998), and mainly at night, since net avoidance by anguilliform fishes during daytime has been reported (Schoth and Tesch 1984; Castonguay and McCleave 1987; Tesch and Wegener 1990). As the RMT8 was not equipped with an opening-closing device, only a rough idea of the optimal fishing depth can be presented, and therefore conclusions on exact depth of capture are not possible.

The size and abundance of specimens in a collection may be influenced by gear selectivity, net avoidance, depth range sampled and trawl pattern (Kleckner and McCleave 1985). Number of catch of European conger eel leptocephali in the present study were too low to permit a detailed analysis of the distribution; qualitative data between the different hauls were not fully comparable and could not be used for absolute abundances estimates. However, 62 % of the larvae were caught in four hauls, suggesting the existence of aggregations. Three of these four hauls were performed at depths ranging from 40 to 60 m, i.e. well above the seasonal thermocline. There is a reported correlation between the mesh size and the length of the larvae captured: 500 and 1800 μm mesh nets are appropriate for capturing leptocephali as small as 5 mm long (Wippelhauser et al. 1985) and 10 mm long (Castonguay and McCleave 1987), respectively. The net used in this work had a 4500 μm mesh, which probably does not efficiently catch conger leptocephali smaller than 50 mm long. So, negative stations at night are used only as evidence for absence of larger leptocephali. However, since there is an overall scarcity of leptocephali, negative stations could also easily occur by chance.

As we have already mentioned, it has been suggested that around November the leptocephali of *C. conger*, with 30 mm length, leave Gibraltar and spread westward and northward (Strehlow et al. 1998). The transfer of European conger eel from the spawning area (Mediterranean), via the Strait of Gibraltar, into the Central and NE Atlantic may be partially explained as passive transport based upon known surface currents.

The warm, saline Mediterranean Water (MW) flows into the Gulf of Cadiz through the Strait of Gibraltar and descends to around 800-1200 m. This water then spreads out as the MW tongue into the North Atlantic, southward towards the Canary Islands, westward to the Azores and northward along the Iberian Peninsula (Daniault et al. 1994). Here, the Portuguese Circulation is characterized by an opposing bizonal current pattern that flows parallel to the Iberian coast, a southward coastal upwelling in summer

and a northward flow of the Portuguese Coastal Counter Current in winter (Fíuza, 1984) (Fig. 10).

If the conger leptocephali leave the Strait of Gibraltar in November, as suggested, they could take advantage of the Portuguese Coastal Counter Current, the prevailing flow in the winter, from which larval dispersal could take place in the northwest direction. However, an unsolved question remains: must all the leptocephali leave the Mediterranean toward the continental Iberian slope, or, on the contrary, can they complete the entire larval life cycle inside the Mediterranean? So, the pathways and orientation mechanisms utilized during the larval migration are not fully understood.

In fact, it is difficult to explain how our specimens reached the Azores area from the Mediterranean based on the prevailing NE Atlantic circulation pattern (Fig. 10). The Azores Current (AC) forms from a southern branch of the Gulf Stream system and passes just south of the Azores Archipelago to the east of the Mid-Atlantic Ridge. It then splits into a northern branch along 35°N, which meanders eastward towards the Gulf of Cadiz, and a second branch, which moves southeastward towards the Canary Islands (Käse and Krauss 1996). The eastward main jet of the AC (around 32-33°N at 28°W and 33-34°N at 26°W) with a transport of 26 sv (near 28°W) and mean speed in the order of 10 $cm.s^{-1}$ (at 200 m depth) is associated further west (26°W) with adjacent westward-flowing countercurrents on each side, resulting in recirculation both north (counterclockwise circulation) and south (clockwise circulation) (Pingree 1997). Some of the water that reaches the Gulf of Cadiz is entrained in the Gibraltar outflow of MW, which spreads a depth away from the Strait of Gibraltar (Baringer and Price 1997), or contributes to a poleward-flowing upper layer of Moroccan slope (Pingree 1997). The southern branch of the AC (27°W; 27°N) as it reaches the Canary Archipelago, joins the southward-flowing coastal Canary Current along NW Africa (Klein and Siedler 1989, Johnson and Stevens 2000). In this region water was flowing south-southwest (mean ~3 cms^{-1}) parallel to the African coast to 25°N west of Canary Islands. This flow boundary in the east, near 20°W, marks the eastern limit of water from the southern part of the AC, extending south from Madeira (Pingree, 1997). The spreading of the core of the MW from the Gulf of Cadiz under the AC at 35°N towards the Azores does not quite reach the Azores or Madeira, but south of Madeira flows nearer to the African coast. However, very stable eddies that form in the Gulf of Cadiz can carry cells of MW greater distances from Gibraltar (Johnson and Stevens 2000).

We can conclude that there is little chance that leptocephali, as small as 51.5 mm, leaving the known Mediterranean spawning ground might reach the Azores Archipelago. This is a distance of about 3000 km, which would have to be covered in about $2_{1/2}$ months (estimated age of the 51.5 mm long specimen), and the known North Atlantic oceanographic currents circulate in the opposite direction. This larval migration pathway could be possible by a westerly dispersal of leptocephali, driven by localized currents generated by mesoscale eddies, but only for the oldest specimens, or if the larvae had an oriented and active swimming behavior. These data, however, suggest that the *C. conger* has another spawning area somewhere near the Azores Islands. Future research on conger eel reproductive biology must include extensive sampling of leptocephali in the Azores and Mediterranean Sea and analysis in light of current physical oceanographic knowledge, to provide new insights into spawning and larval migration of European conger eel.

Acknowledgements

This study was financially supported by the "Fundação para a Ciência e Tecnologia" (Research Project PRAXIS XXI:2/2.1/MAR/1728/95). The first author has a PhD grant (BD18082/98) from the same sponsor. We thank Dr. J. Wilson for English language assistance, and to the crew and support stuff of R.V. "Arquipélago" for their assistance.

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3. Otolith features, microstructural growth and Sr:Ca ratios of the conger eel leptocephali

3.1 Changes in otolith microstructure and microchemistry during larval development of the European conger eel (*Conger conger*)

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Abstract

Otolith microstructure and chemical composition (Sr:Ca ratios) of the European conger eel (*Conger conger*) were examined during the larval developmental stages by scanning electron microscopy and wavelength dispersive spectrometer. Back-calculated hatching dates from the otolith microstructure of the developing leptocephali indicate a protracted spawning season from December to June. The early age of our developing specimens captured south of the Azores Islands suggests that the conger eel has another spawning area closer to Azores than the Mediterranean. Otolith increment width, which was relatively constant and narrow in the developing leptocephalus stage, increased sharply at age 170-250 days. Sr:Ca ratios in the otolith, which increased during the developing leptocephalus stage, showed a rapid drop coinciding with the increase in increment width. These coincidental changes were regarded as the onset of metamorphosis for this species. A close linear relationship between the age at metamorphosis and otolith growth rate indicates that the faster-growing larvae metamorphose earlier, suggesting that somatic growth should play an important role in the timing of metamorphosis. As shown in earlier work, the existence of an otolith marginal zone with unclear rings during the metamorphosis prevents an accurate estimate of the larval stage duration of this species.

Introduction

The European conger eel (*Conger conger* L., 1758) is a marine benthic fish widely distributed in the NE Atlantic, being found from Norway to Senegal, as well as in the Mediterranean and western Black Sea (Bauchot and Saldanha, 1986). However, the knowledge about its early life history, e.g. spawning area(s) and season, distribution and migration of leptocephali is limited (Strehlow et al. 1998; Correia et al. 2002; Antunes and Correia 2003).

Schmidt (1931) caught small conger eel larvae in the Sargasso Sea, Mediterranean and NE Atlantic, suggesting a similar migratory behaviour to the European eel (*Anguilla anguilla* L.), i.e. spawning in the Sargasso Sea, following larval transoceanic migration to the European and North African coasts. However, Schmidt's assumption that C. *conger* spawn in the Sargasso Sea was contested by McCleave and Miller (1994). These authors state that the conger eel leptocephali from the Sargasso Sea were *Conger triporiceps*, a species with an overlapping number of myomeres, described later by Kanazawa (1958). Nowadays the leptocephali of both species, i.e. *C. triporiceps* and *C. conger*, are distinguished based on the catch location, either in the West or in the Central and East Atlantic, respectively (McCleave and Miller 1994; Strehlow et al. 1998).

The spawning ground of *C. conger* is presumed to be in the waters south of the Island of Sardinia in the Mediterranean Sea (Cau and Manconi 1983). This study is supported by the length and otolith analyses of leptocephali collected in the North and Central Atlantic Ocean (Strehlow et al. 1998).

It has been suggested that the leptocephali has a long larval life (Bauchot and Saldanha 1986), taking about 1 or 2 years to drift inshore and to reach the juvenile elver form (Lythgoe and Lythgoe 1971; Wheeler 1985; Strehlow 1992). Back-calculated hatching dates from the otolith microstructure of conger eel developing leptocephali indicate a long spawning season, with one annual peak occurring in summer (Strehlow et al. 1998; Antunes and Correia 2003).

The analysis of metamorphosing larval otolith microstructure suggested that the duration of the developing leptocephalus stage is about 6 to 9 months. Unfortunately, otoliths of those metamorphosing larvae show a peripheral diffuse zone with unclear rings, which prevents an accurate estimate of the individual metamorphosis duration (Correia et al. 2002).

The knowledge of the timing and duration of metamorphosis is essential for understanding the migration mechanism, geographical distribution and the early life history strategy of the conger eel. Combination of otolith microstructure and microchemistry has revealed

considerable information about the early life history of several anguilliform fishes, namely the *Anguilla* species (Otake et al. 1994, 1997; Tzeng and Tsai 1994; Cheng and Tzeng 1996; Arai et al. 1997, 1999ab, 2000b, 2001; Wang and Tzeng 1998, 2000; Marui et al. 2001).

In the present study we examine the ontogenic changes in otolith microstructure and microchemistry (Sr:Ca concentration ratios) that occur during the larval development of *C. conger*. These results form the basis of a discussion about the larval stage duration and the coastal recruitment mechanism for this species.

Materials and methods

Fish collection

A total of 31 fishes were used to examine the otolith growth microstructure and microchemistry: 6 developing leptocephali, 20 metamorphosing larvae and 5 elvers (or juveniles), 4 of which were taken from metamorphosing leptocephali reared in the laboratory.

The developing leptocephali were collected in August 2000 during the cruise of R.V. "Heincke" conducted in the NE Atlantic Ocean (Fig. 1). All larvae were taken with a Young fish trawl (mesh size stretched: 11mm) between the surface and 800 m depth during the night. Water temperature and salinity of the sampling area ranged from 9.3 °C to 24.2 °C and from 34.7 to 36.6 psu, respectively.

The metamorphosing specimens were collected by the glass eel fishery at the mouth of the Minho River (41°N; 08°W; see also Fig. 1), north of Portugal, in April 2000. Fishing, using a stow net, took place in the estuarine area during the night, in the period of the new moon during the flood-tide current (Antunes 1994). The water temperature and salinity in this area ranged from 12.2 °C to 12.4 °C and from 4.6 to 29.8 psu, respectively.

Four metamorphosing conger eels also collected in the Minho River in April 2001, were reared in a 100 l aquarium, under a natural photoperiod and with a water temperature and salinity of 15.0 °C and 31.0 psu, respectively. They successfully completed metamorphosis in about 2 months. We determined the end of the metamorphosis according to the morphological description of D'Ancona (1931). A small conger eel captured by a fisherman on a sandy beach (Praia da Aguda, north of Portugal, about 100 Km south of the Minho River) in November 2000 has also been used.

The external body morphology, pigmentation and myomere counts were used for species identification (Correia et al. 2002). Measurements were made to the nearest 0.1 mm using an

ocular micrometer in a binocular dissecting microscope. Measures and counting procedures described by Smith (1989) were adopted. In elvers, only lengths were measured because their myomeres were not clearly visible. The six developing leptocephali were preserved in 70% ethanol, before the fish measurements. However, the shrinkage caused by the preservation method was not corrected. The ratio of the preanal myomeres to total number of myomeres (PAM/TNM) and the ratio of the preanal length to total length (PAL/TL) were used to define the developmental stage of the conger eel larvae (Tanaka et al. 1987; Yamano et al. 1991; Lee and Byun 1996; Otake et al. 1997; Strehlow et al. 1998; Correia et al. 2002).

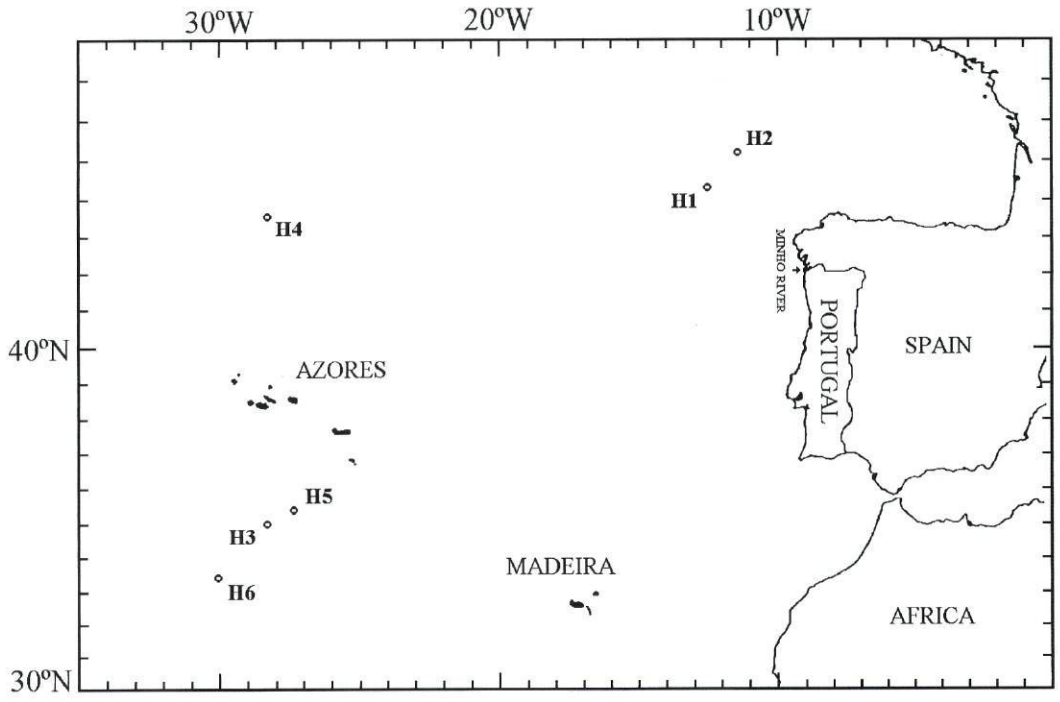

Fig. 1 *Conger conger*. Horizontal distribution of the six conger eel developing leptocephalus collected by the R.V. "Heincke" in the NE Atlantic in August 2000 (*H1-H6* specimens, see Table 3 for details).

Otolith preparation

Sagittal otoliths were extracted, cleaned and embedded in epoxy resin. The otoliths were then ground by hand through the sagittal section with 600, 1200 and 2400 silicon carbide abrasive paper to expose the core. After that, they were polished with 6, 3 and 1 μm diamond pastes and finally with alumina solution (1:20) on an automated polishing wheel (Struers, Planapol V). The surface of the otolith to be examined must be highly polished to prevent large diffractions of x-rays and subsequent analytical error (Radtke 1989). Finally, they were

cleaned with absolute ethanol in an ultrasonic bath for 5 minutes and rinsed with deionised water, prior to analysis.

Otolith x-ray analysis

For electron microprobe analysis the otoliths were gold coated in a high vacuum evaporator. Strontium (Sr) and calcium (Ca) concentrations (% dry weight) were measured along the longest axis of each otolith using a wavelength dispersive x-ray electron microprobe (CAMEBAX SX 50). Accelerating voltage and beam current were 15 kV and 10 nA, respectively. The electron beam was focused on a point approximately 2 μm in diameter, with intervals of 5 and 10 μm, for the developing/metamorphosing and elvers specimens, respectively. The counting time was 60 s (30 s per element, 20 s for the measurement of the counts in the corresponding peak and 10 s for measuring back ground contribution). Apatite [$Ca_5(PO_4)_3$] and celestite ($SrSO_4$) were used as standards. The intentional bombardment by an electron beam with increased absorbed current voltages created a slight burn depression at the sampled location, serving as a convenient marker (Tzeng and Tsai 1994). Thus, microprobe measurements points, which were seen as burn depressions on the otolith surface (Fig. 4B), were assigned to otolith growth increments. The results are presented as the amount of Sr divided by the amount of Ca times 1000. The averages of successive data for Sr and Ca concentrations pooled for every 10 successive growth increments were used for the life-history transect analysis.

Otolith increment analysis

Following electron microprobe analysis, the otoliths were repolished with alumina solution (1:20) to remove the coating, etched for 5-10 s with 0.05 M HCl and vacuum coated with gold for scanning electron microscope observation (SEM, Jeol JSM 630-1F) at 15kV.

In most marine fishes, the deposition of the first daily increment occurs during their first exogenous feeding when larvae have completed yolk-sac absorption (Lough et al. 1982; McGurk 1984; Tzeng and Yu 1988). In the Japanese eel the yolk sac was completely absorbed 4-6 days post-hatching (Umezawa et al. 1989). Like others leptocephalus age estimates (Tzeng 1990; Tzeng and Tsai 1992; Cheng and Tzeng 1996; Wang and Tzeng 1998), 5 days were added to the number of daily increments, although no increments were deposited in the core during the yolk-sac stage.

We assumed the growth increment in the larval otolith of conger to be daily, although daily deposition has not been validated in this species. We base this assumption on the results of several related anguilliform species, e.g. *Conger myriaster* (Mochioka et al. 1989), *Anguilla japonica* (Tsukamoto 1989; Umezawa et al. 1989; Umezawa and Tsukamoto 1991), *A. rostrata* (Martin 1995), *A. celebesensis* (Arai et al. 2000a) and *A. marmorata* (Sugeha et al. 2001), which have been shown to have daily depositions.

All otolith measurements were carried out according to the procedures of Correia et al. (2002). The otolith radius and increment width were measured along the maximum otolith radius. Averages widths of every ten successive increments, from the first feeding check (FFC) to the end of the increment countable zone (ICZ) were used for otolith growth analysis (Fig. 2).

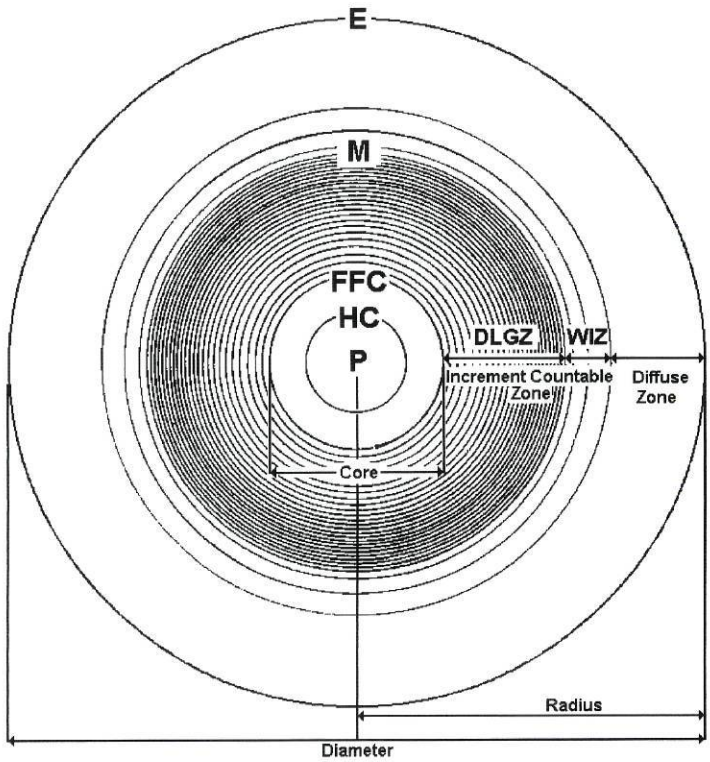

Fig. 2 *Conger conger*. Schematic diagram of the distinct zones and measurements in the otoliths of the metamorphosing leptocephalus (*P* primordium; *HC* hatch check; *FFC* first feeding check; *M* metamorphosis; *E* edge; *DLGZ* developing leptocephalus growth zone; *WIZ* wide increment zone).

Based on previous data on otolith microstructure and Sr:Ca concentration ratios in several anguilliform leptocephali (see "Discussion"), the age at metamorphosis was calculated from the number of daily growth increments in the otolith of the metamorphosing larvae, counted from the outer core (FFC) to the otolith point where otolith increment width suddenly became

wide and the Sr:Ca ratios drastically decrease (onset of metamorphosis), including the additional 5 days yolk-sac larval stage.

The duration from the beginning of metamorphosis to the time of capture, and thus the total age of the larvae, was impossible to establish, because increments are not visible in the diffuse zone (DZ) of the metamorphic otoliths (Correia et al. 2002; present study).

Statistical analysis

All statistical analyses have been carried out according to the procedures described in Zar (1996). Differences among the three larval stages for some otolith parameters (core diameter and core Sr:Ca concentration ratios) were examined by a nonparametric test (Kruskal-Wallis). Significance of the correlation coefficient and regression slope were tested, respectively, by a Fisher's Z-transformation and by an analysis of variance (ANOVA). We have used a level of significance (α) of 0.05. Data are presented as ranges and mean values (± standard deviation).

Results

Size and developmental stage

The morphometric and meristic values obtained for the developing/metamorphosing leptocephali and elvers are presented in Tables 1 and 2, respectively.

The length of the six developing leptocephali ranged from 49.0 to 96.0 mm. In these specimens the PAM/TNM (PAL/TL) values kept an almost constant value of 0.80±0.01 (0.86±0.01). The length of the 20 metamorphosing conger eels ranged between 113.0 and 142.0 mm, with a mean of 126.8±6.8 mm. However, for this metamorphic stage the PAM/TNM (PAL/TL) ratio ranged from 0.32 to 0.48 (0.41-0.54) and presented an average value of 0.41±0.04 (0.48±0.04). The four laboratory-reared larvae had a mean length at capture of 133.0±8.7 mm and an elver size of 88.4±5.8 mm at the end of metamorphosis. The developmental stage (PAL/TL) of the metamorphosing leptocephali at the beginning of the rearing experiment was 0.48±0.01. For the elvers (including the wild-caught specimen) the PAL/TL presented a mean value of 0.37±0.01.

Otolith microstructure

Otoliths in the developing leptocephali were circular, and the growth increments were visible in all sections from the core to the edge (Fig. 3A). The core, constituted of well-calcified substances arranged in a radial form, was observed as a deep hole in the otolith. The primordium located in the core centre was substantially composed of an organic material and appeared as a deep hole after HCl etching. Two rings delimited the core area. The first, being visible as a deep circular groove surrounding the hole, corresponds to the hatching (hatch check, HC). The second deeply etched ring, which separated the first growth increment and the core, probably marks the first feeding of the larva (first feeding check, FFC). These checks were postulated to be the HC and the FFC, since their morphology was similar to those in other anguilliform fishes (Lecomte-Finiger and Yahyaoui 1989; Antunes 1994; Wang and Tzeng 1998, 2000; Arai et al. 2001). Outside this second check, in an outwards direction, daily growth increments were observed (Fig. 3B). The otolith diameter and radius of the six developing leptocephali ranged from 96 to 226 μm and from 56 to 153 μm, respectively.

The otoliths of metamorphosing leptocephali, with a less rounded shape, presented other permanent structures, like a peripheral diffuse zone (DZ), where the rings were unclear, and some accessory growth centres (AGC) (Fig. 4A). The otolith´s increments countable zone (ICZ) presented an inner portion similar to that of the developing leptocephali (developing leptocephalus growth zone, DLGZ); however, its final portion presented a series of wide rings (wide increments zone, WIZ) that became less clear and disappeared when entering the DZ (Fig. 4B). The diameter and radius presented average values of 361±36 and 210±20 μm, respectively. The radial distance from the primordium to the end of the DLGZ ranged between 90 and 161 μm, and was close to the above-mentioned maximum otolith radius (153 μm) in developing leptocephali.

A clear change in otolith microstructure of the conger eel was observed in the elver stage (Fig. 5A). The ground and etched otoliths of the young juveniles were elliptical; the shape changed with growth. The growth of otoliths in the anterior-posterior direction was faster than in other directions. The microstructure of the otolith from the primordium to end of the DZ was also similar to that of the otoliths of metamorphosing eels (Fig. 5B). Several growth checks were observed in the otolith. The otolith diameter and radius of the reared conger eels presented mean values of 1289±89 μm and 714±53, respectively. The wild young conger eel presented a diameter and radius of 833 and 420 μm, respectively.

Table 1 *Conger conger*. Morphometric and meristic characters of the developing (n=6)/metamorphosing (n=20) conger eel leptocephali

Parameter	Abbreviation	Range (mm)	Mean±SD (mm)	Mode
Total length	TL	49.0-96.0/113.0-142.0	70.2±20.7/126.8±6.8	
Predorsal length	PDL	44.0-57.0/32.0-57.0	50.5±9.2/44.2±6.9	
Preanal length	PAL	43.0-82.0/49.0-68.0	60.5±17.5/60.3±5.6	
Head length	HL	3.8-6.1/7.8 -9.3	4.8±1.1/8.5±0.4	
Body depth	BD	4.0-9.0/6.4-9.4	6.2±1.9/7.4±0.7	
Eye diameter	ED	0.8-1.8/1.8-2.2	1.2±0.4/2.0±0.1	
Total number of myomeres	TNM	156-161/154-160		158/156
Last vertical blood vessel	LVBV	59-61/-		61/-
Predorsal myomeres	PDM	77-79/33-60		-/49
Preanal myomeres	PAM	121-126/50-75		126/61

Table 2 *Conger conger*. Sampling date, length, otolith size and developmental stage of the elvers used in this study. Note: for the four reared elvers, MA01, the lengths were final size

Specimen	Sampling Date	Total Length (mm)	Preanal length/ total length	Otolith radius (μm)	Otolith diameter (μm)
AGUN00	13 Nov 2000	210.0	0.38	420	833
MA01(12)	26 Apr 2001	88.5	0.38	712	1300
MA01(14)		82.0	0.35	689	1222
MA01(16)		87.0	0.37	667	1222
MA01(17)		96.0	0.38	789	1411

Table 3 *Conger conger*. Length, otolith radius, sampling date, location, age and hatching date of the leptocephali collected by the R.V. "Heincke" in August 2000 (*, specimens used for electron microprobe analysis)

Specimen	Sampling Date	Catch position	Total length (mm)	Otolith radius (μm)	Age (days)	Hatching date
H6*	11 Aug 2000	33°56'N; 30°08'W	49.0	56	69	4 Jun 2000
H5*	12 Aug 2000	35°39'N; 27°39'W	54.0	67	81	24 May 2000
H3	12 Aug 2000	35°00'N; 28°36'W	54.0	60	85	20 May 2000
H4*	4 Aug 2000	43°57'N; 28°32'W	76.0	93	163	24 Feb 2000
H1*	16 Aug 2000	44°29'N; 12°59'W	92.0	139	240	21 Dec 1999
H2*	17 Aug 2000	45°11'N; 11°46'W	96.0	153	260	2 Dec 1999

There was a good relationship between the diameter (D) and radius (R) of the otolith for the developing leptocephali (r^2=0.99, n=6, P<0.05), metamorphic larvae (r^2=0.66, n=20, P<0.05) and elver specimens (r^2=0.99, n=5, P<0.05). There was a good relationship between the otolith size (D and R) and the fish length for the developing stage (r^2=0.96, n=6, P<0.05 and r^2=0.97, n=6, P<0.05, respectively); however this correlation did not exist during the metamorphosing stage (r^2=0.08, n=20, P=0.22 and r^2=0.00, n=20, P=0.74). There was no correlation between otolith size, expressed as D (r^2=0.20, n=6, P=0.36) or R (r^2=0.21, n=6, P=0.36) and the PAL/TL ratio of the developing-stage leptocephali. During metamorphosis, PAL/TL was negatively correlated with D (r^2=0.20, n=20, P<0.05), but not with R (r^2=0.02, n=20, P=0.53). The cores presented an overall mean value of 21.9±1.4 μm, and no significant differences were found among the different larval stages (P>0.05).

Otolith growth and Sr:Ca ratios change patterns

In the otoliths examined, changes in the Sr:Ca concentration ratios were due mainly to the variation in the amount of Sr, since the Ca content was stable during the larval development (30.8±3.9 %).

Patterns of change in the otolith increment widths and Sr:Ca ratios in the developing leptocephali along the life history transect, from the core to the edge, are shown in Fig. 6. Otolith increment widths increased between the FFC and age 20-40 days, thereafter gradually decreasing and becoming constant toward the edge. The average increment width was 0.54±0.19 μm. Otolith Sr:Ca ratios tended to rise from the core toward the edge. A slight drop in the ratio was found from age 20-60 d. The minimum ratio was recorded in the core (7.1×10^{-3}), with the maximum values ($10.2\text{-}24.0\times10^{-3}$) occurring in the outermost regions.

Figure 7 shows patterns of change in otolith increment width and Sr:Ca ratios along the life history transect of the metamorphosing larvae. The patterns were characterized by drastic changes in both increment width and Sr:Ca ratios in the outer region of the otolith. Otolith increment widths and Sr:Ca ratios showed profiles similar to those in the developing leptocephali. However, in the final portion of the ICZ, otolith increment width sharply increased (WIZ) at the age of 170-250 days, until the microincrements disappeared in the otolith DZ. The mean number of increments in the DLGZ and ICZ was 215±24 and 256±28, respectively. The duration between the onset of width increase and maximum peak (i.e. the number of daily increments in the WIZ) was 39 to 91 d (45±14 days).

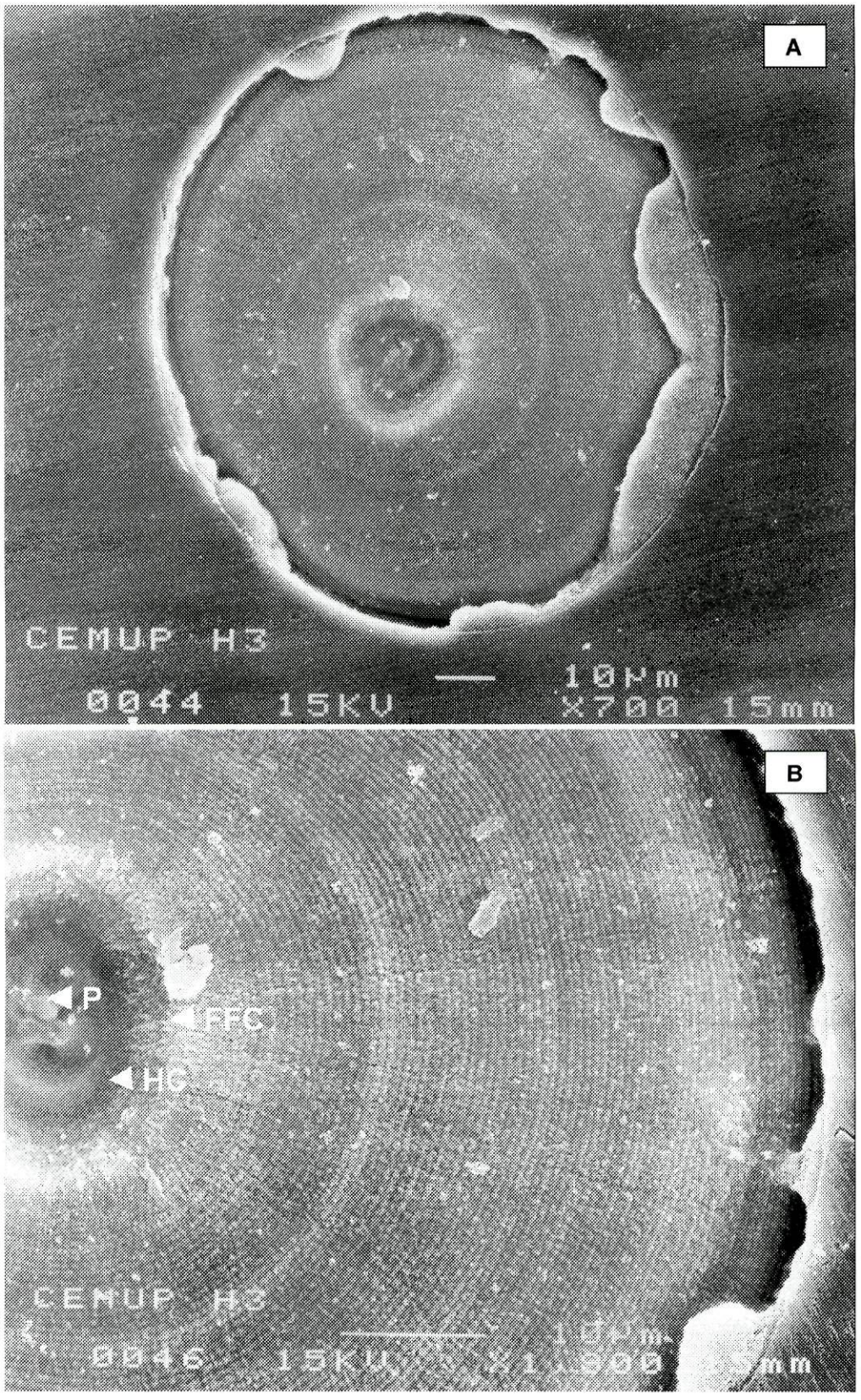

Fig. 3A, B *Conger conger*. SEM microphotographs showing the otolith microstructure of a developing stage leptocephalus (54.0 mm length): **A** whole view; **B** radius (*P* primordium; *HC* hatch check; *FFC* first feeding check; *white dots* daily growth increments).

Sr:Ca ratios reached a maximum level (20.9×10^{-3}) at the end of the otolith DLGZ, decreasing abruptly thereafter, simultaneous with the increase in growth increment width (onset of metamorphosis). The minimum value was recorded in the core (7.1×10^{-3}). After the metamorphosis the Sr:Ca ratios decreased, but never reached the minimum values obtained in the core. The pattern of the changes in Sr:Ca ratios of the otoliths was consistent between the five elvers and was similar for both laboratory-reared and field-caught conger eels (Fig. 8A, B). Sr:Ca concentration ratios were lowest in the primordium (6.3×10^{-3}) and at the edge of the otolith ($4.7\text{-}6.1\times10^{-3}$) in elvers. Profiles of the increment widths and Sr:Ca ratios along the otolith ICZ of the elvers were similar to those observed in the metamorphosing eels (Fig. 8C). No significant differences were found in mean Sr:Ca concentration ratios in the core among different larval stages ($P>0.05$). The core Sr:Ca ratio presented an overall mean value of 7.0×10^{-3}.

Age and hatching time

The ages of the six developing leptocephali ranged from 69 to 260 days (Table 3). Conger eel hatching dates, back calculated from estimated daily ages, indicated that the spawning season lasted 7 months (from early December 1999 to end of June 2000). A strongly significant correlation was found between otolith size (D and R) and age of the developing leptocephali ($r^2=0.98$, $n=6$, $P<0.05$ and $r^2=0.98$, $n=6$, $P<0.05$, respectively). There was also a linear relationship between length and age of the developing leptocephali ($r^2=0.99$, $n=6$, $P<0.05$).

Age at metamorphosis

Based on previous data on otolith microstructure and Sr:Ca ratios in some anguilliform fishes, namely the *Anguilla* species (see "Discussion"), the age at the otolith point where the increment width showed a drastic increase coincidental with a marked decrease in the Sr:Ca ratios (Figs. 2, 4B and 6) was regarded as the onset of metamorphosis for this species (i.e. age at metamorphosis). The duration of the developing leptocephalus stage ranged between 170 and 250 days, with an average value of 215±24 days. The age at metamorphosis was negatively correlated with the mean increment widths of the DLGZ ($r^2=0.56$, $n=20$, $P<0.05$) (Fig. 9).

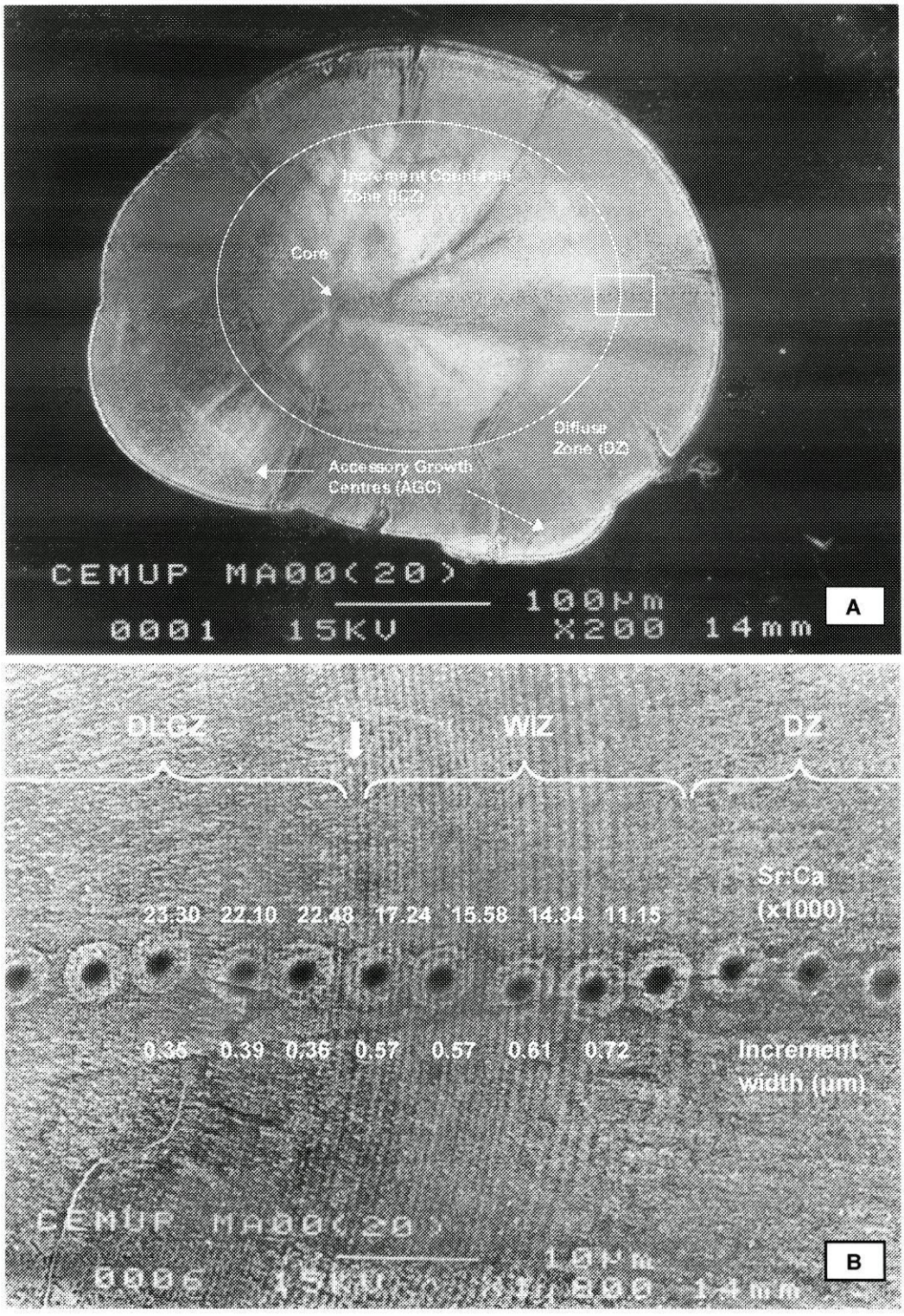

Fig. 4A, B *Conger conger*. SEM microphotographs showing the otolith of a metamorphosing leptocephalus (length: 117.0 mm; preanal length/total length: 0.50): **A** Whole view showing the distinct otolith zones, **B** Detail of the otolith zone indicated in a box in A (*DLGZ* developing leptocephalus growth zone; *WIZ* wide increment zone; *DZ* diffuse zone). Note: the *numbers above and below* burn depressions indicate the Sr:Ca ratios and increment width values, respectively. The *arrow* represents the onset of metamorphosis. See also Fig. 2

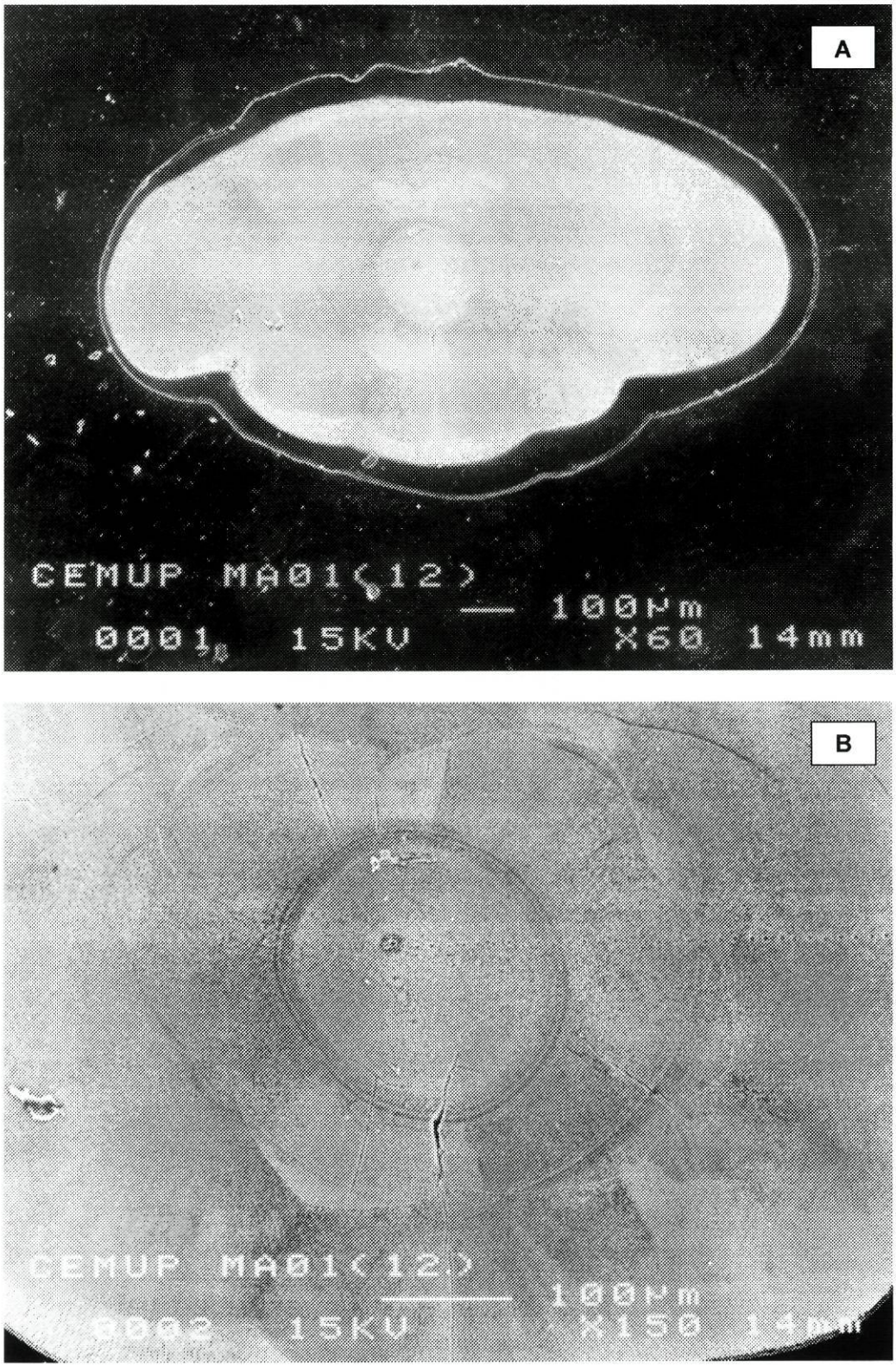

Fig. 5A, B *Conger conger*. **A** Elliptical shape of the otolith of an elver (total length: 85.5 mm); **B** Detail of the central enlarged zone.

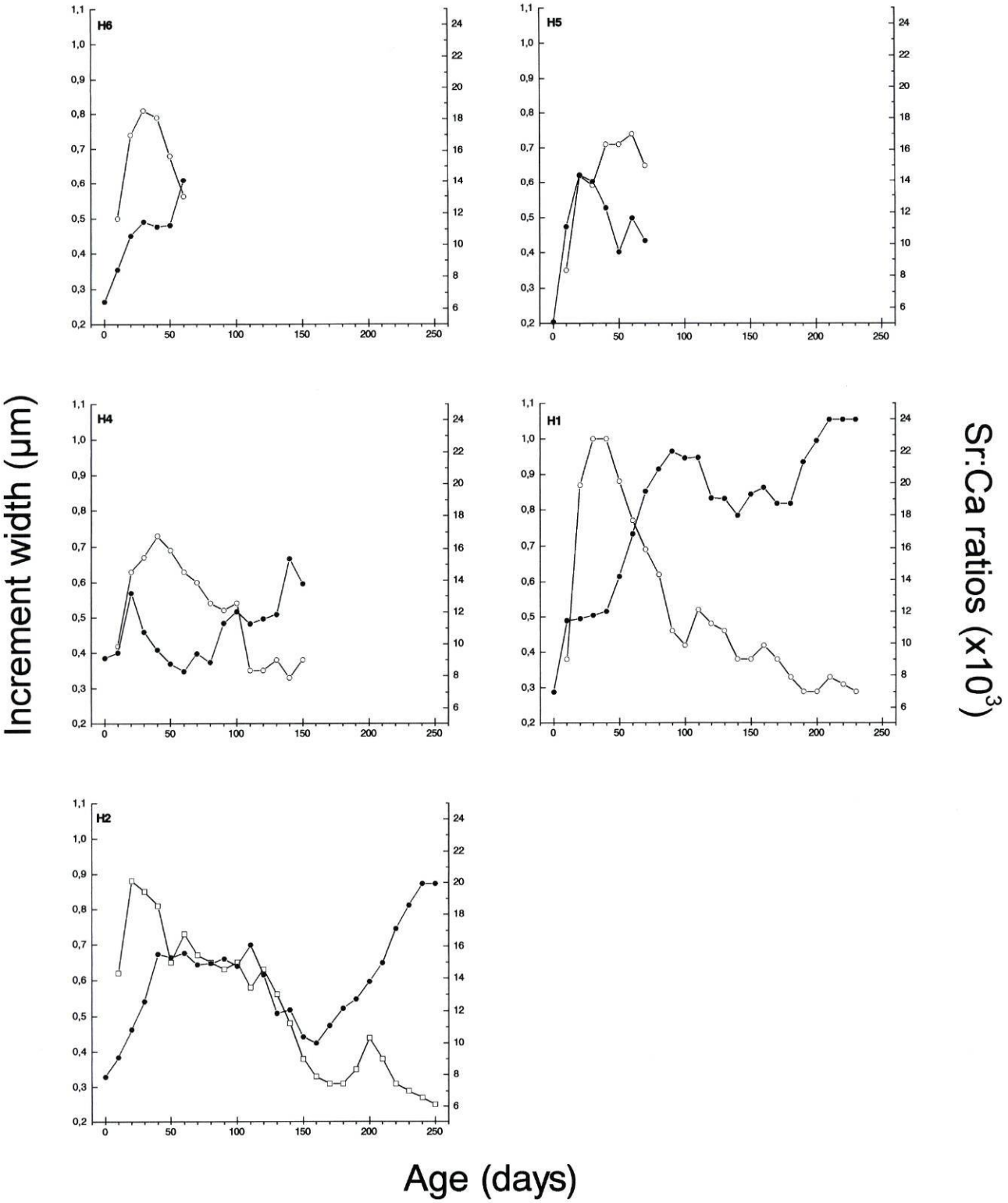

Fig. 6 *Conger conger*. Profiles of the otolith increment width (*open circles*) and Sr:Ca concentration ratios (*closed circles*) measured from the core (age 0) to the edge in the developing leptocephali. *Each point* represents the average of data for every 10 days (*H1-H6* specimens, see Table 3 for details).

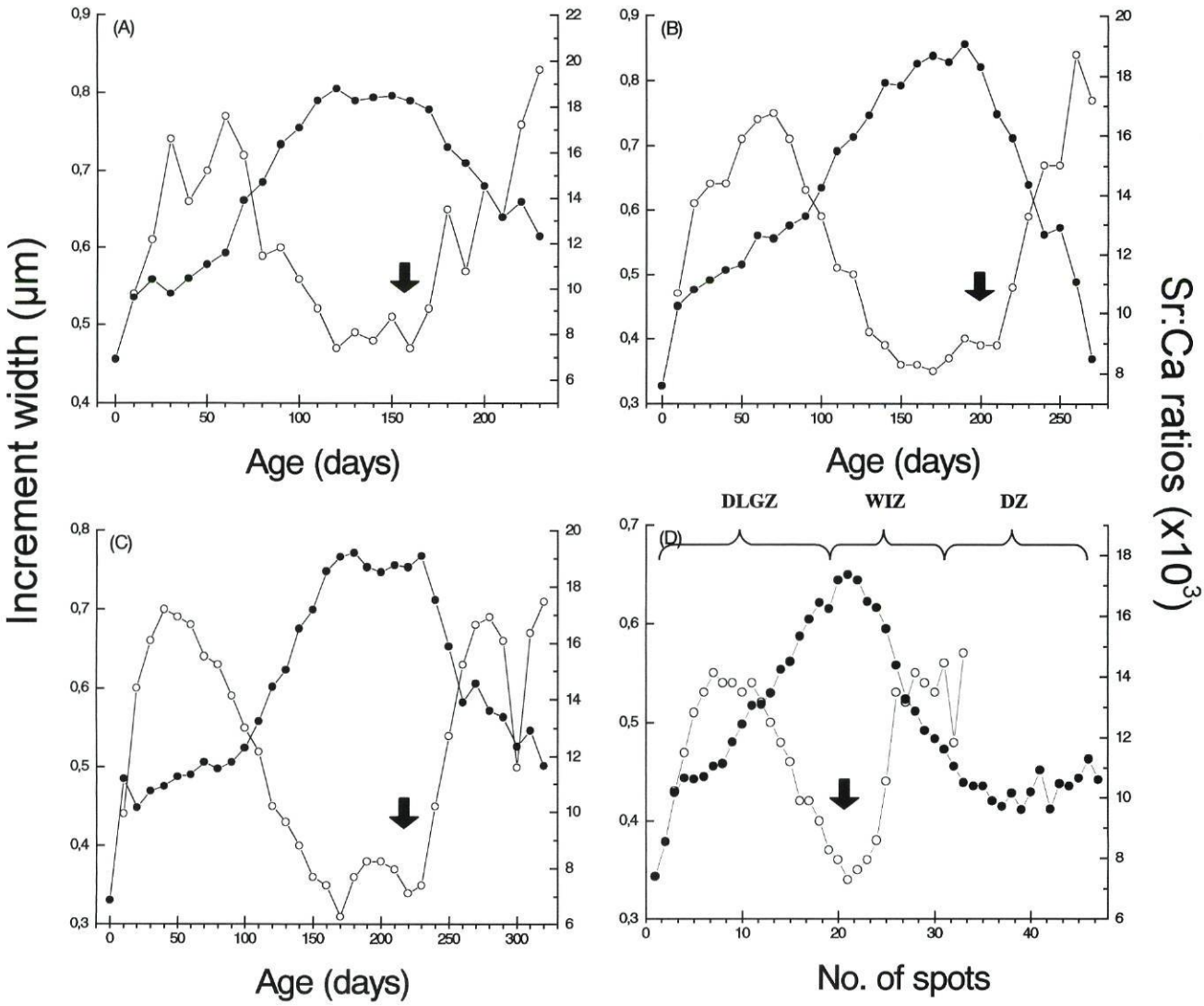

Fig. 7A-D *Conger conger* metamorphosing larvae. **A-C** Profiles of the otolith increment width and Sr:Ca concentration ratios measured from the core (age 0) to the end of the increment countable zone. Specimens were grouped by the time when coincidental changes in increment width and Sr:Ca ratios occurred (**A**: 170-190 days, n=4; **B**: 200-220 days, n=7; **C**: 230-250 days, n=9). **D** Mean values for width increment and Sr:Ca ratios for total specimens (n=20), measured from the core to the otolith edge (*DLGZ* developing leptocephalus growth zone; *WIZ* wide increment zone; *DZ* diffuse zone; *black arrows* approximate locations of the metamorphosis). See also Figs. 2 and 4

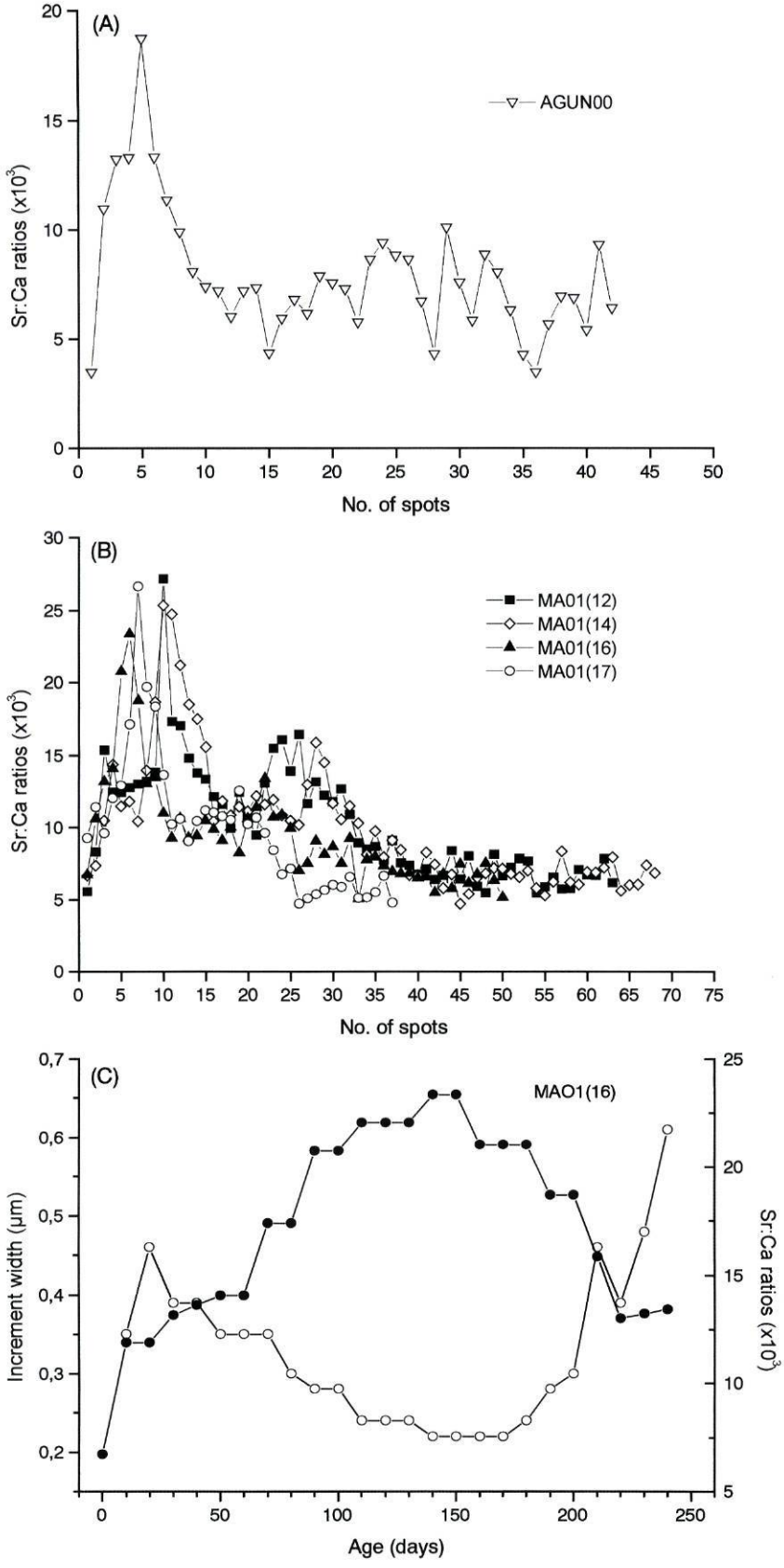

Fig. 8A-C *Conger conger*. **A, B** Sr:Ca concentration ratios measured from the primordium to the otolith edge, respectively, for the wild and reared elvers specimens. **C** Changes in otolith increment width (*open circles*) and Sr:Ca ratios (*closed circles*) in the increment countable zone of one elver (*abbreviations*, see Table 2).

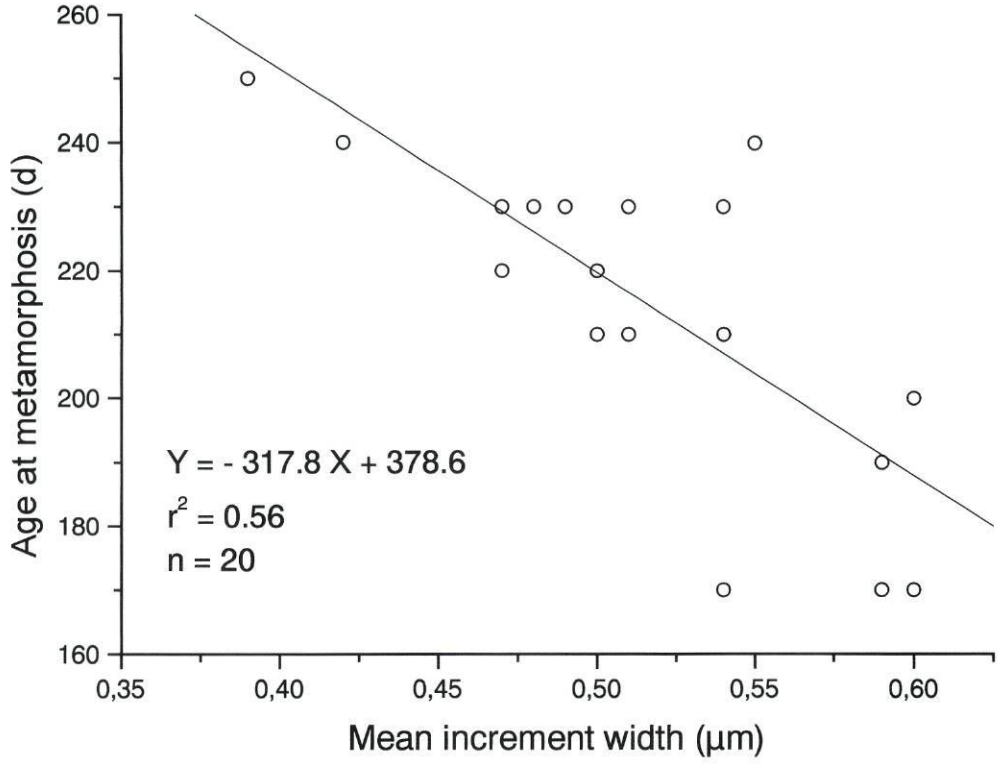

Fig. 9 *Conger conger* metamorphosing larvae. Scatter diagram of age at metamorphosis versus otolith mean increment width. Regression line represents a least-square fit of the linear equation ($P<0.05$).

Discussion and conclusions

The number of myomeres of the conger eel larvae was counted for species identification and was in agreement with the data reported by several authors (D'Ancona 1931; Schmidt 1931; Castle 1970; Strehlow et al. 1998; Correia et al. 2002). The external body morphology and pigmentation pattern of the conger eel leptocephali has been described in detail by D'Ancona (1931) and Correia et al. (2002). The PAM/TNM ratio has been adopted as a criterion for differentiating the developmental stages of conger eel leptocephali (Tanaka et al. 1987; Lee and Byun 1996; Otake et al. 1997; Strehlow et al. 1998; Correia et al. 2002), because it is difficult to define the developmental stages of conger eel larvae from the body proportion, external morphology and pigmentation alone (Tanaka et al. 1987). The PAL/TL index has been used with the same purpose, since it is correlated with the PAM/TNM ratio (Yamano et al. 1991; Otake et al. 1997; Correia et al. 2002), and has the advantage of being more easily obtainable and less affected by counting errors (Correia et al. 2002). The PAL/TL and PAM/TNM mean values obtained for the developing leptocephali (0.86 and 0.80, respectively), metamorphosing larvae (0.48 and 0.41, respectively) and elvers (0.37) where

within the ranges recorded by other authors (Strehlow 1992; Correia et al. 2002). It has been recently suggested that *Conger conger* leptocephali should begin to metamorphose at PAL/TL (or PAM/TNM) values between 0.86 and 0.55 (or 0.77 and 0.42) (Correia, unpublished data). Lee and Byun (1996) reported, for instance, that *C. myriaster* leptocephali began to metamorphose at PAM/TNM values between 0.82 and 0.74.

The otolith morphology of *C. conger* leptocephali has already been described during its developing (Antunes 1994; Antunes and Correia 2003) and metamorphosing stages (Correia et al. 2002), and is similar to that observed in other anguilliform species (Lecomte-Finiger and Yahyaoui 1989; Antunes 1994; Wang and Tzeng 1998, 2000; Arai et al. 2001). Assuming that the increments are deposited on a daily basis, ages of the developing leptocephali were estimated as 69-260 days old after hatching. The estimated hatch dates, back calculated from the sampling dates and ages of the developing leptocephali, ranged from December 1999 to June 2000. Antunes and Correia (2003) reported that the hatching season occurs from April to October. The estimated values for the hatching season were slightly different among these studies, all suggesting an extended spawning season for the conger eel. A long spawning season has been reported in the Japanese eel (Tabeta et al. 1987; Tsukamoto 1990; Tsukamoto and Umezawa 1990), and it has been proposed that this might be due to multiple populations of adult *Anguilla japonica* prolonging the duration of the spawning season (Tsukamoto 1990).

The spawning ground, ecology and migration of leptocephali in the ocean are poorly known at present. Strehlow et al. (1998), based on spatial and temporal distribution of conger eel developing leptocephali collected during several oceanographic cruises, suggested that spawning occurs in the Mediterranean Sea, between July and September. Around November, after a short growth period, the larvae (> 30 mm) start a migration in a NW direction, namely to southern Portugal and Spain, spreading throughout the eastern and central zones of the Atlantic. Here, the conger eels experience a new period of growth, lasting until the beginning of summer (130-150 mm and a maximum of 165 mm length), at which time they resumes migration. It is supposed that this bout of migration, in the direction of the coastal waters of the continental slope, with a possible return to the Mediterranean, induces metamorphosis. However, it is difficult to explain how some specimens reached the Azores area based on this larval migration pathway.

Let us assume, for instance, that our smaller developing specimens collected south of Azores (49.0, 54.0 and 54.0 mm long and 69, 81 and 85 days old, respectively) were in fact born in the Mediterranean Sea (Cau and Manconi 1983). They would have had about 2-3 mo to travel approximately 3000 km from the spawning ground to the catch location. This means

that these larvae would have to swim about 35-44 km day^{-1} against the prevailing circulation pattern of the NE Atlantic (Klein and Siedler 1989; Käse and Krauss 1996; Johnson and Stevens 2000). If conger eel leptocephali migrate actively and directly by, for example, 10 Km day^{-1}, as proposed in the *A. anguilla* larvae (Antunes and Tesch, 1997), they would only be able to cover a distance of about 690-890 Km from the spawning ground. Based on the age of our smaller developing leptocephali and the water current systems of Azores, we propose that conger eel might also spawn in or near the Azores Archipelago. However, further information on the geographical distribution, age and growth of *C. conger* is necessary in order to determine the spawning area(s) of this species and its migration pathways to the European coast and to Azores.

Several studies have indicated that past environmental history of anguilliform fishes can be reconstructed from analysis of otolith Sr:Ca concentration ratios. Changes in otolith Sr:Ca ratios have been considered to be related to environmental factors such as water temperature (Tzeng 1994), salinity (Tzeng and Tsai 1994) and water mass (Otake et al. 1994), as well to endogenous physiological factors (Tzeng 1996; Otake et al. 1997; Arai et al. 1997).

The Sr:Ca concentration ratios and their variation in conger eel larvae are similar to those reported in other anguilliform leptocephali (Otake et al. 1994, 1997; Tzeng 1994, 1996; Tzeng and Tsai 1994; Arai et al. 1997, 1999ab, 2000b, 2001; Wang and Tzeng 1998, 2000; Marui et al. 2001). Sr:Ca ratios content were lowest in the primordium and at the edge of the otolith. Some authors (Tzeng and Tsai 1994; Wang and Tzeng 2000) suggested that the low Sr:Ca ratio in the otolith primordium of the temperate eels (*A. Japonica* and *A. rostrata*) is probably due to the freshwater maternal origin of the yolk sac. Our results, in conjunction with the data obtained by Otake et al. (1997) for the *C. myriaster*, shows that this feature is not restricted to the catadromous species, as it appears to be common in all anguillid leptocephali, including entirely marine species. We think that the low Sr content in the core reflects its chemical composition, since it is substantially composed of an organic material, probably fibroprotein otolin, instead of the typical aragonite matrix. Several investigators (Tzeng 1994; Tzeng and Tsai 1994; Wang and Tzeng 2000) also proposed that the drop in Sr in the eel at and after metamorphosis reflect the sudden change in salinity of the environment of the migratory eel (i.e. the entry into the freshwater habitats less rich in Sr). In the case of conger eel, however, we agree with Otake et al. (1997) who have proposed that the drastic decrease in Sr in the otolith matrix during late metamorphosis reflects the mobilization of body minerals for rapid bone development.

The change in the pattern of otolith growth increments in the metamorphosing eels, as previously described by Correia et al. (2002), could be divided into four phases. The first phase was characterized by a pronounced increase in the width of the daily rings (until a maximum of 0.80 μm), from the FFC to about 30 days. A second phase shows a gradually decreasing increment width, until a relatively constant minimum value (0.35-0.45 μm) is reached, at about 160-180 days. This period of narrow rings can be quite extensive (170-250 days), depending on the specimen. These two phases overlap with those of the developing leptocephali. In a third phase, the increment width changes dramatically and becomes wide (to a maximum of 0.65-0.90 μm) and less clear, until the microincrements disappear, at about 200-320 days, when entering into the fourth phase (diffuse zone). These last two fluctuations are not found in the otolith of the developing leptocephali, in which increment widths remained narrow throughout. Furthermore, Sr:Ca concentration ratios in the otolith show a drastic decrease coincidental with the beginning of the third phase of otolith incrementation, whereas the Sr:Ca ratio does not drop in the larger developing leptocephali.

There is no clear correlation between any of the otolith growth phases (first, second, third and fourth) in metamorphosing conger eel leptocephali and morphological and ecological events. Arai et al. (2001) proposed that the inflection in otolith growth ca. 30 days from hatching, might be related to favourable somatic growth after successfully switching their nutritional source from yolk material to exogenous feeding, which probably occurs after 10 days based on observations that artificially hatched eel larvae exhausted their yolk material during this period (Yamauchi et al. 1976; Tanaka et al. 1995). In the Japanese conger eel, *C. myriaster*, it has been reported that otolith increment widths increased at the onset of metamorphosis (Tanaka et al. 1987; Mochioka 1989; Lee and Byun 1996; Otake et al. 1997), which coincided with a drop in otolith Sr:Ca ratios (Otake et al. 1997). Such relationships between otolith growth, Sr:Ca ratios and metamorphosis seem to be typical in anguilliform fishes, and have been reported in several species, e.g. in *Anguilla* species (Otake et al. 1994; Tzeng and Tsai 1994, Cheng and Tzeng 1996; Arai et al. 1997, 1999ab, 2000b, 2001; Wang and Tzeng 1998, 2000; Marui et al. 2001). Leptocephali contain large amounts of gelatinous extracelular matrix composed of sulphated glycosaminoglycans (GAG) (Pfeiler 1991) that have a high affinity for Sr (Nishizawa 1978). It has been proposed that the rapid GAG breakdown during metamorphosis (Pfeiler 1999) causes a loss of the corporal Sr, resulting in a drastic decrease of otolith Sr content and consequently Sr:Ca ratios (Otake et al. 1997). These considerations lead to the conclusion that the marked increases in otolith increments

widths, coincidental with a dramatic decrease in Sr:Ca ratios, herald the onset of metamorphosis.

Since the number of daily increments from the otolith core to the beginning of the WIZ represents the duration of the developing leptocephalus stage, our results confirm an earlier study (Correia et al. 2002), which indicated that the European conger eel takes about 6-9 months from hatching to reach the onset of metamorphosis. The age at metamorphosis and the duration of the leptocephalus stage appear to be important factors determining the long-distance dispersal of the Japanese eel (Cheng and Tzeng 1996). Wang and Tzeng (2000) showed that the differences in leptocephalus stage duration and growth rate are the principal factors determining the segregation of migrating American (*A. rostrata*) and European (*A. anguilla*) eels.

Whether metamorphosis is triggered by some environmental stimulus or occurs spontaneously at a certain age or size is unknown (Smith 1989). For some species we know that metamorphosis is initiated when pelagic leptocephali migrate to inshore waters, suggesting that the shallow, near-shore environment is somehow involved in the triggering mechanism (Pfeiler et al. 1990). However, in other species, metamorphosis takes place in the open ocean, indicating that factors other than proximity to shore are involved (Pfeiler 1999). Arai et al. (2001) suggested that the environmental sea temperature and somatic growth should play important roles in the timing of metamorphosis in the eels.

The existence of a diffuse zone without distinct rings, in the marginal portion of the otoliths of the metamorphic leptocephalus stage, prevents accurate estimate of the duration of the larval stage in this species (Correia et al. 2002; Antunes and Correia 2003; present study). Tanaka et al. (1987) observed a disturbance of the ring arrangement in the marginal region of the otoliths of Japanese conger eel leptocephali, with a microstructure similar to our diffuse zone. However, these authors assumed it to be an anomalous, non-permanent structure, and did not characterise it as a normal structure produced during the process of metamorphosis. Mochioka et al. (1989) also reported that the otoliths of *C. myriaster* during metamorphosis grew rapidly and with irregularities on the outer surface. Lee and Byun (1996) mentioned that the otolith increments of the outer opaque zone in the metamorphic larvae of Japanese conger eels were not always easily identifiable under light microscopy or scanning electron microscopy. They supposedly solved this problem, by first examining the opaque zone when the ground plane reached the outer margin, and thereafter polishing the otoliths until the core plane was exposed. Curiously, they never showed any picture of this "opaque zone" with clear increments to the otolith edge. Recently, Otake et al. (1997) described the otolith

morphology of the developing and metamorphosing stage of the C. *myriaster* leptocephali, and also reported some difficulties in consistently etching all increments from the core to the edge. These descriptions suggest some subjectivity in the examination of otolith microstructure in the leptocephali of Japanese conger eel.

A diffuse otolith zone was also described in the European eel leptocephali, before the onset of metamorphosis (Antunes and Tesch 1997). Antunes and Tesch (1997) and Williamson et al. (1999) suggested that this otolith portion marks a period of very slow growth and is made up of many daily growth rings that are too thin to be distinguished and counted. Based on this assumption, Antunes and Correia (2003) estimated a total age of about 483 and 736 days, for two metamorphosing conger eels collected in Minho River on April and November 1998, respectively. They used the average increment width of small fragments of ring structure in the diffuse zone to calculate the number of days in that unreadable portion of the otolith. Williamson et al. (1999) suggested that during this period of life the leptocephali have very small food intake and grow very slowly. So, to conserve energy, the eel leptocephali may remain at depths of about 300 m for a long period of time and stop performing diel vertical migrations. Umezawa and Tsukamoto (1991) showed that conditions of prolonged starvation and/or cold temperatures could stop the deposition of daily rings in *A. japonica*. Tabeta et al. (1987) suggested that the daily vertical migration in the leptocephalus stage might be one of the environmental conditions that cause the formation of the daily growth rings. Arai et al. (2000a) suggested that the formation of the diffuse (unclear) increment area in *A. anguilla,* demonstrated by Antunes and Tesch (1997), may simply be due to a technical problem (e.g. overetching), since Lecomte-Finiger (1992) observed clear concentric rings throughout the otolith of this species, including the leptocephalus and metamorphosis stage. Recently, Cieri and McCleave (2000) proposed that the diffuse zone could be produce by a process of calcium resorption in the otolith periphery during the metamorphosis of the eel, as part of calcium metabolism, as skeletal elements are being formed.

We can conclude by saying that the mechanism behind the formation of this structure is not yet understood. If this otolith portion is a visual artefact, resulting from the technical preparation of the otolith, or a different interpretation of the microstructural growth pattern, or even a permanent structure caused by an environmental or physiological stimulus remains open to discussion.

We recently proposed (Correia et al. 2002), based on indirect evidence (duration of the developing leptocephalus stage, time of capture and developmental stage of metamorphosing conger eels) that the duration of the larval phase for this species is about 2 years. However, it

seems that the exact age determination from counting daily growth increments, the values presented for the duration of the metamorphosis and for total larval age, unfortunately, remain speculative for the conger eel.

The estimated duration of metamorphosis in Japanese conger eel is variable among investigators: 23-31 days (Kubota, 1961); 22 days at temperatures of 18-22°C (Asano et al. 1978); 53-75 days at temperatures of 10-16°C (Lee and Byun 1996); and 71 days at temperatures of 11-15 °C (Otake et al. 1997). Our specimens, captured in April 2001, successfully completed metamorphosis in about 2 months at 15°C. However, it is plausible that captive specimens may accelerate metamorphosis (Butler et al. 1996). It is well know, for example, the stress hormone cortisol plays an important role on the metamorphic process of leptocephali (Yamano et al. 1991). As well as the temperature of the sampling area, the difficulty or ambiguity of identifying the beginning and completion of metamorphosis may also result in inaccurate estimation of its duration (Otake et al. 1997).

The diameter and radius of the larval otolith are highly correlated during larval development, suggesting that these otolith measures are helpful in describing the otolith growth rate. The significant correlation between otolith radius (or diameter) and estimated age of the developing larvae suggested the potential use of otolith size to represent fish age. However, during metamorphosis otolith and body growth appear to be uncoupled in conger eel, as shown by Lee and Byun (1996) and Correia et al. (2002). Otolith size from the reared elvers (diameter range: 1222-1300 μm) was twice that of the wild specimen (833 μm), an unexpected result. However, some preliminary studies on reared specimens using fluorescent dyes as otolith time markers have suggested an anomalous high growth of the otolith (Correia, unpublished data), probably as a result of the stress to fish. These observations suggests that the real daily growth rhythm in the otolith diffuse zone cannot be calculated by examining reared larvae

The number of daily growth increments from the outer core to the onset of metamorphosis (i.e. age at metamorphosis) was negatively correlated with the mean increment width, suggesting that fast-growing leptocephali metamorphosed earlier. Several authors (Tsukamoto and Umezawa 1990; Tzeng 1990; Cheng and Tzeng 1996) observed the same phenomenon in *A. japonica* and suggested that the time taken for migration from the oceanic spawning ground to coastal waters was probably shorter for the fast-growing larvae. In general, fast-growing larvae metamorphose early and swim quickly to the preferred habitat (Hunter 1972; Miller et al. 1988). In contrast, slow-growing larvae, which are unable to swim as fast as the fast growing ones, prolong the duration of the larval stage and delay metamorphosis and

recruitment to the estuary (Victor 1986). Furthermore these larvae are more vulnerable to predation, which may cause poor recruitment (Tzeng 1990). Several studies (Tsukamoto 1990; Tsukamoto and Umezawa 1990; Wang and Tzeng 1998; Arai et al. 1999ab, 2000b, 2001; Marui et al. 2001) have also reported a positive relationship between age at recruitment and age at metamorphosis, in temperate and tropical eels, suggesting that early-metamorphosing larvae were recruited to coastal habitats at younger age.

The presence of metamorphosing conger eel larvae in the northern coastal waters of Portugal has been recorded from late October to mid-June, because they sometimes enter in the mouth of the Minho River, by tidal transport, and are caught by the Portuguese glass eel fishery (Correia et al. 2002). These authors showed that the largest larvae, in an advanced metamorphic stage, are recruited early to the northern Portuguese coastal waters. Thus, the migrating mechanism of the conger eel can be summarized as follows: the larvae with a faster growth rate metamorphose and recruit earlier to the coastal areas, probably at a younger age, and with a larger size and an advanced developmental stage. This larval recruitment pathway appears to be common in anguilliform fishes.

Acknowledgments

The Portuguese Foundation for Science and Technology (Research Project Praxis XXI:2/2.1/MAR/1728/95) supported this study. The author has a PhD grant (BD 18082/98) from the same sponsor. We thank Dr. J. Wilson for English language assistance, and Dr. F. Ramos and Dr. P. Piedade for help in preparing and analysing the otolith microchemistry, respectively.

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3.2 Reconstructing the early life history of the conger eel (*Conger conger*) using the microstructure information of otoliths

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Abstract

Otoliths from twenty conger eel metamorphosing leptocephali, collected from the Minho River in February 1999, were analysed by scanning electron microscopy. The total length and the otolith radius of the leptocephali ranged from 104.0 to 133.0 mm and from 202 to 278 μm, respectively. Otolith growth increments were visible along the increment countable zone with an average of 264±28days. In this zone, the otoliths displayed a typical sequential growth pattern with three steps: moderate, slow and finally increasing growth rate. Although we used different etching agents and section planes during the otolith preparation, a permanent peripheral diffuse zone, where the daily increments are unclear, appeared on all otoliths analysed. In an attempt at solving this problem, otoliths of ten other metamorphosing leptocephali reared in aquaria were marked by immersion in tetracycline hydrochloride (400 $mg.l^{-1}$) for 24 h. The distance between the fluorescent mark and otolith edge, measured over a fixed period of time, estimate the diffuse zone grow rate. However, the application of this technique led to an anomalously high otolith growth rate (between 3.5 to 10.3 $\mu m.day^{-1}$), probably as a result of the capture, marking and handling stress. Our knowledge of the conger eel life history, although incomplete, provides us some context to interpret the biological significance of the increment-width inflection points and the microstructural features (e.g. the diffuse zone and the accessory growth centres) observed in the late metamorphosing conger eel otoliths.

Introduction

The European conger eel (*Conger conger* L.) is an important fish of the coastal and outer continental shelf marine ecosystems of the northeastern Atlantic Ocean (Bauchot and Saldanha 1986). However, its early life history, such as spawning area(s), duration of the leptocephalus stage and larval migratory route(s) is poorly known at present.

The Mediterranean Sea is thought to be the spawning place for this species, based on the capture of small (9-20 mm) conger eel leptocephali (Schmidt 1931). This assumption is supported by the capture of sexually mature specimens of *C. conger* in deep waters southeast of Sardinia (Cau and Manconi 1983) and by the length and otolith analyses of leptocephali collected in the North and Central Atlantic Ocean (Strehlow et al. 1998). Other spawning places have been suggested for *C. conger* in the eastern Atlantic Ocean, such as the area between Gibraltar and the Azores (Lythgoe and Lythgoe 1971; Bagenal and Kenney 1973), even though sexually mature specimens have not yet been caught, with the exception of a single mature female caught in the Irish Sea (Fannon et al. 1990). Recently, based on the capture of several small and young leptocephali, we suggested that the conger eel has another spawning ground near the Azores (Correia et al. 2002b, 2003).

The daily pattern of otolith growth, back-calculation of hatching dates and growth rate were studied in field-caught leptocephali of *C. conger*. The spawning season last from December to July, with one annual peak occurring in summer (Correia et al. 2002b, 2003; Antunes and Correia 2003). During the migration from the spawning ground(s) to the waters of the Atlantic continental slope, the leptocephali grow 0.31 to 0.38 mm.day^{-1} (Correia et al. 2002b; Antunes and Correia 2003), reaching a full length of 150 mm (Strehlow et al. 1998). After that the leptocephali start a migration towards the shelf and coastal waters that probably induces metamorphosis (Strehlow et al. 1998). It has been suggested that the leptocephali has a long larval life (Bauchot and Saldanha 1986), taking about one to two years to drift inshore and to reach the juvenile elver form (Lythgoe and Lythgoe 1971; Wheeler 1985, Strehlow 1992). The first half time of the larval stage (i.e. the developing phase) takes between 6 to 9 months (Correia et al. 2002a, 2003). However, preliminary studies on metamorphosing leptocephali showed the presence of a peripheral otolith zone exhibiting a diffuse structure, during which no formation of daily growth increments takes place (Correia et al. 2002a, 2003). For this reason an exact age determination for the metamorphosing conger eel leptocephali by growth increments seems impossible (Correia et al. 2003). A similar region of the otolith, where the morphological features are irregular, was also been described in the

European eel (*Anguilla anguilla* L.) before metamorphosis (Antunes and Tesch 1997). Recently, it has been suggested that this otolith zone is an artefact produced during the preparation of the eel otoliths as a result of an overetching (Arai et al. 2000).

To test if the diffuse zone of the conger eel metamorphosing leptocephali is a permanent structure or an artefact resulting from a bad otolith procedure, we have looked at the effect of different otolith preparation methods, commonly used in anguilliform leptocephalus studies (Table 1), on the final appearance of the otoliths. Additionally, with the purpose of knowing the rhythm of formation of the diffuse zone, we used a fluorescent dye as an otolith time marker, on some reared metamorphosing leptocephali. Finally these new data as been used to make some considerations on the early life stage of the conger eel.

Material and methods

Twenty conger eel metamorphosing leptocephali collected in February 1999 as by-catch of the glass eel fishery at the mouth of Minho River, North of Portugal (Correia et al. 2002a) were used in this study. Both right and left sagittae were dissected from the leptocephali, cleaned of adhering organic tissues, washed with distilled water and air dried. The left otoliths were mounted on to cylindrical stubs with thermoplastic glue, convex side up, and the right otoliths were embedded within an epoxy resin block. The excess resin was removed using a low-speed saw. Both otoliths were hand polished in the sagittal (left sagittae) and frontal (right sagittae) planes with a 2400 silicon carbide abrasive paper and alumina solution (1:20) until the core was revealed. During this procedure we made frequent checks on the core, viewed as a dark spot under a metallographic microscope (Nikon OPTIPHOT-M). Later the otoliths were etched with one of four different chemical agents (five pairs otoliths/etching agent), coated with gold and viewed under a scanning electron microscope (SEM; Jeol JSM 639-1f) at 15 kV. The concentrations and reaction times for each etching agent were established by several trials. The mounting media, the polishing procedure and the etching agents are shown in Table 1. The polished otoliths of five leptocephali, coated with carbon, were also examined at 20 kV using a back-scattered detector (BSD), instead of the secondary electron detector (SED), a procedure described by Waldron and Gerneke (1997). However, these unetched otoliths gave a poorly contrasted image and were excluded from this study. A SEM series of overlapping photographs were taken from the core to the edge of each otolith. The number of daily increments was estimated at ×1800 and ×2200 magnifications, to the sagittal and frontal sections respectively. All otolith measurements were carried out according

to the procedures of Correia et al. (2003). The percentage of the area occupied by the accessory growth centres (%AGCA) on the total otolith surface was also calculated on the sagittal planes (Correia et al. 2002a). Based on previous data on otolith and larval development studies in closely related species (see Discussion), we assumed the growth increments in *C. conger* leptocephali to be daily, and that the beginning of the otolith wide increment zone (WIZ) marked the onset of metamorphosis. We also added 10 days to the number of daily increments to account the time between fertilization and the end of the yolk-sac stage, period during which no increments were deposited.

The otolith growth rate in the diffuse zone (DZ) was studied using wild-caught laboratory-reared metamorphosing leptocephali (n=10) also collected in the Minho River in June 2000 and April/May 2001. After collection, the fishes were transported to the laboratory in aerated containers and reared in a 100 l tank, under an artificial photoperiod (12L/12D) and with a water temperature and salinity of 15 °C and 31 psu, respectively. After 1-day acclimation the leptocephali were anaesthetized with 2-phenoxyethanol (250 $\mu l.ml^{-1}$), weighted and measured to the nearest 0.1 mm length. Otolith marking was performed by immersing, on the 7^{th} and 14^{th} days, the leptocephali into a 5 l aerated seawater aquarium containing 400 $mg.l^{-1}$ of tetracycline hydrochloride ($C_{22}H_{24}N_2O_8.HCl$; Sigma Chemical Co.) during 24 hours in the dark to prevent light-degradation of the fluorescent chemical. We assumed there was no lag-time between exposure to the treatment and incorporation of the chemical into the otoliths (Pitcher 1988). This treatment did not cause any mortality. Near the end of the captive experiment the larvae were fed twice at day with live seawater polychaete. The leptocephali were sacrificed about 2 months after capture and the left sagittal otoliths were removed, cleaned and fixed on microscope glass slides using cyanoacrylate glue. The otoliths were then grounded with 2400 silicon carbide paper and alumina solution (1:20) until the core was revealed. All otoliths were kept in darkness to avoid photo-degradation of tetracycline (Lorson and Mudrak 1987). The detection of the fluorescent band was carried out by viewing the otolith under a Leitz DMRBE microscope (Leica Microsystems) fitted with an ultra-violet (UV) light (568 nm). The presence and location of the fluorescent tetracycline marks were confirmed under transmitted light at ×100 magnification. The otolith growth rate was estimated by measuring the maximum radius between the first and the second tetracycline mark, and between this last mark and the otolith edge and by dividing by the time elapsed. The lengths of the anterior-posterior and dorsal-ventral axes of the otoliths were also measured.

Statistical analyses were performed using Student's t-test and procedures described by Zar (1996). We used a level of significance (α) of 0.05. Data are presented as ranges and mean values (± standard deviations)

Results

Size, developmental stage and morphology

The total length (TL) and developmental stage indicator (preanal length/total length; PAL/TL) of the 20 metamorphosing leptocephali captured in February 1999 ranged from 104.0 to 133.0 mm (117.1±6.5 mm) and 0.39 to 0.54 (0.44±0.04), respectively.

The mean length and weight of the 10 laboratory-reared metamorphosing leptocephali at capture were 108.3±6.4 mm and 1.369±0.382 g, respectively. The PAL/TL at the beginning of the rearing experiment was 0.44±0.10. The average length and wet weight of these leptocephali at the end of the experience were 80.4±8.2 mm and 0.640±0.212 g, respectively, i.e. there was a substantial decrease in weight (53 %) and length (26 %). The PAL/TL presented a mean value of 0.36±0.1 (Table 2).

During the experimental period the leptocephali body become shorter, thicker and deeper. The eyes started to elongate in the antero-posterior direction and the definitive teeth replaced the larval dentition. A series of internal melanophores appeared along the spinal cord, extending from the tail forward. External melanophores appeared dorsally on the head and on the latero-caudal region. The blood become coloured and some internal organs, like the heart, swim bladder and gall bladder, were now visible (Fig. 1).

Otolith preparation

The otoliths from the conger eel metamorphosing leptocephali etched with HCl, CH_3COOH, EDTA and PKb revealed similar results, in both sagittal and frontal sections. Although all the etching agents gave a good enhanced visibility of individual daily growth increments, the EDTA produced better contrast of daily increments. PKb had the disadvantage of being a time-consuming (long-term enzyme digestion) and expensive technique. The increments were broader (paired t-test, $P<0.05$) and were counted more clearly in the sagittal section comparatively with the frontal section (Fig. 3). Daily growth increments

were clearly discernible in the increment countable zone (ICZ) and a peripheral diffuse zone (DZ) was present in all the samples (Fig. 5).

Otolith morphology

The sagittal otoliths of the metamorphosing conger eels were subelliptical in shape, convex on their distal side and somewhat concave proximally (Fig. 2). The longest axis of the otoliths was along the anterior-posterior direction, while the shortest axis was along the proximal-distal direction (Fig. 3). The otolith growth rate was different in each direction, being faster in the anterior-posterior direction due to larger increment width there (Fig. 3). Increment width was narrowest on the distal side and the growth increment was interrupted on the proximal side (Fig. 3B).

All specimens exhibited the same otolith growth pattern. The core, located in the central part of the otoliths, was visible as a deep hole (primordium, P) surrounded by a dark circular groove (hatch check, HC) and a crystalline crown, which was in turn surrounded by a deeply etched ring (first feeding check, FFC) (Fig. 4A). The core had an overall mean diameter of 22±1 μm and no significant differences were found between sagittal and frontal planes (paired t-test, P=0.44). Beyond the FFC a series of growth increments were distinctive along the ICZ. After that, began a DZ, where no increments were visible (Figs. 4B and 5). The majority of the otoliths (75 %) presented one to seven accessory growth centres (AGC) along the edge (Fig. 3), which occupied 3.2 to 21.8 % of the otolith cross sectional surface area.

The increment width showed a characteristic profile along the increment countable zone (Fig. 6). From the FFC to the first 30-60 days, increments were wide (0.68±0.11 μm) (phase I) (Fig. 4A). Then they narrowed and gradually decreased, until a relatively constant minimum value (0.42±0.09 μm) was reached, at about 170 days (phase II). This inner portion of the ICZ that includes phases I and II was named developing leptocephalus growth zone (DLGZ). Between 180 and 260 days, depending of the specimen, the increments abruptly widened to a maximum of 0.86±0.17 μm (phase III). This wide increment zone (WIZ) lasted about 30-70 days. After that the increments became less clear and disappeared, which corresponds to the onset of the DZ (phase IV) (Fig. 4B). As the DZ grew larger the accessory growth centres (AGC) were formed (phase V).

There was no significant difference between either diameter or radius between left (sagittal plane) and right (frontal plane) otoliths (paired t-tests, P=0.87 and P=1.00, respectively). The diameter and radius of the otolith leptocephali ranged from 361 to 494 μm (427±41 μm) and

202 to 278 μm (240±20 μm), respectively. There was no significant difference in increment counts between left and right otoliths (paired t-test, P=0.72). Therefore, these data were averaged for each conger eel. The number of growth increments in the otolith DLGZ, WIZ and ICZ ranged from 185 to 265 (219±26), 30 to 70 (41±11), and 227 to 312 (264±28), respectively. The mean growth rate of the otolith in the DLGZ and WIZ was 0.51±0.15 and 0.65±0.18 $\mu m.day^{-1}$, respectively.

Tetracycline marking

Tetracycline appeared as a distinct bright-yellow ring when viewed under ultraviolet light (Fig. 7A). Incorporation of a fluorescent mark occurred in 100 % of the conger eels leptocephali. The tetracycline mark was also clearly visible as a slight check when viewed with a transmitted light microscope (Fig. 7B). The otolith DZ growth was higher in the first rearing week (9.84±0.57 $\mu m.day^{-1}$) compared with the remaining period (4.84±1.11 $\mu m.day^{-1}$) (paired t-test, P≤0.05). The otoliths showed an elliptical shape and measured 1183±145 and 672±56 μm, on the major and minor axes, respectively (Table 2).

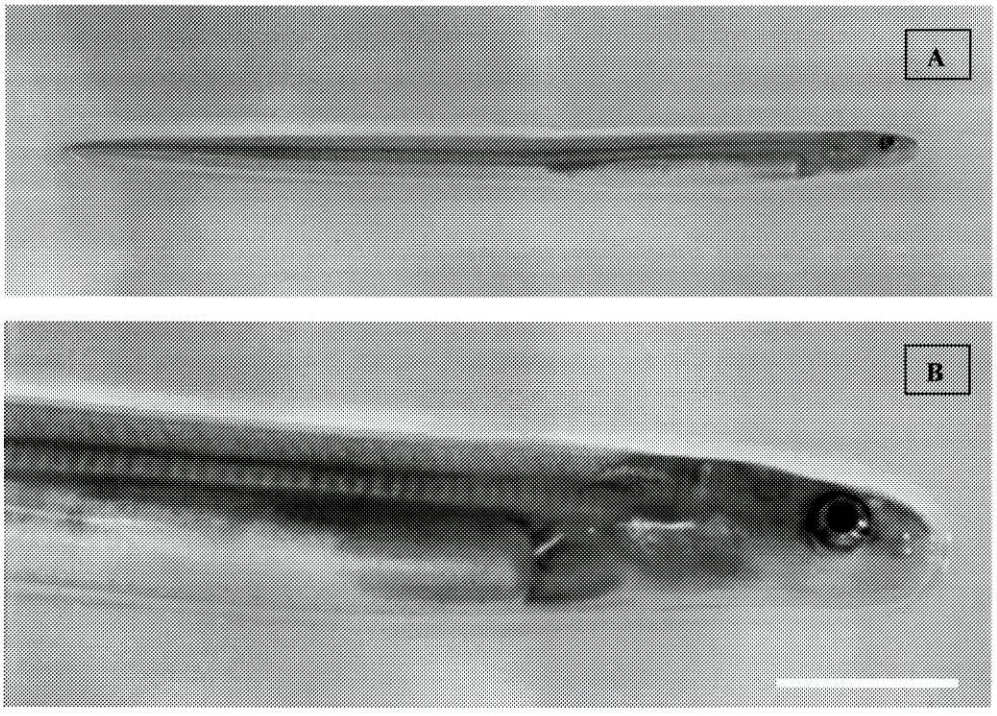

Fig. 1A, B *Conger conger*. **A** Whole view of a late metamorphosing leptocephalus (TL: 86.0 mm; PAL/TL: 0.38). **B** Detail of the anterior portion of the body (*scale bar* 5 mm).

Table 1 *Conger conger*. Review of the most common sagitta procedures from SEM otolith studies on anguilliform fish larvae (abbreviations: TG thermoplastic glue, ER epoxy resin, PR plastic resin, SP spurr; S sagittal, F frontal, T transverse; ? procedure used but not documented; *included also microchemistry analysis; # according with Shiao et al. 1999).

Author(s)	Species	Medium	Plane	Polishing	Etching	Coating
Antunes and Tesch 1997	*Anguilla anguilla*	TG	S	2400 mesh sand paper and aluminium paste	0.5 % HCl (6-8 s)	Au
Antunes and Correia 2003	*Conger conger*	TG	S	2400 mesh sand paper and aluminium paste	0.5 % HCl (6-8 s)	Au
*Arai et al. 1997	*Anguilla japonica*	ER	?	6 and 1 µm diamond pastes	0.05 M HCl (10-30 s)	Pt-Pd
Bishop et al. 2000	*Paraconger caudilimbatus* *Ariosoma balearicum* *Gymnothorax saxicola* *Ophichthus gomesii*	ER	S	?	0.10 N HCl (?)	?
Castonguay 1987	*Anguilla anguilla* *Anguilla rostrata*	ER	S	fine grain sandpaper	5 % EDTA (2-3 min)	Au
Cieri and McCleave 2000	*Anguilla rostrata*	TG	S	12, 9 and 3 µm metal lapping films and 0.05 µm polishing fluid	5 % EDTA (2 min)	Au
Correia et al. 2002a, b	*Conger conger*	TG	S	2400 silicon carbide paper and alumina suspension	0.5 % HCl (8s) 0.05M HCl (10 s)	Au
*Correia et al. 2003		TG	S	600, 1200 and 2400 silicon carbide papers with 6, 3 and 1 µm diamond pastes and aluminium solution	0.05 M HCl (10 s)	Au
Lecomte-Finiger and Yahyaoui 1989	*Anguilla anguilla*	PR	?	600, 1000 and 1500 grit papers	5 % EDTA (1min)	Au
Lee and Byun 1996	*Conger myriaster*	PR	S	600, 800 and 1200 grift silicon carbide papers with 1 and 0.3 µm alumina powders	0.3 % HCl (3-5 s)	Au
Mochioha et al. 1989	*Conger myriaster*	ER	?	waterproof abrasive paper	0.05 N HCl (?)	Au
*Otake et al. 1994, 1997	*Anguilla japonica* *Conger myriaster*	ER	S	polishing papers and 1 µm diamond paste	0.1 N HCl (?)	Au
Shiao et al. 2001	*Anguilla australis*	ER	?	?	0.05 M HCl (15 s)	Au
Sugeha et al. 2001	*Anguilla marmorata*	ER	?	6 and 1 µm diamond pastes	0.05 M HCl (10-30s)	?
Tabeta et al. 1987	*Anguilla japonica*	ER	F	whetstone	0.5 % HCl (?)	Au
Tanaka et al. 1987	*Conger myriaster*					?
Tsukamoto 1989	*Anguilla japonica*	ER, TG	S	# 1200 and #12000 emery papers	1 % HCl (1-3 s)	?
Tzeng 1990	*Anguilla japonica*	SP	F	1000 to 2000 mesh polishing papers and alumina paste	5 % EDTA (3min)	Au
Umezawa et al. 1989	*Anguilla japonica*	ER	S, F, T	rubber stone and 2000 to 8000 grit emery papers	0.1 N HCl (10-20 s)	Au-Pd
Present study	*Conger conger*	ER, TG	S, F	2400 silicon carbide paper and alumina suspension	0.5 M HCl (10 s) 5 % EDTA (2 min) 5 % CH_3COOH (1 min) #PKb (30 min)	Au

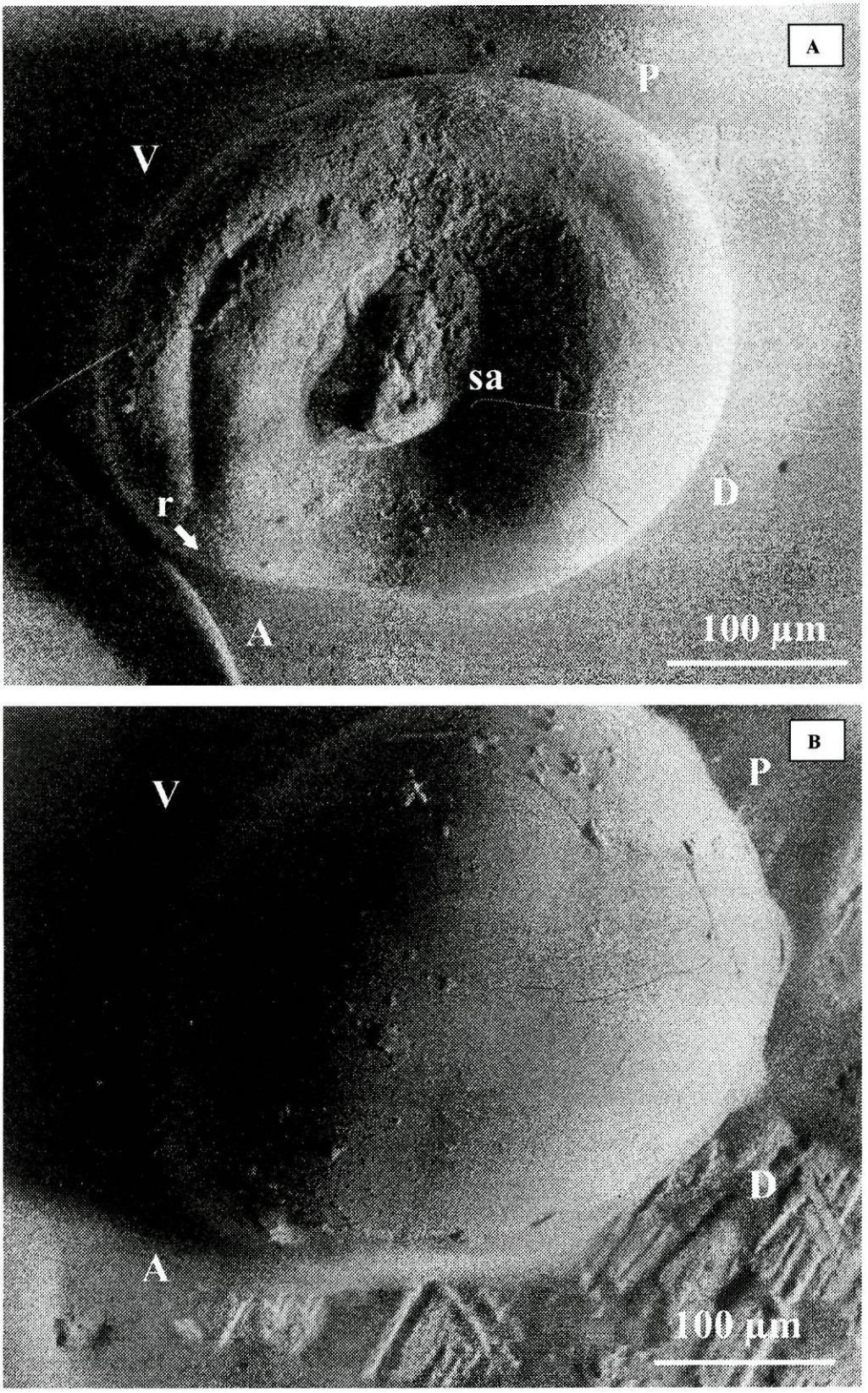

Fig. 2A, B *Conger conger*. SEM photographs showing the external features of the sagittal otolith from a 115.0 mm total length metamorphosing leptocephalus: **A** proximal side of the right otolith; **B**: distal side of the left otolith. Abbreviations: anterior (*A*), posterior (*P*), dorsal (*D*) and ventral (*V*) margins, rostrum (*r*) and sulcus acusticus (*sa*).

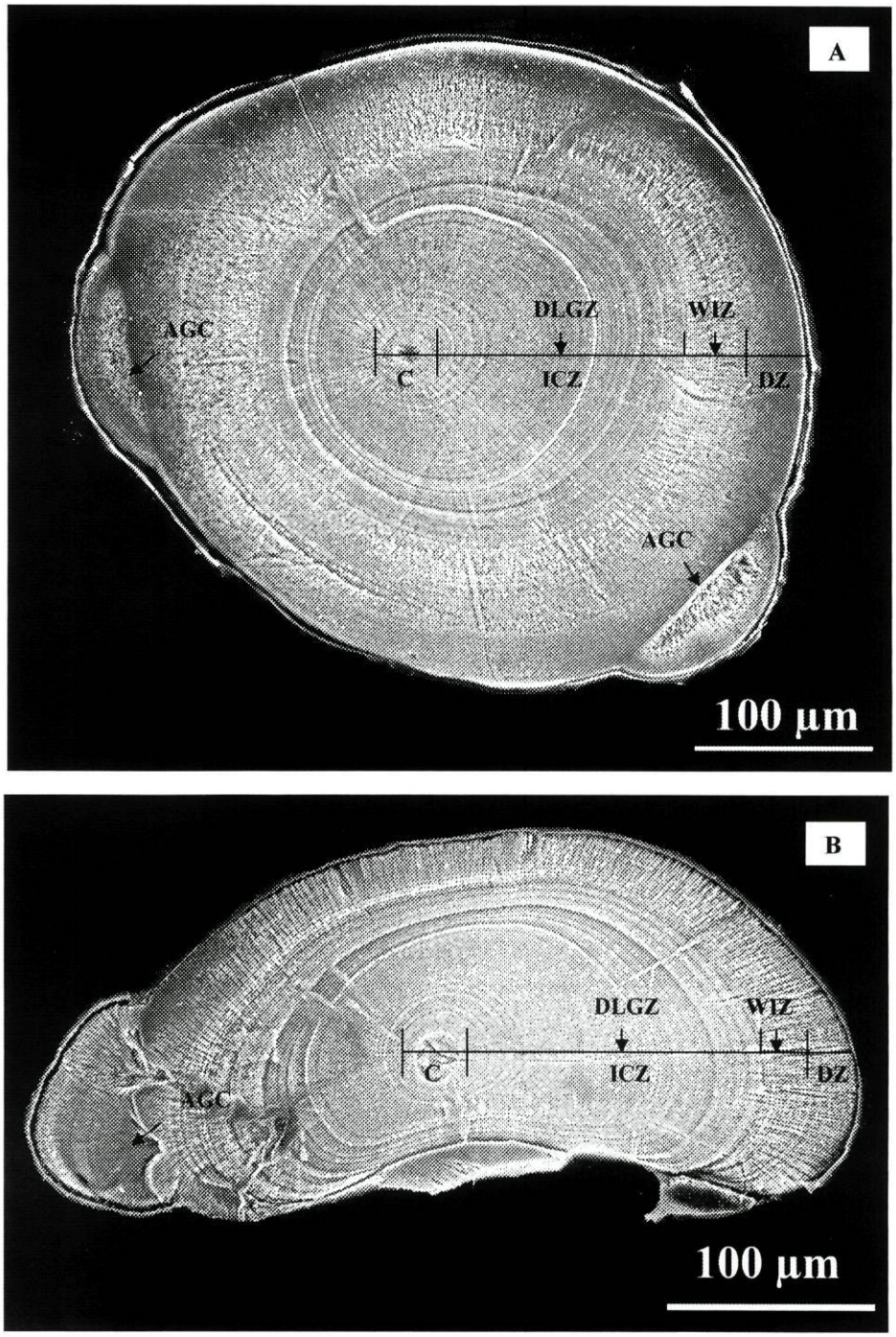

Fig. 3A, B *Conger conger*. SEM photographs showing the microstructure of an otolith on a sagittal (**A**) and frontal (**B**) plane. Legends: *C* core; *DLGZ* developing leptocephalus growth zone; *WIZ* wide increment zone; *ICZ* increment countable zone; *DZ* diffuse zone; *AGC* accessory growth centre.

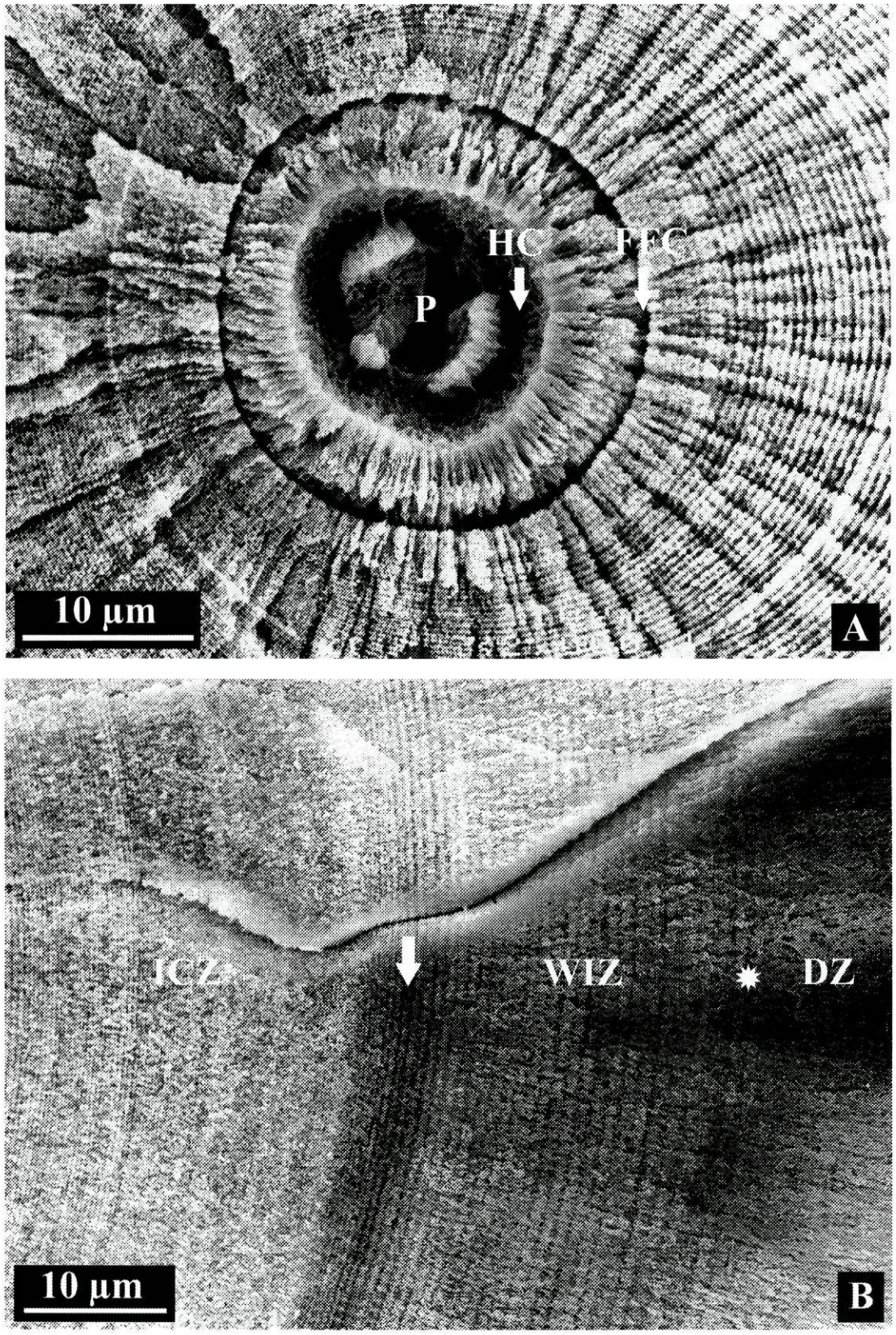

Fig. 4 A, B *Conger conger*. SEM photographs showing some details of the otolith microstructure of a metamorphosing conger eel: **A** area surrounding the core; **B** transition area between the ICZ, WIZ and DZ. Legends: *P* primordium; *HC* hatch check; *FFC* first feeding check; *ICZ* increment countable zone; *DZ* diffuse zone. *Arrow* abrupt change of the increment wide that probably marks the onset of metamorphosis; *Asterisk* last countable daily growth increment. Note: both otoliths are on the sagittal plane.

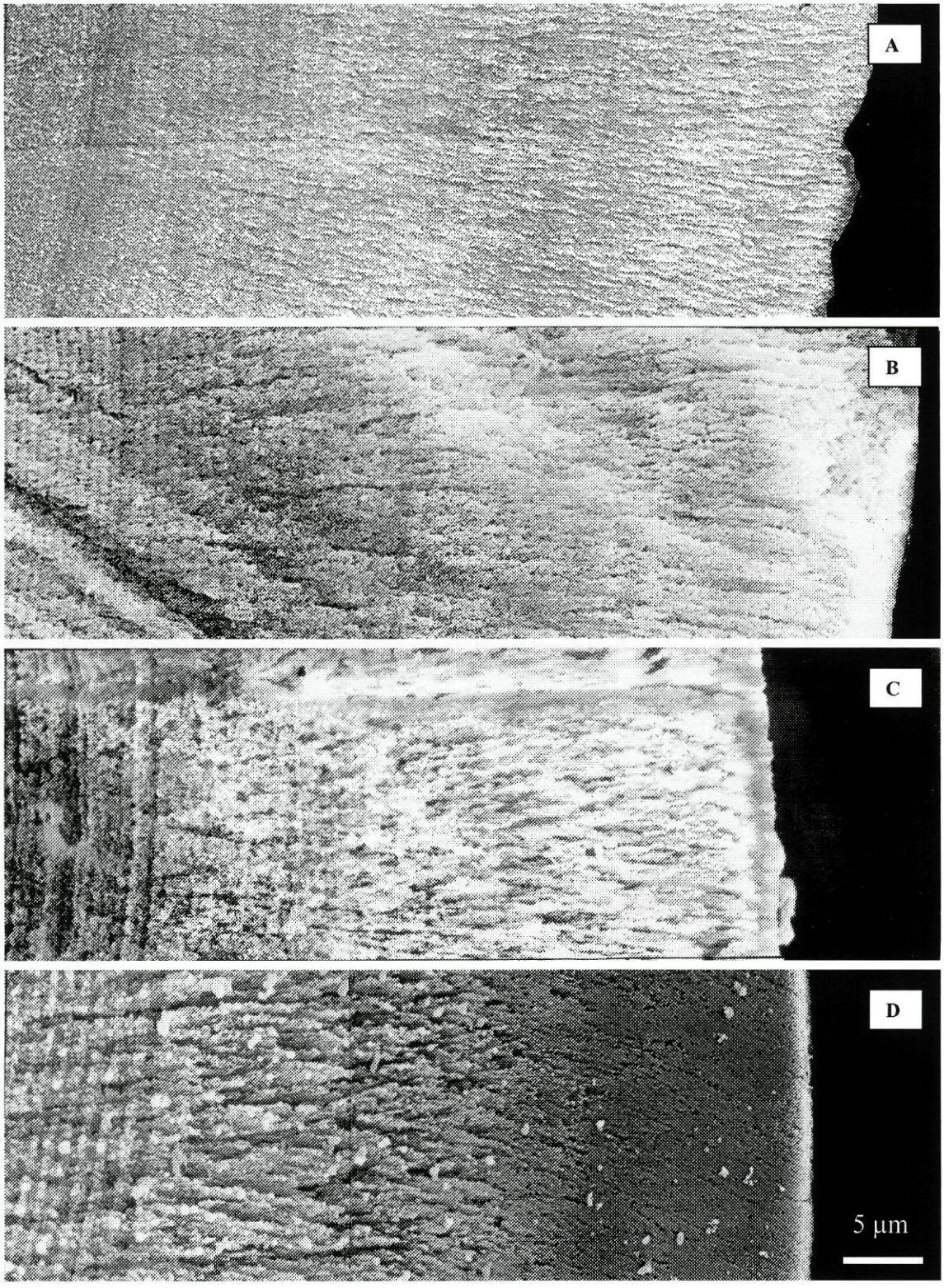

Fig. 5 A-D *Conger conger*. SEM photographs showing the peripheral zone (i.e. end of the WIZ and beginning of the DZ) of four otoliths on the sagittal plane treated with different etching agents (**A** HCl; **B** CH_3COOH; **C** EDTA; **D** PKb).

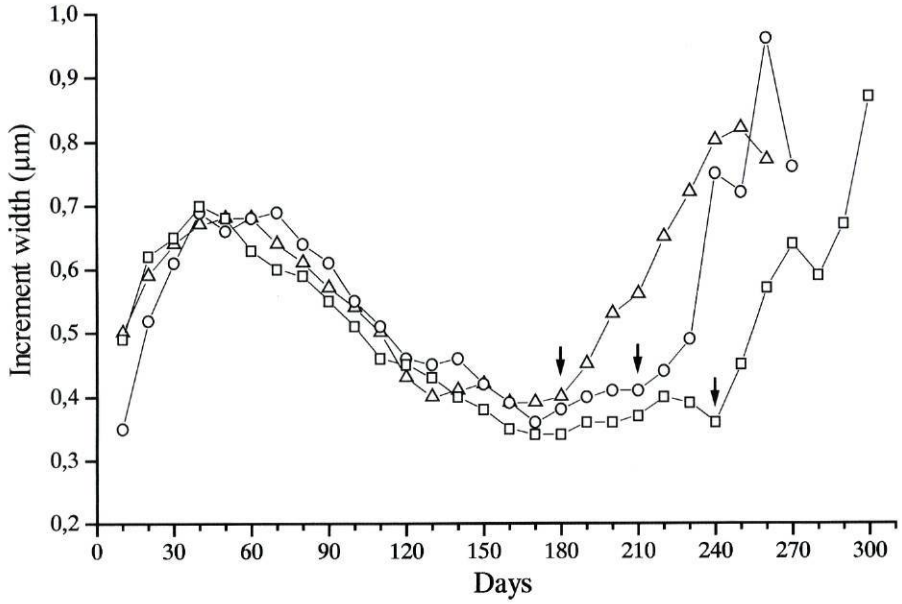

Fig. 6 *Conger conger*. Profiles of the mean increment width from the first feeding check (age 0) to the end of otolith increment countable zone. The specimens have been grouped according by the time when the daily increments abruptly increased (*arrows*), which probably marks the onset of metamorphosis (triangles: 180-200 days, n=10; circles: 210-230 days, n=4; squares: 240-260 days, n=6).

Table 2 *Conger conger*. Collection date, length (TL), weight (W), developmental stage (PAL/TL), major otolith length (OL) and otolith growth rate (OGR) of the ten specimens used for the tetracycline marking at the end of the experiment.

Specimen	Capture date	TL (mm)	W (g)	PAL/TL	OL (µm)	OGR (µm.day^{-1})
M.06.00 (08)	02.06.1999	75.0	0.490	0.36	1240	5.0
M.06.00 (09)		77.0	0.533	0.36	950	3.5
M.06.00 (10)		76.0	0.498	0.36	1260	5.0
M.06.00 (11)		66.0	0.383	0.36	1200	6.7
M.06.00 (12)		80.0	0.647	0.36	1240	4.2
M.06.00 (13)		76.0	0.512	0.36	1210	4.5
M.04.01 (12)	26.04.2001	88.5	0.838	0.38	1300	3.5
M.04.01 (14)		82.0	0.558	0.35	1220	4.0
M.05.01 (01)	24.05.2001	92.0	1.004	0.38	1300	5.8
M.05.01 (02)		91.0	0.941	0.37	1250	6.2

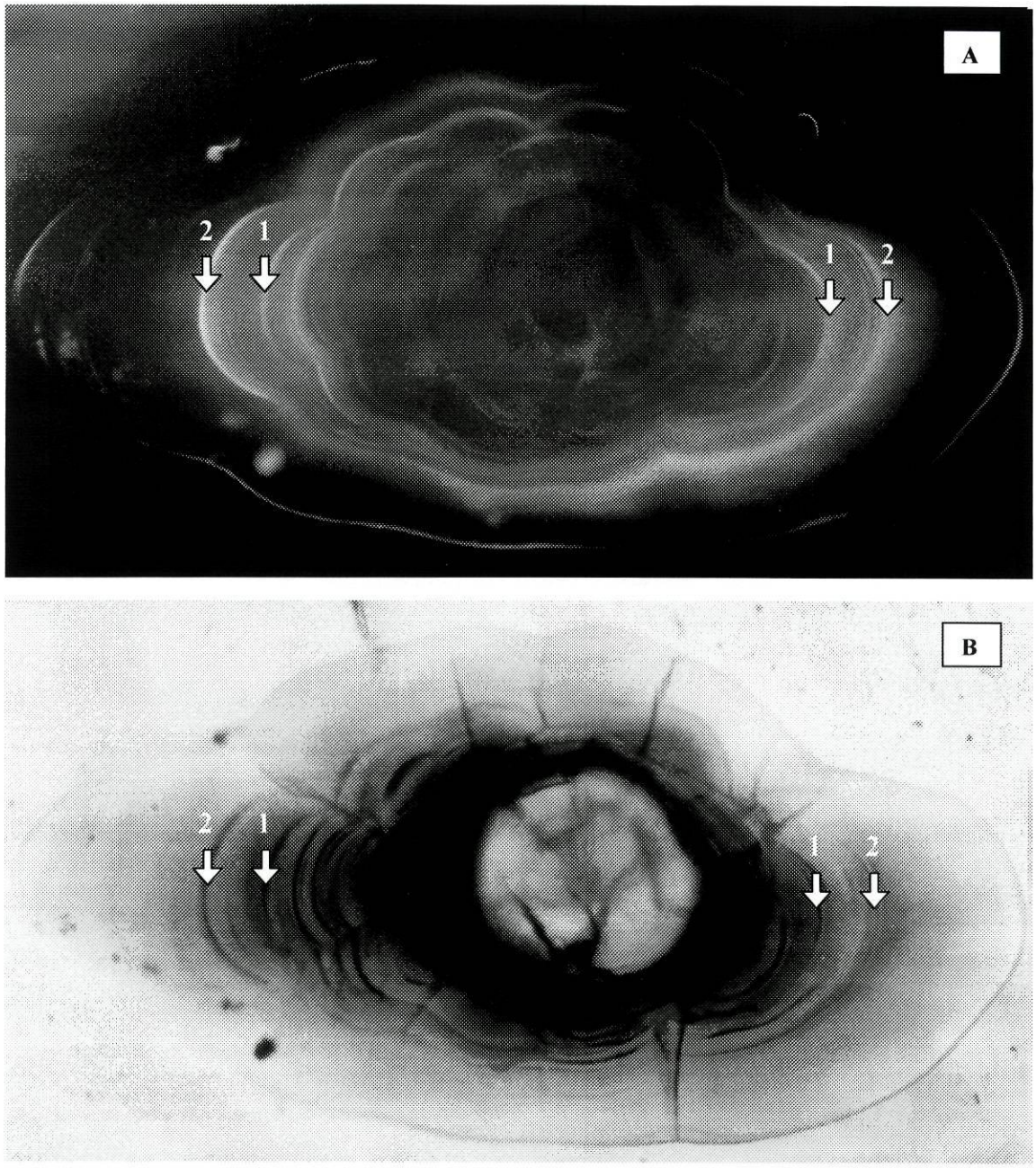

Fig. 7 *Conger conger*. **(A)** UV light photograph of a sagittae treated with 400 $mg.l^{-1}$ of tetracycline in vivo. **(B)** Photograph of the same sample under normal light. *Arrows 1* and *2* indicates respectively the location of the 1st and 2nd tetracycline marks, respectively.

Discussion

Conger eel leptocephali are occasionally caught in the mouth of the Minho River (North of Portugal), from late October until the middle of June, while they are undergoing metamorphosis (Correia et al. 2002a). The metamorphosing leptocephali collected in this study covered the length range and developmental stage previously reported by Correia et al.

(2002a, 2003). The changes in morphology, pigmentation and dentition through metamorphosis were similar to that described by D'Ancona (1931). At the same time there was a substantial decrease in mass and size during metamorphosis, which is typical in leptocephalus fishes (Pfeiler 1986).

Otolith increments of various closely-related anguilliform fishes are deposited daily (Mochioka et al. 1989; Tsukamoto 1989; Umezawa et al. 1989; Umezawa and Tsukamoto 1991; Martin 1995; Arai et al. 2000; Cieri and McCleave 2001; Sugeha et al. 2001). Otolith growth increment refers to a bipartite structure composed of an incremental zone (mainly $CaCO_3$) and a discontinuous zone (organic matrix) formed over 24 h (Mugyia et al. 1981). The age at which daily increment formation is initiated varies widely among fish species (Morales-Nin 2000). Based on the examination of similar published otoliths images of other anguilliform leptocephali (Lecomte-Finiger 1989; Wang and Tzeng 1998, 2000; Arai et al. 2000, 2001), it was assumed that the first distinct increment (FFC), which marks the onset of daily growth deposition, is probably related to the first exogenous feeding, when larvae complete yolk-sac absorption (Correia et al. 2002a, b, 2003). Recently, Horie et al. (2002) showed that for the Japanese conger eel, *Conger myriaster*, hatching occurred 84 h (about 3.5 days) after artificial insemination and that mouth opening occurred on the 7^{th} day after hatching. On the assumption that *C. conger* could have a similar larval development, ten increments were expected to be deposited from fertilization until yolk-sac absorption. However, it is recommended that increment periodicity for *C. conger* be validated in the future, since an appropriate validation of the periodicity of otolith increments formation is still essential for a correct interpretation of the otolith microstructure (Geffen 1992).

The growth history of the metamorphosing conger eels was reflected in the changes of width of the otolith increments. In an earlier study (Correia et al. 2002a), we showed that otolith increment width, which was relatively constant and narrow in the developing leptocephalus stage, increased sharply at age 170 to 280 days. On the other hand, Sr:Ca ratios in the otolith, which increased during the developing leptocephalus stage, showed a rapid drop coinciding with the increase in increment width (Correia et al. 2003). These coincidental changes have been regarded as the onset of metamorphosis and are typical of several anguilliform fish species, including *C. myriaster* (Otake et al. 1997) and *C. oceanicus* (Correia et al. submitted). Based on this assumption, the age of leptocephali in the first half of its larval phase (developing leptocephalus stage) was estimated at 219±26 days (i.e. the average number of days in the DLGZ), including the 10 day hatching/yolk-sac period adjustement. Although the rapid drop in Sr:Ca ratios might be associated with decreasing Sr

levels in the body as a result of catabolism of Sr-rich sulphated glycosaminoglycans (GAG) during metamorphosis (Otake et al. 1997), no explanation has been give for the larger growth increments recorded in the otolith wide increment zone (WIZ). According to D'Ancona (1931), metamorphosis begin in *C. conger* before larval growth is completed, so that the younger semilarvae are larger that the fully developed larvae. Based on this observation, we suggest that the WIZ could be produced by a last short growth period of the leptocephali (lasting 30 to 70 days, i.e. the number of days of the WIZ), before the beginning of the body shrinkage, which should occur at 150 mm length (D'Ancona 1931). Although the leptocephali stop feeding at the onset of metamorphosis (Pfeiler 1986), they could have some remaining energy reserves, which enable them to continue grow for sometime. Some studies have shown that the ability of the otolith to reflect changes in food abundance and growth fluctuations of larger larvae and juveniles may be lag by one week (Moksness et al. 1995), two weeks (Molony and Choat 1990; Folkvord et al. 2000) or even three weeks (Neilson and Geen 1985). The existence of this lag period could be attributable to real physiological events, such as the mobilization and exhaustion of the energy reserves (Molony and Choat 1990). Later on in the metamorphosis process, the wider increments became poorly contrasted and disappeared, when entering the DZ (Correia et al. 2002a, 2003; present study). Based on the same findings, we think that the daily periodicity of increment deposition stop in the DZ, as a result of poor growth conditions, since the metamorphosing leptocephali probably exhausted their energetic reserves allocated for growth and started to shrink. However, other causes like the seawater temperatures encountered by leptocephali could explain in part the variation of the increment thickness along the radius of the otolith ICZ (Antunes and Correia 2003). Furthermore, the relative influences of endogenous and exogenous factors on the scaling of the increment width and the observed transitions in increment-width patterns remain unclear.

In this species AGCs fuse together to form the adult-like otolith shape (Correia et al. 2002a). AGCs appear with or after metamorphosis from larvae to juvenile in several fish species (Campana 1984; Gartner 1991; Sogard 1991; Hare and Cowen 1994; Volk et al. 1995; Modin et al. 1996; Wilson and McCormick 1997; Fischer 1999; Brown et al. 2001; Neuman et al. 2001; Plaza et al. 2001), including other conger eels (Lee and Byun 1996; Correia et al. submitted). Some hypotheses have been proposed to explain the origin of AGCs, e.g. habitat shift (Sogard 1991), ontogenetic dietary shift (Marks and Conover 1993) and/or physiological changes associated with metamorphosis (Hare and Cowen 1994). Congers undergo habitat, morphological and physiological changes at the larval to juvenile transition (Yamano et al. 1991; Bell et al. 2003). During metamorphosis conger eels change from their pelagic

behaviour to more benthic. Such a shift in behaviour and habitat is likely accompanied by significant shifts in ambient temperature and food availability, variables that are known to influence the appearance of the otoliths (Campana and Neilson 1985). Ontogenetic habitat shifts from a larval pelagic to a juvenile benthic stage are often marked by distinct changes in otolith morphology (Thorrold and Milicich 1990; May and Jenkins 1992), including the formation of AGCs, often coincident with physiological changes such as metamorphosis (Secor et al. 1995). Some studies have shown that by the end of the metamorphic stage, leptocephali fishes had begun feeding orally (Rasquin 1955; Mercado and Ciardelli 1972). The presence of AGCs on the otolith surface of conger leptocephali is common in a late stage of metamorphosis (Correia et al. 2002a, 2003; present study). Therefore, both the dietary shift and habitat shift hypotheses for the occurrence of these otolith structures seem reasonable for this species.

The conventional methods generally apply diluted HCl or EDTA to etch the sectioned otoliths for SEM observations (Campana and Neilson 1985). HCl reacts with $CaCO_3$ producing calcium chloride, water and carbon dioxide, whereas EDTA chelates calcium and removes the ions from otoliths. These two chemicals are expected to dissolve only $CaCO_3$ but in practice the proteinaceous discontinuous zones are dislodged prior to the highly calcified incremental zones (Mugiya et al. 1981; Watabe et al. 1982). Sometimes these methods do not discriminate easily between the incremental and discontinuous zones (Shiao et al. 1999). Etching otoliths with proteinase K buffer (PKb) reveal more visible daily increments and enhance the contrast since the PKb removes the organic matrix (proteins) leaving the calcified structure almost intact to reveal conspicuous daily increments (Shiao et al. 1999). In the present study, EDTA was found to be the most efficient and effective etching agent and there were no significant differences in size and morphological features of the otoliths in the two section planes. The anterior-posterior direction of the otolith seemed the most appropriate for examining daily growth increments, with increments on proximal and distal portions of the otolith becoming narrow and difficult to discern. This would indicate that less material is deposited in these areas in relation to the dorsal and ventral parts of the otolith, due to ontogenetic changes in accretion rates onto different portions of the otolith. The otoliths do not form a complete growth sequence throughout the early lifetime of the fish, since a peripheral area of poorly defined increments was present. There were also morphological changes in the manner in which the increments had been deposited, resulting in a change in the direction of crystal growth and thus perhaps obscuring the increments. However, independent of the section plane and etching agent used, all otoliths recorded a peripheral

otolith diffuse zone, showing it not to be an optical artefact resulting from the otolith preparation, but a permanent structure of the metamorphosing otoliths.

Marking otoliths is probably the best method for validation of growth rhythms in otoliths for species, which cannot be reared from larvae to adults in the laboratory (Cermeño et al. 2003). Tetracycline is the most commonly used marking agent and is known to be incorporated into calcifying tissues in fishes during growth, although generally with high mortality rates (Geffen 1992). Tetracycline was very successful in inducing a mark in the otoliths of all conger eels exposed and no mortality was observed during and just after the immersions. Treatment marks in the otolith of the conger eel indicated an asymmetrical growth of the otolith, increasing preferentially in the anterior-posterior axis. During the first controlled period (first captive week), the otolith grows about 10 $\mu m.day^{-1}$. However, during the last captive period the otolith growth was slow, approximately 5 $\mu m.day^{-1}$. In both cases, the daily otolith growth was much higher than the largest increment growths (about 1.20 μm) reported for this species (Correia et al. 2002a, 2003). Our data indicates that in artificial conditions otolith growth rate in metamorphosing conger eel leptocephali was higher that in nature. Indeed, it has recently been reported that reared metamorphosing leptocephali had larger otoliths than wild specimens (Correia et al. 2003). Studies on *C. myriaster* have shown that metamorphosis is accompanied by changes in cortisol and thyroid hormones (T_3 and T_4) (Yamano et al. 1991). Stressed adult fish show typical endocrine stress responses manifested by, for example, activation of the hypothalamic-pituitary-interrenal axis and resultant elevation of plasma cortisol (Donaldson 1981). Changes in tissue cortisol concentrations in Japanese flounder appear to vary depending on the rearing conditions (Tanaka et al. 1995). Cortisol may have a synergistic action with thyroid hormones (TH) in the metamorphosis of flatfish larvae (De Jesus et al. 1991; Tanaka et al. 1995). TH could influence, for example, the skeletal ontogeny of larvae, causing anomalies affecting the caudal fin and cranium (Power et al. 2001). These findings suggest that the aquarium conditions and the handling stress, may produce a disequilibria of the endocrine control of metamorphosis, producing an anomalous high grow rate of the otolith. Future attempts should be made to determine the pathway and mechanisms of the DZ deposition.

In this work we have made a scenario associating some microstructural aspects of the otolith growth of conger, to some ecological, physiological and behaviour events taking place during metamorphosis. Further research is necessary, however, to confirm some of these hypotheses.

Acknowledgements

The author was supported by a PhD grant (BD18082/98) from the Portuguese Foundation for Science and Technology.

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4. OTOLITH MICROSTRUCTURE AND MICROCHEMISTRY OF EUROPEAN CONGER EEL CLOSE-RELATED SPECIES

4.1 Early life history of the American conger eel (*Conger oceanicus*), as revealed by otolith microstructure and microchemistry of metamorphosing leptocephali

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Abstract

The early life history of the American conger eel, *Conger oceanicus*, was studied using otolith microstructure and chemical composition in metamorphosing leptocephali collected from New Jersey estuarine waters. The ages of leptocephali were estimated by counting daily growth increments. Ages of early metamorphosing leptocephali at recruitment to the estuary ranged from 155 to 183 days, indicating that migration of conger eel leptocephali from their oceanic spawning ground to the estuary requires 5 to 6 months. Back-calculated hatching dates suggest that the spawning season lasted 3 months, from late October to mid December. However, in the late metamorphic leptocephali, the presence of an unclear peripheral zone in the otolith prevents the accurate estimate of the larval stage duration. The calcium content was almost constant throughout the otoliths. Both strontium and Sr:Ca ratios increased with age, but dramatically decreased at age 70 to 120 days. The otolith increment width also showed a marked increase at the same ages, indicating the onset of metamorphosis. A negative correlation between age at metamorphosis and otolith growth rate indicates that faster-growing leptocephali arrive at the estuary earlier than slower growing ones. A close relationship was also found between age at recruitment and age at metamorphosis, suggesting that individuals that metamorphosed earlier were recruited to the estuary at a younger age. This larval migration pattern appears to be similar among anguilliform fishes.

Introduction

The American conger eel, *Conger oceanicus* (Mitchill 1818), is a bottom-dwelling fish, frequently found along the east coast of North America, from the Gulf of Maine to northern Florida, and extends into the northeastern Gulf of Mexico (Smith 1989; Collette and Klein-MacPhee 2002). This species occurs from shallow coastal waters, occasionally entering estuaries and bays, to the edge of the continental shelf at depths up 577 m, and is usually associated with structured habitats such as piers, wrecks, jetties, reefs or burrows shared with tilefish (Able and Fahay 1998; Collette and Klein-MacPhee 2002).

There is little information available on the early life history of *C. oceanicus*. Schmidt (1931) found small leptocephali in the Sargasso Sea and concluded that was the breeding place of this species, but he provided no detail of the number and distribution of these larvae. No adult conger eels have been observed spawning in the Sargasso Sea and eggs are undescribed (Able and Fahay 1998). Early-stage leptocephali collected in the Sargasso Sea, east and northeast of the Bahamas islands (Castonguay and McCleave 1987; McCleave 1993; McCleave and Miller 1994; Miller 1995) and larger leptocephali (up to 85 mm) collected from the Florida Current and Gulf Stream south of Cape Hatteras, North Carolina (McCleave and Miller 1994) showed that Schmidt (1931) was clearly correct in stating that conger eel spawns in the western North Atlantic.

Bigelow and Schroeder (1953) suggested that *C. oceanicus* matures during summer and then moves offshore, but they gave no evidence for this pattern. The absence of ripe or spent female conger eels in the inshore waters of the Mid-Atlantic Bight and early signs of maturation in females in late spring and early summer (Hood et al. 1988; Eklund and Targett 1990) suggest that they leave the region to spawn off-shore, probably in the Sargasso Sea (Able and Fahay 1998). The spawning season is apparently long, perhaps from late summer through the winter (McCleave and Miller 1994). The mechanisms used by the leptocephali to exit the Gulf Stream and cross the continental shelf to colonize juvenile habitats are not well understood (McCleave 1993; McCleave and Miller 1994; Bell et al. 2003). The duration of the conger eel larval period is not known.

The examination of otolith microstructure and microchemistry has been used to reconstruct the early life history (e.g. spawning area and season, duration of the leptocephalus phase and distribution and larval migratory route) of several anguilliform fishes, including some conger eels (Otake et al. 1997; Correia et al. 2003).

The present paper examines, for the first time, the otolith microstructural growth and the changes in otolith Sr:Ca ratios in *C. oceanicus* leptocephali during the metamorphic stage, in an attempt to elucidate some aspects of its larval life history.

Material and Methods

The twenty metamorphosing conger eel leptocephali used in this study were collected as part of long-term ichthyoplankton sampling (Able and Fahay 1998; Witting et al. 1999). Fishing took place in the estuary, at night, using a 1m, 1mm plankton net off of a bridge in Little Sheepshead Creek, at Great Bay, in southern New Jersey (Fig. 1), in May 2001, April 2002 and May 2002. Three half hour tows were performed with the net at a mid-water depth (approximately 2 m). Temperature and salinity were recorded at the beginning and at the end of the sampling, using a field thermometer and a hand-held refractometer, respectively. Water temperature and salinity of the sampling area ranged from 8 to 17 °C and 26 to 30 psu, respectively.

After capture the leptocephali were preserved in 95% ethanol, and the general body morphology, pigmentation, morphometric and meristic characters were analysed following the methodology described by Smith (1989). Measurements were made to the nearest 0.1 mm, and myomeres counts were done using a dissecting microscope. There was no correction for shrinkage caused by preservation.

Otoliths were prepared for the examination of microstructure and microchemistry, as described in Correia et al. (2003). Otoliths were embedded in epoxy resin, ground to expose the core with a series of graded silicon carbide papers (600, 1200 and 2400 grit), and further polished with diamond pastes (6, 3 and 1 μm), and alumina solution (1:20). Finally, they were cleaned in an ultrasonic bath, rinsed with deionised water and given a gold coating by high vacuum evaporation. Sr and Ca concentrations (% dry weight) were measured along the longest axis of the otolith using a wavelength dispersive X-ray electron microprobe (CAMEBAX SX 50). Apatite [$Ca_5(PO_4)_3$] and celestite ($SrSO_4$) were used as standards. Accelerating voltage and beam current were 15 kV and 10 nA, respectively. The electron beam was focused on a point about 2 μm in diameter, spacing measurements at 5 μm intervals. The microprobe measurements points, which were seen as burn depressions on the otolith surface, were assigned to otolith growth increments. The results are presented as the amount of Sr divided by the amount of Ca times 1000. The averages of successive data for Sr and Ca concentrations pooled for every 10 successive growth increments were used for the

life history transect analysis. Following microprobe analysis, the otolith surface was repolished with alumina solution (1:20), etched for 15 seconds with 0.05 M HCl and vacuum coated with gold for scanning electron microscope observation (SEM, Jeol JSM 630-1F) at 15kV. Core diameter (C), maximum otolith diameter (D) and maximum otolith radius (R) were measured from the SEM photographs (at magnifications between 300 and 2500x) according to the procedures of Correia et al. (2002a). The otolith radius and increment width were measured along the maximum otolith radius. The averages of every 10-successive increment widths from the Hatch Check (HC) to the end of the Increment Countable Zone (ICZ) were used for otolith growth analysis.

We assumed the growth increment in the larval otolith of conger to be daily, although daily deposition has not been validated in this species. We base this assumption on the results of several related anguilliform species, e.g. *Conger myriaster* (Mochioka et al. 1989), *Anguilla japonica* (Tsukamoto 1989; Umezawa et al. 1989; Umezawa and Tsukamoto 1991), *A. rostrata* (Martin 1995; Cieri and McCleave 2001), *A. celebesensis* (Arai et al. 2000a) and *A. marmorata* (Sugeha et al. 2001), which have been validated as being deposited daily. Based on previous studies on otolith microstructure and microchemistry in some leptocephalus fishes (see discussion), age at which the increment width showed a rapid increase simultaneous with a marked decrease in Sr:Ca ratios was regarded as the onset of metamorphosis for this species. Since all the individuals captured are still in metamorphosis, the duration of this stage could not be determined. For the early metamorphic leptocephali ($PAL/TL \geq 0.63$), the number of increments between the hatch check and the otolith edge was regarded as the age at recruitment. Unfortunately, for the late metamorphic leptocephali ($PAL/TL < 0.63$) the age at recruitment (and also the timing between the onset of metamorphosis and the time of capture) could not be established directly from the otoliths because there was a peripheral diffuse zone with unclear increments.

Statistical analyses were performed using the Microcal Origin 5.0. Significance of the correlation coefficient and regression slope were tested, respectively, by a Fisher's Z-transformation and by an ANOVA (Zar 1996). We used a level of significance (α) of 0.05. Data are presented as ranges and mean values ($\pm$ standard deviation).

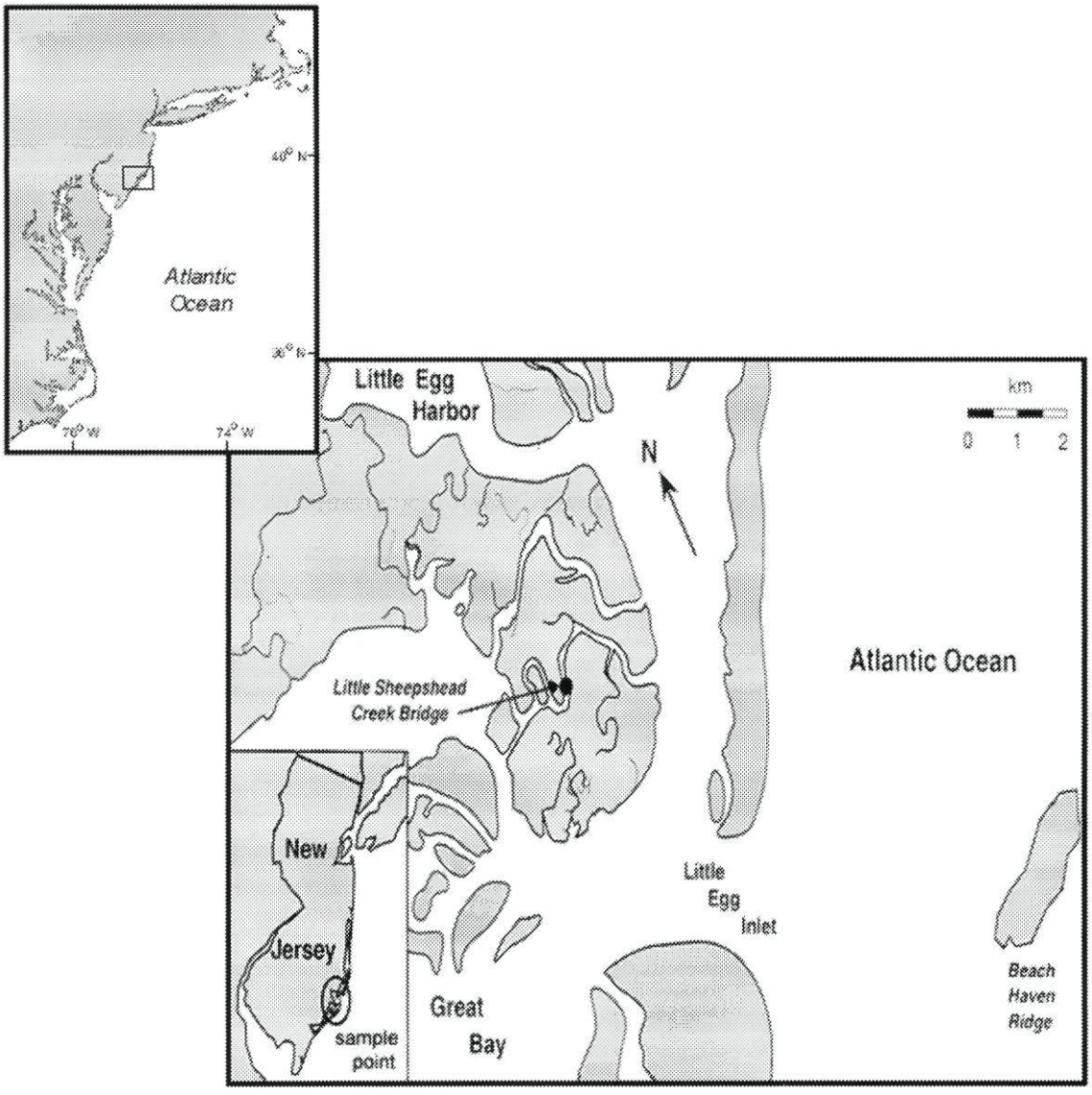


Fig. 1 *Conger oceanicus*. Catch location of the metamorphosing leptocephali in New Jersey, USA.

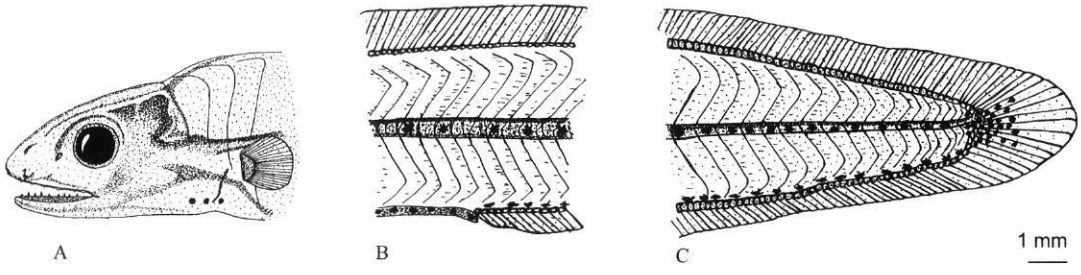


Fig. 2 *Conger oceanicus*. Illustration of the head (**A**), anal region (**B**) and caudal fin (**C**) of a metamorphosing leptocephalus (total length: 84.0 mm; PAL/TL: 0.58).

Table 1 *Conger oceanicus*. Morphometric and meristic characters of the twenty metamorphosing conger eel leptocephali (lengths are expressed in mm).

Parameter	Range	Mean±Standard deviation	Mode
Total length	84.0-104.0	94.2.±5.7	
Predorsal Length	30.0-52.0	40.8±6.0	
Preanal length	42.0-71.0	57.4±8.8	
Head Length	6.0 -7.5	6.8±0.4	
Body depth	6.3-10.0	8.3±1.1	
Eye diameter	1.5-1.6	1.5±0.0	
Total number of myomeres	141-147		145
Predorsal myomeres	44-52		44
Preanal myomeres	58-76		65

Table 2 *Conger oceanicus*. Collection date, length (TL), developing stage (PAL/TL), age at recruitment to the estuary and hatching date of the metamorphosing leptocephali.

No.	Date	TL (mm)	PAL/TL	Age (days)	Hatching date
CO5	May 14, 2001	97.0	0.74	168	November 28, 2000
CO4		96.0	0.63	179	November 18, 2000
CO2	May 24, 2001	94.0	0.52	-	-
CO10	May 30, 2001	93.0	0.58	-	-
CO6		84.0	0.58	-	-
CO8		97.0	0.55	-	-
CO9		90.0	0.53	-	-
CO7		88.0	0.53	-	-
CO3		91.0	0.51	-	-
CO11		89.0	0.47	-	-
CO12	April 04, 2002	99.0	0.71	156	October 31, 2001
CO15	May 01, 2002	104.0	0.68	155	November 28, 2001
CO13		100.0	0.63	182	October 30, 2001
CO14		85.0	0.67	183	October 31, 2001
CO16	May 08, 2002	99.0	0.70	171	November 19, 2001
CO19		101.0	0.67	167	November 23, 2001
CO17		87.0	0.66	149	December 11, 2001
CO21		99.0	0.61	-	-
CO20		93.0	0.60	-	-
CO18		98.0	0.58	-	-

Results

Morphology, pigmentation and dentition

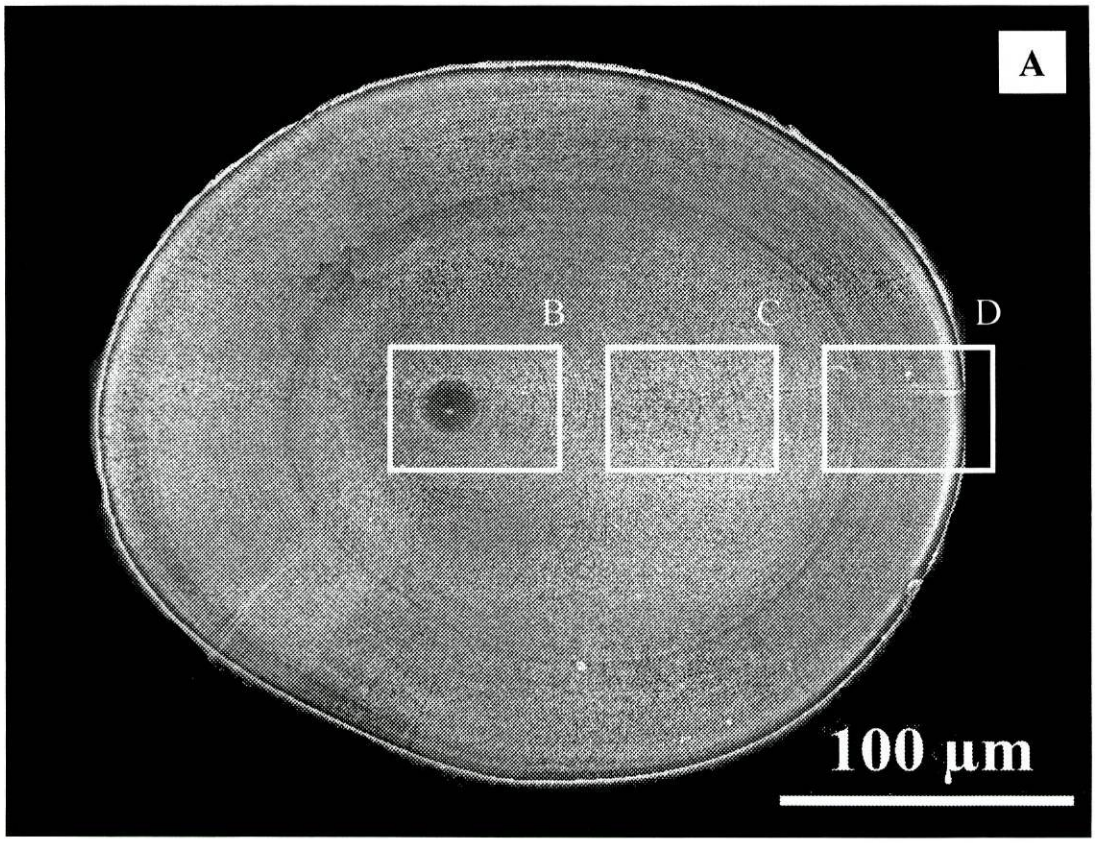
The metamorphosing leptocephali were elongate, laterally compressed, with "w" shaped myomeres and a simple tubular gut along the ventral margin of the body (Fig. 2). The head was a medium size, with rounded eyes, lips and nasal tubes. The dorsal fin was extended anteriorly, but did not reach half of the total length. They had large melanophores along the midlateral line, which became sparser or disappeared altogether anteriorly. Additional smaller melanophores appeared near the tail region. They had two prominent melanophores near the anus and three melanophores at angle of the lower jaw. The crescent-shaped patch of pigment under the eye, characteristic of the developing stage, was absent. Some of these leptocephali had small vestigial euryodontic teeth in both maxillas, however the majority had lost their larval dentition.

Biometric and meristic values

The total length of leptocephali ranged from 84.0 to 104.0 mm, with a mean of 94.2±5.7 mm (Table 1). The developmental stage indicator, preanal length/total length (PAL/TL), ranged from 0.47 to 0.74 with a mean value of 0.61±0.08. The total number of myomeres (TNM), predorsal myomeres (PDM) and preanal myomeres (PAM) ranged from 141 to 147, 44 to 52 and 58 to 76, respectively. The PAL/TL ratio was positively and significantly correlated with the PAM/TNM ratio (r^2 =0.86, n=20, P<0.05).

Otolith growth pattern

Otoliths in the metamorphosing leptocephali were oval (Fig. 3A and 4A) and showed the same concentric ring pattern, except for the margin. Dependent on the larval developmental stage, the otolith showed some permanent structures, like a central Core (C), the Increment Countable Zone (ICZ), the Diffuse Zone (DZ) and the existence of one or more Accessory Growth Centres (AGC). The core, located on the posterior side of the otolith, is composed of an amorphous primordium surrounded and delimited by the innermost discontinuous zone, presumed to be the hatch check (HC) (Fig. 3B). The core had a mean diameter of 19±1 μm. Beyond the HC, clear daily growth increments marked the beginning of the ICZ. In this increment countable zone, the otolith increment width showed a characteristic curve along the



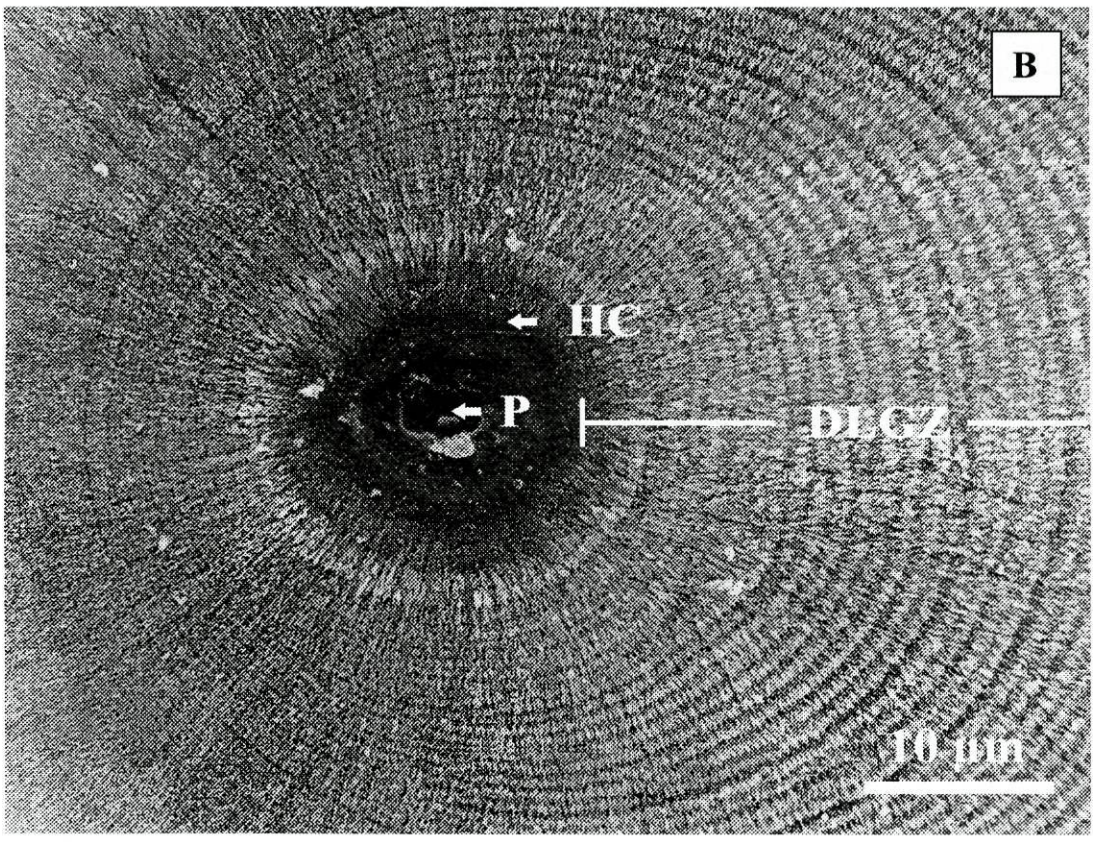


Fig. 3A, B *Conger oceanicus*. SEM photographs showing the otolith microstructure of an early metamorphosing conger eel leptocephalus (total length: 96.0 mm; PAL/TL: 0.63). **A** whole view (boxes with letters correspond respectively to Figs. 3B, C and D); **B** otolith core and surrounding zone.

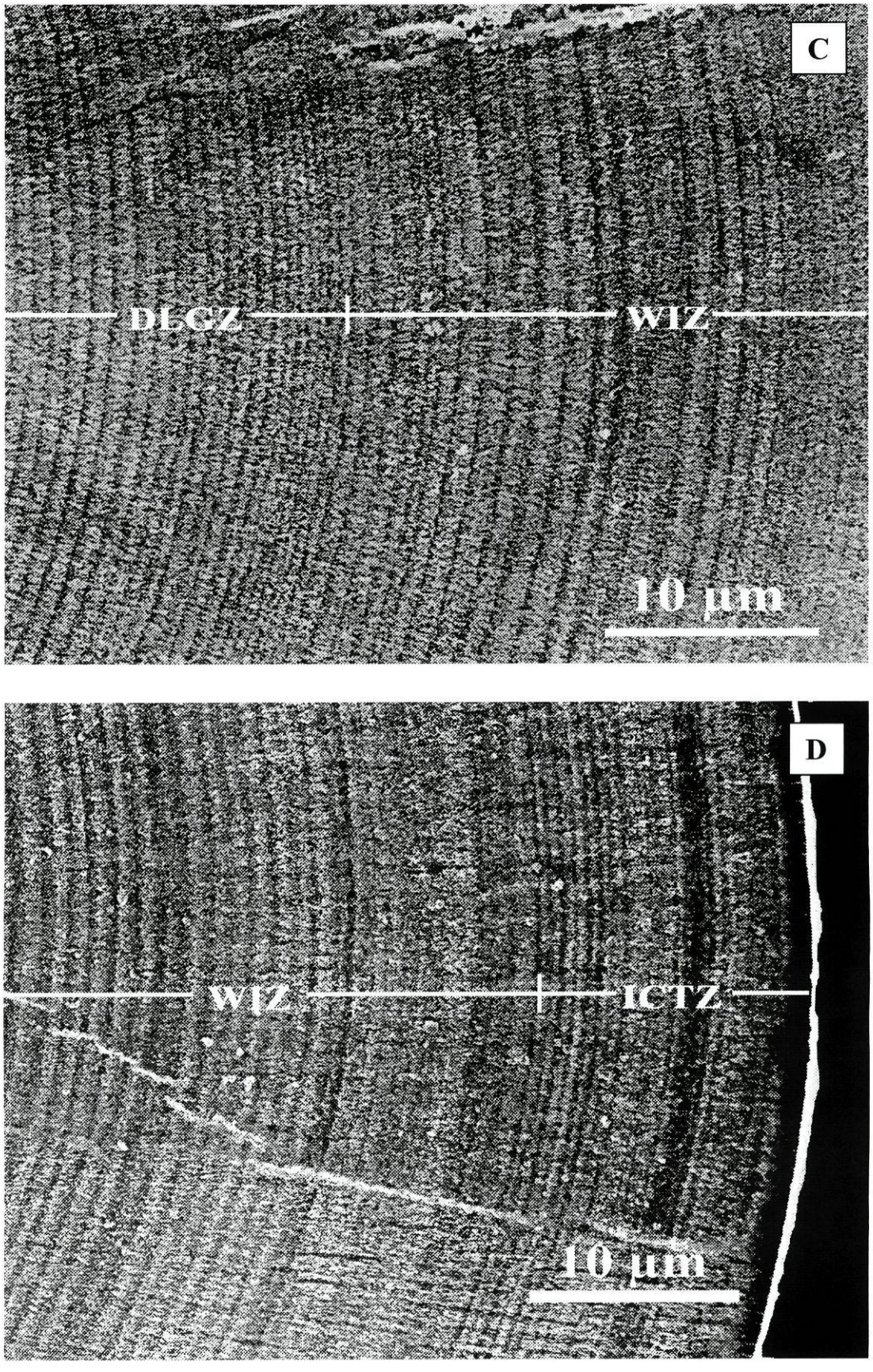


Fig. 3C, D *Conger oceanicus*. **C** boundary between the developing leptocephalus growth and metamorphic zones; **D** otolith edge (Legends: *P* primordium; *HC* hatch check; *DLGZ* developing leptocephalus growth zone; *WIZ* wide increment zone; *ICTZ* increment countable terminal zone).

radius (Fig. 5). Otolith increment widths increased (until a maximum of 1.10 μm) between the HC and age 30-50 days (phase I), thereafter gradually decreasing, until a relatively constant minimum value was reached (0.65 μm), at about 80 days (phase II). This inner portion of the ICZ, that includes phases I and II, is named the otolith Developing Leptocephalus Growth Zone (DLGZ) (Fig. 3C). In a third phase, the increments widen abruptly (to a maximum of 1.20-1.50 μm), which depending on the specimen, could occur between 70 to 120 days. This Wide Increment Zone (WIZ), occurred over about 20 to 40 days, after that the increment width decreased (ICTZ) (phase IV) (Fig. 3C and D). In the late metamorphic leptocephali (PAL/TL<0.63), the increments of the peripheral zone became less clear and disappeared, which corresponds to the onset of the Diffuse Zone (DZ) (phase V). However, clear marks sometimes appeared in the marginal region. As the DZ grew larger, accessory growth centres were formed (phase VI), giving the otolith a fan-like morphology at end of the secondary growth centres (Fig. 4A). In these structures the aragonite needles were arranged at an angle relative to the sagittal plane (Fig. 4B).

There is a good correlation between the diameter and the radius of the otolith (r^2=0.76, n=20, P<0.05). The radius and diameter of the otoliths ranged from 142 to 212 μm and from 250 to 376 μm, with a mean of 173±17 μm and 301±38 μm, respectively. The otolith size (diameter and radius) was not significantly related to total body length (r^2=0.16, n=20, P=0.08 and r^2=0.19, n=20, P=0.06) but it was significantly related to head length (r^2=0.42, n=20, P<0.05 and r^2=0.39, n=20, P<0.05) and to the PAL/TL ratio (r^2=0.57, n=20, P<0.05 and r^2=0.70, n=20, P<0.05). The number of increments in the ICZ ranged from 143 to 202 days, with a mean of 180±18 days. The width of the DZ presented a minimum value of 14 μm and a maximum of 52 μm (28±11 μm).

Otolith Sr:Ca ratios

Sr concentrations dramatically changed along the life history transect. Sr content in the otoliths averaged 0.25±0.08 % in the core and increased outwards to a maximum level of about 0.39±0.06 % at ages 70 to 120 days. Subsequently Sr content decreased rapidly to about 0.28±0.03 % at the outermost regions. Ca content was almost constant (26.2±1.8 %) throughout the otolith, except for the core region, which recorded a low value (25.9±1.8 %). Sr:Ca ratios changed in a similar manner to Sr content (Fig.6) and averaged 8.5×10^{-3} (1.0×10^{-3} SD) in the core and rapidly increased soon after hatching. The ratios reached a maximum

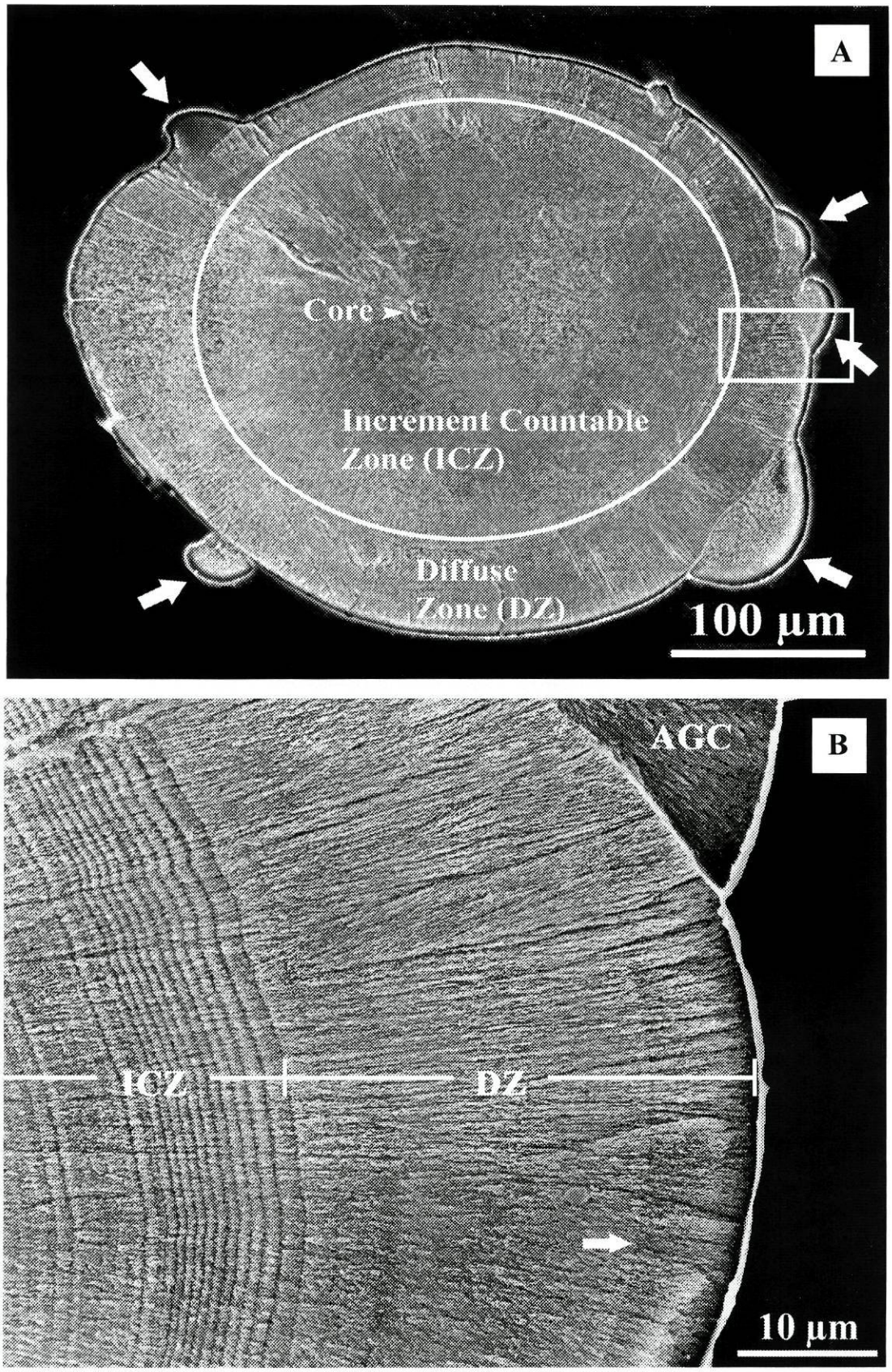


Fig. 4A, B *Conger oceanicus*. SEM microphotographs showing the otolith microstructure of a late metamorphosing conger eel leptocephalus (total length: 88.0 mm, PAL/TL: 0.53): **A** whole view of the otolith with accessory growth centres indicated by *arrows*; **B** marginal region of the otolith (detail of the region indicated by a box in A) showing the disturbed arrangement of the peripheral rings (*AGC* accessory growth centres; *ICZ* increment countable zone; *DZ* diffuse zone; *arrow* indicates a clear mark).

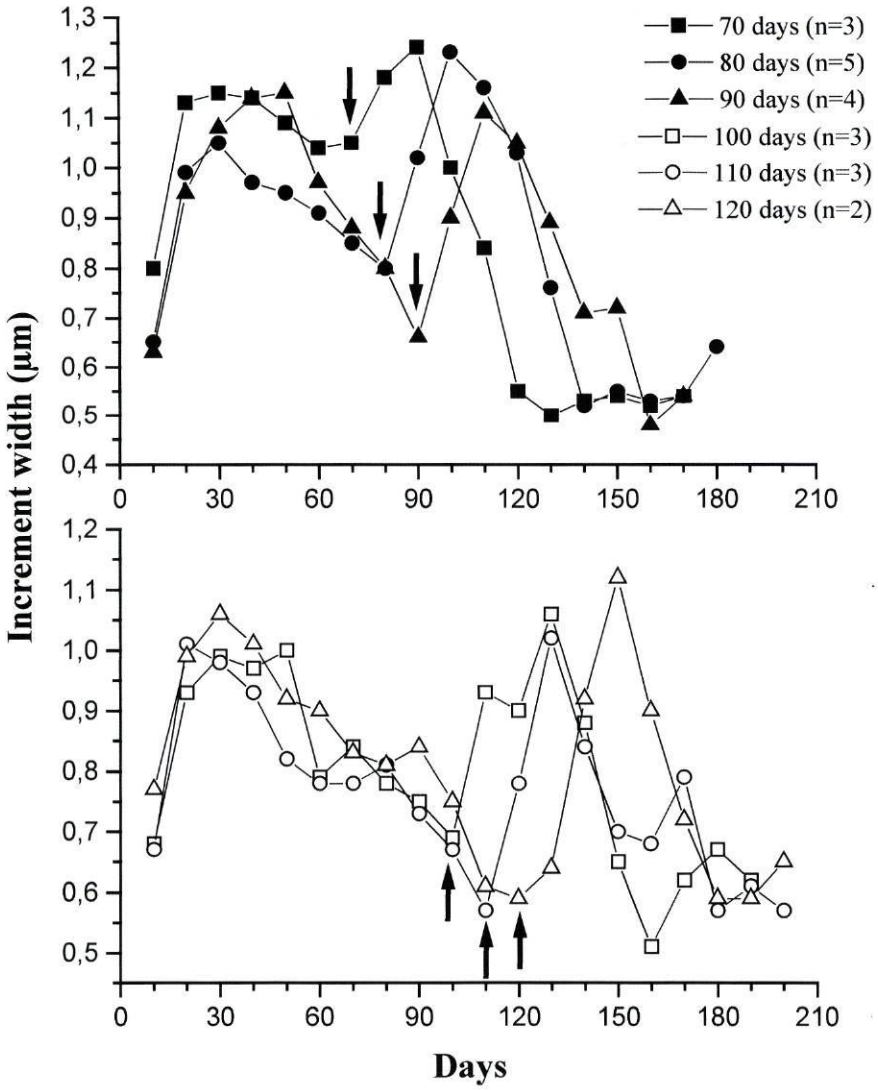


Fig. 5 *Conger oceanicus*. Profiles of the mean increment width throughout the otolith´s increment countable zone. Specimens were grouped by the time when the daily rings abruptly increased (see *arrows*).

level of about 15.4×10^{-3} (1.8×10^{-3} SD), some 70 to 120 days after hatching, i.e. at the end of the DLGZ. Subsequently, the ratios sharply decreased and maintained a stable minimum value of 11.9×10^{-3} (0.9×10^{-3} SD) outwards to the otolith edge (Fig. 7). The sudden increase in otolith increment width (WIZ), at ages 70 to 120 days, coincided with the rapid drop in otolith Sr content and Sr:Ca ratios and marked the onset of metamorphosis (see Discussion) (Fig. 8).

Age and hatching time

The ages at recruitment of the leptocephali to the estuary, while still in an early developmental stage (PAL/TL≥0.63), and thus did not have a DZ in the otolith, ranged from

149 to 183 days (Table 2). Hatching dates, back-calculated from these daily estimates, indicated that the spawning season lasted about 3 months, from late October to mid December.

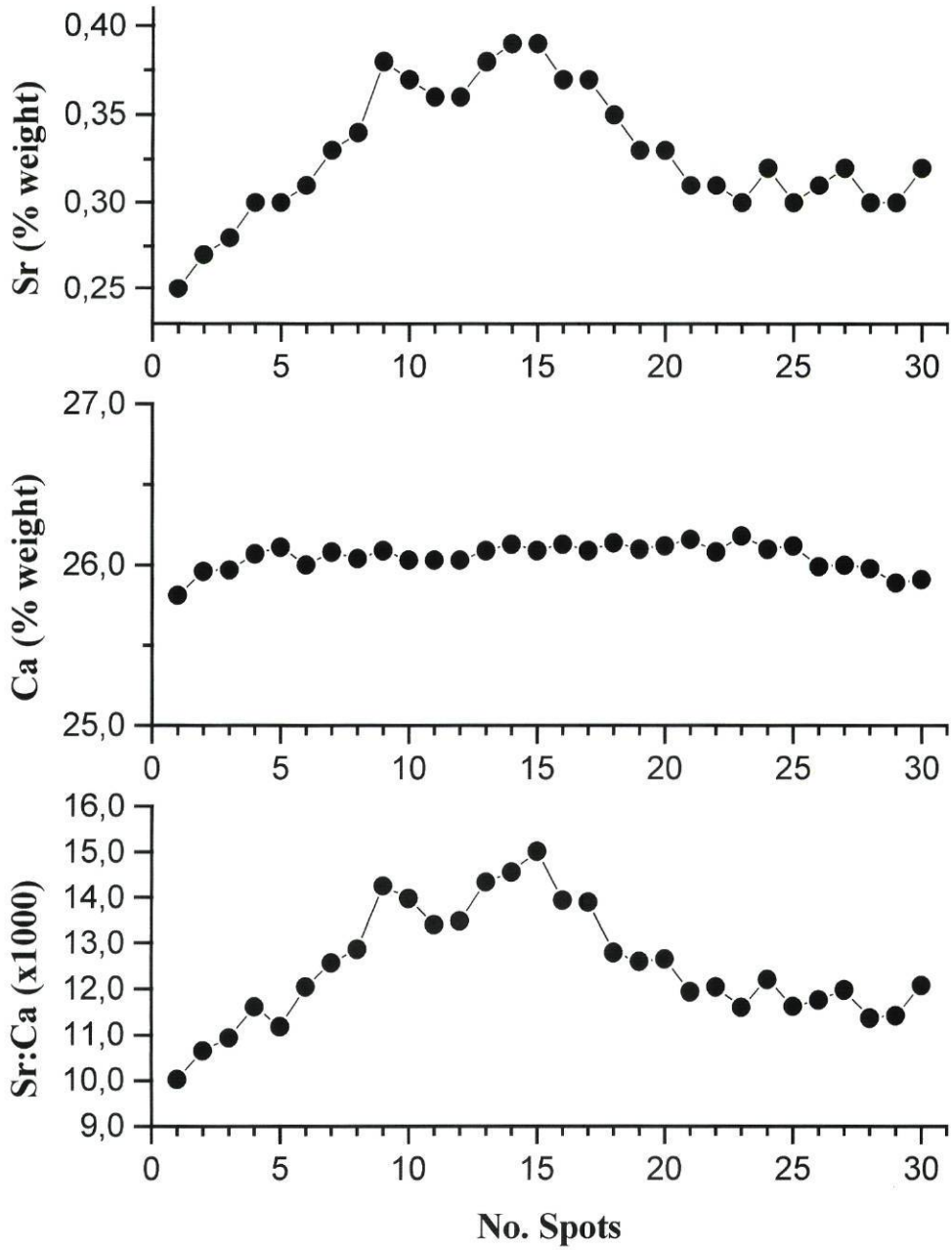


Fig. 6 *Conger oceanicus*. Profile of the mean values for Sr and Ca contents and Sr: Ca ratios from the core to the edge of otoliths determined by wave-length dispersive electron micropobe analysis (sample size: 10 specimens).

Age at metamorphosis

The duration of the developing leptocephalus stage (i.e. the number of increments in the DLGZ) ranged between 70 and 120 days, with a mean value of 92±16 days. The age at metamorphosis was negatively correlated with the mean increment widths of the DLGZ (r^2=0.62, n=20, P<0.05) (Fig. 9). A close linear relationship was also apparent between the age at recruitment and age at metamorphosis for the early metamorphic leptocephali (r^2=0.85, n=9, P<0.05) (Fig. 10). For these specimens, the metamorphosis started 59 to 83 days (67±8 SD) before the arrival in estuarine waters.

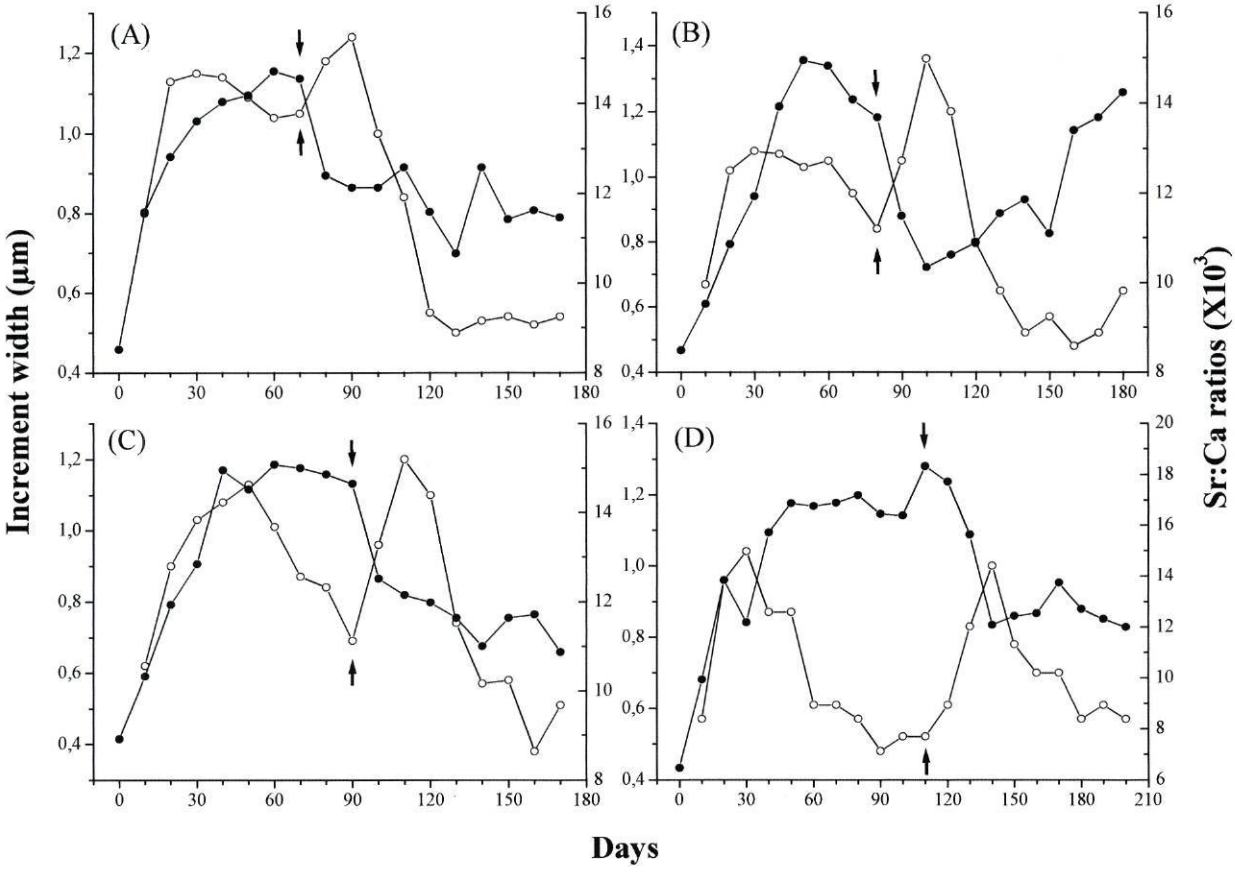


Fig. 7 A-D *Conger oceanicus*. Profiles of the otolith increment width (open circles) and Sr:Ca concentration ratios (solid circles) measured from the core (age 0) to the end of the increment countable zone. Specimens were grouped by the time when coincidental changes in increment width and Sr:Ca ratios occurred (**A**: 70 days, n=3; **B**: 80 days, n=2; **C**: 90 days, n=3; **D**: 110 days, n=2). *Black arrows* represent approximate locations of metamorphosis.

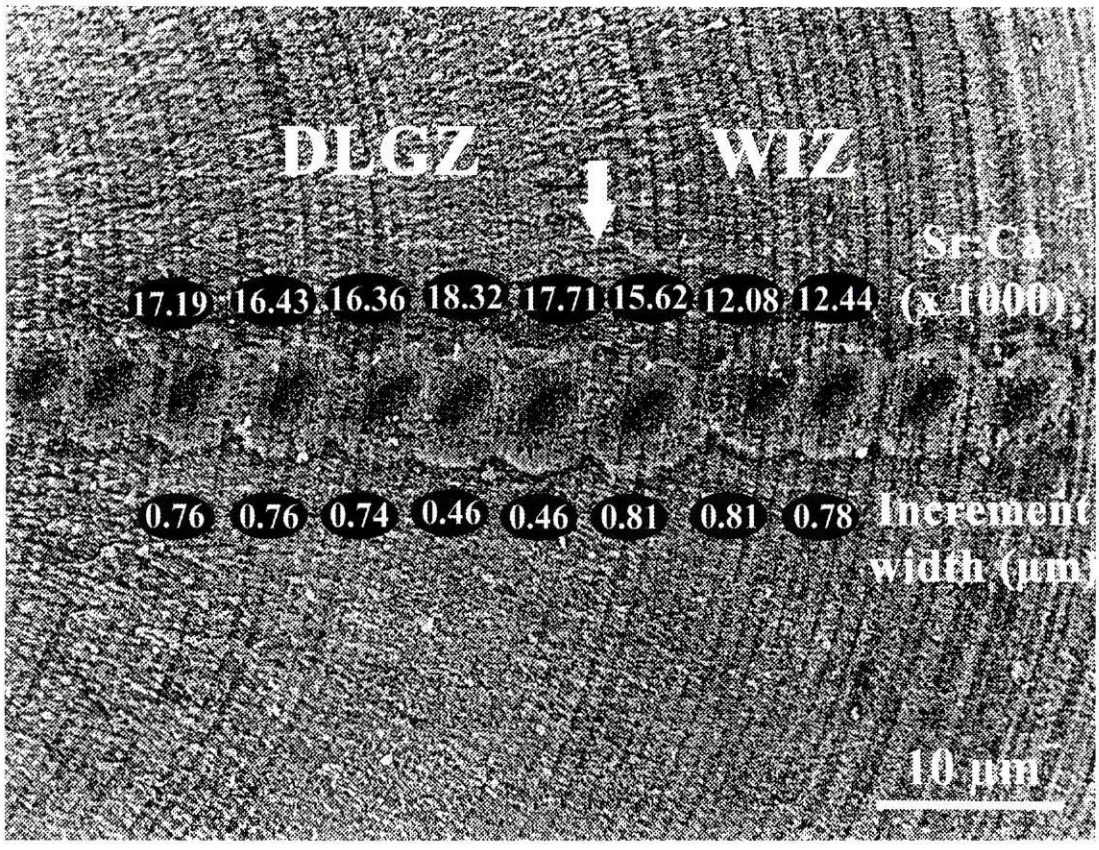


Fig. 8 *Conger oceanicus*. SEM microphotograph showing the Sr:Ca ratios and increment width values, in a otolith of a metamorphosing conger eel leptocephalus (length: 101.0; PAL/TL: 0.67). Abbreviations: *DLGZ* developing leptocephalus growth zone; WIZ wide increment zone; *arrow* onset of metamorphosis.

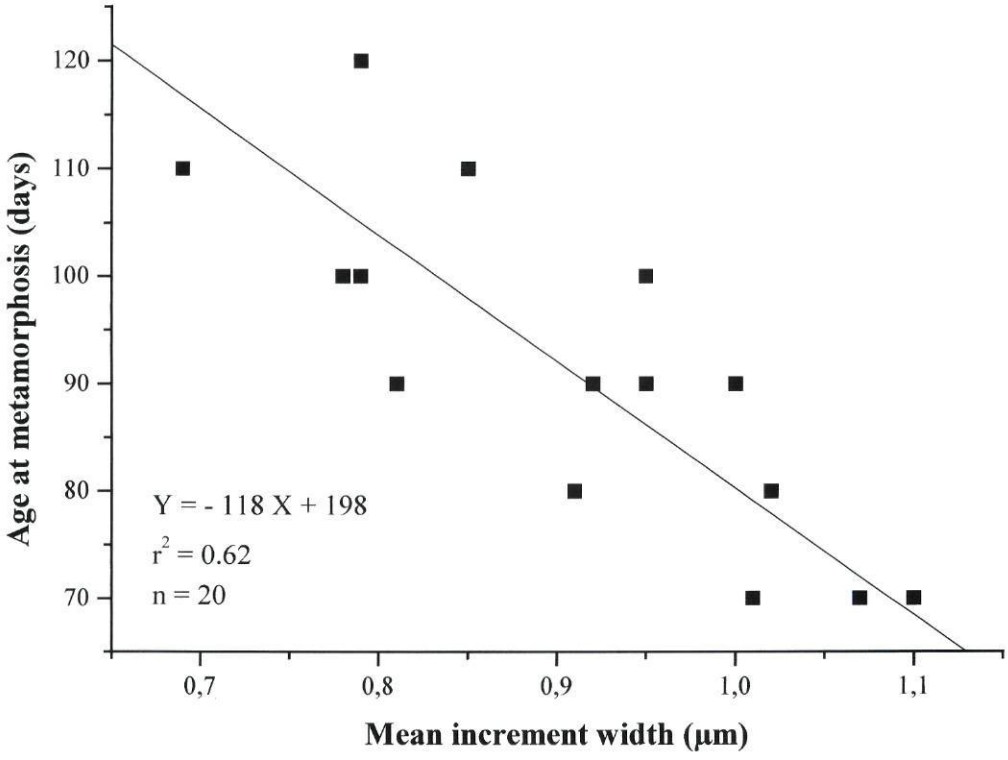


Fig. 9 *Conger oceanicus* metamorphosing leptocephali. Scatter diagram of age at metamorphosis versus mean otolith increment width. Regression line represents a least square fit of the linear equation ($P<0.05$).

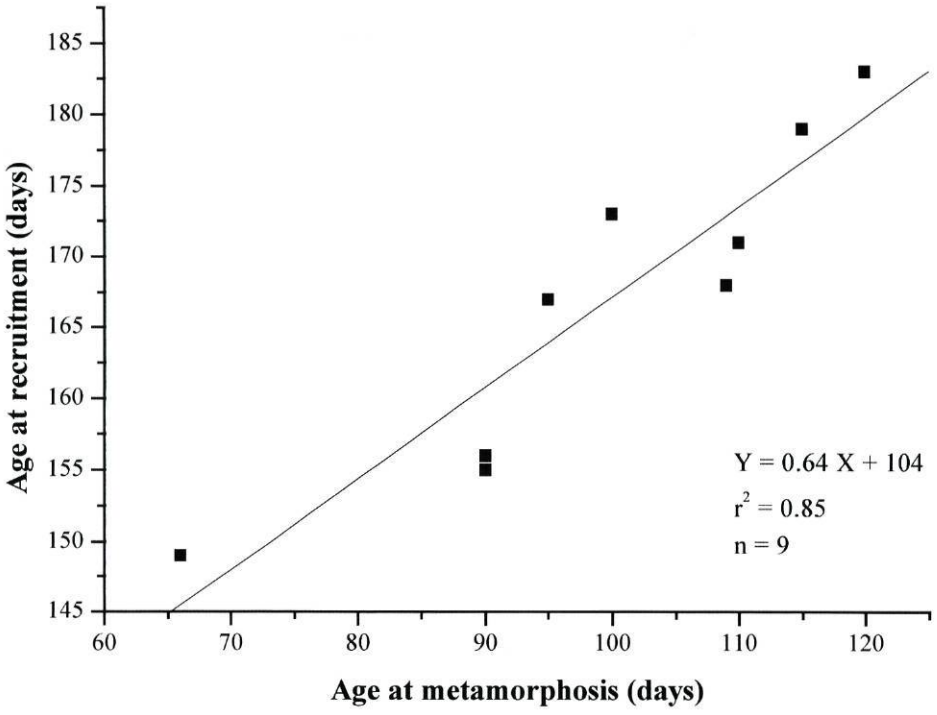


Fig. 10 *Conger oceanicus* metamorphosing leptocephali. Relationship between age at recruitment to the estuary and age at metamorphosis. Regression line represents a least square fit of the linear equation ($P<0.05$).

Discussion

Conger oceanicus arrive in coastal waters while they are undergoing metamorphosis (Able and Fahay 1998; Bell et al. 2003). Metamorphosing leptocephali collected in this study ranged from 84.0 to 104.0 mm TL and covered the length range (70 to 11 mm) reported by Bell et al. (2003). The total number of myomeres (TNM), the principal species identification criterion, ranged between 141 and 147, which is in agreement with the values reported by other authors (Schmidt 1931:140-149; Smith 1989:140-148; McCleave and Miller 1994:137-148). The ranges of the predorsal (PDM) and preanal (PAM) myomeres, 44-52 and 58-76, of our specimens were, as expected, smaller that the counts obtained by Smith (1989) for the developing stage (PDM:67-81; PAM:113-124), since during metamorphosis the anus and the dorsal fin begin to moves anteriorly in congrid leptocephali (Otake et al. 1997; Strehlow et al. 1998; Bell et al. 2003). The use of the PAL/TL, instead of the usual PAM/TNM, as an indicator of the development stage in several studies of eels (Yamano et al. 1991; Otake et al. 1997; Correia et al. 2002ab, 2003; Bell et al. 2003) has the advantage of being more easily obtainable and less affected by measurement errors (Correia et al. 2002a). In our study the

PAL/TL was highly correlated with the PAM/TNM ratio and ranged between 0.47 and 0.74. These values are similar to the ranges recorded recently by Bell et al. (2003) on other transforming leptocephali collected at this location (0.45 to 0.60).

The metamorphosing leptocephali had the body shape associated with the general and identifying features of this species. Based on morphology, pigmentation and dentition these metamorphosing leptocephali correspond to the developmental stage M1, described by Bell et al. (2003). The body is laterally compressed with the formation of the lips and the straightening of the dorsal profile of the head. The body depth decreases and the anus and dorsal fin move anteriorly. They have large individual melanophores along the midlateral line and small melanophores along the top of gut. Additional smaller melanophores may appear near the tail region. Some leptocephali may have vestigial teeth but they disappear before the end of this stage. M1 stage individuals are usually caught in the coastal area and estuaries, on pelagic and demersal habitats (Bell et al. 2003).

The otolith growth pattern of *C. oceanicus* metamorphosing leptocephali is similar to that recorded by Lee and Byun (1996) and Correia et al. (2002a, 2003) for *C. myriaster* and *C. conger*, respectively. The profile of the otolith growth increments could be divided into five stages. From HC to about 30 to 50 days, the growth increments became wide (phase I). This inflection in otolith growth might be related to favourable somatic growth after successfully switching their nutritional source from yolk material to exogenous feeding (Arai et al. 2001). After this peak, the increment width diminished gradually and maintained a low value to about 80 days (phase II). Thereafter, the increments abruptly widened during 20 to 40 days (phase III), and started to decrease in width at the outer margin of the Increment Countable Zone (phase IV). Later in metamorphosis, the otolith continued to grow after increment resolution was lost, at about 180 days, resulting in a peripheral diffuse zone (phase V). The first three phases of the otolith increment width profile were similar to the above mentioned conger species, although the 4th and 5th phases are absent, respectively in *C. conger* (Correia et al. 2002a, 2003) and *C. myriaster* (Lee and Byun 1996). The biological relationship between otolith growth and morphological and ecological events are not fully understood.

The otoliths of *C. oceanicus* developed a peripheral diffuse zone in the metamorphosing specimens, as have already been recorded in *C. conger* (Correia et al. 2002a, 2003; Antunes and Correia 2003). Several authors have described the otolith microstructure of *C. myriaster* during metamorphosis, but they never identified the existence of a peripheral diffuse zone, although they had reported several problems. Tanaka et al. (1987), for instance, recorded a disturbance of the ring arrangement in the marginal region of the otoliths and assumed it to be

an anomalous, non-permanent structure. Mochioka et al. (1989) also reported some irregularities on the outer surface of the otoliths, while Lee and Byun (1996) observed a peripheral opaque zone in the otoliths where the increments were difficult to identify under light and scanning electron microscopy. Finally, Otake et al. (1997) related some technical problems in consistently etching all rings from the core to the edge of the otolith. This unclear increment area, previously observed in the European eel, *A. anguilla* (Antunes and Tesch 1997), could represent a period of a very slow growth made up of several daily rings that are too thin to be distinguished and counted (Williamson et al. 1999), or a process of calcium resorption in the marginal portion of the otolith during metamorphosis (Cieri and McCleave 2000) and even poor otolith preparation, e.g. overetching (Arai et al. 2000a). This unreadable marginal otolith diffuse zone, the meaning of which remains unknown, prevents the accurate estimate of the total age of the late metamorphosing individuals from our collections.

The occurrence of AGCs in some otoliths of *C. oceanicus* appears to common to other congrid eels during metamorphosis (Lee and Byun 1996; Correia et al. 2002a, 2003). These structures are responsible for the secondary growth layers, which will generate the elliptical otolith shape in adults (Correia et al. 2002a). The mechanism resulting in such structures is not fully understood, however it has been suggested that the AGCs reported in flatfish otoliths are related to a change in habitat and behaviour (Sogard 1991; Modin et al. 1996; Neuman et al. 2001). A transforming otolith, which will result in an adult-like form, may also be necessary to navigate through different environments to pursue prey and detect approaching predators (Brown et al. 2001).

Patterns of the strontium (Sr) to calcium (Ca) ratio of otoliths have been used to reconstruct a chronology of environmental conditions related to age and life stage of fish, particularly the habitat use and seasonal migration for various anguilliform fishes, including *Anguilla* spp. (Otake et al. 1994; Tzeng 1995; Cheng and Tzeng 1996; Tzeng et al. 1997; Arai et al. 1997, 1999, 2000b, 2002) and *Conger* spp. (Otake et al. 1997; Correia et al. 2003). In these fishes change in otolith Sr: Ca ratios have been considered to be related to shifts in temperature and water chemistry along possible migratory routes (Tzeng 1994; Tzeng et al. 1997; Tsukamoto et al. 1998; Tsukamoto and Arai 2001; Jessop et al. 2002), as well to ontogenetic variations in otolith elemental composition (Otake et al. 1997).

The pattern of Sr:Ca ratios along the otolith transect in the metamorphosing *C. oceanicus* leptocephali was consistent with the values reported in other anguilliform species (*C. myriaster*, Otake et al. 1997; *C. conger*, Correia et al. 2003). Sr:Ca ratios were lowest in the primordium and at the edge of the otolith. It has been suggested that the low Sr content

observed in the otolith primordium of eels is probably due to the maternal or freshwater origin of the oocyte (Tzeng and Tsai 1994; Wang and Tzeng 2000). However, the results obtained by Otake et al. (1997) and by Correia et al. (2003) for *C. myriaster* and *C. conger*, respectively, two marine species, showed that this feature could not be associated with a change in salinity and probably reflects the organic chemical composition of the otolith primordium (Correia et al. 2003). The rapid decrease in Sr:Ca ratio simultaneous with the increase in otolith increment width appear to occur during metamorphosis and have been observed in several species of anguilliform fishes, including *A. japonica* (Otake et al. 1994; Cheng and Tzeng 1996; Arai et al. 1997), *A. anguilla* and *A. rostrata* (Arai et al. 2000b; Wang and Tzeng 2000), *A. australis, A. celebesensis* and *A. dieffenbachii* (Marui et al. 2001), *A. bicolor pacifica* (Arai et al. 1999; Marui et al. 2001), *A. marmorata* (Marui et al. 2001; Arai et al. 2002), *C. myriaster* (Otake et al. 1997) and *C. conger* (Correia et al. 2003). Tzeng and Tsai (1994) and Wang and Tzeng (2000) proposed that the drop in Sr:Ca ratio in the eel otolith after metamorphosis reflects the sudden change in ambient salinity associated with the migratory behaviour of the eel, i.e. the entry into freshwater habitats less rich in Sr. However, since *C. oceanicus* individuals, in our collections, are often taken at nearly full salinities (approximately 28 psu) (Martino and Able 2003) this likely would not result in a change in strontium:calcium ratios. This phenomenon probably represents the mobilisation of body minerals for rapid bone development that occurs during the late metamorphosis in leptocephalus fishes (Otake et al. 1997). The same author proposed that the rapid drop in Sr.Ca ratios might be associated with a decreasing Sr levels in the body as a result of catabolism of Sr-rich sulphated glycosaminoglycans (GAG) during metamorphosis. Campana (1999) suggested that the elevated Sr:Ca ratios in eel leptocephalus otoliths, namely the peak in Sr:Ca ratio at the time of metamorphosis from larval stage, is due to the extremely low growth rates, even at high temperatures, since metamorphosis is a life history stage with markedly curtailed growth (Pfeiler 1999). These findings suggest that these changes are a common feature in leptocephalus fishes that may be associated with the remarkable morphological and physiological transformations that occurs during development.

The close relationship between the diameter and radius of the otolith indicates that both measures are helpful in describing otolith growth. As already observed for *C. myriaster* (Lee and Byun 1996) and *C. conger* (Correia et al. 2002a, 2003), somatic and otolith growth became uncoupled during the metamorphic stage. However, otolith size was significantly related to the HL, suggesting that the head and otolith continue to grow although fish length decreased during metamorphosis. Assuming that the formation of the first increment

coincided with hatching and that increments are deposited on a daily basis, the ages of the early metamorphosing leptocephali, which did not have the unclear diffuse zone, were estimated as 149 to 183 days old after hatching. These results should be interpreted with caution because of the small sample size. The hatching dates, back-calculated from sampling dates and estimated ages, ranged from late October to mid December, and agree with the proposed spawning period based on the collection of small larvae (McCleave and Miller 1994). These dates also suggest a short spawning period during the winter season. However, the presence of small leptocephali (<30 mm long) of *C. oceanicus* over deep water in the southwestern Sargasso Sea in autumn and winter implies a protracted spawning period there (McCleave and Miller 1994). Back-calculated hatching dates from the otoliths of developing leptocephali of *C. conger* also indicated a long spawning season, from December to July, with one consistent annual peak, occurring in summer (Correia et al. 2002b, 2003). Lee and Byun (1996) estimated birthdates from September through February by analysis of the otolith microstructure of *C. myriaster* leptocephali. An extended spawning period has also been reported in *A. japonica* (Tabeta et al. 1987; Tsukamoto 1990; Tsukamoto and Umezawa 1990) and it has been proposed that this might be due to multiple populations of adult *Anguilla japonica* prolonging the duration of the spawning season (Tsukamoto 1990).

The duration of the developing leptocephalus stage, i.e. the number of daily increments from the HC to the onset of the WIZ, indicates that *C. oceanicus* takes about 2 to 4 months from hatching to reach metamorphosis. Correia et al. (2002a, 2003) reported 6 to 9 months of duration for the developing leptocephalus stage of *C. conger* and Lee and Byun (1996) noted 4 to 8 months in the case of *C. myriaster*. The differences observed in the duration of the developing leptocephalus stage between these *Conger* species, may be related to the different distances from the spawning ground to the juvenile coastal area and/or to the dynamics of the oceans currents in the various areas. *C. conger*, for example, spawns in the open ocean at least 3000 Km from the coast (Correia et al. 2002b, 2003). Takai (1959) proposed a single spawning site for *C. myriaster* at the edge of the continental slope in the East China Sea, but there is also a possibility of multiple spawning sites in the east Asia region (Mochioka et al. 1988). However, these hypotheses concerning the exact location of the spawning site for this species are not based on any substantial data from the population structure or the distribution of larvae and eggs. Spawning of *C. oceanicus* in the Sargasso Sea indicates that adults cross the Florida Current-Gulf Stream and successful leptocephali might cross the current in the opposite direction to colonize juvenile habitat on the continental shelf, a migratory pattern similar to that of the American eel, *Anguilla rostrata* (McCleave and Miller 1994). It could be

also conceivable that leptocephali use deeper countercurrents under the Gulf Stream that apparently occur in this area (Able and Fahay 1998) to recruit to juvenile habitats. Apparently the Japanese eel, *Anguilla japonica*, spawns in an analogous location with respect to the western boundary current of the North Pacific Ocean, the Kuroshio Current (Tsukamoto 1992). The size distributions of leptocephali suggest gyres in the south-western Sargasso Sea, Antilles Current and the Florida Current north of Bahamas are routes of exit for anguillid and congrid eels (McCleave 1993). Most *C. oceanicus* enter the Florida Current north of Bahamas (McCleave and Miller 1994), since there are few data on distribution of leptocephali or early juveniles between the current and the shelf in relation to physical features (McCleave 1993). The mechanisms used by these leptocephali to exit the Gulf Stream and cross the continental shelf are still poorly understood (Able and Fahay 1998). Our data suggests, however, that the migration of leptocephali from their oceanic spawning ground to the estuary requires 5 to 6 months. The lengthy duration of metamorphosis and the timing of metamorphosis are important factors determining the long-distance dispersal of the eels (Cheng and Tzeng 1996) and might be responsible for the segregation of the migrating *A. rostrata* and *A. Anguilla* (Wang and Tzeng 2000). A short duration of the leptocephalus stage might favour oceanic retention, while a long duration might favour emigration (McCleave 1993). The onset of metamorphosis to the juvenile phase may be delayed until appropriate physical conditions are meet (Tzeng 1990), as has been suggested for *C. oceanicus* (Bell et al. 2003). Larvae with long pelagic existences may be more susceptible to being carried by different currents and to being exposed to different growth conditions (Benoît et al. 2000)

The age at metamorphosis is inversely related to the growth rate suggesting that slower-growing fish apparently metamorphosed later. This phenomenon was also found in *Pseudopleuronectes americanus* (Chambers and Leggett 1987), in *A. japonica* (Tzeng 1990), in *Megalops cyprinoides* (Tzeng et al. 1998) and in *C. conger* (Correia et al. 2003). Although it has not usually been considered an estuarine species, rare, single occurrences of young stages in estuaries have been reported through the Middle Atlantic Bight (Hauser 1975; Moring and Moring 1986; Able and Fahay 1998). The ingression of *C. oceanicus* leptocephali into the estuaries occurs regularly from May to July (Able and Fahay 1998), at ages of 168±12 days (this study) and co-occurs with morphological metamorphosis and settlement to bottom habitats (Bell et al. 2003). Metamorphosis appeared to have started several days (68±8) before arriving in the estuary. The positive relationship between the age at recruitment and the age at metamorphosis suggests that *C. oceanicus* that metamorphosed at an earlier stage tended to recruit to estuaries at younger ages, indicating that early-metamorphosing

larvae are recruited earlier. Several authors (Tsukamoto 1990; Tsukamoto and Umezawa 1990; Arai et al. 1999, 2000b, 2001; Wang and Tzeng 1998; Marui et al. 2001) found the same phenomenon in temperate and tropical eels. This relationship between the timing of metamorphosis and the inshore migration of leptocephali seems to be typical for anguillid and congrid eels. However, there is a need for further studies of this critical stage, in order to elucidate the mechanisms used by *C. oceanicus* to optimise the recruitment to coastal areas.

Acknowledgements

The author was supported by a PhD grant (BD18082/98) from the Portuguese Foundation for Science and Technology. We thank Dr. J. Wilson for English language assistance and Angie Muzeni for help with the collection of specimens used in this study.

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4.2 Sagitta microstructure of European conger eel, *Conger conger* (L.), leptocephali compared with leptocephali of the eel, *Anguilla anguilla* (L.)

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Abstract

Sagitta microstructure of the European conger eel (*Conger conger*) was analysed. The study used twenty-one pre-metamorphic leptocephali collected from the Iberian continental slope and the Bay of Biscay, and of two metamorphosing larvae captured on the continental shelf near to the Minho River entrance. The total length and the radii of the sagittae of the leptocephali ranged from 85 to 133 mm and from 113 to 260 μm, respectively. Daily rings were visible along the entire length of the sagitta radii of the pre-metamorphic leptocephali, with an average of 277 rings. A diffuse zone, where no rings were visible, was present in the sagittae of the two larvae which had already metamorphosed. The fact that conger eel larvae grow well up until metamorphosis, could be the reason for the clear visibility of most of the daily rings in the pre-metamorphic leptocephali. This could be useful, when comparing other species, for example the European eel, *Anguilla anguilla*, whose sagittae present a diffuse zone prior to metamorphosis.

Introduction

Daily growth increments (rings) have been shown to occur in the otoliths of anguilliform fishes. They have been demonstrated in the artificially hatched larvae of *Anguilla japonica* during the first 6 days of life (Umezawa *et al.* 1989) and in elvers (Umezawa and Tsukamoto 1991), as well as in the otoliths of leptocephali and the metamorphic stages of *Conger myriaster* (Mochioka *et al.* 1989). In the European eel, *Anguilla anguilla* (L.), determining the age of glass eels is problematic because a diffuse zone is present prior to metamorphosis; this is a region of the sagitta where morphological features are irregular (Antunes and Tesch 1997). However, increments in the sagittae of premetamorphic European conger eel, *Conger conger* (L.), leptocephali are easily counted (Strehlow *et al.* 1998).

The interpretation of the "daily rings" of the economically important genus *Anguilla*, as well as those of other less known Anguilliformes, is important because the number and morphology of the rings can help to increase understanding of their life history. The other alternative method of investigation is bio-oceanographic sampling, which is difficult and expensive for these deep sea fish.

The Mediterranean Sea is thought to be a spawning area for European conger eel, indicated by catches containing small, 9 to 20 mm leptocephali (Schmidt 1931). Schmidt's (1931) assumption that *C. conger* could also spawn in the western Sargasso Sea was contested by McCleave and Miller (1994). They suggested that the small leptocephali from the Sargasso Sea, which had a relatively high number of myomers, were *Conger triporiceps* and that occurrence of *C. conger* leptocephali is restricted to the eastern and central Atlantic. However, nothing is known about the spawning location and time of metamorphosis of *C. conger* (Strehlow *et al.* 1998).

The sagitta microstructure of conger eel leptocephali at two different larval phases was analysed and an initial estimation of larval age, based on otolith microstructure was carried out. Knowledge of microstructure of conger eel leptocephali sagittae was used to aid identification of the phase of *A. anguilla* leptocephali. A comparison was made with the same phase of growth in otoliths of *A. anguilla* in which the daily rings were obscured by the diffuse zone.

Materials and methods

Twenty-one premetamorphic conger eel leptocephali were captured using an Isaacs-Kidd mid-water trawl during an expedition of the R.V. Heincke to the Bay of Biscay and the Portuguese coast between 26th May and 5th July 1989 (Table 1). Two metamorphic conger eel leptocephali were collected in April 1998 and November 1998 as by-catch of the glass eel fishery at the mouth of Minho River.

Table 1: Age determination by daily rings and radius of otoliths of *Conger conger* leptocephali caught in the East Atlantic and in the Minho River (leptocephali data modified from Strehlow *et al.* 1998).

Area	n	Capture date	Position	L_T (mm)	Radius of otolith (μm)	Number of daily rings
Bay of Biscay	7	26.5.1989	47°N/7°W	109	172	368
		5.6.1989		121	191	364
				133	178	299
		4.6.1989	44°N/9°W	120	133	273
				109	135	288
		3.6.1989		102	143	253
				125	167	301
North coast of Portugal	11	3.6.1989	41°N/9°W	98	140	269
				89	147	273
				114	180	313
				123	148	262
		2.6.1989		110	203	313
				108	183	334
				108	119	269
				102	133	250
		30.5.1989		105	126	250
				116	142	315
				109	183	385
Northern Iberian Basin	3	28.5.1989	43°N/13°W	96	130	263
				91	113	208
				85	147	230
Minho River	2	23.4.1998	41°N/8°W	128	195	483
		18.11.1998		133	246	736

The number of myomeres present in the pre-metamorphic leptocephali was counted for species identification. In the metamorphic larvae the total number of myomeres (TNM) and the number of myomeres to the origin of the anal fin (MA) were counted, and the MA/TNM ratio calculated. The ratio was used as in indicator of the stage of development (Tanaka *et al.* 1987).

Both right and left sagittae were dissected from the leptocephali, polished in the sagittal plane with 2400 mesh sand paper and aluminium paste until the core was revealed. Then they were etched for 8 s with a 0.5 % solution of HCl, coated with gold and viewed under SEM (Jeol JSM 35 C) at 15 kV. The diameter (D), radius (R), width of rings in the countable zone (CZ) and diffuse zone (DZ) width were measured (Figure 1). In the diffuse zone of metamorphic leptocephali there were small fragments of ring structure and the narrower spacing of these rings was used for calculating the number of days in that part of the otolith. For premetamorphic leptocephali, the daily growth increments by length of leptocephali (TGL) and by width of sagittae (TGS) were calculated from the total length (L_T), and the radius distance with age ratios, respectively.

Results

The microstructure of conger eel larval otoliths showed well-defined structures: the nucleus (N), the ring-countable zone (CZ) and the diffuse zone (DZ) (Figure 1).

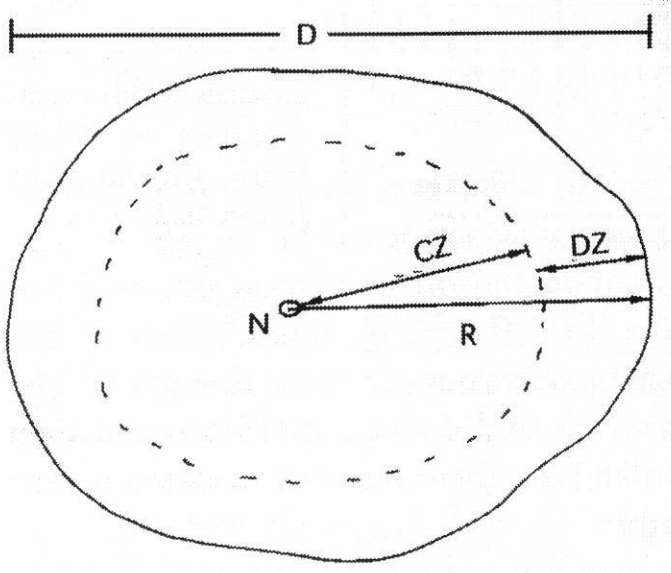

Figure 1: Schematic otolith measurements of conger eel leptocephali. N = nucleus, D = diameter, R = radius, CZ = zone of countable rings, DZ = diffuse zone.

Premetamorphic leptocephali

In the premetamorphic leptocephali, otolith growth increments were visible in all sagittal sections from the nucleus to the edge (Figure 2).

The increment width along the sagittal radius showed a characteristic curve (Figure 3). The width increased (0.5 μm-0.9 μm) from the nucleus up to a distance of 15 μm and then decreased (0.4 μm) up to a distance of 85 μm. Beyond 85 μm there was little variation (0.4-0.6 μm) in increment width to the edge of the otolith.

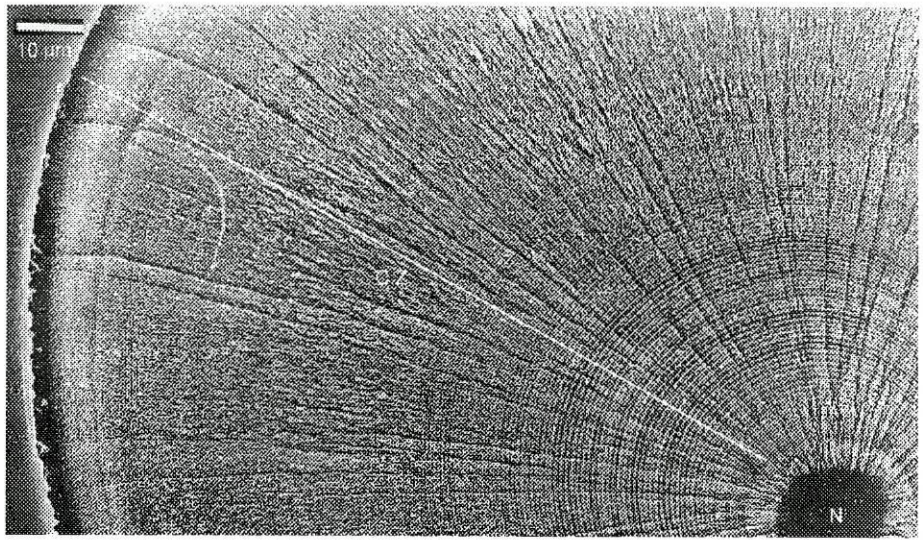

Figure 2: Sagitta of premetamorphic *Conger* leptocephali, May 1989, sampled on the Atlantic continental slope. N = nucleus, CZ = zone of countable rings.

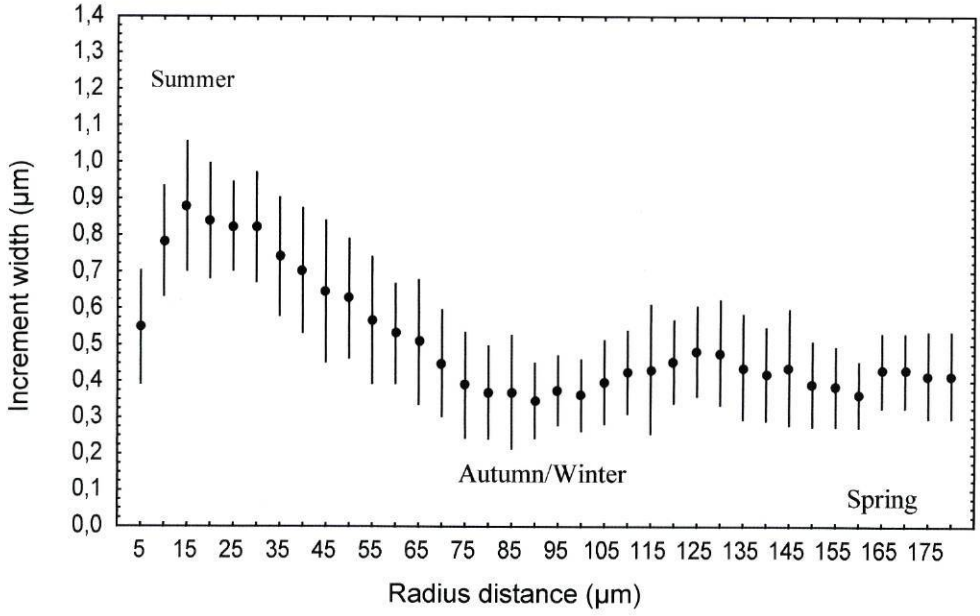

Figure 3: Sagitta increment width versus distance of radius from the nucleus (mean value ± sd).

An average of 277 increments (equivalent to about 9 months) were counted from the nucleus to the edge, giving the date of hatching in the summer, if it is assumed that each increment corresponds to one day (Figure 4).

The number of increments until the end of the decreasing width zone at 85 μm from the nucleus, corresponded to a total of 109±26 days (mean ± s). From back-calculation November was determined as the period with smallest increment widths. A significant correlation was found between the assumed age of leptocephali and the age where increment width ceased decreasing (lowest point) (r^2=0.37; P<0.05; n=21), as well as between sagitta diameter and age (r^2=0.71; P<0.05; n=21).

Significant correlation was found between L_T and sagitta diameter (r^2=0.23, P<0.05, n=21), sagitta radius (r^2=0.16, P<0.05, n=21) and age (r^2=0.22, P<0.05, n=21). The average daily growth increment of leptocephali (TGL) after hatching was 0.38 ± 0.05 mm day^{-1} (n=21), while the average daily growth of sagittae (TGS) was 0.53 ± 0.06 μm day^{-1}.

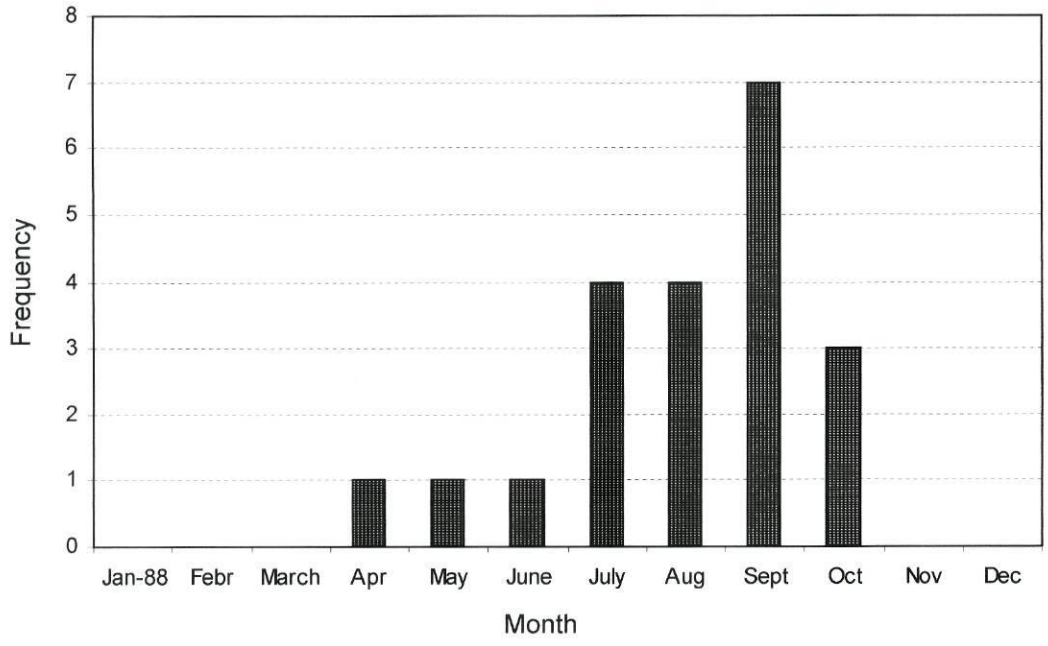

Figure 4: Back-calculated hatch dates of pre-metamorphic conger eel larvae.

A significant negative correlation was found between age and TGL (r^2=0.64, P<0.05, n=21) (Figure 5) but not between age and TGS (r^2=0.14, P=0.24, n=21) (Figure 6). The regression of age and L_T was: x = 69 + 1.956 L_T (Figure 7). Thus, if summer is the time when they hatch, they will be 30 mm at the first winter, and when they reach 160 mm they will be near 380 days of age (13 months).

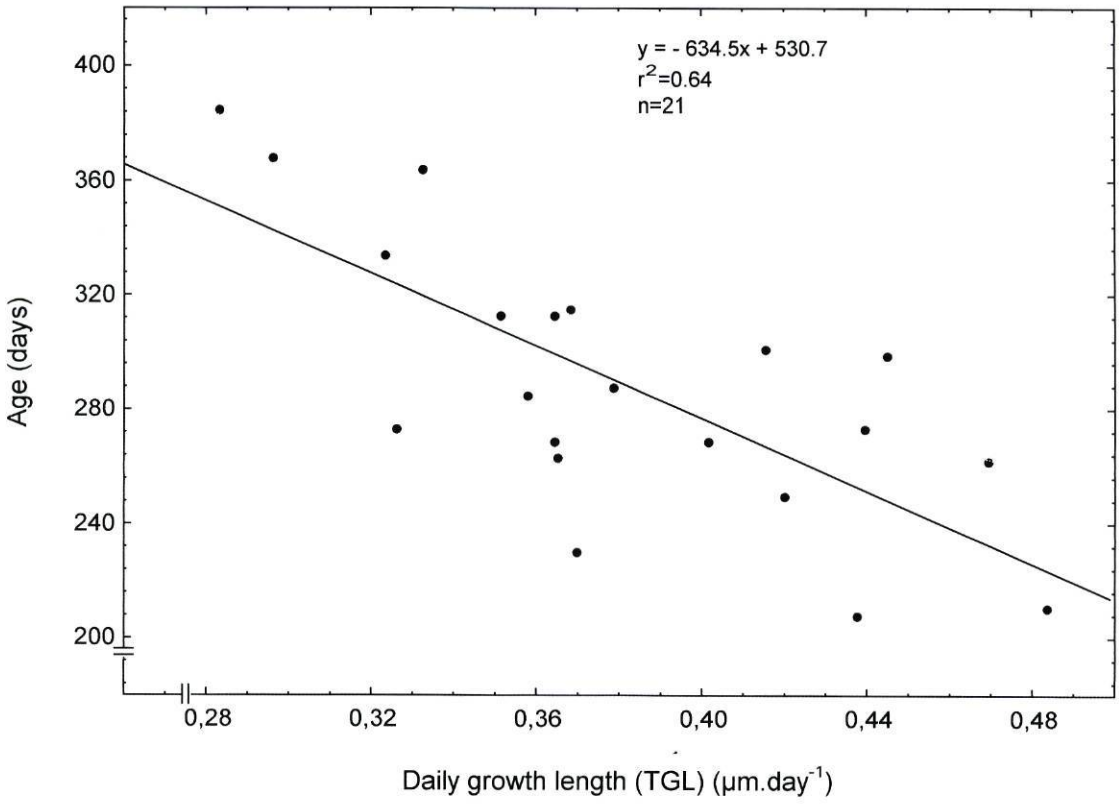

Figure 5: Relationship between age and average daily growth length (TGL) of pre-metamorphic leptocephali.

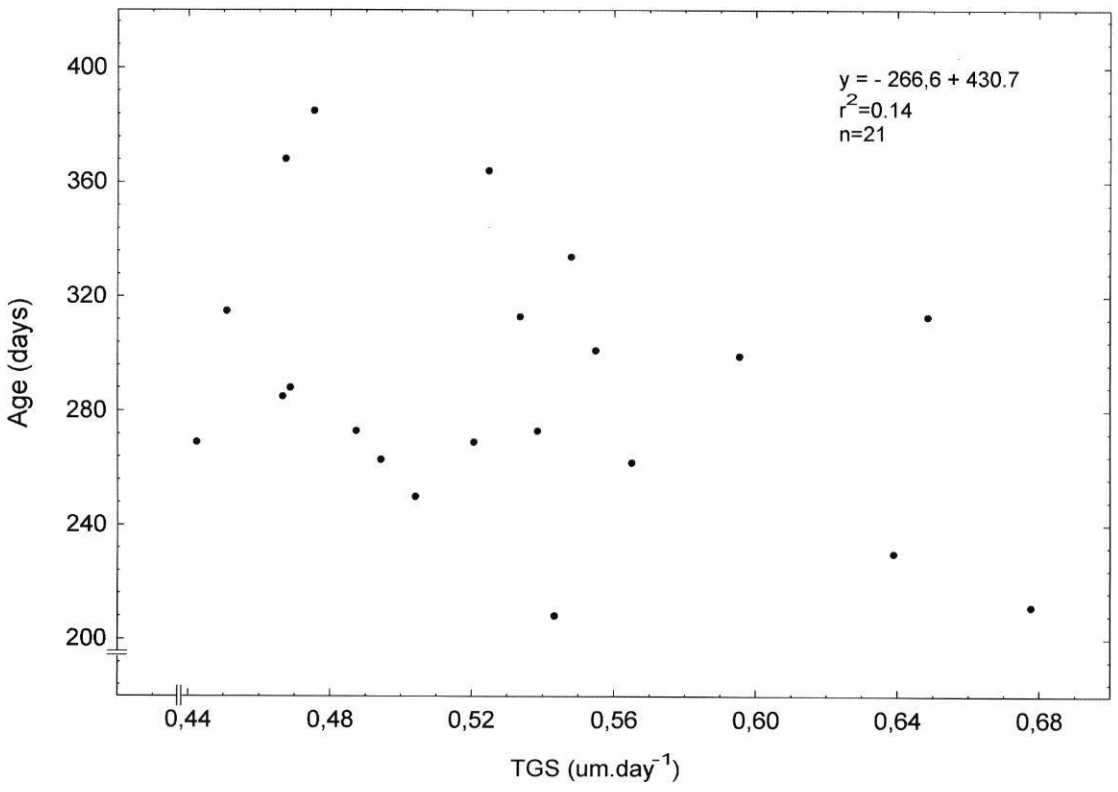

Figure 6: Relationship between age and average daily growth increment (TGS) of pre-metamorphic leptocephali sagitta.

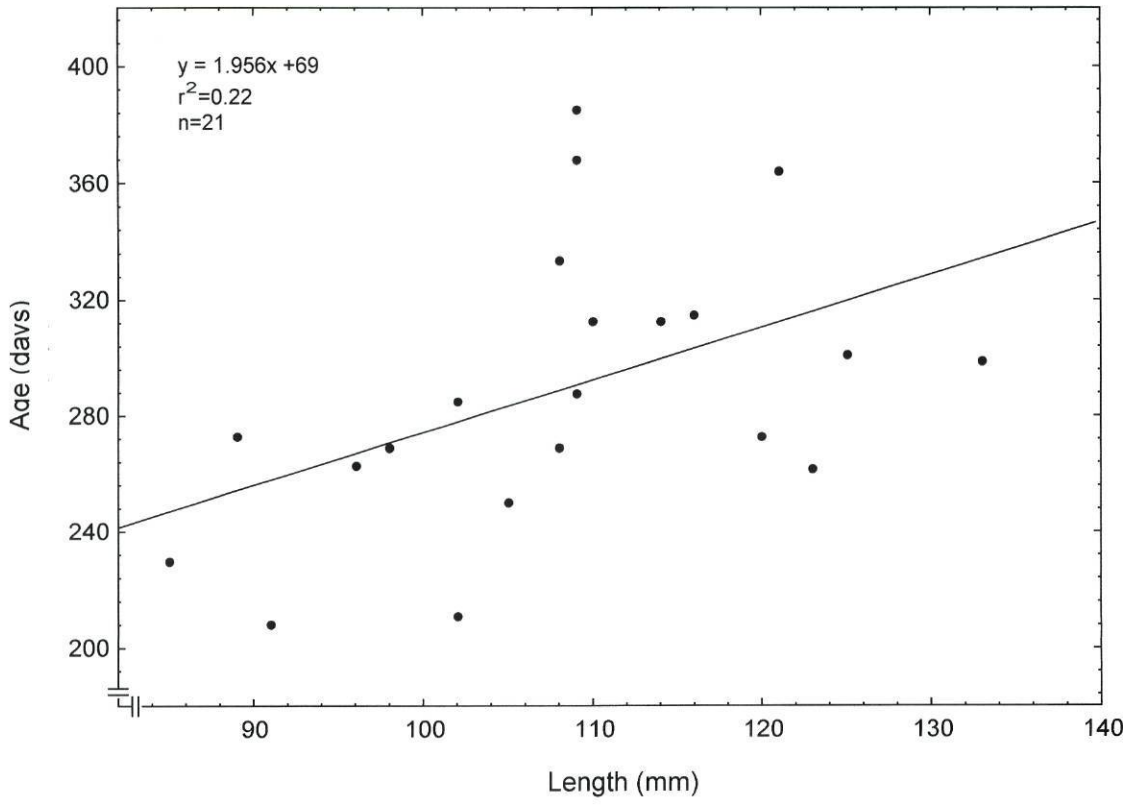

Figure 7: Relationship between age and length of pre-metamorphic leptocephali.

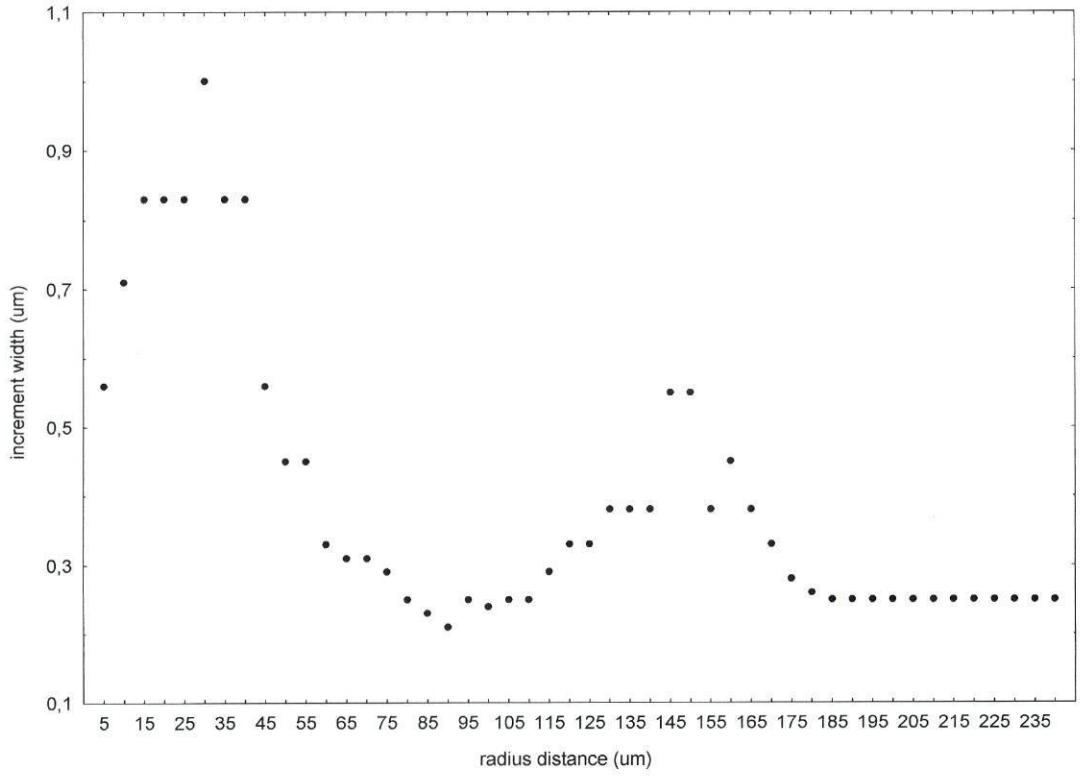

Figure 8: Sagitta increment width versus distance of radius from the nucleus of metamorphic leptocephali.

Metamorphic leptocephali

The lengths of the two leptocephali at metamorphoses were 128 and 133 mm (April and November 1998, respectively). The number of myomeres counted to the origin of the anal fin (MA) was 65 in April and 63 in November. The total number of myomeres (TNM) was 160 in April and 155 in November, giving MA:TNM=0.41 for both leptocephali. These values indicated that the leptocephali were at metamorphosis, compared to that obtained for premetamorphic leptocephali (0.71) by Strehlow *et al* (1998).

The sagittae had radii of 195 and 260 μm and diameters of 366 and 380 μm, respectively. The increment widths were similar to those on the sagittae of premetamorphic stages (Figure 8). The difference was the presence of a diffuse zone beyond 180 μm (Figure 9). The estimated total number of increments after hatching was 483 (April 98) and 736 (November 98), calculated using the narrow "rings" in the D.Z. For these otoliths, a period of 120 days was measured from the first ring until the point where increment width stopped decreasing. It is also important to note that after the 400th ring, a zone appeared which is difficult to read.

Figure 10 shows a sagitta of a 75 mm *Anguilla* leptocephalus prior to metamorphosis (stage I), collected on the same cruise along with premetamorphic conger eel leptocephali. However, at this stage it was possible to detect the presence of the diffuse zone.

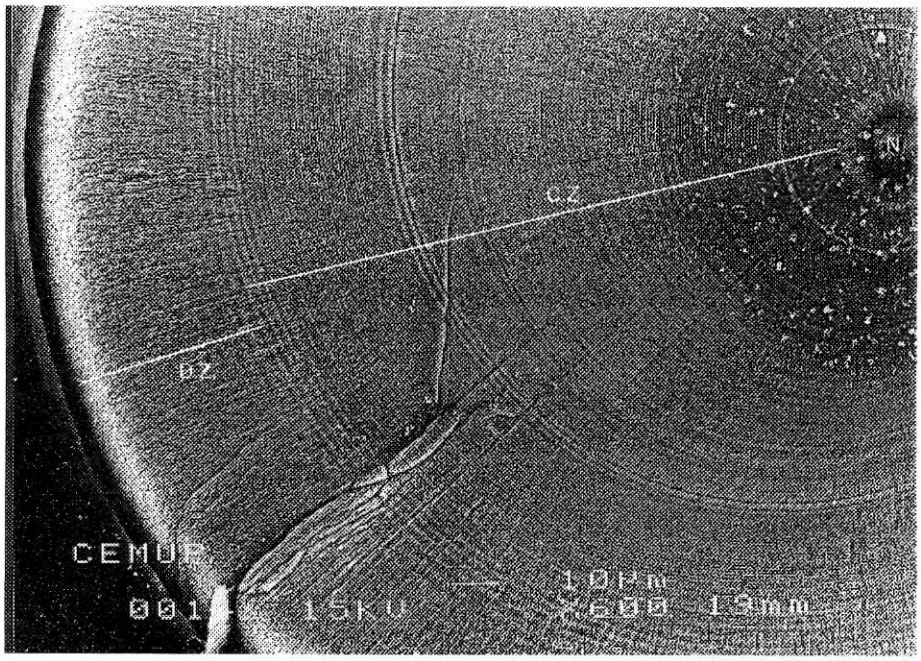

Figure 9: Sagitta of metamorphic *Conger* eel leptocephalus captured at the Minho River mouth. N = nucleus, CZ = zone of countable rings, DZ = diffuse zone.

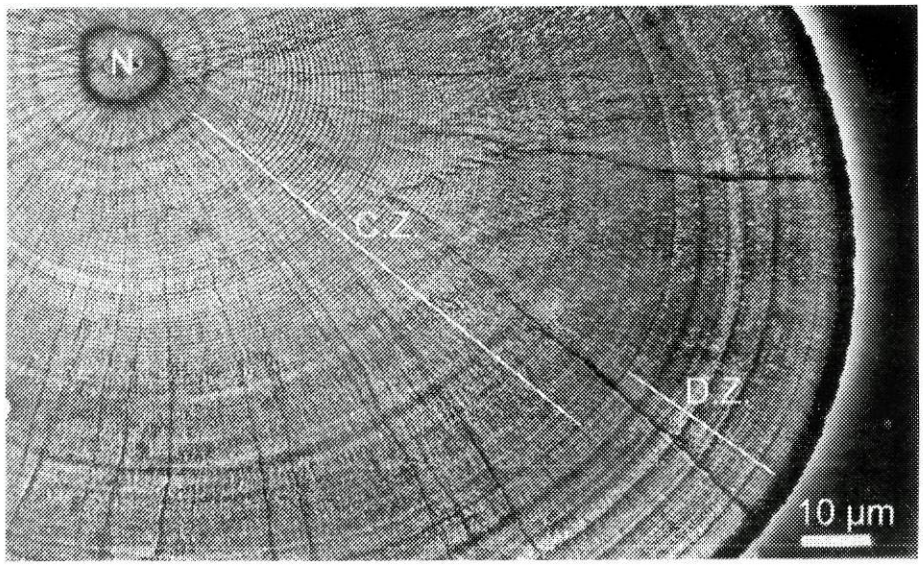

Figure 10: Sagitta of *Anguilla* leptocephalus, stage I, May 1989, captured on the Atlantic continental slope. N = nucleus, CZ = zone of countable rings, DZ = diffuse zone.

Discussion

Beyond the nucleus, it was assumed that the periodic structures were daily growth rings, as demonstrated in other Anguilliform species (Mochioka *et al* 1989; Umezawa *et al.* 1989; Umezawa and Tsukamoto 1991). Based on this assumption, the average total number of increments for the premetamorphic leptocephali was 277, which corresponds to a summer spawning season and agrees with the observations on leptocephali of a smaller body size in the Mediterranean (Schmidt 1931; Strehlow *et al.* 1998).

Increment width increases (reaching 0.9 µm) until the otolith radius (R) is 15 µm, corresponding to summer. The width then decreases to 0.4 µm, when R=80 µm (120 days by back-calculation). This is probably caused by the lower temperatures during autumn and winter. The increasing temperature in spring probably explains the next increase in the width of increments and the continuous larval growth until length at metamorphosis is reached (160 mm).

Older larvae are bigger. For the premetamorphic leptocephali, the correlation between age and the point at which increments width stops decreasing of is probably due to the influence of environmental factors.

The daily growth increment (TGL) of premetamorphic leptocephali (0.38 mm.day^{-1}) is similar to that of small *Anguilla* larvae, as described by Castonguay (1987). However, for *Anguilla* leptocephali with 75 mm length, captured in May 1989, the TGL was only 0.24 mm.day^{-1} (Antunes 1994). These larvae were near to their maximum length prior to metamorphosis and the sagittae already present the "diffuse zone". The TGL is still lower (around 0.2) in the smallest *Anguilla* sp. (Boetius and Harding 1985; Kleckner and McCleave 1985; Tesch 1998).

Otake *et al* (1997) considers that the onset of metamorphosis for *C. myriaster* occurs around about the fourth month (110 rings). In the present study, *C. conger* commenced metamorphosis later, *c.* 9.5 months (120 mm L_T).

The preanal length of the different larval stages is an indicator of their development stage. Strehlow (1992) observed a MA/TNM ratio of 0.77 for larvae in the premetamorphic stage (that includes the specimens recorded in this study). In the leptocephali captured in the Minho River, this ratio was 0.41. *Anguilla* sp. exhibited similar tendencies (Schoth, 1982). Tanaka *et al.* (1987) used this method to describe premetamorphic and juvenile development for *C. myriaster*, and found values of 0.84 and 0.23, respectively.

In the sagitta of metamorphic larvae, the area of the second decrease in increment width corresponded to about the 400th ring. The rings in this area were so thin that it was not possible to count them and the area was termed the "diffuse zone". This zone is probably laid down in late summer and early autumn and could be due to the leptocephali having nearly completed their growth before the commencement of metamorphosis.

Sagittae analysis showed relatively older larvae coming from the Bay of Biscay compared with those from the northern coastal region of Portugal. This was similar to the larvae of *A. anguilla* (Antunes and Tesch 1997) which for other ecological reasons, were even younger at higher latitudes.

Sometimes, metamorphic conger leptocephali are caught in the European glass eel fishery (November to April), in the marine area next to the Minho River mouth. There are some similar reports for *C. oceanicus*, which is found in the coastal and estuarine regions of the Chesapeake Bay between May and August, and commences metamorphosis at 160 mm (Hardy 1978). *C. myriaster* has been recorded in coastal waters between November and July, at the end of its metamorphosis (Tanaka *et al.* 1987).

The comparison between *A. anguilla* and *C. conger* is interesting. Analyses of the microstructure of sagittae of conger eel leptocephali before and during metamorphosis were

used as a reference for the description of the larval phase of *Anguilla* leptocephali. Most larval phases of *A. anguilla* are probably found within the diffuse zone of the sagitta, as it attains a considerable length in the first seven months of life with only a little further growth prior to metamorphosis (Antunes and Tesch 1997). In *C. conger*, using the presented data, the half time of its larval phase could be easily identified in the sagitta, and the reason for this could be the fact that the spawning season is in summer. Note that the maximum larval growth rate is expressed at the end of the following summer.

Data from J. Schimdt (in: Boetius and Harding, 1985) indicate that the spawning peak for *A. anguilla* takes place in March/April. The number of rings in the countable zone (CZ) is about 200 (Antunes 1994). This number corresponds well with the number of days from spawning time to the beginning of winter. The leptocephali may also have reached higher latitudes and lower temperatures. These events could cause a slowdown in growth to the extent that daily rings are no longer formed, giving way to the diffuse zone. This development is favoured by slowdown in growth, and faster growth is not resumed during the next summer (Antunes and Tesch 997). In *A. anguilla*, the diffuse zone of the sagittae appears in the premetamorphic stage, as seen in larvae captured in May/June and October (Antunes 1994; Antunes and Tesch 1997). The appearance of this diffuse zone could coincide with autumn/winter and the non-deposition of visible increments (rings) following these seasons and could be due to the fact that *A. anguilla* reached the length (60 to 70 mm) at which the metamorphosis starts. In *C. conger*, the fact that during its first winter the larva has a length of only 30 mm (metamorphosis at 160 mm), leaves considerable potential for continuing growth, and narrow winter increments are formed and not a diffuse zone, as in *A. anguilla*,. Broader increments are resumed in the next growth phase in spring.

The possible reason of why increment counting is harder from the 400th ring could be that the larvae's length is close to 160 mm, i.e. close to the metamorphosis phase. The diffuse zone could be produced in summer-autumn and the growth rate reduction associated, later on to the metamorphosis, could contribute to difficulties in estimating the duration time of this phase. Recording the onset of metamorphosis and its duration time is problematic. The diffuse zone could appear before the metamorphosis starts, which is similar to *A. anguilla*.

Acknowledgements

The author wishes to thank Dr. F. W. Tesch for support during the 1989 expedition of the R.V. Heincke. The study was supported by Fundação para a Ciência e Tecnologia (PRAXIS XXI: 2/2.1/MAR/1728/95). The co-author has a grant for PhD studies (BD 18082/98) from the same sponsor. We thank Dr. Jonathan Wilson for English language assistance and Dr. Mike Weber for German translation.

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5. PRELIMINARY STUDY ON POPULATION CONGER EEL GENETICS

5.1 Evidence for genetic differentiation in the European conger eel, *Conger conger*, based on mitochondrial DNA analysis

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Abstract

The European conger eel *Conger conger* is an important marine benthic fish in the NE Atlantic; however, little is know about its reproductive biology. In an attempt to gain a better understanding of the conger eel population structure, mitochondrial DNA (mtDNA) sequences were examined. A region with 432 bp of the control region of the mtDNA was sequenced from 40 individuals from six different locations around the central and eastern North Atlantic Ocean. Thirty variable positions defined 28 distinct haplotyptes. The average sequence difference within samples (1.3-4.2 %) was comparable to those between samples (1.4-3.6 %). The mtDNA showed some geographical differentiation between local populations samples, suggesting that the conger eel does not comprise a single panmictic population. However, given the limitations of our sample sizes, the results should be interpreted with caution. These results are preliminary and more individuals from more sites, including the Mediterranean Sea, should be analysed in detail. The genetic variability detected in this study is an initial step to elucidate the genetic background of the conger eel population structure.

Introduction

The European conger eel *Conger conger* (Linnaeus, 1758), an important fisheries resource, is widely distributed in the NE Atlantic, Mediterranean and western Black Sea (Bauchot and Saldanha 1986). Despite being a geographically widespread species, there is relatively little known about the reproductive biology, ecology and migratory behaviour of *C. conger* (Cau and Manconi 1983, 1984; Fannon et al. 1990; Strehlow et al. 1998; Sbaihi et al. 2001; Correia et al. 2002ab, 2003; Antunes and Correia 2003; Sullivan et al. 2003) and the information obtained so far is not enough to elucidate the entire life cycle of this fish, namely the location of the spawning ground(s), the duration of the leptocephalus stage and the coastal recruitment larval mechanism, which is essential for rational stock assessment and management.

From previous studies, several spawning locations have been suggested for the conger eel, although naturally spawning conger eels have not yet been observed and the reports about the capture of maturing specimens are scarce (Cau and Manconi 1983; Fannon et al. 1990). The Mediterranean Sea is thought to be the spawning area for European conger eel, indicated by catches containing small, 9-20 mm, leptocephali (Schmidt 1931). Several authors (Lythgoe and Lythgoe 1971; Bagenal and Kenney 1973; Wheeler 1985) also suggested that conger eels spawn once during the summer, at greats depths (3000-4000m), in the NE Atlantic, between Gibraltar and the Azores, but they did not mention any references to support their assumptions. Until now, the only well known spawning ground for this species is in the waters south of the Island of Sardinia, in the Mediterranean Sea (Cau and Manconi 1983). This assumption is supported by the length and age of developing leptocephali collected in the North and Central Atlantic Ocean (Strehlow et al. 1998). Although the spawning area of *C. conger* in the North Atlantic is still hypothetical, based on the age of leptocephali and water current systems, Correia et al. (2002b, 2003) recently suggested that this species might spawn in an area closer to the Azores Archipelago. However, further information on the geographical distribution, age and growth of *C. conger* leptocephali is necessary in order to determine the actual spawning ground(s) of this population and its migration pathways to the European and North African coasts.

In the present study we investigated genetic variation of the European conger eel from different locations around the Central and Eastern Atlantic Ocean using mitochondrial DNA sequencing, in an attempt to provide additional information to better understand the spawning ground, ecology and migration of conger eel leptocephali in the ocean.

Material and methods

Fish sampling

A total of 40 *C. conger* leptocephali, already used in some ageing studies (Correia et al. 2002a, b, 2003; Antunes and Correia 2003) were collected from six locations in the central and eastern Atlantic Ocean: Azores (n=13; two samples points), North Portuguese Continental Slope (n=8), Bay of Biscay (n=4; two samples points) and Minho River (n=15) (Fig. 1 and Table 1).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from ethanol-preserved muscle tissue of each individual of *C. conger* using proteinase k digestion, ammonium acetate separation of proteins and cold-ethanol DNA precipitation (Sambrook et al. 1989). A fragment of the mtDNA of the control region was first amplified by the Polymerase Chain Reaction (PCR) with a pair of primers designed for this study, L15013 (or CR1) (5´-CGGTTTTGTAATCCGAAG-3') and H15590 (5´-ATAGGAACCAGATGAAAG-3'). These primers were designed based on a control region nucleotide sequences from the mitochondrial genome of *Conger myriaster* (Inoue et al. 2001). To get stronger PCR products a new internal reverse primer CR2 (5´-TTGTCCCTGATTATCAATAAAC-3') was designed based on a mtDNA sequence of *C. conger*, and used in all subsequent amplifications reactions. The PCR amplifications were carried out in a T3 Termocycler (Biometra) under the following conditions: initial 3 min denaturation at 94 ºC, 35 alternating cycles of 30 s at 94 ºC for denaturation, 30 s at 59 ºC for annealing and 30 s at 72 ºC for extension. An additional final extension as made during 2 min at 72 ºC. A total of 2 µl of each PCR product was used for 2 % agarose gel electrophoresis for verifying the amplified fragment length with a standard size marker (Marker 5, Eurogentec, UK). Band characterization under ultraviolet light was made by staining with ethidium bromide. The rest of the PCR products were purified by enzyme cleaning. Purified double-stranded DNA was used as template for automated sequencing reactions performed using a T3 Termocycler and run on an ABI 310 DNA sequencer (Applied Biosystems). The primers used for sequencing were the same as those for PCR amplification.

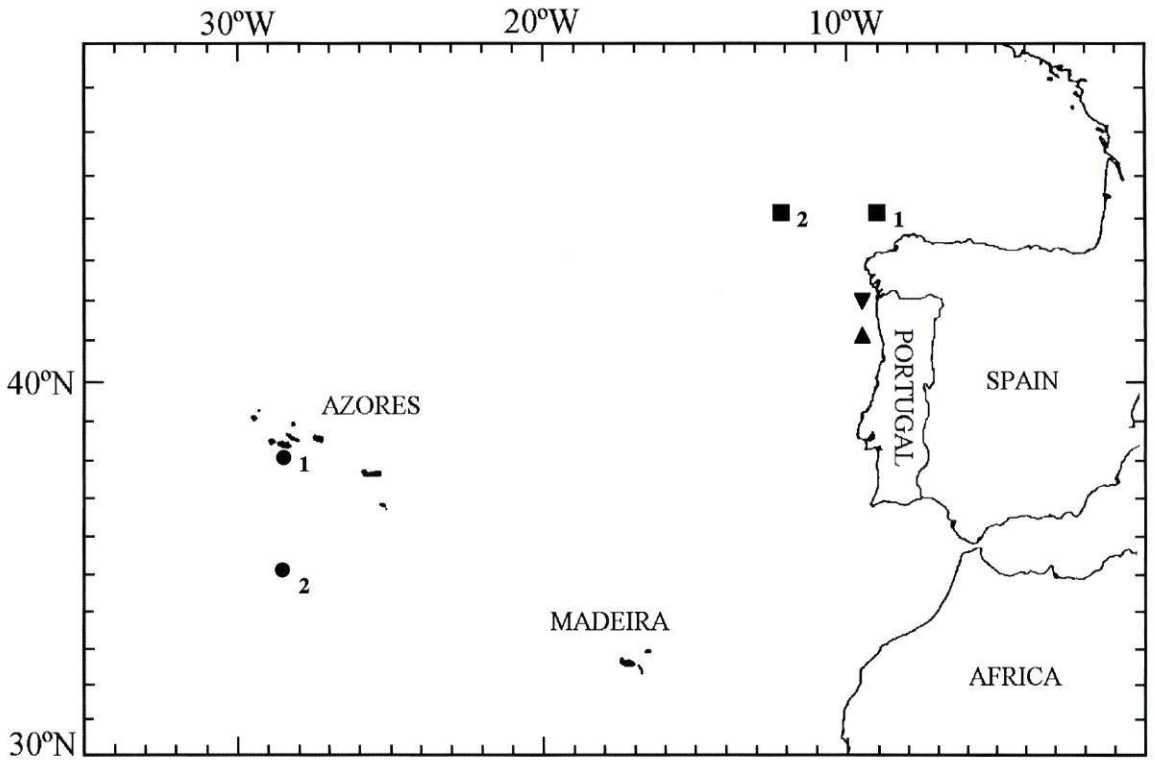

Fig. 1 *Conger conger*. Sampling location of the 40 leptocephali collected in the central and eastern Atlantic Ocean. Legends: *numbered circles* Azores 1 and 2; *numbered squares* Bay of Biscay 1 and 2; *down triangle* Minho River; *up triangle* North Portuguese Continental Slope. See also Table 1.

Table 1 *Conger conger*. Location (area and geographic position), sampling date, number (n), total length (TL), developmental stage (PL premetamorphic and ML metamorphic leptocephali) and age of the leptocephali used in this study (data extracted from Correia et al. 2002a, b, 2003; Antunes and Correia 2003). Legends: *A1* Azores 1; *A2* Azores 2; *MR* Minho River; *NPCS* North Portuguese Continental Slope; *BB1* Bay of Biscay 1; *BB2* Bay of Biscay 2. See also Figure 1.

Area	Position	Capture date	n	TL (range)	Stage	Age (days)
A1	38° N/29° W	October 1999	10	51.5-125.5 mm	PL	76- 275
A2	35° N/29° W	August 2000	3	49.0-76.0 mm	PL	56-93
MR	42° N/9° W	February 1999	15	108.0-136.0 mm	ML	Unknown
NPCS	41° N/9° W	May/June 1989	8	89.0-123.0 mm	PL	250-385
BB1	44° N/9° W	June 1989	2	102.0-125.0 mm	PL	253-301
BB2	44°N/12° W	August 2000	2	92.9-96.0 mm	PL	240-260

Data analysis

All sequences were aligned, using the sequence alignment editor software Bio Edit 5.0.9 (Hall 1999) with subsequent refinement by eye using the Chromas 2.23 program (Technelysium Pty Ltd). Average sequence differences within and among samples were calculated from pairwise sequence differences obtained by MEGA version 2.1 (Kumar et al. 2001). Insertions/deletions were treated as the fifth character and no mutations were weighted for sequence difference estimation. Pairwise sequence differences among samples were tested statistically using permutation tests. A haplotype and a sequence-based statistical tests proposed by Hudson et al. (1992) and Hudson (2000) when haplotype diversity is very high and the samples sizes are small were used for detecting genetic differentiation of subpopulations. Analysis was performed between locations with a sample size ≥8 (Azores 1, Minho River and North Portuguese Continental Slope) using DnaSP 4.0 with 1000 permutations (Rozas et al. 2003). Genetic variation within each population was quantified by mtDNA haplotype diversity (*h*) and nucleotide diversity (π) (Nei 1987). A maximum parsimony network was constructed using the algorithm described by Templeton et al. (1992) that allows phylogenetic estimation with low levels of divergence. TCS version 1.6 (Clement et al. 2000) implements the algorithm and provides a 95 % plausible set for all haplotypes linkages in an unrooted tree.

Results

mtDNA variation

The aligned mtDNA sequence data consisted of part of the control region containing 432 base pairs (bp) (Fig.2). There were a total of 30 variable positions and 28 haplotypes were found in the 40 individuals. The frequency of variable sites in this part of the mtDNA control region was 6.7 % (30/448 bp). Haplotype diversity (h) and nucleotide diversity (π) were highest in the Bay of Biscay (1.000±0.500) and North Portuguese Continental Shelf (0.010±0.006) samples, respectively (Table 2). The average sequence divergence and its standard error in the overall data of all individuals was 2.464±0.516 %. Average sequence divergences in overall data within samples varied from 1.333±0.906 % (Azores, sample point 2) to 4.214±1.060 % (North Portuguese Continental Slope), and those between samples

ranged from 1.400±0.508 % (between Minho River and Azores 2) to 3.650±0.835 % (between North Portuguese Continental Shelf and Azores 1) (Table 3).

ACACGACAT*ATGGACATATAACACA	25
AATAACTGTATAA*TTACATAAATGA	50
TATACAACCTACCT*ATGTGTATGTT	75
GTATTTCACT*TATGTAATATATACA	100
TAAT*ATGTAAATACAACATACACCT	125
ATGTAATCAA*TACATAACATGTATT	150
GATTACAAAGGTGTATGTAAAC*T*AT*	175
CTGT*AAATGTAAACGTTACATACCC	200
ATA*CCAGATTTTAAATC*AATGAAGT	225
ATAACATAC*ATATTAATGG*ACCT*C*T	250
AATAA*C*ATTAATAATGAC*TT*AAAGA	275
AC*TGCAGCAAATAGTATTAAAT*CTA	300
TAAATATTGGACAG*TG*ATT*CATGAT	325
TTGAATGAT*AAATGCAC*AAACCAAG	350
TTTCCATGAAGAATG*ACATTAACTG	375
GACCTAAACCAGCATG*CGCAGTAAG	400
AAACCACCAACCAGCACAAT*TCAGG	425
AAAATAT	

Fig. 2 *Conger conger*. Nucleotide sequence of a 432 pb fragment of the mtDNA control region from one individual collected in the Minho River. *Asterisks* indicate the variable sites in 28 haplotyptes.

Haplotype network

The geographical distribution of haplotypes was distinctly non-random and some samples showed exclusive haplotyptes (Fig. 3). Haplotypes h1, h3-h8, and h11, h12 and h14-h21, and h22, and h23-h28 were only present at Azores 1, Azores 2, Minho River, Bay of Biscay 1 and North Portuguese Continental Slope, respectively. The most frequently observed haplotype (h13, n=8) was found in all samples, with the exception of the Azores 1, Bay of Biscay 2 and North Portuguese Continental slope (Table 2). The network has the features of a recent expansion in star-like pattern with multiple rare alleles and a more ancestral feature such as haplotype h13.

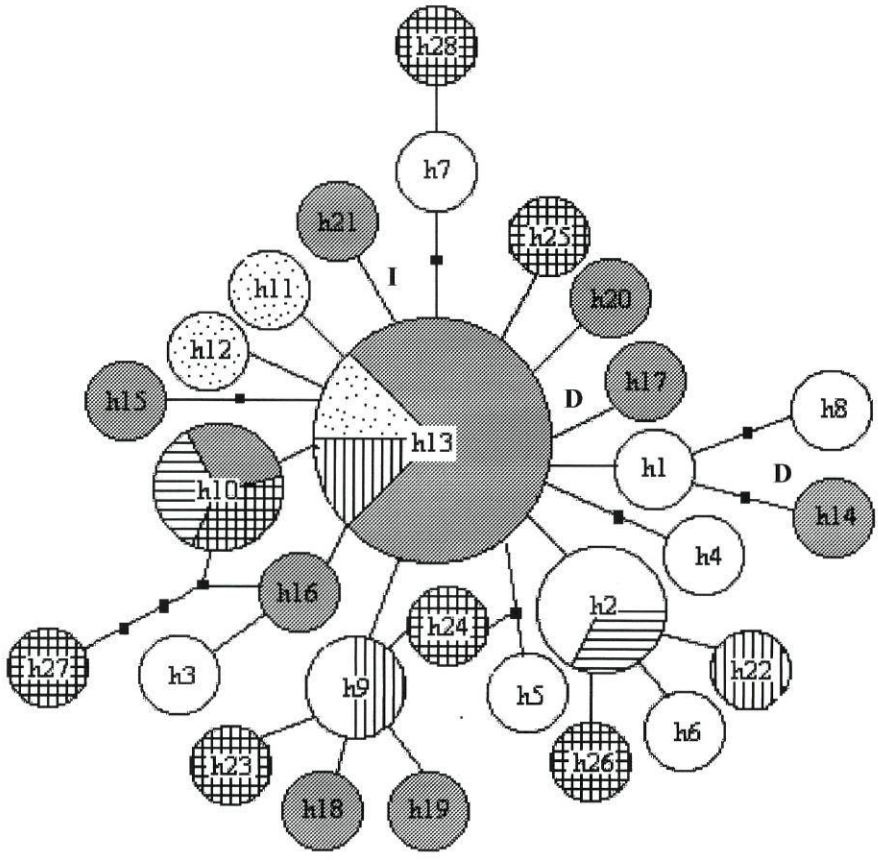

Fig. 3 *Conger conger*. A mtDNA haplotype network estimated with the 95% statistical limits of parsimony using the algorithm in Templeton et al. (1992). Haplotyptes are represented in circles with their size proportional to their frequencies. Each *solid bar* represents a single mutational event. The letters *D* and *I* represent deletions and insertions, respectively. Legend: Azores 1 *white area*; Azores 2 *punctuate area*; Minho River *grey area*; Bay of Biscay 1 *horizontal lines area;* Bay of Biscay 2 *vertical lines area*; North Portuguese Continental Slope *square-lined area*.

Genetic differentiation analysis

The results of the haplotype and sequence-based statistics (Table 4) show the existence of significant genetic differentiation between local populations samples. Azores 1 and North Portuguese Continental Slope subpopulations are not genetically differentiated ($P \geq 0.05$) but there is a significant genetic differentiation between the Minho subpopulation and the other two subpopulations ($P < 0.05$). All sequence-based statistics gave similar results, exception made for the Snn test. However, for unequal sample sizes (our case) the Hst and Z* are the most powerful tests (Hudson et al. 1992). The Snn statistic is preferable in situations with low to moderate variation and equal sample sizes (Hudson 2000).

Table 2 *Conger conger*. Absolute and relative frequencies of the haplotypes, including collection site, number of haplotypes per site (n_h), number of individuals sequenced (n_t), haplotype (h) and nucleotide (π) diversity (± SD). Legends: *A1* Azores 1; *A2* Azores 2; *BB1* Bay of Biscay 1; *BB2* Bay of Biscay 2; *MR* Minho River; *NPCS* North Portuguese Continental Slope. See also Figure 3.

Site/ Haplotype	A1	A2	BB1	BB2	MR	NPCS	Total (Abs.)	Frequency (%)
h1	1	0	0	0	0	0	1	2.5
h2	2	0	0	1	0	0	3	7.5
h3	1	0	0	0	0	0	1	2.5
h4	1	0	0	0	0	0	1	2.5
h5	1	0	0	0	0	0	1	2.5
h6	1	0	0	0	0	0	1	2.5
h7	1	0	0	0	0	0	1	2.5
h8	1	0	0	0	0	0	1	2.5
h9	1	0	0	0	0	1	2	5.0
h10	0	0	0	1	1	1	3	7.5
h11	0	1	0	0	0	0	1	2.5
h12	0	1	0	0	0	0	1	2.5
h13	0	1	1	0	6	0	8	20.0
h14	0	0	0	0	1	0	1	2.5
h15	0	0	0	0	1	0	1	2.5
h16	0	0	0	0	1	0	1	2.5
h17	0	0	0	0	1	0	1	2.5
h18	0	0	0	0	1	0	1	2.5
h19	0	0	0	0	1	0	1	2.5
h20	0	0	0	0	1	0	1	2.5
h21	0	0	0	0	1	0	1	2.5
h22	0	0	1	0	0	0	1	2.5
h23	0	0	0	0	0	1	1	2.5
h24	0	0	0	0	0	1	1	2.5
h25	0	0	0	0	0	1	1	2.5
h26	0	0	0	0	0	1	1	2.5
h27	0	0	0	0	0	1	1	2.5
h28	0	0	0	0	0	1	1	2.5
n_h	9	3	2	2	10	8		
n_t	10	3	2	2	15	8		
h	0.978±0.054	1.000±0.272	1.000±0.500	1.000±0.500	0.857±0.090	1.000±0.062		
π	0.007±0.005	0.003±0.003	0.005±0.006	0.005±0.006	0.004±0.003	0.010±0.006		

Table 3 *Conger conger*. Average number (±SE) of pairwise differences in mtDNA sequences within and between six samples location of the European conger eel. Legends: *MR* Minho River; *NPCS* North Portuguese Continental Slope; *A1* Azores 1; *A2* Azores 2; *BB1* Bay of Biscay 1; *BB2* Bay of Biscay 2.

	MR	NPCS	A1	A2	BB1	BB2
MR	1.448±0.448	2.817±0.698	2.327±0.621	1.400±0.508	1.733±0.707	1.667±0.698
NPCS		4.214±1.060	3.650±0.835	2.917±0.741	3.125±0.878	2.875±0.846
A1			3.133±0.906	2.367±0.663	2.400±0.803	2.400±0.804
A2				1.333±0.906	1.667±0.780	1.667±0.788
BB1					2.000±1.329	1.500±0.794
BB2						2.000±1.359

Table 4 *Conger conger* Analysis of the genetic differentiation between Azores 1 (*A1*), Minho River (*MR*) and North Portuguese Continental Slope (*NPCS*) samples. Values for mean haplotype diversity (Hd) and for haplotype (Hst) and sequence-based statistics (Kst*, Z*, Snn) calculated according to Hudson et al. (1992) and Hudson (2000). Legends: *A1* Azores 1; *MR* Minho River; *NPCS* North Portuguese Continental Slope. Symbols: *ns* not significant; * 0.01<P<0.05; ** 0.001<P<0.01

	Hd	Hst	Kst*	Z*	Snn
A1 vs MR	0.903	0.085	0.049	4.641	0.640
(n_1=10; n_2=15)		(0.004**)	(0.005**)	(0.005**)	(0.025*)
MR vs NPCS	0.885	0.077	- 0.060	4.455	0.630
(n_1=15; n_2=8)		(0.013*)	(0.007**)	(0.005**)	(0.061 *ns*)
NPCS vs A1	0.987	0.000	0.002	4.066	0.513
(n_1=8; n_2=10)		(0.742 *ns*)	(0.387 *ns*)	(0.360 *ns*)	(0.418 *ns*)

Discussion

The mtDNA segment sequenced in the present study comprised a portion of the control region assuming that there is a similarity between the mitochondrial DNA of the European conger eel, *Conger conger* and the Japanese conger eel, *Conger myriaster* (Inoue et al. 2001). A total of 30 variable sites were observed in this conserved region, in a total of 40 animals, defining 28 different haplotyptes. The frequency of variable sites in the control region (6.9 %, 30/432 bp) was lower than that of *C. myriaster* (19.8 %, 89/450 bp) (Ishikawa et al. 2001). Haplotype diversity is very high, but nucleotide divergence is very low, which is a common feature in marine species. The haplotype network has a typical starlike shape as in many marine species, suggesting a high effective population size, a low divergence between haplotypes and a possible recent expansion.

Ishikawa et al. (2000) recently investigated the geographical genetic variation of the Japanese conger eel, *C. myriaster*, using mitochondrial DNA sequencing and nuclear DNA fingerprinting by amplified fragment length polymorphism analysis, and reported that there were apparently no genetically isolated populations in this species. However, since this study has been inconclusive, five polymorphic microsatellite markers are being developed in an attempt to better understand the recruitment mechanisms and population structure of this species, whose spawning sites and migration routes are unknown (Kimura et al. 2003). Concerning the European conger eel, *C. conger*, the only well known spawning ground is in the Mediterranean (Schmidt 1931; Cau and Manconi 1983; Strehlow et al. 1998). However, some evidence (length and age of leptocephali) exists on the early life history of *C. conger* suggesting the possibility of another spawning area near the Azores Archipelago (Correia et

al. 2002b, 2003). Therefore, a first step should be to clarify the population structure of the European conger eel to provide further knowledge about its reproductive biology, which is essential for rational stock assessment and management.

The answer to the question of whether *C. conger* eel includes multiple populations or a single panmictic population is unknown. If the population isolation occurred a short time ago, or if gene flow among local populations continues to occur, little or no genetic difference is expected, even when using modern molecular techniques. Although the exact duration of the leptocephali larval stage of the European conger eel remains unknown and speculative (Correia et al. 2003), the conger eel should take about two years to drift inshore and to reach the juvenile form (Correia et al. 2002a). This long larval life enables the leptocephali to be transported by the ocean currents and to be disperse over a wide area. The large-scale dispersion of the leptocephali and the North Atlantic currents could explain a gene flow among geographically distant areas, like for example between Azores and northern Portugal. In fact, some individuals with the same mtDNA were found in widely separated locations during this study. However, the present mtDNA sequencing analyses provided some evidence for the existence of a significant genetic differentiation among local populations, suggesting that the conger eel does not comprise a single panmictic population. Nevertheless, since the number of individuals examined in the present study was small, and therefore the power for detecting significant genetic difference was somewhat low, these results should be interpreted with caution. Furthermore, the knowledge about the reproductive biology of conger eel, although limited, suggests that males and females could have different pre-migratory behaviors (Cau and Manconi 1983). In that case, nuclear DNA may therefore be preferable for testing for genetic differentiation of subpopulations, since mitochondrial genomes are often only transmitted maternally.

The main purpose of the present work was not to examine in detail the genetic population structure of the Atlantic population of the European conger eel, but to detect molecular genetic variations in *C. conger*, as an initial step for a future more extensive molecular genetic research. Although the oceanographic sampling of leptocephali was difficult and expensive, the genetic composition and the age of leptocephali might reflect the location and time of spawning adults. In conjunction with leptocephali, the analysis of adults specimens collected more intensively and from a wide area of the NE Atlantic (including Madeira and Canary Islands) and Mediterranean, are essential to understand the population structure and the genetic variation of this species.

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6. DISCUSSION AND FINAL CONCLUSIONS

6. Discussion and final conclusions

From late October to mid-June, metamorphosing European conger eel, *Conger conger,* leptocephali enter the estuary of the Minho River (North of Portugal) by tidal transport and are caught using a glass eel fishing net. For the Japanese conger eel, *C. myriaster*, this coastal water presence is recorded between November and July, at the last developmental and metamorphic stages (Tanaka et al. 1987). The ingression of the American conger eel, *C. oceanicus* into the coastal and estuarine waters, for instance, occurs regularly from May to July, while they are undergoing metamorphosis (Able and Fahay 1998).

The *C. conger* leptocephali were identified by their external body morphology, pigmentation and myomere counts. The description of the morphological features and pigmentation pattern of the developing and metamorphosing leptocephali agrees well with those made by D'Ancona (1931). The number of myomeres (TNM, PDM, PAM and LVBV) of our collected specimens falls also within the values reported by other authors (D'Ancona 1931; Castle 1970; Schmidt 1931; Strehlow et al. 1998).

As in other studies (Tanaka et al. 1987; Yamano et al. 1991; Lee and Byun 1996; Otake et al. 1997; Strehlow et al. 1998), the PAM/TNM and PAL/TL ratios had been successfully adopted as a criterion for differentiating the developmental stages of conger eel leptocephali. In *C. conger* these larval developmental indicators (PAM/TNM and PAL/TL ratios respectively) appear to be almost constant throughout the developing stage (0.78 and 0.88), but diminishes to nearly half during the metamorphic stage (0.36 and 0.43). Based on the values observed for the developing and metamorphosing leptocephali our results suggest that *C. conger* should begin to metamorphose at PAM/TNM (PAL/TL) values between 0.77 and 0.42 (0.86 and 0.55). Lee and Byun (1996) reported, for instance, that *C. myriaster* leptocephali begin to metamorphose at PAM/TNM values between 0.82 and 0.74.

The otolith morphology of *C. conger* leptocephali during its developing and metamorphosing stages is somewhat similar to that observed in other anguilliform species (Tabeta et al. 1987; Lecomte-Finiger and Yahyaoui 1989; Antunes 1994; Wang and Tzeng 1998, 2000; Arai et al. 2001a). The larval growth history of the conger eel is reflected in the changes of width of the otolith increments, probably associated with both endogenous and exogenous factors. The otolith increment width, which is relatively constant and narrow in the developing leptocephalus stage, increases sharply at ages 170 to 280 days. On the other hand, otolith Sr:Ca ratios which increase during the developing leptocephalus stage, show a rapid drop coinciding with the increase in increment width. These coincidental changes, also

observed in *C. oceanicus*, have been regarded as the onset of metamorphosis and are common of several other anguilliform fish species, including *C. myriaster* (Mochioka et al. 1989; Lee and Byun 1996; Otake et al. 1997). Although the rapid drop in Sr:Ca ratios might be associated with decreasing Sr levels in the body as a result of GAG catabolism during metamorphosis (Otake et al. 1997), no explanation has been give for the larger growth increments recorded in the otolith wide increment zone (WIZ). According to D'Ancona (1931), metamorphosis begins in *C. conger* before larval growth is completed, so that the younger semilarvae are larger that the fully developed larvae. These wide rings could be produced by a last short growth period of the leptocephali (lasting 30 to 70 days, i.e. the number of increments of the WIZ), before the beginning of the body shrinkage, which should occur at 150 mm length (D'Ancona 1931). Although leptocephalus fishes stop feeding at the onset of metamorphosis (Pfeiler 1986), they could have some remaining energy reserves, which enable them to continue growing for sometime. In fact, increment number appears to be unaffected by food deprivation at least when body energy reserves are sufficient to enable limited skeletal growth to occur (Marshall and Parker 1982; Campana 1983; Volk et al. 1984; Neilson and Geen 1985). Later on during metamorphosis, these wider increments became poorly contrasted and disappeared upon entering the diffuse zone (DZ). Now the daily periodicity of increment deposition stopped, probably as a result of poor growth conditions, since the metamorphosing leptocephali probably exhausted their energetic reserves allocated for growth and started to shrink. Furthermore, the relative influences of endogenous and exogenous factors on the scaling of the increment width and the observed transitions in increment-width patterns remain unclear.

The DZ formed outside the ICZ having unclear rings and associated with a clear change in the otolith growth direction have also been observed in late metamorphosing leptocephali of the American conger eel, *C. oceanicus*. Several authors have described the otolith microstructural growth of *C. myriaster* during metamorphosis, but none of them has identified the existence of a peripheral diffuse zone, although they have reported several problems in the examination of the otolith microstructure (Tanaka et al. 1987; Mochioka et al. 1989; Lee and Byun 1996; Otake et al. 1997). A similar DZ, probably resulting from a very slow growth period and made up of many daily growth rings too thin to be distinguished and counted, has been already described in the European eel leptocephali, *Anguilla anguilla*, before the onset of metamorphosis (Antunes and Tesch 1997). Based on this assumption, we roughly estimated a total age of about 483 and 736 days, for two metamorphosing conger eels collected from the Minho River, respectively in April and November of 1998, using what

supposedly seemed to be small fragments of ring structure within the DZ. We initially suggested that independently of the favorable external factors, namely the high temperatures of the end of summer and early autumn, the diffuse zone could represent a decrease of increment width due to the near-complete growth of the larvae before the start of metamorphosis. However, the examination of the otolith Sr:Ca ratios by WDS showed that this zone is formed during metamorphosis. Other hypotheses have been suggested to explain the formation of this unclear zone in the glass eels otoliths, such as a stop in the diel vertical migrations of leptocephali (Williamson et al. 1999), a process of calcium resorption in the marginal portion of the otolith during metamorphosis as part of calcium metabolism (Cieri and McCleave 2000) and poor otolith preparation (Arai et al. 2000a). It has been show that all otoliths of *C. conger* recorded a peripheral otolith diffuse zone and that this structure is not an optical artifact resulting from the otolith preparation, but a permanent structure of the late metamorphosing leptocephali. The mechanism behind the formation of this structure, be it an environmental or a physiological stimulus, is not yet understood and future attempts should be made to determine the pathway and mechanisms of the DZ deposition.

The occurrence of AGCs in some otoliths of *C. conger* and *C. oceanicus* indicates that they are universal structures in the conger eel otoliths, appearing in the late metamorphosing stage. AGCs appear with or after metamorphosis from larvae to juvenile in several fish species (Campana 1984; Gartner 1991; Sogard 1991; Hare and Cowen 1994; Volk et al. 1995; Modin et al. 1996; Wilson and McCormick 1997; Fischer 1999; Brown et al. 2001; Neuman et al. 2001; Plaza et al. 2001), including *C. myriaster* (Lee and Byun 1996), as foci for further otolith deposition (Campana 1984). The mechanism resulting in such structures is not fully understood, however some hypotheses have been proposed to explain the origin of AGCs, e.g. habitat shift (Sogard 1991), ontogenetic dietary shift (Marks and Conover 1993) and physiological changes associated with metamorphosis (Hare and Cowen 1994). Congers undergo habitat, morphological and physiological changes at the larval to juvenile transition (Yamano et al. 1991; Bell et al. 2003). Therefore, the AGCs could be produced during a significant habitat chance, resulting in a shift in ambient temperature and/or salinity and food availability, variables that are known to influence the appearance of the otoliths. A transforming otolith, which will result in an adult-like form, may be necessary to navigate through different environments to pursue prey and detect approaching predators (Brown et al. 2001). In fact, the particular sound frequencies to which the otolith responds, depends on the shape of the otolith (Gauldie 1988) and intra-species variation of otolith shape have been

observed (Lombarte and Castellon 1991) and attributed to adaptation to different habitats (Wilson 1985).

The scenario that we made associating some microstructural aspects of the otolith growth of conger, to some ecological, physiological and behavior events taking place during metamorphosis need, however, further research to confirm or reject some of the above-mentioned hypotheses.

Otolith increments of various closely related anguilliform fishes are deposited daily (Mochioka et al. 1989; Tsukamoto 1989; Umezawa et al. 1989; Umezawa and Tsukamoto 1991; Martin 1995; Arai et al. 2000a; Cieri and McCleave 2001; Sugeha et al. 2001). Assuming that also in this species the micro-increments are deposited on a daily basis, ages of the conger eel developing leptocephali collected in the Azores ranged between 69 and 275 days, indicating that the hatching dates for this species varies greatly between December and July, with an annual peak in the beginning of the summer season. These results agree with the observations made from the capture of small leptocephali in the Mediterranean Sea (Schmidt 1931) and from the temporal and spatial distribution of the larvae in the North-East and Central Atlantic (Strehlow et al. 1998). Although the estimated values for the hatching season of the developing leptocephali collected in the NE Atlantic were slightly different (from April to October), both studies suggest an extended spawning season for the conger eel. Lee and Byun (1996) estimated birth dates from September through February, also by analysis of the otolith microstructure of *C. myriaster* leptocephali. An extended spawning period has also been reported in *A. japonica* (Tabeta et al. 1987; Tsukamoto 1990; Tsukamoto and Umezawa 1990). On the other hand, the hatching dates for *C. oceanicus*, back-calculated from sampling dates and estimated ages, suggest a short spawning period during the winter season, ranging from late October to mid December. These data agree with the proposed spawning period for this species based on the collection of small leptocephali (McCleave and Miller 1994).

Several studies have indicated that past environmental history of anguilliform fishes can be reconstructed from analysis of otolith Sr:Ca concentration ratios. Changes in otolith Sr:Ca ratios have been considered to be related to environmental factors such as temperature (Tzeng 1994), salinity (Tzeng and Tsai 1994) and water mass (Otake et al. 1994), as well as to endogenous physiological factors (Tzeng 1996; Arai et al. 1997; Otake et al. 1997). The Sr:Ca ratios and its variation in the metamorphosing *C. conger* and *C. oceanicus* larvae are similar to those reported in other anguilliform leptocephali, namely *Anguilla* and *Conger* sp (Otake et al. 1994, 1997; Tzeng 1994, 1996; Tzeng and Tsai 1994; Arai et al. 1997, 1999ab, 2000b, 2001b, 2002; Wang and Tzeng 1998, 2000; Marui et al. 2001). Sr:Ca ratios are lowest in the

primordium and on the edge of the otolith. Although it has been suggested that the low Sr content observed in the otolith primordium of eels is probably due to the freshwater maternal origin of the yolk-sac (Tzeng and Tsai 1994; Wang and Tzeng 2000), our results obtained with two strictly marine species, showed that this feature can not be associated with a change in salinity and that it probably reflects the organic chemical composition of the otolith primordium. The rapid decrease in Sr:Ca ratio simultaneous with the increase in otolith increment width appears to occur during metamorphosis and has been observed in several species of anguilliform fishes, including *C. myriaster* (Otake et al. 1994, 1997; Cheng and Tzeng 1996; Arai et al. 1997, 1999ab, 2000b, 2002; Wang and Tzeng 2000; Marui et al. 2001). Several investigators (Tzeng 1994; Tzeng and Tsai 1994; Wang and Tzeng 2000) proposed that the drop in Sr:Ca ratios in the eel otolith after metamorphosis reflects the sudden change in ambient salinity associated with the migratory behavior of the eel, i.e. the entry into freshwater habitats less rich in Sr. However, since individual *C. conger* and *C. oceanicus* in our collections are often taken at nearly full salinity this likely would not result in a change in Sr:Ca ratios. This rapid drop in the otolith Sr:Ca ratios probably represents the mobilization of body mineral elements for rapid bone development that occurs during the late metamorphosis in leptocephalus fishes, namely a decreasing of the Sr levels in the body as a result of catabolism of Sr-rich GAG during metamorphosis (Otake et al. 1997). These findings suggest that these changes are a common feature in leptocephalus fishes and may be associated with the remarkable morphological and physiological transformations that occur during larval development.

The significant correlation between the otolith radius (or diameter) and the estimated age of the developing larvae, suggested the potential use of the otolith size to represent the fish age. However, as already observed for *C. myriaster* (Lee and Byun 1996), somatic and otolith growth become uncoupled during the metamorphic stage for both *C. conger* and *C. oceanicus*. The somatic growth rate of the developing leptocephali of *C. conger* estimated from the linear regression is 0.31 $mm.day^{-1}$. Although it falls within the ranges reported by several authors for other anguilliform species, namely *Anguilla* sp (Boëtius and Harding 1985; Kleckner and McCleave 1985; Castonguay 1987; Tesch 1998), it appears somewhat low and to assume a simple linear regression is probably not suitable for a leptocephali growth curve. Recently, Horie et al. (2002) observed a total length of 2.5 mm for a newly artificially hatched larvae of *C. myriaster* and reported a curvilinear fish length-age relationship in 19 day-old larvae, with a growth rate of about 0.9 $mm.day^{-1}$ for the first 7 days after hatching.

It has been suggested that the conger eel leptocephalus has a long larval life (Bauchot and Saldanha 1986), taking about one to two years to drift inshore and to reach the juvenile elver form (Lythgoe and Lythgoe 1971; Wheeler 1985; Strehlow 1992). Concerning the exact location for the different larval stages, the developing leptocephali of *C. conger* appear to be restricted to the continental slope, suggesting that the leptocephali do not enter the continental shelf and coastal waters unless they attained the metamorphosing stage. Our results suggest that the leptocephali of the European conger eel, *C. conger*, takes about 6 to 9 months from hatching to onset of metamorphosis. This estimate is shorter than the 15 months (1¼ years) proposed by Strehlow et al. (1998) to the end of this stage. The *C. oceanicus* apparently spend less time, 2 to 4 months, to reach metamorphosis. In *C. myriaster* the onset of metamorphosis occurs between the 4th and 8th month (Otake et al. 1997). The differences observed in the duration of the developing leptocephalus stage between these *Conger* species, may be related to the different distances from the spawning ground to the juvenile coastal area and/or to the dynamics of the ocean currents in the various areas. Based on indirect evidence (duration of the developing leptocephalus stage, time of capture and developmental stage of metamorphosing conger eels) we proposed about two years for the duration of the larval phase for this species. However, the exact age determination from counting daily growth increments in metamorphosing leptocephali seems to be impossible to establish and the values presented for the duration of the metamorphosis and total larval age, unfortunately, remains speculative for the conger eel.

Otolith analysis has been extensively applied worldwide in age determination of teleosts fishes, including *C. conger* (Fannon et al. 1990; Sbaihi et al. 2001; Sullivan et al. 2003). However, even in juveniles and adult specimens of conger, otoliths often proved to be useless for age determination due to multiple banding, and vertebrae were found to be a more efficient and effective structure for ageing conger eels (Sbaihi et al. 2001; Sullivan et al. 2003). Hood et al. (1988) recorded the same problem for *C. oceanicus* and recommended the use of alternative bony structures for ageing conger eels. Takai (1959) and Kubota (1961) reported the age and growth of *C. myriaster* using otolith analysis, although the annuli were barely visible, where the faint opaque zones do not allow age to be estimated using traditional techniques. To solve this problem a new ageing technique by UV light observation of burnt otoliths was successful applied to this Japanese species (Katayama et al. 2002). In our case, the lack of permanent hard structures bearing potential age markers in conger eel leptocephali, suggests the use of other approaches for age determination.

The age at metamorphosis and the duration of the leptocephalus stage appear to be important factors determining the long distributional dispersion of the Japanese eel (Cheng and Tzeng 1996) and might be responsible for the segregation of the migrating *A. rostrata* and *A. anguilla* (Wang and Tzeng 2000). A short duration of the leptocephalus stage might favour oceanic retention, while a long duration might favour emigration (McCleave 1993). The onset of metamorphosis to the juvenile phase may be delayed until appropriate physical conditions are meet (Tzeng 1990), as has been suggested for *C. oceanicus* (Bell et al. 2003). Larvae with long pelagic existences may be more susceptible to being carried by different currents and to being exposed to different growth conditions (Benoît et al. 2000).

Whether metamorphosis is triggered by some environmental stimulus or occurs spontaneously at a certain age or size is unknown. The length at which the *C. conger* leptocephali undergo metamorphosis appears to be between 155 and 165 mm based on the largest reported developing and metamorphosing leptocephali. Metamorphosing conger eel leptocephali captured in the Minho River reared in aquaria at temperatures between 15 and 16 °C completed the metamorphosis at a final length of 70-80 mm, with a mean PAL/TL of 0.37, in about 1½ to 2 months. The estimated duration of metamorphosis in *C. myriaster* is variable among investigators: 23 to 31 days (Kubota, 1961); 22 days at temperatures of 18 to 22 °C (Asano et al. 1978); 53 to 75 days at temperatures of 10 to 16 °C (Lee and Byun 1996); and 71 days at temperatures of 11 to 15 °C (Otake et al. 1997). The temperature of the sampling area, the difficulty or ambiguity in identifying the beginning/completion of metamorphosis and the artificial rearing conditions may result in inaccurate estimation of the duration of the metamorphosis. Our data indicates that under artificial conditions otolith growth rate in metamorphosing conger eel leptocephali is higher that in nature. The aquarium conditions and the handling stress, may produce a disequilibrium of the endocrine control of metamorphosis, producing an anomalously high growth rate of the otolith. Although experimental rearing appears to be one of the best methods for studying early growth of larval fishes, the use of fluorescent dyes as otolith time markers to study the real daily growth rhythm in the otolith diffuse zone by examining reared larvae has been unsuccessful.

The age at metamorphosis is inversely related to the somatic growth rate both in *C. conger* and *C. oceanicus* suggesting that slower-growing fish apparently metamorphosed later. This phenomenon was also found in *Pseudopleuronectes americanus* (Chambers and Leggett 1987), *A. japonica* (Tsukamoto and Umezawa 1990; Tzeng 1990; Cheng and Tzeng 1996) and *Megalops cyprinoides* (Tzeng et al. 1998), suggesting that the time taken for migration from oceanic spawning ground to the coastal waters was probably shorter for the fast-growing

larvae. In general, fast-growing larvae metamorphose early and swim quickly to the preferred habitat (Hunter 1972; Miller et al. 1988). In contrast slow-growing larvae, which are unable to swim as fast as the faster growing ones, prolong larval stage duration and delay metamorphosis and recruitment to the estuary (Victor 1986).

The positive relationship between age at recruitment and age at metamorphosis suggests that *C. oceanicus* that metamorphosed at an earlier stage tended to recruit to estuaries at younger ages, indicating that early-metamorphosing larvae are recruited earlier. Several authors (Tsukamoto 1990; Tsukamoto and Umezawa 1990; Arai et al. 1999ab, 2000b, 2001b; Wang and Tzeng 1998; Marui et al. 2001) found the same phenomenon in temperate and tropical eels. This relationship was impossible to establish in *C. conger* since all the metamorphosing larvae presented a diffuse zone, which prevents the accurate determination of the age at coastal recruitment. However, our data (TL and PAL/TL ratios) shows that the largest larvae, in an advanced metamorphic stage, are recruited early to the northern Portuguese coastal waters. Thus, the migrating mechanism of the conger eel can be summarized as follows: the larvae with a faster growth rate metamorphose and recruited earlier to the coastal areas, probably at a younger age, and with a larger size and an advanced developmental stage. This larval recruitment pathway appears to be common in anguilliform fishes.

While conger eels are an important component of the coastal and outer continental shelf marine ecosystem, very little is known about the spawning ground(s), ecology and migration of *C. conger* leptocephali in the ocean. Although it has commonly been suggested in some textbooks, that the European conger eel, *C. conger*, has a spawning place in the North-East Atlantic, between Gibraltar and Azores (Lythgoe and Lythgoe 1971; Bagenal and Kenney 1973; Wheeler 1985; Hayward and Ryland 1995), there are no references to support this statement. Currently, the only spawning area well known for this species is in the Central-East Basin of the Mediterranean (Cau and Manconi 1983).

Larvae of conger eel, collected over a broad geographic area, can be assigned ages derived from daily increment counts, and this information can be integrated with data on regional water circulation to map patterns of larval drift. Given the duration of the larval stage and approximate currents speeds, back-calculated hatch dates can be used to infer the general area of spawning. Based on spatial and temporal distribution of conger eel developing leptocephali collected in the North-East and Central Atlantic by several oceanographic cruises, Strehlow et al. (1998) suggested that spawning occurs in the Mediterranean Sea, between July and September. Around November the leptocephali, with 30 mm length, leave Gibraltar and

spread westward and northward, namely to southern Portugal and Spain, extending to all the East and Central zone of the Atlantic. Here, the conger eel has a new growth period lasting until the beginning of summer (130-150 mm and maximal of 165 mm length), when they start a new migration. It is supposed that this last migration in the direction of the coastal waters, with a possible return to the Mediterranean, induces metamorphosis.

The transfer of European conger eel from the spawning area (Mediterranean), via the Strait of Gibraltar, into the Central and North-East Atlantic may be partially explained as passive transport by the Portuguese Coastal Counter Current. It is the prevailing flow in the winter, from which larval dispersal could take place in the northwest direction. However, this can not explain how some developing specimens reached the Azores area from the Mediterranean based on the prevailing NE Atlantic circulation pattern. There is little chance that leptocephali, as small as 49.0, 51.5 and 54.0 mm, leaving the known Mediterranean spawning ground might reach the Azores Archipelago. This is a distance of about 3000 km, which would have to be covered in about 2 to 3 months (estimated age of these specimens), and the known North Atlantic oceanographic currents circulate in the opposite direction. This larval migration pathway could eventually be possible for the oldest specimens by a westerly dispersal of leptocephali driven by localized currents generated by mesoscale eddies, or if the leptocephali had an oriented and very active swimming behaviour (35 to 44 $km.day^{-1}$).

These data suggest that the conger eel might also spawn in or near the Azores Islands. The question regarding whether *C. conger* eel has multiple populations or a single panmictic population is at present unknown. However, preliminary mtDNA sequencing analyses made by us on some leptocephali provided some evidence for the existence of significant genetic differentiation among local populations, suggesting that the conger eel does not comprise a single panmictic population.

Future research must include further sampling and analysis of leptocephali over a wide area and time in light of current physical oceanographic knowledge, which might reflect the location and time of adult spawning, and provide new insight into the larval migration of the European conger eel. Additionally, the analysis of adult specimens intensively collected from a wide area of the NE Atlantic (including Madeira and Canary Islands) and Mediterranean, are essential to understand the population structure and the genetic variation of this species. A multidisciplinary approach might also include in the future the use of both otolith elemental fingerprinting and microsatellite DNA for stock identification.

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Picture:
SEM photograph of the otolith microstructure of a metamorphosing conger eel