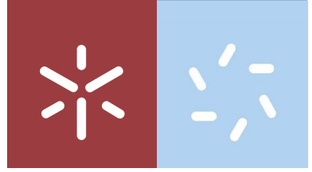
Phenotypic plasticity of habitat use by the European eel, *Anguilla anguilla* (Linnaeus, 1758) in the Minho River

Alexandre Manuel Calheiros Pereira Gomes

UMinho | 2022



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Dissertação de Mestrado
Mestrado em Biodiversidade, Ecologia, e Alterações Globais

Trabalho efetuado sob a orientação do(a)
Doutora Sofia Ester e Sousa de Aguilar Dias
e do
Professor Doutor Ronaldo Gomes Sousa

julho de 2022

AGRADECIMENTOS

Chegado ao final o processo de investigação e escrita desta dissertação de mestrado, apenas me resta agradecer a todos os que, direta ou indiretamente, contribuíram para o sucesso desta etapa.

Em primeiro lugar gostava de agradecer à Doutora Ester Dias e ao Doutor Ronaldo Sousa por toda a ajuda e apoio para que isto fosse possível. Um especial agradecimento para eles, que me acompanharam em todas as etapas deste processo e sempre me motivaram a fazer o melhor possível. Deixo também um enorme agradecimento a todas as equipas que me acolheram e me deram todas as condições para que eu pudesse desenvolver esta investigação, ao CIIMAR, ao Aquamuseu, ao Departamento de Biologia da Universidade do Minho, ao CBMA e também ao IB-S.

Claro que não podia passar sem agradecer à minha família, pai, mãe, sem vocês não tinha chegado até aqui, não podia concluir esta etapa sem o vosso apoio. Para a Or, e aos meus amigos, deixo também um especial obrigado por todo o suporte e pelo incentivo que precisei durante o caminho, quando as coisas não corriam da melhor forma.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Plasticidade fenotípica do uso de habitat pela Enguia europeia, *Anguilla anguilla* (Linnaeus, 1758) no Rio Minho

RESUMO

O decréscimo populacional da enguia europeia (*Anguilla anguilla*), desde a década de 1980, tem origem numa combinação de factores ambientais e antrópicos. Estudos anteriores mostram que as enguias apresentam diferentes estratégias de uso de habitat e este comportamento pode ter um papel chave no estado de saúde das populações. Assim sendo, os objeticos deste estudo foram: i) identificar o padrão de uso de habitat da Enguia Europeia (a partir deste ponto, enguia) no Rio Minho (SO, Europa), ii) avaliar a condição dos indivíduos associado a cada estratégia, e iii) investigar se a forma dos otólitos permite a discriminação entre grupos de indivíduos, de acordo com a estratégia de uso de habitat. Para isso, 80 enguias foram recolhidas em três localizações ao longo do gradiente de salinidade e os rácios de Sr:Ca e Ba:Ca dos otólitos foram usados para reconstruir o uso de habitat. Todos os indivíduos foram classificados como residentes em água salobra, residentes em água doce, nómadas para montante, nómadas para jusante ou nómadas entre habitats. Como indicadores da condição das enguias, o índice de Fulton, índice hepatosomático, conteúdo de lípidos no músculo e a infeção da bexiga natatória pelo nemátode *Anguillicola crassus* foram avaliados. Para distinguir as enguias entre grupos de acordo com a estratégia, a microestrutura dos otólitos foi analisada utilizando os seguintes índices de forma: fator de forma, arredondamento, circularidade, retangularidade elipticidade. Os resultados mostraram que as enguias têm diferentes padrões de uso de habitat, que variam entre táticas residentes e nómadas. A percentagem de residentes (51%) e de nómadas (49%) foram similares entre as enguias analisadas. Apesar disso, a tática dominante foi a nómada entre habitats (39%), seguido das táticas de residência – água salobra (26%) e água doce (25%). Nem todas as enguias entraram em água doce até à data da captura (26%), sugerindo estratégias de ciclo de vida alternativas à catadromia. A estratégia dominante não está associada a uma melhor condição; os grupos que mostraram valores de mediana mais elevados para os índice hepatosomatico e de conteúdo lipídio foram os residentes em água salobra e os nómadas para montante. Estes grupos também mostraram a percentagem mais baixa de prevalência do parasita *A. crassus*. Este estudo realça a importância dos habitats estuarinos na conservação e gestão desta espécie. Os índices de forma dos otólitos foram mais eficazes a distinguir os indivíduos com maior permanência em água doce ou salobra do que a discriminar entre estratégias de uso do habitat.

Palavras-chave: Ecologia, Plasticidade de uso de habitat, Química dos otólitos, Microestrutura, Avaliação da condição.

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ABSTRACT

The European eel (*Anguilla anguilla*) populations declined dramatically since the 1980s due to a combination of environmental changes and a variety of anthropogenic impacts. Previous studies showed that eels can present different habitat use strategies and this behavior can have a key role in populations' health status. Thus, the objectives of this study were to: i) identify the habitat use patterns of European eel (hereafter, eel) in the Minho River (SW- Europe), ii) evaluate the condition of the individuals associated to each strategy, and iii) investigate if otolith shape could discriminate between groups of individuals according to the strategy in habitat use. For that, 80 eels were collected in three locations along the salinity gradient and the ratios of Sr:Ca and Ba:Ca in otoliths were used for habitat use reconstruction. All individuals were classified either as brackish residents, freshwater residents, upstream shifters, downstream shifters or interhabitat shifters. As indicators for eel condition, Fulton index, hepatosomatic index, muscle fat content, and infection with the introduced swimbladder nematode *Anguillicola crassus* were assessed. To discriminate between groups of eels according to strategy, otoliths' microstructure analysis was conducted using the following shape indices: form factor, roundness, circularity, rectangularity and ellipticity. The results showed that eels presented different habitat use patterns with resident and nomadic tactics. The relative percentage of resident (51%) and nomadic strategies (49%) were similar among the eels analyzed. Nonetheless, the dominant strategy was the interhabitat shifting (39%), followed by the resident strategies - brackish (26%) and freshwater residents (25%). Not all the eels entered a fresh water habitat (26%), suggesting alternative tactics to catadromy.The dominant strategy was not associated to a better condition; the groups showing the highest hepatosomatic index and muscle lipid content median values were the brackish residents and downstream shifters. Also, these groups showed the lowest prevalence values of the parasite *A. crassus*. This study reinforces the importance of estuarine habitats for the conservation and management of this species. The otoliths' shape indices were more effective in discriminating between individuals inhabiting brackish and freshwater habitats, than between strategies.

Keywords: Ecology, Habitat use plasticity, Otolith chemistry, Microstructure, Condition assessment.

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LIST OF ABBREVIATIONS

Ba – Barium

BR – Brackish water resident

Ca – Calcium

CI – Circularity

CITES – Convention on International Trade in Endangered Species

CSH – Conditional strategy hypothesis

EU – Europe

DS – Downstream shifters

EL – Ellipticity

FF – Form factor

FR – Freshwater residents

HSI – Hepatosomatic Index

IS – Interhabitat shifters

IUCN – International Union for Conservation of Nature

K – Fulton Index

LH – Life history

LHP – Life history plasticity

Mg – Magnesium

MLC – Muscle lipid content

Mn – Manganese

NW – Norwest

OA – Otolith area

OL – Otolith length

OP – Otolith perimeter

OW – Otolith width

RE – Rectangularity

RO – Roundness

SI – Shape indices

Sr – Strontium

US – Upstream shifters

INTRODUCTION

Biodiversity loss in aquatic environments is at warming levels and is expected to cause negative alterations to the ecosystem processes and services and, consequently, to human well-being. The aquatic ecosystems' biodiversity loss is caused by exploitation, climate change, water pollution, and habitat degradation (Worm et al., 2006; Williams-Subiza and Epele, 2021).

Among the aquatic species impacted by human activities, the migratory species face constantly greater risks due to habitat reduction and fragmentation, pollution, and overfishing (Chapman et al., 2011). Climate change poses additional threats to populations living at the species' southern distribution area, particularly along mid-latitudes (Wigley et al., 1981). Climate change is also disrupting long-established cycles of water temperature, salinity, precipitation, and river flow patterns (Wigley et al., 1981). The disruption of established cycles and the increase of extreme climatic phenomena may induce evolutionary changes in both its strength and direction (Tamario et al., 2019), which putatively will affect fish phenotypic diversity, recruitment, and population connectivity.

Life history plasticity

Life history (LH) is an evolutionary biology theory that describes species' individuals' life cycles from the moment of birth to death, including their development and reproduction (Nylin and Gotthard, 1998).

Diadromous migration assumes that most of the individuals of a population migrate between rivers and the sea at a predictable time through physiologically mediated processes (Morais and Daverat, 2016). However, some species exhibit variations to the predominant migratory tactics (Dodson et al., 2013; Tamario et al., 2019). Fish migration encompasses a broad range of behaviors and LH strategies which includes whether or not to migrate (migration vs residency), migration timing (synchrony vs asynchrony), and distance traveled (Tamario et al., 2019). The LH diversity also includes movements within a mosaic of habitats with asynchronous availability of resources and other biological interactions within a watershed (Brennan et al., 2019). Thus, biocomplexity and diversity of LH strategies can be described as portfolio diversification to increase population stability and resilience (Schindler et al., 2010), which likely resulted from a successful evolutionary process to maximize individual fitness (Dodson et al., 2013). This portfolio effect provides species with an extraordinary adaptive tool to persist in varied and unpredictable environments (Schindler et al., 2010).

The complexity of fish life history plasticity (LHP) has focused mainly on anadromous fish (i.e., migration of adult fish from the sea to freshwater to spawn). Some salmonid species display alternative migratory tactics to anadromy (migratory vs. resident), which co-exist within populations and potentially can be adopted by any individual (Dodson et al., 2013). The variation in the migratory behavior is controlled by

environmental, physiological, and morphological thresholds, and part of that variation is genetically controlled (Dodson et al. 2013). For example, the timing of sexual maturity and the adoption of a resident tactic in male Atlantic salmon, *Salmo salar* (Linnaeus, 1758), is strongly related to body size (Paez et al., 2010). Heritability studies regarding salmonid fishes show that LHP has the potential to show responses to natural selection and constitute an adaptive process (Dodson et al., 2013).

Although most of the studies on LHP have focused on anadromous species, some studies addressed this behavior also in catadromous species (i.e., migration of adult fish from the river to sea to spawn) (Chapman et al., 2012). For example, the European flounder *Platichthys flesus* (Linnaeus, 1758) may use coastal areas, brackish, and freshwater habitats during larval development, spending variable periods in each one (Daverat et al., 2012; Dias et al., 2020). The existence of alternative LHs was also proposed for *Anguilla* species. As in European flounder, species from the genus *Anguilla* show different patterns in habitat use during their continental life (Daverat et al., 2006; Daverat et al., 2011). Moreover, previous studies showed that catadromy can be facultative in these species (i.e., not all individuals enter freshwater habitats) (Tsukamoto et al., 1998, Daverat et al., 2006). The existence of different LHs in the European eel will be detailed in the next section.

European eel, *Anguilla anguilla* (Linnaeus, 1758), the case study

According to the phylogenetic classification system, the *Anguilla* genus (Schrank, 1798) belongs to Family Anguillidae, Order Anguilliformes, Superorder Elopomorpha, and Infraclass Teleostei. The Superorder Elopomorpha includes 157 genera within 25 families, with around 1000 species comprising all eels and relatives (Chen et al., 2014; Van der Laan et al., 2014).

The stock of *A. anguilla* is in critical conditions and has decreased by 90% since the 1980s (Bornarel et al., 2018; Wilson and Veneranta, 2019), which prompted the EU Regulation 1100/2007 for its recovery requiring member states to establish eel management plans to guarantee successful escapement rates of silver eels (spawning migrating individuals). However, eel fisheries across almost all life stages still occur throughout most of Europe, while this species is considered to be critically endangered by the IUCN's Red List and is listed on Appendix II of CITES and Bonn Convention (Wilson and Veneranta, 2019). Among the main anthropogenic factors responsible for this decline are overfishing, riverine constructions (e.g., hydropower or pumping stations), habitat loss, fragmentation and degradation, and pollution. These factors impact their health status, promoting an increase in diseases and parasitism (ICES, 2018; Wilson and Veneranta, 2019).

The *A. anguilla* is a long-lived, semelparous species reproducing in the Sargasso Sea (spawning and hatching). The newly hatched leptocephalus larvae drift (presumably for 2 to 3 years) with the ocean currents

to the continental shelf of Europe and North Africa, where they metamorphose into glass eels and enter continental waters (Schmidt, 1923; Since et al., 2005). The detailed life cycle is presented in Figure 1. The metamorphosis allows brackish water colonization and the possibility to use selective tidal stream transport: during ebb tides the individuals remain close to the benthos, while during flood tides, they swim on the water column. During their upstream migration into freshwater habitats, elvers (growth mid-stage between glass and yellow eel) acquire the capability to use counter-current swimming strategy (McCleave and Kleckner, 1982; Edeline et al., 2007).

The growth stage, known as the yellow eel stage, may occur in marine, brackish, or freshwater environments. The yellow stage typically lasts 2–25 years (but can exceed 50 years) prior to metamorphosis to the silver eel stage and maturation (Wilson and Veneranta, 2019). Several phenotypic and physiological changes characterize the silver eel stage. Phenotypic changes include increasing eye size and body silver pigmentation (Pankhurst, 1982). The physiological changes include an increase in cortisol levels (pointed as the primary factor in the mobilization of energy stores), maturation factors (vitellogenin – main precursor on female gonad maturation, free fatty acids, cholesterol, phospholipids, and triglycerides), and a reduction on intestine weight (Van Ginneken et al., 2007). Silver eels stop feeding and migrate to the Sargasso Sea to spawn, dying afterwards.

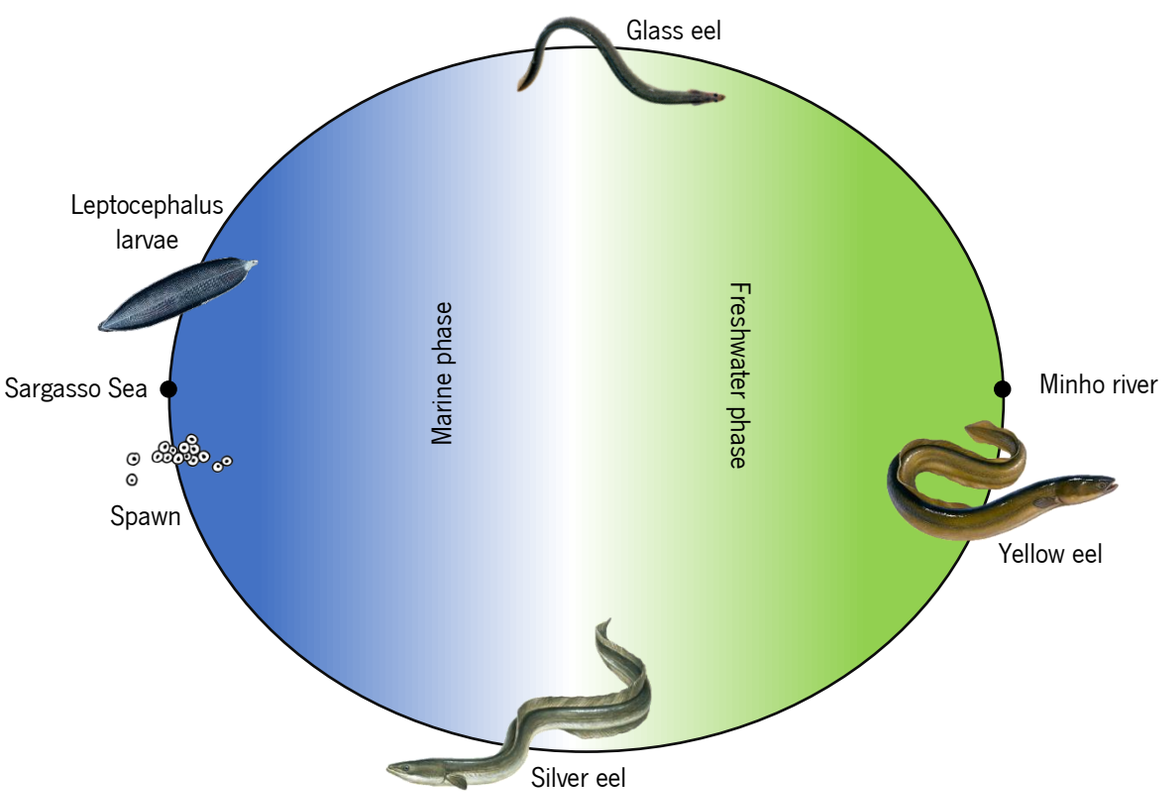


Figure 1. *Anguilla anguilla* catadromous life cycle.

The eel's migration from hatching to arrival into coastal waters, and movement throughout salinity gradients have been justified by intrinsic and environmental factors, respectively (McCleave and Edeline, 2016). During habitat shifting, this species faces drastic changes in environmental factors such as salinity, water column depth, and temperature. In addition, the biotic pressures should be greatly different, such as the available prey and potential predation, and considering this, the position on the trophic chain may shift (Bonhommeau et al., 2008).

Although eels are described as catadromous, previous studies showed they can grow in brackish or marine environments without entering freshwater habitats, which is characterized as a semi-catadromous or facultative catadromous behaviour (Tzeng et al., 2000). Several groups of eels can be distinguished according to habitat use: ocean, estuarine, or freshwater residents, and groups with irregular movements throughout the salinity gradient (i.e., nomads) (Jessop and Iizuka, 2002; Tzeng et al., 2002).

The consequences of each tactic (or combination of tactics) for eel populations remain unclear. The LHP of eels could be analyzed considering the conditional strategy hypothesis (CSH), which states that genetically monomorphic individuals use a specific tactic, depending on their status (size, sex, age) or condition (energy reserves) to acquire higher fitness (Gross, 1996). Since each tactic has unequal fitness, the one that produces better fitness will dominate. This theory was tested for the masu salmon *Oncorhynchus masou*, (Brevoort, 1856), which displays partial migration, being hypothesized that individuals can maximize their fitness by adopting resident tactics under favorable early growth conditions (Morita et al, 2014). However, CSH does not seems to explain the existence of catadromy for the American eel, *Anguilla rostrata* (Lesueur, 1817) in the St. Jean River (Canada) or the *A. anguilla* in the North and Baltic seas, because freshwater eels presented lower growth rates (Thibault et al., 2007) and lower muscle fat content (Marohn et al., 2013), respectively than those that never entered freshwater habitats. Despite this, higher values of fat content were found for coastal residents and downstream nomads than for freshwater residents, even above the revised threshold values necessary for spawning migration (between 13,5 and 28 %) (Marohn et al., 2013). Also, previous studies indicated that primary recruitment of maturated eels, and consequent gene flow, mostly occurs between eels with marine/brackish-based LHs, which suggests that the majority of reproducing eels are semi-catadromous (Tsukamoto et al., 1998). Thus, marine/brackish-based LHs probably result in a higher fitness for eel's populations than the catadromous LHs.

Otoliths as a tool to reconstruct habitat use

The otoliths are metabolically inert structures located in the inner ear of teleost fish and are less vulnerable to post-depositional chemical and structural modification as they grow through uptake from the water masses through which a fish passes during its lifetime, constituting good records of its life (Campana

and Thorrold, 2001; Elsdon and Gillanders, 2003).They are formed by crystalline calcium carbonate concretions located on both sides of a fish brain cavity (Radtke and Shafer, 1992; Brown and Severin, 2009).

Table 1. Otolith's microstructure (adapted from Jensen (1961)).

Structure designation	Description
Nucleus (core)	Otolith's centre opaque dot corresponding to age 0
Annuli (ring)	A pair of complete rings (dense + translucent) – 1 year
Translucent ring	Slow growth ring (usually formed during winter)
Dense ring	Fast growth ring (usually formed during summer)
False ring	Incomplete ring (not used on ageing)
Life stages marks	Otolith growth differentiation due to metamorphosis

Otoliths are widely used for age estimation (Svedang et al., 1998). Considering otoliths' structure (Table 1), formed by summer and winter growth ''rings'', and in order to ageing fishes, in this particular case, eels, several techniques for this application have been developed, widely used, and reviewed in order to evaluate accuracy (Svedang et al., 1998).

Otolith growth patterns combined with chemical composition provide detailed individual chronologies of habitat use which are of great value to ecological studies (Elsdon and Gillanders, 2003). The assumption to use such approach is that there is a significant correlation between the elemental composition in otoliths and the physicochemical properties of the ambient (e.g., Thorrold et al., 1997; Campana et al., 2001). Early studies on strontium incorporation into otoliths of Japanese eel, *Anguilla japonica* (Temminck and Schlegel, 1846), showed that strontium:calcium (Sr:Ca) ratio levels correlate with the salinity of the water (Tzeng 1996; Kawakami et al., 1998). Strontium is taken up in proportion to ambient concentrations (Bath et al., 2000, Kraus and Secor, 2004), and the effect of a shift in habitat on the Sr:Ca incorporation in the otolith of *A. anguilla* was also demonstrated by Daverat et al. (2005). Also, previous studies have already identified a strong relationship between the barium (Ba) in water and its deposition in otoliths (Walther and Thorrold, 2006). Moreover, reconstruction of habitat use patterns and movements, combining otolith chemical analysis and a Bayesian approach has been proved a very powerful tool to classify the eel tactics in a French river basin without any a priori judgment on the expected patterns (Fablet et al., 2007).

The microstructure and microchemistry analyses on *A. anguilla*'s otoliths have provided relevant information not only on their habitat use patterns across its distribution (Antunes and Tesch, 1997), but also enabled answering questions related to their life history in general. For example, in the Minho River, the hatching occurs during late Summer, first metamorphosis at age 198 ± 27.4 days with a duration of 32 ± 13.2 days; also, recruitment occurs at the age of 249 ± 22.6 days (Arai et al., 2000). Through otolith readings it was also possible to correct the information of a transatlantic cross duration of about 3 years, concluding

that leptocephali travels from the Sargasso Sea to coastal European waters within 1 year, or even between 7 and 9 months since hatching (Lecomte-Finiger, 1992).

Parasitism infection, by *Anguillicola crassus* (Kuwahara, Niimi & Itagaki, 1974)

The parasite *Anguillicola crassus*, is an invasive nematode parasite that inhabits the *A. anguilla*'s swimbladder. Its records were first reported in the 1980's in European countries when analyzing *A. anguilla* and species that are part of their diet (Haenen and Van Banning, 1990). Its introduction was originally attributed to imported eels for stocking and commercial purposes (Kennedy and Fitch, 1990).

The eggs are found on the water column and on the swimbladder's lumen, where a small part hatch(Charleroy and Belpaire, 1990). Larvae, normally, leave the cuticle on freshwater and parasite their intermediate host, mainly copepods, moulting to the final larvae stage which grows and reproduce inside the swimbladder on adult eels. Small glass eels can also be infected, but the parasite remains outside the swimbladder. The copepods could also be ingested by other larger species, which act as a paratenic hosts, being then ingested by an adult eel, where the parasite completes its life cycle (Charleroy and Belpaire, 1990), as shown in Fig. 2.

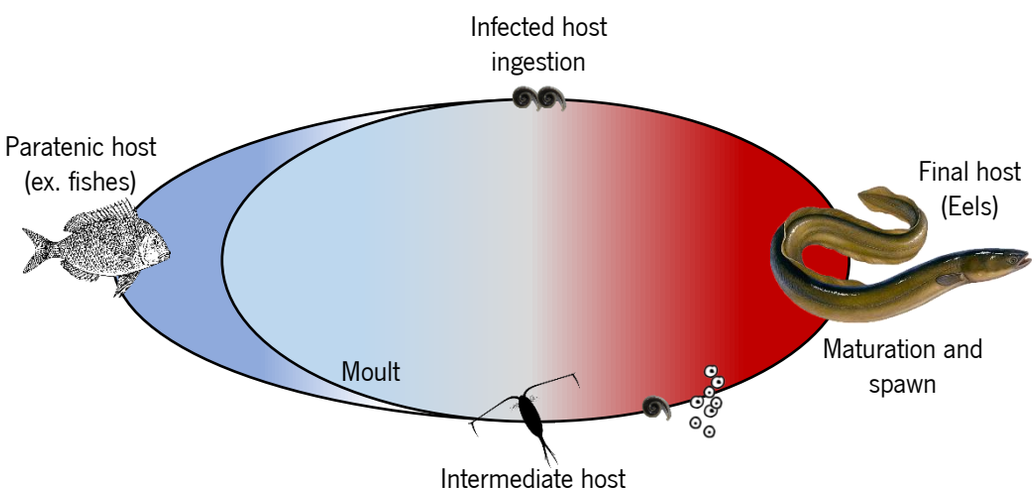


Figure 2. *Anguillicola crassus* life cycle.

This nematode is native in East Asia (Japan, Korea, Taiwan, and China), and its effects on *A. japonica* do not seem problematic (Nagasawa et al., 1994). On the other hand, their effects on *A. anguilla* (final host) are serious, both on wild and farmed eels (Nagasawa et al., 1994). The damage is mostly on the swimbladder wall, such as inflammation, clouding, thickening, and even changes in all four layers of the wall (Würtz and Taraschewski, 2000). Alterations in the lumen's gas composition were also reported, presumably, due to

these physical alterations caused by *A. crassus* infection (Würtz et al., 1996). The differences in the severity of infections between European and Japanese eels could be explained due to a lower specific immune response of *A. anguilla* to nonindigenous parasitism when compared with the intrinsic immune capability of the native host *A. japonica* (Nielsen, 1999). However, the associated problems with the infection in the *A. anguilla* may go further than this because this species makes a transatlantic journey to spawn, and the health status of the swimbladder should be a factor influencing the swimming capacity and a concern for the *A. anguilla* population decline (Palstra et al., 2007).

Objectives

The impact of fisheries and climate change on fish conservation stresses the need to disclose fish LH diversity because current conservation and management plans may inadvertently impose selection on certain phenotypes that can be unsuited for future climatic conditions. This is particularly relevant in the case of the *A. anguilla,* which is of great conservation concern and yet, economically explored.

For the adult eel fisheries in Portugal, the values were reduced from an average of 20 tons/year (1991-2000) to under than 10 tons/year (2003-2008), and for the glass eel fisheries in Minho River, the values were reduced from an average of 24 tons/year (1974-1984) to an average of 10 tons/year (until 2000) (Estado Português, 2012). Eels have an estimated value of 80 million euros impact on European economy but this value could rise up to 360 million euros if the stock levels was as in the past (Moriarty and Dekker, 1997).

Thus, this study aimed to investigate LH diversity in the *A. anguilla* of the Minho River by identifying their habitat use patterns and evaluating the condition associated with each strategy. This river was selected as the case study because eels have a high ecological and economic importance, since it is the only river where glass eels fisheries are still allowed at the national level. Moreover, the current habitat available for diadromous species in now *ca*. 6% than the historically available habitat (Mota et al., 2016).

Assuming the conditional strategy hypothesis, each strategy is expected to have unequal fitness; thus, the one that produces better fitness will dominate (Gross, 1996). Measuring fitness (i.e., reproductive success) in eels is difficult because they form a single randomly mating population, reproducing in an unknown area in the Sargasso Sea (Schmidt, 1923). However, evidence is growing that poor silver eel quality (i.e., low-fat content) significantly impacts reproductive success, implying that habitat quality plays a key role in stock decline (Marohn et al., 2013). Thus, this study hypothesized that the strategy that results in eels with better condition would dominate. To investigate this hypothesis, habitat use patterns were identified through otolith chemical analysis. The condition was assessed by calculating the Fulton index and the hepatosomatic index and estimating the percentage of lipids in the muscle.

METHODOLOGY

Study area: Minho River

Minho river is located in NW Iberian Peninsula (SW Europe; Fig. 3) and drains a hydrological basin of 17,080 km^2, 95% of which is in Spain and 5% in Portugal. The last 70 km serve as a natural border between Portugal and Spain (Antunes et al., 2011).

The annual average river discharge is 300 $m^3 s^{-1}$ (Ferreira et al., 2003); it can vary between 100 $m^3 s^{-1}$ during summer, and over 600 $m^3 s^{-1}$ during winter (Confederación Hidrográfica del Miño-Sil, 2018). During low river discharge, the salinity intrusion is maximized (*ca.* 25 km upstream), and the contrary happens during high flow (Dias et al., 2016).

The Minho River estuary has an area of about 23 km^2, where 9% is the intertidal section. This estuary is categorized as mesotidal with a tidal range varying between 0.7 m and 3.7 m, and the tidal influence occurs up to 40 km where the uppermost 30 km are tidal freshwater wetlands (Vilas and Somoza, 1984). The minimum and maximum depths are set between 2.6/4 m up to 23/26 m, respectively (Souza et al., 2013; Dias et al., 2020).

Veiga da Mira river is a tributary of the Minho River, located at 28 km from the river mouth (Fig. 3). This river has a total length of 12.6 km, and the basin area is about 46.5 km^2. The land use within this basin is distributed as 60 % on forest and natural grassland, 30 % for agriculture and 10 % as urban and industrial occupation (Dias et al., 2019).

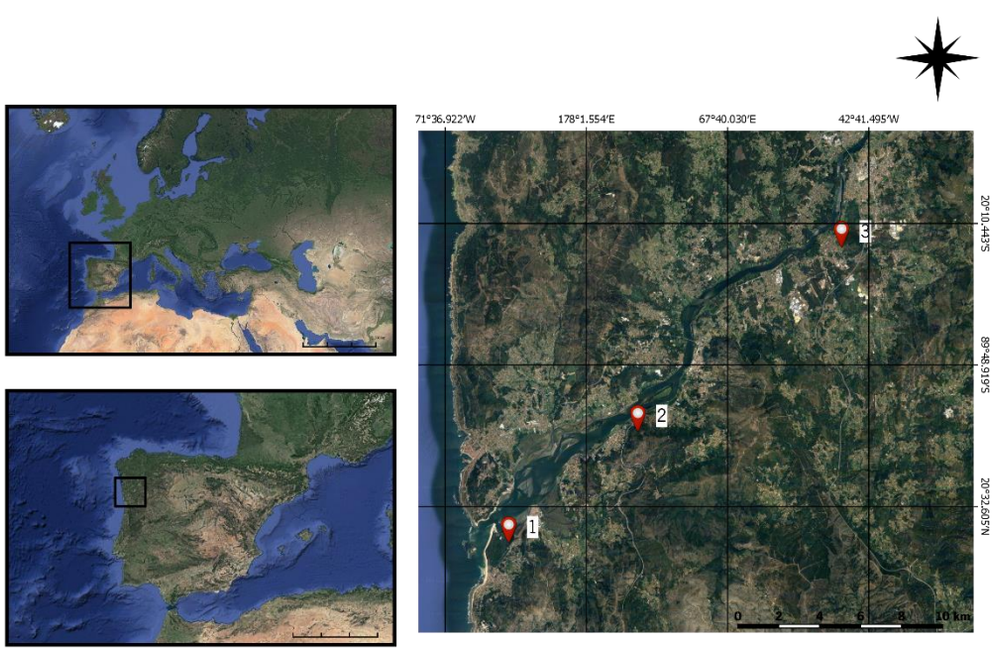


Figure 3. Sampling locations in the Minho River : stations 1 – Low estuary, 2 – Middle estuary, and 3 – Tributary (Veiga da Mira).

Data collection

Eels were collected in three stations along the salinity gradient during 2019: station 1 is located near the river mouth (meso- to euhaline), station 2 at 12 km from river mouth (poly- to oligohaline), and station 3 is located at Veiga da Mira River (fresh water).

In stations 1 and 2, yellow stage eels were collected using fyke nets of 10 mm mesh size, 7 m total length, 0.7 m mouth diameter, and 3.5 m central wing (Moura et al., 2022). At station 3, eels were collected by electrofishing, using an Electrocatch International model WFC911 (continuous current [DC], 200–400 V; 1–3 A; Electracatch International, Dublin, Ireland). Electrofishing was conducted, in delimited areas of 100 m^2, with one or two passages along the area. All eels were kept alive with aeration during transportation. In the laboratory, eels were anesthetized, measured (±0.1 cm), weighted (±0.1 g), and then kept frozen at −20℃ until processing. For eels above 30 cm, additional biometric measures were recorded for the Durif Silvering Index estimation, including the pectoral fin length and the vertical/horizontal eye diameter (Durif et al., 2005; Durif et al., 2009).

Eels were dissected to retrieve de swim bladder for parasite inspection and the liver to calculate the hepatosomatic index. A piece of dorsal muscle was removed to estimate lipid content, and otoliths were excised for microstructure and chemical analysis (Fig. 4).

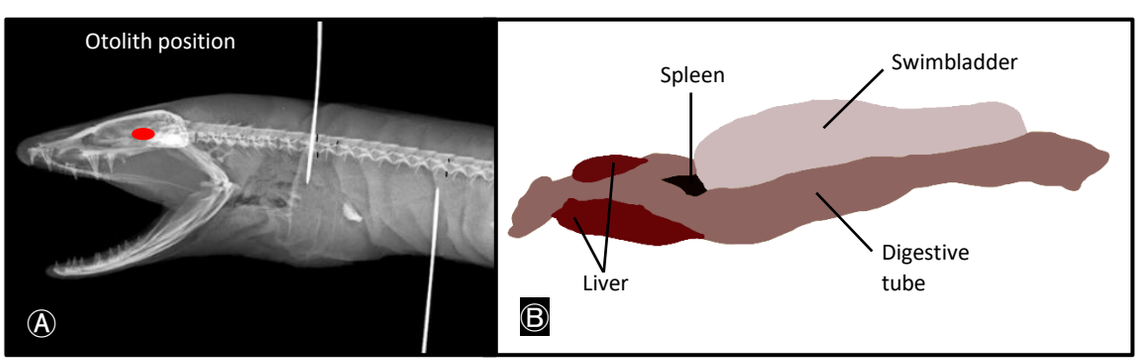


Figure 4. Ⓐ Eel head x-ray with otolith position (Image: CSIRO Australian National Fish Collection) Ⓑ Internal organs of interest.

The procedures for each part of the laboratory work and data analysis are detailed in the following sections.

Laboratory work

After extraction, otoliths were cleaned using ultrapure water and photographed with microscope Nikon SMZ800. The left otolith was used for microstructure analysis, and the right otolith was used for chemical analysis.

Eel age was assigned by counting the annual growth increments on the left sagittal otoliths following Moura et al. (2022). All otoliths were immersed in a clearing agent (ethanol and glycerol, 1:1) to enhance their transparency during reading, with the proximal surface (*sulcus acusticus*) turned up and examined using a stereomicroscope (Nikon SMZ800) against a dark background with reflected light. An age of 0 years was attributed to glass eels so only the continental age was considered. For eels older than 5 years, otoliths had to be treated to enhance rings' visualization. The process consisted of embedding the otolith in epoxy resin and mounting it on a glass slide for sagittal grinding. Each otolith was grounded manually using a decreasing range in the coarseness of silicon carbide wet-dry papers (600 – 4000 grit). Afterwards, the otolith plane was etched with 5% EDTA, stained with a drop of 1% Toluidine blue, and rinsed with running water. Each otolith was observed with the stereomicroscope with reflected light, and age was estimated (Figure 5). Three independent readings were undertaken, and only otoliths with consistent concordance in age were used.

Another complementary information of the microstructural analysis was collected, the distance from the nucleus to the mark of the first metamorphosis (i.e., zero band).

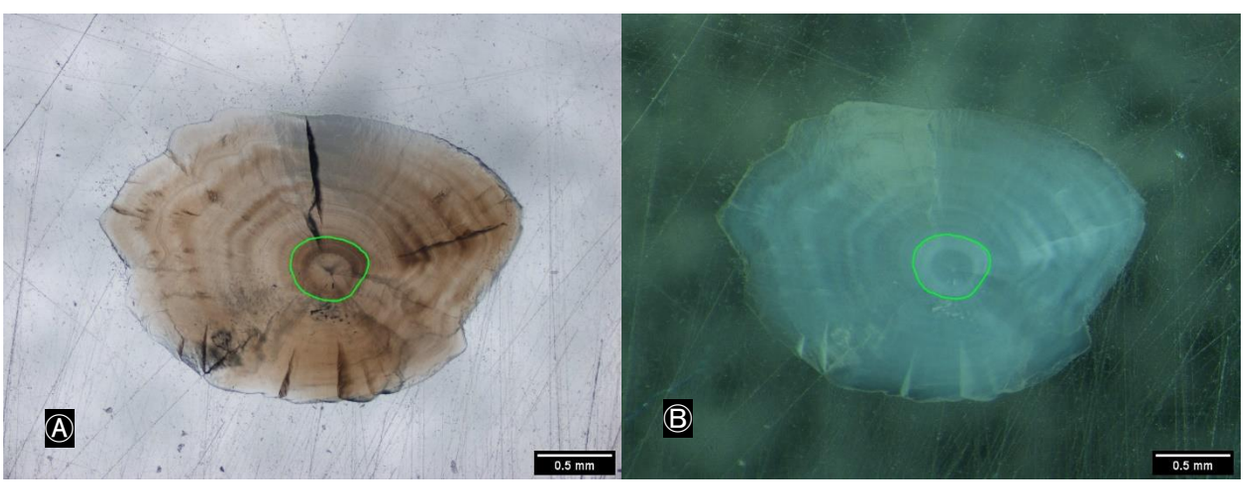


Figure 5. Otolith on white Ⓐ and black Ⓑ background (nucleus expose plan). The identified area in green corresponds to the zero band.

Table 2. Otolith dimensions and shape indices description.

Otolith dimensions	Shape indices
A – Area	$FF = \frac{4BA}{P^2}$
P – Perimeter	$RO = \frac{4A}{BL^2}$
L – Length	$CI = \frac{P^2}{A}$
W – Width	$RE = \frac{A}{LW}$
	$EL = \frac{L-W}{L+W}$

The images retrieved during the procedure conducted above were treated with the software PaintNet to transform it into binarized images. Binary otolith images were measured using the program ImageJ (v. 1.50) to assess the morphometric size parameters, otolith length (OL, mm), otolith width (OW, mm), otolith area (OA, mm^2), and otolith perimeter (OP, mm) (Rasband, 2009). With these variables, it was possible to calculate and access the Shape Indices (SI): Form factor (FF), Roundness (RO), Circularity (CI), Rectangularity (RE) and Ellipticity (EL), which describe the otolith plane (Tuset et al., 2003) (Table 2).

The right otoliths of eels were cleaned and embedded with epoxy resin (Araldite® 2020) in a standard probe-mount. The individually embedded otoliths were then ground by hand through the sagittal plane with 600, 1200, and 2400 silicon carbide abrasive paper to expose the otolith core. They were then successively polished with 1 μm diamond paste and stored in clean plastic sealable vials until analysis. The eel otoliths were sampled across a transect perpendicular to the growth marks from the core to the edge (Figure 6). The isotope concentrations were measured with ICP- MS- Elan DRC II (Perkin Elmer) together with high repetition rate infra-red femtosecond laser ALFAMET (Alfamet, Novalase SA Amplitude Systems), and the definitions were a pulse rate of 3KHz, 115Mw power and an ablation spot size of 20μm. To reduce the signal peak broadening and also to boost the signal-to-noise ratio a low volume ablation cell was used ($3.7cm^3$).

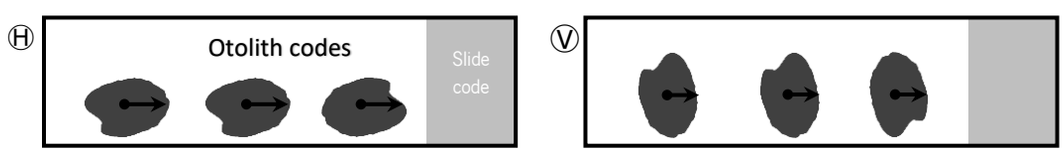


Figure 6. Assembly of otoliths on microscope slides Ⓗ horizontal and Ⓥ vertical transects.

For the external calibration of the standards, NIES glass 610/ 612/ 614 were used and these scans were performed multiple times, depending on the day of analysis, the characteristics of the samples, the time required to each sample, and the time available at the day that it was performed. The software GLITTER was used to process the trace elements concentrations, standard deviations and detection limits.

Resorted to external calibration with series of 13 coprecipitated carbonate pellets ranging from 0.1 to 500 $\mu g.g^{-1}$ to trace elements quantification. The matrix matching calibration was preferred over NIST to best accuracy. For the calibration curve was utilized one otolith reference material (NIES 22) pelletized. The calibration curve was repeated every 12 samples using 3 enriched pellets and CRM NIES 22 to correct low-frequency drift to adjust the environment because of the changes in the working area, such as changes in temperature, plasma, and electronics. The LA-ICP-MS analysis was conducted at Institut des Sciences Analytiques et de Physico-Chimie pour l'Environnement et les Matériaux (IPREM, Université de Pau et des Pays de l'Adour, France).

After collection of isotopes concentrations elemental analysis, the data was treated with a package running on Microsoft® Excel® for Microsoft 365 MSO (version 2205 Build 16. 0. 15225. 20028) 32-bit. At the end of this process was possible to obtain the concentration of the isotopesSr_{86}, Ba_{138}, and Ca_{43} as well as its ratios.

To determine the prevalence of *A. crassus* infection (i.e., the percentage of infected hosts in a population) and the number of parasites per infected host eels' swim bladders were opened with a longitudinal cut under a stereomicroscope (Nikon SMZ800). This method allows to flatten this tubular organ to observe and quantify *A. crassus* inside each eel specimen.

The condition assessment was done by calculating the Fulton index, the hepatosomatic index, and by estimating the percentage of lipids in eels' muscle tissue.

The Fulton Index (K) estimates the relation between length (mm) and weight (g) at the time of capture; condition increases with increasing K. The weight increase occurs at a higher rate than length in most fishes, so equation (Eq.) 1 uses the cube of total body length, as presented (Nash et al., 2006; Silm et al., 2017).

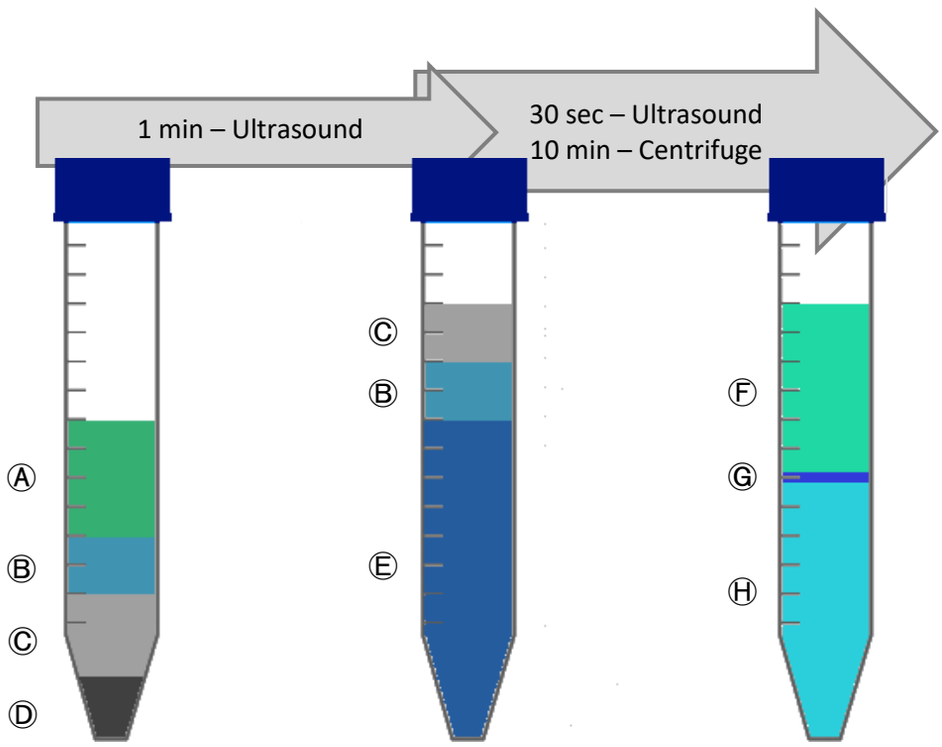
$$\textbf{Eq. 1: K} = \frac{\text{Total body weight [g]}}{(\text{Total body length [cm]})^3} \times 100$$

The Hepatosomatic Index (HSI) allows to calculate the relation between liver weight (g) and total body weight of the individuals (g) at the time of capture, with expected higher HSI values in individuals in a better condition Equation (Eq) 2 outputs the percentage of liver weight in relation to the total weight of the individual (Rankin, 2009).

$$\textbf{Eq. 2: HSI} = \frac{\text{Liver weight [g]}}{\text{Total body weight [g]}} \times 100$$

The lipids were extracted from a piece of lyophilized dorsal muscle, which was ground to a homogeneous powder with a mortar and pestle. After that, lipids were removed following a modified procedure by Bligh and Dyer (1999). A mixture of 2:1 chloroform and methanol and 0.8 ml of deionized water were added to each glass vial containing 0.030g of muscle tissue. This solution was then homogenized for 1 minute in an ultrasound machine (Bioblock Scientific Vibra Cell, model 75043) using 10 seconds pulses with 2 seconds pause, while on ice. The next step was to add a 1: 1 ratio of chloroform: deionized water and homogenized in an ultrasound machine again, for 30 seconds, while on ice. Afterward, the solution was centrifuged (Sigma, model 4-16KS) for 10 minutes at 3000 rpm at a temperature of 15 ℃.

At the end of this process, the solution was separated into 3 different sections, the supernatant (aqueous solution), the interphase (solid) and the inferior phase (aqueous solution). The last is the section that contains the lipids, the target of this analysis.



Ⓐ Methanol
Ⓑ Chloroform
Ⓒ Deionized water
Ⓓ Dehydrated dorse muscle
Ⓔ Previous solution
Ⓕ Supernatant
Ⓖ Interphase
Ⓗ Inferior phase

Figure 7. Lipid extraction protocol.

To finalize the protocol, the inferior phase containing the lipids was transferred into empty tubes that were previously weighed, and left 48h undisturbed for solvent evaporation. After that, the tubes were weighted again, and the muscle lipid content was estimated following Eq. 3.

$$\textbf{Eq 3: MLC (\%)} = \frac{\text{Final}_{\text{weight}} - \text{Initial}_{\text{weight}}}{\text{Sample}_{\text{weight}}} \text{x} 100$$

Data analysis

The analysis of Sr, Ba transects was based on the method developed by Fablet et al. (2007), expanding the Bayesian labeling method from a monoelemental transect (Sr:Ca) to a multielemental (Sr:Ca, Ba:Ca) transect to assign retrospective eel habitat use (Daverat et al., 2011, Daverat et al., 2012). Briefly, for each otolith, annual ring positions along the transect axis were acquired with respect to the distance to the "zero band", and used as time references to transform Sr:Ca and Ba:Ca series to time series using a linear interpolation (Fablet et al., 2007, Daverat et al., 2012). The zero band distance was used to relate to the beginning of the yellow eel stage. There was no direct validation because the sample did not contain any eel that could be geolocated with a direct method throughout their entire life, nor water Sr:Ca and Ba:Ca were estimated during the study. Thus, we assumed that coupled Sr:Ca and Ba:Ca measures could be regarded as a proxy of the habitat or saline compartment, as it was previously inferred in this ecosystem (Daverat et al., 2012). Then, a hidden Markov chain model was used to account for the nature of each individual eel movement. This meant that the model considered each eel Sr:Ca and Ba:Ca time series as a whole. The principle was that the probability of an eel going from one habitat to another habitat at time t was dependent on the habitat used at time t – 1 and on the assigned habitat at time t + 1 (Daverat et al., 2012). The hidden Markov chain algorithm (Rabiner, 1989) retrieved the most probable individual habitat used (or sequences of habitats) using a backward and forward fitting (each time step was dependent on the subsequent and previous steps). There is no p-value associated with the results because the data concern the most probable habitat sequence for each eel, nor there is any level of confidence since there was no comparison between otolith microchemistry and the habitats used by eels during their life (Daverat et al., 2012). The habitat use patterns were defined as the sequence of the successive habitats visited by each eel (e.g., the movement pattern issued from habitat sequence BBBFFFFFFFFBBBBB was BFB). Given the whole otolith set, the automated and unsupervised classification of individual habitat use patterns could be determined as well as the relative frequencies of these categories of habitat use patterns. The unsupervised categorization does not exploit any prior knowledge and is issued only from data characteristics and statistics (Daverat et al., 2012).

Based on the results from the unsupervised classification, eels were categorized based on their patterns of habitat use (Figures 8 to 12) as follows: brackish resident (BR: never entered freshwater until capture), freshwater residents (FR: after entering freshwater for the first time, never returned to a brackish habitat), downstream shifter (DS: remained in a brackish environment after an initial phase in freshwater), upstream shifter (US: spent some time in a brackish environment before entering in a freshwater environment), and interhabitat shifters (IS: moved more than once between habitats until capture) (Marohn et al., 2013).

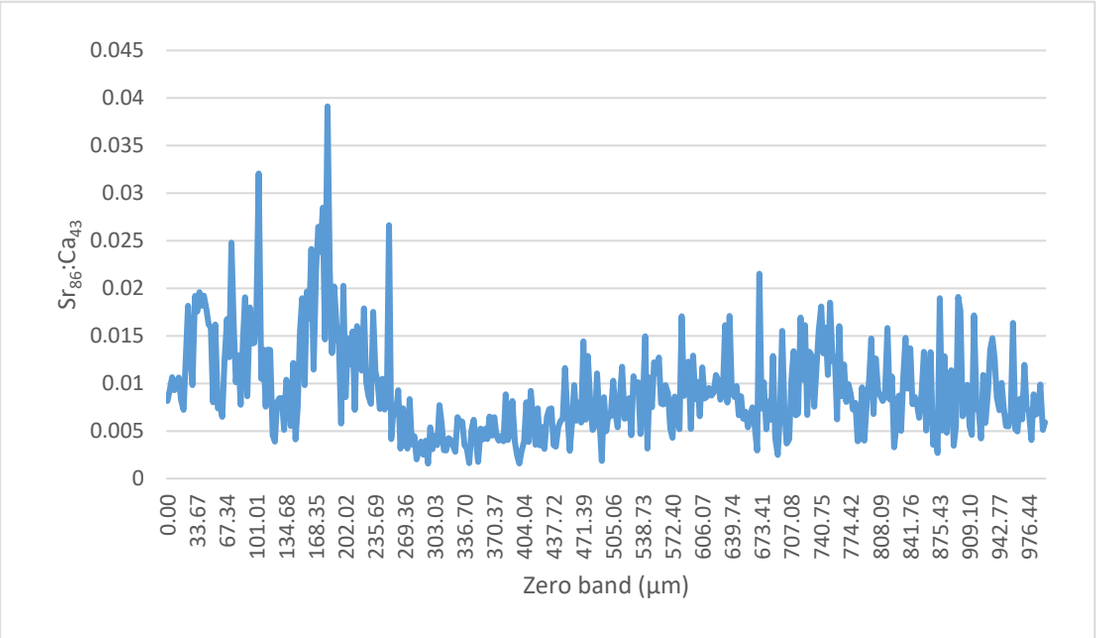


Figure 8. Brackish water resident graphical Sr_{86}:Ca_{43} pattern example.

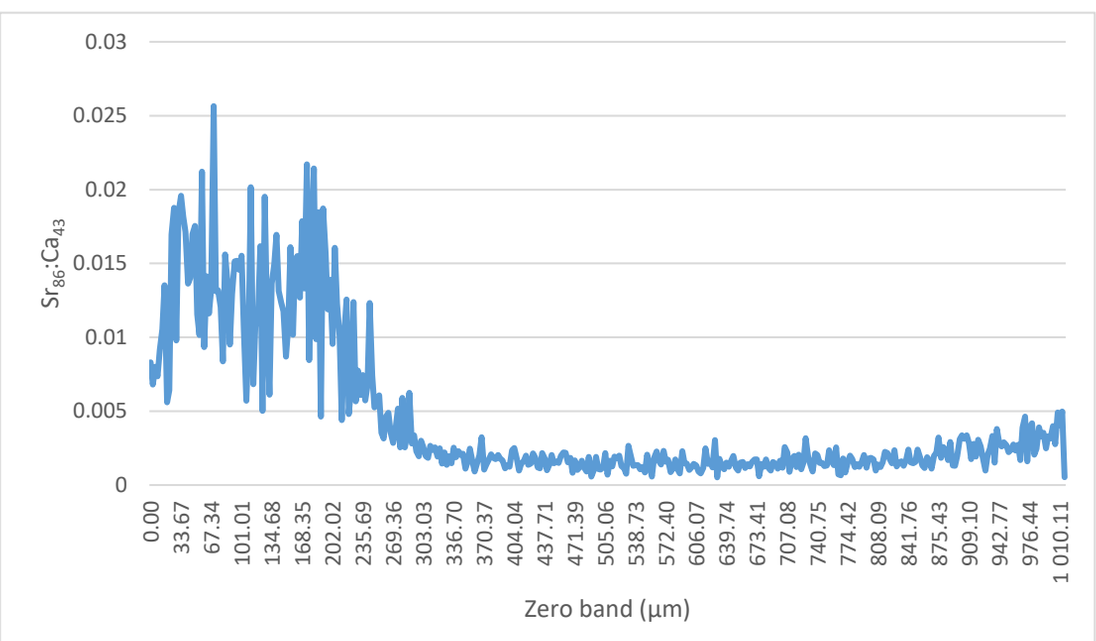


Figure 9. Freshwater resident graphical Sr_{86}:Ca_{43} pattern example.

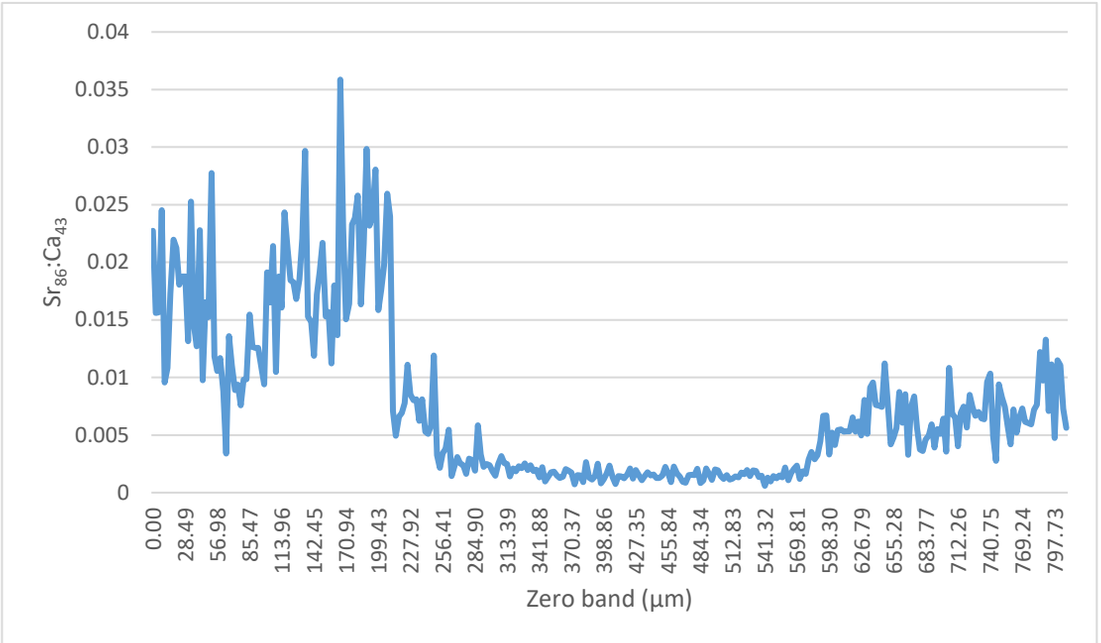


Figure 10. Dowstream shifter graphical Sr_{86}:Ca_{43} pattern example.

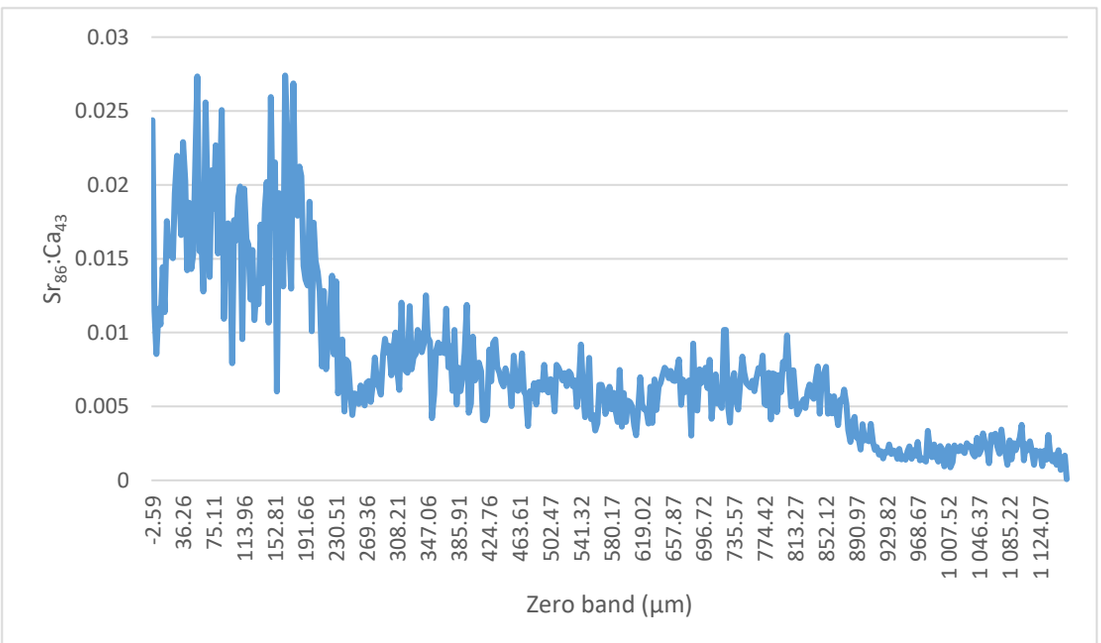


Figure 11. Upstream shifter graphical Sr_{86}:Ca_{43} pattern example.

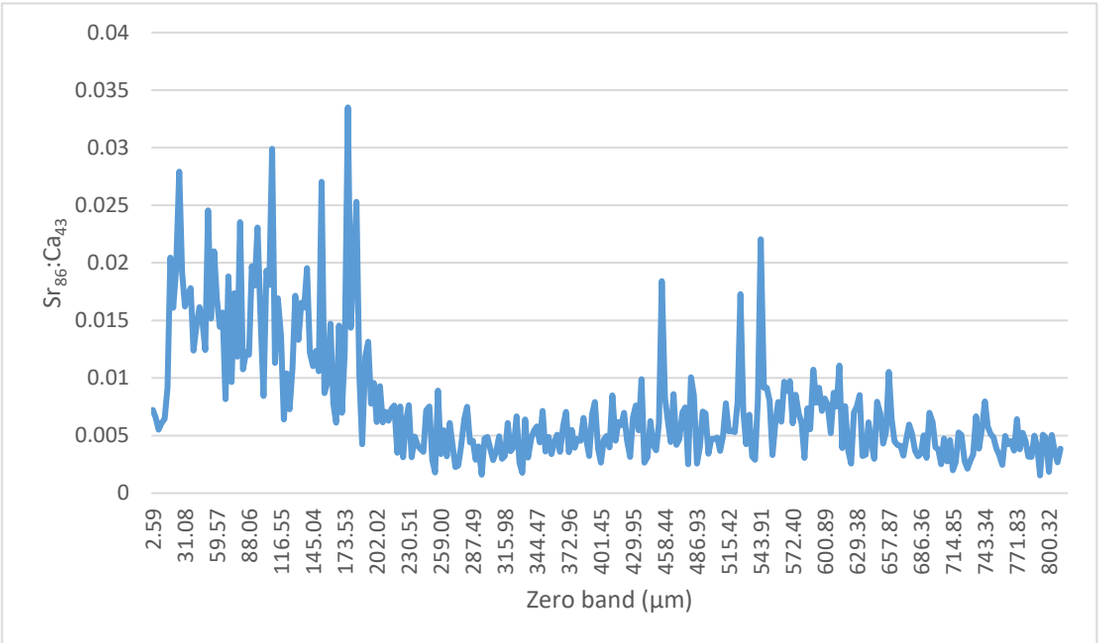


Figure 12. Interhabitat shifters graphical Sr_{86}:Ca_{43} pattern example.

Analyses of variances (ANOVA) and co-variances (ANCOVA) were used to test for differences in condition indices and shape indices between habitat use patterns or sampling locations, followed by a posterior multiple comparison tests, after testing for the assumptions. Each variable was checked for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) and was transformed accordingly using the best transformation method proposed by the function bestNormalize available in the package *bestNormalize*. Additional assumptions for each parametric test were checked. When the assumptions to conduct a given parametric test were not met, the equivalent non-parametric test was used (i.e., Kruskal–Wallis test, nonparametric regression comparisons). The relationship between each variable of interest and eel length was previously investigated using Spearman correlations and linear regressions to ensure that differences in eel length among samples did not confound group-specific differences. Groups with less than five individuals were not included in hypothesis testing. A significance level of $p < 0.05$ was used for all tests.

The statistical analyses presented in this study were performed using the software R version 4.0.4 (2021-02-15) and RStudio (2022.02.3+492) with previous data preparation using the software Microsoft® Excel® for Microsoft 365 MSO (version 2205 Build 16. 0. 15225. 20028) 32-bit as spreadsheet.

RESULTS

Analysis by sampling location

A total of 80 eels was studied in this project. Their age varied between 0 and 10 years. The smallest eel was collected at station 3 (9.4 cm) and the largest at station 2 (91.81 cm); 48% of the eels collected were larger than 30 cm. The body weight varied between 1.25 g (station 3) and 143.47 g (station 2).

The lowest value Fulton index (K) value was recorded at station 2 (0.0044) and the highest at station 1 (0.5340). The lowest hepatosomatic index (HSI) value was registered at station 3 (0.165%) and the highest at station 2 (5.924%). Muscle lipid content (MLC) was analysed only for 42 individuals and varied between 2.87% and 39.25%, at stations 3 and 2, respectively. The number of *A. crassus* varied between 0 (61,6% of the individuals analyzed) and 21 at station 1 (1.25% of the individuals analyzed).

Regarding the otoliths' shape indices, the form factor values were lower and higher at stations 2 and 3, respectively; the roundness lower value was recorded for station 1 and the higher for station 3; the lowest circularity value was recorded at station 3 and the maximum at station 2; the minimum and maximum rectangularity values were registered in stations 1 and 2, respectively; the ellipticity at station 3 was the lowest, where the highest was at station 1. For the last otolith's microstructure data, the zero band, the minimum and maximum values were recorded at station 2, and in 98% of the eels, this distance was greater than 170 μm, contrary to the expected average of 170 μm (ICES, 2011).

Table 3. General characterization of the individuals collected in each sampling location in the Minho River: 1 - low estuary, 2 - middle estuary, and 3 – tributary; Data include: sample size (n), minimum (min), maximum (max), median (M), standard deviation (σ) and the result of the statistical test performed.

Sampling location	Age (yrs.)			
	n	Min	Max	M (±σ)
1	28	3	10	5 (±1.43)
2	22	2	6	4 (±0.93)
3	31	0	7	3 (±1.82)
	ANOVA, F (2,53) = [6.963], p = $2X10^{-3}$			
	Length (cm)			
	n	Min	Max	M (±σ)
1	27	16.27	78.22	40.36 (±16.76)
2	22	20.66	45.0	30.75 (±14.13)
3	31	9.4	30.8	20.30 (±6.14)
	Kruskal-Wallis, H (2) = 16.209, p = $3X10^{-4}$			
	Weight (g)			
	n	Min	Max	M (±σ)
1	37	23	64.6	30.70 (±7.48)
2	22	14.781	143.47	41.70 (±29.51)
3	31	1.25	47.8	11.20 (±12.88)
	Kruskal-Wallis, H (2) = 16.048, p = $3X10^{-4}$			

Overall, the eels collected at station 1 were older and larger than those collected in the other stations (Table 3). The opposite was observed for the eels collected at station (Table 3; Tukey HSD: $p < 0.05$).

Length was correlated with all the condition indices determined during this study, increasing with increasing HSI (linear regression: $R^2 = 0.29$, $F_{(1,78)} = 33.86$, $p < 0.001$) and muscle lipid content (linear regression: $R^2 = 0.36$, $F_{(1,40)} = 23.62$, $p < 0.001$), and decreasing K (Fig. 13). No significant relation was found with the *A. crassus* infection (Fig. 13).

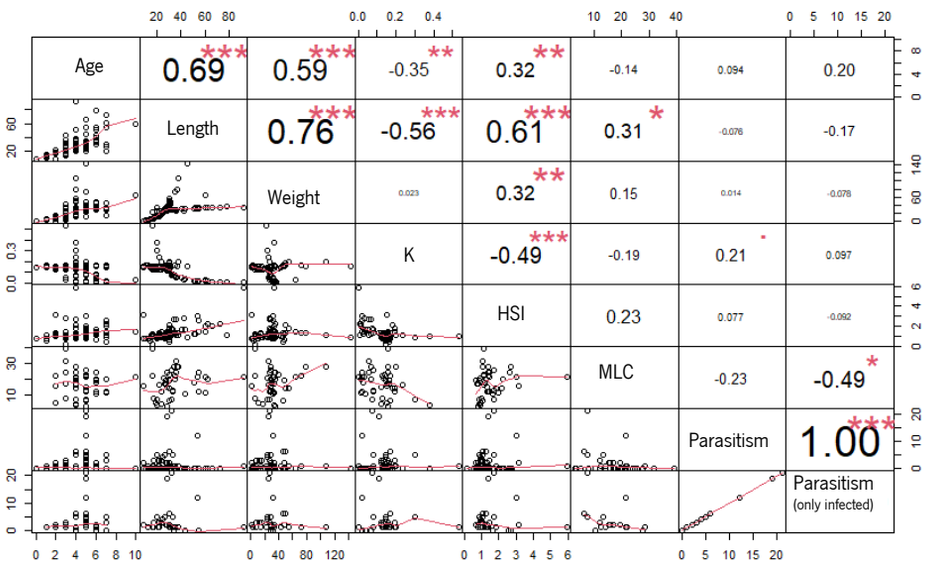


Figure 13. Spearman correlation between age, length, weight, the condition indices (K – Fulton index, HSI – Hepatosomatic index and MLC – Muscle lipid content), and the number of *A. crassus* of the eels collected in the Minho River. The significant level is $p<0.05$: *, $p<0.01$: ** and $p<0.001$: ***. A correlation value of 0 means no correlation and a value of ±1 means total correlation.

Table 4. Condition assessment of the eels collected in each sampling location: 1 - low estuary, 2 - middle estuary and 3 – tributary; Data include: sample size (n), minimum (min), maximum (max), median (M), standard deviation (σ) and the result of the statistical test performed.

Sampling location	Fulton index ($g.mm^{-3}.100$)			
	n	Min	Max	M (±σ)
1	27	0.0076	0.5340	0.049 (±0.115)
2	22	0.0044	0.3785	0.156 (±0.070)
3	31	0.1204	0.1681	0.146 (±0.014)
	Hepatosomatic index (%)			
	n	Min	Max	M (±σ)
1	27	0.898	2.997	1.46 (±0.59)
2	22	0.815	5.924	1.22 (±1.09)
3	31	0.165	3.175	0.92 (±0.47)
	smANCOVA, H (2) = 0.23, p = 0.01			
	Muscle lipid content (%)			
	n	Min	Max	M (±σ)
1	19	7.36	27.75	16.85 (±6.24)
2	13	3.39	39.25	22.12 (±10.03)
3	10	2.87	22.58	16.73 (±7.62)
	ANCOVA, F (2,38) = 2.015, p = 0.10			

Eels collected in station 1 presented lower K values than those in stations 2 and 3 (Table 4). Although K relates with length, no statistical test was conducted on this data, due to the fact that the slopes between K and length were different between stations (following Cone, 1989). Eels collected at stations 1 and 2 presented higher HSI median values than those collected in station 3 (Table 4). The highest median values for muscle lipid content were observed for the eels collected at station 2, but no significant differences were found between stations (Table 4).

Table 5. Infection by *Anguillicola crassus* in the eels collected in the Miho River for each sampling location: 1 - low estuary, 2 - middle estuary and 3 – tributary; data presented: sample size (n), number of infected individuals, prevalence of infection, minimum (Min), maximum (Max), median (M) and standard deviation (σ), for parasitism infection by *A. crassus*.

Sampling location	n	Infected (n)	Prevalence (%)	Min	Max	M (±σ) (only infected)
1	25	9	36	0	21	5 (±7.76)
2	20	6	30	0	6	1 (±2.26)
3	28	13	46	0	5	2 (±1.36)

The prevalence of infection by *A. crassus* was higher in the eels collected at sampling location 3, than in the other sampling locations. The median intensity values were higher for eels collected at station 1 than at stations 2 and 3 (Table 5).

Table 6. Otoliths' microstructure data description by sampling location: 1 - low estuary, 2 - middle estuary and 3 – tributary; data presented: sample size (n), minimum (min), maximum (max), median (M), standard deviation (σ) and the result of the statistical tests.

Sampling location	Form Factor (FF)			
	n	Min	Max	M (±σ)
1	28	0.6905	0.8036	0.771 (±0.034)
2	19	0.6733	0.7984	0.747 (±0.034)
3	31	0.7453	0.8544	0.802 (±0.025)
	smANCOVA, H (2) = 2.756, p = $4.7x10^{-3}$			
	Roundness (RO)			
	n	Min	Max	M (±σ)
1	28	0.5604	0.7156	0.654 (±0.038)
2	19	0.5751	0.7166	0.643 (±0.038)
3	31	0.6400	0.8283	0.702 (±0.045)
	smANCOVA, H (2) = 2.756, p = $4.1x10^{-2}$			
	Circularity (CI)			
	n	Min	Max	M (±σ)
1	28	15.64	18.20	16.30 (±0.76)
2	19	15.74	18.67	16.82 (±0.79)
3	31	14.71	16.86	15.68 (±0.48)
	smANCOVA, H (2) = 2.756, p = $4.8x10^{-3}$			
	Rectangularity (RE)			
	n	Min	Max	M (±σ)
1	28	0.7000	0.7749	0.745 (±0.016)
2	19	0.7194	0.7855	0.743 (±0.016)
3	31	0.7067	0.7677	0.740 (±0.015)
	ANOVA, F (2,50) = [0.537], p= 0.588			
	Ellipticity (EL)			
	n	Min	Max	M (±σ)
1	28	0.1465	0.2654	0.190 (±0.028)
2	19	0.1381	0.2382	0.188 (±0.026)
3	31	0.0621	0.1860	0.148 (±0.028)
	smANCOVA, H (2) = 2.756, p = $1.2x10^{-2}$			

The otoliths of eels collected at station 3 presented higher values for form factor and roundness values than those in the estuarine stations (i.e., stations 1 and 2) (Table 6). On the other hand, eels collected in stations 1 and 2 presented higher values for the variables circularity and ellipticity, than those collected at station 3 (Table 6).

The rectangularity of otoliths was statistically similar between sample groups.

Analysis by habitat use classification

A subsample of 56 eels was studied in this section. Five strategies were found, after the otolith chemical analysis, which consisted of two broader groups, residents and shifters (or nomads). Residents included brackish water residents (BR) and freshwater residents (FR). If the individuals have shown movements between habitats according to salinity, they were considered as shifters: upstream shifters (US), downstream shifters (DS) and interhabitat shifters (IS).

Table 7. Number of individuals (n) by habitat use pattern, according to microchemistry analysis, by sampling location. Habitat use classification: BR – brackish; FR – freshwater; US – upstream shifter; DS – downstream shifter and IS – interhabitat shifter.

Sampling location	Microchemistry analysis (n)				
	Residents		Shifters		
	BR	FR	US	DS	IS
1	15	-	-	2	2
2	-	3	2	1	12
3	-	11	1	-	8

Brackish residents were only found at sampling location 1, freshwater residents were found mostly at sampling location 3, and interhabitat shifters at sampling location 2 (Table 7). The dominant strategies found among the eels analysed were interhabitat shifting (39%), followed by residency in brackish (26%) and freshwater environments (25%) (Table 7).

Table 8. General characterization of individuals by habitat use strategy, BR – brackish water resident, FR – freshwater resident, US – upstream shifter, DS – downstream shifter and IS – interhabitat shifter; data presented: sample size (n), minimum (min), maximum (max), median (M), standard deviation (σ) and the result of the statistical test performed. Hypotheses testing did not include the US and DS due to the small sample size per group.

Strategy	Age (yrs.)			
	n	Min	Max	M (±σ)
BR	15	4	7	5 (±1.11)
FR	14	2	7	4 (±1.44)
US	3	4	7	5 (±1.53)
DS	3	4	6	5 (±1.00)
IS	21	3	7	4 (±0.97)
	Kruskal-Wallis, H (4) = 14.37, p = $6.2x10^{-3}$			
	Length (cm)			
	n	Min	Max	M (±σ)
BR	15	20.16	60.33	34.80 (±13.05)
FR	14	17.60	31.20	25.05 (±4.22)
US	3	26.80	45.00	30.05 (±9.62)
DS	3	28.38	43.78	30.40 (±8.37)

IS	21	16.27	37.60	28.40 (±15.36)
	Kruskal-Wallis, H (4) = 13.71, p = 8.3x10^{-3}			
	Weight (g)			
	n	Min	Max	M (±σ)
BR	15	24.50	33.80	28.7 (±2.90)
FR	14	7.36	47.80	22.09 (±12.81)
US	3	28.00	143.47	45.67 (±62.20)
DS	3	25.60	54.91	31.60 (±15.49)
IS	21	11.20	107.13	29.34 (±22.18)
	Kruskal-Wallis, H (4) = 5.23, p = 0.265			

The BR were older than FR and IS (pairwise test: $p<0.05$) (Table 8). The US and DS showed a similar age range as the BR (Table 8) The FR individuals were smaller than the BR (pairwise test: $p<0.05$) and no significant differences were found between these groups and IS (pairwise tests: $p> 0.05$) (Table 8). The US and DS presented similar length ranges (Table 8). No significant differences were found between the weights of BR, FR, and IS (Table 8). The highest weight values were found for US and DS (Table 8).

As previously found, length is correlated with all the condition indices (Fig. 14). As length increases, K and HSI decrease (linear regression: $R^2=0.29$, $F_{(1,54)}= 23.46$, $p< 0.001$), and the muscle lipid content increases (linear regression: $R^2=0.36$, $F_{(1,40)}= 23.62$, $p< 0.001$).

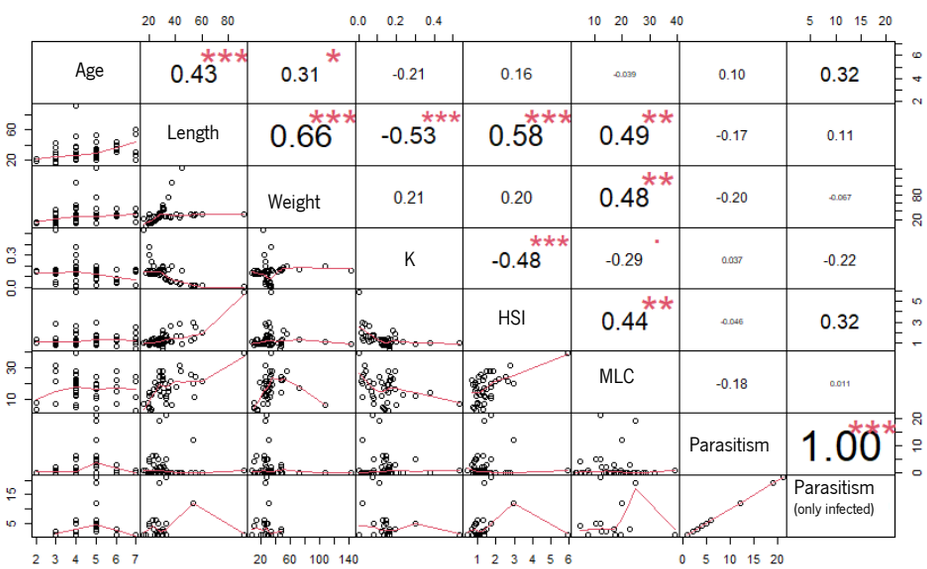


Figure 14. Spearman correlation between age, length, weight, the condition indices (K – Fulton index, HSI – Hepatosomatic index and MLC – Muscle lipid content) and the number of *A. crassus* of the eels collected in the Minho River. The significant level is $p<0.05$: *, $p<0.01$: ** and $p<0.001$: ***. A correlation value of 0 means no correlation and a value of ±1 means total correlation.

As found in previous studies (e.g., Moura et al 2022), length correlates with the majority of shape indices (SI) (Fig. 15). No correlation was found between eel length or weight and rectangularity (Fig. 15).

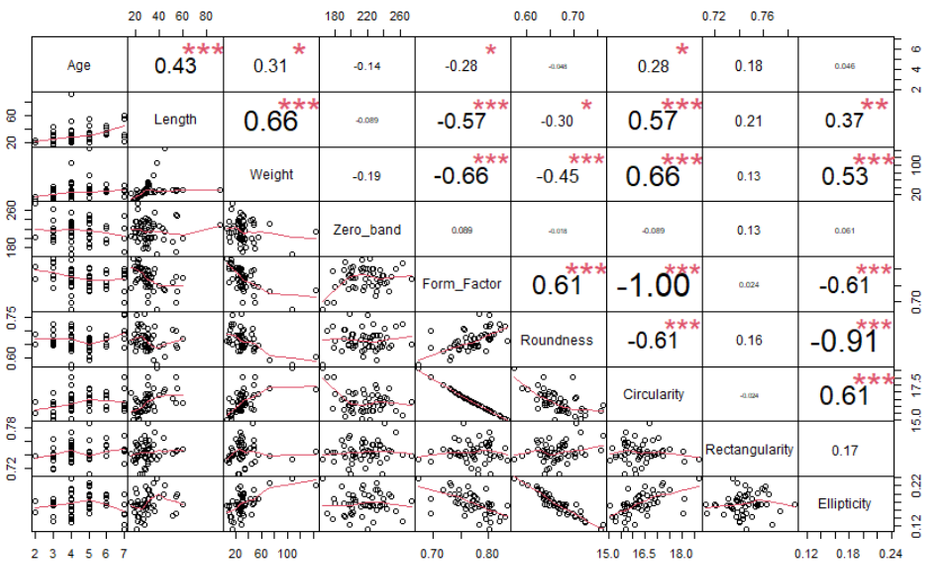


Figure 15. Spearman correlation between age, length, weight, otoliths' zero band and shape indices data of the eels collected in the Minho River. The significant level is p<0.05: *, p<0.01: ** and p<0.001: ***. A correlation value of 0 means no correlation and a value of ±1 means total correlation.

The maximum and minimum Fulton index (Fig. 16) were also recorded for IS; the maximum and minimum hepatosomatic index (Fig. 17) were recorded for IS and US, respectively; and the maximum and minimum muscle lipid content (Fig. 18) were registered for the IS.

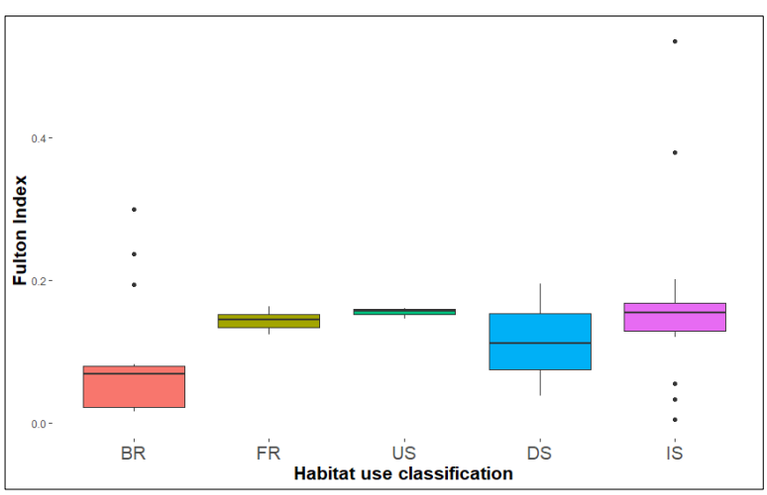


Figure 16. Graphical description of Fulton index data, relative to microchemistry selection sub-sample (56 eels) collected on Minho river, by habitat use classification: BR – brackish water resident, FR – freshwater resident, US – upstream shifter, DS – downstream shifter and IS – interhabitat shifter.

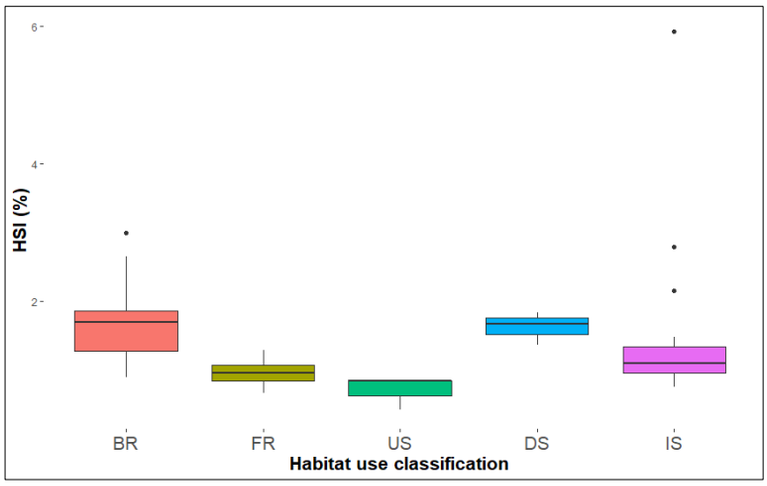


Figure 17. Graphical description of hepatosomatic index (HSI) data, relative to microchemistry selection sub-sample (56 eels) collected on Minho River, by habitat use classification: BR – brackish water resident, FR – freshwater resident, US – upstream shifter, DS – downstream shifter and IS – interhabitat shifter.

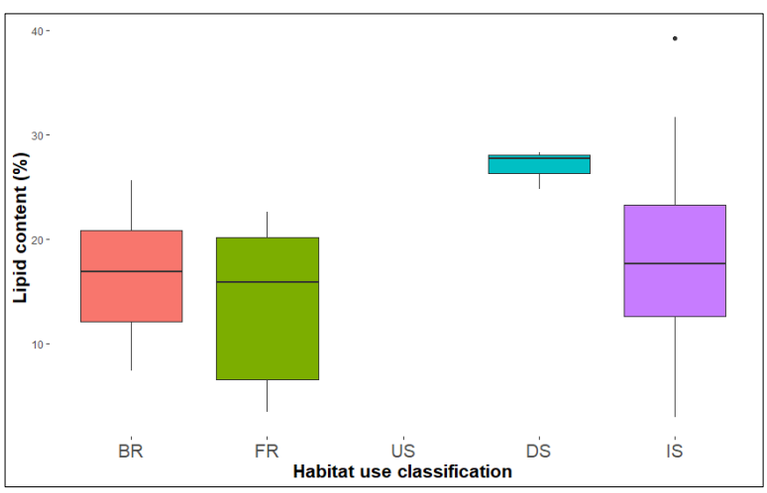


Figure 18. Graphical description of muscle lipid content, relative to microchemistry selection sub-sample (56 eels) collected on Minho River, by habitat use classification: BR – brackish water resident, FR – freshwater resident, US – upstream shifter, DS – downstream shifter and IS – interhabitat shifter.

Overall, the eels belonging to the group shifters, presented higher values for all the condition indices evaluated during this study than the brackish or freshwater residents (Figs 16-18). The K values were higher for IS and US than for the other strategies (Fig. 16). The HSI values were similar between strategies (smANCOVA, $H(2)= 0.27$, $p= 0.07$) (Fig.17). The DS (27.75±1.89 %) and IS (17.60±9.86 %) showed the highest values of muscle lipid content (Fig. 18). No significant differences were found in the muscle lipid content among the dominant strategies (ANCOVA: $F(2,35)= 2.22$, $p= 0.12$).

Table 9. Infection by *Anguillicola crassus* in the eels collected in the Miho River by habitat use classification: BR – brackish water resident, FR – freshwater resident, US – upstream shifter, DS – downstream shifter and IS – interhabitat shifter; data include: sample size (n), number of infected individuals, prevalence of infection, maximum (Max), median (M) and standard deviation (σ), for parasitism infection by *A. crassus*.

	Total (n)	Infected (n)	Prevalence (%)	Max	M (±σ) (only infected)
BR	14	6	43	21	5 (±7.4)
FR	13	8	62	5	2 (±1.7)
US	2	0	0	-	-
DS	3	2	67	19	10 (±12.7)
IS	21	10	48	6	2 (±1.9)

Eels belonging to the groups DS and FR showed the highest prevalence values of *A. crassus*, while US and BR showed the lowest values (Table 9). However, the BR and the DS were those presenting the highest levels of infection, that is a higher number of parasites per individual when compared to FR and IS (Table 9). The group US showed a prevalence of 0%, but the number of eels analysed belonging to this group is too low to draw any conclusion.

Regarding the distance of the zero band to the otolith's nucleus, the highest median values were found for the individuals belonging to the groups IS (220.78 ± 23.71 μm), followed by FR (219.97 ± 23.46 μm), and BR (215.91 ± 23.04 μm) (Fig. 19), and no statistical differences were found between these three groups (ANOVA: $F_{(2,47)}$= 0.129, p=0.89).

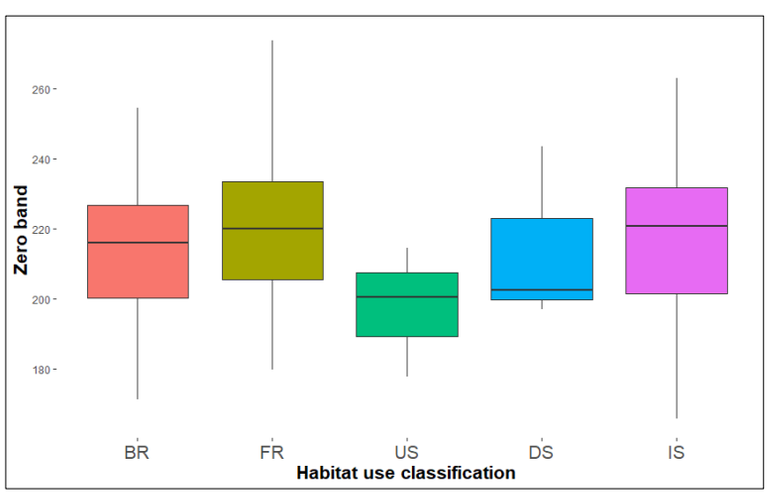


Figure 19. Graphical description of the zero band (μm), relative to microchemistry selection sub-sample (56 eels) collected on Minho River, by habitat use classification: BR – brackish water resident, FR – freshwater resident, US – upstream shifter, DS – downstream shifter and IS – interhabitat shifter.

The otoliths of eels classified as FR presented higher values for form factor and roundness values than the eels classified with other habitat use patterns (Fig. 20 and 21). On the other hand, eels classified as FR presented lower values for the variables circularity and ellipticity (Figs. 22 and 24) , than the eels classified with other habitat use patterns (IS also presented similar lower values for the variable ellipticity).

Mathematical differences were found for form factor (smANCOVA, H(2)=2.004, p=0.020), circularity (smANCOVA, H(2)=2.004, p=0.019) and ellipticity (smANCOVA, H(2)=2.004, p=0.036), and were not found for roundness (smANCOVA, H(2)=2.004, p=0.101). The rectangularity (Fig. 23) of otoliths is statistically similar between sample groups (ANOVA, H(2,44)=0.368, p=0.694).

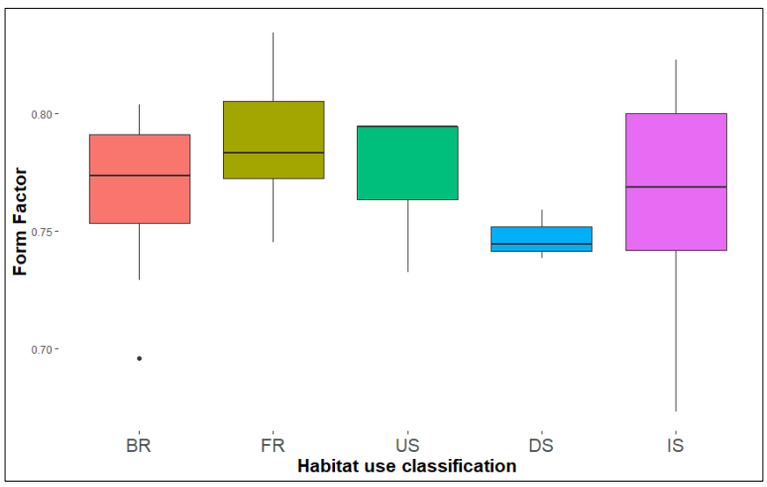


Figure 20. Graphical description of the form factor (FF), relative to microchemistry selection sub-sample (56 eels) collected on Minho River, by habitat use classification: BR – brackish water resident, FR – freshwater resident, US – upstream shifter, DS – downstream shifter and IS – interhabitat shifter.

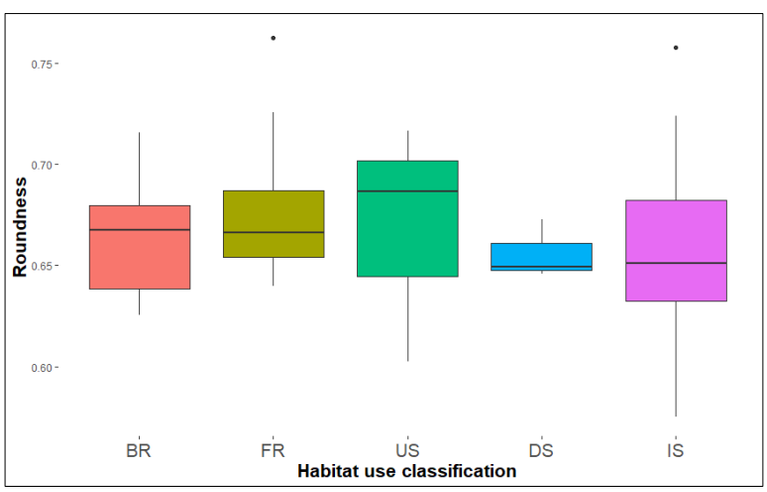


Figure 21. Graphical description of the roundness (RO), relative to microchemistry selection sub-sample (56 eels) collected on Minho River, by habitat use classification: BR – brackish water resident, FR – freshwater resident, US – upstream shifter, DS – downstream shifter and IS – interhabitat shifter.

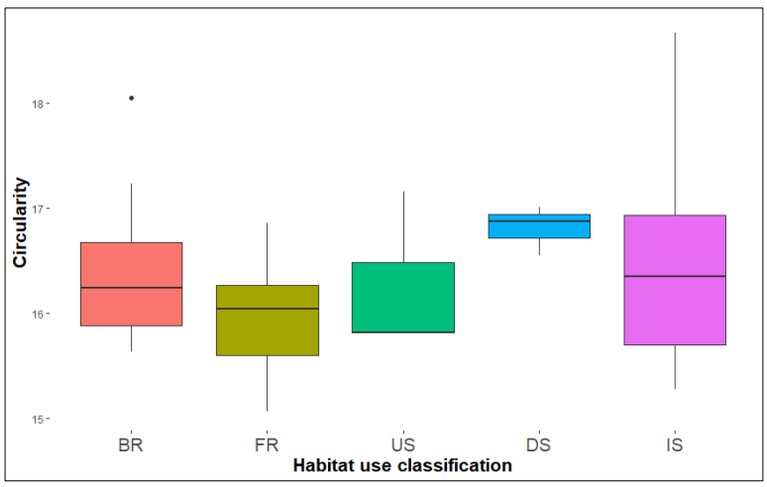


Figure 22. Graphical description of the circularity (CI), relative to microchemistry selection sub-sample (56 eels) collected on Minho River, by habitat use classification: BR – brackish water resident, FR – freshwater resident, US – upstream shifter, DS – downstream shifter and IS – interhabitat shifter.

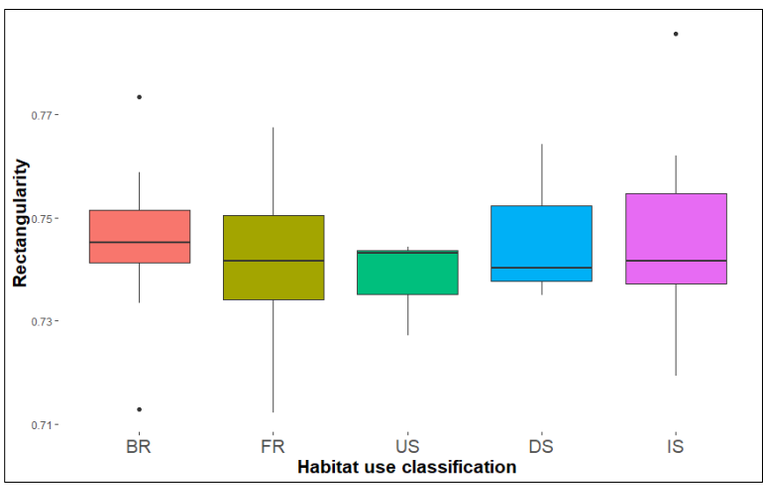


Figure 23. Graphical description of the rectangularity (RE), relative to microchemistry selection sub-sample (56 eels) collected on Minho River, by habitat use classification: BR – brackish water resident, FR – freshwater resident, US – upstream shifter, DS – downstream shifter and IS – interhabitat shifter.

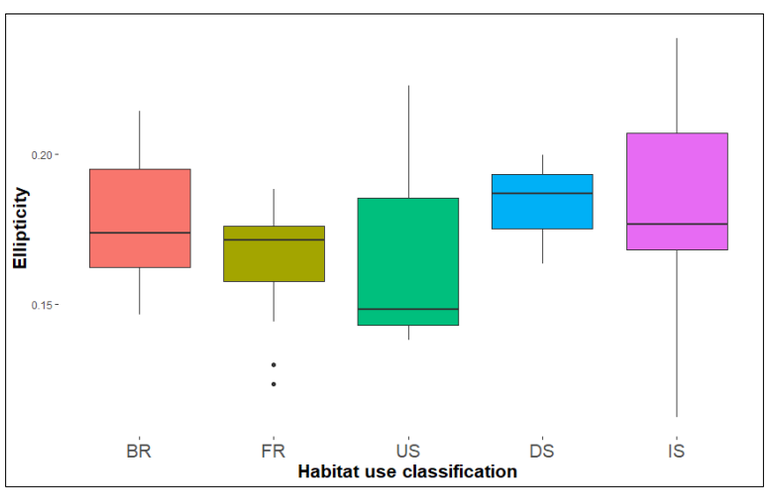


Figure 24. Graphical description of the ellipticity (EL), relative to microchemistry selection sub-sample (56 eels) collected on Minho river, by habitat use classification: BR – brackish water resident, FR – freshwater resident, US – upstream shifter, DS – downstream shifter and IS – interhabitat shifter.

DISCUSSION

The *A. anguilla* displayed a wide repertoire of habitat use patterns in the Minho River, which included two resident (brackish or freshwater) and three nomadic strategies (upstream, downstream, or interhabitat shifters). The relative percentage of resident (51%) and nomadic strategies (49%) were similar among the eels analysed. Nonetheless, the dominant strategy was the interhabitat shifting (39%), followed by the resident strategies - brackish (26%) and freshwater residents (25%). Previous studies showed that eels tend to enter freshwater environments at an early age (Daverat et al. 2011), and that shifting to the marine estuary is a common tactic in temperate ecosystems (Daverat et al. 2006). This was attributed to the fact that in *A. anguilla*, the gut develops for osmoregulation in freshwater whatever the ambient salinity (Ciccotti et al. 1993), indicating ontogenetic programming for osmoregulation in freshwater. During this study, the chronologies in habitat movements were not established, but our results indicate that at least 26% of the eels analysed never entered a freshwater habitat until the moment they were captured, and that the upstream shifters (5%) spent a relatively high proportion of their life in brackish habitats before entering a freshwater environment. It was proposed that settlement of eels in brackish habitats is condition-dependent, with low-body-condition glass eels preferring high-salinity habitats (Edeline et al., 2006). Early migrants usually present better body condition and migrate further upstream than the late migrants, which are usually in the worst conditions and settle in the lower estuary (e.g., Edeline et al., 2009).

Although this study does not allow to draw firm conclusions about the condition of eels during settlement, the freshwater residents were among the eels showing the highest distance values between the nucleus and the zero band, which relates to the size of the individuals at the time of metamorphosis (ICES, 2009). On the contrary, the brackish residents, and especially the upstream shifters, showed lower values for this variable when compared to the freshwater residents. This could indicate that the individuals moving to freshwater after entering the river were those arriving in a better condition. A similar relationship was also recently found for eels from the Gironde estuary kept in captivity and wild eels collected in the Medoc watershed (Gaillard et al. 2022). However, whether the highest distances between the nucleus and the zero band correspond to eels in a better condition at entry (as a result of a faster growth) or if it relates to time spent in the coastal habitat (or offshore) before entry is a topic that deserves further attention.

The relative percentage of each strategy was unevenly represented across sampling locations. The individuals collected in the lower estuary were predominantly brackish water residents (79%), in middle estuary were mainly interhabitat shifters (67%), and the individuals collected in the tributary were mainly freshwater residents (55%), although the interhabitat shifters comprised 40% of the eels analysed in this habitat. This means that the eels that moved into freshwater, after entering the estuary, stayed in a freshwater habitat until capture, and most of the eels that experienced the highest salinities at the time of recruitment

appeared not to have entered a freshwater habitat until they were captured. A similar pattern was observed for *A. japonica* in Hamana Lake system of central Japan (Yokouchi et al., 2012). While the eel colonization strategies can be influenced by several factors such as condition, as discussed above, environmental variables such as temperature and salinity (Edeline et al., 2004; Edeline et al., 2006), or the development stage (Crean et al,. 2005). Yokouchi et al. (2012) also proposed that time of arrival to freshwater can also determine the strategies of habitat use later during development. Early arrival into freshwater enables eels to successfully a establish river residency, while many later arrivals tend to shift back to the estuarine habitats (Yokouchi et al 2012). The later arrivals encounter many elvers that already established their residence and started their growth earlier. If they were slow to enter freshwater due to a lower body condition, this could reduce their ability to compete with the eels that entered the river earlier with a higher condition level (Yokouchi et al 2012). This could partially explain the patterns in habitat use found for the interhabitat shifters. These eels showed two distinct behaviours after metamorphosis: entered a brackish environment, moved to freshwater and then back to estuary (alternating between habitats afterwards), or stayed in a brackish environment for a relatively long time before moving into the freshwater habitat (alternating between habitats afterwards). Competition for space and food may occur not only with eels from the same cohort but also with older resident yellow eels, because the existence of the Frieira dam at 76 km from the mouth of the estuary, along with other obstacles contributed to a habitat reduction of ca. 90% for diadromous species in this basin (Moura et al. 2022). Thus, further research on the relationships between available habitats in the river system and the migratory patterns of young eels is needed. Another not mutually exclusive explanation may be that eels do not actively move between habitats with different salinities but stay in a habitat where salinity varies throughout the year. Most interhabitat shifters were collected at station 2, a tidal freshwater area located in the middle estuary. Although salinity tends to be low, it can increase during the summer due to an increase in the salinity intrusion (Dias et al. 2016). Thus, some eels may settle in this habitat and experience seasonal variations in the salinity values, which are then reflected in the Sr concentrations in the otoliths. To further investigate this topic, complementary tracking studies are necessary, such as those that use acoustic telemetry, to confirm if changes in otoliths' Sr:Ca and Ba:Ca are the result of active movements or if they result from changes in habitat environmental conditions. Another important source of validation would have been measuring these elements in the water because this would allow discriminating between habitats on a seasonal basis.

Contrary to hypothesized, the interhabitat shifters (dominant strategy found in the Minho River) did not present an overall better condition than the eels using other strategies, except for the Fulton index. In fact, the interhabitat shifters and upstream shifters were the strategies associated to higher values of the Fulton index. No firm conclusions can be drawn from the interpretation of this index because as the slopes were

different among the eels collected in the different sampling locations (Cone, 1989) and the representation of the different strategies within each habitat was poor, no statistical analysis was possible in the scope of this study. Although no significant changes were found between strategies according to the hepatosomatic index and muscle lipid content, these indexes were higher in eels belonging to the brackish resident and downstream shifter strategies. This indicates that eels spending relatively more time in brackish habitats were those presenting an overall better condition. Previous studies already suggested that, in general, the growth of yellow eels in estuaries is higher than that of eels in freshwater habitats (*A. anguilla*, e.g., Arai et al., 2006; Melia et al., 2006; *A. rostrata*, e.g., Morrison and Secor, 2003, Jessop et al., 2008; *A. reinhardtii* in Australia, Walsh et al., 2006). Moreover, a recent study conducted in the Minho River found that the eels from the tributaries showed smaller length-at-age and lower body condition than those collected in the estuary (Moura et al., 2022). Also, studies of silver-phase *A. anguilla* found that individuals that exclusively inhabited freshwaters had significantly lower muscle fat contents than eels that never entered freshwaters (Marohn et al., 2013). Although there are no estimates for the productivity in the tributaries of the Minho River, previous studies indicate that the food webs in the tributaries are mainly supported by aquatic plants- and terrestrial-derived detritus (Dias et al., 2019). The detrital pathway is usually considered less efficient when compared to the phytoplankton pathway (Rooney et al., 2011), more associated with the estuary (Dias et al., 2016). Moreover, extreme hydrological regimes in the tributaries, characterized by droughts during the summer and torrential flooding during the winter, can impact fish responses, promoting stress conditions (Rytwinski et al., 2020). Additionally, the infection by *A. crassus* was previously associated with eels growing in freshwater environments because the prevalence of this parasite tends to be higher in these habitats (e.g., Marohn et al 2013). The highest prevalence values were found for freshwater residents and downstream shifters in this study, although brackish residents presented higher median intensity values than the freshwater residents.

The otoliths' shape indices discriminated poorly between habitat use strategies, but eels spending relatively more time in brackish environments presented lower form factor and roundness values and higher circularity and ellipticity values, than those that spent relatively more time in freshwater. These findings are similar, but not totally in agreement, with recent findings for eels collected in the Minho River where the otoliths of eels from the tributaries were round and circular, whereas the otoliths of eels from the estuary were elliptical and rectangular (Moura et al., 2022).

This study showed that eels presented diverse habitat use patterns in the Minho River and confirmed that catadromy is facultative in this species. The reasons for that were not investigated during this study but could include the body condition on arrival to the river, local environmental conditions, or both. Moreover, as high-fat contents are considered essential for a successful transoceanic spawning migration (Larsson et al., 1990),and *A. crassus* has a negative impact on condition, this study highlights the importance of brackish

waters for eels in the Minho River, and thus regional conservation and management plans must accommodate the variability in habitat use shown by this species. Future studies would also benefit from a broader understanding of the factors responsible for this variability to help mitigate the effects of climate change or other long-term changes on populations and ecosystems.

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