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BIOLOGY AND ECOLOGY OF THE ALLIS SHAD, *Alosa alosa* (LINNAEUS, 1758), IN THE MINHO RIVER

Tese de Candidatura ao grau de Doutor em Ciências do Mar e do Ambiente, Especialidade em Planeamento e Gestão Ambiental submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Para Ti

Onde estiveres, estás

sempre comigo

AUTHOR'S DECLARATION

The author states that she provided a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published or under publication articles included in this thesis.

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Hybridization between sympatric shads *Alosa alosa* (L.) and *Alosa fallax* (L.) in an Iberian Peninsula river (Minho River, NW of Portugal): first evidences inferred by morphological and molecular characterization. *Manuscript in final preparation*.

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> *"Toda a grande e profunda dificuldade carrega em si a sua própria solução. Ela obriga-nos a mudar nosso modo de pensar de forma a encontrá-la."*
>
> Niels Henrik David Bohr

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PREAMBLE

It is generally agreed that, in recent years, the majority of migratory fish species suffered a striking decline. Diadromous species such as sea lamprey, *Petromyzon marinus* (Linnaeus, 1758), Atlantic salmon, *Salmo salar* Linnaeus, 1758, European eel, *Anguilla anguilla* (Linnaeus, 1758), Allis shad, *Alosa alosa* (Linnaeus, 1758), Twaite shad, *Alosa fallax* (Lacepède, 1803) and Sea trout, *Salmo trutta* Linnaeus, 1758, have a high commercial value in the river systems along its distribution area as, for example, in the Iberian Peninsula. Yet, many populations persist with dramatically reduced levels of abundance. Underlying reasons for this decline are loss of habitat, overfishing, pollution and climate change. Due to the significant reduction of the effectives and the extinction of migratory fish populations in several geographic areas, many species are now classified as threatened or endangered.

Among the above-mentioned species, the Allis shad is one of the most important commercial species in Europe (Aprahamian et al., 2002). The Allis shad commercial fishery is generally carried out at the time of the migration from their feeding grounds, in marine environment, to their spawning grounds, in continental waters, with few recorded catches at sea or along the coast. Recently, an activity of sport fishing in France, Great Britain and Portugal, namely in Minho River, was also reported. In the current distribution range of Allis shad, and between 1978-1998, the approximate values for captures per fishing boat ranged from 355 to 1 198 tones. About 94% of this total was recorded in France, with 89% of those belonging to the Gironde-Garonne-Dordogne system (Aprahamian et al., 2002). Until the late 20th century, this population was considered as being the largest (Baglinière, 2000) and as being of reference (Martin Vandembulcke, 1999) in European but, starting in the early 2000s, we assisted to the collapse of this population (Rougier et al., 2012). Currently there is evidence that the population of Minho river, in Portugal, is one of the most important in the southern part of their distribution range. However, like the other European populations, from the mid-twentieth century the Minho river Allis shad population suffered a decline in the order of 90% of their actual spawners.

The widespread decline of Allis shad throughout Europe and the vanishing of rivers where there was a thriving exploitation was cause for concern within the scientific community, which led to investigations in several research lines. At European level, several studies, as the latitudinal variations in the spawners' age of maturity (Lassalle et al., 2008b), the status of the spawners' stocks, implications for their conservation (King and Roche, 2008) and management of stocks (Woillez and Rochard, 2003), the population dynamics (Martin

Vandembulcke, 1999; Rougier et al., 2012), the migration patterns in the early life stage (Lochet et al., 2008; 2009), the discrimination of the natal origin (Thomas et al., 2005), the osmoregulation in juveniles (Lenguen et al., 2007; Bardonnet and Jatteau, 2008) and the evolutionary history based on molecular phylogenetic (Faria et al., 2006; 2007) were performed.

In Portugal, scientific information is scarce, relying mainly on general knowledge of Douro river's population biology and some of its protection measures (Eiras, 1977; 1981a) and interspecific differentiation and hybridization with *A. fallax* (Alexandrino, 1996). Some aspects of the biology and ecology of landlook populations were also assessed (Eiras, 1981b; 1983; Collares-Pereira et al., 1999).

Given the high ecological and commercial importance of the Allis shad and the enormous scientific gap concerning this species in this geographical area, the central objective of this study was, within a broad spectrum, to investigate the biology and ecology of Minho river Allis shad, in order to bridge this gap and provide basic scientific knowledge that can be applied to the management and conservation of the species, as well as to future scientific research.

With this study it was sought to answer several questions, being the thesis structured around three main objectives:

1) to investigate the general aspects of the biology and ecology of the species, in terms of:

   a) spawners: biometry and age structure of the spawning stock, their migratory behavior and reproductive biology;
   b) juveniles: distinction between *Alosa* spp. species, recognition and characterization of the habitat use at the time of their seaward migration and diet.

2) to assess the existence and the extent of hybridization between the two sympatric species of the genus *Alosa* present in Minho river.

3) to understand the migration patterns by studying the microchemistry of otoliths in order to:

   a) discriminate the natal origin;
   b) estimate the period of the freshwater phase and recognize the role of the estuary during the seaward migration.

ABSTRACT

The Allis shad, *Alosa alosa* (L.), is an anadromous migratory fish with great commercial importance in Europe. This species is gradually disappearing from European rivers, a fact which is especially induced by anthropogenic disorders. From the mid 20th century there has been a marked decline in the number of spawners and in the available habitat, mainly due to dam construction, sand extraction, pollution and overfishing. The striking reduction of their natural distribution range led to the disappearance from most of the northern Europe river basins, and so, to it inclusion in the red list of the International Union for Conservation of Nature (IUCN), in the Directive Habitats and also in the Appendix III of the Bern Convention. In Portugal, the Allis shad is classified as an endangered species in the Red Book of the Vertebrates of Portugal and the reduction of populations led to its legal protection, among others, under Decree-Law Nº. 140/99 of April 24th, Decree-Law Nº. 316/89 of September 22nd, Law Nº. 2097 of June 6th 1959, Regulatory Decree Nº. 43/87 of July 17th, Regulatory Decree Nº. 7/2000 of May 30th and Decree-Law Nº. 8/2008 of April 9th.

At the national level, and more specifically in the geographic area of the Minho river basin, the Allis shad takes a major cultural role, thus elevating the knowledge about its population to a level of utmost importance towards its conservation, given its socio-economic, historical, ethnographic and gastronomic value. There is evidences that the Minho river Allis shad population is one of the most important in the southern limit of their actual distribution range, with a clear need to collect basic biological information, aiming at their management and conservation.

The first chapter describes, based on current knowledge, the status of the migratory fish populations in the Iberian Peninsula, focusing on the Minho river populations.

Chapter 2 addressed general issues of the species biology and ecology, with a preliminary characterization of the populations' demography, migration and reproduction of adults, spawners' age and biometric structure and its comparison with other European populations (section 2.1). Ecological aspects in the juvenile stage were also considered, with a preliminary recognition of their freshwater habitat use, with particular attention to the diet (section 2.2). The data collection of a longer period of time allowed for a deeper understanding of the biology and ecology of the species on the continental stage of its life cycle, which brought information related to parasitology and hybridization, thus enabling us to point out some guidelines towards conservation of the species in Minho river (section 2.3). Specific aspects of reproductive biology such as fecundity and oogenesis of female gonads during the spawning migration, both at macroscopic and histological level,

and the relation of the Gonadosomatic index with the different stages of oocyte development (section 2.4) were addressed.

In Chapter 3 was assessed the extent of hybridization between the two sympatric species of the genus *Alosa* present in Minho river, the Allis shad (*A. alosa*) and the Twaite shad (*A. fallax*). To this purpose, genetic analyzes were performed, whose resulting genotypic classification is compared with the phenotypic classification found by meristic and morphometric characterization, both for spawners and juveniles.

In chapter 4, one of the emerging issues in the study of the biology of migratory fish species was addressed: the knowledge of their migration patterns. It was intended to clarify some aspects of the Allis shad migratory history, by studying otoliths' microchemistry. Because homing behavior is a common feature of the life cycle of many anadromous species, it was explored the chemical composition of otoliths for detecting their natal origin. Thus, the geochemical signatures of otoliths (microelemental and isotopic) of spawners and juveniles from several rivers were analysed in order to discriminate, especially, the populations of rivers Minho, Lima and Mondego (section 4.1). For the Minho river, the migration patterns of the species and the role of the estuary during the seaward migration were also evaluated, by analysing the Sr:Ca ratio along the major growth axis of the otolith. The otoliths of spawners were analysed as an attempt to find out, in retrospective, the timing of the seaward migration. The freshwater phase was also analysed in otoliths from juveniles caught in the tidal freshwater wetlands, before the migration towards the sea (section 4.2).

Finally, Chapter 5 discusses the main results of the previous chapters, forming a general conclusion, with the aim that the knowledge acquired in this work may be taken into account in conservation and management measures for the species. Because this work is the first approach to the biological and ecological aspects of Minho river Allis shad, this chapter also proposes some future research areas which seem to be relevant for a deeper understanding of the life cycle of this species.

RESUMO

O sável, *Alosa alosa* (Linnaeus, 1758), é uma espécie migradora anádroma com grande importância comercial a nível europeu. Esta espécie tem vindo progressivamente a desaparecer dos cursos de água europeus, induzido especialmente por distúrbios antropogénicos. A partir de meados do século 20 verificou-se uma acentuada regressão do número de reprodutores e da área de habitat disponível devido, principalmente, a factores como a construção de barragens, a extracção de inertes e a poluição. A marcada redução da sua área de distribuição natural conduziu, actualmente, ao seu desaparecido da maioria das bacias do norte da Europa, o que levou esta espécie a ser incluída na lista vermelha da União Internacional para a Conservação da Natureza (IUCN), na Directiva Habitats e no anexo III da Convenção de Berna. Em Portugal, o sável está classificado como espécie em perigo no Livro Vermelho dos Vertebrados de Portugal e a redução das populações levou à sua protecção legal, entre outra, ao abrigo do Decreto-Lei nº 140/99 de 24 de Abril, do Decreto-Lei nº 316/89 de 22 de Setembro, da Lei nº 2097 de 6 de Junho de 1959, do Decreto Regulamentar nº 43/87, de 17 de Julho, do Decreto Regulamentar nº 7/2000 de 30 de Maio e do Decreto-Lei nº 8/2008 de 9 de Abril.

No contexto nacional, e mais especificamente na área geográfica da bacia do rio Minho, o sável assume um papel cultural preponderante, revestindo-se o conhecimento sobre a sua população de extrema importância no sentido da sua conservação, dado o seu valor socio-económico, histórico, etnográfico e gastronómico. Há evidências da população do rio Minho ser uma das mais importantes do limite sul da sua área de distribuição actual, sendo clara a necessidade de recolher informação biológica de base com vista à sua gestão e conservação.

No primeiro capítulo, com base no conhecimento actual, descreve-se o estado das populações de peixes migradores no contexto ibérico e tendo como foco as populações do rio Minho.

No capítulo 2, são abordadas as questões gerais da biologia e ecologia da espécie, com uma caracterização preliminar da população em termos demográficos, da migração dos adultos e reprodução, da estrutura etária e biométrica, e respectiva comparação com outras populações europeias (secção 2.1). É feita uma abordagem a aspectos ecológicos na fase juvenil do seu ciclo de vida, com um reconhecimento preliminar das áreas de ocupação, dando particular atenção ao regime trófico (secção 2.2). A análise de uma série temporal mais alargada, permitiu um conhecimento mais profundo da biologia e ecologia da espécie na fase continental do seu ciclo de vida, dando-se a conhecer informação relacionada com a parasitologia e hibridização e apontam-se algumas

diretrizes no sentido da conservação da espécie no rio Minho (secção 2.3). Abordam-se ainda aspectos específicos da biologia reprodutiva, como a fecundidade e a evolução oogénica da gónada feminina durante a migração reprodutiva, tanto a nível macroscópico como histológico, e a relação do Índice Gonadossomático com os diferentes estágio de desenvolvimento do oócito são igualmente abordados (secção 2.4).

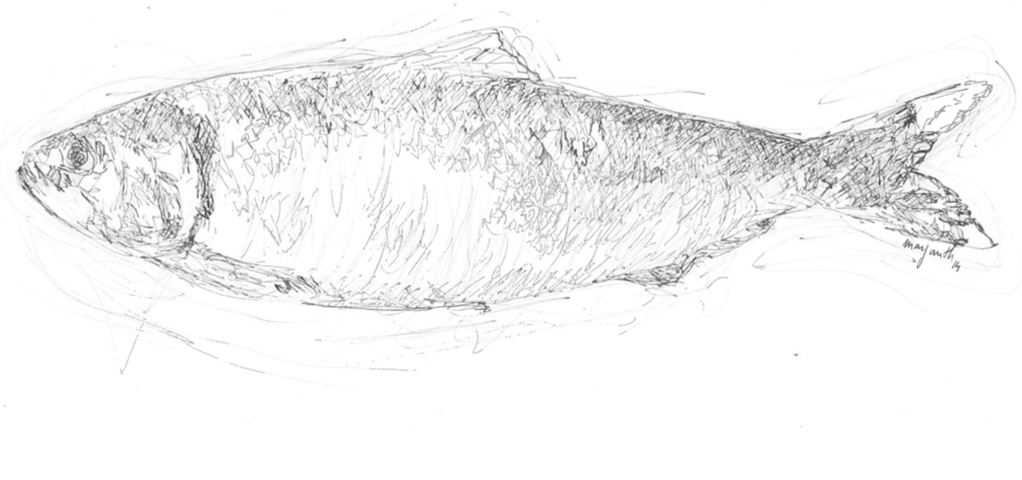
No capítulo 3 avalia-se a extensão da hibridação entre as duas espécies simpátricas do género *Alosa* presente no rio Minho, o sável (*A. alosa*) e a savelha (*A. fallax*). Para tal, recorreu-se a análises genéticas, cuja classificação genotípica resultante é comparada com a classificação fenotípica atribuída pela caracterização merística e morfométrica, quer para reprodutores, quer para juvenis.

No capítulo 4 aborda-se uma das questões emergentes no estudo da biologia de espécies ícticas migratórias, nomeadamente o conhecimento dos seus padrões de migração. Tenta-se esclarecer alguns aspectos da história migratória do sável através do estudo da microquímica de otólitos. Sendo o "homing" uma característica comum do ciclo de vida de várias espécies anádromas, explora-se a composição química dos otólitos como via para detecção do rio natal. Analisam-se, assim, as assinaturas geoquímicas de otólitos (microelementares e isotópicas) de adultos e juvenis de sável de vários rios, de forma a discriminar, especialmente, as populações dos rios Minho, Lima e Mondego (secção 4.1) No rio Minho, os padrões migratórios da espécie e o papel do estuário na fase da migração para o mar foram avaliados por análise do rácio Sr:Ca ao longo do maior eixo de crescimento do otólito. Analisaram-se os otólitos de reprodutores numa tentativa de encontrar, em retrospecção, a altura de migração para o mar, sendo que a duração da fase de água doce foi analisada em otólitos de juvenis capturados em zona tidal de água doce, antes da sua migração em direção ao mar (secção 4.2).

Finalmente, no capítulo 5 discutem-se os principais resultados dos capítulos anteriores, numa conclusão geral, onde se pretende que o conhecimento adquirido neste trabalho possa ser tomado em consideração em medidas de conservação e de gestão para a espécie. Tendo sido este trabalho a primeira abordagem dos aspectos biológicos e ecológicos do sável do rio Minho, neste capítulo são ainda propostas futuras áreas de investigação que parecem ser relevantes para um conhecimento mais profundo do ciclo de vida desta espécie.

CHAPTER 1

STATUS OF THE DIADROMOUS FISH OF THE IBERIAN PENINSULA



1. STATUS OF THE DIADROMOUS FISH OF THE IBERIAN PENINSULA: PAST, PRESENT AND TRENDS

M. Mota, C. Antunes and E. Rochard (*Accepted*) Status of the Diadromous Fish of the Iberian Peninsula: Past, Present and Trends. Limnetica.

ABSTRACT

We examined the past, present and predictable status of diadromous fishes on the Iberian Peninsula, with a focus on the species inhabiting the Minho River. New data on six diadromous species (five anadromous, one catadromous) were collected.

Due to population extinction and abundance reductions many species are now classified as threatened or endangered. Many populations persist at only drastically reduced abundance levels. Habitat loss (especially damming), overfishing, pollution, and, increasingly, climate change contributed to this decline of diadromous fish. Although there is still limited information on the conservation status, migratory behaviour, biology and ecology of diadromous fish in the Iberian Peninsula, it is clear that the general trend of these populations is towards decline.

As most of the larger Iberian rivers cross international borders, the management and the conservation of migratory fish species on transboundary rivers should be based on international cooperative and concerted efforts.

Key words: Diadromous fishes, Iberian Peninsula, decline, habitat loss

1.1. INTRODUCTION

Although diadromous fishes represent less than 1% of the world's fish fauna, their value to humans exceeds this proportion by far (Limburg and Waldman, 2009). The diadromous fish species Sea Lamprey *Petromyzon marinus* Linnaeus, 1758, Atlantic salmon *Salmo salar* Linnaeus, 1758, European eel and glass eel *Anguilla anguilla* (Linnaeus, 1758), Allis shad *Alosa alosa* (Linnaeus, 1758), Twaite shad *Alosa fallax* (Lacepède, 1803) and Sea trout *Salmo trutta* Linnaeus, 1758 have a huge economic and cultural importance in several Iberian Peninsula (IP) basins, especially in the Minho River (NW Iberian Peninsula). Apart from the mentioned species, other diadromous species are present on the IP (e.g. Doadrio, 2002; Cabral et al., 2006; Doadrio et al., 2011). The thin lipped mullet *Liza ramada* (Risso, 1827) and the European flounder *Platichthys flesus* (Linnaeus, 1758) are exploited species, despite their lower economic value while the River Lamprey *Lampetra fluviatilis* (Linnaeus, 1758) is an unexploited species. The European Sturgeon *Acipenser sturio* Linnaeus, 1758 was formerly a commercially important species and present in several IP rivers. Its two last records were an individual near the mouth of the Guadalquivir (Spain) in 1992 (Almaça and Elvira 2000, Doadrio et al., 2011) and a large specimen at Gijon (Asturias) in 2010 (B. Elvira pers. com).

Diadromous fish use successively freshwater, estuarine and marine habitats, which exposes them to environmental impacts and fishing in all three habitats. But this also gives them opportunities to disperse. The successful completion of such life cycle requires favourable conditions in both freshwater and marine environments. The transition from fresh to marine habitats, and back, is particularly critical due to urban and industrial developments in coastal zones, which are the most intensively modified and threatened ecosystems (McDowall, 1999).

During the last century, we observed a drastic decline in the abundance of diadromous fish populations (Lobón-Cervia, 1999; Groot, 2002; Baglinière et al., 2003; Hendry and Cragg-Hine, 2003; Beaulaton et al., 2008; OSPAR, 2008; OSPAR, 2009). Several basins have been cleared of most of their diadromous fish species as a result of habitat fragmentation due to dams construction (Nicola et al., 1996; Larinier, 2001) and associated variability in flow regimes (Lundqvist et al., 2008), deterioration in water quality in industrialized areas (Baglinière et al., 2003; Maes et al., 2008) and destruction of spawning habitats caused by sand and gravel extractions (Table 1.1). Their migrations through estuaries increase diadromous fish vulnerability, as their physical concentration increases their catchability, with the associated risks of overexploitation (McDowall, 1999) and unsustainable fisheries (Rochard et al., 1990).

In Galicia, the available area for diadromous fish represents only 12.5% of their potential original distribution area (Hervella and Caballero, 1999), and it is even less for the rest of the Iberian Peninsula (Figure 1.1). Nowadays, in Portugal, only 3.8% of the total length of a drainage basin is available for the eel (Eel Management Plan -EMP, 2010), and even less for other diadromous fish species with more limited capacity to get over obstacles and more restricted favourable habitats like Allis shad. In the Minho basin, the accessible area to diadromous species decreased from about 17 000 km^2 (at the beginning of the twentieth century) to around 1 000 km^2 (6%) nowadays. For species using only the main channel of the Minho River to migrate (e.g. Allis shad), the accessible habitat represents no more than 26.6 % of the pristine habitat.

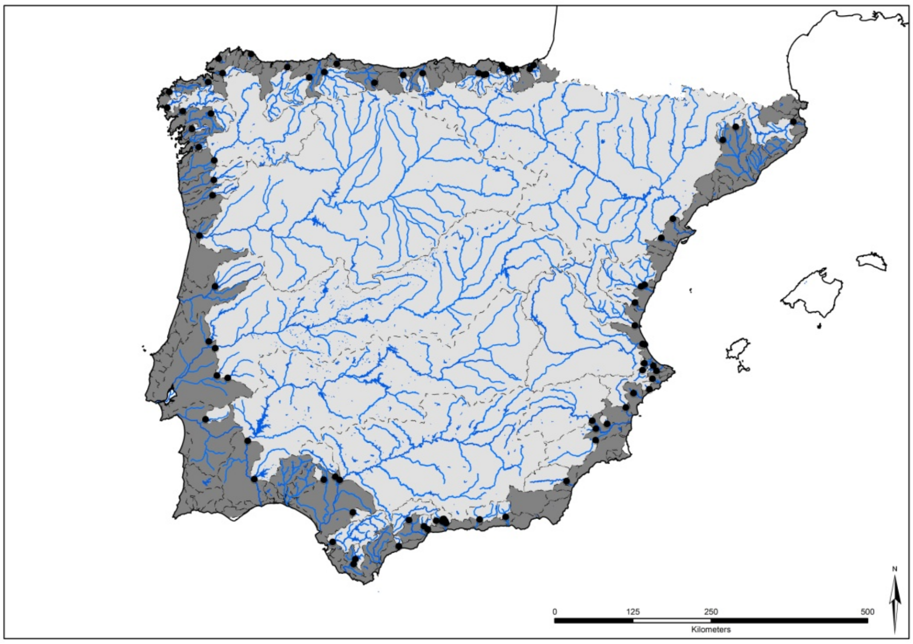


Figure 1.1. Iberian Peninsula hydrographical network showing the available habitat to diadromous species. Spots: first obstacle in the main course of the rivers; Dark grey: available habitat for diadromous fish; Bright grey: inaccessible habitat for diadromous fish.

Recently, climate changes are reported to be an additional factor contributing to diadromous extinction (Boisneau et al., 2008; Jonsson and Jonsson, 2009; Lassalle and Rochard, 2009; Lassalle et al., 2010). Béguer et al. (2007) pointed out that, at the European scale, the temperature is the main factor structuring the diadromous fish assemblages through hydrographical basins.

During their life histories, diadromous species pass through and use a wide range of freshwater, marine and estuarine habitats and often cross numerous international borders. Identifying appropriate protective measures for poorly known species is practically unfeasible (McDowall, 1999), nevertheless, measures as providing healthy habitats and restoring connectivity are valuable whatever the migratory species.

In this paper, we compiled information from literature, official statistics of captures and unpublished data, in order to summarize the past and present status of the commercially most important and exploited diadromous fish of the IP: Sea lamprey (*P. marinus*), Atlantic salmon (*S. salar*), European eel and glass eel (*A. anguilla*), Allis shad (*A. alosa*), Twaite shad (*A. fallax*) and Sea trout (*S. trutta*). We aim to summarize information at the IP scale, giving the Minho River system a particular focus. Apart from being one of the most well preserved large Iberian rivers, there is a good long-term series of fisheries data and recent scientific research allows better knowledge about diadromous species.

1.2. Search of Iberian Peninsula Diadromous Fish Information

For the IP, data on historical and present numbers of diadromous fish are scarce and the availability of information seems associated with their commercial relevance. All diadromous species regularly encountered in the study area were considered. This list was based on the Red Books of Portugal and Spain.

We synthesized WEB information on the current status of IP diadromous fish using the following databases and search tools: Google Scholar was used for general bibliographic methods using citations and other more general referencing as the common and scientific name of the diadromous species; ScienceDirect, Scopus and PubMed were used for advanced search, using keywords and back search. To refine results, the search was restricted to “cited reference” search.

However, as data were variable in quality and quantity and difficult to obtain for most species, information from books, unpublished literature and other documents was gathered from several sources, such University and museum libraries. Additional published and unpublished data were provided by scientists working in this field (see Acknowledgments).

Minho River time-series data sets were collected mostly from official data of the local Maritime Authorities, which had an especially rich and long set of diadromous species landings (from 1914 for Allis shad, Sea lamprey and Salmon and from 1975 for glass eel). These data sets refer to the labile fishing periods for each fish species, which may vary over the years. This information is however inaccurate and underestimates the real numbers of captures, which are screened of by fishermen.

Although the quality and quantity of time series data sets vary, the trend is almost always the same, with diadromous fishes in decline, often to historic lows.

Since the second half of the 20th century, the data for diadromous species in the Minho River are restricted to official statistics of fishery landings (Table 1.1; Figure 1.2). As this information is imprecise and subject to bias, there is an urgent need for additional information concerning these species.

1.3. IBERIAN PENINSULA DIADROMOUS FISH STATUS

1.3.1. *Petromyzon marinus*

Sea lamprey is widely distributed on both sides of the North Atlantic (Quintella, 2006; Beaulaton et al., 2008), occurring also in Mediterranean and Atlantic Canadian basins (Halliday, 1991). As other migratory fish, this species is common along large Iberian rivers, occurring in several Spanish basins along the Cantabrian, Atlantic, and Mediterranean River (Figure 1.3). Although the Sea lamprey inhabits most Cantabrian rivers, it is missing in some areas of País Vasco, Cantabria and in some Galician rivers (Doadrio et al., 2011; Perea et al., 2011). According to Cobo (2009), this species is still present in several Galician rivers and in Asturias (Rodríguez-Muñoz, 1992). More recently, juvenile specimens were collected from the Guadiaro River, confirming the occurrence of sea lamprey reproduction in southern Spain (Perea et al., 2011). In Portugal, this species is present in all of the main basins, being more abundant in the northern Sado River (Almeida et al., 2002a; Quintella et al., 2003; Almeida et al., 2008). The southern natural distribution limit is near the Guadiana River (Hardisty, 1986; Almeida et al., 2008).

As well as other migratory fish common along large Iberian rivers, Sea lamprey is currently declining due to the construction of dams, which represent a loss of habitat of around 80 % (Mateus et al., 2012) because, in general, the fish pass systems in dams do not work. For example, the access to the main channel of the river Tagus (which flows through Portugal and Spain) by the Sea lamprey is nowadays restricted to the Portuguese portion of this river because of two dams blocking the access to its Spanish part (Assis, 1990). In Portugal, the usable habitat does not exceed 588 km (Mateus et al., 2012).

The main threats to Galician sea lamprey populations are also the construction of dams, as well as gravel extraction in the freshwater larval-growth areas, overfishing and poaching (Cobo, 2009).

Table 1.1. Migratory fish species occurring on the Iberian Peninsula, the major threats affecting them, conservation status attributed by IUCN, Portugal and Spain and future trends. PT: Portugal; SP: Spain; LC: Least concern; VU: Vulnerable; CR: Critically endangered; EN: Endangered; DD: Data deficient; NA: not available; NP: Not present; IN: Increase; DC: Decrease.

	Confirmed presence in Iberian Peninsula			Main threats	Present conservation status			Trends
	North IP	West IP	South IP		IUCN	PT	SP	
P. marinus	Cantabrian coast (Asturias, Galicia)	Galicia, West coast of Portugal	Algarve and Mediterranean Spain	Rivers fragmentation pollution	LC	VU	VU	IN
S. salar	Cantabrian/Galician coasts (Bidassoa, Asturias, Bask Country)	Galicia, and Minho river	NP	Pollution, dams, overfishing	LC	CR	EN	DC
S. trutta	Asturias (Cantabria and Galicia)	North of Portugal	NP	Pollution, dams	LC	CR	VU	DC
A. alosa	Bidassoa, Asón	North and centre of Portugal	NP	Dams, habitat loss, pollution, overfishing	LC	EN	VU	DC
A. fallax	DD	Galicia, north and centre of Portugal	Guadiana, Ebro, Guadalquivir	Habitat loss, pollution	LC	VU	VU	DC
A. anguilla	All rivers	All rivers	All rivers	Habitat loss, pollution, parasites and diseases, predation, overfishing	CR	EN	VU	DC

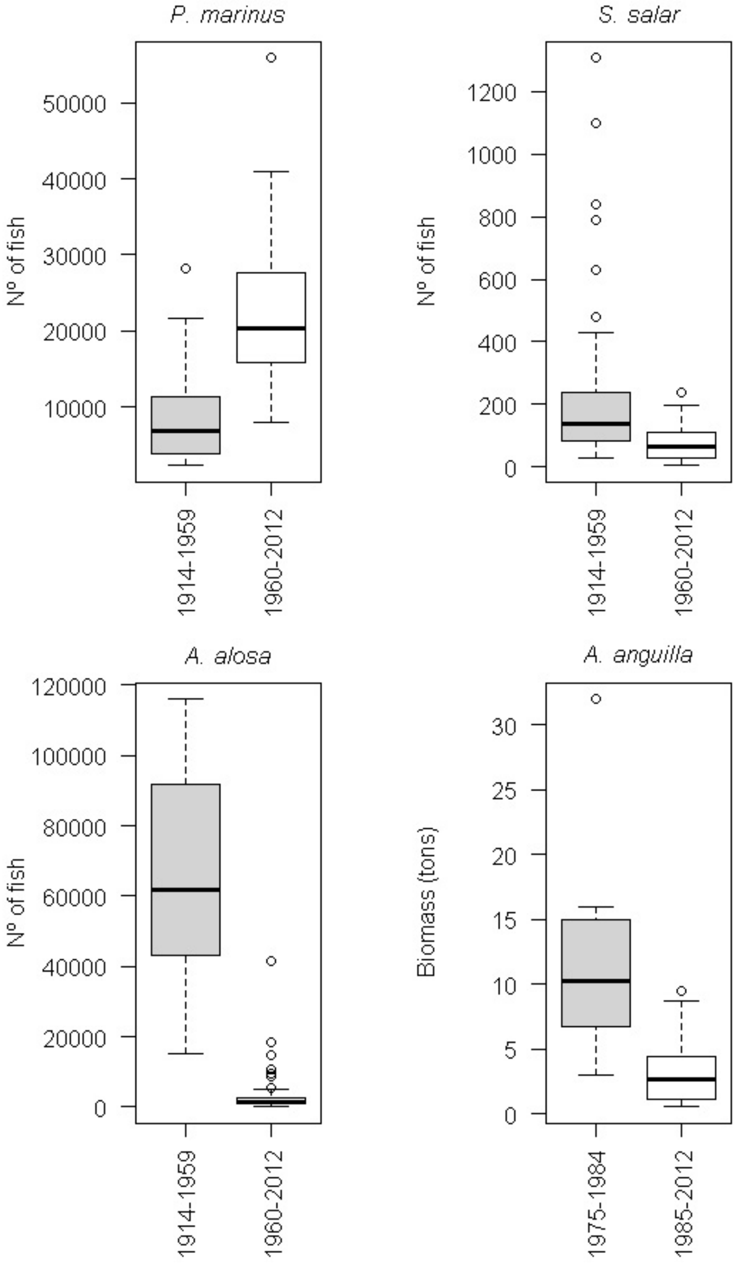


Figure 1.2. Minho River landings (in nº of fish) of Sea lamprey, Atlantic salmon and Allis shad and (in tonnes) of Glass eel. Data are shown separately for the first half of the 20th century (1914-1959) and the second half of the 20th century (1960-2012) for Sea lamprey, Atlantic salmon and Allis shad. The Glass eel data were separated according to the first major drop observed in the landings. Boxplots present the median (central line) and the interquartile range from the first to the third quartile (box). Whiskers indicate the minimum and maximum values excluding outliers, which are presented as separate circles.

The Sea lamprey has been considered for quite a long time a gastronomic delicacy in Europe (Kelly and King, 2001; Maitland, 2003), which has lead to intense commercial exploitation in some countries. It is a commercially important and valuable species in Spain and Portugal (Kelly and King, 2001; Maitland, 2003); in Portugal, a single fish can cost as much as 45 € (Andrade et al., 2007) every year in the beginning of the fishing season. During the peak of migration, the main Portuguese estuaries and rivers are crowded with fishermen and poachers (Almeida et al., 2002a). Next to the reduction of suitable habitat due to dam construction, this intense fishery constitutes the main threat to the survival of this species in Portuguese river basins (Almeida et al., 2002b; Andrade et al., 2007).

At the European level, the Portuguese, Spanish and French (OSPAR, 2009) Sea lamprey populations are not considered as endangered but are facing, in the medium term, a high risk of extinction in the wild (Lassalle et al., 2008a). The status of Spanish Sea lamprey populations remains poorly known but at the Spanish autonomous region level, as well as in Portugal, this species benefits of several conservation regulations (Table 1.2).

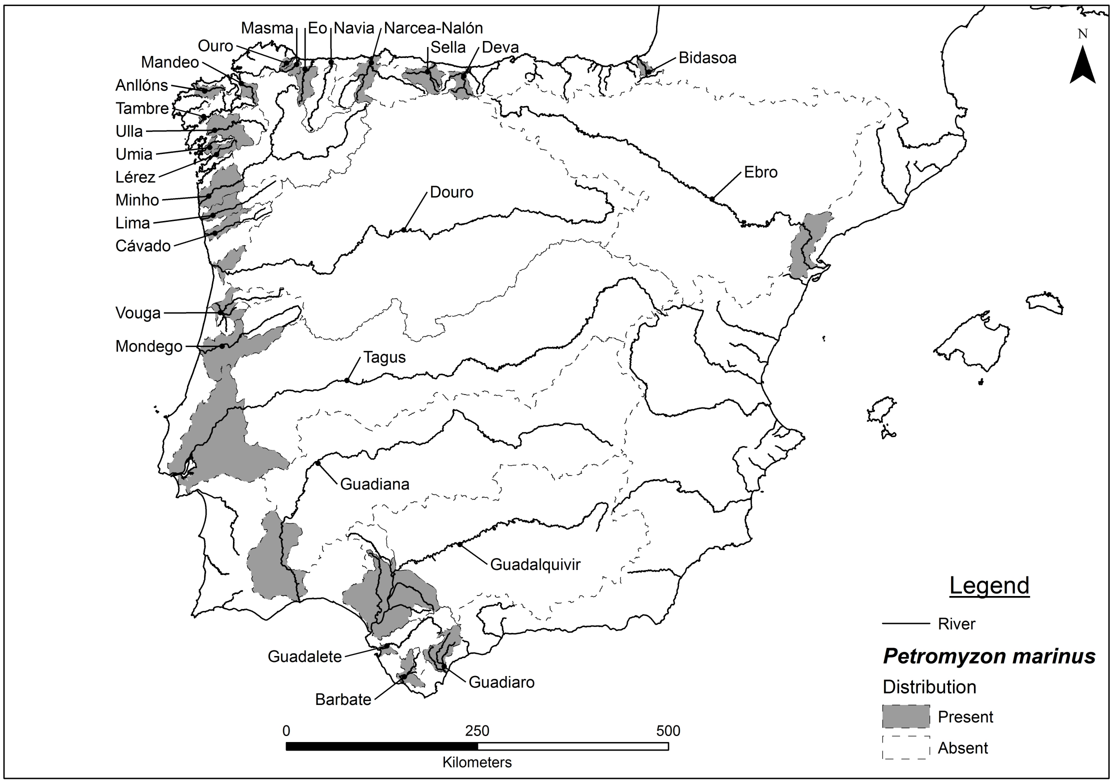


Figure 1.3. Sea Lamprey distribution no the Iberian Peninsula, showing areas where the species is present or absent.

Table 1.2. Sea Lamprey conservation status at Portuguese (national) and Spanish (autonomous region) levels.

	Region	Conservation status	Regulation document
Portugal	National	Vulnerable	Red Book of endangered species
Spain	Galicia autonomous region	Vulnerable	Galician Catalogue of Endangered Species
	Asturias autonomous region	Vulnerable	Regional Catalogue of Endangered Vertebrate Fauna of the Principality of Asturias
	Extremadura autonomous region	Endangered	Annex I of Regional Catalogue of Endangered Species of the Extremadura
	Navarra autonomous region	Special interest	Navarra Registration of the Vertebrate Wildlife

Even though the current abundances of Sea lamprey are still far below the historic levels (OSPAR, 2009), some European rivers, such as the Garonne, Adour, Vilaine and Rhine, have shown a recent raise in the population size since the 1990s (Beaulaton et al., 2008).

The Portuguese Sea lamprey populations have decreased dramatically since the 1980s (Collares-Pereira et al., 2000). In the Tagus River, the decline of catches after the 1950s was mainly due to the huge reduction of the original spawning areas related to construction of dams, but the recruitment of this species has also been affected by the pollution of some tributaries (Ferreira and Oliveira, 1996).

Cobo et al. (2010) has described an increase in Sea lamprey ammocoetes' density between 2007 and 2009 in the Galician rivers, with higher densities and biomass in Cantabrian Slope rivers. In the Minho River, there is little knowledge about the larval phase, but we have noticed an increase in the abundance of adults captured from 2002 on (Figure 1.4). Official statistics of the Minho River small-scale fisheries indicate that the Sea lamprey is the only diadromous species that has been exhibiting increasing captures in recent years (Figure 1.2 and 1.4).

Although future trends remain unclear, Lassalle et al. (2008a), using a niche modeling approach, predict a decline in Iberian Sea lamprey populations. According to this study, the northern Spain and Adour basins could become in the near future the southern limit of the distribution of the sea lamprey, which could disappear from Portuguese basins.

As the Sea lamprey adult phase is parasitic, feeding on large fish, it seems possible that the abundance of Sea lamprey may be associated to the abundance of suitable preys, particularly shad and salmon. According to Henderson (2003) the re-establishment of large populations of these migratory species in the rivers of a given region could help in the re-establishment of large populations of Sea lamprey. For example Silva et al. (2013) has documented haematophagus feeding of postmetamorphics on Twaite shad and salmon. Yet further research is needed concerning these phenomena in order to explore these relationships, as prey increase could be a way of preventing the disappearance of the Iberian Sea lamprey populations.

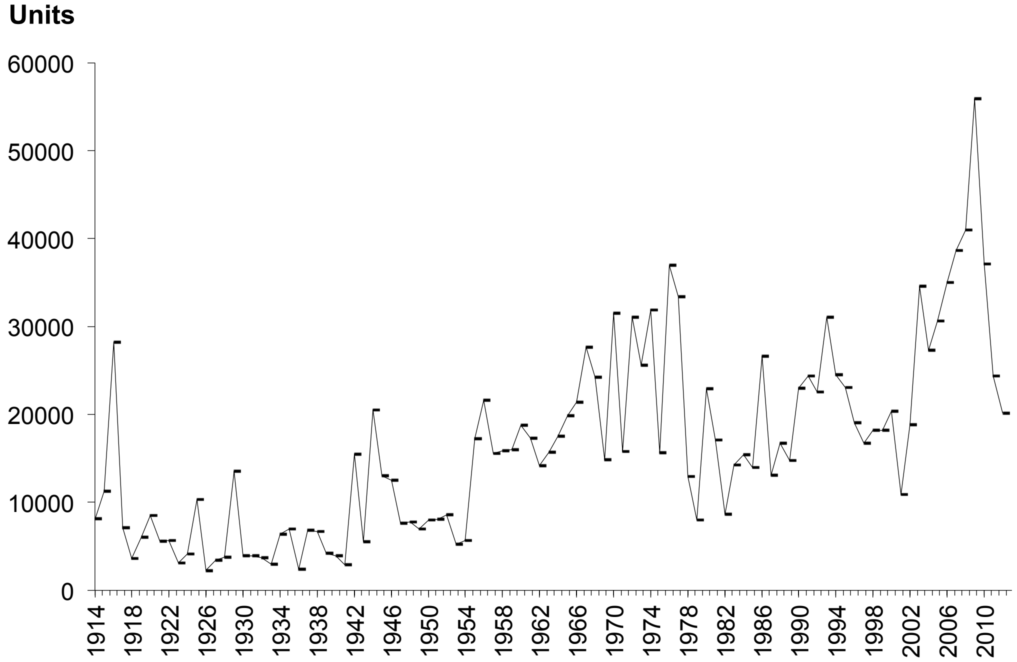


Figure 1.4. Time series of sea lamprey catches (number) in the Minho River, reported by Portuguese fishermen to the local Maritime Authorities (source: Maritime Authority of the Caminha Fishing Harbour).

Based on recent genetic analyses that suggest differentiation between European and North American Sea lamprey populations (Rodríguez-Muñoz et al., 2004), Mateus et al. (2012) recommend to manage both populations independently and proposed to revise the IUCN status of the European species. The status attributed to the Sea lamprey in the Annex II of the Habitats Directive should also be revised.

1.3.2. *Salmo salar*

The current distribution of the Atlantic salmon (*S. salar*) ranges from Northern Portugal to North America. In the East Atlantic, the species can be found from northern Portugal (42°N) to Petjorskoye in northern Russia (Jonsson and Jonsson, 2009). Its range includes rivers in Spain, France, UK, Ireland, Norway, Sweden, Finland and other countries draining into the Baltic, as well as Iceland and Greenland (Hendry and Cragg-Hine, 2003). Wild Atlantic salmon has disappeared from Germany, Switzerland, the Netherlands, Belgium, the Czech Republic and Slovakia basins. Comparatively healthy populations are known to be found in only four countries: Norway, Ireland, Iceland and Scotland (OSPAR, 2010). In the last 50 years, the Iberian rivers where salmon were present ranged from the Minho River (NW Portugal), the species' southern limit in Europe (Hervella, 2002; Álvarez et al., 2010), to the Bidassoa river (Basque country, North Spain). Formerly the species could be found as far south as the Douro River. Presently, except for wandering migration events which can occur in small N-Portuguese rivers, such as the Lima River, its southern limit of distribution is in Minho River. Thus, in Iberian Peninsula the species inhabits, in a relatively permanent way, about twenty rivers covering the Cantabrian, Galician and Northern Portugal coasts, between the Minho River and the Bidassoa River (Doadrio et al., 2011).

Álvarez et al. (2010) pointed out several negative impacts on the "salmonids'" habitat: damage to riverine vegetation that causes insulation increase and, consequently, water temperature rising; disappearing of spawning grounds and alevin habitats; modification and disappearing of ponds where adults can rest during migration and change in the estuarine conditions. These threats affected particularly the Oria, Urumea, Pas and Nálon-Narcea basins.

The IUCN Red List of Threatened Species states the Atlantic salmon globally as "Least concern" species (IUCN, 2013). However, this conservation status needs to be updated, in Spain it is considered as "Endangered" (Doadrio, 2002) and in Portugal it is considered as "Critically endangered" (Cabral et al., 2006) (Table 1.1).

On the Iberian Peninsula, as well as in other European regions, the first phase of the decline of salmon populations started in the 19th century, continuing during the 20th century (Álvarez et al., 2010). Cumulative and synergistic factors are recognized to be responsible to the populations' regression. The decrease of the water quality due to industrialization, the development of hydropower at the end of the 19th century and during the 20th; as well as overfishing and illegal fishing with explosives had devastating results and led to the disappearance of Atlantic salmon from many rivers of the North Iberian Peninsula (Álvarez et al., 2010).

In the Ulla and Lérez rivers, located in the southern part of the European distribution of wild Atlantic salmon, the populations have been seriously endangered since the late 1970s; by 1991 the River Lérez population was close to extinction (Saura et al., 2006). At the beginning of the 21st century, Atlantic salmon migrated along only 32 Iberian rivers (Álvarez et al., 2010).

Official data of small-scale fisheries start to be available from the second half of the 20th century. Since the 1970s, the Iberian Peninsula has been facing a severe drop in the catches. The Galician rivers have contributed with more than half of the salmon catches of all Spain, being the “salmonid” region *par excellence* (Hervella, 2002); they provided 12 000 salmons per year in the 1920 and 1930 decades. In the beginning of the second half of the 20th century, the total yearly catches in Galician rivers, including Minho (border between Portugal and Galicia, Spain), reached 2 000 individuals in the most productive years. Since 1980 the average yearly catch has dropped to about 200. The worst year was observed in 1998 with only 26 salmons caught in all of Galicia (Álvarez et al., 2010) and North Portugal. In the latter, until the 1990s salmon populations were described for the Minho and Lima Rivers (Antunes and Weber, 1990; Valente et al., 1991). It was estimated that less than 250 mature fish exists and 90% of them belong to the Minho population. The decline has continued along the last three generations, and accentuated fluctuations in the number of mature individuals are admitted (Rogado et al., 2006a). Nowadays the Lima population is residual or even extinct.

The Minho River was the largest “salmonid” river of the Cantabrian-Atlantic slope and also the one with the largest production capacity. But due to the construction of dams, the area used by the salmon represents nowadays only 6% of the pristine favourable growth habitats (Hervella, 2002; Álvarez et al., 2010). Data supplied by fishermen indicate catches of about 1 000 salmons per year in the 1st half of 20th century. Since 1950, the catches in the Minho River dropped to 200-400 individuals and, in the last decades, did not exceed 100 fish per year (Álvarez et al., 2010). More recent statistics supplied by Maritime Authorities, shown that catches in the Portuguese part of the Minho River did not exceed 64 individuals in the last ten years, with a minimum of only 5 individuals caught in the year 2000 (Figure 1.2 and Figure 1.5).

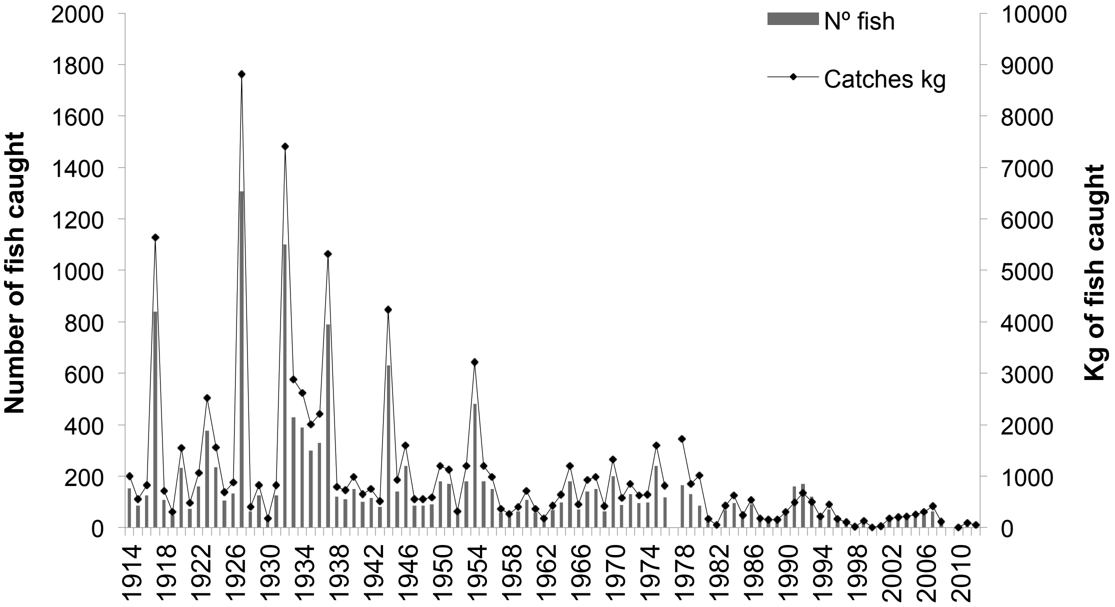


Figure 1.5. Time series of salmon catches (weight and number) in the Minho River, reported by Portuguese fishermen to the local Maritime Authorities (source: Maritime Authority of the Caminha Fishing Harbour).

Although data from fisheries are now established more seriously, the state of salmon populations remains unclear for most of the IP Rivers. That is partly because few Iberian rivers have migration control devices and, partly due to the lack of knowledge regarding fundamental aspects of population structure, such as spawning population and juvenile growing areas (Álvarez et al., 2010). However, a comprehensive restoration program for Atlantic salmon in some Galician and international rivers, including the Ulla, the Lérez and the Minho, began in 1995 (Caballero, 2002a).

The results of stocking programs are variable and have been amply documented (see Fraser, 2008), whereas cases of natural recolonization by Atlantic salmon are rare and have been described only in small- or medium-sized river systems (Vasemägi et al., 2001; Saura et al., 2006; Saura et al., 2008).

As Atlantic salmon is a cold water species, it is likely that populations inhabiting the natural southernmost edge of the distribution range will be extremely endangered or even become extinct, due to the combination of several threats experienced over the past centuries and the recent climate changes effects (Lassalle and Rochard, 2009; Álvarez et al., 2010; Valiente et al., 2010).

1.3.3. *Salmo trutta*

The Sea trout (*S. trutta*), anadromous form of brown trout, is a species of high commercial and ecological value in Europe. This species has a wide range of distribution, being found throughout the range of the Atlantic salmon in continental Europe, on the British Isles, in Scandinavia and Iceland. The species occupies rivers in the northwestern quadrant of the Iberian Peninsula, with the Minho River (latitude 42º), North Portugal, considered to be the southern limit of its natural distribution (Caballero et al., 2006; Milner et al., 2006a; Okumus et al., 2006; Caballero, 2002b; Elliott, 1989). In the first half of the 20th century, Sea trout also occurred in the Cávado River (Southern of the Minho River), but populations have disappeared or have been reduced to extremely low numbers as a result of various pressures (Valente et al., 2000) and, today, the only functional population of Sea trout is for sure the one in the Minho River.

Sea trout is reasonably abundant and widespread in European rivers, supporting important sport and commercial fisheries (Euzenat et al., 2006). Although not all aspects have been addressed, it is an extensively studied species (e.g. Nikolic et al., 2011; Snoj et al., 2002; Pettersson et al., 2001; Euzenat et al., 1999; Lyse et al., 1998; Johnstone et al., 1995; Solomon, 1995; Lund and Hansen, 1992; Hindar et al., 1991; Dellefors and Faremo, 1988). Long-standing studies have been performed on the North Esk, Scotland (Pratten and Shearer, 1983), Black Brows Beck in Cumbria, England (Elliott and Elliott, 2006), and in Burrishoole, Co. Mayo, Ireland (Poole et al., 2006). These studies were afterwards complemented by studies on the Rivers Bresle (Euzenat et al., 2006) and Oir (Acolas et al., 2008), France. However, these studies mainly focused on the North-East Atlantic populations, and there is a lack of scientific literature on Iberian populations. To date, few references can be found on the Iberian stock's characteristics (Toledo *et al.*, 1993; Caballero *et al.*, 2006). Indeed, scientific studies situate the Sea trout's natural distribution range is situated between latitude 48ºN and 62ºN (Jonsson and L'Ábée-Lund, 1993; Richard, 1986), leaving a large gap between the latitudes 42º (Minho River mouth) and 48º North (Normandy coasts) (Caballero, 2002b). For the Iberian Peninsula, only few studies were done. There are some references to the Asturian population's life cycle (Alvarez-Riera, 1995; Toledo et al., 1993). Caballero (2002b) describes for the first time the Iberian Sea trout life cycle and Caballero et al. (2002) compares some Sea trout biological variables with salmon populations.

The status of Sea trout stocks and fisheries varies across its range, depending on the influence of local factors (Milner et al., 2006b). Sea trout stocks seem to be healthy in some regions, but there have been major collapses in others (Harris and Milner, 2006), with a decline in trout populations found in numerous places (e.g. Aarestrup and Jepsen, 1998; Jutila et al., 2006; Okumus et al., 2006).

Worldwide many Sea trout stocks are extinct or are facing extinction because of anthropogenic impacts: environmental degradation, damage of spawning areas and interruption of spawning migrations (dam constructions), deforestation, drainage, straightening and channelling of watercourses, overfishing and poaching (see Landergren, 1999; Lundqvist et al., 2006; Okumus et al., 2006).

Sea trout collapsed in the Midwest Irish region and Scotland in 1988–1990 (Butler and Walker, 2006; Gargan et al., 2006). These same occurred in many other western Sea trout fishery areas, where populations collapsed happened in 1989 (Gargan et al., 2006). The areas, which have seen the greatest declines in Sea trout, coincide geographically with the development of salmon aquaculture. Although no direct link has yet been established, there is an increasing concern about the possible links between the sea lice disease and its effect on wild salmonid host stocks.

Threats to Sea trout should be considered seriously. There is an urgent need for the development of an integrated management and conservation strategy. Prevention of further decline through effective fishery enforcement is essential to preserve the remaining stocks, but this will require integration between different agencies and countries and that requires shared aims and a common strategy (Okumus et al., 2006).

The high variability of life-history patterns of *S. trutta*, their iteroparity and, especially, the lack of understanding regarding the relationships between resident and anadromous brown trout stocks within the same catchment, have hindered attempts to establish realistic stock–recruitment relationships (Poole et al., 2006).

An effort has to be made to preserve and prolong long-term studies, so that essential background data for the interpretation of short-term fluctuations and trends can be obtained and made available (Euzenat et al., 2006). Baglinière and Maisse (2002) demonstrated that, from a functional point of view, headwater streams are very important in the recruitment process of Sea trout in a basin population and claim the need to maintain their physical integrity. They have also demonstrated that ensuring a free movement of fish between the main stem and tributaries of a stream is important for growth and breeding.

Regarding the Iberian Peninsula, Sea trout is especially important in Galician and North Portuguese (particularly in the Minho River) rivers. Here, Sea trout are ubiquitous and represent an important natural resource, as well as an important sport fishery species (Hervella and Caballero, 1999; Caballero et al., 2006). Populations have been described for the Minho and Lima rivers (Antunes and Weber, 1990, Valente and Alexandrino, 1990). Information collected from fishermen indicates that the number of mature individuals is extremely limited and that the species (both sea and brown trout) are in continuous decline in these two basins (Rogado et al., 2006b).

In Portugal, the reduction of Sea trout populations over the past 10 to 15 years have reached 98% of mature adults and it is expected that this declining trend could persist for the next 10 to 15 years (Rogado et al., 2006b). The causes for the reduction, though generally understood, are neither reversible nor have they been eliminated. The evaluation of the population decline is based on abundance data, reduction of the area of occupancy, extent of occurrence and habitat quality and also on the expansion of non-native species (Rogado et al., 2006b), such as the rainbow trout (*Oncorhynchus mykiss*). The realization of any stocking programs in these basins should preferably use the eggs or alevins from the same population or, at least, close populations, preventing the import of non-indigenous populations that are genetically different (Antunes et al., 2001).

As a natural resource, Sea trout have a socioeconomic value that exceeds that of salmon in some areas (Milner et al., 2006b). Given the social and economic value of Sea trout fisheries, stocks need to be conserved and associated fisheries need to become sustainable (Walker et al., 2006). Stocking programs, as those performed in Scotland (see Lundqvist et al., 2006; Hay and Hatton-Ellis, 2006) should hence be considered into account to maintain and preserve Iberian sea trout populations.

1.3.4 *Alosa alosa* and *Alosa fallax*

Historically, the distribution of *Alosa* species covered the eastern Atlantic from Norway to Morocco and the Western Mediterranean Sea. From the middle of the 20th century on, a clear contraction of the natural distribution range was observed, and, nowadays, Allis shad (*A. alosa*), is restricted to the Atlantic coast of France and Portugal (Baglinière, 2000). Currently, the northern limit of spawning stocks areas is the Vire Basin in Normandy and the southern limit is the Minho, located at the boundary between Portugal and Spain (unpublished data).

On the Iberian Peninsula shad populations are more frequent in Cantabrian and Atlantic coasts than at the Mediterranean coast (Table 1.1). Allis shad, once found in all major Iberian rivers (Doadrio, 2002, Doadrio et al., 2011), is nowadays rare, much reduced or virtually extinct in most of them (Figure 1.6). In Portugal, the Minho River population seems to be one of the most important populations of the southern part of its distribution, although Allis shad has been described to be present in the Vouga and the Mondego (Faria, 2007) rivers, for which little data are available.

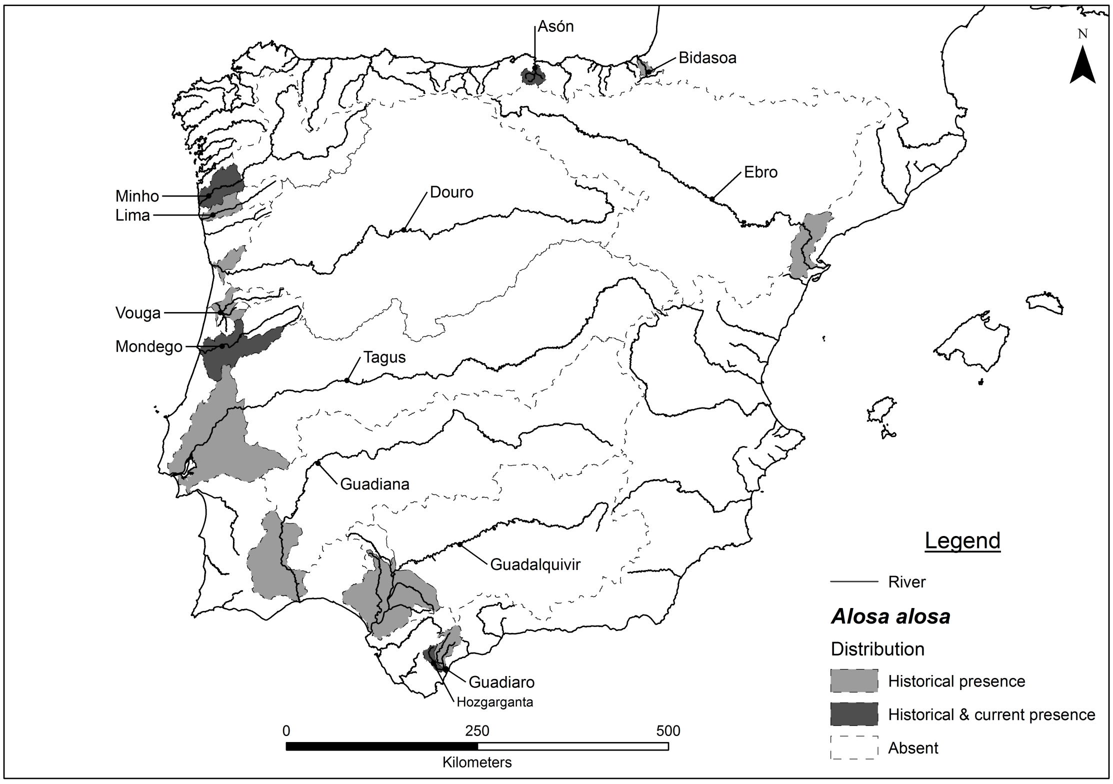


Figure 1.6. Historical and current distribution of the Allis shad on the Iberian Peninsula.

General literature reports the presence of *Alosa* spp. in Galicia (Spain) but detailed information about which species are present in the river systems is restricted to the presence of *A. alosa* in the River Minho (Mota and Antunes, 2011; 2012) and of *A. fallax* in the river Ulla (Silva et al., 2013; Nachón et al., 2013). In addition, the Spanish sport fishermen report the existence of important *Alosa* spp. populations in some Northern Spanish rivers that were never studied.

Historically, Allis and Twaite shads were of great economic importance, but nowadays the Galician populations are severely impacted (Hervella and Caballero, 1999). Portuguese shad populations suffered an evident decline since the middle of the 20th century, particularly in the Minho and Lima rivers.

The main reasons for this decline have been: the construction of dams which, next to preventing access to spawning grounds, prompt the hybridization with Twaite shad; the permanent loss of suitable habitat; the generalized pollution of aquatic systems, especially in the lower reaches and estuaries; and overfishing (Aprahamian et al., 2003, Rougier et al., 2012).

The decline of Portuguese Allis shad populations has also been related to the reduction of river flows and overfishing of the most important stocks (Costa et al., 2001). Legal and illegal by-catch of juveniles by glass eel fisheries and other fisheries (Antunes and Weber, 1996; Assis et al., 1992) and hybridization between *A. alosa* and *A. fallax* (Alexandrino, 1996) were also reported as a major constraint to Portuguese shad populations.

The gradual decrease of *Alosa* spp. in several European basins, caused essentially by anthropogenic pressures, led to its listing in the Red List of the International Union for the Conservation of the Nature (IUCN), in Annex III of the Bern Convention, and in the Habitats Directive (Annex II).

In Spain *Alosa* species (*A. alosa* and *A. fallax*) are classified as vulnerable in the Red List of the Vertebrates of Spain (ICONA, 1986), in the Red Book of the Vertebrates of Spain (Blanco and González, 1992) and in the Atlas and Red Book of the Continental Fish of Spain (Doadrio, 2002). At the regional level, in Galicia they are listed as endangered species (Viéitez and Rey, 2005). In Portugal *A. fallax* is classified as a vulnerable species and *A. alosa* as endangered (Cabral et al., 2006), being now under legal protection.

Within the Iberian Peninsula, the Minho was once the river with the highest catches of *Alosa* species (Baldaque da Silva, 1892), and *A. alosa* still has a great commercial and heritage value in the Minho River basin (Mota and Antunes, 2011).

Official statistics from the Portuguese Maritime Authorities for the Minho River small-scale fishery show yearly mean catches of 200 tonnes of *A. alosa* during the first half of the 20th century, with peaks of 300 tonnes (Mota and Antunes, 2011). After the 1950s catches decreased by about 90% (Fig. 1.7), coinciding with the building of the first dams (Alexandrino, 1996; Baglinière et a*l*., 2003). The last dam was built in 1969, confining the accessible main course of the river Minho to 80 km; this dam is nowadays the first physical barrier faced by Allis shad.

Even though the captures in the last decades never rose to the original values, the same source shows peak catches of about 18 tonnes in 1980 and 16 tonnes in 2009. However, it is broadly known that these data could be underestimates due to the lack of reliable official statistics. Unofficial Portuguese and Spanish data over the last eight years indicates that catches may have been twice as high (Mota and Antunes, 2011), but the general trend is the drop of long-term catches (Figure 1.2 and Figure 1.7). Nonetheless, the Allis shad of the Minho River is still of high commercial and heritage value and information from fishermen confirms the existence of a shad population worth preserving.

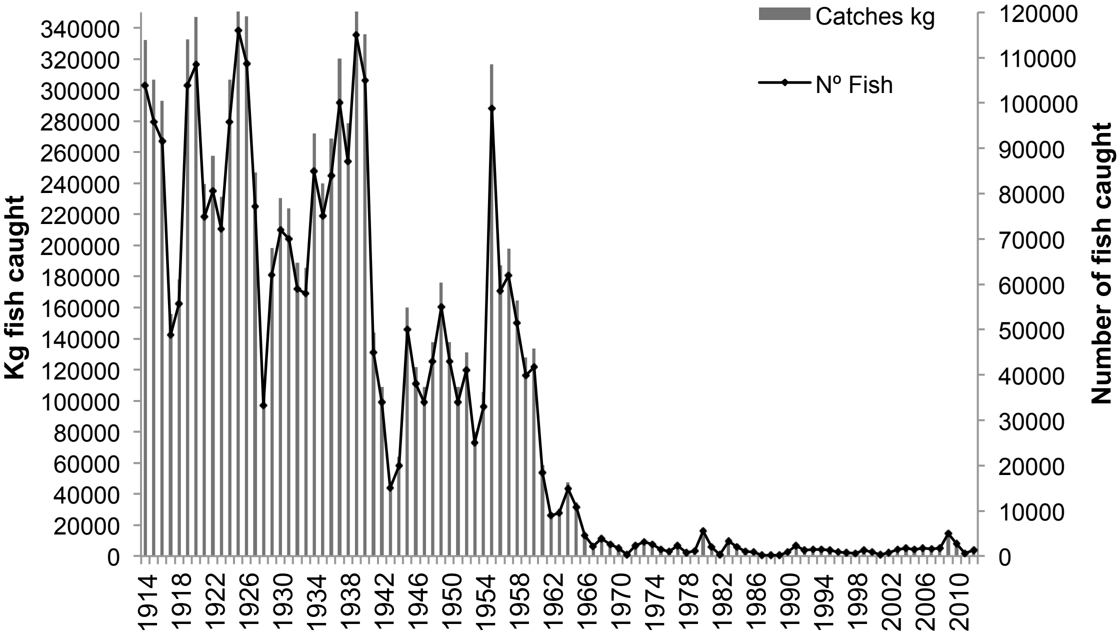


Figure 1.7. Time series of Allis shad catches (weight and number) in the Minho River, reported by Portuguese fishermen to the local Maritime Authorities (source: Maritime Authority of the Caminha Fishing Harbour).

Next to the loss of habitat, one of the major threats to the Minho River Allis shad population are the overfishing and poaching. In this sense, it could be important to improve our knowledge of the species marine phase, since there is evidence of by-catch by marine fisheries. On the other hand, the habitat loss imposed by the first Minho River dam limits the river’s carrying capacity and can lead to hybridization with *A. fallax*. Alexandrino (1996) reported high levels of introgression in a close population, the Lima River population. Recent morphometric works on the Minho Allis shad population demonstrate an average of 5% of hybrids among spawners and 19% of hybrids among young of the year (YOY) (Mota and Antunes unpublished data).

Preliminary and pioneer biological and ecological studies of Allis shad in the Minho River (Mota and Antunes, 2011; 2012) already provide some guidelines for future management and conservation of this species, which should primarily be based on cross-border cooperation protocols and joint measures. As habitat loss is one of the major causes for population decline, the knowledge of YOY Allis shad growth areas could be considered a key factor determining its abundance (Mota and Antunes, 2012). The location of spawning grounds and juvenile growth habitats allowed identification of "sanctuary areas". The restriction of sport fishing in these areas and of commercial fishing in other critical areas, as the river mouth and locations with reduced distances between banks, may help in the success of recruitment. On the other hand, as the spawning grounds of this population are below the first dam (Mota and Antunes, 2012), the regulation of the dam flow is essential for the survival and recruitment this River's Allis shad population. Unfortunately, ecological values of water discharge are managed by the hydro-power company and the dam's fish ladder does not work in terms of upstream fish passage.

Furthermore, a better understanding of the introgression level, of the specific upstream and estuarine habitat requirements for the growth of YOY Allis shad and reliable marine and riverine catch statistics would allow managers to plan conservation and protection strategies.

1.3.5. *Anguilla anguilla*

The European eel (*A. anguilla*) is present in all watersheds, but strongly limited by dams. On the Iberian Peninsula, the eel has disappeared from most of the Ebro, Tagus, Douro and Guadiana catchment areas (Doadrio, 2002). Large-scale surveys by the early 80s showed that the eel became extinct in more than 80% of the Iberian rivers (Lobón-Cervia, 1999) and it is presently absent from central Spain (Nicola *et al.*, 1996) and Portugal.

The abundance of riverine stocks of European eel depends entirely on the annual recruitment of the youngest juveniles (i.e. glass eels) reaching continental waters. In general, the eel density decreases with distance to the river mouth (Lobón-Cervia et al., 1995) and varies inter annually and between different rivers. Bernat et al. (1987; 1988) found 1 535 – 1 891 ind./ha and 3 031 – 4 063 ind./ha in the Esva basin. In the River Esva eel, density was estimated 1 300 – 2 120 ind./ha (Lobón-Cerviá et al., 1995). In Minho River tributaries the average density of yellow eels was 1 083 ind/ha (Antunes et al., 2012).

Although European eel still seems to be common in many areas (mainly due to transfer of individuals) (Antunes et al., 2012), the species is subjected to several threats. Main threats include: fisheries, stream migration blockages, loss of habitat, pollution, parasites and diseases, predatory birds as well as climatic changes in their environment especially during their larval marine migration (OSPAR, 2008). During the 1980s commercial fishing declined in the whole of Europe (Figure 1.8).

In the Atlantic, the most important glass eel fishery basins are the Minho, the Asturian basins, the Basque river basins and the Guadalquivir. Whereas, in the Mediterranean, the most important glass eel fishing points are the Delta of the Ebro and the Valencian Albufera (Díaz and Castellanos, 2007; Domingos and Antunes, 2011). Indeed, contrary to what happens in Portugal where the glass eel fishery was forbidden in 2000, in all river basins except for the Minho River where it is still allowed, in Spain the glass eel fishery exists in all Region Basin Demarcation (RBDs). However, there are different status among fishermen. For instance, Basque fishermen cannot sell their catches and should therefore be classified as non-professional. In Cantabria both professional and recreational glass eel fishery exists, mainly located in the Nansa (Pasand). The glass eel fishery is a very traditional fishery in Asturias and Galicia/North Portugal region (Minho River) (Domingos and Antunes, 2011; Andonegi et al., 2011). Despite the efforts of the Portuguese authorities, resulting in the confiscation of a large number of nets, poaching remains a problem all over the country, especially in the northern and central parts of Portugal (Domingos and Antunes, 2011).

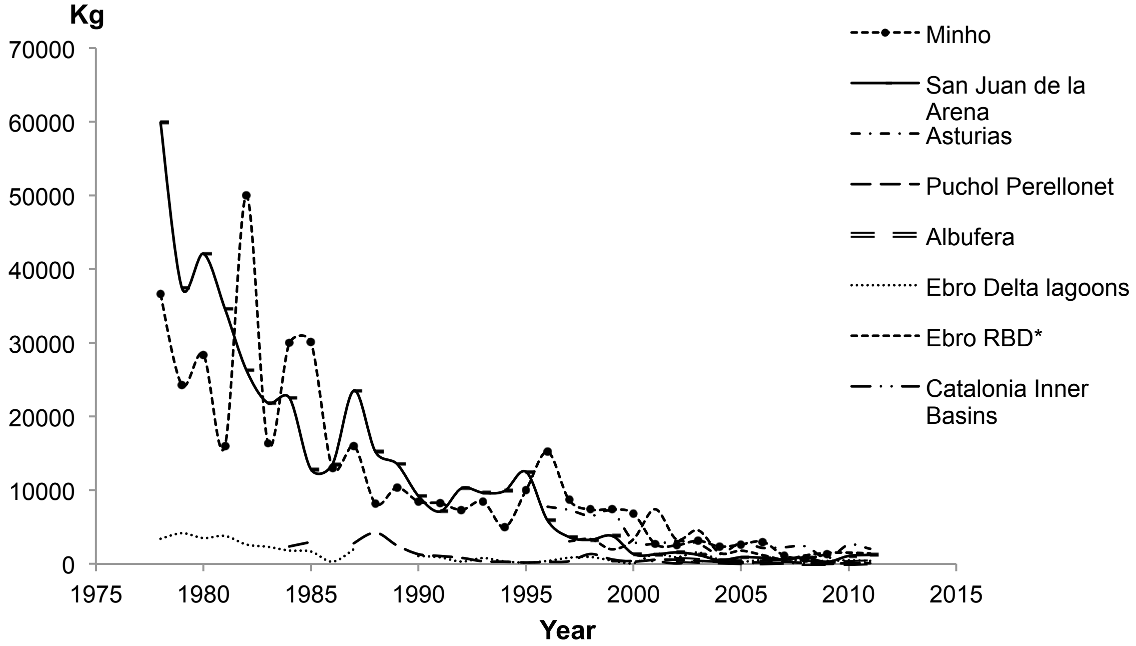


Figure 1.8. Time series of glass eel fishery (in kg) for the Iberian Peninsula (modified from Andonegi et al., 2011) (*RBD-Region Basin Demarcation).

In addition to that, there is an important yellow and silver eel fishery in Galicia, C. Valenciana, Catalonia and Portugal. There is no professional yellow or silver eel fishery in the Basque Country, Asturias, the international section of the Minho River and Cantabria. In these regions, recreational fishery was furthermore forbidden in 2009, 2007 and 2011, respectively, and it is negligible in Cantabria. In Portugal, the yellow eel fishery is ruled by eleven specific byelaws applied to eleven fishing areas in coastal waters (estuaries and coastal lagoons) and nine other byelaws, which are applied to specific fishing areas in inland waters. These define the river stretches where fishermen are allowed to fish and lay down fishing rules (gears and mesh sizes, the size limit of the species, hour restrictions and species restriction).

Considering chemical pollution, eels presenting a high contaminant burden, including heavy metals, and low energy stores may reveal migration failure and/or reproduction impairment (Belpaire and Goemans, 2007), and they may also be more susceptible to diseases (Langston et al., 2002). A few studies were done about heavy metals in eel from the Iberian Peninsula, considering the risk for human consumption (Establier, 1975; Rico et al., 1987; Sánchez et al., 1994, 1998; Linde et al., 1999; Usero et al., 2004; Urena et al., 2007). In general the values obtained in the edible portion of eels stayed below the maximum limits allowed according to EU regulations, and consequently, regarding possible effects of the studied metals, human consumption of these fish should be safe (Eira et al., 2009; Neto et al., 2011). Different IP basins reveal pesticides as Albufera, Valencia (Ferrando et al., 1992), organochlorine residues in Doñana (Rico et al., 1987), in Cantabria (Guitart et al., 2005), Ebro delta (Ruiz and Llorente, 1991) and River Turia (Bordajandi et al., 2003), mercury in Cadiz (Establier, 1975), PCB's in Ria de Aveiro (Antunes and Gil, 2002) and River Turia (Bordajandi et al., 2003).

Concerning parasite infection, the nematode *Anguillicoloides crassus* is a pathogenic helminth potentially representing an additional factor in the decline of eel populations (Münderle et al., 2004; Kennedy, 2007). In fact, *A. anguilla* is unable to develop an effective immune response against *A. crassus* (Taraschewski, 2006). The nematode is widespread on the Iberian Peninsula. Data were reported on parasites and pathogens in Spanish Mediterranean basins, Asturias, in five Portuguese brackish water systems (Aveiro Lagoon, Óbidos lagoon, Tejo estuary, Santo André Lagoon and Mira estuary) and the Minho River. The *A. crassus* prevalence values ranged from 1.7% to 100% (Andonegi et al., 2011; Domingos and Antunes, 2011). Pathogenic bacteria can play a leading role in the decline of such eel population because all bacterial diseases were found to be as prevalent as swim-bladder disease; however, bacterial pathogens are strongly virulent for European eels. Youngest eels are the most susceptible to acute bacterial diseases and so they may suffer mortality in natural habitat (Esteve and Alcaide, 2009).

Apart from the fish species Lusitanian toadfish (*Halobatrachus didactylus*) that can predate on eels (Costa et al., 2008) and the European eel, which can display cannibalistic behavior (Domingos et al., 2006), the main predators of eels in Iberian Peninsula aquatic systems include the great cormorant, *Phalacrocorax carbo*, and the European otter, *Lutra lutra* (Trigo, 1994; Beja, 1996; Cerqueira, 2005; Dias et al., 2012). Dias (2007) estimated a consumption of 2.2 tonnes of eels by great cormorant in the Minho River for the period between October 2005 and March 2006.

The eel is considered as a "Vulnerable" species in Spanish and “Endangered” in Portuguese Red Data Book (Blanco and González, 1992, Cabral et al., 2006) (Table 1.1) and Annex II of CITES since 2007.

The assessment of glass eel fisheries by local administrations, reinforced by the activities of the ICES/EIFAC Working Group on Eel (Braum and Tesch, 1990), has been instrumental in detecting long-term trends in glass eel catches. Analysis of these data sets also revealed a steep decline in the capture of glass eels in rivers flowing on the two sides of the Atlantic Ocean (Hagstrom and Wickstrom, 1990; Moriarty, 1990; Castonguay et al., 1994; Moriarty, 1996; Moriarty and Tesch, 1996; Antunes and Weber, 1996).

In Spain, each autonomous government is in charge of the control, regulation and management of eel fishery and population. The only information that is compiled routinely corresponds to fisheries. In addition to that, each autonomous region has its own methodology to compile fishery data (Andonegi et al., 2011; Domingos and Antunes, 2011). Portugal has delivered two EMP's (Eel management Plan) to comply with the needs set by the Eel Regulation 1100/2007 (Domingos and Antunes, 2011). One of those plans was established at the national level for the entire country, and the other one was the transboundary EMP for the international part of the Minho River and both were already approved by the European Union.

1.4. CONCLUSION

During their life histories, diadromous species pass through and use a wide range of freshwater, marine and estuarine habitats and often cross numerous international borders. Identifying appropriate protective measures for poorly known species is practically unfeasible (McDowall, 1999). Nevertheless, general measures, such as providing healthy habitats and restoring connectivity, are valuable whatever the migratory species.

From an ichthyological point of view the Iberian Peninsula is extremely attractive. Apart from the great variety of endemic freshwater fish species (e.g. Elvira, 1995), the IP comprises several diadromous fish species in numerous rivers, which are economically valuable species, especially those here described. As seen, in the last decades, there has been a global declining trend in abundance and distribution of diadromous fish in the IP, following the tendency also observed in the rest of Europe and worldwide.

It is clear that the decline of numerous diadromous species cannot be attributed to a single factor acting individually, but that it is due to several factors acting simultaneously. Barriers to migration, habitat destruction (e.g. gravel extraction), overfishing and pollution have caused a loss of suitable space and of connectivity between vital habitats.

Furthermore, climate change, pointed out as a disturbance to the thermal regime of aquatic habitats, can alter the distribution of fish species and their assemblages as well as species richness in each basin (Taverny and Elie, 2009). Representing the southern limit of the distribution of several species, the IP will undoubtedly experience the most marked effects of climate change in terms of river assemblages.

Although information on the conservation status, migratory behaviour, biology, genetics and ecology of diadromous fish still remains deficient (e.g. the Minho River shad and sea lamprey populations have been studied only recently by Araújo et al. (2013a; 2013b; 2013c) and Mota and Antunes (2011; 2012) and further research is needed, it is obvious that the general trends for these Iberian populations are declining and that most of the species may disappear from most of their natural Iberian distribution range.

Additionally, most of the larger Iberian rivers, such as the Minho, Lima, Douro, Tagus and Guadiana, cross international borders, requiring the management and conservation status of migratory fish species to be improved through international cooperative efforts. Cooperation between the range countries in promoting research, awareness and cross-border management of migratory fish, is of vital importance, as these activities may further enhance conservation results for this group of species.

As pointed out by Cooke et al. (2012), when looking for migratory fish that cross international borders or that are part of shared stocks in international river basins, one of the most important management requirements is to develop concerted actions to regulate fisheries and maintain river health. If these actions are not effective, transboundary migratory fish populations may be particularly vulnerable to several threats such as overfishing (often occurring in nursery and breeding areas, and on migration routes), loss of habitat and connectivity between vital habitats, and alteration of river base conditions, such as water quantity, flow, and temperature (Cooke et al., 2012). One of the best examples of concerted actions aimed at ecological restoration was developed by the states bordering the Rhine river and included restocking of extinct fish species such as Atlantic salmon and Allis shad (Van Dijk, 1995; Beeck et al., 2008).

In the Iberian context, the Standing Committee of the International Stretch of the Minho River is an example of international fishery management cooperation. It regulates the small-scale fishery and sport fishing activities, although the regulations refer only to the international segment of the Minho River. Considering the effectiveness of conservation actions, governance structures are likely to be more successful if they recognize the connectivity of systems regarding shared hydrological basins.

1.5. ACKNOWLEDGMENTS

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CHAPTER 2

GENERAL BIOLOGY AND ECOLOGY OF THE MINHO RIVER ALLIS SHAD *Alosa alosa* (L.)

2.1. FIRST REPORT ON THE STATUS OF ALLIS SHAD *Alosa alosa* (L.) IN THE MINHO RIVER (NORTHWESTERN IBERIAN PENINSULA)

M. Mota and C. Antunes 2011. First report on the status of Allis shad (*Alosa alosa*) in the Minho River (Northwestern Iberian Peninsula). J. Appl. Ichthyol. 27 (3): 56–59.

2.1.1. INTRODUCTION

The Allis shad, *Alosa alosa* (Linnaeus, 1758), is an anadromous member of the family Clupeidae. Like other diadromous fish (see Limburg and Waldman, 2009), this species has been progressively disappearing from European rivers due to river fragmentation, increasing sediment load and pollution. In Portugal a marked decline in the shad populations was noticed since the middle of the 20th century, particularly in the rivers Minho and Lima, while in the rivers Tagus and Guadiana only a residual population still exists (Faria, 2007). The anthropogenic pressures that seem to cause the gradual disappearance of *A. alosa* in several European basins, has resulted in its listing in (i) the Red List of the International Union for the Conservation of the Nature (IUCN), (ii) in Annex III of the Bern Convention, and in the Habitats Directive. The drastic decline in the spawning stock biomass and the restrictions of usable habitat area prompted its classification at national level as a species in danger (Cabral et al., 2006), now being under legal protection. As other Portuguese rivers (Lima, Cávado, Douro, Vouga, Mondego, Tejo, Sado and Guadiana), the Minho was one of the rivers with the highest catches of the species (Baldaque da Silva, 1892) and, in the Minho River basin, *A. alosa* still has a great commercial and cultural value. Portuguese official statistics of the small-scale fishery confirm mean catches of 200 tonnes during the first half of the 20th century, with peaks of 300 tonnes. After the 1950s catches decreased by about 90% (Figure 2.1), coinciding with the construction of the first dam (Alexandrino, 1996; Baglinière et al., 2003). In the last 20 years mean annual catches reached about four tonnes, while in 1980 the catch peaked with about 18 tonnes. However, these values seem to be underestimates due to the lack of good official statistics and unofficial Portuguese and Spanish data over the last eight years indicates that yields may have been twice as high, pointing to the existence of a noticeable population worth studying to develop conservation and restoration strategies. Furthermore, this species has become rare in Southern Europe (Limburg and Waldman, 2009). The objectives of this study, therefore, were to identify key parameters of the general biology of the species in the Minho River and assess the actual state of the *A. alosa* population in this specific river.

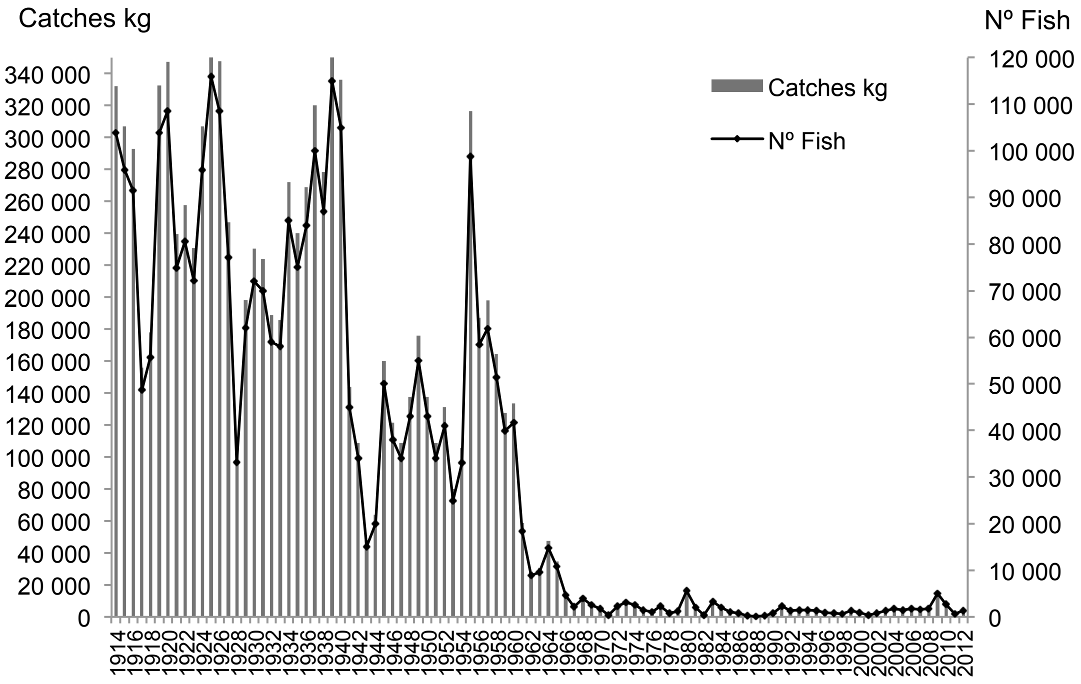

Figure 2.1. Time series data for half a century of *A. alosa* catches (kg and numbers) in the Minho River reported by Portuguese fishermen to the local Maritime Authority of Fishing Port of Caminha.

2.1.2. MATERIALS AND METHODS

Specimens were sampled by experimental fishing and from fishermen of the international part (Spain and Portugal) of the Minho River between April and August 2009. Two sampling stations (estuary and spawning area) were selected (Fig. 2.2). A typical trammel net (140 m long; 140 mm loose inner layer mesh size) was used.

Total length (TL) was measured to the nearest 0.1 cm (measuring board) and total weight (TW) was determined to the nearest 5 g (digital hanging Kern HDB balance). The TL and TW statistics were expressed as means and standard deviations, separately according to sex, as well as the respective maximum and minimum values.

Gonads were removed from both sexes and weighed using a digital A&D EK-610i balance with a precision of 0.01 g. The gonadosomatic index (GSI) was calculated for each sex in order to document changes in fish maturity and spawning state during the upstream migration, using the equation GSI = 100*(Wg ⁄ Wt) where Wg is the gonad weight (g) and Wt is the fish total weight (g).

Condition factors with gonad weight (K) and without gonad weight (K') and gonadal factor condition (ΔK) were estimated as $K = Wt/Lt^b$; $K' = Wc/Lt^b$; $\Delta K = K - K'$ where *Wt* is the fish total weight (g), *Wg* is the gonad weight (g), $Wc = Wt - Wg$; *Lt* is the total length (mm) and *b* is the alometry coefficient obtained through the length-weight relationship.

Scales of 111 specimens were removed and treated according to Baglinière et al. (2001) for ageing and identification of spawning marks.

Figure 2.2. Map of the study area showing the location of the two experimental fishing sites (stars).

2.1.3. RESULTS AND DISCUSSION

The available habitat for the *A. alosa* in the Minho River during freshwater migration covers a stretch of approximately 80 km in the international Minho River section (from the estuary to the first dam). Their estuary upstream migration began in March and ended in June. The earlier spring migration is related to the latitude: south populations migrate earlier in the year than further north populations (Mennesson-Boisneau et al., 2000a; Aprahamian et al., 2002; Baglinière et al., 2003).

The highest percentage of males occurred in March and April (63.6 and 69.6% respectively), while females dominated May and June (61.2 and 80%, respectively). This migratory behaviour is in accordance with the life history described for this species (Baglinière et al., 2003; Maitland and Hatton-Ellis, 2003), where males migrate upstream first, followed by females. The presence of post-spawner shads in the upper international Minho River, in early August, may suggest the spawning season to extent until this month, as reported for other *A. alosa* populations (Baglinière et al., 2003).

From the total samples, 49.5% were females and 50.5% were males, indicating no significant difference ($p > 0.05$) from a 1:1 sex ratio as also described for the Douro River *A. alosa* population (Eiras, 1981a). However, other *A. alosa* populations showed different sex ratios (Table 2.1) and it seems difficult to explain whether annual fluctuations of this parameter reflects a biological principle or represent a bias in the sampling methods, periods and ⁄ or locations (Mennesson-Boisneau et al., 2000b).

The results on biometrical and demographical structure of the 200 specimens sampled are given in Table 2.1 and Fig. 2.3. Comparing with northern populations, the Minho River *A. alosa* population presents the highest growth rate. This pattern was also demonstrated by Lassalle et al. (2008b), where comparisons of Portuguese and Moroccan populations with northern population showed larger lengths to the first ones (negative latitudinal gradient). In contrast, allometric coefficient b was lowest than in the Portuguese and Moroccan populations considered by Mennesson-Boisneau et al. (2000b). However, the Douro River population (Froese and Pauly, 2005) showed an allometric coefficient close to the one given for the Minho River population. Although the Minho and Douro populations are larger in length, they present lower weights compared to length-weight relationships to the rest of the populations elsewhere and thus may explain the difference.

Table 2.1. Total length and weight results for the Minho River *A. alosa* sampled between March and July 2009 and comparison with European and Moroccan populations (adapted to Mennesson-Boisneau et al., 2000b).

	Minho River Present Study		Lima River Alexandrino (1996)		Loire River Mennesson-Boisneau (1990)		Gironde River Taverny (1991)		Adour River Prouzet (unpublished data)		Sebou River Sabatié (1993)	
	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males
b	2,81 (mixed)		3,1139 (mixed)		3,019	3,114	3,3429	3,2252	2,671	3,116	3,4105	3,2587
N	99	101	86	97	1041	1172	191	152	101	118	317	482
%	49,5	50,50	47	53	47	53	56	44	46	54	40	60
Length (mm)												
$\bar{X}$	664	607	634	537	556	484	585	533	539	492	636	549
SD	34	25	44	61	39	39	26	29	25	32	3,8	4,4
Min	540	530	480	400	414	339	509	383	475	415	490	445
Max	740	670	733	666	680	635	650	597	600	605	720	670
Weight (g)												
$\bar{X}$	2678	2208	2864	1663	1712	1067	2185	1572	2066	1411	3019	1800
SD	819	408	611	575	489	342	391	299	329	324	58	47
Min	915	890	1237	668	500	174	1245	610	1400	800	1250	850
Max	4355	3450	4400	3215	3800	2487	3250	2775	2900	2800	4525	3275

N, nª of individuals; b, allometric coefficient; %, percentage; $\bar{X}$, average; SD, standard deviation; Min, minimum and Max, maximum.

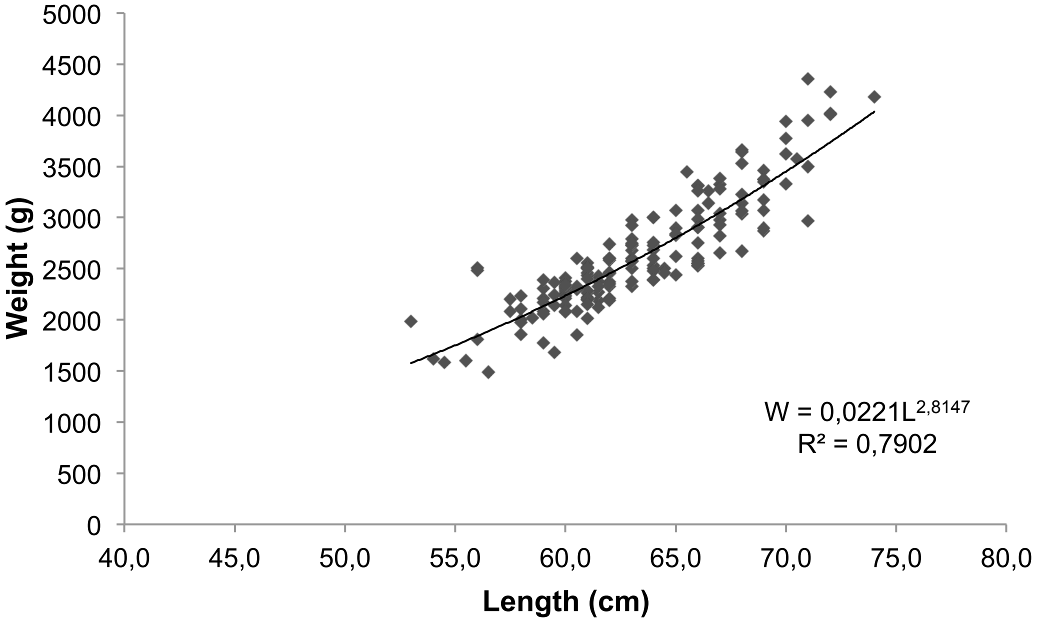

Figure 2.3. Length-weight relationship for the *A. alosa* population migrating for spawning into the Minho River. The allometric equation is given as: W = total weight (g), L = total length (cm); a = 0.0221 and b = 2.8147. Data are combined for both sexes. The low b value may have been caused by spent fish.

GSI revealed highest values for females and, with the exception in April when we observed a slight decrease in the GSI values. GSI increased until June and strongly decreased in July for both sexes, indicating an increase of gonadal weight during the upstream migration and the presence of postspawners in July (Fig. 2.4).

Condition factor (K) decreased along the five migratory months for both sexes. In contrast, gonadal condition factor (ΔK) was the same from March to May and slightly decreasing in June and July for males and, for the females, slightly decreased from March to April, rose from May to June, and fell again in July (Fig. 2.5). The high female (ΔK)observed in June indicates fish are ready to spawn.

The age structure of migratory adults is given in Table 2.2. The scales reading revealed an average age of 6.7 years for the total fish sampled. For males, 57.4% of the specimens were 6 years old and 58.1% of females were 7 years old. As seen in other studies (Mennesson-Boisneau et al., 2000b; Aprahamian et al., 2002; Lassalle et al., 2008b) the majority of females matured one year later than males, however, the majority of the Minho River adults spawner population seems to be one year older than those of other European and Mediterranean populations. Further, 5.4% of the spawners had spawning marks on their scales. These results show the evidence of a few fish returning for a second spawning migration and thus, as other European populations (see Mennesson-Boisneau et al., 2000b; Aprahamiam et al., 2002; Maitland and Hatton-Ellis, 2003), this population is considered semelparous.

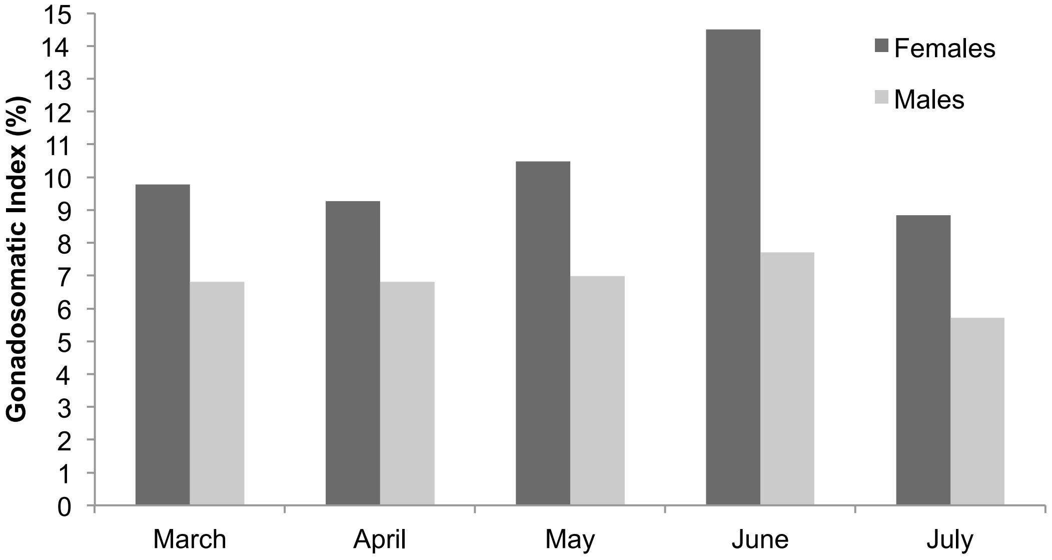

Figure 2.4. Changes of the Gonadossomatic Index (%) (GSI) in *A. alosa* specimens during the spawning upstream migration in the Minho River, shown separately for males and females. Total n = 200 specimens.

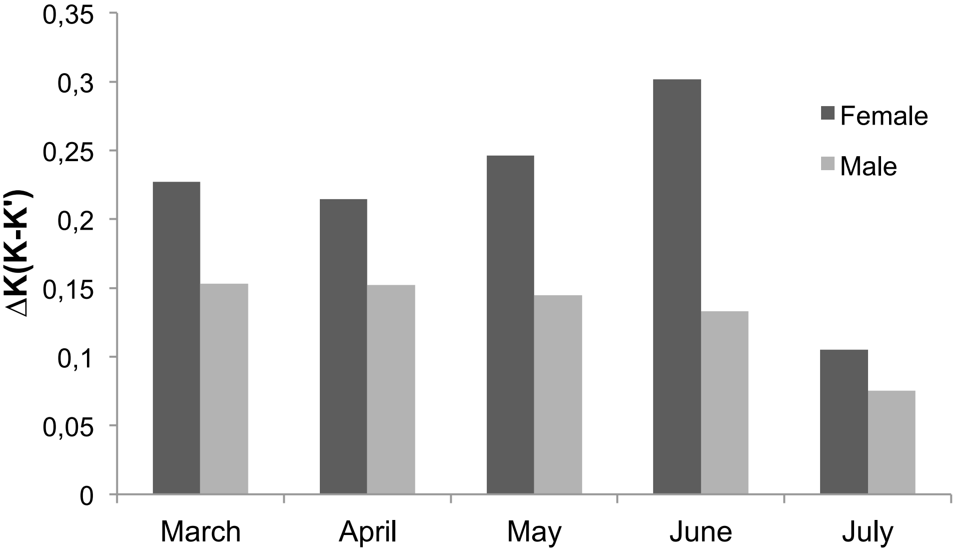

Figure 2.5. Variation in the Gonadal factor condition (DK) of males and females of *Alosa alosa* during the months of the spawning migration in the Minho River.

Table 2.2. Age structure of Minho River *A. alosa* population by sex and length.

	Males				Females			
			Length (mm)				Length (mm)	
Age	N	%	$\bar{X}$	SD	N	%	$\bar{X}$	SD
5	7	6,93	604,3	35,64	2	2,15	620,0	84,85
6	58	57,4	604,7	23,53	9	9,68	655,6	17,40
7	36	35,66	614,6	21,66	54	58,06	663,1	35,89
8	---	---	---	---	28	30,11	667,3	33,95

In the last 30 years two peaks were revealed concerning the data of Portuguese fishermen (1980 and 2009 spawning seasons). Although fluctuations in the abundance of Alosa populations are a reality (Baglinière et al., 2003), future investigations will be carried out in the way of enhance knowledge of the status and dynamic of the Minho River *A. alosa* population.

2.1. 4. ACKNOWLEDGEMENTS

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2.2. A preliminary characterisation of the habitat use and feeding of Allis shad *Alosa alosa* (L.) juveniles in the Minho River tidal freshwater wetlands

M. Mota and C. Antunes 2012. A preliminary characterisation of the habitat use and feeding of Allis shad (*Alosa alosa*) juveniles in the Minho River tidal freshwater wetlands. Limnetica, 31 (1): 165-172

ABSTRACT

The availability of high-quality rearing areas and survival during the early life-history stages are considered key factors determining the number of recruits produced and are prerequisites for the successful recruitment of several fish species. To identify the freshwater habitat use of Allis shad, several samples were collected in the tidal and non-tidal freshwater areas of the Minho River during the autumn of 2009. The two places where juveniles were found are substantially different in terms of sediment texture and organic matter but similar in depth and water current velocities. The food items found in the stomach contents may indicate that movements of juveniles occur in the estuary.

Key words: Allis shad, Juveniles, Habitat, Freshwater stage, Conservation.

2.2.1. INTRODUCTION

Diadromous fishes have suffered dramatic population declines over the past century in Europe as well as in North America (Ross and Bennett, 1997; Limburg and Waldman, 2009). The Allis shad *Alosa alosa* (Linnaeus, 1758) is an anadromous fish of the family Clupeidae. This species has been progressively disappearing from European rivers. The main causes of such declines are dam construction, sediment loading, pollution and habitat alteration or loss. The larvae and juveniles rear in freshwater, and juveniles migrate to the ocean before age 1 and mature in the ocean. Young-of-the-year (YOY) Allis shad migrate downstream in schools during the summer and fall of the first year of life. The migration lasts from 3 to 6 months (Taverny et al., 2000; Baglinière et al., 2003). In Europe, early life stage studies have been performed on the estuarine phase and discrimination of the natal origin of YOY Allis shad (Tomás et al., 2005; Lochet et al., 2009) and on the feeding ecology of juvenile Twaite shad *Alosa fallax* (Lacépède, 1803) habitat (Aprahamian, 1989; Assis et al., 1992; Oesmann and Thiel, 2001). However, habitat use and requirement studies have not yet been specifically addressed for pre-migratory YOY Allis shad in riverine habitat. Indeed, at the European level, little is known about the distribution and characterisation of the freshwater and estuarine habitats that are important to the rearing of Allis shad in its early life stage.

Because habitat loss is one of the main causes for population decline, the survival and availability of rearing areas in the early life stages could be considered a key factor determining the abundance of YOY Allis shad.

Therefore, the aims of this study were to perform a preliminary study of freshwater habitat use by YOY Allis shad to investigate the following topics: (1) to identify the Minho River's freshwater and tidal freshwater wetland (TFW) habitats occupied by juvenile Allis shad; (2) to characterise the habitats of importance for the growth of the species in its early life in freshwater, including such physical aspects as sediment texture (granulometry) and water current velocity and depth; and (3) to examine the diet of the juvenile stages in those habitats.

2.2.2. MATERIALS AND METHODS

2.2.2.1. Study area

The Minho River is an international water body on the northwestern Iberian Peninsula. Its watershed covers 17 080 km^2, and its main course is approximately 300 km long. The last 77 km of the river forms the natural northwestern boundary between Portugal and Spain and is currently the only free segment for the upstream migration and spawning of Allis shad and other diadromous fish (between the river mouth and the first dam, located in Spain). The Minho estuary is 40 km long and has a maximum width of slightly greater than 2 km near the river mouth. The salt intrusion in the estuary extends from approximately 11.3 km and up to 16.8 km inland during higher spring tides (September). Tidal Freshwater Wetlands (TFWs) are found in the 29 km upstream stretch. The study area was located between the Minho River TFWs and the spawning area (personal observation) approximately 80 km from the river mouth, near the first dam (Fig. 2.6).

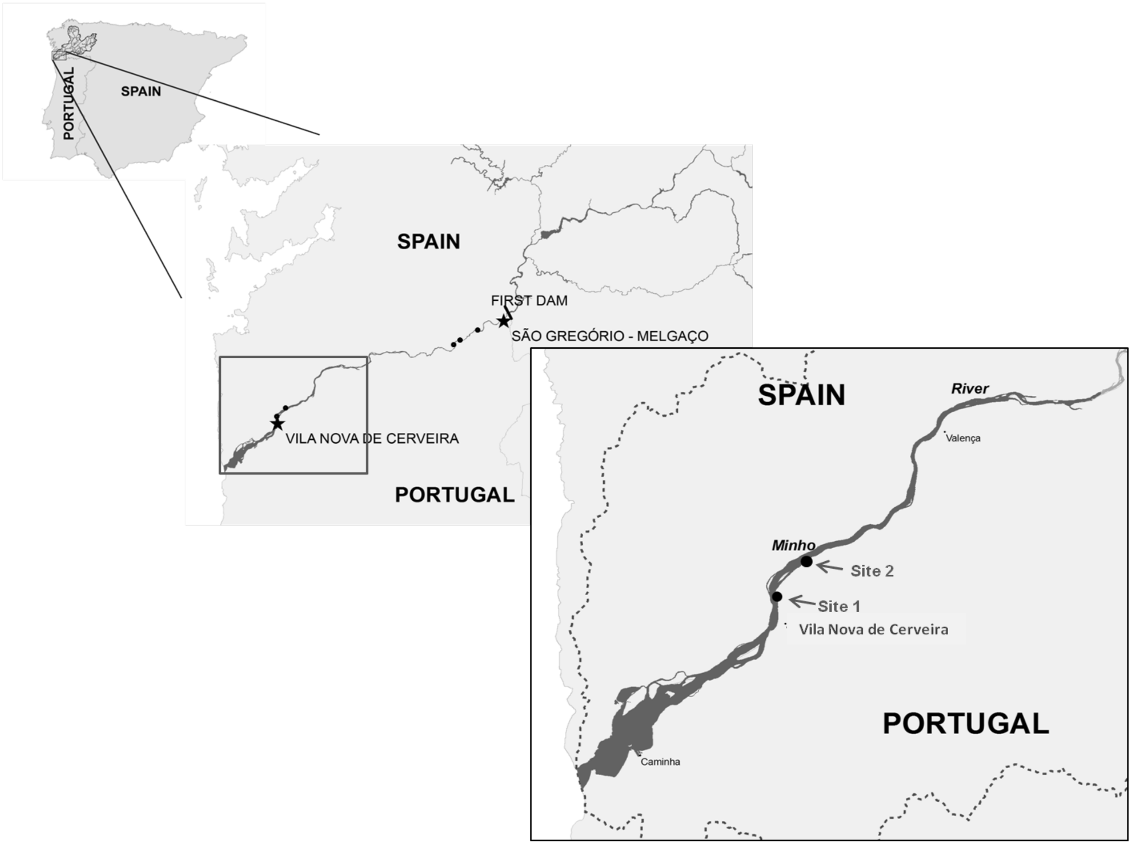

Figure 2.6. Map of the study area showing the location of the non-tidal and TFW sampling sites (dots). Site 1 and Site 2 are the two TFW sites at which YOY Allis shad were found (arrows).

2.2.2.2. Sampling strategy

The potential rearing habitats of juvenile *A. alosa* were investigated along the Minho River TFWs and the spawning area during autumn 2009. At three sites near the spawning grounds and two sites in the TFWs, a beach seine net (10 mm mesh, bag 4.8 m in length, 3 m mouth width, wings 6 m in length and 2.4 m in height) was used to sample the YOY Allis shad. The sampling effort was distributed over the daylight hours and the night. During each sampling period, the water temperature was recorded with an YSI 6820 multiparameter meter (USA).

2.2.2.3 Habitat characterisation

In the field, YOY Allis shad were collected and quickly stored under refrigeration before transport to the laboratory. The fish were labelled, measured (±0.1 mm; fork length (FL) and total length (TL)) and weighed (±0.01 g wet mass). The length-class frequencies were calculated for the total sample of fish. A two-way ANOVA was performed to evaluate differences in total lengths between months and sampling sites. The catch per unit effort (CPUE) was calculated for each sampling site. The CPUE was obtained by the standardisation, to each month and site, of the number of individuals caught per unit area (ha).

The digestive tracts of all YOY caught were excised and preserved in 70 % alcohol for stomach content analysis, and food items were counted and identified up to the lowest taxonomic level possible. The relative importance of each prey item in the diet was evaluated by two indices (data from the two sampling sites separately): the numerical index (NI) – the percentage of the number of individuals of a prey over the total number of individuals of all prey, and the occurrence index (OI) – the percentage of non-empty stomachs in which a prey occurred over the total number of occurrences (Hyslop, 1980). The remaining biological material was frozen for subsequent isotopic analysis of muscle and for otolith extraction.

The two sampling sites where the presence of YOY Allis shad was verified (site 1 and site 2, both located in the TFWs) were characterised in relation to the following physical variables: the texture of the sediment, current velocity and depth. Transects perpendicular to the edge, 7 meters apart, were performed in an area of approximately 1 100 m2 at sampling site 2 and 600 m^2 at sampling site 1. At 3-meter intervals within each transect, sediment samples for granulometry and organic matter content analysis were collected and the depth was recorded. The granulometry was analysed after oven drying the sediment for 72 hours. The dried sediment was placed on a column of sieves with different mesh sizes, in descending order from top to bottom, according to the Wentworth scale (Mudroch and Azcue, 1995) and sieved. The frequency of each sieved fraction was expressed as a percentage of total weight, and the cumulative percentage was calculated. The organic matter content of the sediment was determined after combustion in a muffle furnace for 4 hours at 550 ºC. The organic matter was expressed as a percentage of the weight loss of each sample after incineration.

For the first and last transect of each sampling site, the current velocity was recorded with a flow meter (Valeport BFM001) at the bottom, middle and surface of the water column.

2.2.3. RESULTS

YOY Allis shad were only caught in November and December 2009, during the night, at the two sampling sites located in the Minho River TFWs (Fig. 2.6): site 1 (41º57'100'' N; at 14 km from the river mouth) and site 2 (41º58'501'' N; at 16.7 km from the river mouth). YOY Allis shad were not found in the sampling sites located near the spawning grounds or in any sites sampled during the day.

A total of 228 fish were caught. Of these fish, 215 were from site 1, and 13 were from site 2 (Fig. 2.6). Accordingly, the CPUE is higher for site 1 in both months (Fig. 2.7).

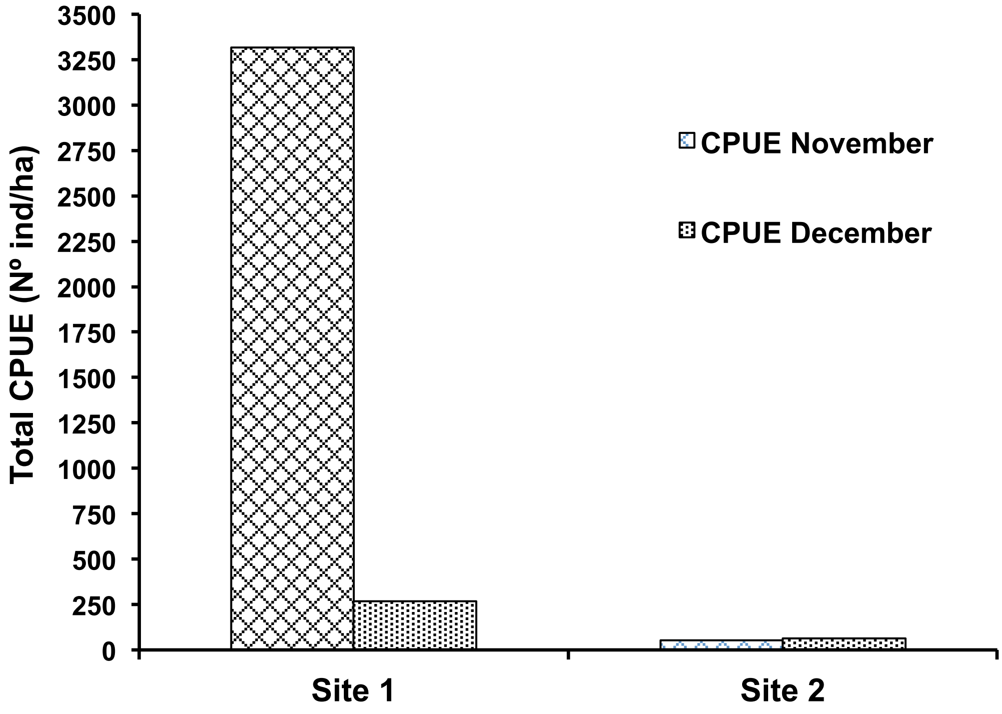

Figure 2.7. Comparison of the Total CPUE (number of individuals/ha) between months and sampling sites.

The average total length of the fish at each site and in each month is shown in Figure 2.8. The average total lengths of the YOY Allis shad differ significantly between months (*p*=0.009). The average total lengths are higher in November than in December. However, the differences among sites as well as among sites and months are not significant (*p*>0.05). The length-class frequencies calculated for the entire sample are shown in Figure 2.9. The most common length class is 9.1-10 cm, followed by 8.3-9.1 cm.

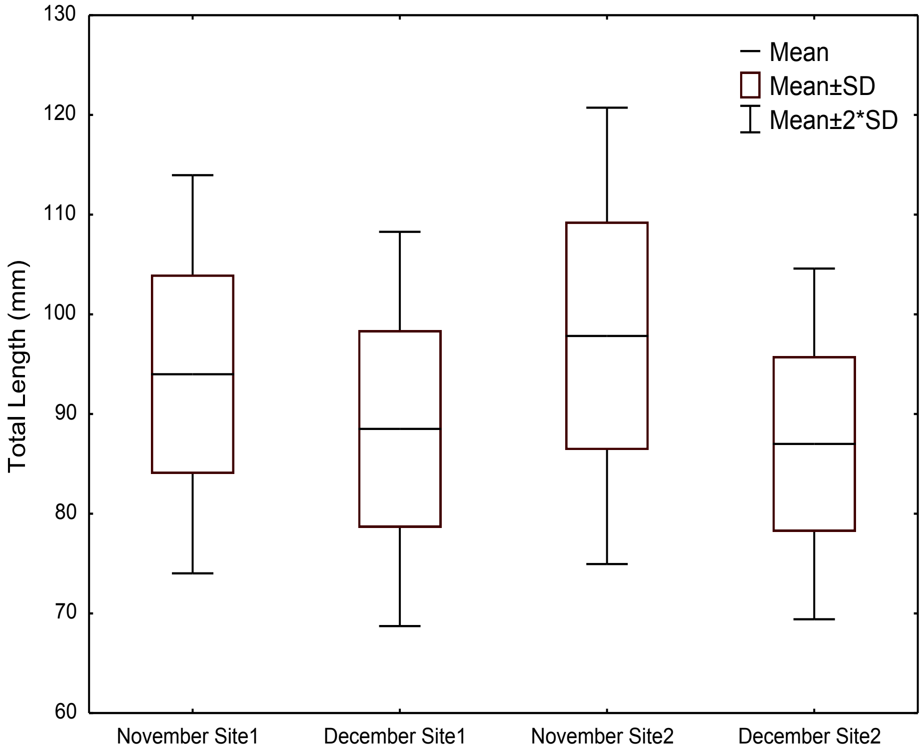

Figure 2.8. Boxplot of the average total length of YOY Allis shad by site and month.

Table 2.3 lists the prey items found in the stomach analyses at each site and the corresponding values of the diet indices. The numerical and occurrence indices show *Corophium* sp. as the most important food item at site 1, whereas it is the second most important at site 2 according to the numerical and occurrence indices. According to the numerical index, dipteran nymphs are also the second most important group at site 2. According to the occurrence index, dipteran nymphs, insect adults, *Corophium* sp. and Collembola are the second most important groups at site 2. At site 2, the Chironomidea group occurs in all of the stomachs. However, the Collembola group is the most numerous at site 2, followed by *Corophium* sp. and dipteran nymphs.

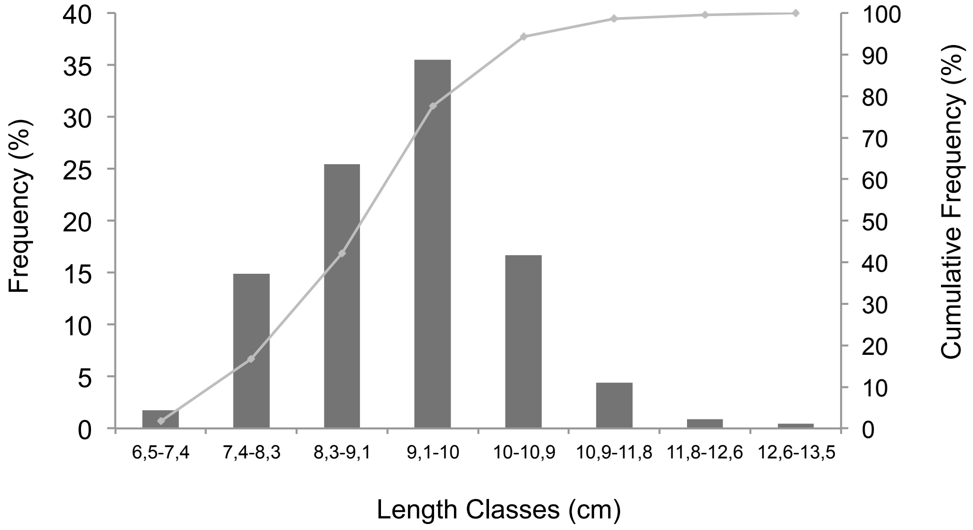

Figure 2.9. Length-class frequencies (%) of all the fish caught at both sampling sites. N = 228.

Table 2.3. Composition of food items in the stomachs of YOY Allis shad. Site 1 N = 215; Site 2 N = 13. NI-Numerical Indices; OI-Occurrence Indices.

	Site 1		Site 2	
	NI	OI	NI	OI
Crustacea				
Amphipoda (not identified)	0.1	2.1	0.0	0.0
Atyaephyra desmarestii	4.1	29.8	0.0	0.0
Corophium sp.	90.7	100.0	13.3	66.7
Gammaridae (not identified)	2.3	42.6	0.0	0.0
Gammarus sp.	2.2	23.4	11.1	33.3
Saduriela sp.	0.1	2.1	0.0	0.0
Not identified	0.0	0.0	4.4	33.3
Arachnida				
Not identified	0.0	0.0	2.2	33.3
Insecta				
Collembola (not identified)	0.0	0.0	26.7	66.7
Chironomidae	0.3	10.6	11.1	100.0
Coleoptera	0.0	0.0	2.2	33.3
Ephemeroptera	0.0	0.0	2.2	33.3
Plecoptera	0.1	4.3	2.2	33.3
Simuliidae	0.0	0.0	4.4	33.3
Trichoptera	0.1	2.1	0.0	0.0
Nymph not identified	0.1	4.3	13.3	66.7
Adult not identified	0.0	0.0	6.7	66.7

The physical characteristics of the habitat at site 1 and site 2 show both similarities and dissimilarities. The water at both sites is quite deep. The water depth at site 2 is slightly greater than the depth at site 1 (8 m maximum and 7 m maximum, respectively). The current velocity at the sampling sites is lower than the current velocity in the main channel (average 0.3 m/s at site 1 and 0.02 m/s at site 2, compared with 0.85 m/s in the channel). In addition, both sites include an upstream area with semi-emergent rocks. This feature gives the sites the appearance of a pool.

The sediment grain size and organic matter content differ substantially between the two sites. The two sites differ greatly in the dominant granule fraction (Fig. 2.10). The dominant fraction is 1 mm-0.5 mm at site 1 and 0.25 mm-0.125 mm at site 2. As expected, the percentage of organic matter in the sediment is higher at site 2 (3.85 %) than at site 1 (0.67 %).

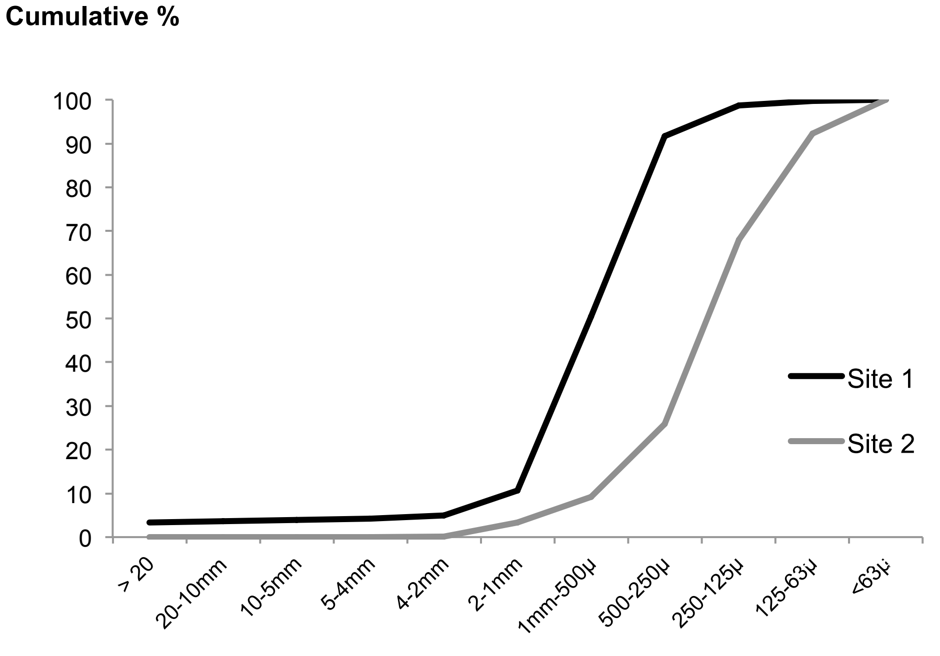

Figure 2.10. Cumulative percentages of sediment grain-size fractions at Site 1 and Site 2.

2.2.4. DISCUSSION

Since the middle of the 20th century, the habitat available for the upstream migration of the Minho River *A. alosa* has decreased substantially due to dam construction and currently includes only 80 km (from the estuary to the first dam) of the existing 300 km. As the loss of habitat is a reality, it is of extreme importance to identify and preserve the areas appropriate for the high-quality development of the early life stages of Allis shad.

The absence of juveniles in the samples collected at the 3 upstream sites, near the spawning area, might be explained by the sites selected, the hour of the day or the sampling methodology. Intensive research on rearing habitat in the upstream area should be performed. Sampling at night should be emphasised because catchability appears to be greater at night.

The CPUE was exceptionally high at site 1 in November relative to the other capture data. It is possible that gregarious behaviour may facilitate a greater catch in the preferred habitats. Isolated captures of juvenile Allis shad are described as a by-catch component in the glass eel fishery (Antunes and Weber, 1996).

The results of this study describing diet and food preferences agree with those reported by Taverny et al. (2000). The YOY Allis shad appear to be euryphagous, using a broad range of trophic resources accessible in the upstream and estuarine environments. Ross and Bennett (1997) demonstrated that American shad (*Alosa sapidissima*) prey selection is opportunistic, as this species feeds on a variety of aquatic and terrestrial prey taxa. Crustaceans appear to be an important item in the juveniles' diet. Oesman and Thiel (2001) described copepods, mysids and fish as the principal food items of juvenile Twaite shad in the Elbe estuary. Aprahamian (1989) reported copepods, mysids and gammarids as the principal food items of juvenile Twaite shad in the Severn Estuary. The results of the current study suggest that amphipods are the preferred item, but this tendency is not statistically significant. Fish were absent from the diet of the YOY Allis shad. The subtidal macrozoobenthic study performed by Sousa *et al.* (2008) in the Minho River did not detect brackish-water organisms (e.g., *Corophium* sp.) in the upper TFWs. The presence of food items typical of brackish areas in the stomach contents of the juveniles sampled at site 2 may indicate that the juveniles move within the estuary. However, this conclusion should be viewed with caution due to the low number of fish caught at this sampling site. It is necessary to recognise that the hypothetical movements of the juveniles can only be confirmed through isotopic analysis.

The two TFW locations at which the juveniles were found differ substantially in sediment texture and organic matter but are similar in depth and low current velocities compared with the main channel. Although juveniles were captured at few sites, the study showed that YOY Allis shad occur in deep pools close to the bank, where the faster river currents can be avoided. Aprahamian (1981) found similar behaviour in young Twaite shad (*A. fallax*). Because YOY Allis shad appear to be generalists and/or opportunists in their prey selection, the physical characteristics of the habitat, such as the water column depth and current velocity, may be a more important aspect of the juveniles' choice of rearing area than the availability and/or types of prey items present in these areas.

Future investigations will be conducted to integrate the variables describing the biotic processes (e.g. the availability and abundance of prey and competitors) and to estimate the residence times of the YOY Allis shad in freshwater, tidal freshwater and brackish habitats. These investigations will be performed by determining the changes in the stable isotope ratios that occur when the juveniles migrate seawards from the freshwater habitat to a new habitat. The ultimate objective is to determine the most important ecological characteristics of the freshwater habitat and to map these characteristics as well as the geological and topographic characteristics of the habitat.

A better understanding of the specific abiotic and biotic upstream and estuarine habitat requirements for the growth of YOY Allis shad should allow managers to plan conservation and protection strategies for such areas.

2.2.5. ACKNOWLEDGEMENTS

Micaela Mota is supported by a PhD grant from the Portuguese Foundation for Science and Technology – FCT (SFRH/BD/44892/2008). We especially thank Eduardo Martins, Eugénio Pereira, Rita Carvalho, Vânia Gonçalves and António Roleira for field assistance, Sandra Fernandes and Sofia Ribeiro for laboratory assistance, Claudio Ramos for producing the map of the study area, Jonathan Wilson for helpfully providing an English version of the manuscript and Pedro Seixas for helpfully providing a Spanish version. The authors thank associate editor García-Berthou and the reviewers for their constructive comments.

2.3. NEW INSIGHTS INTO BIOLOGY AND ECOLOGY OF THE MINHO RIVER ALLIS SHAD *Alosa alosa* (L.) – CONTRIBUTION TO THE CONSERVATION OF ONE OF THE LAST EUROPEAN SHAD POPULATIONS

Part of this section was accepted for publication in the Reviews in Fish Biology and Fisheries journal as: M. Mota, A. Bio, M. Bao, S. Pascual, E. Rochard and C. Antunes 2013. New insights into biology and ecology of the Minho River Allis shad *Alosa alosa* (L.) – contribution to the conservation of one of the last European shad populations.

ABSTRACT

In spite of shrinking populations, the Allis shad *Alosa alosa* (Linnaeus, 1758) is a species of commercial importance in Europe. On the Iberian Peninsula, especially in the international Minho River, it plays also an important cultural role. From the mid-20th century on, a marked decrease in the number of spawners occurred in that river, following the reduction of available habitat due to dam construction. We investigated the biology and ecology of the Minho River's Allis shad population, considering: spawners age structure and migration behaviour, reproductive biology, hybridization with Twaite shad *Alosa fallax* (Lacepède, 1803), juvenile growth, habitats and diet, as well as parasites. Results suggested that males migrate one year earlier than females and sooner in the season. Female's GSI increased with time and was higher within the spawning area. According to gill rakers numbers, the level of hybridization is important with a higher percentage for juveniles than for adults (17% and 3.6% respectively). To the best of our knowledge, we present the first report of *Anisakis pegreffii* and *Rhadinorhynchus pristis*, as well as other mouth and visceral cavity parasites. The achieved results can provide guidelines for future management and conservation of one of the last stable European shad populations.

Key words: Allis shad, biology, ecology, conservation, management.

2.3.1. INTRODUCTION

As broadly known, since the middle of the 20th century, Allis shad *Alosa alosa* (L.) populations have suffered a significant decline in abundance throughout their geographic range. Formerly found in the eastern Atlantic from Norway to Morocco and in the Western Mediterranean Sea, the range of Allis shad distribution is nowadays restricted to the Atlantic coast of France and Portugal (Baglinière, 2000), being the northern limit of the spawning areas the Vire Basin in Normandy and the southern limit the boundary between Portugal and Spain (unpublished data).

The main causes of Allis shad population decline are habitat alteration or loss, mostly due to dam construction, altered sediment loading, pollution and overfishing. In Portugal, the decline of this species has also been attributed to river flow depletion and overfishing of the major stocks (Costa et al., 2001). Additionally, hybridization between *A. alosa* and *A. fallax* (Alexandrino, 1996) and by-catch of the species by glass eel and other fisheries (Antunes and Weber 1996; Assis et al., 1992) are known constraints to Portuguese shad populations. In this sense, Allis shad was included in Annex III of the Bern Convention and Annex II of the European Union’s Habitats Directive of 1992. Whilst Allis shad is classified as ‘least concern’ on the red list of the International Union for the Conservation of Nature (Freyhof and Kottelat 2008), some European countries consider the species as “vulnerable” (e.g. IUCN-France, MNHM, SFI, and ONEMA 2010; Doadrio, 2002) or even as an “endangered” species (e.g. Cabral et al., 2006).

Allis shad commercial and recreational fisheries have been of economic and social importance for centuries. Regarding the Iberian Peninsula, the Minho River was once the river with the highest landings of *Alosa* species (Baldaque da Silva, 1892).

At the European level, until the end of the 20th century, the Gironde population was considered the largest (Baglinière, 2000) and a reference Allis shad population (Martin Vandembulcke, 1999). After the collapse of the Gironde Allis shad population from the beginning of the 2000s (Rougier et al., 2012), one of the largest populations in the southern part of the species’ distribution seems to be the Minho river population (unpublished data), where Allis shad still has important commercial and heritage values (Mota and Antunes 2011). However, after the 1950s, Minho River catches decreased by about 90%, compared to the catches of 200 tonnes reported in the first half of the 20th century (Mota and Antunes 2011). In the light of these results, knowledge on the biology and ecology of the Minho River Allis shad population is needed to evaluate its current status, to assess possible threats and to support management actions.

Several studies have clarified some aspects of the biology, ecology and conservation (King and Roche, 2008; Lassalle et al., 2008b), early life history (Lochet et al., 2008;

Lochet et al., 2009; Tomás et al., 2005), osmoregulation (Bardonnet and Jatteau, 2008; Leguen et al., 2007) and genetics (Faria et al., 2006; Faria 2007, 2012) of the European *Alosa* species. There is, however, still a lack of knowledge about the Allis shad of the Northern Iberian Peninsula. In Portugal, only few works were done concerning the biology and ecology of *A. alosa* in the Douro (Eiras, 1977; 1981a), landlocked populations (Collares-Pereira et al., 1999; Eiras, 1981b; 1983), genetics (Alexandrino, 1996) and phylogeny (Faria, 2007). More recently, preliminary studies took place on the Minho River Allis shad population, i.e. the most stable population of the southern limit of the distribution of this species, analysing the spawners' biology (Mota and Antunes, 2011) and juveniles' habitat use in freshwater (Mota and Antunes, 2012). The present work aims to continue and deepen that preliminary work and to contribute to the management and preservation of the population and to the conservation of the species in general. Our objective was to clarify the life traits and the biological characterisation of the Minho River population.

Firstly, we investigated characteristics of spawner's population, such as size (weight-length relationship, Condition Factor *K*), age structure, migratory behaviour (male and female migration patterns), reproductive biology (semelparity, spawning season and ovarian development), parasites and the overlap of *A. alosa* and *A. fallax* spawning areas and hybridization. We analysed the intra and inter-annual variation in weight, length, gonadosomatic (GSI) and hepatosomatic (HSI) index and age, per sex and per capture zone (considering the upper, tidal freshwater and lower estuary zones). We established a model to assess sex for individuals of unknown gender and evaluated the weight loss during the upstream migration.

Secondly, we examined juveniles, giving attention to species identification (*A. alosa*, *A. fallax* and hybrids), size distribution and relationships between fish size, weight-length relationship and diet. We discriminated groups by modelling the number of gill rakers depending on size, and tested the monthly and inter-annual size variation.

And thirdly, we provide some guidelines that could be included in future conservation management plans, based on the gathered knowledge about this population.

2.3.2. MATERIALS AND METHODS

2.3.2.1. Study area

The Minho River is an international water body located in the northwest of the Iberian Peninsula (Fig. 2.11A). Its main course is 300 km long, comprising a total basin area of 17 080 km^2. The last 77 km includes its international section (the natural boundary between Portugal and Spain) and is the free stretch of the river for the upstream migration and spawning of Allis shad (Mota and Antunes 2012).

The Minho estuary is 40 km long and has a total area of 23 km^2 with a maximum width of just over 2 km near the mouth. The influence of spring tides extends approximately 40 km upstream. Salt intrusion expands to around 11.3 km and up to 16.8 km inland during higher spring tides in September. The Tidal Freshwater Wetlands (TFWs) are located in the upper 30 km, reducing in 10 km during higher spring tides in the summer.

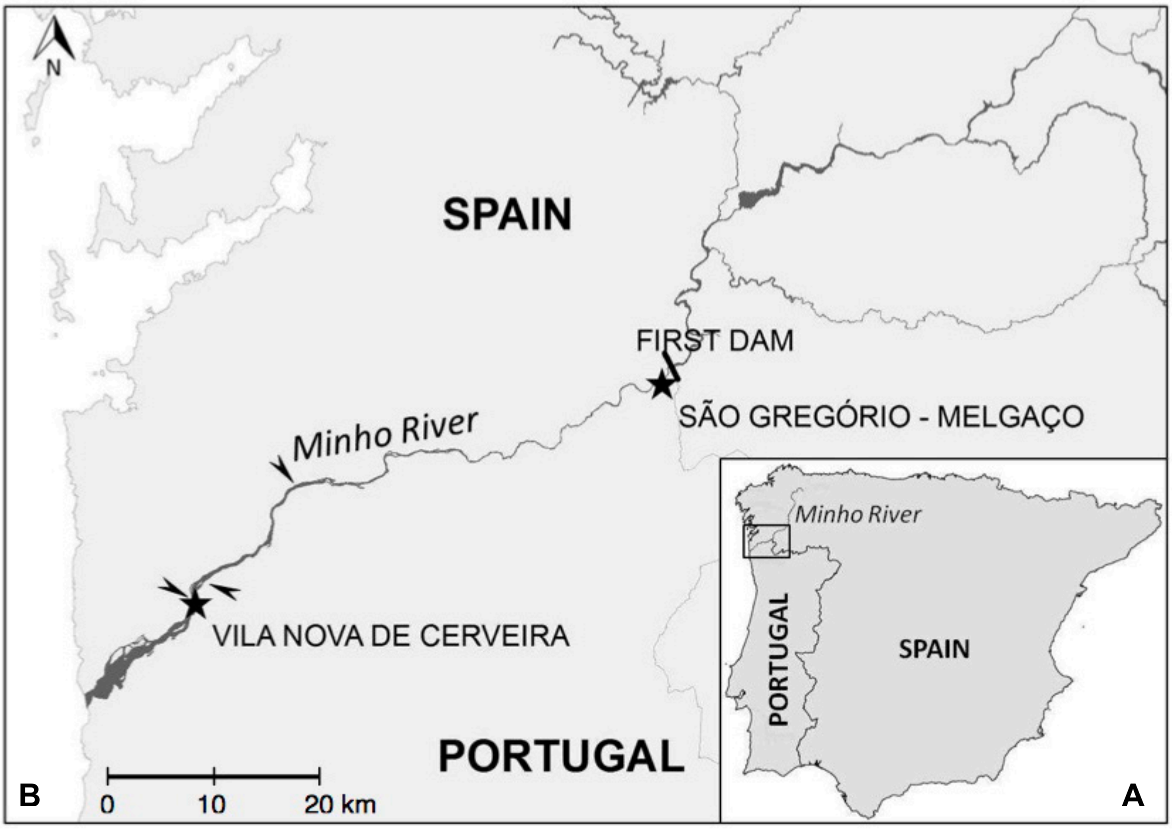

Figure 2.11. Map of the study area showing the location of the international Minho River in northern Portugal (A) and the experimental fishing sites (B); stars: spawners' sampling sites; arrows: juveniles sampling sites.

2.3.2.2. Source of biological material and sampling strategy

Samples of adults and juveniles were obtained in 2009, 2010 and 2011, from the existent small-scale fishery and from experimental fishing in the international Minho River. The samples supplied by fishermen were either adults from traditional Allis shad fishery or juveniles caught as by-catch of the glass eel fishery.

Experimental fishing of adults took place during the spawning migration season (March-August), using a typical shads' trammel net (140 m long; 140 mm loose inner layer mesh size). Two sampling stations were selected: one located in a TFW (near Vila Nova de Cerveira), the other in the spawning grounds (near São Gregório – Melgaço) reported by Mota and Antunes (2012) (Fig. 2.11B).

Experimental fishing of the young of the year (YOY) took place between September and January (YOY seaward migration season), during the night, period in which the catches were more efficient (Mota and Antunes, 2012), using a beach seine net (10 mm mesh; bag: 4.8 m long, 3 m mouth width; wings: 6 m long and 2.4 m high). Sampling sites were chosen based on results from a preliminary investigation of potential rearing habitats (Mota and Antunes 2012) (Fig. 2.11B).

2.3.2.3. Data collection

After capture, specimens were measured for fork length (FL), total length (TL) and total weight (TW). Spawners FL and TL were measured to the nearest 0.1 cm (on a measuring board) and TW determined to the nearest 5 g (digital hanging Kern HDB balance). The YOY FL and TL were measured to the nearest 0.1 mm and TW to the nearest 0.01 g (digital A&D EK-610i balance). To sort spawners into species and to establish the extent of hybridisation between Allis and Twaite shads, the first gill arch was removed and gill rakers counted under a binocular microscope (Nikon SMZ800), which allowed counting even the not completely developed rakers present at each extremity. A sample of adults' scales (N=385) was collected and subsequently mounted for ageing and identification of spawning marks, following the Baglinière et al. (2001) protocol.

Spawners of Allis shad were dissected to check gender, and to extract gonads, livers and otoliths. Gonads and livers were weighed (±0.01 g) using a digital A&D EK-610i balance.

Digestive tracts were removed and preserved in 70% ethanol for further stomach content analysis and internal metazoan parasites analysis. Oral cavity and gills were also inspected under stereomicroscope to remove isopoda parasites. Parasite identification was well fitted by diagnostic morphological characters under light microscopy and when necessary subjected to PCR-diagnosis for identification at the species level. Genomic DNA was isolated using Qiagen DNeasy^{TM} Tissue Kit according to manufacturer's instructions. DNA quality and quantity was checked in a spectrophotometer Nanodrop® ND-1000 (Nanodrop technologies, Inc) and in 1% agarose gel. 18S and ITS (ITS1, 5.8S rDNA and ITS2) genes were amplified and the obtained sequences were subjected to a homology search through Basic Local Alignment Search Tool (BLAST) searches in the National Center for Biotechnology Information (NCBI) database.

After measuring, the YOY were dissected for stomach, gills and otoliths excision. Allis shad, Twaite shad and hybrids were distinguished on the basis of the gill raker numbers of the first gill arch, as described for the adults. The YOY digestive tracts were preserved as those of the adults. Food items were counted and identified up to the lowest taxonomic level possible and the relative importance of each prey item in the diet was evaluated by the numerical index (NI) (the percentage of the number of individuals of a prey over the total number of individuals of all prey) and the occurrence index (OI) (the percentage of non-empty stomachs in which a prey occurred over the total number of occurrences) (Hyslop, 1980). The vacuity index (VI) (percentage of empty stomachs) was also evaluated.

2.3.2.4. Data analysis

2.3.2.4.1. Spawners

Individuals were identified to species level based on their morphology and on the number of gill rakers of the first branchial arch, i.e. less than 61 rakers: *A. fallax*, from 61 to 115: hybrids, and more than 115: *A. alosa*, as described in Alexandrino et al. (2006), and the TL-gill rakers number relationship was assessed for the three groups.

Allis shads (N=467) were separated according to their sex, age and capture zone, i.e. upper (spawning grounds), mid (TFWs) and low (estuary). The groups were compared in terms of weight and length distributions and weight loss along upstream migration, and inter-annual variation in weight, length and age per sex were analysed.

The gonadosomatic index (GSI) and the hepatosomatic index (HSI) were determined using the following formulae: $GSI = 100\times(Wg/Wt)$, where Wg is the gonad weight (g) and Wt is the fish total weight (g); and $HSI = 100\times(W_l/Wt)$, where W_l = liver weight (g). Differences in GSI and HSI were addressed per sex, between years and capture zones, and between months in each capture zone. All spawned individuals (caught after they reproduced) were excluded from this analysis.

Since most samples (particularly those referring to females) failed the Shapiro-Wilk Normality test and Levene's test for homogeneity of variance across groups, and samples differ in size (making these failures even more relevant), non-parametric methods were applied. Differences between groups were assessed through a distribution free analysis of variance using the Kruskal-Wallis test, to determine if any group was different from the others, and post-hoc pair-wise Wilcoxon rank sum tests, corrected for multiple testing, to see which groups differed (Hollander and Wolfe, 1973). Spearman correlation was applied to assess GSI-HSI relationship.

For several samples from small-scale fishery only TL, FL and TW data and scales could be collected for ageing. To estimate the probability of being male or female for these samples of unknown gender, the data of known sex were used to develop a zone-specific model relating sex to fish length and weight. Starting with a logistic regression model containing sex as binomial response variable and TL and TW, as well as their interaction, as explanatory variables, a stepwise model selection procedure based on BIC (Bayesian Information Criterion; Schwarz 1978) was applied to eliminate non-significant model terms. The final model was subsequently used to predict sex (with pointwise CI) for the samples of unknown gender.

All test results refer to an $\alpha=0.05$ significance level. Analyses were done in R (R Dev. Core Team 2009).

2.3.2.4.2. Young of the year

Juveniles *Alosa* spp. are difficult to distinguish by external morphological analysis. In order to separate the *Alosa* spp. specimen (N=560) into Allis shad *A. alosa*, Twaite shad *A. fallax* and hybrids, the number of gill rakers per TL was analysed. Model-based hierarchical clustering was applied, using the MCLUST package in R (Fraley and Raftery 2002; Fraley et al. 2012), allowing selection of 1 to 3 clusters and choosing the best model from among all available parameterizations using the BIC. For each of the resulting three clusters, or groups, linear models of the number of gill rakers depending on fish TL were computed.

The identified groups were separately tested for monthly size variation (pair-wise Wilcoxon test) and inter-annual TL variation per month. The hypothesis of one of the groups being more frequent in certain months was also assessed.

2.3.2.4.3. YOY and spawners TL-TW relationship and Condition Factor (K)

Length-weight relationships (LWR) were calculated for the pooled (YOY and adult) Allis Shad samples and for adult female, adult male and YOY fish separately. For adult fish two LWR were calculated: one for all fish, a second one pooling the data of the low and mid zones and excluding the upper zone data to eliminate potential bias due to weight loss of individuals caught in this area which are likely to have spawned.

We used the equation TW = aTL^b (Bagenal and Tesch, 1978), being TW the total weight of the fish (g), TL its total length (cm), *a* the equation's constant and *b* its slope. The parameters were determined through log-log linear regression analysis, i.e. log TW = log *a* + *b* log TL. The coefficient of determination R^2 was used to evaluate the strength of the LWR (Yılmaz and Polat, 2011).

The Condition Factor K (K= 100×(TW/TL^3); (TW=total weight in gr, TL=total length in cm) was calculated per year, for males and females separately, excluding fish from the upper river zone. For the YOY, only the 2009 and 2011 data were considered to calculate K, as only two individuals were sampled in 2010.

2.3.3. RESULTS

2.3.3.1. Spawners catch, gender, weight, length and age distributions

The classification of samples into species depending on the gill rakers number revealed 3.6% of hybrids in the spawner's population. The TL-Number of gill rakers relationship is given in Figure 2.12. The numbers of gill rakers ranged between 115 and 159 (Median 138) for *A. alosa*, for between 34 and 60 (Median 46) for *A. fallax* and between 63 and 104 (Median 84) for hybrids.

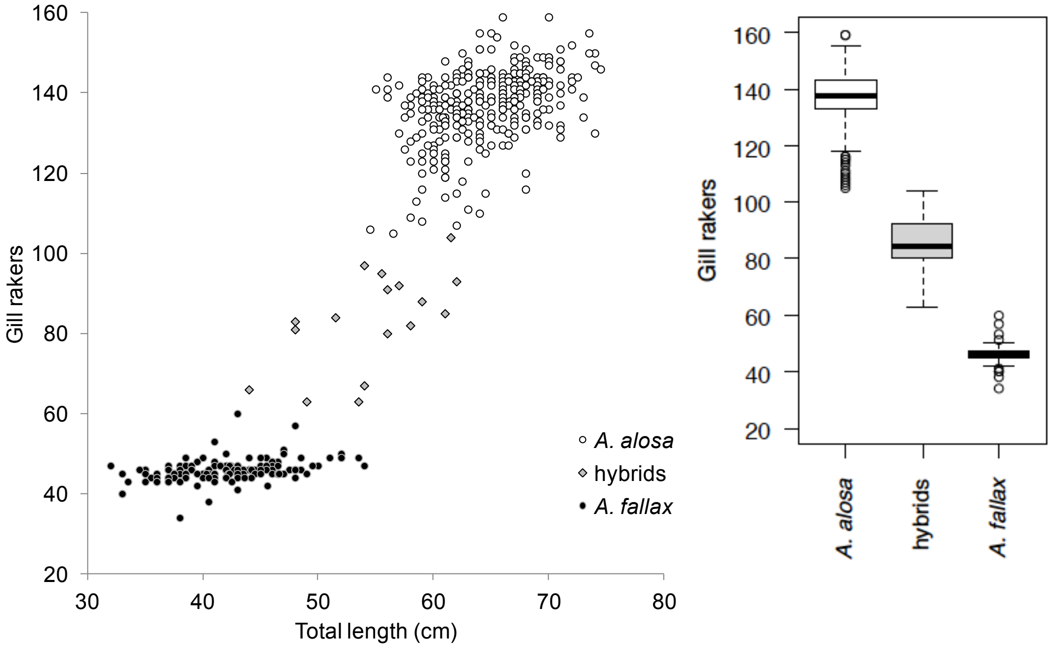

Figure 2.12. Relation between the number of gill rakers and the total length of adult *Alosa* sp. from the Minho River.

The number of Allis shad spawners caught in the Minho River by experimental fishing and fishermen varied over the 3-year study period (Table 2.4). Higher catches were obtained in 2009, followed by 2010 and 2011. Overall, the highest catches were observed in April and May, with an exceptionally high number of fish caught in the central zone during April 2009 (105 individuals). The catches per zone showed a predominance of fish in lower and central zones from March to May and in the upper zone from June onwards.

Table 2.4. Numbers of *A. alosa* caught during the 3-years study per month and sampling area (low: lower estuary; mid: mid zone, TFWs; upp: upper zone, spawning ground).

Year	March			April			May			June			July			August			Sum
	low	mid	upp	low	mid	upp	low	mid	upp	low	mid	upp	low	mid	upp	low	mid	upp	
2009	7	24	2	18	105	4	0	36	13	0	1	19	0	1	30	0	0	1	261
2010	6	11	0	14	9	0	0	47	1	1	5	20	1	0	10	0	0	8	133
2011	7	16	0	0	5	2	1	11	6	0	0	16	0	0	8	0	0	1	73
Sum/zone	20	51	2	32	119	6	1	94	20	1	6	55	1	1	48	0	0	10	
Sum/month	73			157			115			62			50			10			467

Table 2.5. Distribution of the number of spawners per year. gender and month (F: females; M: males; NI: unidentified gender).

Year	March			April			May			June			July			August			Sum
	F	M	NI	F	M	NI	F	M	NI	F	M	NI	F	M	NI	F	M	NI	
2009	8	14	11	25	59	43	30	19	0	16	4	0	21	9	1	1	0	0	261
2010	5	12	0	13	10	0	24	20	4	18	8	0	7	4	0	5	3	0	133
2011	8	15	0	3	4	0	12	6	0	9	7	0	6	2	0	1	0	0	73
Sum/gender	21	41	11	41	73	43	66	45	4	43	19	0	34	15	1	7	3	0	
Sum/month	73			157			115			62			50			10			467

Table 2.5 shows the distribution of spawners by gender per month and year. With the exception of April 2010, more males than females were caught in March and April, and more females than males in the rest of the observation months, in all study years. Most of the non-identified gender specimens are from spring 2009, when more samples were obtained from fishermen.

Regarding the length distribution of spawners per gender and year (Fig. 2.13A), females were generally larger than males, with sizes ranging from 54.0 to 74.5 cm and from 53.0 to 70.0 cm, respectively. Females also tended to be heavier than males (Fig. 2.13B), with weights ranging from 915 to 4515 g and from 590 to 3878 g, respectively. The heaviest female was 735 mm long, the heaviest male 700 mm. Weight and length (TL and FL), as well as TW/TL and TW/FL relationship differences between sexes were all highly significant (*p*<0.001), with females heavier, longer and higher TW/TL and TW/FL values. Overall and for each gender, TW did not differ significantly (*p*>0.05) between years, whereas TL significantly increased from year to year (overall and for males) and between 2009/10 and 2011 (for females).

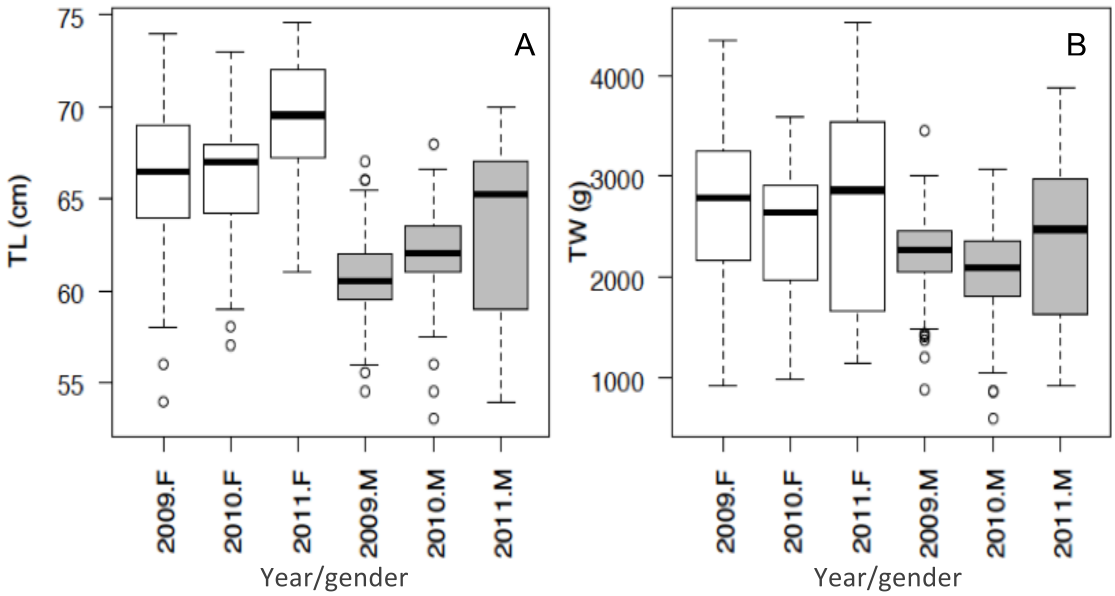

Figure 2.13. Adult *A. alosa* total length (TL) and weight (TW) distributions per gender and year; F: female, M: male. Boxplots: central line – mean; interquartile range from the first to the third quartile (box). Whiskers: minimum and maximum values excluding outliers. Outliers – circles.

The mean age of first maturity was 6.12 years for males and 6.96 for females. The age structure of spawners and respective mean weights and lengths by capture year are given in Table 2.6. Males tended to be 1 year younger than females, and this age difference is significant (*p*<0.001). Most of the females were 7 years old whereas most males were 6 years old (except for the year 2010, when 45% of the males were 7 years old and 43% 6 years old). Accordingly, females showed no significant differences in age between years, whereas males showed significant ($p<0.05$) inter-annual differences, with lower age in 2011 and higher age in 2010.

Spawning marks were found in 20 individuals (7 males; 13 females), representing 5.19% of all analysed fish and 3.76% of the male and 6.53% of the female fish. With the exception of one single 8 year old female (from 2010) that presented the spawning mark aged two years before the capture date, all specimens presented spawning marks from the year before the capture date. The youngest male with spawning mark was 5 years old and the oldest 7 years old; the youngest and oldest females were 6 and 8 years old, respectively (Table 2.6).

Table 2.6. Age structure of the Minho River *A. alosa* spawners per sex and year. The number of specimens (N) and percentage of the yearly catch, as well as mean total length (TL), weight (TW) and the number of individuals presenting spawning marks (Spw) are given.

Year	Age (years)	Male				Female			
		N	Mean TW (g)	Mean TL (mm)	Spw	N	Mean TW (g)	Mean TL (mm)	Spw
2009	5	11 (11%)	2115	606		2 (2%)	2380	620	
	6	68 (66%)	2237	608	2	14 (15%)	2389	666	
	7	24 (23%)	2152	608		57 (60%)	2696	666	
	8	0 (0%)	–	–		22 (23%)	2912	665	
2010	5	7 (13%)	2145	610	2	2 (3%)	1732	625	
	6	23 (42%)	1932	626	1	16 (25%)	2446	657	3
	7	25 (45%)	2115	620	1	31 (48%)	2536	664	2
	8	0 (0%)	–	–		15 (23%)	2717	670	4
2011	4	1 (4%)	1925	590		0 (0%)	–	–	
	5	9 (33%)	2452	634	1	4 (12%)	3202	688	
	6	15 (56%)	2400	647		6 (18%)	2510	677	
	7	2 (7%)	2322	625		17 (50%)	2823	702	2
	8	0 (0%)	–	–		7 (21%)	3103	692	2

Except for the single small 4-year male caught in 2011, TL medians and means are similar for different ages though more variable for younger (5-year) spawners (Table 2.6); for the same sex, TL was not significantly different ($p>0.05$) between ages.

Regarding the weight loss along the upstream migration, TW and the TW/TL relationship were significantly ($p<0.001$) lower in the upper zone then in the low or mid zones, for both female and male (no significant differences were found between the low and mid zones). There was a significant weight loss between the mid and upper zones (females 33%, males 30%), after a slight (non-significant) increase of TW for females and (non-significant) decrease for males between the low and mid zones (Table 2.7).

Table 2.7. Male (M) and female (F) mean total weights and their standard deviation (SD) per sampling area (low: lower estuary; mid: mid zone, TFWs; upp: upper zone, spawning ground) and weight loss between zones (low→mid: weight loss between the lower estuary and the tidal freshwater wetland zone, etc.). Note that no low→mid weight loss was observed in females.

Sex	TW (g) per zone			Difference in TW	
	low mean (SD)	mid mean (SD)	upp mean (SD)	low→mid	mid→upp
F	2977 (502)	3069 (598)	2066 (745)	92g/3%	-1003g/-33%
M	2430 (465)	2330 (380)	1642 (527)	-100g/-4%	-688g/-30%

2.3.3.2. Spawners gender assignment

Since fish sizes and weights did not differ significantly between the low and the mid zones, and given that all fish of unknown gender were captured there, regression modelling was based on the fish of known gender captured in the mid zone or in both low and mid zones. Fish TL was the most significant variable (p<0.001), and the only variable included in the final logistic linear gender models. Its inclusion in the models rendered the TW and TL:TW interaction terms non-significant. The model for the pooled low and mid zones explained 33.8% of the deviance, the model for the mid zone 36.5% of the deviance. Both models were used to estimate the probability (and point-wise SE) of being female or male for the samples of unknown gender (Fig. 2.14). The low and mid zone model predicted 42 females and 9 males; for 7 specimens the CI (i.e. prediction ±2×SE) span the decision threshold (0.5) and no gender could be assigned. The mid zone model assigned the same gender to the specimens of the mid zone data.

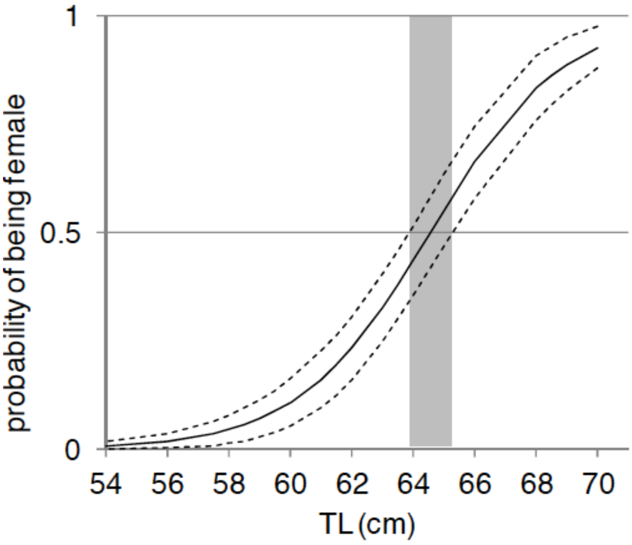

Figure 2.14. Predicted probability (+/- 2xSE, dashed lines), for the specimens of unknown gender, of being an *A. alosa* female, depending on fish total length (TL) and according to the logistic low-and-mid zone model. The threshold for being considered female was a probability of 0.5. No gender was assigned to the specimens with sizes in the grey shaded range, since confidence intervals include the specified threshold value.

2.3.3.3. GSI and HIS

The GSI of males were frequently less than those of females. Differences between zones per sex showed GSI to be significantly higher in upper zone females than in low and mid zone females (Fig. 2.15A). No significant differences were observed between GSI of low and mid zone female, nor between GSI of males from different zones.

When looking for GSI differences between months per sex, the females' GSI increased with time, being significantly ($p<0.05$) higher in May than in April and in June than in April. The difference between June and May is non-significant ($p=0.058$). No significant difference was observed between July and previous months (NB: there were only 4 females captured in July) (Fig. 2.15B). No significant differences between months were observed for male GSI (NB: only 1 male in July) (Fig. 2.15B). Comparison of GSI between months per zone and per gender did not showed significant differences, as there were too few samples per month and zone.

Regarding HSI, female HSI was significantly different between all zones, whereas male HSI was only significantly lower in the upper zone, compared to the low and mid zones, and no significant difference between low and mid zones was observed (Fig. 2.15C). Comparing the HIS between months per sex, female HSI increased significantly from March to May, but no other significantly difference was observed for this gender (Fig. 2.15D). In males, HSI increased between March and May, being significantly higher in April and May than in March, though it was significantly lower in June than in April (Fig. 2.15D). Looking for differences between months per zone and sex, the mid zone males' HSI was lower in March than in April and May; no other significantly differences were found.

Concerning the GSI-HSI relationship, in females, no significant change in HSI due to changes in GSI could be found (none of the linear models had a significant slope parameter). Analogous results were obtained for the inverse relationship, i.e. GSI depending on HIS. Regarding males, GSI decreased significantly with increasing HSI in the upper zone; there were no significantly changes in the other zones. Again, the same results were obtained for the inverse relationship, i.e. GSI depending on HIS.

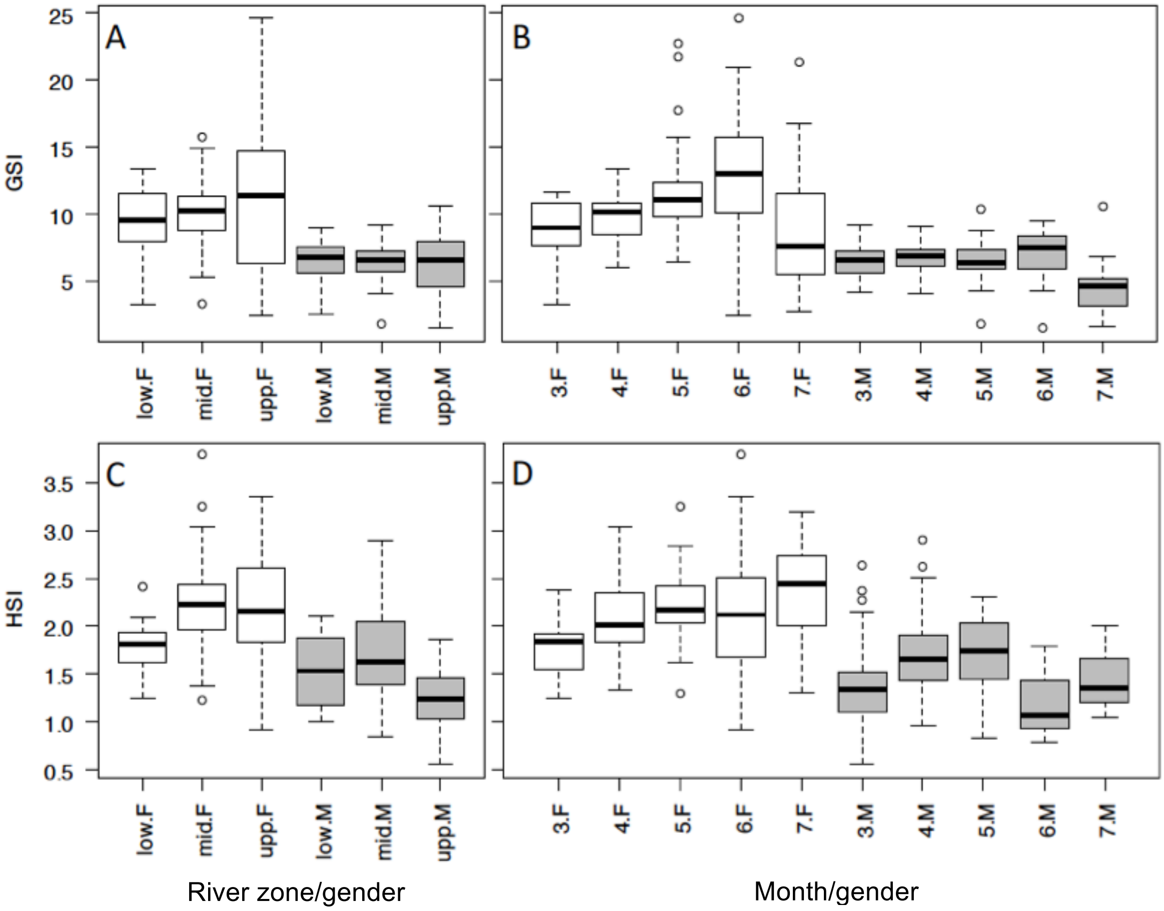

Figure 2.15. Changes in *A. alosa* Gonadossomatic Index (GSI) and Hepatosomatic Index (HIS) during the upstream spawning migration. A: GSI variation per gender and river zone; B: GSI variation per gender and month; C: HIS variation per gender and river zone; D: HIS variation per gender and month (F: female, M: male).

2.3.3.4. Spawners parasitology and diet

According to the ITS amplified regions of 750 bp and their sequence homology searches (Blast values of 100%) the two **Anisakidae** species commonly infecting the visceral cavity of Allis shads analyzed belong to *Anisakis pegreffii* and *Hysterothylacium aduncum*. According to the 18S amplified regions other parasite species infecting the Allis shads belong to trematodes of Hemiuridae, the accidental acanthocephalan parasites of *Rhadinorhynchus pristis* and the isopoda which likely belongs to the family Cymothoidae (Fig. 2.16).

The analysis of stomach contents revealed that the overwhelming majority of spawners did not feed during migration. The exception were two individuals caught in the low estuary zone (one male and one female) and two males caught in the TFWs (mid) zone showing stomachs completely filled with Mysidacea crustaceans, one of the latter with digested content. In addition, four individuals from the TFWs zone (two males and two females) and five from upper zone (two males and three females) showed vestiges of plant fragments in their stomachs and two upper zone fish (one male and one female) fragments of fishing lines.

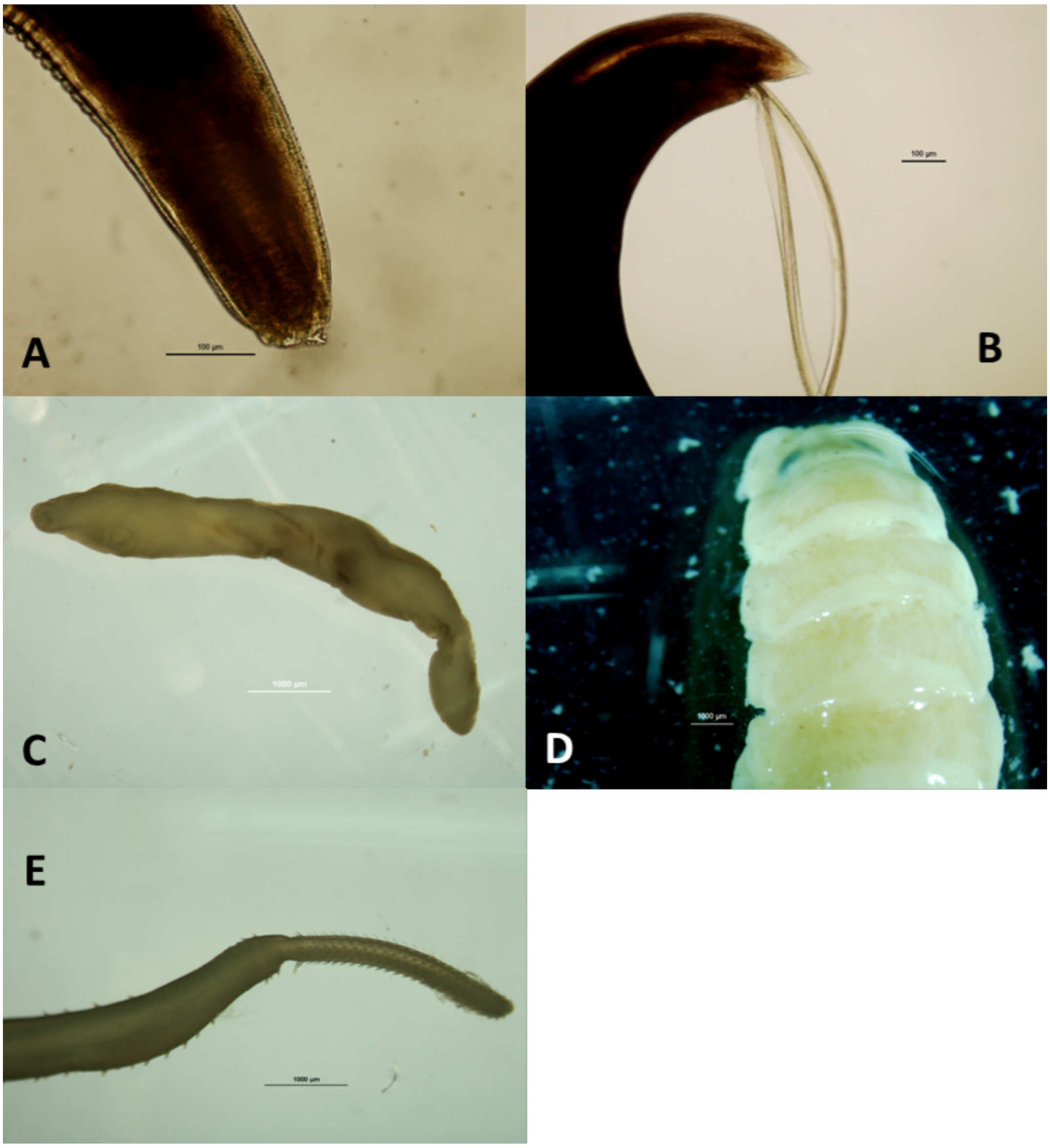

Figure 2.16. Metazoan parasites identified in *A. alosa* from the Minho river. A: *Anisakis* sp. nematode, B: *Hysterothylacium* sp. nematode, C: Digenean trematode of the Hemiuridae, D: Isopoda resembling a Cymothoidae, E: *Rhadinorhynchus* sp. Acanthocephalan

2.3.3.5. Young of the year species identification

The best hierarchical clustering model relating the number of gill rakers with YOY TL was an unconstrained ellipsoidal model, with 3 components. This model was used to classify our samples into Allis shad, Twaite shad and hybrids. Linear models of the number of gill rakers depending on TL were: 32.54+2.56×TL for Allis shad (R^2=0.63); 19.70+1.27×TL for Twaite shad (R^2=0.52); and 29.47+1.46×TL (R^2=0.23) for hybrids. Model predictions, confidence and prediction intervals are presented in Table 2.8 and Figure 2.17.

Most of the 560 YOY were collected in November 2009 (n=204) and September 2011 (n=220) (Fig. 2.18). According to our classification, 61% were Twaite shad, 29% Allis shad and 10% hybrids. Hybrid percentage ranged from 6 to 17%.

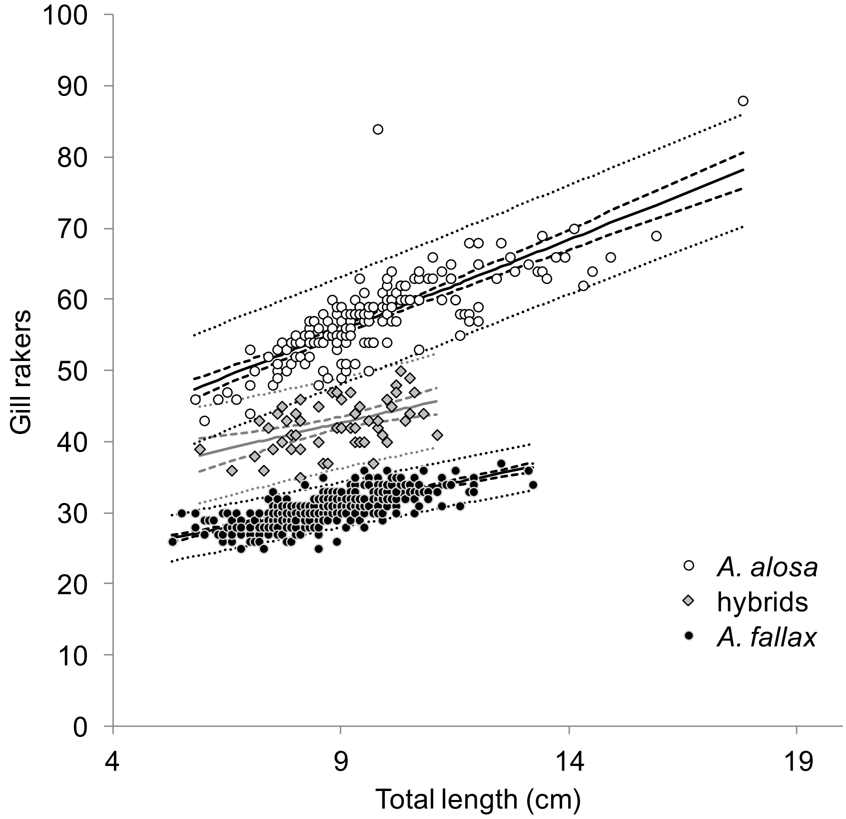

Figure 2.17. The sampled young of the year species, according to the cluster analysis, and fitted linear models relating gill rakers numbers to total length (TL) (continuous lines: model prediction, dashed lines: confidence intervals, dotted lines: prediction intervals).

Table 2.8. YOY predicted number of gill rakers (and confidence intervals) for different total lengths (TL).

TL (cm)	Number of gill rakers		
	A. alosa	*A. fallax*	hybrids
5	45.3 (43.7–47.0)	26.0 (25.5–26.6)	36.8 (33.8–39.8)
6	47.9 (46.6–49.2)	27.3 (26.9–27.7)	38.2 (36.0–40.5)
7	50.5 (49.4–51.5)	28.6 (28.3–28.9)	39.7 (38.1–41.3)
8	53.0 (52.2–53.9)	29.8 (29.6–30.0)	41.2 (40.1–42.2)
9	55.6 (54.9–56.2)	31.1 (30.9–31.3)	42.6 (41.8–43.5)
10	58.2 (57.6–58.8)	32.4 (32.1–32.6)	44.1 (42.9–45.2)
11	60.7 (60.0–61.4)	33.6 (33.3–34.0)	45.6 (43.8–47.3)
12	63.3 (62.4–64.2)	34.9 (34.4–35.3)	47.0 (44.6–49.4)
13	65.8 (64.7–67.0)	36.2 (35.6–36.7)	48.5 (45.4–51.6)
14	68.4 (67.0–69.8)	37.4 (36.7–38.1)	49.9 (46.1–53.7)
15	71.0 (69.2–72.7)	38.7 (37.9–39.5)	51.4 (46.9–55.9)
16	73.5 (71.5–75.5)	40.0 (39.0–40.9)	52.9 (47.6–58.1)

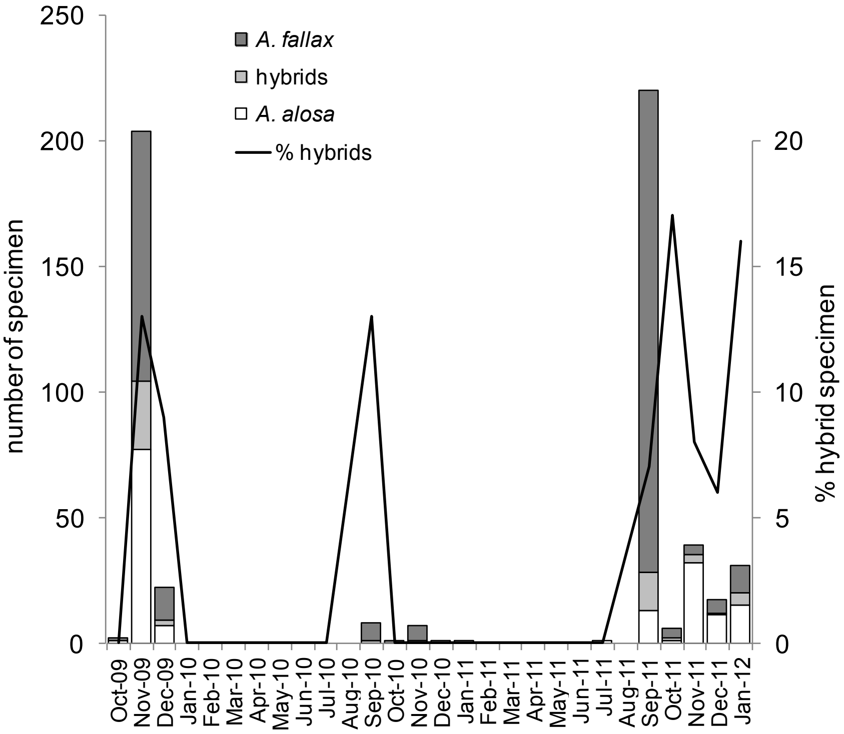

Figure 2.18. Number of captured young of the year per identified species and percentage of hybrids per month.

2.3.3.6. Young of the year size distributions

Allis shad tended to be bigger and heavier than Twaite shad, and hybrids presented intermediate values (Fig. 2.19). Differences were significant between Allis shad and Twaite shad ($p<0.001$ for TL and TW) and between Allis shad and hybrids ($p=0.014$ for TL and $p=0.026$ for TW), but non-significant between Twaite shad and hybrids ($p>0.05$).

The monthly size distributions (Fig. 2.19) showed an overall increasing trend in TL from September to January, although there were some very large Allis shad juveniles in November. TW increased towards November but stabilized or decreased afterwards.

Allis shad TL differed significantly between September and January ($p=0.002$) and between September and November ($p=0.021$). Twaite shad TL differed significantly between September and January, November, and December (all $p<0.001$), and between November and January ($p=0.019$). Hybrid TL differed, like those of Allis shad, between September and January ($p=0.015$) and between September and November ($p<0.001$). There were too few October samples to test for differences between groups. In terms of TW, differences were significant for Allis shad between September and November ($p=0.003$), for Twaite shad between September and November ($p<0.001$), December ($p<0.001$), and January ($p=0.001$), and for hybrids between September and November ($p<0.001$), and January ($p=0.026$).

2.3.3.7. Young of the year diet

The lists of the prey items found in the stomach content analyses of YOY Allis shad, Twaite shad and hybrids, and the corresponding values of diet indices are given in Table 2.9. Both occurrence (OI) and numeric indices (NI) identified *Corophium* sp. as the most important food item for all the groups. The second most important item was **Gammaridae** in terms of OI for all groups, and in terms of NI for *A. alosa* and *A. fallax*; Hybrids preferred *Atyaephyra desmarestii*. The third most frequent item was *Atyaephyra desmarestii* for *A. alosa* and hybrids, and *Gammarus* sp. (almost together with *Atyaephyra desmarestii*) for *A. fallax*. According to the NI, *Atyaephyra desmarestii* was the third most important food item for *A. alosa* and *A. fallax*; **Gammaridae**, closely to Plecoptera, came third for hybrids. Concerning the vacuity index (VI), hybrids exhibited the emptiest stomachs (8.33%), followed by *A. fallax* (6.65%) and *A. alosa* (6.15%).

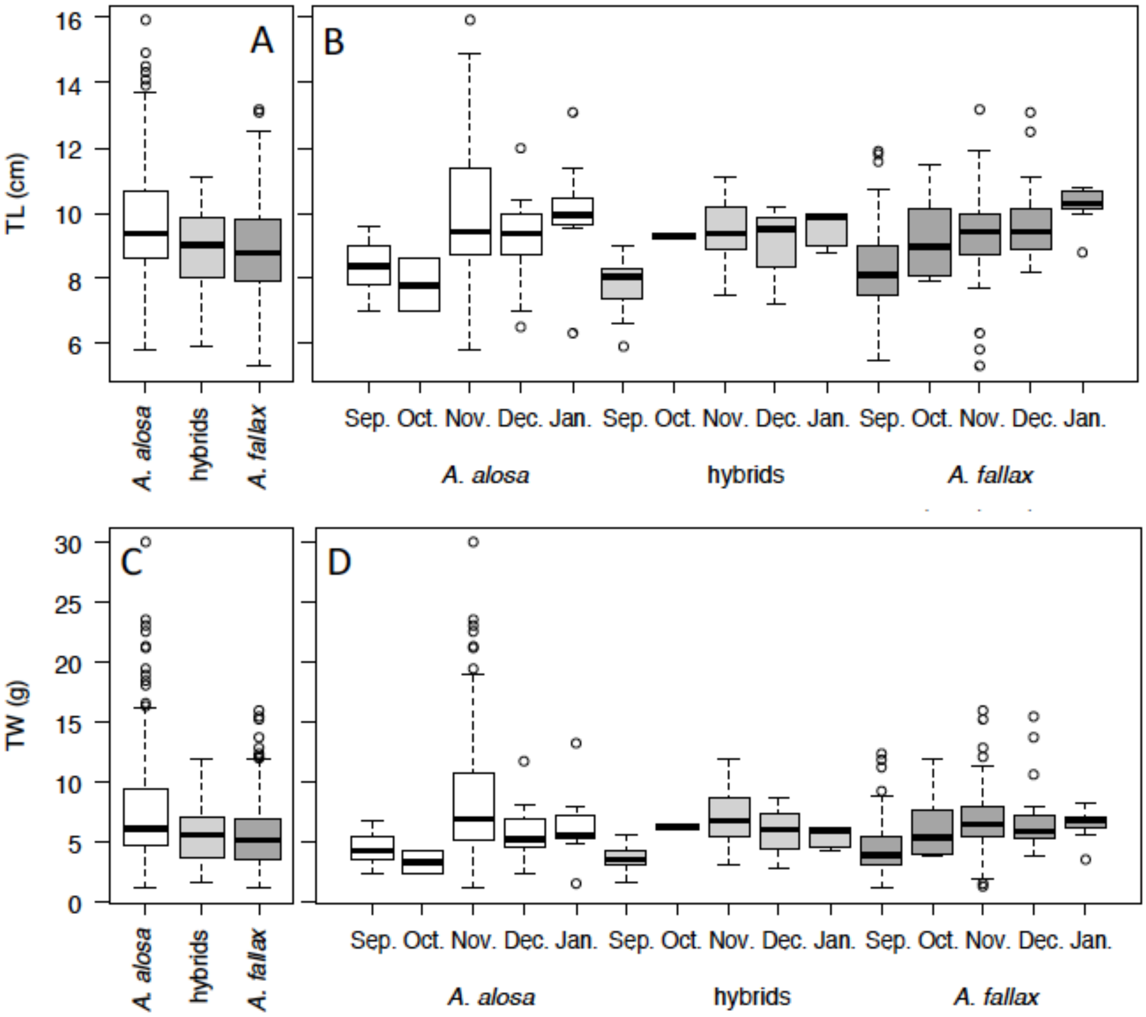

Figure 2.19. Total length and total weight distribution of the young of the year samples, per identified species (A, C) and per species and capture month (B, D). The single July sample of *A. alosa* is not shown. The boxplots present the median (central line) and the interquartile range (IQR), from the first to the third quartile (box). Whiskers indicate the minimum and maximum values excluding outliers (i.e. points that differ more than 1.5 times the IQR from the respective quartiles), which are presented as separate circles.

Table 2.9. Diet composition for *A. alosa* (N = 130), *A. fallax* (N=329) and hybrid (N=84) YOY (NI: Numerical Index; OI: Occurrence Index).

Class	*A. alosa*		*A. fallax*		Hybrids	
	OI	NI	OI	NI	OI	NI
Crustacea						
Not identified	0	0	0.65	0.11	1.30	0.16
Amphipoda (not identified)	9.02	0.22	11.00	2.53	11.69	1.28
Atyaephyra desmarestii	25.41	1.16	12.30	3.28	29.87	5.90
Corophium sp.	87.70	21.71	89.97	73.62	87.01	77.99
Gammaridae (not identified)	43.44	2.40	40.45	11.71	41.56	4.31
Gammarus sp.	11.48	0.33	12.94	2.26	12.99	2.02
Saduriela sp.	2.46	0.03	2.59	0.23	6.49	0.27
Mollusca						
Bivalvia (not identified)	0.82	0.05	3.24	0.52	3.90	0.16
Arachnida						
Not identified	0	0	0.32	0.02	1.30	0.05
Insecta						
Collembola (not identified)	0	0	0	0	2.60	0.64
Chironomidae	5.74	0.18	9.06	2.44	11.69	0.69
Coleoptera	0.82	0.01	0.32	0.02	0	0
Empididae	0	0	0.32	0.05	0	0
Ephemeroptera	2.46	0.03	0.97	0.20	2.60	0.11
Heteroptera	0	0	0.32	0.02	0	0
Magaloptera	0	0	0.97	0.07	0	0
Plecoptera	4.92	0.08	1.94	0.14	3.90	3.93
Simuliidae	0	0	0.65	0.05	1.30	0.11
Tabanidae	0	0	0	0	1.30	0.05
Trichoptera	2.46	0.03	0.97	0.07	2.60	0.11
Nymph not identified	0.82	0.02	0.32	0.02	5.19	0.80
Adult not identified	2.46	0.14	6.15	1.56	3.90	0.21
Not identified	9.84	0.14	4.53	0.88	9.09	1.06

2.3.3.8. Allis shad length-weight relationship

The descriptive statistics and parameters of the length-weight relationships of Minho river Allis shad are given in Table 2.10.

Combining low and mid zone data, for males and females separately (Fig. 2.20), the TW-TL model equations were $\text{TW} = 0.0290\times\text{TL}^{2.739}$ for males (R^2=0.61) and $\text{TW} = 0.0069\times\text{TL}^{3.090}$ (R^2=0.62) for females. Female Allis shad tended to be lighter than males up to a length of 59.45 cm, and heavier than males when bigger. For the YOY Allis shad data, the TW-TL model equation was $\text{TW} = 0.0076\text{xTL}^{2.995}$ (R^2=0.95) (Fig. 2.21).

The Condition Factor (*K*) was slightly higher for females in all years (the 3-year means were 1.013 for females and 0.993 for males). Both male and female *K* decreased from 2009 (male mean 1.026; female mean 1.063) to 2010 (male 0.919; female 0.954) and slightly increased from 2010 to 2011 (male 1.003; female 1.028) (Fig. 2.22). YOY *K* also decreased from 2009 to 2011 (0.82 and 0.68, respectively) and the mean value of the two-years study was 0.76 (Fig. 2.22).

Table 2.10. Descriptive statistics and estimated parameters of length-weight relationships for *Alosa alosa* from the Minho River.

Sample	Zone	N	TL (cm)			TW (g)			LWR parameters			
			min	mean	max	min	mean	max	a	b	95% CI of b	R^2
All	All	564	5.8	48.9	74.5	1.2	1726	4515	0.0064	3.070	3.045–3.095	0.990
All	low&mid	423	5.8	43.5	74.5	1.2	1661	4515	0.0054	3.148	3.136–3.161	0.998
YOY		160	5.8	9.7	15.9	1.2	7.7	29.9	0.0076	2.995	2.884–3.107	0.947
Adult F	All	212	54.0	66.9	74.5	915	2623	4515	0.0778	2.467	1.592–3.342	0.128
Adult F	low&mid	120	54.0	66.8	74.5	1050	3049	4515	0.0069	3.090	2.651–3.529	0.622
Adult M	All	192	53.0	61.6	70.0	590	2167	3878	0.0060	3.097	2.426–3.768	0.304
Adult M	low&mid	143	54.5	61.7	70.0	1255	2347	3878	0.0290	2.739	2.373–3.104	0.609

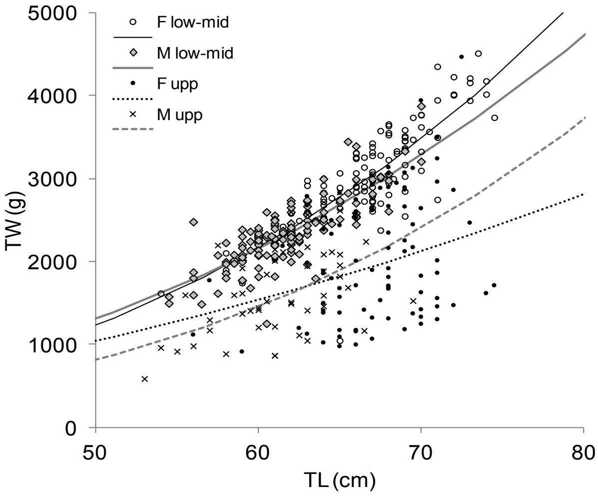

Figure 2.20. *A. alosa* spawning population weight-length relationships, for each gender, for the pooled low and mid zone data and for the upper (upp) zone data (F: female, M: male).

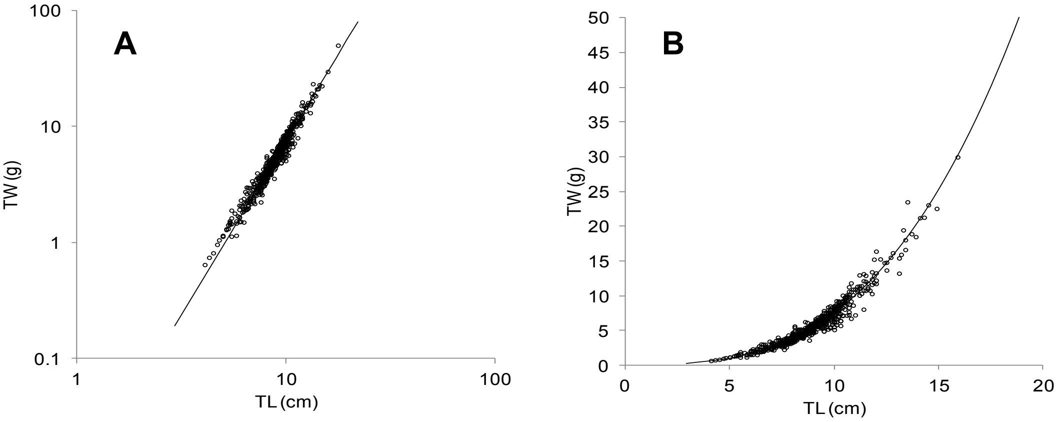

Figure 2.21. *A. alosa* young of the year population weight-length (TW-TL) relationship; A: on a log-log scale; B: on the observation scale.

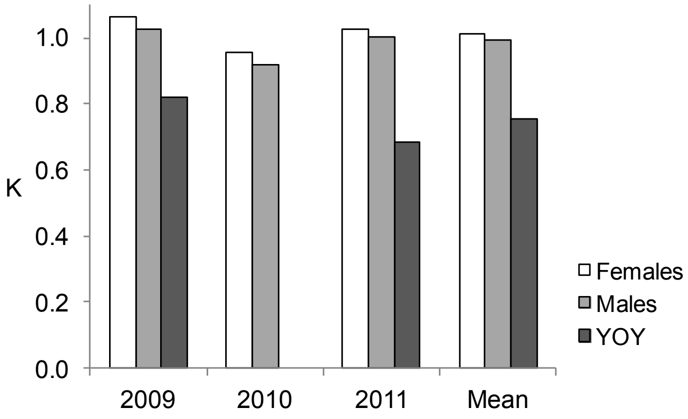

Figure 2.22. Yearly variation of the Condition Factor (*K*) for male and female spawners and young of the year (YOY).

2.3.4. DISCUSSION

The present study follows the first and preliminary study of Mota and Antunes (2011) on the biology and ecology of Allis Shad in Minho River, nowadays thought as the most stable population in the southern part of the species' distribution.

It is clear that the shad catches in the Minho River vary over the study years, being 2009 an exceptionally successful year. Official data of the small scale fishery reported by Marine Authorities showed two abundance peaks in the last 30 years, one in 1980 and the latest in 2009 (Mota and Antunes, 2011). Although the experimental fishing effort was not constant among the years (in increased over the 3-years study), the same trend was verified in our data and reflects the common fluctuations in abundance of *Alosa* populations, as reported by Baglinière et al. (2003). Nonetheless, attention must be done regarding the long-term decrease of the population size and the Allee effect (Allee, 1931), as the depensation in fish stock productivity can accelerate population decline in an extreme way and lead to the population extinction or seriously block its recovery (Walters and Kitchell, 2001).

The spawning migration of adults in the Minho system occurs between March and June, with peaks of migration in April and May, reflecting the common pattern of the species' migratory dynamics, when a dense influx of fish occurs one or two times per migration season (Mennesson-Boisneau et al., 2000a; Rochard, 2001). Nonetheless, indications from fishermen and experimental fishing show that the migration season may, occasionally, extend from January to July. Furthermore, the latitudinal south-north gradient (Mennesson-Boisneau et al., 2000a; Aprahamian et al., 2002; Baglinière et al., 2003) is here well explicit, since the Minho Allis shad population starts migration earlier in the spring than northern European populations and latter than Moroccan populations. The diurnal migratory pattern described for other European populations, where migratory activity is more intense during the daylight (Mennesson-Boisneau et al., 2000a; Baglinière et al., 2003), could not be found in this study. In fact, diurnal experimental fishing was ineffective, likely because fish are able to avoid the gears that are visible due to water transparency.

The catches per zone openly reflect fish migration pattern. Fish abundances in the lower and central zones are higher in spring and decrease towards earlier summer, increasing at the same time in the upper zone. Moreover, gender-specific migratory behaviour is evident. As previously described by Mota and Antunes (2011) for the Minho population, males migrate upstream earlier, predominantly during March and April, while females prefer the months of May and June-July, confirming the history pattern that was previously described for the species (Baglinière et al., 2003; Maitland and Hatton-Ellis, 2003).

Like in other European populations (Mennesson-Boisneau et al., 2000b; Aprahamian et al., 2002; Lassalle et al., 2008b) the majority of the Minho system male spawners are one year younger than the female ones. In addition, few spawners return for a second spawning migration and the overwhelming majority of these returns a year after the first spawning. As seen by Mota and Antunes (2011), the Allis shad population of the Minho River is semelparous.

Notice, that the inter-annual differences found in the males' ages between 2010 and 2011 can be partly due to the difficulties to interpret the winter rings on scales (Sabatié and Baglinière, 2001) and also because scales of the males are more difficult to interpret than those of females (Baglinière et al., 2001).

Weight loss after spawning migration is high (33% for females and 30% for males) but not as marked as that described by Mennesson-Boisneau et al. (2000a), who reported a maximum weight loss of 59%, depending on the length of spawning migration. In fact, in the Minho system, the stretch available for the upstream migration and spawning is only 77 km long; quite bellow the 125 km in the Dordogne system and the 576 km in the Loire system (Mennesson-Boisneau et al., 2000a). Besides, the weight loss along migration is more pronounced between the mid zone and the upper zone than between low zone and the mid zone, where the tidal cycle assist in the migration (Rochard, 2001).

The TL regression model for spawners gender assignment may be useful for future joint monitoring programmes with fishermen since, in our experience, this cooperation is based mainly on fish number, TL and TW data. Although the inter-sexual differences in morphology are little, external difference such as the size of the cloaca (Aprahamian et al., 2002) and milt release in males, combined with the regression modelling, might help to assign spawners gender and, thus, improve the knowledge of the Minho River Allis shad's demographic characteristics.

The condition factor (*K*) is associated to the body weight and length relationship. The ratio of the weight for a given length can vary intra specifically depending on the environment, the season (with great influence of the gonadal maturation stage) and the individuals' ontogenetic development (Froese, 2006), and it is expected that a fish in better condition has higher growth rates, higher reproductive potential and survival (Pope and Krus, 2001). In this study, we considered for the *K* estimation YOY in seaward migration (age 0+) and spawners at the beginning of the upstream migration. The high reproducer *K* value in comparison to juvenile *K* value can result from gonad development, since, as postulated by Bengen et al. (1991), gonadal development starts at sea before the upstream migration. On the other hand, the growth rate at early stages in freshwater may differ from adults' growth rate at sea, thus influencing the weight-length relationship and *K* parameter. The slight 2009-2010 decrease of the spawners' *K* may be due to the relatively lower weights observed in 2010, for females with identical lengths and males with bigger lengths than in the previous year.

When comparing age and TL at maturity with those of southern Portuguese (Eiras, 1981a; Alexandrino, 1996) and Moroccan populations (Sabatié et al., 1996), we observe that in the Minho the population (males and females) matures one year latter and to individuals tend to be larger. This aspect departs somewhat from the negative latitudinal gradient described by several authors (Mennesson-Boisneau et al., 2000b; Aprahamian et al., 2002; Lassalle et al., 2008b), who however did not consider Allis shad data from the Minho River. For example, Lassalle et al. (2008b) demonstrated statistically the relationships between latitude and life history variables for *A. alosa* without data from the Minho River. In the same way, the growth per age seems to be higher when compared with southern populations, as the Lima and Sebou (Sabatié et al., 1996), Douro (Eiras, 1981a) and Mondego (Alexandrino, 1996). Yet, the negative latitudinal gradient seems to be valid in relation to northern populations (e.g. Maitland and Lyle, 2005; Lassalle et al., 2008b).

The weight growth (TW) is strongly correlated with the length growth (TL), both for male and female spawners and juveniles. The allometric coefficient (*b*) found for the male spawners was lower than that for females (considering the low and mid zone samples). The slope of the male and female TW/TL curves suggests a difference between the weight growth rates, with female Allis shad being lighter than males up to a length of 59.45 cm, and heavier than males when bigger. Compared to other European populations (e.g. Mota and Antunes, 2011), the males' *b* value is quite similar to those of the Douro population (e.g. Froese and Pauly, 2013) but lower than all the others. Female spawners showed a *b* value closer to that of other populations, with the exception of the Adour female (e.g. Mota and Antunes, 2011), which presented a *b* value < 3. In short, spawners from the Minho population seem to be one year older and to have a higher growth rates than those from other European and Moroccan populations, although Moroccan females have quite similar weights, but smaller TL. The "discontinuity" of the negative latitudinal gradient regarding southern Allis shad populations could be the result of the position of the marine growing area. Although seasonal upwelling occurs in summer along the western Iberian coast and along the western African coast up to Morocco (Nykjaer and Van Camp, 1994), the northern coast of Africa and the southern coast of Portugal are only softly or intermittently affected by coastal upwelling (Barton, 1998), and it seems that in Portugal local winds induce a greater amount of upwelling than the winds of Northwest Africa (Detlefsen and Speth, 1980). Thus, it could be possible that the marine growing zone of the Minho Allis shad population is located in an area of upwelling episodes, which are more intense than in northwest Africa, and probably with greater abundance of food resources than in northwest Africa. Given the proximity of the Minho (41º 51' N) and Lima (41º 40' N) Rivers, this fact does however not explain the growth rate difference between these two populations. And the higher growth rates may also result from the food richness available to juveniles during their downstream migration (Sabatié and Baglinière, 2001). However, as postulated by several authors (e.g. Sabatié et al., 1996; Mennesson-Boisneau et al., 2000b; Lassalle et al., 2008b), explanations and comparisons between populations must be cautious, owing to the lack of knowledge of the marine sector occupied by Portuguese populations, the absence of long-term studies, diversity of years and sampling methods and sample sizes.

Contrasting with the Atlantic salmon that at the beginning of spawning migration shows slight developed gonads (Greene, 1926), Allis shad arrives to freshwater in stages II and III of gonadal development, being the early stages of gonadal development started at sea (Bengen et al., 1991). Although our work has not addressed the maturation of gonads, GSI provides an indication of the sexual maturity and, the Minho Allis shad GSI in the low estuary (female mean 9.61; male mean 6.63), confirms the advanced maturity stage at the beginning of the spawning migration. Nonetheless, as found by Bengen et al. (1991) for the Garonne population, it seems that the complete maturation of the gonads occurs only in the spawning zone, where GSI values are significantly higher than those in the mid and lower zones (though less evident in males) (with female means of 14.41, 10.08 and 9.61, and male means of 7.42, 6.58 and 6.63, respectively, for the upper, mid and lower zones). Although GSI values for spawned fish were not included in this study, a preliminary GSI analysis revealed a decrease in GSI values in the upper zone during the summer (from 12.58 in June to 8.73 in July and 5.22 in August for females, and 6.97 in June to 4.54 in July and 2.97 in August for males). This drop of the GSI values in July and August seems to indicate June as the most favourable month for the reproduction of Allis shad in the Minho.

During the freshwater phase of the spawning migration, Allis shad spawners do not feed (Aprahamian et al., 2003; Mennesson-Boisneau et al., 2000a). Minho River spawners are no exception as the majority of the stomachs were empty of food items and contained, as also found by Quignard and Douchement (1991), frequently a whitish or greenish fluid. The few specimens presenting non-digested marine crustaceans (mysidacea crustaceans) in their stomachs may indicate a fast migration in the estuarine zone. In fact, the distance between the mouth of the Minho River and the TFWs (mid zone) is around 16 km, suggesting a speed of migration similar to those estimated by Rochard (2001) for an estuary (ranging between 17 and 23 km/day). The plant fragments found in a few stomachs are unlikely food items of this species (Maitland and Lyle, 2005) and were, certainly, ingested by accident. The fishing lines found in the stomach contents of the upper zone specimens may have been ingested by accident as well, since sport fishing is very common in this area and such fragments should be frequent in the water column.

The larval ascaridoid nematodes of the genus *Anisakis* spp. are known as the etiologic agent of a fish-borne zoonosis called anisakiasis (EFSA 2010). In fact, *A. pegreffii,* which is here reported for the first time in Allis shad, was recently identified as the zoonotic agent of a human intestinal case of anisakiasis within an eosinophilic granuloma (Mattiucci et al., 2011). This new emerging parasite-host relationship might lead to a geographical expansion of *Anisakis* spp. from marine to freshwaters ecosystems, which may involve emerging health risks for wildlife. This was suggested by Shields et al. (2002) regarding the first report of *A. simplex* in *Alosa sapidissima* from two Oregon rivers and also by Bao et al. (2013) concerning the first report of *A. simplex* in *Petromyzon marinus* in the Minho river. The other marine parasite nematode *H. aduncum* and the Hemiuridae, were previously reported in a few scientific researches (Aprahamian et al., 2002). And, although another member of the **Rhadinorhynchidae** acanthocephalan (*Rhadinorhynchus lintoni*) was well known in Allis shad (Aprahamian et al., 2002), to the best of our knowledge this is the first report of *Rhadinorhynchus pristis* in this anadromous fish. This parasite was reported infecting mackerels (*Scomber scombrus* and *Scomber japonicus*) from the Portuguese coast and other scombroid fishes and swordfish (*Xiphias gladius*) from both sides of the North Atlantic (Costa et al., 2004), and also found in the stomach of conger eel (*Conger conger*) from Madeiran waters (Costa et al., 2009). Furthermore, the finding of an ectoparasite Cymothoidae isopoda agrees with previous records of this crustacean in marine, freshwater, or brackish water teleost fishes (Öktener and Trilles, 2004). The parasitic isopods are blood feeding; several species settle in the buccal cavity of fish, others live in the gill chamber or on the body surface including the fins. There are few previous records about these isopoda infecting shads, specifically Cymothoidae parasites were found infecting *Alosa fallax* (Öktener et al., 2009).

Considering Allis shad juveniles, their gregarious behaviour may lead to a greater catch in the estuarine preferred habitats (Mota and Antunes, 2012). Actually, during the 3-years study, highest catches were always obtained in November (2009 and 2011). As found by Sabatié (1993) for the Sebou, it was rare to find YOY in the Minho estuary during the summer, yet numbers increased by early autumn. Moreover, physical factors, such as first autumn floods and decreases in water temperature, seem to induce downstream migration (Cassou-Leins and Cassou-Leins, 1990; Sabatié, 1993), though YOY stayed in the Minho's TFWs after these events. The few studies concerning the juvenile freshwater phase lead to fragmented information. They do not provide information at a consistent level (Taverny et al., 2000) and comparisons between populations are thus difficult to achieve. Due to the concise analysis of Northern and Southern populations, the latitudinal pattern for the YOY seawards migration is unclear. Indeed, the largest YOY arrive in the estuary between late August to mid-September in the Loire (Baglinière et al., 1988), between October to January in the Gironde (Taverny, 1991), between October and December in the Sebou (Sabatié, 1993) and between November and January in the Minho. As suggested by Lochet et al. (2009), the different size of entry in estuary may suggest a different age at which habitat shift occurs. Moreover, the estuarine time window could be affected by several conditions as space, temperature, salinity and food resources (Lochet et al., 2009), which can vary between different estuaries and, thus, reflecting the different times of habitat shift.

In the Minho River, the inconsistency of catches during the 3-years study can be attributed to two hypothetical causes. First, high numbers of spawners can cause high recruitment. This could explain the great YOY catches in 2009 but not in 2011, when the number of spawners was less. Given the low number of spawners in 2011, the better reproduction success compared to 2010 could be due to physical conditions. The second factor affecting the reproduction success in the Minho Allis shad population may be river flow, influenced by high dam discharge fluctuations. In fact, the spawning ground of Minho's Allis shad is located immediately below the first dam (Mota and Antunes, 2012) where great oscillations in the water column can occur. If, on the one hand, heavy discharges and associated floods can drag the eggs and larvae from the suitable habitat, on the other hand, severe water retention and an abrupt drop in water height during several hours can expose nests, rendering eggs unviable, hence constraining the reproduction success and recruitment.

The number of the gill rakers along the first gill arch is one of the meristic characteristics that effectively distinguish Allis shad from Twaite shad (Sabatié et al., 2000; Baglinière et al., 2003) and determine the extent of hybridization between the two species (Maitland and Lyle 2005). Whereas in adults it is assumed that Allis Shad normally have more than 90 rakers and Twaite Shad less than 60 rakers (hybrids with intermediate numbers), in juveniles the differentiation is done through the relationship between the number of gill rakers on the first gill and the total length (TL), which, in anadromous juvenile shad, are positively correlated (Hillman et al., 2003; Taverny, 1991). Our model of the number of gill rakers depending on TL show that Minho's Allis shad juveniles have higher numbers of gill rakers per TL than Aude River and Sebou River juveniles (the same applies to adults in the latter river; Sabatié et al., 1996) and lower numbers of gill rakers per TL than Adour River juveniles (see Aprahamian et al., 2003). In comparison to those of the Aulne River, and although the number of gill rakers are lower for smaller individuals, our linear model suggests a difference between gill raker growth rates in favour of Minho's juveniles from

13 cm TL on (Minho: 32.54+2.56×TL, R^2=0.63; Aulne: 45.6+1.58×TL) (see Aprahamian et al., 2003).

Hybridization between *A. alosa* and *A. fallax* is a well-known phenomenon among European and North Africa shad populations (see Alexandrino and Boisneau, 2000). As we have seen, morphological confirm the presence of hybrids in the Minho shad population. River dams are one of the factors that can lead to hybridization, as they may disturb migration and induce an overlap of the spawning areas (Boisneau et al., 1992). In the Minho River, five dams were built between the late 1950s and the early 1970s, but overlap of spawning areas is not clear. In fact, *A. alosa* and *A. fallax* spawning grounds were quite apart and, even after dam construction, it seems that the two species' spawning areas remain substantially separated (by about 30 km) (personal observation). Although is not clear how hybridization happens when spawning grounds are clearly separated (Maitland and Lyle, 2005), the reduction of water flow imposed by dams could cause some *A. fallax* individuals to migrate up in search of original water volume/flow (Faria, 2007) where a certain hybridization will occur. Further research is needed concerning this issue, mainly supported by genetic data, in order to better understand the pathways of hybridization in the Minho shads.

Albeit with some degree of uncertain due to the inconsistency of catches in the 3-years study, some insights on the migratory pattern of Minho shads' YOY may be hypothesized. The observed general increasing trends in size (TL) from September to January in the TFWs sampling site may reflect a size-dependence to the seaward migration. Lochet (2006) found that, for the Gironde-Garonne-Dordogne Allis shad YOY, the entry into the estuary is non-size-dependent but the exit to marine habitats is size-dependent. According to Limburg (1996a), the American shad (*Alosa sapidissima*) YOY downstream movement seems to be age-dependent and larger individuals seem to move downstream earlier than smaller individuals form the same age cohort.

In freshwater and brackish water, the diet of Allis and Twaite YOY shads is quite similar, based mainly on zooplankton (Cassou-Leins et al., 1988). As found by Mota and Antunes (2012), the present work also shows that the diet of Minho's Allis shad YOY in the TFWs, as well as that of Twaite shad and hybrids, is mainly composed of brackish crustaceans. Although crustaceans seem an important diet item (Mota and Antunes, 2012), YOY shads appear to be euryphagous and opportunistic, as they resort to a wide variety of trophic resources and to the most accessible preys existing in the estuarine environments. Actually, the macrozoobenthic community composition reported by Sousa et al. (2008) nearby our YOY sampling station seems to be, apart from *Corbicula fluminea*, mainly composed of brackish crustaceans such as *Corophium multisetosum*. Consequently, it could be hypothesised that the two YOY shad species compete with each other for the

TFWs food resources. However, it is suggested that closely related species require different ecological niches (Chase and Leibold, 2003) and, when they share the same food resources, their coexistence involves habitat partitioning in time and space (e.g., Hesthagen et al., 2004; Lecomte and Dodson, 2005; Sandlund et al., 2010). In fact, Lochet et al. (2009) argued that, during seaward migration, the two sympatric species exhibit different pattern of estuarine use. One the other hand, the difference in the branchial filter morphology points to an ontogenetic variation in diet, as Twaite shad is able to feed on fish even at a small size (Oesmann and Thiel, 2001). The ontogeny and the expected residence time difference in the estuary indicate that the competition for food resources between sympatric shads at young stages should be minor in the Minho.

The fact that the Minho's YOY hybrids presented the highest Vacuity index seems to us a curious aspect. Once hybrids have intermediate numbers of gill rakers, we can speculate that they have a selective disadvantage in foraging. In fact, as previously seen, the adult hybrids population is much lower than YOY hybrid population, leading us to question the fitness of the hybrids.

2.3.5. CONCLUSION

As broadly known, effective management plans promoting sustainable human use and conservation of species should be based on solid scientific information. This study has increased our knowledge on the structure and migration of the Minho River Allis shad population, giving biological and ecological insights to adequately manage and conserve this important resource. It furthermore provides significant information that affords managers with some level of predictableness of migration patterns.

For Minho's Allis shad, an effective conservation plan should attempt to maintain current stocks. The goal of a successful conservation plan should be to restore or maintain a sustainable natural ecosystem while sustaining the community services needs (e.g. Wilzbach et al., 1998). Likewise, dam water discharges should be managed to ensure adequate water flow and speed throughout the spawning grounds and the YOY freshwater habitat or, at least, during potential peak periods of migration.

The time series data of the population dynamics provided in this study may be useful to be applied in model-based analysis and recruitment prediction. Furthermore, understanding cues for adult and juvenile fish migration could help managers to recognize critical migration periods and associated factors so that informed decisions can be taken.

2.3.6. ACKNOWLEDGMENTS

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2.4. ASPECTS OF THE REPRODUCTIVE BIOLOGY OF FEMALES *Alosa alosa* (L.) IN THE INTERNATIONAL MINHO RIVER (NORTHWESTERN IBERIAN PENINSULA)

Submitted to the Journal of Applied Ichthyology: M. Mota, A. Bio, R. Carvalho, A. Alves, E. Oliveira, A. Lobo-da-Cunha, E. Rochard and C. Antunes. Reproductive biology of females *Alosa alosa* (L.) in the international Minho River (Northwestern Iberian Peninsula).

ABSTRACT

Ovarian development, fecundity and GSI of female *Alosa alosa* were studied during the spawning migration season in the International Minho River. Samples were collected throughout three years on a monthly basis in three different sections of the river: low estuary, upper estuary and spawning grounds. GSI increased with time and was higher within the spawning area. Mean Relative Fecundity was 87×10^3 eggs/kg and the increase of eggs per kg extra body was around 135 000 eggs/kg. The oocyte development of *A. alosa* showed an asynchronism of maturation. Histological analysis shows that females spawning in batches over a period of days or weeks since oocytes at different stages of development (recently developed post-ovulatory follicles, older post-ovulatory follicles and advanced yolked oocytes) are observed simultaneously. The state of oocytes varies along the migratory route, since more advanced state of maturation (VI oocytes) was observed in summer than in spring.

Key words: Allis shad, Female, Spawning, Fecundity, GSI, Oocyte developmental stages.

2.4.1. INTRODUCTION

Several diadromous fish populations around the world have been reported as endangered species (Groot, 2000; Wolff, 2000) particularly due to anthropogenic pressure. Anadromous fish mature and overwinter in the sea and spawn in fresh water. and their life cycle successful entails beneficial conditions in both freshwater and marine habitats.

The Allis shad *Alosa alosa* (Linnaeus, 1758) is an anadromous fish of the family Clupeidae, and its fishery has a long history in places where the species occurs. In France and Portugal, Allis shad fishery is carried out since the Middle Ages, and in Morocco it has been a secular tradition that started long before the 18th century (Elie et al., 2000).

The shad’s stock has declined dramatically since the 1950s throughout its geographic range. The gradual decreasing of *Alosa* species in several European basins, caused essentially by anthropogenic pressures, led to its listing in the Red List of the International Union for the Conservation of the Nature (IUCN), in Annex III of the Bern Convention, and in the Habitats Directive (Aprahamian et al., 2003).

The Allis shad is a commercially important fishery resource in the Minho River (NW Iberian Peninsula). Compared to the catches of 200 tonnes described for the first half of the 20th century (Mota and Antunes, 2011) catches decreased by about 90% after the 1950s, and even in the best years of the last decades, captures never rose to these original values again. However, this species still migrates into the Minho River, which holds what is thought to be one of the last stable European shad populations (unpublished data).

Despite the important ecological role of *A. alosa* and its high commercial value, reproductive biological and gonad development information is very scarce. Several studies have clarified some aspects of the biology, ecology and conservation (e.g. King and Roche, 2008; Lassalle et al., 2008b). In Portugal, only few works were done concerning the biology and ecology of *A. alosa* in the Douro River (Eiras, 1977; 1981a). More recently, primary studies took place on the Minho River Allis shad population, analysing the spawner’s biology (Mota and Antunes, 2011) and juveniles’ habitat use in freshwater (Mota and Antunes, 2012). In this river, the spawning migration occurs between March and June and may extend occasionally from January to July and June seems to be the most favorable month for the reproduction of Allis shad in the Minho (Mota and Antunes, 2011).

There are only few published studies on ovarian maturation in *Alosa* species. In Portugal, Pina et al. (2003) described the oogenesis stages for *A. fallax*. gross and microscopic descriptions of ovaries and oocytes were reported for the anadromous Allis shad in the Garonne river by Bengen et al. (1991) and for freshwater populations of the Moroccan shad by Lahaye (1960) and by Clift (1872), and by Mylonas et al. (1995) for American shad (*A. sapidissima*).

However, as reproductive strategy is a key mechanism determining recruitment and population dynamics (McGraw and Caswell, 1996), comprehension of spawning migration and reproductive biology is crucial to establish successful conservation and management for this species.

In the present work, we investigated the histological gonad development in anadromous Allis shad females during the spawning migration. This work contributes to deepen the knowledge on the Minho River Allis shad reproductive biology, complementing a comprehensive study on the biology and ecology of this species, reported by Mota et al. (unpublished data), in which the authors focused on gross aspects of the species reproductive biology, such as semelparity, spawning season and ovarian development according to the gonadosomatic index (GSI).

We studied for the first time the histological aspects of oocytes of the Minho River Allis shad, in order to analyze the gonads evolution during the spawning migration, regarding the oogenesis stages in different capture zones (low estuary, tidal freshwater wetlands and spawning grounds) along the migration route and the developmental stages on a monthly and inter-annual basis. We examined the correlation between GSI and each developmental stage and fecundity. The results of our work may be used to define management and conservation measures to cease and invert the decline in the Minho's Allis shad population and to improve its conservation status.

2.4.2. MATERIALS AND METHODS

2.4.2.1. Data collection

Specimens were sampled by experimental fishing and obtained from fishermen practicing small-scale fishery in the international part (Spain and Portugal) of the Minho River, between March and August of 2009, 2010 and 2011. For the experimental fishing, two sampling stations were selected: the estuary and the spawning area. A typical trammel net (140 m long; 140 mm loose inner layer mesh size) was used.

Fish were kept refrigerated immediately after capture and brought to the laboratory. Total length (TL) was measured to the nearest 0.1 cm (measuring board) and total weight (TW) was determined to the nearest 5 g (digital hanging Kern HDB balance). Gonads were removed and the gender was subsequently determined. Female gonads were weighted using a digital A&D EK-610i scale with a precision of 0.01 g. A sample of approximately 1cm^3 of ovary was collected from 60 females and preserved in 70% alcohol, to count the eggs and calculate fecundity. Ovary samples of 54 females (14 from 2009, 23 from 2010 and 17 from 2011) were collected and preserved in in Bouin liquid during 24h, for histological analysis. After fixation, samples were dehydrated in ethanol, embedded in paraffin and sectioned at 6 µm using a Leica RM 2255 microtome. Sections were stained with Hematoxylin-Eosine (H-E) and, afterwards, covered with DePex mounting medium for microscopy.

2.4.2.2. Data analysis

Females Allis shad were separated according to the capture zone, i.e. upper (spawning grounds), mid zone (tidal freshwater wetlands - TFWs) and lower estuary.

The gonadosomatic index (GSI), was calculated as: GSI = 100*(Wg ⁄ Wt), where Wg is the gonad weight and Wt is the fish total weight, to assess changes in fish maturity and spawning stage during the upstream migration. Differences in GSI were addressed between months, between sampling zones, and between months in each capture zone. The analysis was performed both including and excluding the post-spawning females (i.e. females presenting ovaries at least partially spent).

Fecundity was estimated by direct counts of fixed mature ovarian subsamples. Individual fecundity was computed as: F = N×Wg/GSW, where N = total number of oocytes, Wg = gonadal weight and GSW = gonadal subsample weight. F was divided by the weight of the fish to estimate relative fecundity (FR). The linear model of fecundity dependent on fish weight was computed.

Histological characteristics were used to classify oocyte development and complement the information of gonads maturity given by GSI. The average number of oocytes for each developmental stage according to size, cytoplasm staining and follicular cell differentiation was estimated in six randomly selected microscopic fields of each of the three slides per individual. The oocytes development stages were categorized according to Pina et al. (2003) and Olney et al. (2001). The maximum diameter of oocytes was measured for each developmental stage to the nearest 0.01µm.

The different developmental stages were tested for differences in frequencies of the developmental states, differences between zones, for Inter-annual differences per capture zone and for differences between months per capture zone. The seasonal variation of the frequency of developmental stages was also addressed as well as the relationship between cell diameter and developmental stage, between the number of atretic (AR) oocytes and capture zone and between the number of AR oocytes and GSI including spawned females.

Finally, the correlation between the developmental stages and GSI of spawned and non-spawned females was analyzed per month and per capture zone.

For each developmental stage, oocyte diameters were divided into frequency classes (with class intervals of 50 µm for the LP stage, 100 µm for CA and PV, and 200µm for VI). Samples were compared in terms of oocyte developmental stage frequencies (counts and percentages, using stacked barcharts) and differences for each oocytes type were tested for significance. Data were tested for normality using the Shapiro-Wilk Normality test. Given that many groups failed the normality test, treatment effects were assessed through a distribution free analysis of variance using the Kruskal-Wallis test to determine if any group was different from the others, and post-hoc pair-wise Wilcoxon rank sum tests, corrected for multiple testing, to see which groups differed (Hollander and Wolfe, 1973). A Kruskal-Wallis rank sum test was also applied to test for differences between the frequencies of the developmental states. All test results refer to an α=0.05 significance level. Analyses were done in R (R Dev. Core Team).

2.4.3. RESULTS

The females' GSI excluding spawned females, increased with time, being significantly (*p*<0.05) higher in May than in April and in June than in April (Fig. 2.23A). The difference between June and May is non-significant (*p*=0.058). No significant difference was observed between July and previous months (NB: there were only 4 females captured in July). When looking for GSI differences between months including spawned females, a decrease in GSI values in July and August was observed (Fig. 2.23B).

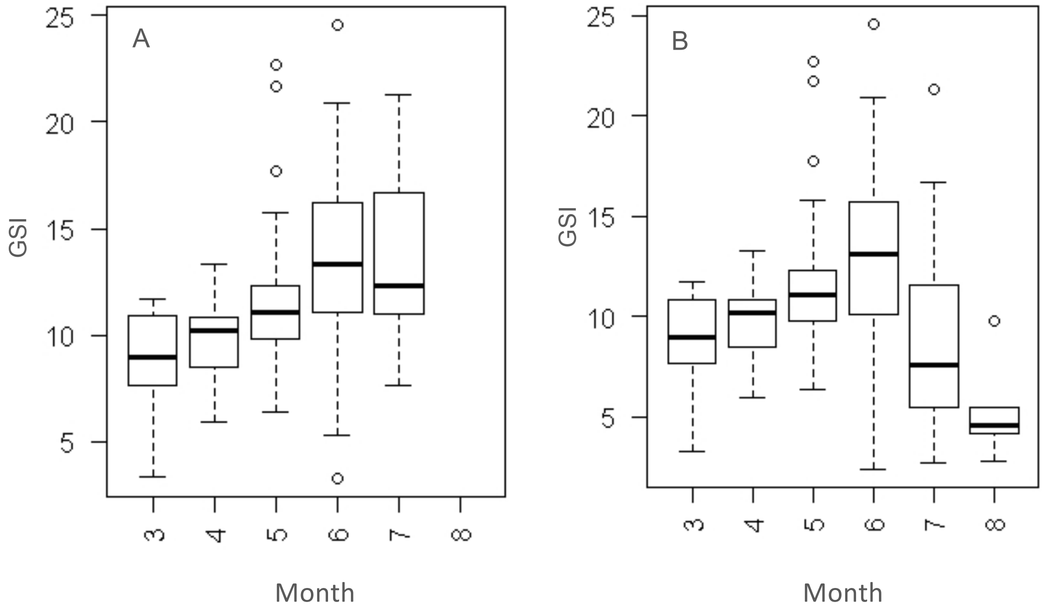

Figure 2.23. Monthly changes in *A. alosa* Gonadosomatic Index (GSI) during the upstream spawning migration. A: GSI variation per month (March to July) excluding spawned females; B: GSI variation per month (March: 3 to August: 8) including spawned females. The boxplots present the median (central line) and the interquartile

range (IQR), from the first to the third quartile (box). Whiskers indicate the minimum and maximum values excluding outliers (i.e. points that differ more than 1.5 times the IQR from the respective quartiles), which are presented as separate circles.

Excluding spawned females, the GSI was higher in the upper zone (spawning zone), but no significant differences were observed between the GSI of the low and mid zones (Fig. 2.24A). When spawned females were included in the analysis, no significant differences in GSI were observed between zones (Fig. 2.24B). The GSI per month and capture zone including spawned females showed GSI to be lower in July and August in the upper zone (Fig. 2.25) but significant pair-wise differences were found only between July and May and June GSI. No significant differences were found in monthly GSI in the low and mid zones both including and excluding spawned females.

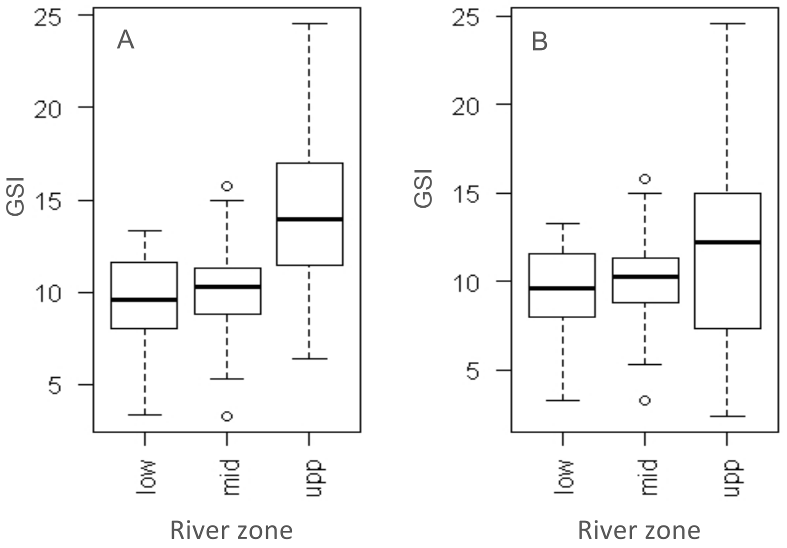

Figure 2.24. Changes in *A. alosa* Gonadosomatic Index (GSI) during the upstream spawning migration. A: GSI variation per river zone excluding spawned females; B: GSI variation per river zone including spawned females. The boxplots present the median (central line) and the interquartile range (IQR), from the first to the third quartile (box). Whiskers indicate the minimum and maximum values excluding outliers (i.e. points that differ more than 1.5 times the IQR from the respective quartiles), which are presented as separate circles.

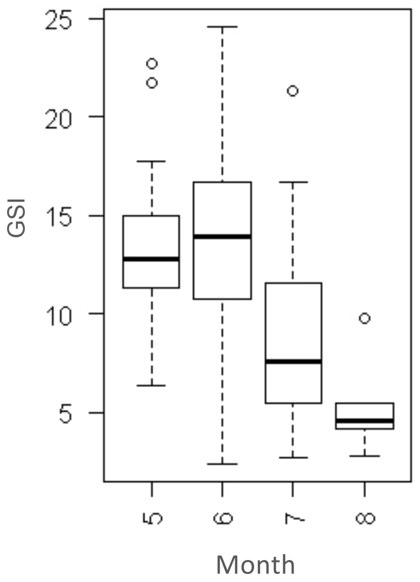

Figure 2.25. Monthly changes in *A. alosa* Gonadossomatic Index (GSI) in the upper zone including spawned females. (from May: 5 to August:8). The boxplots present the median (central line) and the interquartile range (IQR), from the first to the third quartile (box). Whiskers indicate the minimum and maximum values excluding outliers (i.e. points that differ more than 1.5 times the IQR from the respective quartiles), which are presented as separate circles.

Mean fecundity (F) for the 3-year study was 230×103 eggs/ovary (min. 36×10^3, max. 564×10^3 eggs/ovary), and mean FR was 87×10^3 eggs/kg, (min. 23×10^3, max. 155×10^3 eggs/kg). The linear model of F dependent on females weight showed that fish weight is a good and very significant predictor of F (R^2=0.67, p<0.001) (Fig. 2.26). The increase in the number of eggs was about 135 000 per kg extra body weight.

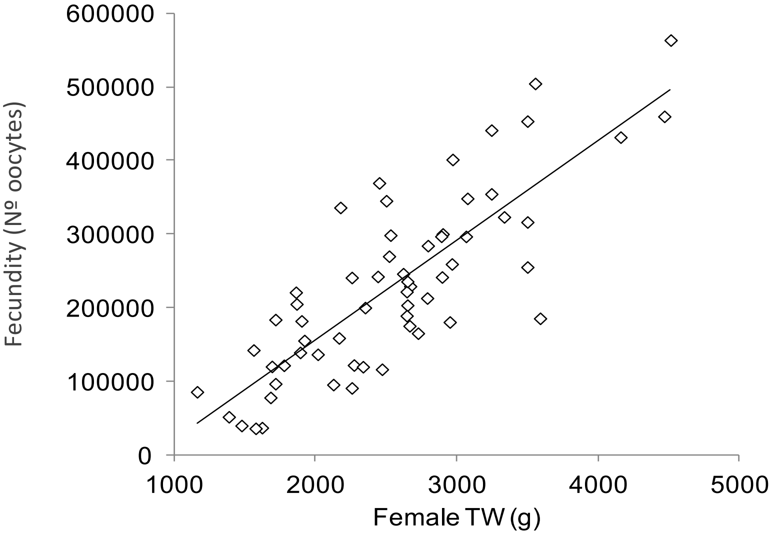

Figure 2.26. Linear model of Fecundity (N oocytes) depending on *A. alosa* females' weight.

Histology revealed Allis shad gonads maturity during the spawning migration to have five oocytes development stages (Fig. 2.27), distinguishing: (1) Late perinucleolus stage (LP) - oocytes without yolk, nucleoplasm with granular appearance, several small nucleoli dispersed in the periphery of the nuclear membrane, basophilic cytoplasm and high nucleus/cytoplasm ratio; (2) Cortical alveoli stage (CA) - appearance of variably sized cortical alveoli and the vitelline envelope becomes prominent; (3) Pre-vitellogenesis stage (PV) - advanced coalescence of lipid drops and yolk globules forming a sole large oil droplet and a continuous yolk mass, the nuclear membrane breakdown and the nucleus is not distinguishable in the cytoplasm; (4) Vitellogenesis stage (VI) - oocytes showing a continuous mass of yolk and a large oil droplet, resulting from the complete coalescence of yolk globules and the lipids; (5) atretic oocytes (AR) - the oocyte and surrounding follicular cells degenerate and are replaced by connective tissue.

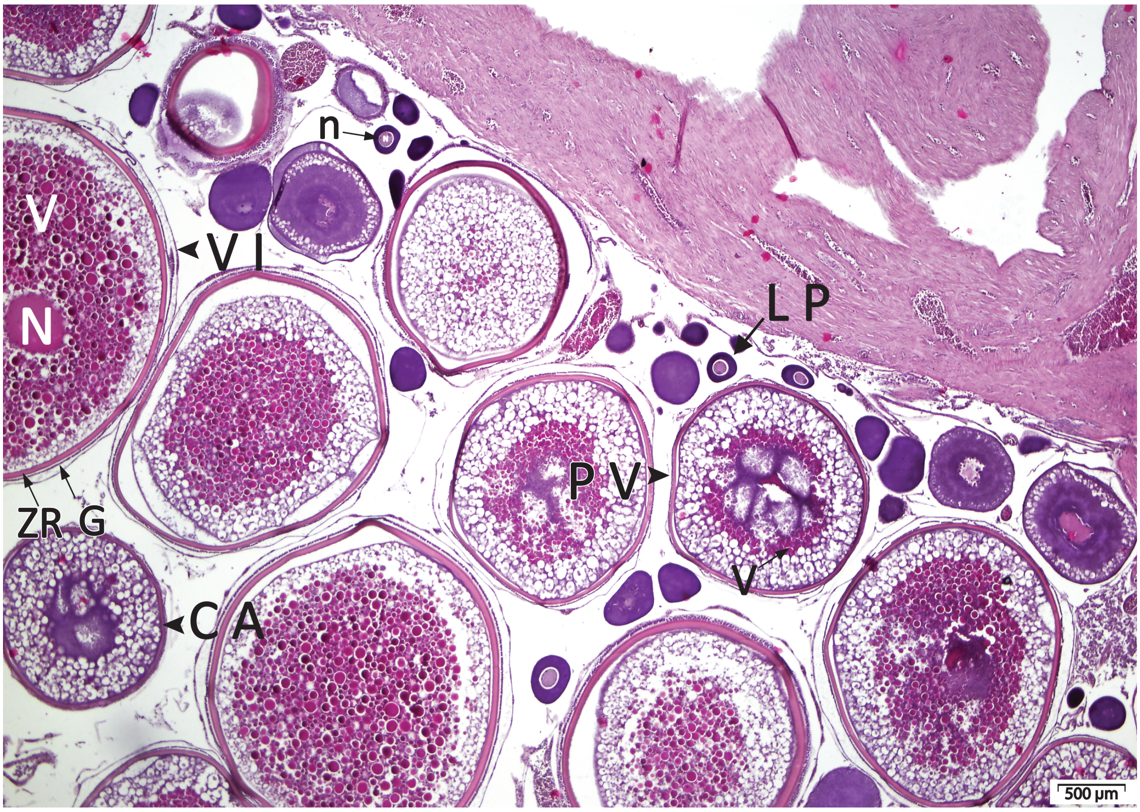

Figure 2.27. Oocyte developmental stages of Minho's *Alosa alosa*. LP - Late perinucleolus stage; CA - Cortical alveoli stage; PV - Pre-vitellogenesis stage; VI - Vitellogenesis stage; N – Nucleos; n – Nucleoli; ZR – Zona radiata; G – Granulosa cells; V – Yolk.

According to the Kruskal-Wallis rank sum test, differences between the frequencies of the developmental states were not significant (p=0.067); i.e. data do not show any frequency to be significantly different from the others.

Regarding differences in developmental stages between zones, AR is significantly different in all zones (gradually increasing); LP is significantly different in the upper zone, compared to both the low and the mid zones. No differences were found for the other developmental stages per zone. No significant Inter-annual differences were found for the developmental stages with the exception of AR in year 2010, which was significantly different from the AR in 2011. Percentages per capture zone showed increasing AR and VI from the lower to the upper part of the estuary, and decreasing CA and LP (Fig. 2.28A). In spite of the overall significant difference between years in oocytes developmental stages of the mid zone samples, no pair-wise difference was significant. No other significant differences between years per zone were found. The LP stage in April was significantly different from August and July LP, and March LP is significantly different from August and July LP. Despite the overall significant difference in PV and AR between months, no pair-wise difference was significant for these developmental stages between months. No other significant differences between months were found.

Considering the seasonal variation in the frequencies of developmental stages, there were higher percentages of VI and AR oocytes, and lower percentages of LP, in summer than in spring (Fig. 2.28B). Seasonal differences were significant for AR (*p*=0.007), PV (*p*=0.021) and LP (*p*=0.0002), and non significant for VI (*p*=0.36) and CA (*p*=0.062).

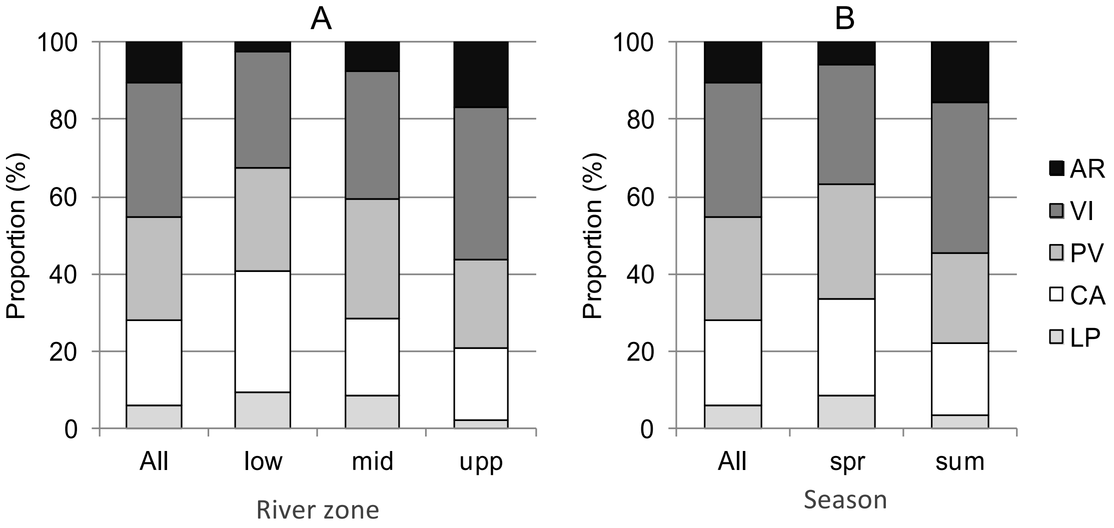

Figure 2.28. Proportion of oocytes in different development stages (LP: late perinucleolus stage, CA: cortical alveoli stage, PV: pre-vitellogenesis stage, VI: vitellogenesis stage, and AR: atretic oocytes). A: for all females and per zone, B: for all females and per season.

Spearman rank correlation analyses of developmental stages with GSI, per capture zone and excluding spawned females, showed significant negative correlation between GSI and CA in the lower zone (*p*=0.019). No other significant correlation was found. When including spawned females, the only significant correlations found were between GSI and LP (*p*=0.0017), and between GSI and PV (*p*=0.0012), in the upper zone. Considering correlations of developmental stages with GSI per month, including spawned females, GSI was significantly negatively correlated with AR in April. No other significant correlation was found, both including and excluding spawned females.

The relationship between cell diameter and developmental stage showed that all pairwise differences were significant; i.e. all oocytes types had significantly different diameters (Fig. 2.29A). Observing the relationship between the number of AR and the capture zone, once again all pairwise differences were significant, as all zones showed significantly different AR frequencies (Fig. 2.29B).

The relationship between the number of AR and GSI was no significant both for analyses per month and per capture zone, and both including and excluding spawned females.

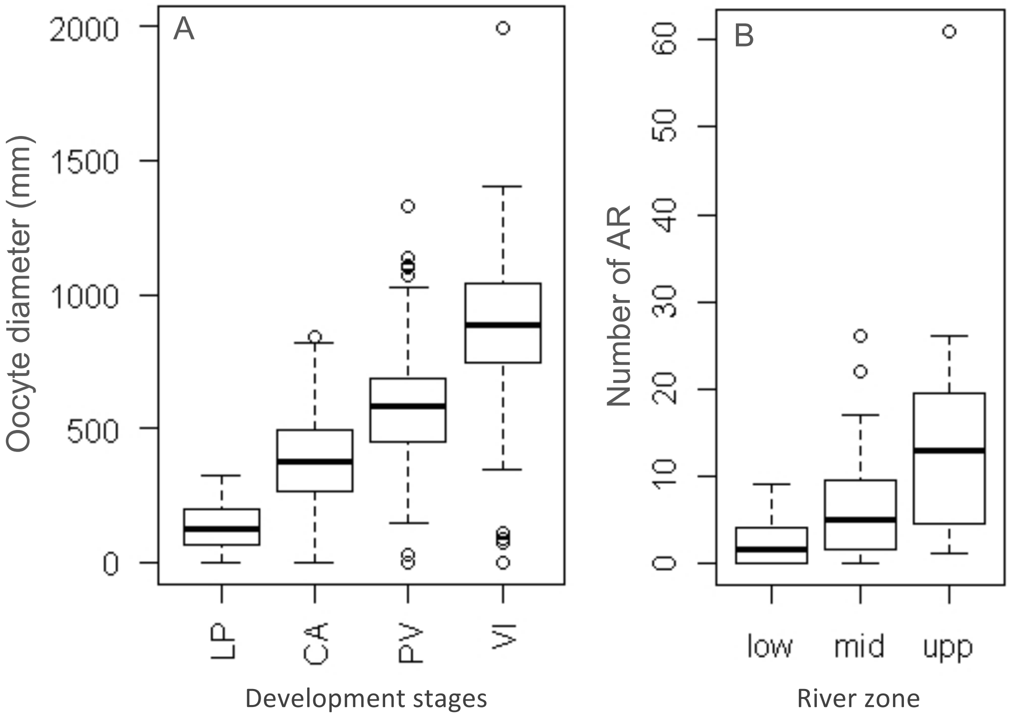

Figure 2.29. A: Diameters of oocytes in different development stages (LP: late perinucleolus stage, CA: cortical alveoli stage, PV: pre-vitellogenesis stage, VI: vitellogenesis stage, and AR: atretic oocytes); B: Number of AR per capture zone. The boxplots present the median (central line) and the interquartile range (IQR), from the first to the third quartile (box). Whiskers indicate the minimum and maximum values excluding outliers (i.e. points that differ more than 1.5 times the IQR from the respective quartiles), which are presented as separate circles.

Figure 2.30 shows the oocyte diameter frequency classes for each stage. The histogram of the LP stage cell diameters is polymodal, with high frequencies of diameters between 50 µm and 250 µm. For the PV stage diameters between 400 µm and 800 µm were predominant. The other maturity stages have a single mode, with diameters between 300 µm and 400 µm for CA, and between 800 µm and 1000 µm for VI.

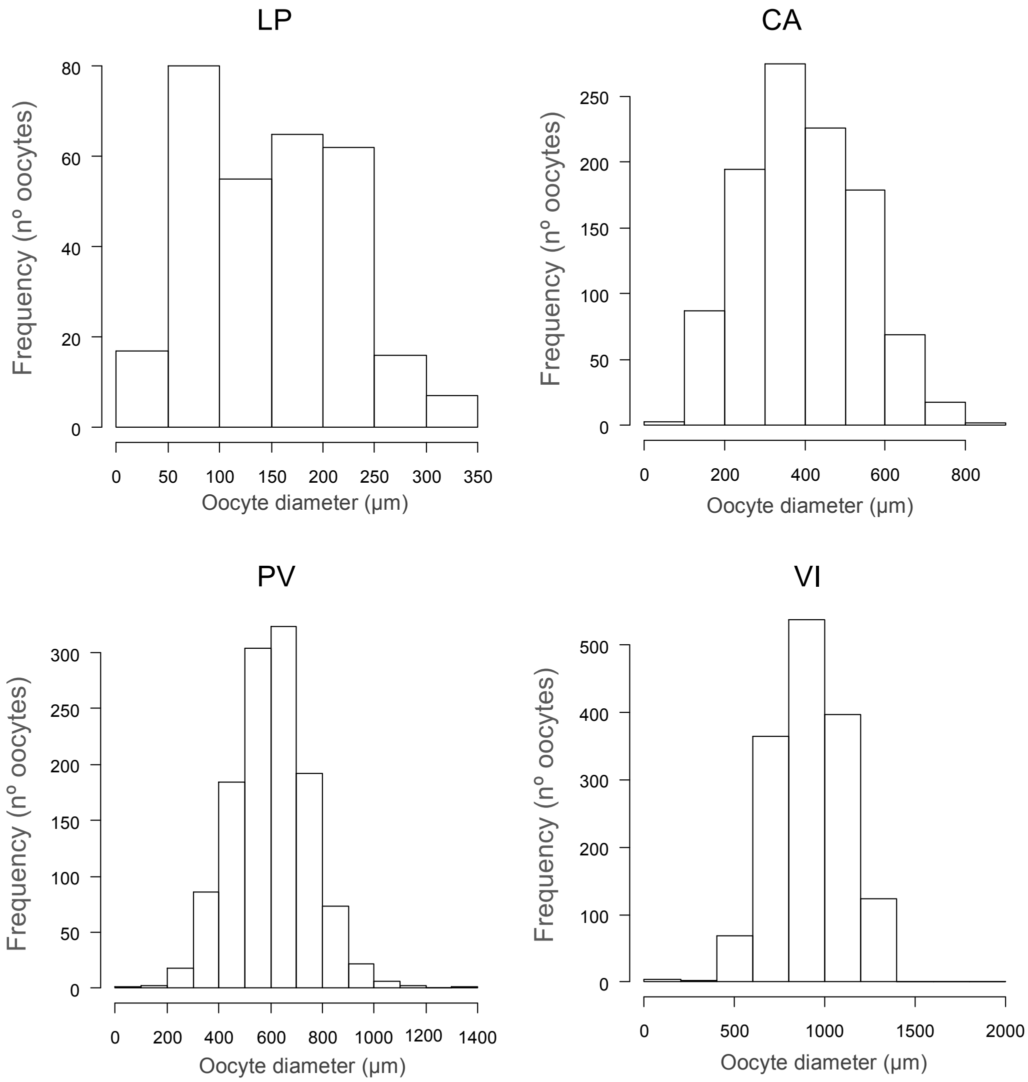

Figure 2.30. Oocyte diameter frequency classes for each development stage (LP: late perinucleolus stage, CA: cortical alveoli stage, PV: pre-vitellogenesis stage VI: vitellogenesis stage.

2.4.4. DISCUSSION

The Gonadosomatic Index (GSI) usually provides an indication of the sexual maturity of fish. Maturing Minho's Allis shad migrate around 80 km up the estuary to the spawning grounds and ovary size increases during the migration route. As found by Bengen et al. (1991) for the Garonne population, it seems that the complete maturation of the Minho's' Allis shad gonads occurs only in the spawning zone, where GSI values are significantly higher than those in the mid and lower zones. But, in the low and mid estuary, the high values of GSI confirm the advanced maturity stage at the beginning of the spawning migration.

The decline of the GSI values in July and August in the upper zone indicates June as the most favourable month for the reproduction of Allis shad in the Minho. Our study did not detected relevant correlations between GSI and oocytes developmental stages. Though GSI could reflect the developmental stage of oocytes and give a gross indication of the microscopic ovary maturation along the spawning migration that was only confirmed for the (negatively correlated) CA stage in the lower capture zone.

The negative GSI-AR relationship found for April when spawned females were included is difficult to explain, as (as seen above) the best month for the Minho's Allis shad seems to be June and it is unlikely the presence of many spawned females in April, just as the positive relationship GSI and LP in the upper zone. The positive relationship between the upper zone GSI and PV may be more understandable, as PV seems to be, along with VI, the most frequent developmental stages (see below), with larger oocyte diameters than early stages. Yet, this does not explain why the same relationship is not seen for the VI stage. In short, the found relationships between GSI and the oocytes stages do not accurately represent the microscopic maturation stage.

The females' relative fecundity is lower than that described by Baglinière et al. (2003) (i.e. 100–150×10^3 eggs/kg), but well within the ranges of southern populations (Sabatié et al., 1996; Eiras, 1981a) (76.8×10^3 eggs/kg and 79,9×10^3 respectively). As for other European and Moroccan populations (Eiras, 1981a; Aprahamian et al., 2003), a significant correlation was found between absolute fecundity and the females' total weight. The linear model of F dependent on weight predicts a considerable increase in the number of eggs per additional kg of body weight, indicating that bigger females are more productive.

Although the relative fecundity values found are within the clinal gradient described for the species (decreasing in southern stocks) (Cassou-Leins et al., 2000; Aprahamian et al., 2003), precautions should be taken when comparing and interpreting data, since the methodology applied for fecundity estimation may vary (Sabatié et al., 1996; Cassou-Leins et al., 2000; Aprahamian et al., 2003). On the other hand, comparisons of absolute fecundity become even more complicated as the majority of published studies only refer to extreme values or to the relationship between absolute fecundity and ovarian weight and total lengths and weights of the fish (e.g. Cassou-Leins et al., 2000; Aprahamian et al., 2003). We observed an unremitting distribution of oocytes stages in mature ovaries and, as suggested by Olney et al. (2001) for American shad, Allis shad probably exhibit indeterminate fecundity and, thus, previous evaluations of fecundity reported for shad's populations might be imprecise. Moreover, batch fecundity, spawning frequency and spawning duration remains unknown.

The activity of oocyte development of Allis shad showed to be similar to those reported by Pina et al. (2003) to *A. fallax*, by Olney et al. (2001) for *A. sapidissima* and by Bengen et al. (1991) for the Garonne *A. alosa*. During the spawning season, Allis shad oocyte development is a continuous activity comprising several stages of oocytes, with a new spawning batch maturing many times in highest spawning months. Oocyte ultrastructure and development stages of *Alosa* are typical of other clupeiform fishes (Hunter and Maciewicz, 1985). They show an asynchronous oocyte development, where oocytes in many stages of development appear simultaneously in reproductively active ovaries (Wallace and Selman 1981). The ovaries of mature *Alosa* contain perinucleolus, unyolked, partially yolked, and yolked oocytes, corresponding to the LP, CA, PV and VI stages respectively. But, though not significantly, the most frequent developmental stages observed within the basin were PV and VI. Same observations were assigned to the Garonne Allis shad population by Bengen et al. (1991).

Females are batch spawners (they spawn repetitively during the spawning season) since ovaries of partially spawned individuals showed PV and VI oocyte stages, as well as postovulatory follicles. The multiple stages found are an indicator of earlier recent spawns, representing the developing oocytes, the next batches of oocytes. For *A. fallax*, Pina et al. (2003) demonstrated that all oocytes stages were present in the ovary simultaneously, from late immature onwards and during all year, so called a "reserve fund", consisting in oocytes that in certain point seems to stop their development for some time and resume growth towards maturation, seeming a regeneration of the oocytes in the perinucleous stage at the beginning of each reproductive cycle. The same was not observed for the Minho's Allis shad, but probably because, unlike the *A. fallax* that have an iteroparous life cycle, Allis shad is semelparous, spawning only once in its life cycle.

The higher percentages of VI and AR oocytes in summer than in spring correspond to more mature ovaries in upper zone (summer months) that in the lower zone (spring months), as Allis shad abundances in the lower and central zones are higher in spring and decrease towards earlier summer, increasing at the same time in the upper zone (unpublished data). Unlike verified for the Garonne Allis shad (Bengen et al., 1991), this fact might indicate that the state of maturation of oocytes is not constant along the migratory route. It may be constant in the lower and mid zone, but in the upper zone we assist to an increase in the percentage of VI oocytes stage.

With the exception of LP stage where no predominant oocyte frequency class is evident, all the other stages showed a predominant frequency class diameter. No gap in the diameter frequency distributions was found between the CA and PV/VI development stages. The same was found by Pina et al. (2003) for *A. fallax* and, as thought by the author, it must represent the ability to push oocytes throughout vitellogenesis from a previtellogenic stage during the spawning season. This kind of oocyte development has relevant implications in fecundity, as accurate fecundity evaluations depends on estimates of spawning frequency and batch fecundity (Pina et al., 2003).

Although all capture zones showed oocytes with different AR frequencies and this development stage was more frequent in the upper zone, the AR development stage was present in all sampling zones. The fast upstream migration can induce atresia, since yolk may be an energetic resource when food supplies stops (Pina et al., 2003) and, thus increases atresia rates (Bengen et al., 1991). Olney et al. (2001) also reported that energy reserves of partially spent ovaries could be recovered by resorption of yolked, un-spawned oocytes. As upstream migration is energetically expensive, tissues where energy is saved could apparently be used to improve recovery from migration (Leonard and Mccomick, 1999).

According to Olney et al. (2001), both semelparous and iteroparous populations of American shad are batch spawners. The author also affirms that populations exhibiting both life history strategies are resilient to unpredictable reproductive environments. Transposing to the European populations, this aspect may have been taken into account in the survival of the scarce landlocked populations (Castelo do Bode and Aguieira reservoirs in Portugal and River Guadalquivir in Spain) after construction of dams. Although we did not observed the oocyte “reserve fund” reported by Pina et al. (2003) for *A. fallax*, this may be also present in *A. alosa* (we just studied the ovary during the spawning migration) and regenerate at the beginning of the reproductive cycle. Moreover, Olney et al. (2001) hypothesize that funds in partly spent ovaries could intensify somatic energy sources and enhance survival as post-spawning females and, so, partially spent fish may have a better chance than spent fish to become repeat spawners in next seasons.

The annual or lifetime estimations of fecundity is based in the basic assumption of the fixed number of oocytes at the beginning of the spawning season, and that there is no production of new yolked oocytes after spawning begins (Hunter and Leong, 1985). Thus, future studies should focus in specify batch fecundity and spawning frequency, as there are no accurate estimates of spawning duration of Allis shad.

Evaluation of fecundity is a key step to quantify the reproductive potential of individual fish and to understand the stock-recruitment relationship, increasing the knowledge of the state of a stock and improving the assessment of the spawning stock biomass (Murua et al., 2003; Witthames et al., 2009).

Batch spawning has important ecological implications since an individual can spread its gametes over a large spatio-temporal scale, thus increasing the chances that offspring will find healthy conditions for survival (Olney et al., 2001). Upcoming studies should focus on identifying the postovulatory follicles and on the determination of their age, to obtain accurate fecundity estimates and better understand spawning frequency and the number of batches. Another point that should be taken into account in future researches is the direct effect of atresia on oocytes production and on fecundity estimates (Pina et al., 2003).

The present study provides initial information concerning the reproductive biology of Minho’s Allis shad and may serve as a basis for future researches in this field. Comprehension of reproduction and recruitment is crucial to establish preventive and compensatory measures for the successful conservation and management of the species.

2.4.5. ACKNOWLEDGEMENTS

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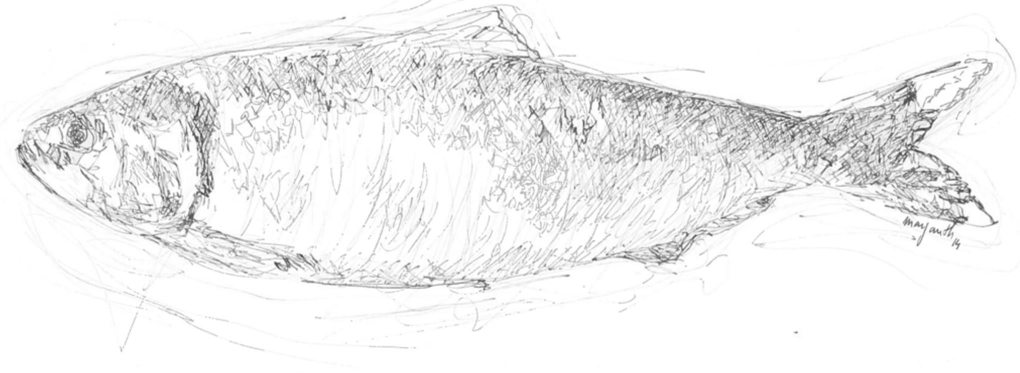
CHAPTER 3

HYBRIDIZATION PATTERNS BETWEEN ALLIS SHAD *Alosa alosa* (L.) AND THE SYMPATRIC TWAITE SHAD *Alosa fallax* (L.)



3. HYBRIDIZATION BETWEEN SYMPATRIC SHADS *Alosa alosa* (L.) AND *Alosa fallax* (L.) IN AN IBERIAN PENINSULA RIVER (MINHO RIVER, NW OF PORTUGAL): FIRST EVIDENCES INFERRED BY MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION

Manuscript in final preparation

ABSTRACT

Allis shad and Twaite shad colonize the Minho River but their populations are scientifically little explored. In order to verify the extent of hybridization between the two anadromous *Alosa* species of the Minho River, Allis and Twaite shads, morphological and genetic characterization were performed by meristic and morphometric counts and by genetic analysis. A Model-based hierarchical clustering was applied to separate the juveniles *Alosa* spp. specimen according to TL-N gill rakes. The spawners' mean number of gill rakers of the first branchial arch for Allis shad was 134.9, 46.5 for Twaite shads, 102.4 for *A. alosa* backcrosses and 68,4 for *A. fallax* backcrosses. For both species, the females had a mean size higher than males. Spawning activity in Allis shad occurs in late June and July and in late May and June for Twaite shad. Morphometric and genetic confirm the presence of hybrids in the Minho shad populations, presenting intermediate morphological and genetic compositions. Most of the Minho hybrids were F2s and backcrossed individuals. A good fit between the morphological and genetic classifications was verified between juveniles' model classification and microsatellite loci analysis. Mismatch between genotype classification and phenotypic classification was low; higher % of mismatches was found among hybrids' classification. The juveniles' model classification of samples into species based on phenotype indicated a higher percentage of hybrids between to juveniles than to adults. The percentage of hybrids among the Minhos' shad populations does not seem to be important to affect their genetic integrity.

Key words: *Alosa alosa, A. fallax,* morphometry, hybridization.

3.1. INTRODUCTION

Is generally known that human activities weaken the organisms' ability to adapt to environmental changes and endangers biodiversity. Genetic conservation, enhanced by recent developments in molecular biology, has emerged as a useful toll, providing new insights into evolutionary and ecological processes (Saura and Faria, 2011).

Hybridization between species provides experimental material for studies of processes involving divergence and speciation (Hewitt, 1988; Harrison, 1990), constituting an evolutionary process of particular relevance to conservation. Unlike "natural hybridization" that can contribute to increase the genetic variability, hybridization resulting from anthropogenic pressure, as habitat modification and fragmentation or species introductions, is gradually increasing, compromising the genetic integrity of species and, ultimately, leading to the extinction. Thus, distinguishing the natural hybridization of the anthropogenically induced hybridization is of utmost importance for the implementation of appropriate conservation strategies (Allendorf et al., 2001).

The implementation of conservation and management strategies is especially challenging regarding diadromous species (Saura and Faria, 2011). These species cross successively freshwater, estuarine and marine habitats, being exposed to threats and degradation in all three habitats. The adaptations acquired for one of these environments do not guarantee their overall success (Saura and Faria, 2011).

Atlantic shad (*Alosa alosa* and *Alosa fallax* sp.) are considered as vulnerable species in Europe (IUCN criteria), mainly due to the drastic reduction of their distribution range and to the decrease of the quality of freshwater habitats. Allis and Twaite shads are included in the Red Book of endangered species in Spain, France and Portugal. In addition, they are listed in Appendix III of the Bern Convention and Appendices II and V of the Habitat Directive. The conservation, protection and management of these heritage species pass through a precise knowledge of the remaining population regarding abundance and biological and genetic characteristics (Véron et al., 2001).

Studies of these two anadromous Clupeid fish species, based on the analysis of morphological characters, allozymes and mtDNA, support the existence of two distinct lineages, corresponding to the two described species (Alexandrino et al., 2006; Faria et al., 2006). As these species co-exist in many Atlantic rivers and there are no complete reproductive barriers between them, hybridization has been extensively documented (Baglinière, 2000).

The hybridization between these two species appears to be closely related to the habitat disorders and/or decline the parental populations, leading to disruption of spatial and behavioural barriers (Boisneau et al., 1992). Among these disorders, the construction of dams and other barriers to migration seems to take a leading role by preventing individuals from *A. alosa* reach their usual breeding areas and leading to the overlap of breeding sites with *A. fallax*, thus facilitating the occurrence of hybridization.

The two species of Atlantic shads, Allis shad and Twaite shad, colonize the Minho River but their populations are scientifically little explored contrary to those of the largest European rivers. Studies regarding the Allis shad spawner's general biology and ecology were conduced by Mota and Antunes (2001), as well as primary characterisation of the juveniles' habitat use and feeding (Mota and Antunes, 2012). It is reported a decreased by about 90% (Mota and Antunes, 2011) in the catches of the Allis shad migrant population, coinciding with the building of the first dams (Alexandrino, 1996; Baglinière et al., 2003). Recent morphometric works on the Minho Allis shad population demonstrate an average of 5% of hybrids among the spawners population (Mota and Antunes, 2011).

Although recognized the reduction of the population numbers far below to the original effectives, the Allis shad of the Minho river still to be explored and still have a great commercial and cultural interest. Thus, in order to clarify the taxonomic status of Minho's shads' populations, we accomplish the morphological and genetic characterization of these populations.

The objective of this work was to examine for the first time the extent of hybridization between anadromous Allis and Twaite shad of Minho River, both morphologically and genetically. For these propose we analysed Allis and Twaite shads spawners and juveniles and compared morphological and genetic characteristics.

3.2. MATERIALS AND METHODS

3.2.1. Study area

The Minho River is located in the northwest of the Iberian Peninsula, comprising a total watershed of 17080 km^2 and the main course is approximately 300 km long. The international section, which corresponds to the last 77 km, is a natural northwest boundary between Portugal and Spain and is, currently, the only free passage for Allis shad and other diadromous fish upstream migration and spawning, between the Atlantic Ocean and the first dam, located in Spain (Mota and Antunes, 2012). The Minho estuary, with an extension of 40 km, has a maximum width of just over 2 km near the river mouth and salt intrusion extends from around 11.3 km and up to 16.8 km inland during higher spring tides, in September (Mota and Antunes, 2012).

Rainfall in the Minho River basin shows a high variability in the discharge regime as result of the strong seasonal and inter-annual variability. River flow exhibits a typical pattern for Iberian rivers, increasing usually in autumn or early winter, with a gradual decline during late spring and summer.

The study area is located between the Minho River mouth and the first dam. Two sampling stations (estuary and spawning area) were selected for the experimental fishing.

3.2.2. Sampling Strategy

The adults' samples were obtained from experimental fishing and fishermen of the international section of the Minho River (Portugal and Spain) during the period of March 2009 to August 2012. Experimental fishing was carried out using different typical trammel nets according to the species and sampling station: drift trammel nets (140 m long and 140 mm loose inner layer mesh size and 140 m long and 70 mm loose inner layer mesh size) were used to catch Allis and Twaite shad in the estuary and fixed trammel nets (25 m long and 140 mm loose inner layer mesh size and 25 m long and 90 mm loose inner layer mesh size) were used in the spawning area to catch Allis and Twaite shad.

Sampling sites of juveniles were chosen based on results from a preliminary investigation of potential rearing habitats (Mota and Antunes, 2012). The samples were obtained by experimental fishing, between September and January (seaward migration season), during the night, period in which the catches were more efficient (Mota and Antunes, 2012), using a beach seine net (10 mm mesh; bag: 4.8 m long, 3 m mouth width; wings: 6 m long and 2.4 m high).

3.2.3. Data collection

Spawners shads (Allis shad N=467; Twaite shad N=189) total length (TL) were measured to the nearest 0.1 cm (on a measuring board) and total weight (TW) determined to the nearest 5g (digital hanging Kern HDB scale). The juveniles' (N=560) FL and TL were measured to the nearest 0.1 mm and TW to the nearest 0.01 g (digital A&D EK-610i balance). A sample of spawners Allis shad (N=377) and Twaite shad (N=174) scales was collected and prepared for ageing and identification of spawning marks, following Baglinière et al. (2001) protocol.

To classify adults into species and firstly distinguish hybrids, the first gill arch was removed and gill rakers counted under a binocular microscope (Nikon SMZ800), which allowed counting even the not completely developed rakers present at each extremity. Juveniles Allis shad, Twaite shad and hybrids were distinguished on the basis of the gill rakers numbers of the first gill arch, as described for the adults.

Both adults and juveniles blood, muscle or fin tissue were collected and preserved in 95% ethanol or immediately frozen. All samples were stored at -80C for posterior genetic analysis. DNA was extracted from tissue samples using EasySpin TM 96-well extraction plates according the manufacturer's recommendations.

3.2.4 Genetic analysis

Adults Allis shad (N=75) and Twaite shad (N=29) and juveniles (N=86) were screened at 24 microsatellite loci. The loci used were designed for *A. alosa* and *A. fallax* (Faria et al. 2004). The forward primer of each locus was made with an M13 tail on the 5' end to allow a second fluorescently labeled primer to be ligated to it during PCR amplification. The 24 primers sets were combined into 4 different multiplexes (M) to group loci by like PCR annealing temperatures and different allele size ranges as follows: M1:(Aa14, Af13, Af20, Af6, Aa16) M2: (Asa2, Asa9, Asa6, Af11, Asa8, Af15) M3: (AsaD312, AsaC059, Aa20, AsaB020, AsaC334, AsaD055) M4: (AsaD429, AsaC010, AsaD042, AsaC051, AsaD278, AsaD021, AsaD392). Qiagen master mix was used for PCR amplification according to the manufactures recommendations. A touchdown PCR temperature cycling protocol was used for all PCRs ranging from 60C to 55C and using 33 cycles. Multiplexed PCR produces were run in an ABI Prism Analyzer with an internal size ladder (Genescan 350 ROX) and allele sizes were calculated using Genescan software (Applied Biosystems). Two researchers independently estimated the size of each allele and any ambiguities were amplified and run on the ABI Prism Analyzer a second time.

3.2.5. Data analysis

The adults' gills were counted and morphologically-identified *A. alosa* were considered as those with more than 115 gill rakers along their first gill arch, while *A. fallax* had less than 61, and hybrids had intermediate counts, according with Alexandrino (1996). To separate the Juveniles *Alosa* spp. specimen (N=560) into Allis shad *A. alosa*, Twaite shad *A. fallax* and hybrids, the number of gill rakers per TL was analysed. Model-based hierarchical clustering was applied, using the MCLUST package in R (R Dev. Core Team 2009), allowing selection of 1 to 3 clusters and choosing the best model from among all available parameterizations using the BIC. For each of the resulting three clusters, or groups, linear models of the number of gill rakers depending on fish TL were computed.

We used NewHybrids (Anderson and Thompson, 2002) to identify hybrids based on 20 microsatellite loci. Baseline data for each species contained 30 individuals of known phenotype from the Minho. Individuals chosen for the baseline dataset had gill rakers numbers and sizes near to the average or higher for each species. We used a model where individuals could be identified as pure *A. alosa*, pure *A. fallax*, F1 hybrids, F2 hybrids, *A. alosa* backcrosses or *A. fallax* backcrosses. We categorized individuals to one of these classes based on which had the highest probability calculated using NewHybrids.

3.3. RESULTS

The spawning migration of Minho river shad populations occurs more intensely between March and June for Allis shad and between April and June for Twaite shad. Peak of migration occurs in April for Allis shad and in May for Twaite shad (Fig. 3.1).

The spawning activity is more intense in late June and July for Allis shad and during late May and June for Twaite shad (personal observation).

The Allis and Twaite shad females showed higher size than males both for TL and TW. Dimorphism is close to 6 cm for both Allis and Twaite shad and 800 g and 350 g for Allis and Twaite shad respectively (Table 3.1). The averages of TL and TW show no significant differences according to the species ($p > 0.01$).

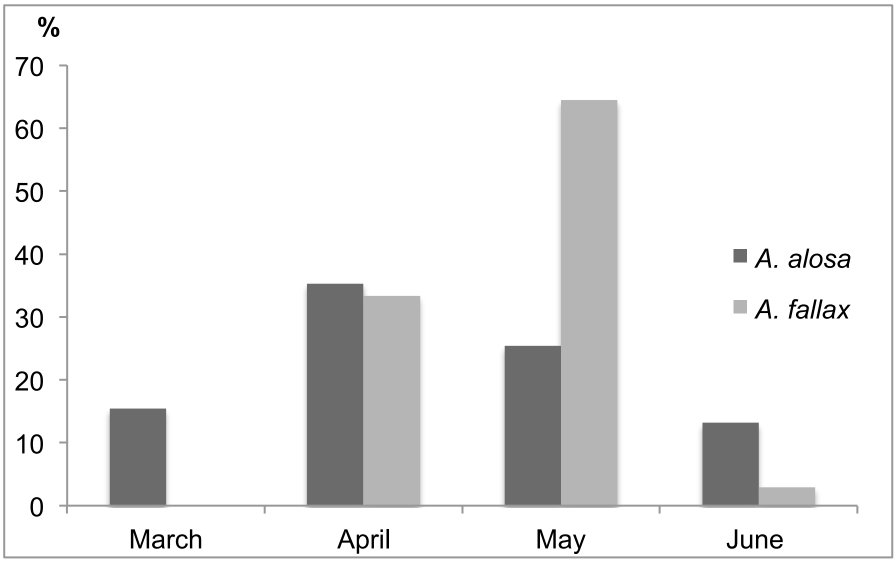


Figure 3.1. Distribution of the percentage of caught shads' spawners per species and month.

Table 3.1. Allis shad and Twaite shad average total length (TL) and total weight (TW), standard deviations (SD), number of samples (N), minimum total length and total weight (TL min; TW max) and maximum total length and total weight (TL max; TW max) by sex.

River	Species	Sex	TL (mm)	SD	N	TL (mm) min	TL (cm) max
Minho	*Alosa alosa*	M	620	27	162	560	700
		F	681	26	116	630	745

			TW (g)	SD	N	TW (g) min	TW (g) max
Minho	*Alosa alosa*	M	2364.9	346.6		1800	3878
		F	3258.6	476.5		2705	5295

			TL (mm)	SD	N	TL (mm) min	TL (mm) max
Minho	*Alosa fallax*	M	393	34	115	320	515
		F	457	45	64	380	540

			TW (g)	SD	N	TW (g) min	TW (g) max
Minho	*Alosa fallax*	M	545.4	158.2	115	290	1370
		F	898.8	293.8	64	445	1515

Allis shad females may reach sexual maturity as younger as 5 years and reach the 8 years old (Table 3.2). Most females were 7 years. In males, sexual maturity can be reached at 4 years, with older individuals of 7 years. Most males were 6 years. Regarding Twaite shads, most of the females were 4 and 5 years; the youngest presented 3 years and the oldest 7 years. Males were mostly 4 years. The males' age ranged between 4 and 6 years.

Regarding the spawning marks, 13 females and 7 males of Allis shad exhibited spawning marks, which represent 5.19% for the all individuals sampled. In turn, 49.4% of Twaite shads showed spawning marks, which 54.5% of females have spawned once, mostly at ages 4 and 5 and 20.5% spawned a second time frequently at ages 3 and 4. Only one female spawned a second time at age 6 and 15.9% of the females spawned a third time, normally at ages 3, 4 and 5. Looking at Twaite shad males, 47.6% had spawned once, in the average, at age 3. Males with two spawning marks (30.9%) spawned mostly at ages 3 and 4 and with 3 spawning marks (19 %) mostly at ages 3, 4 and 5. One single male presented 4 spawning marks at ages 2, 3, 4 and 5.

Table 3.2. Age structure of the Minho River spawners *A. Alosa* and *Alosa fallax* per sex. N: number of specimens and percentage in brackets; Spw: percentage of individuals presenting spawning marks; TL: mean total length; TW: mean total weight.

	Age (years)	*Alosa alosa*				*Alosa fallax*			
		N	Spw (%)	TL (mm)	TW (g)	N	Spw (%)	TL (mm)	TG (g)
Males	3	--	--	--	--	33 (29.7)	---	360 (3.1)	417.1 (11.4)
	4	1 (0.5)	--	590 (-)	1925 (-)	52 (46.9)	42.9	390 (3.2)	518.7 (14.2)
	5	27 (14.6)	1.6	652.8 (28.1)	2389.2 (362.1)	15 (13.5)	33.3	429.3 (5.6)	718.1 (43.9)
	6	106 (57.3)	1.6	640.7 (27.9)	2424.3 (349.5)	11 (9.9)	23.8	445.5 (9.1)	790.1 (64.8)
	7	51 (27.6)	0.5	651.8 (27.2)	2384.9 (345.7)	--	--	--	--
Females	3	--	--	--	--	2 (3.2)	--	399 (4)	658.0 (31)
	4	--	--	--	--	20 (31.6)	18.2	431.8 (5.6)	768.8 (39.2)
	5	8 (4.2)	--	649.9 (22.1)	2413.1 (401.0)	24 (38.1)	36.4	453.2 (4.5)	854.4 (34.1)
	6	36 (18.7)	1.5	651.2 (23.2)	2373.5 (391.5)	14 (22.2)	29.5	495 (6)	1124.3 (38.7)
	7	105 (54.4)	2	641.4 (25.3)	2436.3 (436.1)	3 (4.8)	6.8	515 (16.1)	1344.3 (92.3)
	8	44 (22.8)	3	640.9 (25.0)	2431.2 (443.8)	--	--	--	--

Females Allis shad were longer than males only at age 6 (Fig. 3.2). Both males and females Allis shad sizes are quite similar at same age. Twaite shad females, at the same ages, were longer than male. At the same age, Allis shad were longer than Twaite shads.

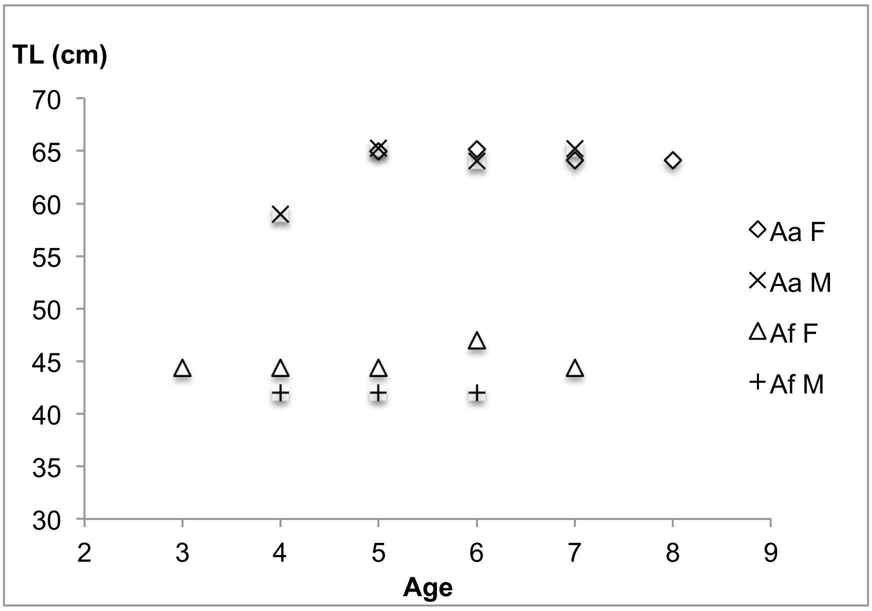


Figure 3.2. Relationship between the mean length at age of the Allis shad (Aa) and Twaite shad (Af) populations of Minho River. Aa F: *Alosa alosa females*; Aa M: *Alosa alosa males*; Af F: *A. fallax* females; Af M: *A. fallax* males.

The classification of samples into *Alosa* species depending on the gill rakers number revealed 3.6% of hybrids in the spawner's population. The numbers of gill rakers ranged between 115 and 159 (Median 138) for Allis shad, between 34 and 60 (Median 46) for Twaite shad and between 63 and 114 (Median 84) for hybrids.

The best hierarchical clustering model relating the number of gill rakers with juveniles TL was an unconstrained ellipsoidal model, with 3 components. This model was used to classify our samples into Allis shad, Twaite shad and hybrids. Linear models of the number of gill rakers depending on TL were: 32.54+2.56×TL for Allis shad (R^2=0.63); 19.70+1.27×TL for Twaite shad (R^2=0.52); and 29.47+1.46×TL (R^2=0.23) for hybrids. Model predictions, confidence and prediction intervals are presented in Table 3.3.

Table 3.3. Juveniles predicted number of gill rakers (and confidence intervals) for different total lengths (TL).

TL (mm)	Number of gill rakers		
	A. alosa	Hybrids	*A. fallax*
50	45.3 (43.7–47.0)	36.8 (33.8–39.8)	26.0 (25.5–26.6)
60	47.9 (46.6–49.2)	38.2 (36.0–40.5)	27.3 (26.9–27.7)
70	50.5 (49.4–51.5)	39.7 (38.1–41.3)	28.6 (28.3–28.9)
80	53.0 (52.2–53.9)	41.2 (40.1–42.2)	29.8 (29.6–30.0)
90	55.6 (54.9–56.2)	42.6 (41.8–43.5)	31.1 (30.9–31.3)
100	58.2 (57.6–58.8)	44.1 (42.9–45.2)	32.4 (32.1–32.6)
110	60.7 (60.0–61.4)	45.6 (43.8–47.3)	33.6 (33.3–34.0)
120	63.3 (62.4–64.2)	47.0 (44.6–49.4)	34.9 (34.4–35.3)
130	65.8 (64.7–67.0)	48.5 (45.4–51.6)	36.2 (35.6–36.7)
140	68.4 (67.0–69.8)	49.9 (46.1–53.7)	37.4 (36.7–38.1)
150	71.0 (69.2–72.7)	51.4 (46.9–55.9)	38.7 (37.9–39.5)
160	73.5 (71.5–75.5)	52.9 (47.6–58.1)	40.0 (39.0–40.9)

Comparing meristic characteristics of the Atlantic shad population by river (Table 3.4), the spawner population of Allis shad of Minho river (males and females) are bigger, both in TL and TW that populations located on the northernmost rivers and close to the Lima and Sebou rivers, for both sexes. Males always showed lower mean TL and TW than female. Regarding Twaite shads, TL and TW were close to Charente and Gironde rivers.

The Minho river Allis shad population had a number of gill rakers average of 134.8, higher than the most of the Atlantic coast populations and close to Lima river. Minho river Twaite shads showed a mean number of gill rakers (46,5) relatively close to that of the Mondego Twaite shads (42.4).

Table 3.4. Meristic characteristics of Atlantic shad population throughout species range. TL: Total length; GR: gill rakers. Standard deviation (SD) values are given in brackets. Adapted from Véron et al., 2001.

	Alosa alosa					*Alosa fallax*					Authors
	Sex	Mean TL (mm)	Min TL (mm)	Max TL (mm)	GR	Sex	Mean TL (mm)	Min TL (mm)	Max TL (mm)	GR	
Minho	F	681.3 (26)	630	745	134.9 (12.7)	F	457 (45)	380	540	46.5 (5.6)	Present work
	M	620 (27)	560	700		M	393 (34)	320	515		
Orne	--	--	--	--	112 (13)	--	--	--	--	--	Véron, 1999
Vire	--	--	--	--	123 (8)	--	--	--	--	--	Véron, 1999
Aulne	F	552.5 (12.5)	540	575	112 (11)	--	--	--	--	--	Véron, 1999
	M	459.3 (29.5)	420	515		--	--	--	--	--	
Blavet	--	--	--	--	121 (4)	--	--	--	--	--	Véron, 1999
Vilaine	F	549.3 (36.9)	520	605	119 (16)	--	--	--	--	--	Véron, 1999
	M	473.0 (39.5)	425	517		--	--	--	--	--	
Loire	F	556 (39)	414	680	122 (10.2)	F	391 (27)	367	515	49 (4.8)	Mennesson-Boisneau, 1990
	M	484 (39)	339	635		--	--	--	--		
Gironde	F	585 (26)	509	650	--	F	437 (27)	392	509	--	Taverny, 1991
	M	533 (29)	383	597	--	M	383 (27)	320	450	--	
Garonne	--	--	--	--	127 (5.6)	--	--	--	--	42 (1.6)	Douchement, unpublished data
Charente	F	570.2 (40.8)	485	660	125 (10)	F	438.2 (27.3)	405	510	42 (4)	Verón et al., 2001
	M	527.1 (44.5)	410	595		M	376 (29.2)	340	415		
Adour	F	539 (25)	475	600	124 (4.8)	--	--	--	--	48 (2.3)	Prouzet, unpublished data; Douchement, unpublished data
	M	492 (32)	415	605		--	--	--	--		
Lima	F	634 (44)	480	733	133 (9.8)	F	421 (38)	342	560	50 (4.6)	Alexandrino, 1996
	M	537 (61)	400	666		M	391 (47)	284	468		
Douro	--	--	--	--	123 (10.7)	--	--	--	--	42 (2)	Alexandrino, 1996
Mondego	--	--	--	--	124 (12.4)	--	--	--	--	46 (2.6)	Alexandrino, 1996
Sebou	F	636 (3.8)	490	720	110 (6.8)	F	382 (5)	300	460	42	Sabatié, 1993
	M	549 (4.4)	445	670		M	318 (3)	240	390		
Rhône	--	--	--	--	70 (12)	F	533 (33)	415	657	40 (2.4)	Douchement, unpublished data
	--	--	--	--		M	465 (47)	363	556		
Aude	--	--	--	--	72 (4)	--	--	--	--	--	Douchement, unpublished data

The results of genetic analyses performed on Minho river shads' populations revealed that the alleles found for the different loci are the normally present in both of Atlantic shad populations. Allelic richness of anadromous *A. alosa* and *A. fallax* was found to be similar. The NewHybrids analysis to estimate the probability of genetically sampled individuals fall into each group (*A.alosa*, *A. fallax* and hybrids) showed a total percentage of hybrids among spawners fish of 31%. When looking for hybrids among Allis shad and Twaite shad separately (Table 3.5), 24% and 62% of hybrids were found, respectively. Among juveniles, the percentage of hybrids was 21%. Considering hybrids, most of them were *A. alosa* backcrosses (70%), whereas 56% were F1 hybrids.

Table 3.5. Morphometric characteristics of hybrids *A. alosa* backcrosses and *A. fallax* backcrosses.

	A. alosa backcrosses				*A. falax* backcrosses			
	N	TL (mm)	TW (g)	GR	N	TL (mm)	TW (g)	GR
Male	6	590 (35.2)	1900 (499.9)	101 (19.8)	4	471 (38.1)	972.5 (318.9)	74.8 (12.6)
Female	12	599.4 (36.4)	2041.2 (484.9)	103.8 (20.7)	2	512.5 (22.5)	1355 (115)	62 (-)

Mismatch between genotype classification and phenotypic classification occurred in few samples (Table 3.6). The higher percentage of genotype and phenotypic classification mismatch was verified in spawners for samples classified genotypicaly as hybrids. Among these, mismatches occurred mainly (76.9%) for *A. alosa* genotypicaly classified as hybrids.

Table 3.6. Number and percentages of mismatch between genotype classification and phenotypic classification found in the Minho river shad population.

	Alosa alosa	*Alosa fallax*	Hybrids
Spawners	3 (6.4%)	0	13 (31%)
Juveniles	1 (1.2%)	3 (3.5%)	4 (4.7%)

3.4. DISCUSSION

The two populations shads of the Minho river are well distinguished by morphological and meristic characteristics. As for the most of the European populations (e.g. Mennesson-Boisneau et al., 2000b; Aprahamian et al., 2002), the Minho River Allis shad have a larger size, both in length and weight, and higher number of gill rakers. Reproductive migration differs for the two species, where Allis shad migration begins earlier than the Twaite. The spawning activity also differs between the two species, with the most intense spawning month for Allis shads being in July is and in June for Twaite shads. As described for other European populations (e.g. Mennesson-Boisneau et al., 2000a; Aprahamian et al., 2002), the migration and spawning season of Twaite shad is shorter than in the Allis shad.

Genetically, the separation of *A. alosa* and *A. fallax* as distinct species is supported by great differences in allozyme and microsatellite allele frequency between them (Faria, 2007; Alexandrino et al., 2006; Jolly et al., 2011). The occurrence of morphological hybrids between *A. alosa* and *A. fallax* species has been widely documented (Aprahamian et al., 2002). Furthermost evidences for hybrids formation caused by anthropogenic disruption results from the breakdown of reproductive isolation between species that have recently diverged in sympatry or from introgression between allopatric species after posterior contact (e.g. Walters et al., 2008; McDevitt et al., 2009; Behm et al., 2010; Vonlanthen et al., 2012). Although sometimes the two species hybridize at high frequencies, the observed level of genetic divergence between the two species indicates they have remained distinct lineages despite recent hybridization (e.g. Jolly et al. 2011).

Our morphological and genetic analyses confirm the presence of hybrids in the Minho shad populations. As reported for other European populations, the *Alosa* hybrids of Minho river presented intermediate morphological and genetic compositions. Previous studies (e.g. Alexandrino et al., 2006) also had showed a correlation between genotypes based on nuclear markers and the number of gill rakers, with the hybrids presenting intermediate morphological and genetic compositions. These studies strongly support the occurrence of hybridization.

River dams are one of the factors that can lead to hybridization, as they may disturb migration and induce an overlap of the spawning areas (Boisneau et al., 1992). The replenishment of the spawning migration corridors by dam removal has been widely discussed (e.g. Hasselman and Limburg 2012). For example, Hasselman et al. (2014) provided indications that dam removal could help maintain alewife and blueback herring species integrity. In the Minho River, five dams were built between the late 1950s and the early 1970s, but overlap of spawning areas is not clear. Prior to the dam construction it was observed that *A. alosa* and *A. fallax* spawned several hundred kilometres apart. After the dam construction it appeared that the two species' spawning areas remained separated by about 30 km (personal observation). It is not clear how hybridization happens when spawning grounds are clearly separated (Maitland and Lyle, 2005). One possibility is the reduction of water flow imposed by dams could cause some *A. fallax* individuals to migrate up in search of original water volume/flow (Faria, 2007) where hybridization occurs. According to our genetic analysis, most of the Minho hybrids (both juveniles and adults) seem to be F2s and backcrossed individuals (mainly *A. alosa* backcross), suggesting the occurrence of introgression between the species and that hybrids backcrossing primarily with *A. alosa* in their spawning grounds.

Although our genetic findings indicate higher percentages of hybrids than those found by meristic classification, these results couldn't be transposed to the general population, as the sample chosen for genetic analysis did not take into account the representativeness of the population but rather the validation of the meristic classification. In this sense, one can say that our meristic classification is quite accurate, since mismatch between genotype classification and phenotypic classification was low and higher percentages of mismatches were found among hybrids' classification.

When comparing our model for juveniles' classification with the microsatellite loci analysis we noted a good fit between the morphological and genetic classifications. The model for juveniles is probably least accurate when used to classify individuals at the limits of the prediction intervals, especially for *A. alosa* at the lower limit of the prediction interval. The few discrepancies between morphological and genetic classification concerned juveniles classified as *A. alosa* by the model, fitting the lower limit of its prediction interval, that were genotypically classified as hybrids.

The juvenile model classification of samples into species based on phenotype indicated a higher percentage of hybrids between to juveniles than to adults (10% juvenile hybrids against 3.6% adult hybrids). Faria (2007) suggests that underestimation of hybridization rates may occur owing to several factors, such as non-representative sampling, disturbance of hybrids' migratory behaviour or weakness in the detection of late generation backcrosses. The discrepancy in the proportions of juveniles and adult hybrids may also be attributed to a selective disadvantage of hybrids compared with the parental species.

While no studies on the hybrids' fitness compared to that of pure parental species have been conducted (Faria, 2007) and assumptions must be cautious, it is clear that hybrids are viable and have a nonzero reproductive success in the Minho and other drainages.

Although natural hybridization does not require special conservation measures and may, instead, be a source of genetic variability (Rieseberg and Burke, 2001), caution must be taken when introgression has anthropogenic causes, since it can lead to the loss of local genetic diversity and adaptive potential (García de Leániz et al., 2007). The construction of barriers in the natural migration routes has been assumed as the main cause of hybridization between European shad species, that may result in the overlapping of spawning grounds of the two species (Alexandrino et al., 2006). Nonetheless, it must be taken into account that natural hybridization can also occurs and the distinction between these different circumstances, though essential for conservation, can be very challenging (Jenneckens et al., 2000).

In the Minho River, the causes of hybridization between the two shad populations are not very clear. The number of hybrids among the shad population does not seem to be important to affect the genetic integrity of the two *Alosa* species, nor for that special management and conservation measures have to be taken in this direction.

3.5. ACKNOWLEDGEMENTS

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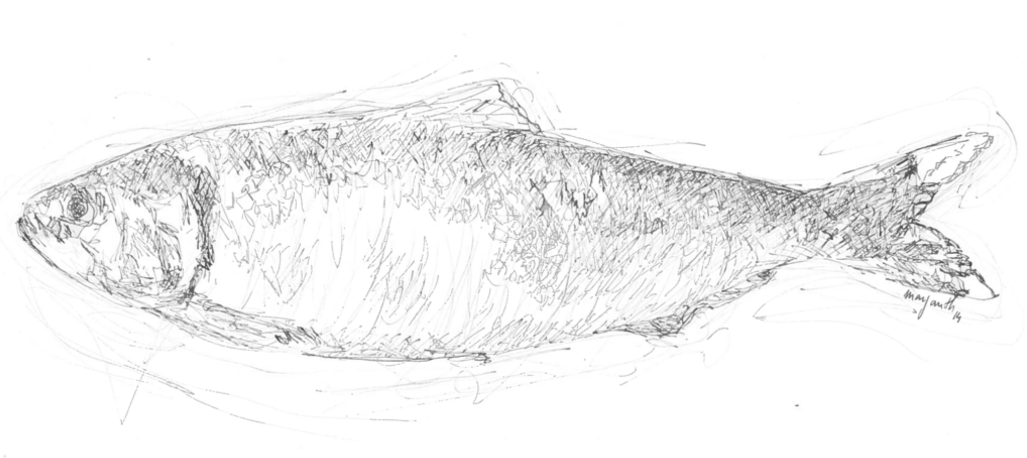
CHAPTER 4

MIGRATORY PATTERNS OF ALLIS SHAD *Alosa alosa* (L.) INFERRED FROM OTOLITHS MICROCHEMISTRY



4.1. OTOLITH ELEMENTAL SIGNATURES ASSESSING THE NATAL ORIGIN OF EUROPEAN ALLIS SHAD *Alosa alosa* (L.) POPULATIONS

Part of this section was submitted to the Canadian Journal of Fisheries and Aquatic Sciences as: J. Martin, S. Launey, H. Drouineau, Q. Rougemont, P. Jatteau, G. Bareille, S. Berail, C. Pécheyran, E. Feunteun, S. Roques, D. Clavé, D. Nachón, M. Mota, E. Réveillac, F. Daverat. *Dispersal capacities of anadromous Allis shad population inferred from a coupled genetic and otolith approach*.

ABSTRACT

Geochemical signatures in otoliths of adults and juveniles Allis shad (*Alosa alosa*) captured in 13 rivers around the European range of the species were used to distinguish the natal origin of the populations. The relationship between water elemental composition and juveniles' otolith elemental composition of 5 rivers was also assessed. A LA ICP-MS was used to quantify ^{63}Cu, ^{6}Li, ^{7}Li, ^{43}Ca, ^{86}Sr, ^{111}Cd and ^{138}Ba in otoliths and a MC-ICP-MS was employed to determine ^{87}Sr:^{86}Sr isotopic ratios. Water samples were analysed with an ICP-MS to quantify Sr, Ba, Cu, Cd and Li concentrations and an ICP-AES was used to determine Ca concentrations. Except for Ba:Ca ratios in adults' otoliths, elemental and isotope ratios in otoliths and water samples varied substantially among rivers. It was verified a significant positive linear regressions ($p<0.01$) between water and juveniles otolith microchemistry for Sr:Ca, Ba:Ca and Li:Ca. CDA analysis of otoliths geochemical signatures showed strong geographical separation of adults shads. The goundtruthed atlas of the geochemical signatures in the otoliths of juvenile Allis shad and water samples was used in a Bayesian model for determine adult natal river. Allis shad captured in the Blavet, Nivelle Minho and Dordogne rivers were classified as having been born in the same river in which they were sampled. Among Portuguese populations, geochemical signatures in otolith of Allis shads captured in Minho river were assessed as having the same natal origin, but the geochemical signatures of the Allis shads captured in the Mondego and Lima rivers were classified in 80% and 50% respectively as have their natal origin in the Minho river. The present study suggests that water chemical elements can be used to predict otolith chemistry, as strong correlation was found between the two variables.

Key words: Allis shad, homing, otoliths, geochemical signatures.

4.1.1. INTRODUCTION

The homing behaviour, i.e. the return of spawners to the same location where they were born, is supposed to be a common characteristic of the life cycle of many aquatic vertebrates, including anadromous fishes. Knowledge on natal origins of individuals across extensive geographic areas where populations potentially mix is one of the most challenging aspects of the connectivity for migratory species and populations structure (Barnett-Johnson et al., 2008). Despite the scientific significance, distinguishing origins of migratory fish populations is of great interest for fisheries management. The determination of an individual's home area also identifies the stock to which it belongs among mixed or straddling stocks (Thorrold et al, 1998).

As migration and dispersal are believed to be fundamental processes defining populations (Hanski and Gilpin, 1997; Rieman and Dunham, 2000), tracking migratory fish movement is a crucial issue in the biology and conservation management of stream fishes. Understanding linkages between areas occupied along the life history of migratory fish can direct conservation efforts more appropriately for the breeding season, wintering, and rest sites (e.g., Myers et al., 1987).

Direct examinations of homing in aquatic organisms are unusual and involve various difficulties. Few tagging studies have confirmed the return of anadromous fish species to the natal river to spawn (e.g Labelle, 1992). Such studies are possible when extensive hatchery releases exist and the tag recovery programs are effective, but it is significantly more difficult and expensive in wild fish due to financial constraints and because young stages of fish are difficult to catch and tag with a satisfactory levels of mortality (Thorrold et al, 1998). Furthermore, the main difficulty in tag recovery programs is not to assess the number of fish coming back in the origin river but to count the strayer going in other rivers.

Elemental and isotopic analysis of fish otolith has been widely used as a potentially powerful technique to provide a natural tag for fish habitat assessment. Chemical signatures in otolith have been used to discriminate and identify stocks or nursery areas (Campana et al., 1994; Campana and Gagné, 1995; Campana et al., 2000; Thorrold et al., 1998) and to detect migratory patterns (e.g. Limburg, 1995; Limburg et al., 2001). Otolith elemental fingerprinting is based on two important properties of otoliths. Firstly, otoliths grow throughout the life of the fish and, unlike other fish hard parts, are metabolically inert and are not reabsorbed (Campana and Neilson, 1985), providing thus the most complete chemical record. Otoliths are formed by the deposition of calcium carbonate crystals, primarily aragonite, within a protein matrix. The otolith material accretes and incorporates trace elements from the environment that have been absorbed across the gills or gut and entered the fishes' bloodstream (Campana, 1999). The differences in water chemistry between locations, whether due to natural or anthropogenic effects are reflected in the otolith microchemistry, and these differences can be used to identify fish that were in different habitats during their lives (Brazner et al., 2004; Thorrold et al, 1998). This otoliths' ability may also be useful as a natural marker of natal spawning area of individual fish (Thorrold et al, 1998).

The use of isotopes in otoliths, particularly Strontium isotope (^{87}Sr : ^{86}Sr) ratios, has been broadly useful in identifying natal freshwater habitats (e.g. Kennedy et al., 2000; Hobbs et al., 2005; Hobson and Wassenaar, 2008). The age and composition of watersheds' bedrocks have been found to be stable across years, providing a reflex of Sr isotope ratios in fish tissues (Kennedy et al., 2000).

Historically, Allis shad (*Alosa alosa*) was of great economic importance but, nowadays, European populations are severely impacted. During the last century, we observed a drastic reduction in the species populations and the ancient European distribution range (from Norway to Morocco and the Western Mediterranean Sea) is now reduced to the range between the Vire Basin, in Normandy and the Minho river, located at the boundary between Portugal and Spain (unpublished data). The drastic reduction of the European spawning population needs effective conservation and urgent management measures.

To date, most methods used to discriminate Allis shad population structure in Europe, using genetics (Alexandrino and Boisneau, 2000) and meristics (Sabatié et al., 2000) failed to solve possible differences between groups of fish. To the best of our knowledge, studies reporting discrimination of natal origins of European Allis shad populations using otolith microchemistry are restricted to the Gironde population, where Tomás et al. (2005) could discriminate fish from Garonne and Dordogne rivers.

In this study we examined the ability of otolith chemical composition to act as natural tags of natal river in Allis shad. We investigated otolith geochemical signatures of adults and juveniles Allis shad (*A. alosa*) of 13 rivers around the European range of the species. We also investigated the relationship between water elemental composition and juveniles' otolith elemental composition in 5 European rivers. We used elemental Sr:Ca, Ba:Ca, Cd:Ca, Cu:Ca and Li:Ca ratios and isotope ^{87}Sr:^{86}Sr ratios as tags to discriminate natal origins.

4.1.2. MATERIALS AND METHODS

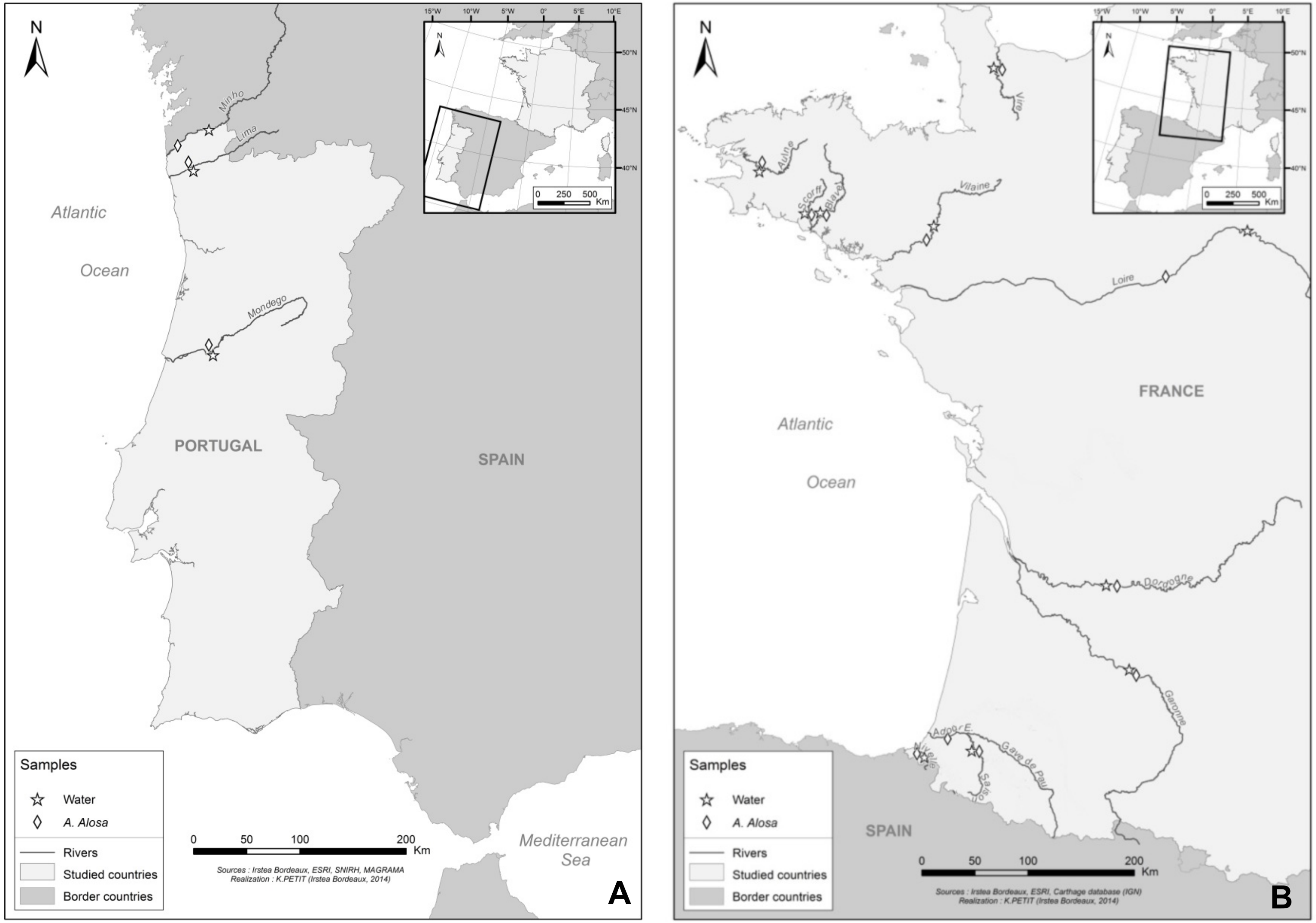
4.1.2.1. Fish and Water sampling

Mature Allis shad (N=429) were collected during their spawning migration from 13 European Rivers (Fig. 4.1), between April and June 2009 to 2013.

The samples were obtained from the commercial fishery and by experimental fishing, using a trammel net. Adult fish were also collected upriver either by sport fishing or found dead after spawning. The samplings cover all existing native range of the species.

Juveniles were sampled in 5 French rivers and one Iberian Peninsula River (Minho River). Samples from French rivers were obtained between June and October 2013 and from the Minho River between 2009 and 2012 from September to January. French samples were captured using a bongo net and Minho's' samples using a beach seine net (10 mm mesh; bag: 4.8 m long, 3 m mouth width; wings: 6 m long and 2.4 m high). The total (TL) and fork (FL) lengths were measured to the nearest millimetre. For some fish sampled after spawning, it was not possible to collect biometric data due to their advanced state of decomposition.

Spatial variability in water chemistry was investigated across the same rivers where Allis shad were sampled (except Adour E., since being lower part of the estuary does not represents a potential spawning ground) and plus 5 potential natal rivers that flow through a variety of geological formations from intrusive rocks to sedimentary deposits of paleozoic to cenozoic ages (BRGM French Geological Survey). Water samples were collected from late May to September 2013, close to the historic spawning area of *Alosa alosa*. At each location, 100 ml of river water was collected for Cu:Ca, Cd:Ca, Sr:Ca, Ba:Ca, Li:Ca and ^{87}Sr:^{86}Sr analyses. Syringes were used to transfer water samples to acid-washed and acidified (2% ultrapure HNO^{3} JT Baker, Ultrex II) low-density polyethylene (LDPE) bottles, through 0.45 µm Nalgene® polytetrafluoroethylene filters. In the field, water samples were kept on ice and kept refrigerated in the laboratory.



Sources : Irstea Bordeaux, ESRI, SNIRH, MAGRAMA
Realization : K.PETIT (Irstea Bordeaux, 2014)

Sources : Irstea Bordeaux, ESRI, Carthage database (IGN)
Realization : K.PETIT (Irstea Bordeaux, 2014)

Figure 4.1. Map of the Portuguese (A) and French (B) rivers where Allis shad and water samples were collected.

4.1.2.2. Otolith preparation and analysis

After dissection, Allis shad sagittal otoliths were rinsed in distilled water and air-dried. One *sagitta* per fish was embedded in epoxy resin (Araldite 2020, Escil) with the sulcus side down. Resin blocks were ground with ultrapure water and sandpaper (1200 – 4000 grit) until the *primordium* was reached. Finally, otoliths were rinsed with ultrapure water and air-dried before being stored in individually labelled plastic vials. Cleaned otoliths were analysed for Cd:Ca, Li:Ca, Sr:Ca, Ba:Ca and Cu:Ca ratios on an Elan DRC 2 (Perkin Elmer, USA) inductively coupled plasma quadrupole mass spectrometer. The ICP-MS is coupled to a high repetition rate Infra Red femto-second laser ablation system (Alfamet-Novalase, France).

A C-shaped ablation trajectory (60µm), was applied 40µm (±1) distant from the *primordium* of Allis shad otoliths' (Fig. 4.2) in order to avoid the maternal influence on the chemical signature (Kalish, 1990; Rieman et al., 1994). We assume that the first otolith feeding mark was at 40 µm from the *primordium* (Lochet, 2008).

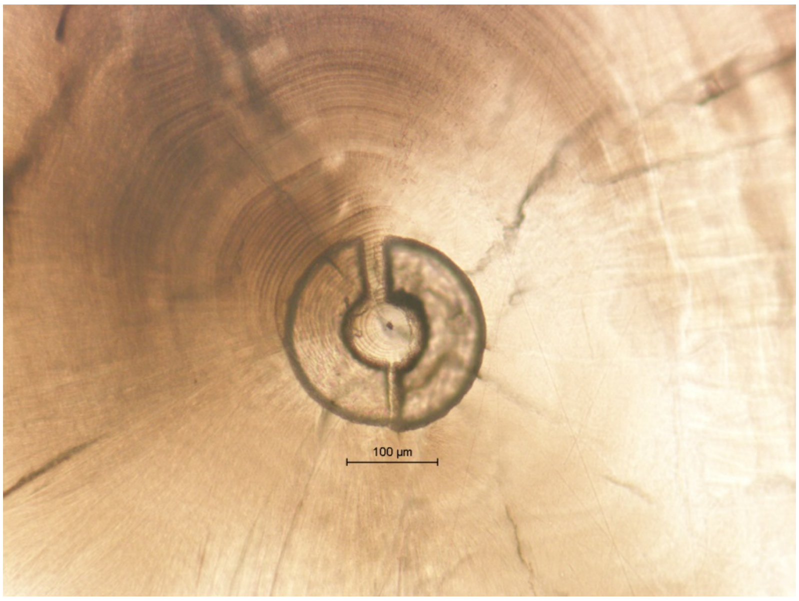


Figure 4.2. Otolith of *Alosa alosa* showing the opposite C-shaped ablation trajectories (at 40 µm from the *primordium*; 60 µm ablation) performed by femto-second laser ablation for both elemental (left) and Sr isotope analysis (right). Photograph courtesy of Jean Martin.

A He gas stream transported ablated material to the ICP-MS (gas flow rate 0.68 l·min-1), where it was mixed with an Ar gas sample and a wet aerosol (2% HNO^3) supplied by a self-aspirating (1mL·min-1) conical nebulizer in the concentric region of the quartz dual inlet spray chamber. Elemental ratios were quantified by monitoring ^{63}Cu, ^{6}Li, ^{7}Li, ^{43}Ca, ^{86}Sr, ^{111}Cd and ^{138}Ba. Calcium was used as an internal standard to improve the consistency of the measurement of the concentrations (Campana, 1999). Elements were standardized to calcium based on the stoichiometry of calcium carbonate (389 000 µg Ca·g-1 otolith) (Brown and Severin, 2009): Sr:Ca (mg·g-1), Cu:Ca (µg·g-1), Ba:Ca (µg·g-1), Li:Ca (µg·g-1) and Cd:Ca (µg·g-1). To ensure the best accuracy, the quantification of trace elements in otoliths was achieved by external calibration using both carbonate pellets (Barats et al., 2007) and 3 NIST glass standards (610, 612, 616). An otolith Certified Reference Material (NIES 22, National Institute for Environmental Studies) was also pelletised and used in the quality control of the analysis of selected trace elements in the fish otolith. Except for Cd element, all elemental concentrations in the otolith were above the detection limits.

After elemental analysis, the otoliths of the same adults were used for determine the ^{87}Sr:^{86}Sr ratio, using a Nu-Plasma multicollector inductively-coupled-plasma mass-spectrometer (MC-ICP-MS, Nu Instruments, UK), coupled to a Lambda 3 femtosecond laser ablation system (Nexeya SA, Canejan, France). This laser is fitted with a diode-pumped Yb:KGW crystal laser source (HP2, Amplitudes Systemes, Pessac, France) transmitting 360 fs pulses. Of the three available wavelengths (1030 nm - fundamental; 515 nm - 2nd harmonic; 257 nm - 4th harmonic), in this study the 257 nm wavelength was used. This laser source is a new approach in analytical applications, since operates within a wide range of repetition rate (1–100 kHz) and at low energy (from 200 mJ per pulse below 1 kHz to 1.5 mJ per pulse at 100 kHz at this wavelength), contrasting with the commonly used lasers that operate at high energy and low repetition rate. The laser beam is focused with a 100 mm objective, which can be rapidly moved (up to 2 m·s-1) with high repositioning precision due to a 2D galvanometric scanning module attached to the optical line. The laser beam covers an optical field of 16x 16 mm^2.

The ablated material was transferred by a He gas stream (gas flow rate 0.68 L•min-1) from the laser cell to the MC-ICP-MS, where it was mixed in a double entry plasma torch with a wet aerosol of a 2% HNO^3 solution. The aerosol was produced by combining a pneumatic nebulizer (200 µl·min-1 micro-concentric) with a cyclonic spray chamber. The contribution of ^{87}Rb to ^{87}Sr intensity was deduced from the ^{85}Rb signal (Barnett-Johnson et al. 2010). ^{83}Kr intensity was monitored to control any potential interference of ^{84}Kr and ^{86}Kr on ^{84}Sr and ^{88}Sr respectively. The ^{86}Sr:^{88}Sr invariant ratio was used to correct the ^{87}Sr:^{86}Sr ratio from instrumental mass bias using an exponential law (Walther and Thorrold, 2008). The accuracy of this approach was checked through the analyses of a NIES Certified Reference Material (Nº 22) obtained from otoliths of a marine fish *Lutjanus sebae*. In order to isotopically certify this material, a solution of CRM Nº 22 in 2% HNO^3 was first analysed using a Nu-Plasma MC-ICPMS (^{87}Sr:^{86}Sr = 0.70924±0.000021 2 SD, N =54). Secondly, we analysed CRM Nº 22 pellets (N=113) with the same ablation strategy applied to otoliths and an average of 0.70927 (±0.00011 2 SD) were obtained for ^{87}Sr:^{86}Sr. The ^{87}Sr:^{86}Sr ratios in both pellets and CRM Nº 22 solutions were in well agreement, fitting on the expected range for nearly constant current sea water (0.70917) (Allègre et al., 2010). Finally, the ^{87}Sr:^{86}Sr ratio of CRM Nº 22 pellets was used as an in-house marine carbonate standard and was measured every 6 samples as an external check of reproducibility.

Vaterite disturbance

Because vaterite profiles systematically exhibited lower Sr:Ca and Ba:Ca ratios than aragonitic ones, all otoliths were screened under optical microscopy for vaterite and aragonite inclusions. The result was the exclusion from analyses of 2% of adults that showed vaterite inclusion in their otoliths.

4.1.2.3. Water sample preparation and analysis

In order to quantify Sr, Ba, Cu, Cd and Li concentrations, water samples were diluted 10-fold with 2% HNO^3 and analysed using solution-based ICP-MS (X7 series CCT ICP-MS Thermo Electron). Ca was determined using ICP-AES (Inductively Coupled Plasma Atomic Emission Spectrometry - ACTIVA, Jobin Yvon). Internal standard of indium (2 µg·l−1) was used to calibrate the instrument drift. Blanks were regularly performed, using the same protocol as for the samples, with 18·2 MΩ MQ water (Millipore). The general performance of the procedure was checked for every 10 samples using the certified reference freshwater SLRS-4 (NRCC). Mean Sr:Ca (mg·g−1), Ba:Ca (µg·g−1), Cu:Ca (µg·g−1), Cd:Ca (µg·g−1), and Li:Ca (µg·g−1) ratios were calculated for each site. For the quantification of ^{87}Sr:^{86}Sr in river water, the appropriate volume of each sample (7–50ml) was evaporated to dryness to ultimately get 4 µg of Sr and re-dissolved in 3M HNO^3. Sr was separated from the rest of the matrix using columns containing Sr resin SR-B25-S Eichrom® and sequential elutions with ultrapure water and 3M ultrapure HNO^3 (Prohaska et al., 2002). After Sr separation, the sample was then diluted in 2% HNO^3 (Sr final concentration of 200 µg·l−1) and Sr isotope analysis was performed using the Nu-Plasma MC-ICP-MS. Liquid samples were introduced via a pneumatic nebulizer associated with a cyclonic spray chamber. Isotope intensities were quantified and interferences, as well as instrumental mass bias, were corrected as described above for otolith analyses. Accuracy and precision were monitored with a Standard Reference Material (SRM 987). The $\bar{X} \pm \mathrm{SD}$ value of ^{87}Sr:^{86}Sr in SRM 987 (N=54) found throughout the analyses was 0·71034±0·00003, which properly match with the accepted value of 0·71034 ± 0·00026.

4.1.2.4. Data analysis

As data were not normally distributed, the quantifications of elemental and Sr isotopic ratios in water samples were statistically analysed using non-parametric multivariate tests. Differences in water chemistry among sites were tested using the non-parametric Mann–Whitney U-test and Wilcoxon test with a Bonferroni adjustment.

Except for ^{87}Sr:^{86}Sr, correlations (Pearson's correlation tests) between water and otolith chemistry were examined using juvenile otoliths and river water samples. Linear regressions were fitted to scatterplots of mean ratios of juvenile otoliths and mean river water ratios for all chemical signatures. Linear regressions were used to predict mean otolith values based on mean water sample ratios for those rivers where no juveniles were collected. Based on the assumption of the equilibrium deposition of Sr isotopes in fish otoliths (Kennedy et al., 1997; 2000), water ^{87}Sr:^{86}Sr ratios were directly used to predict means and standard errors of otolith ^{87}Sr:^{86}Sr ratios.

The elemental comparison between water and juveniles otoliths was used to select the most relevant elements to discriminate individual from different watersheds. Geographic differences in multivariate signatures among locations were computed using canonical discriminant function (CDA) analysis.

Canonical variate coefficients are a useful toll, allowing the quantification of the relative importance of each variable to the observed distance among rivers. Since this procedure does not assume homogeneity of covariance matrices and tolerates modest deviations from normality (McGarigal et al., 2000), a quadratic discriminant function analysis (QDFA) was performed to determine the accuracy of an individual *Alosa alosa* be assigned to its capture river. The discriminant function analysis used a jackknife cross-validation procedure to determine classification accuracy. Data analyses were performed using R software (R Dev. Core Team, 2009).

We used Bayesian finite mixture model to determine adult natal stream. This model was based on a goundtruthed atlas of geochemical signatures in the otoliths of juvenile Allis shad and water samples.

4.1.3. RESULTS

The number of adults and juveniles Allis shad samples collected around its European geographical range is given in Table 4.1. The more representative river was Minho river, both for juveniles and adults, and both in number of samples and distribution by years, followed by the Garonne river for adults and the Blavet river for juveniles.

Table 4.1. Number of samples of Allis shad adults and juveniles collected by river and capture river within it European range. Adour R. = upper Adour River, Adour E.= lower Adour Estuary.

	Adults						**Juveniles**					
Rivers	**2009**	**2010**	**2011**	**2012**	**2013**	**Total**	**2009**	**2011**	**2012**	**2013**	**Total**	**Total**
Adour E.			3		29	**32**						**32**
Aulne					12	**12**						**12**
Blavet					7	**7**				16	**16**	**23**
Dordogne				6	69	**75**				3	**3**	**78**
Garonne				27	37	**64**						**64**
Lima					4	**4**						**4**
Loire			4		24	**28**				4	**4**	**32**
Minho	26	29	27		18	**100**	10	4	6		**20**	**120**
Mondego					15	**15**						**15**
Nivelle	16					**16**						**16**
Scorff					10	**10**						**10**
Vilaine		3	10		6	**19**				1	**1**	**20**
Vire			7		28	**35**						**35**
						417					**44**	**461**

Regarding biometrics, in average, Minho river showed the longest adult Allis shad (Table 4.2). The Lima River presented the heaviest individuals and was the second river presenting the longest adults. Longest juveniles were found in the Loire river, but heaviest individuals were from Minho river.

4.1.3.1. European scale elemental and isotopic analysis in river water and Allis shad otoliths

We restricted analyses of geochemical signatures among the rivers to Sr:Ca, Ba:Ca, Li:Ca and ^{87}Sr:^{86}Sr ratios, for which otolith values could be predicted from water samples. Cu:Ca and Cd:Ca ratios were excluded from the analysis, first because there were no variations for these elements between rivers and second because there were no linear relationship between Cu:Ca and Cd:Ca ratios in river waters and otoliths.

Table 4.2. Adults and juveniles Allis shad $\bar{X}$ total weights and fork length, respective standard deviation (SD) and number of individuals per sampling river.

Rivers	Fork length (mm)			Weight (g)			N
Adults							
Adour E.	505.7	±	31.5	2081.3	±	293.7	32
Aulne	480.8	±	52.1	—		—	12
Blavet	—		—	—		—	7
Dordogne	483.4	±	41.9	1365.8	±	495.3	75
Garonne	487.3	±	72.0	1403.3	±	466.3	64
Lima	583.8	±	73.9	2632.5	±	963.4	4
Loire	511.2	±	48.3	1998.5	±	575.0	28
Minho	596.4	±	46.2	2434.1	±	742.3	100
Mondego	494.0	±	30.0	1675.0	±	228.9	15
Nivelle	484.7	±	39.4	1565.0	±	554.7	16
Scorff	516.7	±	59.8	—		—	10
Vilaine	525.9	±	28.2	1161.6	±	245.9	19
Vire	486.4	±	40.6	1430.1	±	355.4	35
Juveniles							
Blavet	70.0	±	10.2	—		—	16
Dordogne	72.7	±	22.0	4.1	±	4.01	3
Loire	278	±	28.8906	—		—	4
Minho	9.785	±	1.2106	8	±	3.15	20
Vilaine	350	±	—	—		—	1

Moreover, our analyses were restricted to variables for which otolith composition could be accurately predicted based on the water chemistry of ambient natal environments. This, in turn, allowed us to groundtruth geochemical signatures in rivers where it was only possible to obtain water samples.

The relation between water and juveniles' otolith chemistry are given in Figure 4.3. Cu:Ca and Cd:Ca in juvenile otoliths were uncorrelated with the corresponding metal:Ca ratios in the water (p>0.05). Sr:Ca, Ba:Ca and Li:Ca showed significant positive linear regressions (p<0.01) between water and otolith microchemistry.

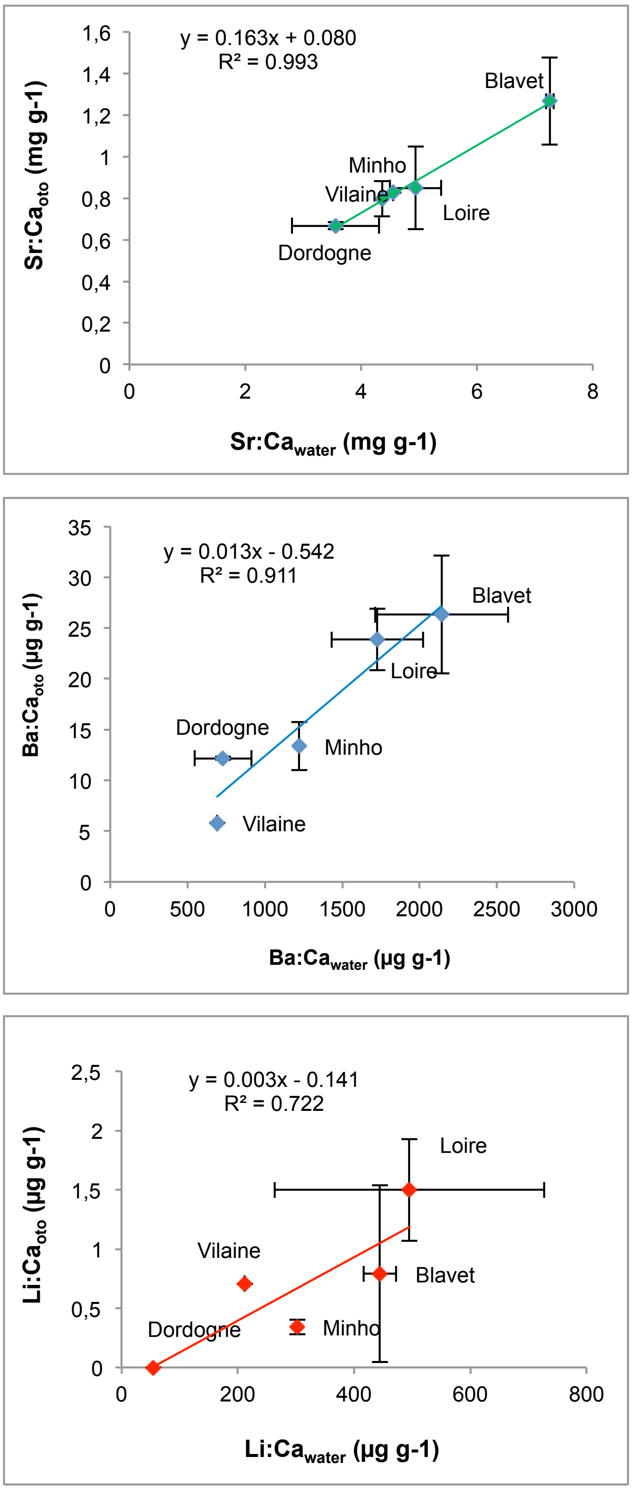


Figure 4.3. The relationship between $\overline{X} \pm SD$ elemental ratios in the juveniles' *Alosa alosa* otolith and river water. Values are given for the five rivers where both otoliths and water samples were collected.

It was verified a considerable variation on elemental and isotope ratios quantified in otoliths and water samples among rivers (Figs 4.4 and 4.5). Regular variations were found in ^{87}Sr:^{86}Sr values along the latitudinal gradient of the distribution range. Ba:Ca ratios in otoliths showed lower variation among samples with no obvious latitudinal gradient.

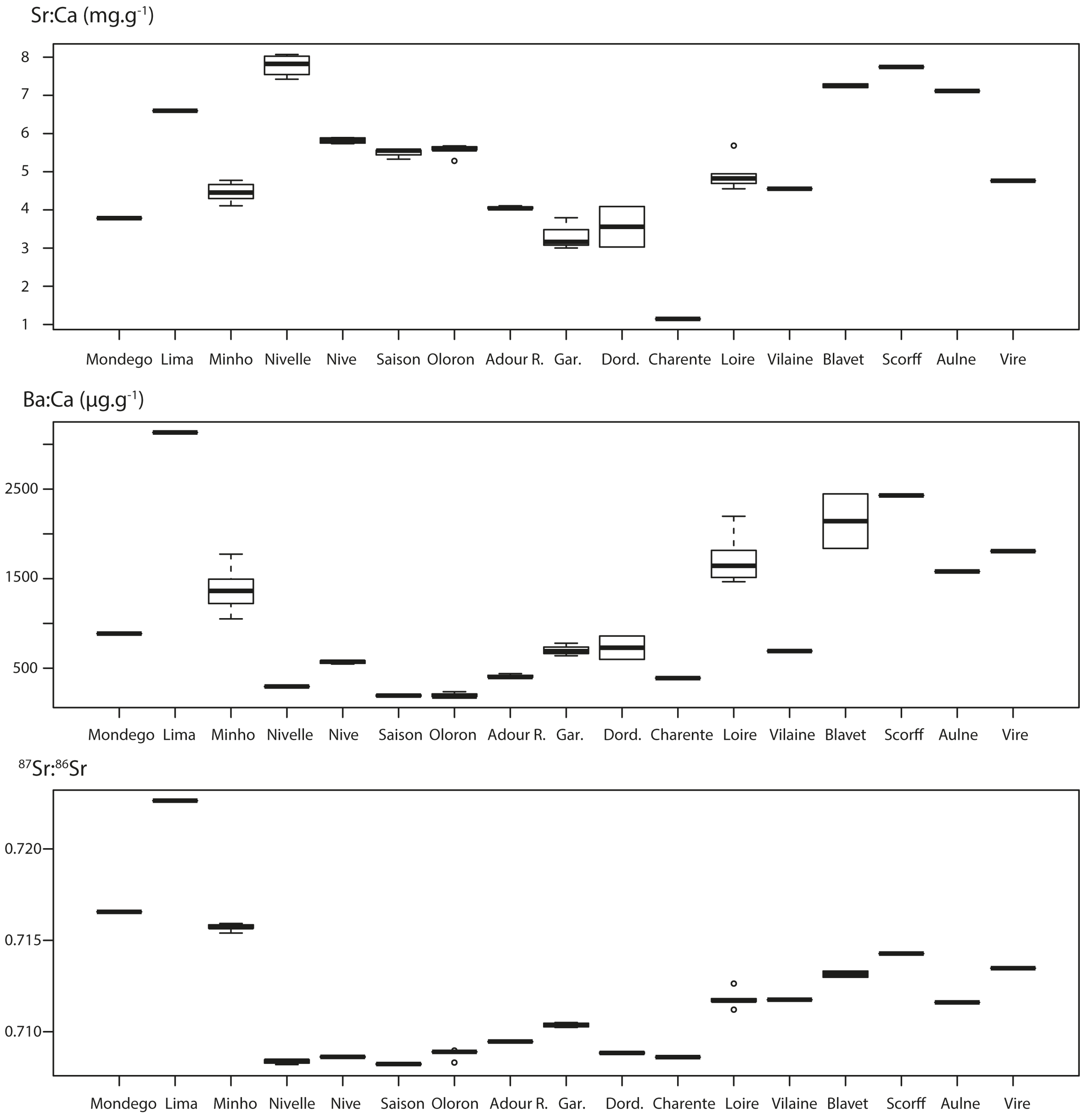


Figure 4.4. Box plot of $\bar{X}$ (a) Sr:Ca (mg·g−1), (b) Ba:Ca (µg·g−1), and (c) ^{87}Sr:^{86}Sr water ratios from 17 potential natal rivers. Extent of boxes: interquartile (25th and 75th percentile); horizontal lines: medians (50th percentile); whiskers: 10th to 90th percentiles; circles: outsiders.

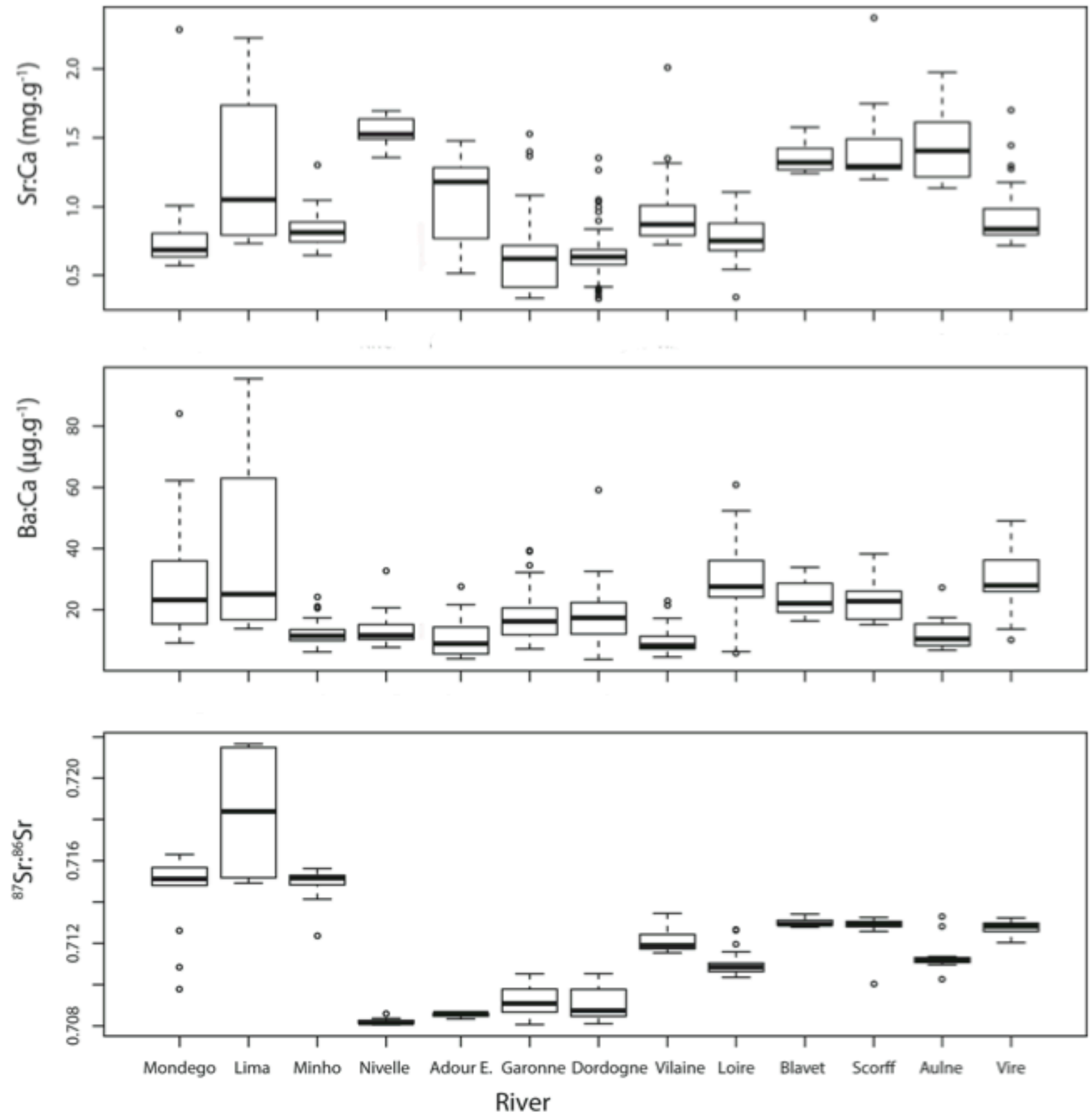


Figure 4.5. Box plot of $\overline{X}$ ratios of (a) Sr:Ca (mg·g−1), (b) Ba:Ca (µg·g−1), and (c) ^{87}Sr:^{86}Sr in the otolith core of adult Allis shad collected from 15 rivers. Extent of boxes: interquartile ranges (25th and 75th percentile); horizontal lines: medians (50th percentile); whiskers:10th to 90th percentiles; circles: outsiders.

The CDA showed strong geographical separation of adults based on the geochemical signatures in otolith cores (Fig. 4.6). Canonical coefficients indicate the relative importance of the six variables to the separation of the geochemical signatures among the rivers. Individuals grouped per capture river were generally separated along the first canonical axis. The absolute value of the coefficient indicates the relative importance of the ratio in directing combined signature along the axis, while the sign describes the direction of the relationship (Table 4.3). The first two canonical variables explained >80% of the variation in the data (Wilks' $\lambda=0{\cdot}419$, $p<0{\cdot}01$). The first canonical variable was primarily conduced by ^{87}Sr:^{86}Sr, followed by Ba:Ca. The second canonical axis was dominated by the variation in Sr:Ca followed by Li:Ca ratios, with small contributions from ^{87}Sr:^{86}Sr and Ba:Ca ratios. Cu:Ca and Cd:Ca ratios had limited expression in the CDA.

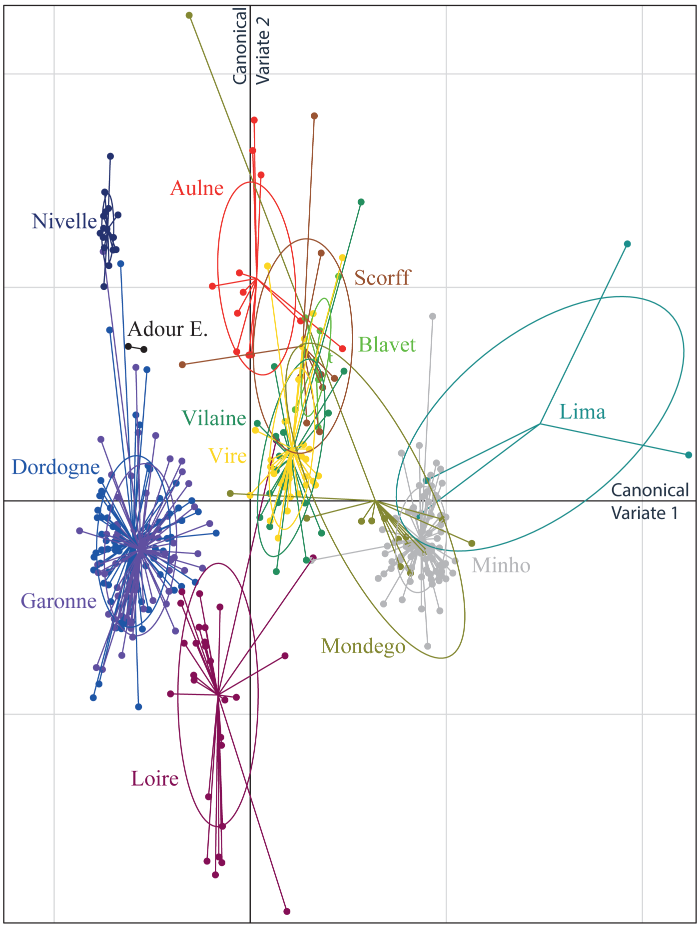


Figure 4.6. Canonical discriminant analysis of Allis shad otolith core signatures from all adult fish collected between 2009 and 2013 grouped by river of capture. Symbols represent individual fish, and ellipses are 95% confidence intervals around each group.

Table 4.3. Total canonical coefficients for canonical discriminant analysis based on elemental and Sr isotopic ratios of adult *Alosa alosa* otoliths from the 13 rivers over 5 years. Values in bold represents $p < 0{\cdot}001$.

Ratios	CV1	CV2
^{87}Sr:^{86}Sr	**0.999**	-0.256
Sr:Ca	0.026	**0.824**
Ba:Ca	**-0.118**	-0.107
Li:Ca	0.020	**-0.544**
Cu:Ca	-0.005	-0.187
Cd:Ca	0.034	-0.010

The adults' cross-validation classification accuracies based on geochemical signatures in otolith cores ranged from 14 to 100% (Table 4.4). Among the 13 rivers, misclassifications occurred in four rivers, with lowest classification success in the Blavet River. Most of these misclassifications were from adjacent tributaries as Garonne- Dordogne rivers.

Bayesian model to determine adult natal rivers based on the goundtruthed atlas of geochemical signatures in the otoliths of juvenile Allis shad and water samples are given in Table 4.5. The model indicated that samples from Blavet and Nivelle were accurately classified in 100% of the cases as having been born in the same river in which they were sampled. The Minho and Dordogne rivers have been classified, into more than 90%, as belonging to the respective rivers where they were captured (93.06% and 94.80% respectively). The Allis shad of the remaining rivers were not classified in more than 50% as from the rivers where they were captured.

Table 4.4. Cross-validation summary from the quadratic discriminant function analysis based on signatures from adults' otolith cores (N=369). Groups are categorized by capture river. Values are percentages and the numbers of individuals are given in parentheses (). Accuracy of group of origin is shown on the diagonal (in bold); accuracies sum 100% across a column for each group. – indicate no classifications.

River	Adour E. (2)	Aulne (12)	Blavet (7)	Dordogne (71)	Garonne (64)	Lima (4)	Loire (28)	Minho (87)	Mondego (15)	Nivelle (16)	Scorff (10)	Vilaine (19)	Vire (34)
Adour E.	**100 (2)**	–	–	–	1.6 (1)	–	–	–	–	–	–	–	–
Aulne	–	**83.3 (10)**	–	–	–	–	–	–	–	–	10 (1)	–	–
Blavet	–	–	**14.3 (1)**	–	–	–	–	–	–	–	50 (5)	5.3 (1)	2.9 (1)
Dordogne	–	–	–	**60.6 (43)**	65.6 (42)	–	–	–	–	–	10 (1)	–	–
Garonne	–	–	–	36.6 (26)	**28.1 (18)**	–	10.7 (3)	–	6.7 (1)	–	–	–	–
Lima	–	–	–	–	–	**50 (2)**	–	–	–	–	–	–	–
Loire	–	–	–	–	1.6 (1)	–	**78.6 (22)**	–	–	–	–	–	–
Minho	–	–	–	–	–	50 (2)	–	**97.7 (85)**	33.3 (5)	–	–	–	–
Mondego	–	–	–	–	–	–	–	1.1 (1)	**40 (6)**	–	–	–	–
Nivelle	–	–	–	2.8 (2)	3.1 (2)	–	–	–	6.7 (1)	**100 (16)**	–	–	–
Scorff	–	16.7 (2)	57.1 (4)	–	–	–	–	–	–	–	**30 (3)**	10.5 (2)	2.9 (1)
Vilaine	–	–	14.3 (1)	–	–	–	10.7 (3)	1.1 (1)	6.7 (1)	–	–	**84.2 (16)**	8.8 (3)
Vire	–	–	14.3 (1)	–	–	–	–	–	6.7 (1)	–	–	–	**85.3 (29)**

Table 4.5. Summary of Bayesian model for determine adult natal stream based on the goundtruthed atlas of geochemical signatures in the otoliths of juvenile Allis shad and water samples. Groups are categorized by the percentage of individuals whose geochemical signatures match in ≥ 50% with the goundtruthed atlas of geochemical signatures for a given river. Values are percentages and the numbers of individuals are given in parentheses ().

	Potential Natal Rivers							
River of capture	Vire	Blavet	Vilaine	Loire	Dordogne	Nivelle	Minho	Lima
Loire	–	–	–	**89,28(25)**	–	–	–	–
Garonne	–	–	–	–	**92,19 (59)**	–	–	–
Dordogne	–	–	–	–	**93,06(67)**	–	–	–
Vilaine	–	15,79(3)	21,05(4)	10,53(2)	–	–	–	–
Blavet	–	**100(7)**	–	–	–	–	–	–
Scorff	–	**80(8)**	–	–	–	–	–	–
Aulne	–	–	–	–	–	**58,33(7)**	–	–
Vire	35,29(12)	11,76(4)	–	–	–	–	–	–
Nivelle	–	–	–	–	–	**100(16)**	–	–
Minho	–	–	–	–	–	–	**94,80(91)**	
Lima	–	–	–	–	–	–	**50(2)**	**50(2)**
Mondego	–	–	–	–	–	–	**80(12)**	–
Adour E	–	–	–	–	–	**51,61(16)**	–	–

4.1.3.2. Portuguese elemental and isotopic analysis in adults Allis shad otoliths

As for the European scale elemental and isotopic analysis here we also restricted analyses of geochemical signatures among the rivers to Sr:Ca, Ba:Ca, Li:Ca and ^{87}Sr:^{86}Sr ratios. As well, analyses were restricted to variables for which otolith composition could be accurately predicted based on the water chemistry of ambient natal environments.

Kruskal wallis test revealed that natal signatures, investigated in the otoliths' core of adults, were significantly different for Ba, Li and Cu (p<0.05). Otolith elemental and isotopic ratios were significantly different for Ba:Ca ratios between Minho-Lima and between Minho-Mondego rivers, for Li:Ca ratios between Minho-Mondego and for Cu:Ca ratios between Minho-Mondego and between Minho-Lima.

Regarding the CDA analysis, unlike the CDA analysis among European range, the geographic distance between rivers were generally separated along the second canonical axis (Fig. 4.7). Adult Allis shads from Minho, Mondego and Lima showed natal geochemical signatures that are partially isolated, but some individuals shared the same natal geochemical signature. Geochemical signatures separated geographically the Minho and Mondego rivers. The Lima river ellipse interconnects with both Minho and Mondego rivers. This river showed 50% of the individuals with natal geochemical signature far different from the Minho and Mondego rivers and 50% of the individuals with natal signatures close to the natal signatures that have been found from fish captured in the Minho and in the Mondego rivers.

The first canonical axis were dominated by the variation in ^{87}Sr:^{86}Sr ratios followed by Ba:Ca ratios and Li:Ca ratios (Table 4.6). Loadings on the second canonical variate were dominated by variation in ^{87}Sr:^{86}Sr and Sr:Ca followed by Cu:Ca ratios. Cd:Ca ratios was of limited use in the CDA.

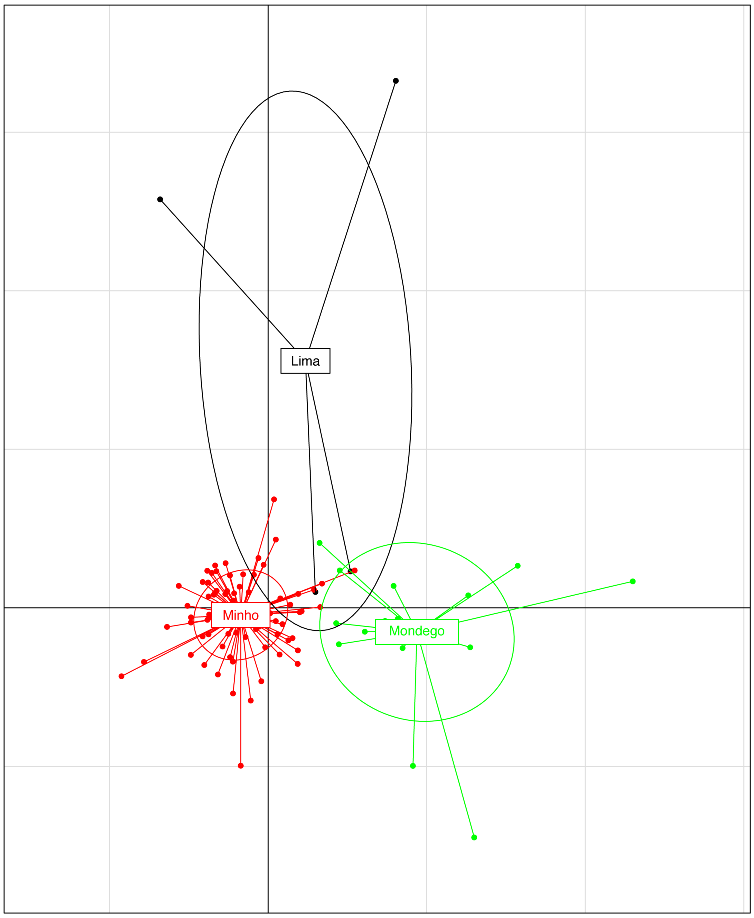


Figure 4.7. Canonical discriminant analysis of otolith core signatures from adult Portuguese Allis shad, grouped by river of capture. Symbols represent individual fish, ellipses are 95% confidence intervals around each group.

Table 4.6. Total canonical coefficients for canonical discriminant analysis based on elemental and Sr isotopic ratios of adult Portuguese Allis shad otoliths. Values in bold represents $p < 0.05$.

Ratios	CV1	CV2
^{87}Sr.^{86}Sr	**0.5286708**	**0.8394859**
Sr.Ca	0.2205894	**0.4977442**
Ba.Ca	**0.8179318**	0.1665923
Li.Ca	0.4541584	0.1144619
Cu.Ca	0.2856112	0.1973485
Cd.Ca	0.1194614	0.1468158

Cross-validation of the quadratic discriminant function (Table 4.4) showed misclassifications for the three rivers. The lowest classification successes were for Lima and Mondego rivers (50% and 40% respectively). The Minho was the river with best classification (97,7%).

Regarding Portuguese rivers, Bayesian model attributed 94,80% of the Allis shad captured in the Minho river as having otolith geochemical signatures similar to those assessed for juveniles' otoliths and water samples from Minho river. For the other two rivers, Bayesian model were based on the elements for which otolith compositions were predicted based on the water geochemical signature of each natal river. Adults captured in the Lima river showed elemental ratios close to those assessed both for Lima and Minho rivers. With the exception of three individuals, the adults captured in the Mondego river showed otolith geochemical signatures identical to the Minho's elemental ratios.

5.2.4. DISCUSSION

Although few studies have been leaning on the mechanisms that led to the differences in fingerprints and how stable chemical marks are processed, it has been suggested that the geochemical signatures can be tags of short-term geographical separation (Campana et al., 2000).

In the present study, results suggest that elemental fingerprints can distinguish Allis shad European natal streams. Elements that were the most important explanatory variables have also been important in other elemental fingerprinting studies (e.g. Walther and Thorrold, 2008; Tomás el al., 2005; Barnett-Johnson et al., 2008). Geochemical signatures in otoliths and water collected from 13 European rivers showed that they could be used as natural tags of natal origins of Allis shad in a large scale of the species' European range.

The positive linear regressions presented for Sr:Ca, Ba:Ca and Li:Ca suggests that these elements are incorporated in the otolith in proportion to the corresponding ratios in the water. Our results suggest that water elemental composition can be employed to predict otolith microchemistry as, in natural environments, a strong correlation between the two variables exist. We found that Sr:Ca, Ba:Ca and Li:Ca ratios in juvenile Allis shad otoliths were correlated with environmental levels of river waters. Apart from the study of Walther and Thorrold (2008) that found resembling results for geochemical signatures in juvenile American shad (*Alosa sapidissima*), several other works has confirmed a strong effect of environmental Sr and Ba concentrations on otolith composition (e.g. Elsdon and Gillanders, 2003; 2005; Kraus and Secor, 2004).

In our study, the Cu:Ca and Cd:Ca ratios in otoliths were not correlated with dissolved levels of the environmental water. This find can result from the fact that fish may regulate Cu and Cd concentrations to physiologically optimum levels, decoupling otolith composition from that of environmental waters (Campana, 1999). Despite the potential role of metabolism in controlling otolith Cu and Cd, these two elements can still be used successfully as stock identifiers if significant differences in elemental concentrations among groups of fish exist. But, the non-significant relationship between otolith and water composition for both Cu:Ca and Cd:Ca ratios leads us to be cautionary when predictions are based on water samples. Thus, water samples may not replace direct analyses of otolith composition for studies that require such elements for habitat-specific signatures of Allis shad.

Accuracies of the individual cross-validation classification averaged 65,5% and were 100% for 2 rivers, 97,7% for one river and exceeding 70% for 4 rivers. However, CDA is a representative/descriptive analysis to display differences among Allis shad caught in different rivers, i.e. to show differences in natal signature from the otolith core of adults but do not assign the natal origin. Cross validation is a predictive analyse to evidence misclassifications.

Bayesian model was used to assign natal origins of adults with some probabilities per river. The model assigned geochemical signatures of most of the Allis shads captured in the Loire, Dordogne, Blavet and Minho similar to the geochemical signatures assigned for the water and juveniles' otoliths of the respective rivers. For the other rivers, the model assigned the natal origin of adults based in water elements, which, in turn, were chosen tanking in to account the linear relationship found between juveniles' otoliths and water chemistry of ambient natal environments. In this analysis, most of Allis shad sampled in Garonne had geochemical signatures identical to the Dordogne signatures. Tomás et al. (2005) well discriminated these two population using elemental fingerprints in juveniles, but the possibility of mixed fish sampling was eliminated. Where we used adult fish, whose natal origin actually could be the Dordogne river entering in Garonne river to spawn, as these two rivers are adjacent tributaries. The same can be assumed for the Scorff river samples, since they were assigned to be born in the adjacent Blavet river.

When looking for the Portuguese rivers, discrimination of the natal origin was quite clear to the Minho river samples: the vast majority were born in the Minho river. The most difficulty was found between the Minho river and the Lima river, where cross-validation classification of the canonical discriminant function showed the latest to be 50% similar with the Minho river. Actually, Bayesian model classified half of the Lima's river Allis shad as having geochemical signatures similar to those reported for the Minho river Allis shad. Even the low number of Lima river samples, we can speculate the geochemical signatures similarity. Lima river is quite near from Minho river (around 30 km) and it Allis shad population is thought to be very unstable in the last decades, mainly due to the block of spawning migration. Despite the decrease of Minho's Allis shad population since the second half of the 20th century, after the collapse of the Gironde population (Rougier et al., 2012), the Minho's population seems to be the most stable population of the European distribution range (unpublished data) and errant fish may spawn in Lima river. In fact, in anadromous migratory fish, a part of the population spawns in the river they born (homing), while some individuals may spawn in different watersheds (straying) (Howard, 1960; Rieman and Dunham, 2000).

According with the CDA cross-validation, Mondego river population seems to be a population apart from Lima river, but quite close from Minho population. In fact, Bayesian model classified 80% of the Allis shad captured in Mondego river as having similar geochemical signatures to Allis shad from Minho river. These two rivers are around 200 km apart from each other and it appeared that it were distinct Allis shad populations. As in this study the relationship of ^{87}Sr:^{86}Sr ratios between juveniles' otoliths and water samples were not assessed, the accuracy of the natal origin of Portuguese populations should be more investigated. A better knowledge of the natal origin of these two populations may provide valuable information for the stocks' management. Confirming Allis shad from Mondego belong to the Minho river population, then the stock of the Minho river is much higher than previously thought.

With better understanding of environmental and biological factors contributing to the variation in otolith fingertips, spatial maps at the correct temporal scales can be made to achieve fish movement (Harrington et al., 1998; Warner et al., 2005). To the present work it was not possible to analyse juveniles ^{87}Sr:^{86}Sr ratios. Because we used water samples to characterize otolith composition where juveniles were unavailable, we could only use elements and Sr isotopes that accrete in otoliths in proportion to their ambient concentrations.

The use of strontium isotopes in otoliths provided a powerful supplement to the range of elemental ratios to determine the natal origins of other anadromous fish as *Alosa sapidissima* (Walther and Thorrold, 2008). Previous studies have highlighted the utility of ^{87}Sr:^{86}Sr ratios to distinguish natal habitats at a local level (Kennedy et al., 1997; Ingram and 1999; Hobbs et al., 2005). Firstly, otolith ^{87}Sr:^{86}Sr values directly reflect environmental water chemistry (Kennedy et al., 2000). Secondly, the geological composition of bedrock within a watershed and the age of geological formations determine the ^{87}Sr:^{86}Sr ratio of dissolved Sr in river water (Palmer and Edmond, 1992). Because the rivers included in this study drained heterogeneous surface geologies (from Palaeozoic to Mesozoic), river-specific ^{87}Sr:^{86}Sr ratios were highly variable and an important component of signature separation.

Barnett-Johnson et al. (2008) suggests that watersheds' geological and hydrological analyses may be a valuable tool in assessing which sites may be most similar in their Sr isotopic values. It should be highlighted that Sr isotopes exhibit low within-population variability and thus some sites with relatively similar geology may still be distinguishable (Barnett-Johnson et al., 2008). This information should be taking into account in future small-scale studies, such as Portuguese populations.

^{87}Sr:^{86}Sr ratios proved to be a strong variable in discriminate Allis shad populations among Portuguese and other European rivers. As systematic variations in ^{87}Sr:^{86}Sr ratios among rivers were found, water samples may be generally an acceptable alternative of otolith analyses to characterize habitat-specific ^{87}Sr:^{86}Sr signatures on both small and large geographic scales.

The use of otoliths and water geographic signatures, if properly explored, can be an accurate method in determining mixed stock composition of migrating Allis shad in their

European range. This methodology should provide fundamental information to the effectiveness of management and conservation of this extremely exploited species. At the Portuguese level, it could be an important tool giving significant information to the management of the Minho, Lima and Mondego rivers Allis shad population. Minho river population is highly exploited and Lima river population, even with low number of spawners, still have a commercial exploitation. As they are two very close populations and certainly a marine mixed stock, the marine dynamic could be investigated effectively by using records from elemental fingerprints methodologies that identify natal origins of migrants. Tracking natal sources is important in understanding the status and trends of declining European stocks as Gironde, Minho and Lima Allis shad population.

5.2.5. ACKNOWLEDGEMENTS

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4.2. SEAWARD MIGRATORY PATTERNS OF THE ANADROMOUS ALLIS SHAD *Alosa alosa* (L.) OF AN IBERIAN RIVER INFERRED FROM INTRA-OTOLITH SR:CA RATIOS

Manuscript in final preparation

ABSTRACT

The Minho river Allis shad early life history was reconstructed using otoliths Sr:Ca ratios, otoliths measurements and timings of juveniles' entrance in the upper estuary. The migratory patterns were estimated by analysis of Sr:Ca ratio of spawners in upstream migration and the freshwater phase addressed by the Sr:Ca ratios recorded in otoliths of juveniles and by count of daily increments. The relationships between the age, fish lengths and otolith sizes of juveniles were also used to address the role of the estuary for the Minho river Allis shad. The correlations between otoliths size, fish length and age were all highly significant. The Sr:Ca ratios in spawners and some juveniles showed an abrupt increase in Sr:Ca value corresponding to the migration through the estuary from freshwater to seawater. The Sr:Ca ratios, both in juveniles and spawners, were significantly different ($p<0.0001$) between the freshwater and marine phases, being the mean value for the first marine Sr:Ca ratio 2.7×10^{-3} and for freshwater 0.91×10^{-3}. The juveniles appear to concentrate in upper estuary until they reach the optimal conditions to shift compartment, as larger juveniles showed a gradually increasing of Sr:Ca values in otoliths and an increasing trend in monthly otolith length and identical monthly dominant otolith size class were found. The positive correlation between the distances from the otoliths core and the first measurement of marine Sr:Ca ratio, the non-significant differences in the Sr:Ca ratios at the first marine measurement by cohorts and the strong correlation between the preliminary assessed age of juveniles and otoliths size suggest a concerted seaward exit and the existence of environmental window at the time of seaward migration.

Key words: *Alosa alosa*, seaward migration, estuary, otoliths, Sr:Ca ratios

4.2.1. INTRODUCTION

Diadromous fish spend part of their life cycles partially in fresh water and partially in salt water, moving across salinity gradients and representing important coastal and inland species (MacDowall, 1988). Catadromous species spend most of their life in fresh water, but breed in saltwater, whereas anadromous fish mature and overwinter in the sea and spawn in freshwater. Their life cycles successful depend on the quality and connectivity of these different ecosystems. Diadromous species bring beneficial conditions in both freshwater and marine habitats, as they provide energy flow and links biological material. Besides, most of these fish species provide commercial and recreational economic benefits.

As broadly known, throughout the last century a drastic decline on the diadromous fish abundances occurred, mainly due anthropogenic pressure (e.g. Lobón-Cervia, 1999; Groot, 2002; Baglinière et al., 2003; Hendry and Cragg-Hine, 2003; Beaulaton et al., 2008; OSPAR, 2008; OSPAR, 2009). Among these species, the anadromous Allis shad (*Alosa alosa* Linnaeus, 1758) have suffered a significant decline on it populations throughout their geographic range since the second half of the 20th century. The ancient distribution covered the eastern Atlantic from Norway to Morocco and the Western Mediterranean Sea, being nowadays restricted to the Atlantic coast of France and Portugal (Baglinière, 2000). Consequently, Allis shad was included in Appendix III of the Bern Convention and Appendix II of the European Union's Habitats Directive. After the collapse of the Gironde population (Rougier et al., 2012), one of the largest Allis shad populations in the southern distribution range seems to be the Minho river population, North-western Iberian Peninsula (unpublished data), where an important commercial and heritage meaning still remains since the beginning of the 20th century (Mota and Antunes, 2011).

As an anadromous fish, Allis shad pass most of it life cycle at the sea. Minhos' river population spend a period of 6-7 years on the sea and spawns in freshwater between latte June and early August (Mota and Antunes, 2011). Juveniles appear in the tidal freshwater wetlands (TFWs) between November (Mota and Antunes, 2012) and January (unpublished data).

An understanding of movement patterns of animals, in both space and time, is essential to the study of animal ecology (Pittman and McAlpine, 2003). Identifying ecologically important habitats and resources, services and functions is critical and decisive for effective conservation and management strategies. The lack of consider stock complexity by fishery managers leads many times to underestimate important components with ecological significance (Stephenson, 1999), which can grievously affect the long-term stability and sustainability of whole stocks (Fritsch et al., 2007).

Regarding Allis shad, at the European level, little is known about freshwater and estuarine phase (Mota and Antunes, 2012). The behaviour and dispersion of young stages of Allis shad is based on experimental conditions and wild ontogenetic events were rarely observed (Lochetet al., 2008).

The Allis shad freshwater and estuarine phase duration is poorly known. Until recently the estuarine phase was estimated as being between the first arrival of juveniles in the estuary (in January) and last record in the estuary (in March) (Lochet et al., 2009), comprising a seaward migration period of 3 to 6 months (Taverny et al., 2000; Baglinière et al., 2003). Lately, based on otolith Sr:Ca and daily growth increments, Lochet et al. (2008) estimated the Gironde system Allis shad estuarine phase lasted from 4 to 36 days. For the Minho system, studies on the spawners' biology (Mota and Antunes, 2011) and juveniles' habitat use in freshwater (Mota and Antunes, 2012) were recently performed, but no information is available concerning migratory patterns and time spend in freshwater and estuary by juveniles.

It is broadly known that of the various hard structures in fishes, ear-stones or otoliths are best appropriated for clarify many issues of fish life history (Gillanders, 2005). Current studies using natural tags as elemental composition in otoliths show great ability for studying populations' movements. Otolith microchemistry is, nowadays, one of the most important fields in otolith research, such as inferring the migratory patterns in diadromous fish.

The most common marker used for tracing salinity history and to reconstruct migratory history of diadromous fish is strontium (Sr), particularly the Sr:Ca ratio gradient in the water (Limburg et al., 2001; Kraus and Secor 2004; Milton et al., 2008).

In order to better understand the habitat use of Allis shad along the Minho system, investigations to reconstruct it life history were performed using the potential of geochemical tags in the otoliths. The objectives of the present study were to identify significant events occurring in the early stage of Allis and address the importance of the estuary for the Minho river Allis shad, using both adults and juveniles otoliths. The migratory patterns were estimated by analysis of Sr:Ca ratio of spawners in upstream migration and the period of the freshwater phase was addressed in juveniles caught in the TFWs before moving seaward through the count of otoliths' daily increments. The distances between both spawners and juveniles otoliths' core and the first measurement corresponding to the marine Sr:Ca ratios were used to address the migratory patterns. The relationships between the juveniles' age, lengths and otolith sizes were also used to this propose. Other factors, as sex for spawners and size for juveniles, were also tested for differences in Sr:Ca records.

4.2.2. MATERIALS AND METHODS

4.2.2.1. Sampling strategy

Allis shad sampling was performed in the international part of the Minho River, North-western Iberian Peninsula. Minho River is a watershed comprising around 17 000 km^2, a main course of just 300 km long and an estuary 40 km long, where salt intrusion is limited to 16 km and TFWs located in the upper 30 km.

Spawners samples were obtained in 2009, 2010 and 2011 and juveniles' samples in 2009, 2010, 2011 and 2012 from traditional Allis shad fishery and as by-catch (N=7) of the glass eel fishery, respectively and by experimental fishing, according to the methodology and sampling sites reported by Mota and Antunes (2011, 2012). Water temperature was recorded every sampling campaign. Spawners were sampled during the spawning migration, between March and August. The individuals were separated according to sex and the total length (TL) recorded for each fish. A sample of spawners' scales was collected to assess fish age. The scales were sampled and treated according to the protocol of Baglinière et al. (2001). Juveniles were sampled in the TFWs during the seaward migration (between September and January) and the TL recorded.

4.2.2.2. Otolith preparation and Sr:Ca ratios quantification

Both spawners and juveniles' sagittal otoliths were extracted and cleaned of organic tissues using Milli-Q water and plastic tools, air- dried and stored in plastic vials. Before the otoliths preparation for Sr:Ca ratios quantification, both spawners (N=221) and juveniles (N=134) whole otoliths were measured. For juveniles, Allis shad, Twaite shad and hybrids were considered. Left and right sagittal otoliths were weighed in a digital Kern ARS 220-4 scale and photographed sulcus side up, for length (µm) and width (µm) measurements. Otolith length was defined as the longest axis between anterior and posterior otolith edge, and otolith width as a distance from dorsal to ventral edge. Juveniles' sectioned otoliths (N=30) were also measured for length of the curvilinear axis of the ventral side of the otoliths.

For Sr:Ca ratios measurements, spawners (N=30) and juveniles (N=30) Allis shad sagittal otoliths were mounted in epoxy resin (Struers, Epofix) with the sulcus acusticus down.

The blocks were sectioned near the otolith margins by cutting away the excess resin with a low speed diamond saw (Buehler, IsoMet) at 6000 rpm. Otoliths were ground in the transversal plane to expose the core with 800, 1200 and 2400 silicon carbide papers (Hermes), making regular optical inspections under a metallographic microscope (Meiji, ML7100), and further polished with 6, 3 and 1 µm diamond pastes (Buehler, Metadi II). Finally, otoliths were cleaned in an ultrasonic bath with pure ethanol, rinsed with deionised water and given a carbon coating by high vacuum evaporation. Sr and Ca concentrations (% dry weight) were measured along the longest axis of the otolith using a X-ray Electron Probe Micro-Analyzer (EPMA, JEOL JXA-8500F). Apatite [Ca5(PO4)3] and celestite (SrSO4) were used as standards. Accelerating voltage and beam current were 15 kV and 20 nA, respectively. The electron beam was focused on a point about 10 µm in diameter, spacing measurements at 20 µm intervals. The acquisition time was 180s (30s per element, 30s for the measurement of the counts in the corresponding peak and 30s for measuring background contribution) per point. Quantitative data were calibrated by the ZAF method (Z, atomic number; A, absorption; and F, fluorescence correction). Limit of detection were 100 ppm. The microprobe measurement points, which were seen as burn depressions on the otolith surface, were assigned latter to otolith growth increments. The results are presented as the amount of Sr divided by the amount of Ca times 1000. Following microprobe analysis, the otolith surface was repolished with alumina solution (1:20) for light microscope observation (Olympus, CX41) at a magnification of 40x. Micrographs were taken using a digital camera (Olympus, SC30). The successive micro-increments from the core to the otolith edge were used for otolith growth analysis. A preliminary count of juveniles' otoliths daily increments were performed in order to estimate the fish age at the tidal freshwater wetlands.

4.2.2.3. Data analysis

Differences between the left and right whole otoliths' measurements (weight, length and width) were assessed using Kruskal-Wallis tests. Otolith size (left and right) depending on fish TL was analysed through linear regression modelling. In addition, differences between months of the whole left otolith size of juveniles were assessed using the Kruskal-Wallis and post-hoc pair-wise Wilcoxon rank sum tests, corrected for multiple testing. The relationship between the total length of juveniles, the whole otolith and sectioned otolith measurements and age were assessed using Spearman correlation test.

To identify the Sr:Ca ratio corresponding to the estuarine phase, we proceeded according to the method of Lochet et al. (2008, 2009) as following. Based on the positive relationship between otolith Sr:Ca and environmental Sr:Ca (Secor and Rooker 2000;

Kraus and Secor 2004), two thresholds were defined, one corresponding to the Sr:Ca ratios at end of the freshwater phase and other corresponding to seaward exit, with intermediate values corresponding to estuarine phase values. The juveniles were sampled in the upper limit of the estuary (TFWs) and the 95th percentiles of Sr:Ca ratios measurements were used as the threshold of freshwater exit. To define the threshold of seaward exit we considered only the peripheral part (clearly marine phase) of spawner's otoliths sampled in the estuary during the spawning migration; i.e. the values of Sr:Ca ≥ 0.001 after an abrupt jump to a level of higher Sr:Ca levels (> 0.002). The selecting values were done individually for each fish and the 5th percentiles of the measurements defined as the seaward exit.

The Shapiro-Wilk test was used to check normality between the Sr:Ca ratios in freshwater and marine phases. Although histogram shows a very balanced distribution of data, the Shapiro-Wilk test did not confirm the normality of data. Thus, parametric (t-test) and nonparametric (Kolmogorov-Smirnov test) were used to search for significant differences in the Sr:Ca ratios between freshwater and marine phases.

The distances between the spawners' otoliths core and the first measurement corresponding to the marine Sr:Ca ratios were taken in order to assess the correlation between them. The relations between the number of the probe measurement and the corresponding marine Sr:Ca ratios were also assessed. These distances represent the migration time according to the Sr:Ca ratio. Parametric (Pearson) and nonparametric (Spearman) tests were performed to assess such correlations. Post-hoc pair-wise Wilcoxon rank sum tests were performed to search for significant differences, both in distances and number of the probe measurement, between sexes and between cohorts.

As some juveniles (N=8) showed a Sr:Ca pattern indicating seaward migration, the TL of these juveniles and the TL of the rest of the juveniles (without seaward migration Sr:Ca pattern) were tested (T-test) for significance differences. Note that these juveniles were not included in the study to determine thresholds of Sr:Ca ratio between environmental phases.

To determine if the juveniles' migration between freshwater and the upper limit of the estuary occurs along a salinity gradient, linear regressions were performed. A linear regression with a positive slope and statistically significant was considered to indicate a "continuous/slow" migration along the estuary. Regression models were carried out for all juveniles. However, in the case of juveniles indicating passage for seawater (i.e. juveniles that showed a jump in Sr:Ca ratios to similar values reported in the marine phase), only the Sr:Ca previous to "jump" were considered. In addition, tests of significance for differences in TL between juveniles with and without significant slopes were performed.

Statistical analysis was performed using the R software (R Development Core Team 2008).

4.2.3. RESULTS

The period of presence of the juveniles Allis shad in TFWs and respective monthly mean water temperature are given in Figure 4.8. The presence of juveniles in the TFWs ranged from the late September to the beginning of January.

The preliminary result for the mean juveniles' age at the TFWs was 105 (SD ± 24) days. The youngest juvenile was 68 days and the older 164 days.

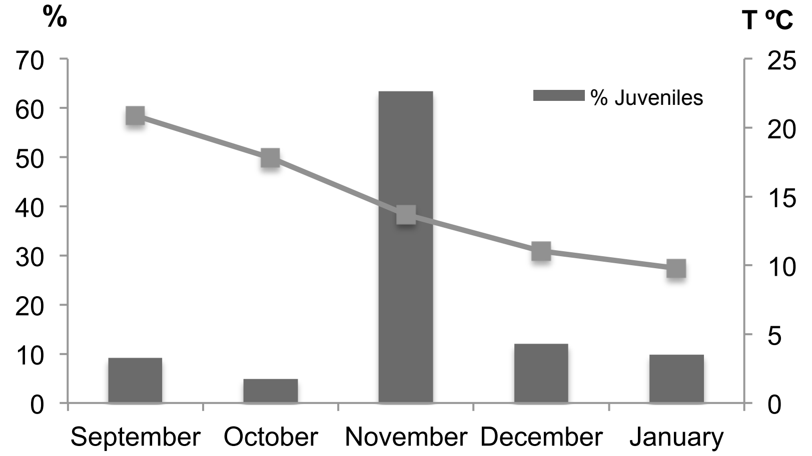

Figure 4.8. Monthly presence of juveniles Allis shad (N=142) in the TFWs and mean water temperature in the TFWs. The results are presented in percentage of fish caught per month (primary vertical axis) and water temperature in Celsius degrees.

Biometric characteristics of both juveniles and spawners analysed for Sr:Ca ratios are presented in Table 4.7.

Table 4.7. Characteristics of spawners and juveniles Allis shad. N: number of samples; TL: mean total length and standard deviation in brackets; Cohort: year of birth and number of samples (in brackets); YOY: young of the year.

	Year of sampling	Sex	N	TL (mm)	Cohort
Adults	2009	Females	5	678.3 (6.9)	2001 (2); 2002 (3)
		Males	5	592.3 (15.8)	2002 (1); 2003 (3); 2004(1)
	2010	Females	5	684.4 (5,5)	2003 (4); 2005(1)
		Males	5	598,1 (15,3)	2003 (1); 2004 (3); 2005(1)
	2011	Females	5	683,3 (6.6)	2004 (4); 2006(1)
		Males	5	598.8 (16.8)	2004 (1); 2005 (2); 2006 (2)
Juveniles	2009	-----	9	88,9 (29,7)	YOY
	2010	-----	3	65 (5)	YOY
	2011	-----	11	106.4 (33.5)	YOY
	2012	-----	7	921.4 (22.5)	YOY

4.2.3.1. Otolith measurements

Otolith measurements are summarized in Table 4.8. There were statistically significant differences between the measurements of the adult left and right otoliths. The left otoliths were significantly (*p*=0.018) longer (mean left−right difference was 79.31 µm = ~1.8%) yet significantly (*p*<0.001) less wide (mean left−right difference 28.52 µm = ~1.2%) than the right ones. In terms of weight, left otoliths were significantly (*p*<0.001) heavier than the right ones (mean left−right difference 0.04 mg = ~0.9%). In juveniles, left and right otoliths differed only significantly in weight (*p*<0.001 for Allis and Twaite shad, *p*=0.017 for hybrids). Left otoliths were heavier (mean left−right differences were 0.0400 mg = ~7% for Allis shad, 0.0028 mg = ~0.6% for Twaite shad and 0.0760 mg = ~14% for hybrids).

Linear models showed significant (*p*<0.001) positive linear relationships between fish TL and otolith length (Table 4.9). Models for adult Allis shad explained little of otolith length variation, compared to the models for juvenile fish. Furthermore, in juveniles, otolith lengths tended to increase with the sample month (although sample size per month does not allowed for many statistical comparisons).

Excluding months with no or only a single sample with otolith measurements, monthly mean (left) juveniles' otolith lengths were: for Allis shad, 1476.6 µm (N=11), 1654.4 µm (N=72), 1529.8 µm (N=14) and 1714.2 µm (N=13), for September, November, December and January, respectively; for Twaite, 1444.7 µm (N=79), 1545.0 µm (N=41) and 1624.1 µm (N=2), for September, November and December, respectively; and for hybrids, 1412.0 µm (N=12), 1618.6 µm (N=24), 1568.1 µm (N=3) and 1620.5 µm (N=6), for September, November, December and January, respectively. For Allis shad, otoliths were significantly (*p*<0.05) shorter in September than in January; for Twaite shad, they were significantly shorter in September than in November; and for hybrids, significantly shorter in September than in November or January. Dividing otolith lengths into 200 µm-size classes, the dominant size classes for Allis shad were 1500-1699 µm for September (with N=7, corresponding to 64% of the monthly observations), November (N=27; 38%) and December (N=6; 43%), and 1700-1899 µm (N=7; 54%) for January. The dominant size classes for Twaite shad were 1300-1499 µm for September (N=39; 49%) and 1500-1699 µm for November (N=19; 46%), and for hybrids 1300-1499 µm for September (N=7; 58%) and 1500-1699 µm for November (N=9; 38%).

Table 4.8. Right (R) and left (L) otolith measurements for Allis shad spawners and for Allis shad, Twaite shad and hybrid juveniles (one single very large Allis shad juvenile specimen was excluded). N: number of individuals.

Sample	N	Measure	Side	Minimum	Mean	Maximum
Adult Allis shad	221	Length (µm)	R	3612	4454	5191
			L	3856	4547	5268
		Width (µm)	R	2033	2442	3866
			L	2086	2409	2833
		Weight (mg)	R	2.00	4.43	6.50
			L	2.80	4.48	6.20
Juveniles Allis shad	49	Length (µm)	R	1072	1631	2213
			L	1134	1626	2137
		Width (µm)	R	663	935	1206
			L	687	950	1219
		Weight (mg)	R	0.10	0.53	1.20
			L	0.10	0.56	1.30
Juveniles Twaite shad	45	Length (µm)	R	955	1474	1846
			L	964	1481	1891
		Width (µm)	R	567	812	1064
			L	593	833	1026
		Weight (mg)	R	0.10	0.48	1.20
			L	0.10	0.50	1.20
Juveniles hybrids	40	Length (µm)	R	1092	1588	2190
			L	1107	1561	1964
		Width (µm)	R	665	887	1238
			L	691	889	1050
		Weight (mg)	R	0.20	0.48	0.90
			L	0.10	0.52	1.30

Table 4.9. Linear models of right (R) and left (L) otolith length depending on fish total length (TL); intercept and TL were significant terms (p<0.001) in all of the models.

Sample	Side	Model	R^2
Adult Allis shad	R	2547.5+29.5 TL	0.193
	L	2660.5+29.2 TL	0.216
Juveniles Allis shad	R	533.7+112.0 TL	0.807
	L	486.8+117.4 TL	0.856
Juveniles Twaite shad	R	473.6+115.9 TL	0.786
	L	508.2+112.6 TL	0.835
Juveniles hybrids	R	574.3+111.7 TL	0.439
	L	295.3+140.8 TL	0.760

The correlations between the whole otolith-sectioned otolith (R^2=0.94), whole otolith-age (R^2=0.82), sectioned otolith-TL (R^2=0.92), sectioned otolith-age (R^2=0.88) and TL-age (R^2=0.76) were all highly significant (p<0.0001).

4.2.3.2. Otolith Sr:Ca ratios

The transects of Sr:Ca ratios in spawners (Fig. 4.9A) and some juveniles (Fig. 4.9B) showed an abrupt increase in Sr:Ca value understood as the migration through the estuary from freshwater to seawater (Fig. 4.10).

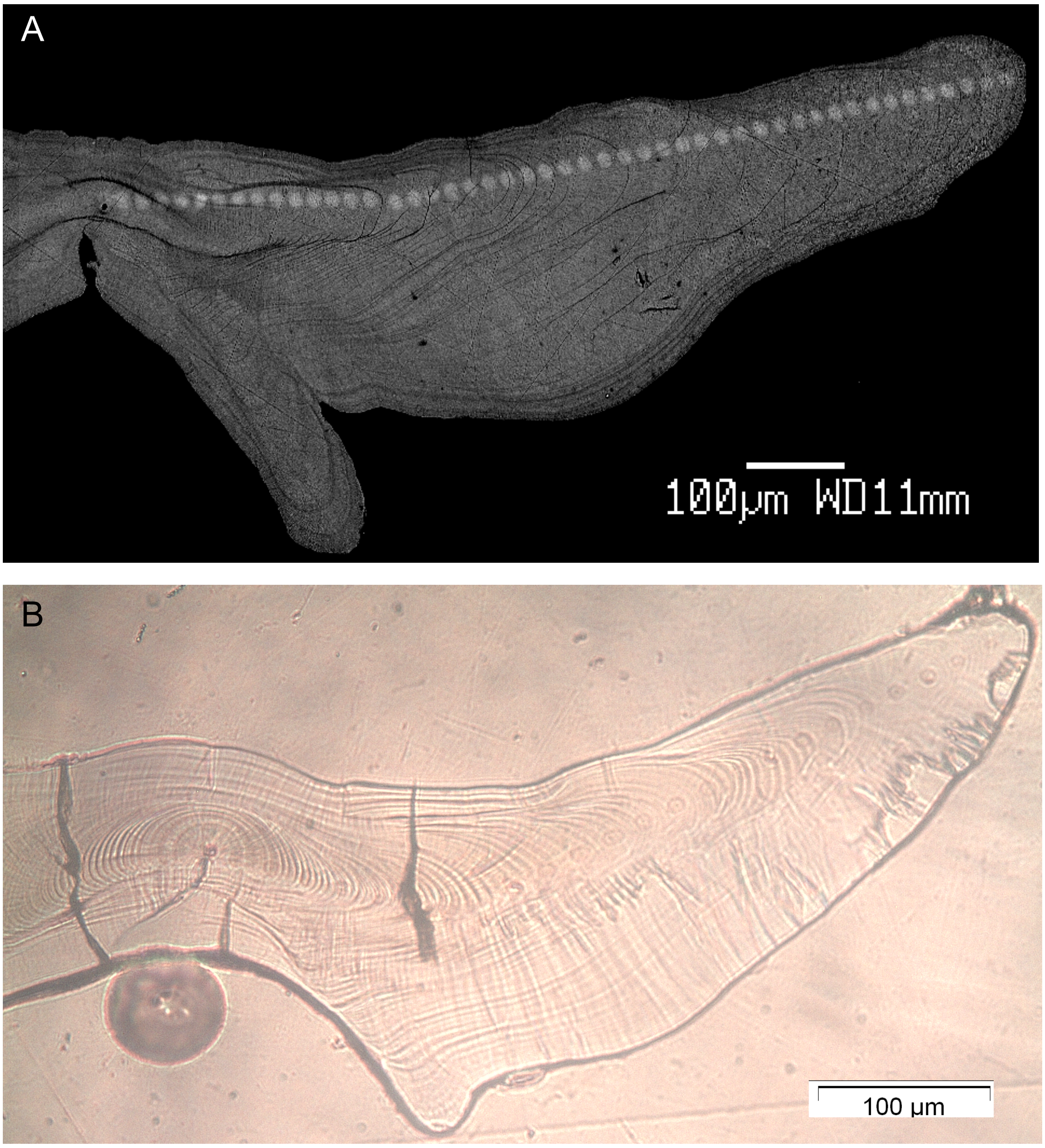

Figure 4.9. Transverse section of a spawner (A) and a juvenile (B) Allis shad otolith exhibiting the electron microprobe burn depressions (light marks in the spawner otolith and deep marks in juvenile otolith).

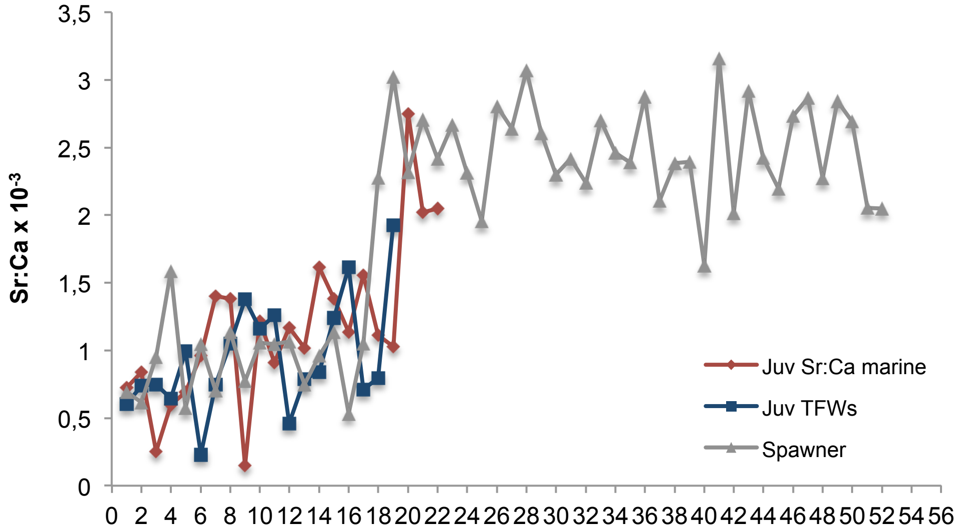

Figure 4.10. Three examples of Sr:Ca ratio profiles according to the distance from the otolith's core to the entrance in upper estuary (Juv TFWs) and to the seaward exit in spawners (Spawner) and juveniles (Juv Sr:Ca marine).

The Sr:Ca ratios measured in juveniles and spawners otoliths were significantly different ($p<0.0001$) between the freshwater and marine phases. The mean value for the first marine Sr:Ca ratio was of 2.7×10^{-3} (SD±$0{,}58\times10^{-3}$), the minimum value of 1.9×10^{-3} and the maximum value of 5.3×10^{-3}. The average for the freshwater Sr:Ca ratios was of 0.91×10^{-3} (SD±$0{,}32\times10^{-3}$), the minimum value of 0.005×10^{-3} and the maximum value of 1.7×10^{-3}.

The limit of Sr:Ca ratio estimated by juveniles' otoliths for the migration between freshwater and estuary was 1.53×10^{-3}, but our results revealed, by retrospective approach in spawners otoliths, the estimated limit of Sr:Ca ratio for migration between the estuary and the sea as 1.39 $\text{X}10^{-3}$, i.e. below to the limit found to the freshwater exit.

Considering the parametric test, there is a significant correlation ($p<0.05$) both between the distances from the core of the spawners' otoliths and the first measurement of Sr:Ca ratio corresponding to marine values and between the number of the probe measurement and the corresponding marine Sr:Ca ratios. Nonparametric test showed almost significant correlations ($p=0{,}051$).

Considering the distance of the first Sr:Ca marine measurement to the core, there was no significant difference ($p>0.05$) between the time of migration of females and males, either in all cohorts together or for the cohorts separately. The same was found when analysing differences in the number of the first Sr:Ca marine measurement. Both for the number and distance of the first Sr:Ca marine measurement, there were no significant differences ($p>0.05$) between cohorts, nor in the total fish nor for sexes separately.

When looking for differences in the TL of juveniles showing seaward migration Sr:Ca pattern and the TL of juveniles without evident seaward migration Sr:Ca pattern, significant differences ($p<0.05$) were found between the two groups, being the later smaller in TL. Regarding the juveniles' migratory patterns between freshwater environmental and the TFW, the linear regressions showed only 8 juveniles having a gradually and significantly increasing values of Sr:Ca in their otolith (i.e. a significant slope in the linear model). Five of them showed this increasing trend in the Sr:Ca ratios and a subsequent jump to marine values. These were, generally, the larger juveniles. The significance tests indicated difference ($p<0.05$) between the TL in juveniles with and without significant positive slope of Sr:Ca ratios.

4.2.4. DISCUSSION

Age estimates using otolith sectioning and interpretation is most often a time consuming process (Boehlert, 1985). As the relationship between fish age and otolith length and/or weight can be used for age estimation, the validation of otolith length/weight-at-age relationships may be a useful tool in determining the young-of-the-year age through otolith measurements and help estimating freshwater and estuarine timings.

In the present work we performed a first and a preliminary approach of the seaward migration patterns of the Minho river Allis shad using juveniles' otoliths size and TL and Sr:Ca ratios in both juveniles and spawners otoliths. Unlike the study of Lochet et al. (2009), where the presence time in the estuary was estimated by the Sr:Ca analysis of returning spawners otoliths, in the present study we combined several information as the monthly presence of juveniles in the TFWs, the relationship between juveniles TL and otoliths size and age, and the Sr:Ca ratios of juveniles and spawners in order to understand the seaward migration patterns of juveniles Allis shad.

Our results showed highly significant correlations both between the size of otolith and juveniles' length and age and between juveniles' length and age. The results also showed a strong and significantly difference between the freshwater and marine Sr:Ca ratios, but it was not possible to address the precise timing of the estuarine phase. The Sr:Ca values in freshwater and seawater were relatively close to those found for American shad and the Gironde Allis shad. American shad freshwater and seawater values was around $0.79\text{x}10^{-3}$ and $3.62\text{x}10^{-3}$, respectively (Limburg, 1995) and the Gironde Allis shad freshwater value was up to $1.1\text{x}10^{-3}$ and seawater values over $1.9\text{x}10^{-3}$ (Lochet et al., 2008; 2009).

Applying the method of Lochet et al. (2008, 2009) to estimate the estuarine phase during seaward migration to the Minho river Allis shad, we obtained an estimated limit of Sr:Ca for the migration between estuarine and marine environmental below to the Sr:Ca limit estimated of for the migration between freshwater and estuary, which may not be realistic. Actually, the minimum value found for the first marine Sr:Ca measurement was higher than those found for the limit of Sr:Ca ratio between freshwater and estuary. On the other hand, although the Sr:Ca values were significantly different for freshwater and marine phases, there was a great variability in the Sr:Ca values both for freshwater and seawater phases. There are several possible explanations for this fact. Movements, both pre-and post-migration, of fish in the estuary and the mouth of the river may occur. Still, strong variations in environmental salinity due to alternating great influence of river flow/ocean flow for extended periods of time can influence the rate of Sr and Ca incorporation or even the ontogenic incorporation of the elements may vary. Thus, in the case of Minho river Allis shad, it will be necessary to search for a more accurate percentile that better reflects the limit of Sr:Ca ratio for the seaward exit. Moreover addressing the age at the estuary entrance and at the seaward exit both in spawners and juveniles will allow estimate the durability of the estuarine phase and better understand the role of estuary for the species. Nonetheless, the present study provides initial insights concerning this issue. The use of otoliths of juveniles at the entrance in the estuary can be an advantage and provide a more detailed analysis in the search of the limits of Sr:Ca ratios for the movements between the freshwater compartment and the estuarine compartment.

The Minho river Allis shad juveniles appear to concentrate in TFWs until they reach the optimal size (and age) to shift compartment (late fall/ early winter, mainly in November). We observed, on the one hand, an increasing trend in monthly otolith length (and consequently in TL, as otolith length and fish TL show positive linear relationship) and, on the other hand, verified that, for *A. alosa*, the dominant otolith size class was identical for all months (except January). The identical TL increasing patterns of hybrids and Allis shad may suggest hybrids to move downstream starting from Allis shad locations and, thus, the sharing of the breeding sites. As hybrids seem to be mainly *A. alosa* backcross (unpublished data) this hypothesis should not be excluded. Moreover, the non-significant differences between Twaite shad and hybrids TL should be further investigated.

We can therefore hypothesise that most of the individuals reach the TFWs approximately at the same age but not yet with the optimal age to change compartments, since the monthly TL increasing trend may reflect a stay in the TFWs up to ideal size/age for the seaward migration. The seaward exit of Minho juveniles Allis shad seems hence to be size-at-age dependent. In fact, Sr:Ca ratios in spawners' otoliths indicated a correlation between the distances from the otoliths core and the first measurement of marine Sr:Ca ratio, suggesting a concerted seaward exit. Moreover, the strong correlation between the preliminary assessed age of juveniles and both sectioned and whole otoliths size reinforces the hypothesis of an environmental window at the time of seaward migration. On the other hand, the non-significant differences in the Sr:Ca ratios at the first marine measurement by cohorts also points to the same fact. In addition, this environmental window does not seem to be related with sex either.

The larger juveniles also showed a gradually increasing of Sr:Ca values in their otolith. This fact may underline the above assumption of permanence in the TFWs until the optimal age to the seaward migration be reached. This pattern was not found in younger individuals, since they probably just arrived to the TFWs, having a short stay in this ambient. Although the saline influence on the TWFs is unlikely, given the time of year (rain season) and the associated strong river discharges, the Sr water values may differ from those of the birth sites and, thus, inducing a gradual Sr otolith chemical signature. But this assumption can only be validated when the water Sr profiles in this river system were further investigated.

Juveniles where the Sr:Ca ratio of a migration pattern was found tend to be larger and older than the others. The fact that larger juveniles have an evident seawater signature recorded by Sr:Ca ratios in otoliths may indicate that these individuals have more complex migratory behavior that include multiple movements between the river mouth and the TFWs. The distance between the river mouth and the TFWs where juveniles were captured is around 16 km apart and juveniles may eventually be prompted to move with the tides or, alternatively, search for more favourable environmental conditions.

The "environmental windows", in ecology, is frequently related with environmental conditions, as temperature (Limburg, 1996b; McCormick et al., 1998), food availability (Cury and Roy, 1989; Sirois and Dodson, 2000) and physiological stage (Zydlewski et al., 2003). The seaward migration strategies of juveniles Allis shad in the Minho river seems to have a very short estuarine duration, with a particular time window. Actually, the Sr:Ca ratios recorded in spawners otoliths showed an abrupt variation in the Sr:Ca values between the freshwater and marine environments.

The presence in the TFWs before seaward migration can be limited by temperature. Although the limited knowledge concerning the temperature tolerance for Allis shad, Backman and Ross (1990) demonstrated that American shad stop feeding at about 9 ºC, with consequent negative growth rate and increased mortality. The low temperature in the estuary during winter and the higher temperature in sea water can explain the time window (Lochet et al., 2009). In fact, higher percentages of juveniles present in the Minho river TFWs were found in November, before a drop of water temperature to values around 11 ºC. Nonetheless, the environmental window may also be associated to the physiological condition of the fish. For the American shad, it was verified that the seawater acclimation for late migrants has great physiological constraints (Zydlewski et al., 2003). However, there are evidences that the Allis shad only enter in the estuary after the full acclimation to seawater (Lochet et al., 2009). Thus, in addition to the temperature, the size-at-age dependence in TFWs can also be related to physiological processes of osmoregulation; i.e. the complete acquire of osmoregulatory abilities in the upper estuary (TFWs), where no saline influence is verified at this time of year. For the Sr:Ca ratios recorded in spawners otoliths, it not seems to have a gradual passage through the estuary. We can then hypothesize that the role of the estuary to the species may just represent a passway between the freshwater and marine environments. A long presence in the estuary can bring disadvantageous conditions, such as competition for food resources and predation (Lochet et al., 2009).

In the present study we demonstrated the first evidence that there is an environmental window for the Minho river Allis shad seaward migration. As a better understanding of juveniles' movements between different compartments (freshwater, estuarine and marine habitats) may be helpful to ensure the feasible management of the species (Lochet et al. 2008), future investigations will be conduced in order to properly correlate the Sr:Ca ratios with age, by counting daily increments, and search for patterns of juveniles' entrance in the tidal freshwater wetlands. The thresholds of Sr:Ca ratio values corresponding to the estuarine phase will be further investigated by searching for the most accurate Sr:Ca ratio for the seaward exit.

4.2.5. ACKNOWLEDGEMENTS

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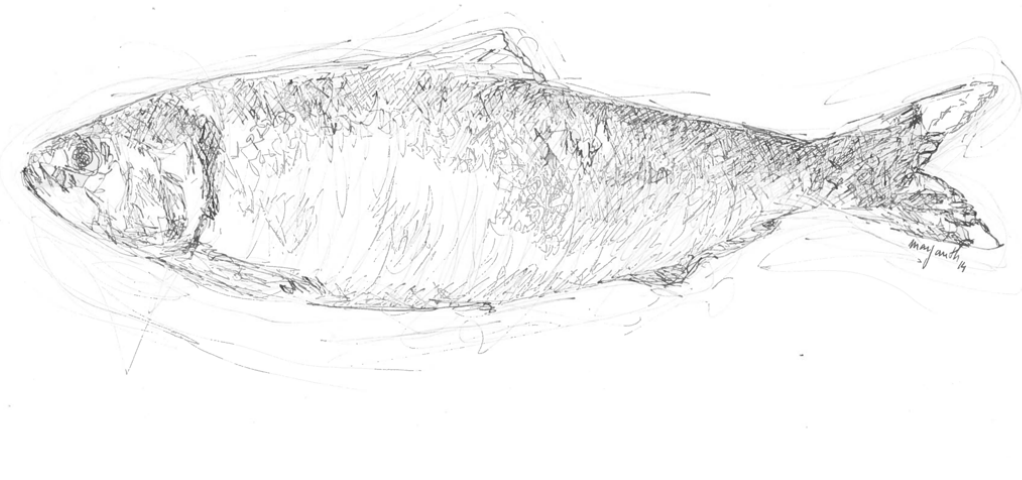
CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS



5. FINAL CONSIDERATIONS

The importance of diadromous fish species largely exceeds the proportion they represent in relation to the total fish fauna in the world. In addition to the tremendous value they represent to humans, these species play an important ecological role in aquatic ecosystems. Their peculiar life cycle, with migratory routes through various marine, estuarine and freshwater habitats, makes them important intermediates in the energy flow between these habitats (Willson and Halupka, 1995). The characteristics of their life cycles not only have important ecological consequences, but also have implications for their conservation and management. If on one hand the use of diverse habitats offers them the opportunity to spread, on the other hand exposes them to a wide range of environmental impacts. In many cases, their migration routes cross international borders, leading to often ineffective efforts of international cooperation in their management and conservation, due to lack of coordination between the different stakeholders and, also, resulting often from the lack of biological and ecological information.

Being the Minho an international river and the Allis shad an important species in terms of exploitation of their stock as well as under the conservationist point of view, the present work aimed to provide information on key ecological and biological aspects, by providing eco-demographic and migratory understanding, as well as useful spatial and temporal occupation indications for the management of the species.

Knowledge of the age structure, fecundity, migration timings and migration behavior are fundamental tools for managing populations, providing some level of predictability of recruitment and stock-recruitment relationship in each year, as well as of migration patterns. This information may be relevant and provide guidance to the resource planning and management, including in the definition of fishing effort per season for this species.

Identification of the spawning areas and the preliminary recognition of the habitat use and feeding of the juveniles' continental phase was a significant advance in this river system, and the information gained may be transposed to other systems. Regarding the spawning areas, which are located immediately downstream of the Minho rivers' first dam, prevails the regulation of water dam discharge in order to ensure adequate water flow and speed for the survival of recruits. Regarding the freshwater habitat occupied by juveniles, this preliminary recognition provided information of the physical characteristics of these locations, which may be taken into account in the search for identical sites and their protection at appropriate times. It is noteworthy that, even at European level, to date, very little is known about the distribution of juveniles in freshwater environments. The first approach taken in this study has provided relevant information to this level.

The study of the otoliths microchemistry provided first indications on the discrimination of natal origin and marine occupation and distribution of mixed stocks. This tool can be further explored in the future, in order to correctly recognize different stocks and their respective size. Two of the three addressed populations, Minho and Lima rivers, are geographically very close, and the Mondego river, though not so close, appeared in this study with similar geochemical signatures to those of Minho river. This should be explored in order to understand the real extent of the stock of Minho River.

On the other hand, we also inferred, in a preliminary way, on the time of the freshwater and estuarine phases by juveniles, and about the role the estuary plays for the species. First evidence points to the existence of an environmental window at the time of the seaward migration and being the estuary a passway linking the freshwater and marine environment. This information, coupled with knowledge of the timings of the spawning season and of the arrival of juveniles to tidal freshwater wetlands, seems especially relevant to the Minho river population, as the migration towards the sea coincides with the glass eel fishery season, legally allowed on this river system, and from which juvenile shads are subject to by-catch.

The issue of the supposed overlapping of the spawning grounds of Allis shad and Twaite shad induced by dam construction and subsequent hybridization between the two species can, in this specific case, be controversial. Despite the higher proportion of juvenile hybrids, the ratio of spawner hybrids in this river system seems quite low. In fact, the presence of low numbers of spawner hybrids seems to bring few ecological and conservationist implications. On the other hand, looking at the proportion of juvenile hybrids, the question arises regarding their viability or even their return to the natal river. This also shows that there is effectively hybridization, but not necessarily that there is an overlapping of the spawning grounds.

In addition to the acquired knowledge, this work defines several lines of research that deserve to be followed and deepened. It would be important to analyze the time series so as to modeling the population dynamics, which is essential for a more feasible management. This would imply the need for data from at least one generation, since the models are designed based on information such as spawners' sex and age, as well as biological parameters such as growth, sexual maturation, reproduction and natural and anthropogenic mortality (Martin Vandembulcke, 1999). Moreover, an accurate estimate of the number of recruits each season is of great importance in order to predict the number of spawners that will migrate in year n, based on the estimated number of juveniles in the estuary for years n-4 to n-8.

The adaptation of a management tool similar to the one developed by Woillez and Rochard (2003) for Allis shad populations in the Gironde-Garonne-Dordogne system could be evaluated for the population of Minho river. It represents a tool for decision support, prepared as a set of indicators that inform decision-makers about the state of the system, allowing them to act in a concerted manner. Therefore, an active cooperation of fishing communities and marine authorities is essential, since for its development, in addition to biological information, it is also necessary knowledge on the actual population size. This information is based largely on a thorough knowledge of the professional fishing activity, mainly regarding the fishing effort. In the present work, this cooperation has been introduced and it would be important to keep it for the support of future research lines.

Finally, it would be crucial, at national level, to extend the geographical area of knowledge concerning this species. Thus, the present study may be used as an example for the investigation of the Allis shad populations such as those of Lima, Mondego and Guadiana rivers.

This work provided a basis understanding for the biology and ecology of the Minho river Allis shad, which may allow for a better monitoring of its recruitment, as well as stimulate the realization of coordinated conservation actions and provide support for the management of the species. From the scientific point of view, this work opens new perspectives for future research lines.

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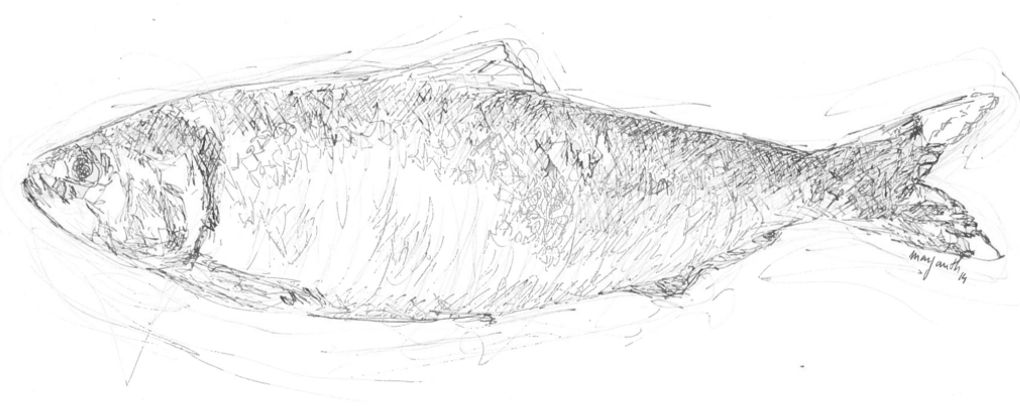

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