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# Shifts in the European Shag diet along the Portuguese coast

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**Mestrado em Biologia Marinha**

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## Declaração de autoria de trabalho

**Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída**

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## Title

Shifts in the European shag diet along the Portuguese coast

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## Abstract

### a. Portuguese

A galheta, ou corvo-marinho-de-crista *Gulosus aristotelis* é uma ave marinha residente em Portugal Continental, especialmente em habitats costeiros. Apesar de ser uma espécie com uma ampla distribuição na Europa, a menor densidade populacional em Portugal torna-a classificada como espécie vulnerável a nível nacional. O núcleo reprodutor mais importante localiza-se no Arquipélago das Berlengas, onde se estima que existam mais de 60 casais. Porém estas aves apresentam outras populações mais ao sul do país, como em Sagres e Sesimbra. Para além dos elevados valores de conservação com a criação de Áreas Marinhas Protegidas (AMP's), a costa portuguesa é também um importante local de actividades antropogénicas como a pesca comercial e o turismo, o que torna a interacção entre humanos e aves mais expressiva e perigosa. Estudos baseados na composição da dieta dos principais predadores, como as aves marinhas, podem indicar a qualidade do ambiente, dando uma resposta indireta da riqueza, a abundância de espécies, a relação entre a composição do habitat e as espécies. Para investigar a dieta alimentar da galheta ao longo da costa, foram seleccionadas três Áreas Marinhas Protegidas (AMP), nomeadamente a Reserva Natural das Berlengas, o Parque Marinho da Arrábida e o Parque Natural do Sudoeste Alentejano e Costa Vicentina. O objectivo geral do presente trabalho foi caracterizar a dieta das populações de galhetas em três locais designados como AMP na costa portuguesa e avaliar se a dieta da população nacional apresenta uma variação espacial ao longo da sua distribuição. Para isso, foi realizada (i) a análise morfológica e molecular da dieta por meio da coleta de regurgitações / egagrópilas; (ii) a comparação de possíveis variações espaciais da dieta associadas a diferentes habitats e, (iii) a caracterização da comunidade de peixes presente em cada área. Das 25 espécies consumidas pelas galhetas, 75% foram encontradas na dieta da subpopulação das Berlengas, sendo considerada a dieta mais diversa comparativamente às outras duas. Apesar da grande riqueza, 57% da biomassa total encontrava-se na Arrábida. As principais presas da dieta em geral foram a galeota Ammodytidae, o peixe-rei *Atherina presbyter* e espécies da família Labridae. Este resultado corroborou os estudos anteriores realizados sobre a população de galhetas no Atlântico Norte e na Península Ibérica, os quais revelaram uma plasticidade na dieta da galheta, sendo a galeota (Ammodytidae) a presa mais numerosa e frequente. A respeito da variação espacial, o presente estudo não apontou diferenças estatisticamente relevantes na composição de presas. Porém, foi possível observar que Ammodytidae foi o grupo mais abundante (63,2%) na dieta da população

da Arrábida, enquanto nas Berlengas foi *Atherina presbyter* o peixe-rei (25%) e em Sagres foram os sargos *Diplodus sp* (33%) e o peixe-rei *Coris julis* (22,2%). Esta variação pode ser reflexo da diferente disponibilidade de presas em locais distintos. A contribuição de biomassa e o tamanho médio dos indivíduos das quatro espécies mais representativas variaram: Ammodytidae teve a sua maior biomassa e tamanho médio registados na Arrábida, enquanto nas Berlengas foram a piarda, o peixe-rei e o peixe-aranha *Echiichthys vipera*. Estes resultados são típicos de uma espécie com comportamento oportunista e generalista, com uma dieta de grande plasticidade variando de presas demersais a pelágicas. Devido à pequena amostra, Sagres não foi considerada fiável em termos comparativos e a sua abundância e diversidade de espécies parecem ter sido subestimadas. Apesar disso, uma experiência piloto com barcoding confirmou a presença de peixe-rei na dieta da subpopulação de Sagres. Apesar da galeota ter sido em geral a presa com maior importância relativa na dieta da população das galhetas em Portugal, nas Berlengas este não foi o grupo predominante, mas sim o peixe-rei, um pequeno peixe pelágico. Este resultado contrasta com estudos realizados em anos anteriores. Apesar de não ter sido testado estatisticamente, sugerimos a hipótese de que possa haver uma variação interanual na disponibilidade de presas. Esta flutuação interanual já foi registada no Atlântico Norte e Península Ibérica, onde há três décadas o consumo de galeotas pelas aves vem sendo reduzido, enquanto o consumo de Gadidae, Gobidae e Labridae aumentaram. Já se sabe que a disponibilidade de presas varia sazonalmente ao longo do ciclo anual das espécies-presa, mas com as mudanças climáticas e a sobrepesca, a tendência é que a disponibilidade de presas seja continuamente alterada, e as dinâmicas das aves sejam afetadas a longo prazo. Numa perspectiva de curto prazo, são esperadas mudanças no comportamento alimentar (por exemplo, substituições na preferência das presas, uso da pesca para detectar maiores abundâncias de presas) e sucesso reprodutivo das aves devido às variações sazonais na disponibilidade das presas. A longo prazo, as mudanças na distribuição das presas e baixo recrutamento de galeotas e pequenos peixes pelágicos, devido à pesca excessiva e mudanças ambientais, tendem a reduzir drasticamente as populações de predadores, a nível global.

A captura acidental em artes de pesca é outra ameaça que tem vindo a afectar significativamente a sobrevivência de diversas populações de aves marinhas, incluindo a galheta. Neste estudo foi registada a presença de um material semelhante a linha de pesca numa única amostra, indicando que em Portugal também esta população se encontra vulnerável a tal ameaça. Apesar de ser apenas um registo, estudos continuados e detalhados são necessários para analisar se há um aumento no efeito potencial das interações entre galhetas e a pesca comercial. O nosso estudo é o primeiro a descrever a composição da dieta da galheta

na Arrábida e a fornecer informações preliminares sobre a dieta em Sagres. A alta contribuição, em geral, da galeota e do peixe-rei coincide com os registos anteriores em populações do Atlântico Norte e Peninsula Ibérica. Assumindo que as dietas das populações de galhetas no Mediterrâneo são compostas pelas famílias Labridae e Trachinidae, existe a possibilidade de que as populações mais ao sul de Portugal tenham um padrão alimentar semelhante às do Mediterrâneo. De forma a aprofundar a posição trófica destas populações localizadas geograficamente numa zona intermédia é imprescindível que que se mantenha a monitorização e análise das dietas destas populações, incidindo ao longo de todo o ciclo reprodutor da galheta em anos sucessivos, a fim de verificar possíveis mudanças temporais na disponibilidade de presas e as suas consequências para a dinâmica das populações da galheta. Sabendo que a identificação por otólitos está sujeita a erros devido aos seus processos erosivos e ao seu tamanho reduzido, uma abordagem holística considerando também DNA barcoding parece ser uma estratégia para tentar superar esse obstáculo.

Palavras-chave: **ecologia alimentar; área marinha protegida; otólito; ave marinha; ADN**

## b. English (300 words)

The European Shag *Gulosus aristotelis* is a seabird resident in mainland Portugal. The diet composition of shags and its spatial variation among marine protected areas (the Berlengas Nature Reserve, the Marine Park of the Arrábida Natural Park and the Southwest Alentejo and Costa Vicentina Natural Park) were studied through morphological and molecular analysis of regurgitated pellets. A total of 29 fish species were identified, with 75% of them present in the Berlengas. Despite the great richness of Berlengas, 57% of the total biomass was comprised in Arrábida. The major prey of the diet were sand eels Ammodytidae, sand smelt *Atherina presbyter* and species from the Labridae family. Sand eels were the most abundant (63.2%) in the diet at Arrábida, while in the Berlengas it was sand smelt (25%) and in Sagres were *Diplodus* sp (33%) and Mediterranean rainbow wrasse *Coris julis* (22,2%). The biomass contribution and mean size of the four most representative species varied: Ammodytidae had its highest biomass and mean size recorded in Arrábida, while Berlengas had the largest sand smelt, Mediterranean rainbow wrasse and the lesser wrasse *Echiichthys vipera*. Our study characterised the shag as opportunistic, with a broad plasticity diet ranging from demersal to pelagic species. Due to the small sample size from Sagres, species abundance and diversity were underestimated and not comparable, but a pilot test using DNA barcoding confirmed Mediterranean rainbow wrasse there. This is the first description of diet composition of the European shag in Arrábida and preliminary information about it in Sagres. The high contribution of sand eels and sand smelt coincides with previous records in North Atlantic populations. Assuming that the diets of shag populations in the Mediterranean are composed of Labridae and Trachinidae families, we raise the hypothesis that the southernmost shags of Portugal have a feeding pattern similar to the Mediterranean ones.

Keywords: **feeding ecology; marine protected area; otolith; seabird; DNA**

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Chapter 1

## 1) The European Shag

### *1.1 Biology and ecology*

The European shag, *Gulosus aristotelis* (Linnaeus 1761), is a seabird species belonging to the Suliforms and the family Phalacrocoracidae, also commonly known as crested cormorant. In Europe, three species of this family are usually found, the Great Cormorant *Phalacrocorax carbo*, the European Shag, and the Pygmy cormorant *Microcarbo pygmaeus*. Contrary to the other two species of cormorants, the European shag is highly associated with marine areas (del Hoyo et al., 1992). It is a medium-large bird with an approximate weight of 2.3 kg with coastal habits, mainly found at rocky shores (Wanless & Harris., 1997; del Hoyo et al., 1992). The male adult European shag, during the breeding season, change its plumage to a black colour with greenish reflections and a prominent crest (see Figure 1). The shag is mainly a sedentary species, although some dispersive movements and short-distance migrations may occur (del Hoyo et al., 1992). They perform prospecting movements for food in areas close to their colonies, with distances around 5 km during the breeding season, not exceeding 20 km in the rest of the year (Velando et al., 2005; Wanless et al., 1991). Despite being rated as the least concern species in a global threat term, the global population of the European shag is officially declining but not fast enough to be considered vulnerable (BirdLife International., 2021).

European shags usually breed in colonies, with nests built on cliffs, platforms, steep areas, and caves (del Hoyo et al., 1992). The clutch size varies between 1 to 8 eggs, with an average of 2.14 – 2.57 eggs per nest and average production of 1.3 chicks/nest, and both parents share parental care until the offspring is fully developed (Silva 2015; Neto 1997; Wanless & Harris., 1997). The reproduction season for the shags occurs between January and August, presenting a wide range (Silva 2015). The reproductive success depends on the age/experience of the couples, and the quality of the nesting site chosen. Experienced couples tend to start breeding earlier so that the hatch date of the eggs coincides with the greatest abundance and availability of food to ensure the offspring's highest possible survival rate (Potts et al., 1980). In addition to the number of experienced couples, reproductive success still depends on external factors such as disturbance near the nests, availability of food - especially high-energy foods (i.e., sardine), – and predation (Silva 2015). The Berlengas Archipelago, in Portugal, holds 60 to 75% of the European Shag national population. Despite the low population size there compared with the global population, the breeding pairs in the Berlengas have remained stable (Silva 2015).

The European shags are visual predators, capturing and chasing their prey underwater, hunting individually or in groups (Wanless and Harris., 1997). They are excellent swimmers that can dive to average depths of 40 m, owing to an adaptation of their partially permeable feathers, which enables the bird to perform rapid and deep dives without experiencing a decrease in body temperature (Grémillet et al., 1998; Wanless et al., 1991). In the late 1980s and early 1990s, the diet of shags in the North Atlantic (i.e., Isle of May) consisted primarily of sand eel, with limited evidence of seasonal differences in diet composition indicating specialised behaviour (Harris & Wanless 1993, 1991). However, with climate change and increased overfishing, the availability and distribution of prey have gradually changed in recent decades, influencing bird dynamics. It shows a great plasticity in its diet, feeding on several benthic, demersal, and pelagic fish species (Hillersøy & Lorentsen., 2012; Velando & Freire., 1999; Álvarez 1998; Harris & Wanless., 1993). However, few species dominate their diet: the sandeels (Ammodytidae family) and the herring *Clupea harengus* are the main prey species during the breeding period in most studies in North of Atlantic (Lilliendahl & Solmundsson., 2006; Velando et al., 2005; Velando & Freire., 1999; Álvarez 1998; Aebischer & Wanless., 1992; Harris & Wanless., 1991). These species are of relatively high calorific value, which is essential to the breeding success of seabirds (Kitaysky et al., 2006; Barrett et al., 1987). For populations along the Norwegian coast, Gadoid species such as the saithe *Pollachius virens* and the cod *Gadus morhua* become important prey species during the breeding period, along with sandeels (Barrett 1991; Barrett et al., 1990).

Figure 1:European shag at Berlengas. Photo by SPEA staff.

### *1.2 Range and geographic distribution*

Europe concentrates around 65% of the global population of the European shag with a vast distribution ranging from the North Atlantic to the Mediterranean (Adlard & Fagundes., 2020; BirdLife International 2015). Although it is classified as a least concern species in the worldwide conservation status, the European population has been decreasing (IUCN 2017; BirdLife International 2021). In the Berlengas Archipelago in Portugal, breeding pairs have declined by 40% since 2002 (Del Moral & Oliveira., 2019).

The population of the European shag is divided into two subspecies in the Iberian Peninsula: The Mediterranean subspecies (*Gulosus aristotelis desmarestii*) and the Atlantic subspecies (*Gulosus aristotelis aristotelis*) (Adlard & Fagundes., 2020). Approximately 1,993 breeding pairs have been estimated (Del Moral & Oliveira 2019), of which 95% are in Spain. In Portugal, the largest colonies are in the Berlengas archipelago, with more than 60 breeding pairs (Del Moral & Oliveira 2019), and in Cabo Espichel, an area of relative importance, representing the second-largest colony with 20 breeding pairs. There are also records of nesting areas in Cabo Carvoeiro, Cabo da Roca, Cabo Raso, Cabo de Sines and Cabo de São Vicente (Del Moral &

Oliveira 2019). Despite its vast distribution, the European shag population in the Iberian Peninsula is considered vulnerable or endangered (Cabral et al., 2005; Velando et al., 2005; Muntaner., 2004).

### *1.3 Threats*

The threats reported for cormorants, as for other seabirds, include mortality caused by commercial fishing, pollution, invasive species, habitat degradation, (micro)-plastic ingestion, and human disturbance (Croxall et al., 2012). The interaction with commercial fisheries is associated with an overlap of interests, which means looking for the same resources, which are fish taxa of economic importance (Żydelis et al., 2013; Velando & Freire, 2002). As a result, the increase in fishing activity in conjunction with the overexploitation of resources can decrease prey availability and affect the populations of shags (Furness 2002).

Changes in the distribution of sardine and anchovy in South Africa, driven by overfishing and environmental changes, contributed to a significant reduction in the number of breeding couples of Cape Cormorant *Phalacrocorax capensis* (Crawford et al., 2014; Okes et al., 2009; Pichegru et al., 2009). Another impact of the interaction with commercial fishing is the accidental capture, known as bycatch (Oliveira et al 2020; Oliveira et al., 2019; Žydelis et al., 2013; Anderson et al., 2011; Okes et al., 2009; Žydelis et al., 2009). Environmental disasters, such as hydrocarbon spills (oil spills), can, directly and indirectly, affect the populations of European Shag, through incorporation of contaminants and the reduction in food availability (Velando et al., 2005). Despite the challenging access or even inaccessibility of nesting areas (i.e., cliffs and caves), disturbances are still observed. Intense maritime tourism activities in feeding areas, for instance, can result in stress to the species and possibly a temporary or permanent abandonment of the nest (Velando & Munilla., 2011).

## 2) Diet studies

### *2.1 General approach and applications*

Studies focusing on diet are one of the most holistic approaches for understanding the direct and indirect effects of environmental changes on different marine species (Hillersoy 2011). In seabirds, for instance, reproductive success and diet are correlated, which means that better reproductive success is directly proportional to specific prey availability and abundance (Barret 2002). In the North Atlantic, the breeding success of shags was lower when the abundance of sandeels was lower (Rindorf et al. 2000). Shags present behavioural adaptations driven by changes in prey abundance, such as increasing time spent feeding due to changes in prey abundances, reducing breeding success (Watanuki et al. 2008; Wanless et al. 2005; Frederiksen et al. 2004). Information about recruitment and breeding success of commercial important fish (e.g., sardines, anchovies) is an indirect approach ensured by diet studies of seabirds, even though this information are often inaccessible to researchers especially at its early life stages (Litzow et al. 2000; Barrett 1991).

### *2.2 Stomach and Faeces*

Seabirds stomach analyses are the most common approaches to investigate diet composition and feeding ecology. However, this is an invasive method, meaning that it relies on the dead birds, either from bycatch or natural causes (Donnelly-Greenan et al., 2014). The analysis of the stomach content allows to obtain an instant result of the birds' feeding behaviour. However, this analysis is limited because identifying digested fish remains is difficult (Gagliardi et al., 2007), and stomachs are sometimes empty (Holst et al., 2001) due to bird stress at that time. Sampling of faeces are used as complementary to other methodologies since it preserves hard parts. Even though faeces can be obtained without killing the individuals, it is not found in the same amounts as pellets (Ross & Johnsson 1995) due to the digestion process.

### *2.3 Regurgitate pellets*

Regurgitated pellets are natural structures constituted by indigestible remains of prey, commonly analysed in studies of seabird diet (Swann et al., 2008; Lorentsen 2004; Velando & Freire., 1999; Johnstone et al., 1990). The identification of contents is based mainly on the visual examination of skeletal structures such as pre-jaws, vertebrae, and otoliths, and therefore requires the use of identification keys (Tuset et al., 2008; Barrett et al., 2007). Otoliths are non-fully digestible structures composed mainly of calcium carbonate which are commonly used to identify species ingested by predators, such as seabirds and fishes (de Assis et al., 2018). Every fish has six otoliths arranged in pairs and called – according to their size – lapillus, asteriscus, and sagitta, which have a crucial function in body balancing and hearing of Osteichthyes (Campana 2004). Due to its largest size, the sagitta is most suitable for systematic and ecological studies (de Assis et al., 2018; Harvey et al., 2000). However, the otoliths can suffer degradation inside the stomach of birds, which may lead to underestimations of the importance of some species in the diet and the length and weight of prey (Harris & Wanless., 1993; Jobling & Breiby., 1986).

Pellets are usually easy to collect in large numbers and without causing any disturbance to the birds. For European shag, the regurgitation occurs at least once a day for each individual (Johnstone et al., 1990; Duffy & Laurenson 1983; Ainley et al. 1981). This methodology allows a long-term analysis and monitoring of the diet and feeding behaviour; since it is not invasive for the species, it is possible to collect pellets practically every day. Thus, changes in shag feeding behavior over one or more years can be detected. Despite the problems associated with identifying prey species, this methodology is considered a suitable method for determining the composition of the diet (Barrett et al., 2007). Furthermore, relationships between fish length and otolith measures elucidate predators' feeding ecology/behaviour, providing information for species management, especially commercially important species (Battaglia et al., 2010; Lorentsen et al., 2004).

## 3) Marine protected Areas (MPA's)

### *3.1 Importance*

Marine Protected Areas (MPAs) are an important ecological tool for conserving marine ecosystems established by governments. Their major aim is to improve the conservation of species and their respective habitats and sustainably manage fisheries activities to reduce their possible negative impact on the ecosystem (Batista & Cabral, 2016; Horta and Costa et al., 2016). There are several types of MPAs and levels of protection according to their management objectives. When well applied and actively monitored, the expected effects within MPAs are an increase in richness, stock/abundance, size and biomass of marine species, especially those of high commercial value (Lester et al., 2009; Tetreault and Ambrose, 2007; Russ, 2002; Mosquera et al., 2000). Marine protected areas can also preserve the structure of habitats, thereby preventing a loss of species diversity or extinction (Zupan et al., 2018; Horta e Costa et al., 2016). The main marine predators such as marine mammals and seabirds have been considered good indicators of the quality/state of marine ecosystems (Sydeman et al., 2017). Their trophic webs tend to comprise, directly or indirectly, several species of other taxa, such as marine fish, which usually are species of commercial value, such as sardines. Therefore, seabirds are an excellent example of key species, which can efficiently identify candidate regions for the implementation of MPAs (Ronconi et al., 2012).

### *3.2 Portugal*

The Southwest Alentejo and Costa Vicentina Natural Park (37º55'N, 9º00'W to 36º59'N, 8º40'W), here mentioned as Sagres, is one of the protected areas studied here. It covers more than 100km of the Portuguese coast and extends 2 km into the Atlantic Ocean, crossing through the regions of Alentejo (southwestern coast) and Algarve (south and southwestern coasts) (Pereira et al., 2017). The Park was created in 1995 characterised by a great diversity of coastal habitats, including beaches, cliffs, and isolated rocks that constitute the perfect habitat for several unique species of fauna and flora. The Arrábida Marine Park (AMP), hereafter as Arrábida, is a reserve located on the west coast of Portugal, considered a biodiversity hotspot due to the structural complexity of the habitat, consisting of shallow waters and rocky bottom. It is an area commonly used for fishing and nautical tourism (Gonçalves et al., 2002). This reserve has different levels of protection including a fully protected area (FPA); partially protected areas (PPA); and buffer areas (BA). The fully protected area is a "no-take, no-go area", with an exception for researching, monitoring and education activities. Most PPAs allow local commercial fishing with traps and jigs for cephalopods but only beyond 200 m from shore and no extractive recreational activities are permitted. In the Buffer areas, some fishing vessels and recreational fishing activities are allowed.

Located on the Portuguese continental shelf, the Berlengas Nature Reserve aim to conserve the marine ecosystem and those related to it (Faria., 2014). The Berlengas archipelago is located at the top of Nazaré Canyon, an important submarine canyon in the transition zone between the Mediterranean and European regions. The presence of this canyon and the upwelling season

(April-September) make this environment rich in nutrients, increasing the biodiversity, especially the fish assemblage (Haynes et al., 1993). It is an important place for seabirds used as a migratory passage, resting and nesting, for example, most of the Portuguese population of European shags (Silva 2015; Neto 1997) the target species of this study.

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Chapter 2

# **Title**: Shifts in the European shag diet along the Portuguese coast

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Keywords: **feeding ecology; marine protected area; otolith; seabird; DNA**

**Abstract:** The European Shag *Gulosus aristotelis* is a seabird resident in mainland Portugal especially in coastal habitats. We studied the diet composition of shags and its spatial variation among marine protected areas (the Berlengas Nature Reserve, the Arrábida Marine Park and the Southwest Alentejo and Costa Vicentina Natural Park), through morphological and molecular analysis of regurgitated pellets. A total of 29 fish species were identified, with 75% of them present in Berlengas. Despite the great richness, 57% of the total biomass was comprised in Arrábida. The major prey of the diet were sand eels Ammodytidae, sand smelt *Atherina presbyter* and species from the Labridae family. Ammodytidae was the most abundant prey (63.2%) in the diet at Arrábida, while in Berlengas was *Atherina presbyter* (25%) and in Sagres *Diplodus* sp (33%) and *Coris julis* (22,2%) were the most important prey. The biomass contribution and mean size of the four most representative species varied: Ammodytidae had its highest biomass and mean size recorded in Arrábida, while in Berlengas it was *Atherina presbyter*, *Coris julis* and *Echiichthys vipera*. Our results characterised the shag as opportunistic, with a wide plasticity diet ranging from demersal to pelagic species. Due to the smaller sample size, the abundance and diversity of species in Sagres were underestimated and not considered comparable. Despite this, a pilot test with DNA barcoding confirmed *Coris julis* in Sagres. Our study is the first to describe the diet composition of the European shag in Arrábida and provide preliminary information about the diet in Sagres. The high contribution of the Ammodytidae and *Atherina presbyter* coincides with previous records in North Atlantic

populations. However, we raise the hypothesis that the southernmost shags of Portugal have a feeding pattern similar to the Mediterranean ones, due to predominance in the diets of Labridae and Trachinidae families.

## Introduction

The European shag *Gulosus aristotelis* has a plastic diet composed of a wide range of prey species, mostly fishes (Howells 2018; Grémillet et al., 1998; Barrett et al., 1990). It is already known that their diet varies thoughtout the year, with an increase of high energy valuable species during the breeding season, such as sand eels (Xirouchakis et al., 2017; Velando & Freire., 1999). It also varies depending on the prey availability, which fluctuates among seasons and location as well. This fact illustrates that the studies based on diet composition of top predators can indicate the environment quality, giving an indirect response of richness, the abundance of species, the relationship between habitat composition and species (Frederiksen et al., 2006). Despite this variation, Howells et al. (2018) showed a decreasing trend of the population of *Ammodytes sp*– one of the most relevant prey groups for shag's diet - in the North Atlantic, which results in a prey replacement and may affect the quality of the diet. This evidence is correlated with increasing threats: climate change and overexploitation (e.g., overfishing), both intensified by human demand, which are activities in direct conflict with seabirds, since they exploit the same resources.

As its name suggests, the European shag is widely distributed throughout the Western Europe. Since they do not prospect far from their nest, understanding the shag diet and its variation provides information about the composition of the ichthyofauna in the environment. Morphological analysis - based on species identification by otoliths, vertebrae, and other hard bone components from regurgitates or faeces - is one of the most common methods used in studies of diet description. Although it is an affordable and accessible technique, it has some complications that may lead to misidentification due to the degradation of otoliths (Barret et al., 2007). In conjunction with morphological analysis, molecular analysis as barcoding, can guarantee the validation of identifications and the complementation of species that morphological techniques cannot ensure.

In Portugal, the European shag can be found in marine protected areas such as the Berlengas Archipelago, where the largest population of European shags is concentrated, being an attractive spot for interdisciplinary studies (Nascimento et al., 2021; Oliveira et al., 2019). Studies on its diet indicated that this species has a massive preference for sand eels, especially during the breeding season (Nascimento et al., 2021; Howells et al., 2018; Liliendahl & Solmundsson., 2006; Velando & Freire., 1999). However, information about the diet of small populations seen in the extreme south (e.g., Sagres) is scarce. For this reason, this study aims to fill this gap using two complementary approaches for prey identification: conventional morphological analysis (using fish vertebrae and otoliths) and DNA barcoding of the 12S mitochondrial gene - focusing on populations of European shag that inhabit marine protected areas. In addition, our study expects to determine whether the diets of the shag populations vary spatially along the coast.

## Objectives

The current study aims to characterise the diet of the European shag in three different locations along the Portuguese coast, (ii) to assess whether the diets of the shag populations vary spatially. Therefore, this work aims to improve the understanding of the feeding preferences and prey dependencies of the European shag.

## Materials and Methods

### Study area

This study took place in three different sites located in Portugal's mainland, designated as protected areas under national and European Union legislation. The Southwest Alentejo and Costa Vicentina Natural Park, hereafter designated as Sagres, was created in 1995, and is characterised by a great diversity of coastal habitats. It represents a suitable habitat for many species of fauna and flora, despite suffering high anthropogenic pressure from fishing activities. The Arrábida Marine Park (AMP), hereafter designated as Arrábida, is a reserve located on the west coast of Portugal, considered a biodiversity hotspot due to the structural complexity of the habitat, consisting of shallow waters and rocky bottom. This reserve has different levels of protection including a fully protected area (FPA); partially protected areas (PPA); and buffer areas (BA). The fully protected area is a "no-take, no-go area", with the exception for research, monitoring and education activities. Located on the Portuguese continental shelf, the Berlengas Natural Reserve is an important place for seabirds, used for nesting, feeding, resting, and migratory passage.

### *Samples collection*

Fieldwork occurred in September 2020 and March/April 2021, with samples from Sagres only collected in 2020. The regurgitate pellets were collected directly from the rocks, slabs and rocky shores where shags rest. Fifty pellets were collected, 22 from the Berlengas, 22 from Arrábida and 6 from Sagres (see Figure 2). Each sample was stored individually, in a small ziplock and kept frozen for later analysis. The date and place were recorded during collection, and a unique identification number was assigned to each sample.

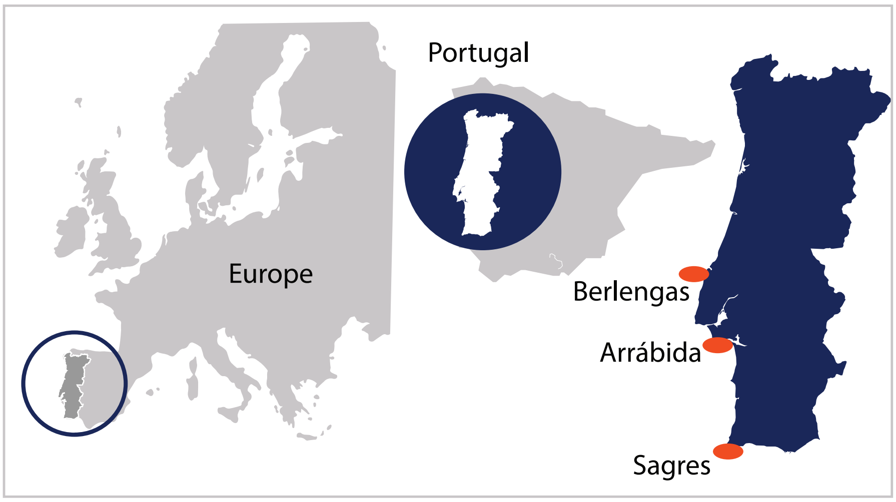


Figure 2:Map of sampling sites along the Portuguese coast.

### *Sorting and macroscopy analysis*

In the laboratory, each pellet was defrosted and placed in a detergent solution for the disintegration and removal of fat and non-bony liquid debris and then washed under running water on a 0.5 mm mesh sieve. As a first step, the remains were clustered based on the type of structure in one of the following categories: otoliths, vertebrae, pre-maxillary bones, pharyngeal teeth, secondary items. After that, they were identified to the lowest possible taxonomic level, counted, and measured. The identification of prey was based on the identification of otoliths (Tuset et al. 2008; Campana 2004; Assis 2000) vertebrae and pre-maxillary bones (Watt et al., 1997). Otoliths were sorted in pairs based on matching sides (left/right), length was measured to the nearest 0.1 millimeters using a stereomicroscope and millimeter paper. The taxonomic level of identification was different for some types of prey and dependent on the level of erosion of the otoliths. In the case of the Ammodytidae family, the distinction of species – with macroscopy technique- is difficult due to the small size of the otoliths and their similar shape. For this reason, we decided to be conservative with this group identification. After identification, the items were stored individually according to their type of structure. Subsequently, items that do not belong to the shag diet (e.g., sand and small stones) were removed.

### Morphological parameters

After the previous steps, each length of otolith/pair of otoliths was registered to estimate the size and biomass of consumed fish based on regression equations available in specialized literature (see Appendix 1). The length of otoliths was measured using graph paper, and pictures were taken to build a database with the species recorded. For species for which it was impossible to obtain regression equations, we tried to use equations for the genus or family. For cases where it was only possible to identify the family or genus, we considered the average length and biomass of a species belonging to the same genus or family with similar total length and biomass. Each species was divided into groups according to their ecological preference, being demersal, benthopelagic, reef associated or pelagic (see Appendix 2).

Several parameters were calculated to quantify preys. The total number of fish in each pellet was estimated as half of the total number of otoliths present or by the presence of other unique structures. The percentage of numerical frequency of each taxon (FN%) was estimated as the number of individuals of same taxon/species in the total number of individuals identified. It was also calculated the percentage of frequency of occurrence of each taxon (FO%) as the proportion of pellets that contain each of taxon in the total of samples analysed. The frequency of biomass (FB%) was given by the proportion of biomass consumed by a given species of the total biomass consumed by the bird. The index of relative importance (IRI%; Pinkas et al.,1971) was used to reduce biases associated with any measure calculating as IRI (%) = FO% * (FN% + FB%). Moreover, prey-specific abundance (PI%) was calculated to quantify the abundance of certain prey items in respect of all pellets as the ratio of abundance of certain prey item and the total number of prey items in all pellets containing the certain item, multiplied by 100. Afterwards, FO% and PI% were plotted (see Figure 3) and analysed together following Amundsen et al (1996) as a modification of the Costello method (Costello, 1990). A holistic approach containing all those parameters cited above was used to infer the prey importance, diet composition and feeding strategy of European shag.

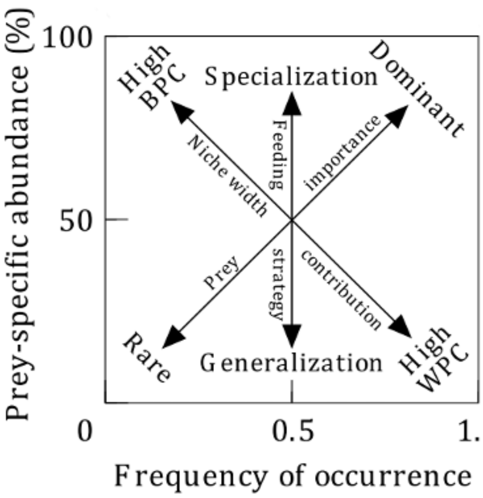


Figure 3:Modified Costello-Plot (after Amundsen et al.,1996), in which the prey-specific abundance is plotted against the frequency of occurrence.

*DNA barcoding*

A total of 14 pellets in good condition for molecular analysis were examined using DNA barcode (12S) - one of the most conservative information markers- to confirm morphological identifications. Eight of them collected in Arrábida (September 2020 - March 2021), three from Sagres (September 2020) and three from Berlengas (September and November 2020 and March 2021). DNA was extracted using the DNeasy Blood and Tissue Extraction Kit (Qiagen), with DNA from individual regurgitated prey amplified using the universal primers MiFish-F and MiFish-R (Miya et al., 2015). These primers were targeted to amplify a hypervariable region of the 12S rRNA gene, with an expected amplicon length of about 163-185 bp. Polymerase Chain Reaction (PCR) was performed under the following conditions: initial denaturation at 95 °C for 2 minutes, followed by 35 cycles (denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 1 sec) and a final extension at 72 °C for 5 minutes. PCR volumes of 10 µl included 1 µl of genomic DNA, 0.8 mM (=0.1 µl) dNTPs (Promega, Madison, WI, USA), 1.25 U (=0.06 µl) GoTaq polymerase (Promega, Madison, WI, USA), 5 mM de Gotaq Buffer (5x) (=2 µl), 2.5 mM (=0.5 µl) MgCl2 and 0.55 mM (=0.11 µl) of forward and reverse primers. PCR amplicons were purified using ethanol/sodium acetate precipitation and directly sequenced with the corresponding PCR primers. Sequencing was performed in an automated sequencer (ABI PRISM 3700) using the BigDye Deoxy Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and following the manufacturer's instructions. The resulting sequences were compared with those in GenBank using the BLAST algorithm (Altschul et al. 1990), and similarity values greater than 98% were considered as a positive identification for the consulted sample.

### *Data analyses*

Species diversity was expressed by the Shannon-Wiener index (H'; Shannon & Weaver, 1963), which is a quantitative measure of diversity that combines species richness in a given area and their relative abundance. This index reflects the number of different species and how the specimens are distributed among them. Spatial variation in diet composition across the different locations were examined with non-metric multidimensional scaling (nMDS) ordinations, using Bray-Curtis measure of similarity on presence-absence data under the R-package' vegan' (Oksanen et al., 2020). Analysis of similarities (ANOSIM) was conducted to examine the significance of spatial variation of diet composition (Clarke & Warwick 1994). Therefore, the R-statistic values tested the null hypothesis that the prey composition in shag's diet did not vary spatially. For that reason, possible meaningful differences between locations can be determined by comparisons of R-statistic values, where values close to 1 correspond to a disparate composition, whereas values bordering on 0 represent slight or no difference (Clarke 1993). To identify the prey items contributing the most to differences in each location we used the Similarity Percentage Analysis (SIMPER; Clarke 1993), by estimating the average contribution of each species to the similarity and dissimilarity between locations. In other words, SIMPER analyses come up with the contribution of each species to the observed similarity (or dissimilarity) between samples, and it uses Bray-Curtis as a measure of similarity (Bray-Curtis 1957). All statistical analyses were performed with RStudio software (Version 4.0.5) and the level of significance was set to $p < 0.05$.

## Results

### *Morphological parameters*

A total of 3,287 food items were found among the 50 diet samples, of which five were incomplete. In total, 1,100 otoliths were obtained, and 610 individuals were identified as belonging to 29 different fish species, 25 genera and 15 families (see Table 1). The most abundant groups (i.e., FN%) were the sand eel Ammodytidae family (46%) followed by sand smelt *Atherina presbyter* (16.4%), Mediterranean rainbow wrasse *Coris julis* (3,6%), *Gobius* sp. (3.6%), pouting fish *Trisopterus luscus* (3.1%). Regarding different study areas, sand eels Ammodytidae had the most representative contribution (63.2%) in the diet composition of European Shags in Arrábida Marine Park, followed by *Atherina presbyter* (12.9%). In Berlengas, this pattern changes, with *Atherina presbyter* being the most numerous fish species (25%) followed by Ammodytidae (12.1%). In contrast, in Sagres, the most representative group was *Diplodus* sp. (33%) followed by *Coris julis* (22,2%), while Ammodytidae were not found. The most common species in Arrábida and Berlengas were Ammodytidae (FO=77.3 and 38.1%, respectively), while in Sagres*,* *Coris julis* (50%).

Table 1:Identification of prey and its qualitative analysis (F.N=numerical frequency; F.O = frequency of occurrence; F.B = frequency of biomass; IRI = index of relative importance)

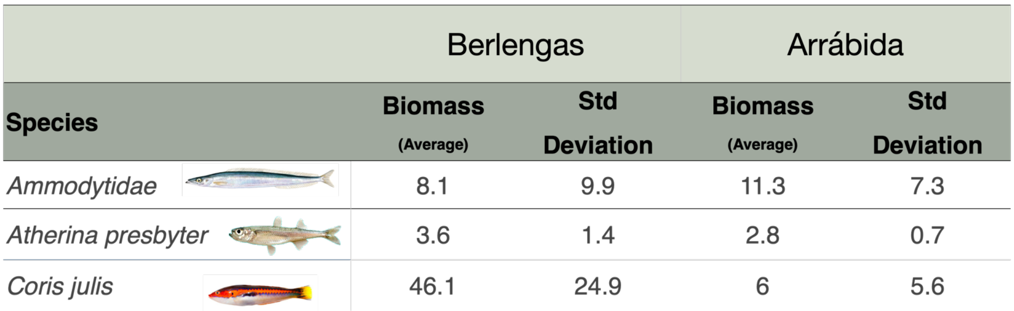
| FAMILY | SPECIES | SAGRES | | | | ARRÁBIDA | | | | BERLENGAS | | | | TOTAL | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **F. N (%)** | **F. O (%)** | **F. B (%)** | **IRI** | **F. N (%)** | **F. O (%)** | **F. B (%)** | **IRI** | **F. N (%)** | **F. O (%)** | **F. B (%)** | **IRI (%)** | **F. N (%)** | **F. O (%)** | **F. B (%)** | **IRI** |
| Atherinidae | | | | | | | | | | | | | | | | | |
| | *Atherina boyeri* | | | | | 0.7 | 4.5 | 0.4 | 5.2 | | | | | 0.5 | 2.2 | 0.1 | 1.4 |
| | *Atherina presbyter* | 5.6 | 25 | 4.9 | 260.6 | **12.9** | **13.6** | 4.6 | **238.8** | **25.3** | 9.5 | 2.9 | **268.6** | **16.4** | **15.6** | **3.6** | **310.3** |
| Ammodytidae | | | | | | | | | | | | | | | | | |
| | Ammodytidae units | | | | | **6.2** | **77.3** | 86.2 | **11543** | **12.1** | **38.1** | 3.1 | **579.1** | **46.1** | **55.6** | **33.9** | **4440.6** |
| Blenniidae | | | | | | | | | | | | | | | | | |
| | *Parablennius pilicornis* | | | | | 0.2 | 4.5 | 0.3 | 2.6 | | | | | 0.2 | 2.2 | 0.1 | 0.6 |
| | *Parablennis sp* | | | | | | | | | 1.6 | 14.3 | 0.2 | 26.3 | 0.3 | 6.7 | 0.1 | 2.9 |
| | *Blenniidae units* | | | | | 0.2 | 4.5 | | | | | | | 0.2 | 2.2 | | |
| Bothidae | | | | | | | | | | | | | | | | | |
| | *Bothidae units* | | | | | | | | | 1.6 | 4.8 | | | 0.5 | 2.2 | | |
| Clupeidae | | | | | | | | | | | | | | | | | |
| | *Clupeidae units* | | | | | | | | | 1.1 | 4.8 | | | 0.3 | 2.2 | | |
| | *Sprattus sprattus* | | | | | | | | | 0.5 | 4.8 | | | 0.2 | 2.2 | | |
| Gadidae | | | | | | | | | | | | | | | | | |
| | *Trisopterus luscus* | | | | | 2.93 | **9.1** | 0.7 | 33.3 | 3.8 | **19** | 2.6 | 122.7 | 3.1 | 11.1 | 1.9 | 55.5 |
| | *Trisopterus minutus* | | | | | 0.2 | 4.5 | | | 4.4 | 4.8 | 6.6 | 52.2 | 1.5 | 4.4 | 4.1 | 24.7 |
| | *Trisopterus units* | | | | | | | | | 0.5 | 4.8 | | | 0.2 | 2.2 | | |
| Gobiidae | | | | | | | | | | | | | | | | | |
| | *Gobius cruentatus* | 5.6 | 25 | 7.9 | 336.3 | | | | | | | | | 0.2 | 2.2 | 0.1 | 0.5 |
| | *Gobius sp* | | | | | 4.9 | 4.5 | 0.3 | 23.4 | 1.1 | 9.5 | 0.1 | 11.9 | 3.6 | 6.7 | 0.2 | 25.3 |

| FAMILY | SPECIES | SAGRES | | | | ARRÁBIDA | | | | BERLENGAS | | | | TOTAL | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **F. N (%)** | **F. O (%)** | **F. B (%)** | **IRI** | **F. N (%)** | **F. O (%)** | **F. B (%)** | **IRI** | **F. N (%)** | **F. O (%)** | **F. B (%)** | **IRI (%)** | **F. N (%)** | **F. O (%)** | **F. B (%)** | **IRI** |
| Labridae | | | | | | | | | | | | | | | | | |
| | *Acantholabrus palloni* | | | | | | | | | 0.5 | 4.8 | | | 0.2 | 2.2 | | |
| | *Coris julis* | **22.2** | **50** | **20.9** | **2157.2** | 3.2 | **18.2** | 2.3 | **100** | 2.7 | **19.0** | 4.1 | 129.6 | **3.6** | **22.2** | 3.6 | **159.1** |
| | *Ctenolabrus rupestris* | | | | | | | | | 3.8 | **19.0** | 2.0 | 110.9 | 1.1 | 8.9 | 1.2 | 21.1 |
| | *Labrus bergylta* | | | | | | | | | 1.6 | 4.8 | 1.4 | 14.6 | 0.5 | 2.2 | 0.9 | 3.0 |
| | *Labrus merula* | | | | | | | | | 1.1 | 9.5 | 3.3 | 42 | 0.3 | 4.4 | 2.1 | 10.6 |
| | *Labrus mixtus* | | | | | | | | | 1.6 | 9.5 | 0.6 | 21.4 | 0.5 | 4.4 | 0.4 | 3.8 |
| | *Labrus sp* | | | | | | | | | 0.5 | 4.8 | | | 0.2 | 2.2 | | |
| | *Symphodus cinereus* | | | | | | | | | 0.5 | 4.8 | 0.3 | 4.3 | 0.2 | 2.2 | 0.2 | 0.8 |
| | *Symphodus melops* | | | | | | | | | 1.6 | 9.5 | 1.6 | 31.1 | 0.5 | 4.4 | 1.1 | 6.9 |
| | *Symphodus roissali* | | | | | | | | | 1.1 | 4.8 | 0.5 | 8 | 0.3 | 2.2 | 0.2 | 1.2 |
| | *Symphodus mediterraneus* | | | | | | | | | 1.6 | 4.8 | 0.6 | 11.1 | 0.5 | 2.2 | 0.4 | 2.0 |
| | *Symphodus* | | | | | | | | | 2.2 | 14.3 | 1.0 | 46.6 | 0.5 | 6.7 | 0.4 | 5.9 |
| | *Labridae units* | | | | | | | | | 3.3 | 14.3 | | | 1.0 | 6.7 | | |
| Lotidae | | | | | | | | | | | | | | | | | |
| | *Ciliata mustela* | | | | | 0.5 | 9.1 | | | 2.7 | 19.0 | | | 1.1 | 13.3 | | |
| Phycidae | | | | | | | | | | | | | | | | | |
| | *Phycis sp* | | | | | | | | | 0.5 | 0.48 | 0.3 | 4.0 | 0.2 | 2.2 | 0.2 | 0.7 |
| Scorpaenidae | | | | | | | | | | | | | | | | | |
| | *Scorpaena elongata* | | | | | 2.7 | 9.1 | 0.3 | 27.3 | | | | | 1.8 | 4.4 | 0.1 | 8.5 |
| Serranidae | | | | | | | | | | | | | | | | | |
| | *Serranus hepatus* | | | | | 0.5 | 4.5 | 0.1 | 2.3 | | | | | 0.3 | 2.2 | 0.1 | 0.7 |
| | *Mycteroperca sp* | | | | | | | | | 1.1 | 4.8 | | | 0.3 | 2.2 | | |
| Soleidae | | | | | | | | | | | | | | | | | |

| FAMILY | SPECIES | SAGRES | | | | ARRÁBIDA | | | | BERLENGAS | | | | TOTAL | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **F. N (%)** | **F. O (%)** | **F. B (%)** | **IRI** | **F. N (%)** | **F. O (%)** | **F. B (%)** | **IRI** | **F. N (%)** | **F. O (%)** | **F. B (%)** | **IRI (%)** | **F. N (%)** | **F. O (%)** | **F. B (%)** | **IRI** |
| | *Microchirus sp* | | | | | 2.7 | 4.5 | 1.0 | 16.6 | | | | | 1.8 | 2.2 | 0.4 | 4.8 |
| | *Solea sp* | | | | | | | | | 0.5 | 4.8 | 0.1 | 3.1 | 0.2 | 2.2 | 0.1 | 0.5 |
| | *Pegusa lascaris* | | | | | 0.2 | 4.5 | 0.6 | 3.7 | | | | | 0.2 | 2.2 | 0.2 | 0.8 |
| | *Soleidae units* | | | | | 1.2 | 9.1 | | | | | | | 0.8 | 4.4 | | |
| Sparidae | | | | | | | | | | | | | | | | | |
| | *Diplodus sp* | **33.3** | **25** | 20 | **1333.3** | | | | | 1.1 | 4.8 | 7.1 | 39.1 | 1.3 | 4.4 | **4.6** | 26.2 |
| | *Diplodus annularis* | | | | | | | | | 1.1 | 4.8 | 0.6 | 8.1 | 0.3 | 2.2 | 0.4 | 1.7 |
| | *Diplodus vulgaris* | | | | | | | | | 1,6 | 9,5 | 0,3 | 18,7 | 0.5 | 4.4 | 0.2 | 3.0 |
| | *Pagrus pagrus* | | | | | 0.2 | 4.5 | 2.3 | 11.4 | | | | | 0.2 | 2.2 | 0.8 | 2.2 |
| | *Spicara smaris* | | | | | | | | | 1,1 | 4,8 | 0,5 | 8,0 | 0.3 | 2.2 | 0.4 | 1.5 |
| | *Spondyliosoma cantharus* | 11.1 | **25** | **21.3** | **810.7** | | | | | 1,1 | 4,8 | 0,7 | 8,7 | 0.7 | 4.4 | 0.6 | 5.7 |
| | *Sparidae units* | | | | | | | | | 0.5 | 4.8 | | | 0.2 | 2.2 | | |
| Trachinidae | | | | | | | | | | | | | | | | | |
| | *Echiichthys vipera* | 5.6 | **25** | **25** | **764** | 0.7 | 4.5 | 1.0 | 7.7 | 1.6 | 4.8 | 1.9 | 17.2 | 1.1 | 6.7 | 1.8 | 19.6 |
| | *Trachinus draco* | | | | | 0.2 | 4.5 | 0.3 | 1.2 | 1.1 | 4.8 | 0.2 | 6.6 | 0.5 | 4.4 | 0.2 | 3.0 |
| Unidentified | | **16.7** | 50 | | | 2.4 | 27.2 | | | 10.4 | 42.9 | | | 5.2 | 37.7 | | |

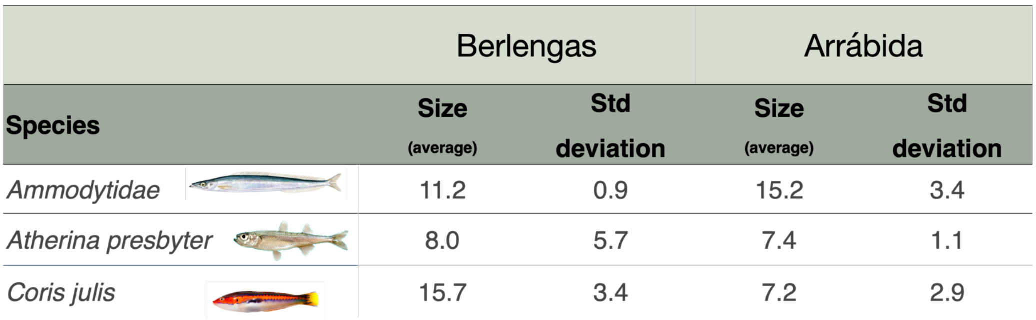
Arrábida was well represented in terms of total biomass, comprising 57% (i.e., 3,386.2 g) of the estimated total mass of the sample studied, followed by Berlengas (41.5%) and Sagres (1.2%). The lowest biomass found in Sagres is a result of few samples collected and analysed. In Arrábida, 89.7% of estimated biomass was represented by demersal species, while in Berlengas and Sagres, benthopelagic was the most representative group comprising around 42% each of estimated biomass. Regarding biomass of species, the most representative in abundance and frequency had their morphological parameters compared (see Table 2; 3), except Sagres due to the low sample number. The shags from Berlengas consumed higher biomass of these species when compared to the population of Arrábida. Although not statistically analysed, these biomasses may be directly associated with fish size, which means that in Berlengas, shags feed on heavier and larger individuals of *Atherina presbyter*, *Coris julis*.

Table 2:Fish biomass (average) of the most common species found in the diet of shags from Berlengas and Arrábida



| | Berlengas | | Arrábida | |
|---|---|---|---|---|
| **Species** | **Biomass** (Average) | **Std Deviation** | **Biomass** (Average) | **Std Deviation** |
| *Ammodytidae* | 8.1 | 9.9 | 11.3 | 7.3 |
| *Atherina presbyter* | 3.6 | 1.4 | 2.8 | 0.7 |
| *Coris julis* | 46.1 | 24.9 | 6 | 5.6 |

Table 3: Fish size(average) of the most common species found in the diet of shags from Berlengas and Arrábida



| | Berlengas | | Arrábida | |
|---|---|---|---|---|
| **Species** | **Size** (average) | **Std deviation** | **Size** (average) | **Std deviation** |
| *Ammodytidae* | 11.2 | 0.9 | 15.2 | 3.4 |
| *Atherina presbyter* | 8.0 | 5.7 | 7.4 | 1.1 |
| *Coris julis* | 15.7 | 3.4 | 7.2 | 2.9 |

Fish species recorded in the diet were mainly demersal (60.3% of the total), followed by pelagic species (17.4%, see Table 4). The average estimated length for demersal and pelagic species were 13.2 cm and 7.7 cm, respectively. In Arrábida, the diet was predominantly composed of demersal species (76.8%) with 13.5 cm, followed by pelagic species (13.7%) with 7.3 cm. In Berlengas, the ecological groups were more balanced, with no dominance of a particular group, with 28% demersal with 10.9 cm, followed by 26.9% pelagic with 8.1 cm, 19.2% reef-associated with 14 cm and lastly, 13.7% of benthopelagic with 12.5cm. In Sagres, 40% of the species were benthopelagic with 5.8 cm, followed by 20% reef associated with 6.4 cm.

Table 4:Percentage of prey – in terms of its ecology- composing the diet of European shag on each location.

| *Ecology* | *Arrábida (%)* | *Berlengas (%)* | *Sagres (%)* | *Total (%)* |
|---|---|---|---|---|
| Demersal | **76.8** | 28.0 | 10 | **60.3** |
| Pelagic | 13.7 | **26.9** | 5 | 1.4 |
| Reef-associated | 3.7 | 19.2 | **20** | 8.9 |
| Benthopelagic | 3.4 | 13.7 | **40** | 8.9 |

The feeding strategy and the importance of individual prey items in the diet of European shag along Portugal visualized in Amundsen diagram. The major concentration of points observed at lower-left quadrant indicated more presence of rare species (Figure 4). However, the presence of Ammodytidae in the upper right quadrant indicates some dominance of this prey for many individuals from Arrábida.

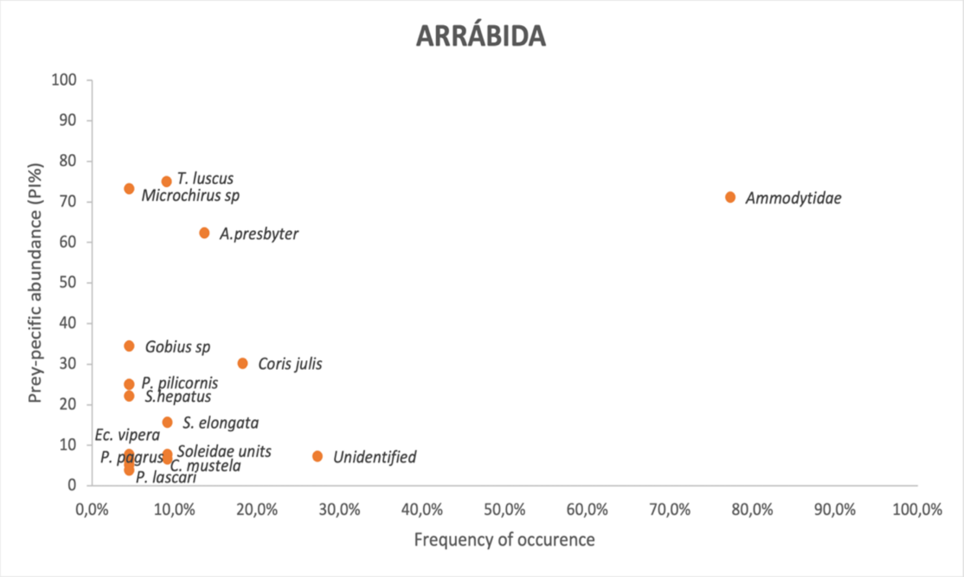


Figure 4: Amundsen diagram for prey species found on pellets from shags in Arrábida.

A similar pattern was observed in Berlengas, but there is no prey dominance. On the other hand, the presence of Atherina presbyter in upper left quadrant indicates that this prey was important for diet of some individuals (Figure 5).

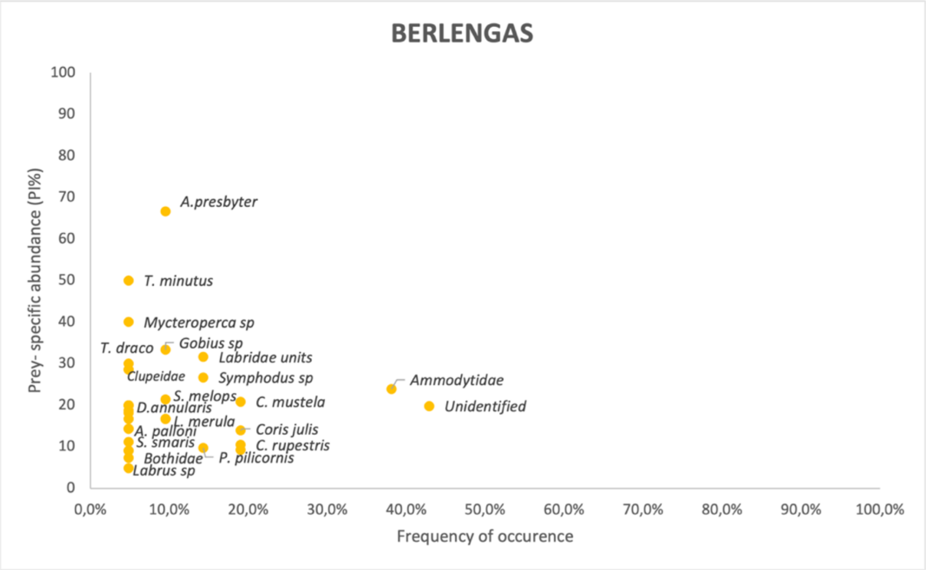


Figure 5:Amundsen diagram for prey species found on pellets from shags in Berlengas.

Sagres, however, did not show any pattern and we infer that this is a result of low sampling (Figure 6).

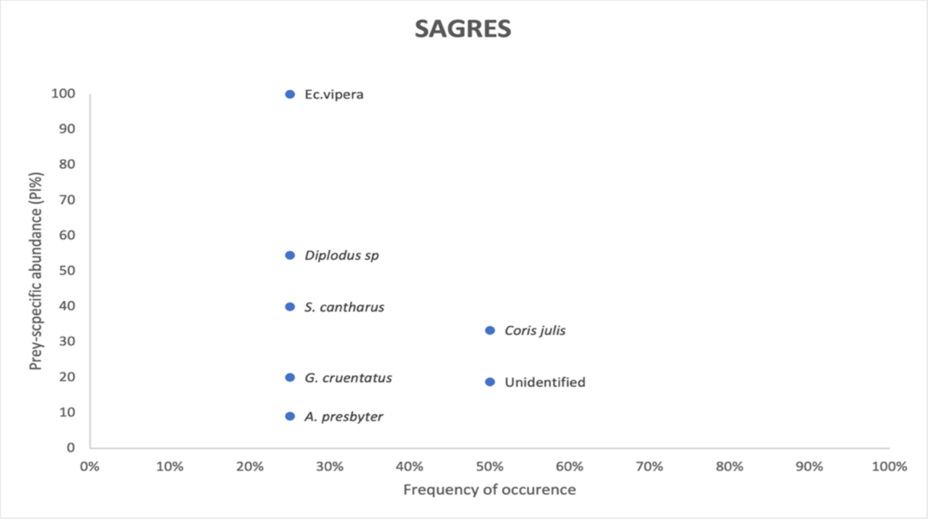


Figure 6:Amundsen diagram for prey species found on pellets from shags in Sagres.

As for feeding strategy, most prey items ingested by European shags are represented in the lower part of the graphs, suggesting a generalist feeding behavior. Surprisingly, a plastic in a pellet was recorded (see Figure 7). It seemed to belong to fishing material, which shows how vulnerable birds can be to one of the most emerging and worrying environmental problems nowadays.

Figure 7:Material similar to nylon found in one sample from Berlengas.

### *Prey species diversity*

Shannon-Wiener diversity index ranged between 1.31 (at Sagres) and 2.97 (at Berlengas), with mean diversity value of 1.73. The greatest diversity value for Berlengas can be understand looking at the presence of exclusive species such as Pouting *Trisopterus luscus,* European sprat *Spattrus spattrus,* Cuckoo wrasse *Labrus mixtus,* Brown wrasse *Labrus merula,* Ballan wrasse *Labrus bergylta,* genera *Symphodus,* and Picarel *Spicara smaris.* The diet of shags from Berlengas and Arrábida were highly diverse, whereas those from Sagres were less diverse with occurrence of only few species (i.e., *Atherina presbyter*, Lesser weever *Echiichthys vipera,* red-mouthed goby *Gobius cruentatus, Coris julis, Diplodus* sp., and Black seabream *Spondyliosoma cantharus*.

### *Spatial variation on shag diet*

The spatial variation of species composition in the shag diet has not been proven. The distribution of species in MDS plot did not result in any pattern (Figure 8). However, the analysis of similarity (ANOSIM) showed dissimilarity in diet composition of shag among locations, however with no significant difference (R-statistical= 0.78; p=0.07). Despite not being statistically significant, the R value = 0.78 can suggest a possible spatial variation.

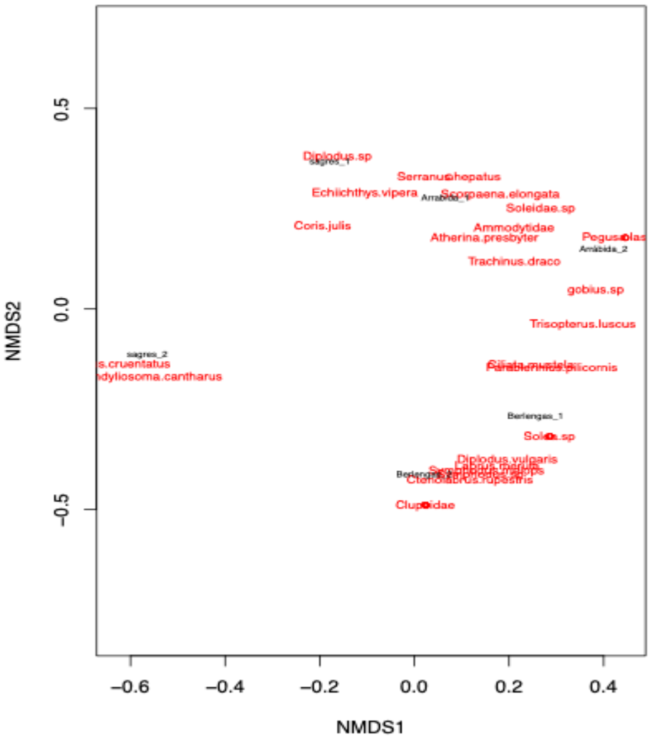


Figure 8:Non-metric Multidimensional scaling ordination plot performace on the prey items found in European shag pellets at three MPAs of Portugal: Berlengas, Arrábida and Sagres (stress= 0.074)

According to SIMPER results, Ammodytidae was a significant element that contributes for highly dissimilarity between Sagres and Arrábida ($p < 0.05$; see Table 5). *Ctenolabrus rupestris* showed as the prey contributing for dissimilarity between Sagres and Berlengas ($p < 0.05$). No significant dissimilarities were recorded between Berlengas and Arrábida, though ($p = 0.13$).

Table 5:Average dissimilarity and discriminating fish species in each station using SIMPER analysis.

| SIMPER ANALYSIS | | | | |
|---|---|---|---|---|
| Locations | Average dissimilarity (%) | Most discriminating species | Contribution (%) | p-value |
| Sagres vs. Arrábida | 97 | Ammodytidae | 60 | < 0.05 |
| Sagres vs. Berlengas | 90 | *Ctenolabrus rupestris* | 4 | < 0.05 |
| Arrábida vs. Berlengas | 77 | Ammodytidae | 40 | 0.13 |

*DNA barcoding*

DNA barcoding of the 12S ribosomal RNA gene produced fragments in teleost fish of approximately 180 base pairs. DNA sequences were submitted to GenBank and BLAST comparisons of the sequences to the species level. We confirmed the presence of *Coris julis* in the diet of European shag in Arrábida and Sagres. Due to not using blocking primers, eight of ten of those sequences amplified *Phalacrocorax* itself.

## Discussion

This is the first study describing the diet composition of European shag in Arrábida and in the southwesternmost point of Portugal, Sagres. Our study characterised the shag diet on the Portuguese coast as opportunistic / generalist, with a wide range of species from demersal to pelagic, focusing on *Atherina presbyter,* Ammodytidae family, and *Coris julis*. This behavior was also registered by Barret (1991), as well as its spatial variation in diet composition, which was directly related to the geographic variation in prey availability. The diversity of target species reflects the flexibility of the shags in feeding strategies, prospecting, and exploring various substrates (Watanuki et al., 2008; Grémillet et al., 1996).
There is no easy and accurate way to assess the diet of seabirds in general, as well as for shags (Carss et al., 1997; Harris & Wanless., 1993; Duffy & Jackson., 1986). Because of this, a holistic approach consisting of different types of identification methods (e.g., morphology; barcoding, isotopes, etc.) is important for a detailed description (Lorentsen et al., 2004).

Previous studies had already recorded a preference for consumption of the Ammodytidae family in other populations in the Atlantic, mainly during the reproductive season (Nascimento et al 2021; Hillersøy and Lorentsen, 2012; Swann et al., 2008; Harris and Wanless, 1993; Barrett et al., 1990). This preference can be a result of greater availability of sand eels in summer as they come out of the sand, dig up and form schools in the water column (Watanuki et al., 2008; Winslade 1974), but can also be due to preference by the birds to consume a more energetic value food in the reproductive and nesting period. However, there is insufficient information about fish communities available, especially about sand eels (Ammodtytidae family) and *Atherina presbyter* because these species are not captured by trawl nets used in most research samplings. Our prey composition results were -for the most part- similar to the results of Nascimento (2021), except for *Boops boops*, *Trachurus trachurus*, for example, which were not present in our analyses. Our results of wide plasticity of the diet - in relation to the consumption of species and feeding habitats - corroborate with previous studies carried out both in the North Atlantic and in the Mediterranean populations (Velando & Freire 1999; Barret 1991).

In addition to seasonal variation, studies showed differences in species composition in the European shag diet in different areas (Howells et al., 2018; Michelot et al., 2017; Cosolo et al., 2011; Velando and Freire, 1999). Although our study did not have a statistically significant result, it suggested that there are variations on diet depending on the geographic area and local environmental characteristics, as suggested by Velando &Freire (1999). Due to the low number of pellets collected, we believe that the abundance and diversity of species in the diet in Sagres was underestimated. Compared with the other two shag populations in this study (i.e, Arrábida and Berlengas), the shag population in Sagres is small, composed of 5 breeding couples. There is no previous published information about their diet when compared with Berlengas and Arrábida. Despite being a pilot experiment for identification, molecular analysis through barcoding identified *Coris julis* in pellets from Sagres. The use of an additional non-invasive method, such as barcoding, was demonstrated to be able to provide detailed information on the identification of prey that may not have been reached by morphological analysis and reduces identification errors. However, our study showed difficulties in amplifying the DNA of the species due to (i) not using block primers or (ii) already very deteriorated pellet. Although not detailed and statistically significant, it is the first insight that the diet composition of birds located further south in Portugal may tend to be composed of species with reef-associated habits, especially labrids, which corroborates to the increase in the consumption of labrids already recorded by Velando & Freire (1999) in populations in Mediterranean. Morat el al (2014) had showed that the diet found in Mediterranean subspecie *Phalacrocorax aristotelis desmarestii* was composed by 25 species, having *Coris julis* as a well-represented prey.

Berlengas was the most diverse in terms of species when compared to the other places, which can be explained by the presence of marine currents from deep waters (i.e., upwelling) that contribute to the Berlengas region having high productivity, marine habitats, and species diversity (Queiroga et al., 2008, 2007). Other explanation that reinforces the highest diversity in diets of shags in Berlengas comes from the fact that rocky habitat tends to be positively correlated with wide range of prey and feeding success (Watanuki et al., 2008). Our results differed from others carried out in Berlengas, where the Ammodytidae family was dominant (Nascimento et al., 2021), while in ours there was an expressive contribution of pelagic species (i.e., *Atherina presbyter*). However, it should be noted that it was not possible to identify the species of sand eels present and its otoliths were very small which may have underestimated the total representation of the Ammodytidae family. We infer that this predominance of *A. presbyter* may be associated with their high availability since – in summer- they reproduce. These variations in proportions can be linked to temporal fluctuations of fish stock or prey availability, which was observed for Gadidae and Ammodytidae in Norwergian waters (Barett et al., 1990). Regarding long interannual fluctuations, Howells et al (2018) had registered a dramatic reduced contribution of Ammodytidae over the past few decades in Scotland, while Gadidae and Gobiidae increased. As the location with the highest population density of shag in Portugal, Berlengas has been monitored and studied (Nascimento 2021; Oliveira et al., 2020; Oliveira et al., 2019) for the last years. The reduced presence of sand eels in shag diet in Berlengas turned out in this study may reflect the reproductive behavior of birds, since there are records of adults - selectively - feeding their chicks with high-energy prey species, such as sand eel and leaving to themselves other less-energetic preys (Lilliedahl & Solmundsson., 2006; Harris & Wanless 1991, 1993).

The advantage of marine vegetation as habitat for prey has already been recorded by Lorentsen et al. (2004) for the Great cormorant, which fed on juvenile gadoids that sheltered in kelp forests in Norwegian waters. Seafloor exploration behavior was evident in this study, with over 60% of fish consumed being demersal and associated with reefs. The main site in Arrabida where European shags could be seen was above the kelp forests. Arrábida's marine forests of macroalgae and seagrass are likely to play an important ecological role for several species, serving as an area of feeding, reproduction, and refuge - which is expected to be an environment rich in species diversity and a possible source for the European shag. The low diversity and the predominance of demersal species, mainly Ammodytidae, in the diet of these birds, highlights the availability of sand eels, once the pellets analysed in this study were from early summer and the sand eels typically feed in the water column in the morning and spend all night buried in the sand (Wislade 1974). Another factor may be associated with the origin of the pellets. Previous studies carried out on the Ciés Islands have pointed to the differences in diet composition between adults and chicks. During breeding season, for instance, adults prefer to feed their chicks with “high energy prey “such as sand eels or sardines. The pellets from adults were more diverse, with the chicks being predominantly composed of sand eels, implying that the parents feed on prey different from the chicks (Velando & Freire, 1999). The presence of families such as Labridae and Scorpaenidae is explained by the type of habitat structure present in Arrábida: mostly rocky reef (Gonçalves et al.,2002). These groups of fish live associated with an environment of structural complexity, composed of rocks of different sizes (Nascimento 2018).

An opportunist behavior had already been registered in studies (Barret 1991), as well as the occurrence of other seabirds taking advantage of commercial fishing to be able to feed (i.e., eating the waste from industrial fisheries) (Tasker et al., 2000). The interaction between fish and seabirds goes beyond the direct opportunity to obtain food, the trophic overlap of interest. The elimination of large fish and predators, species of high commercial value by overfishing, is an indirect effect on the feeding ecology of seabirds. In the North Sea, for example, fishing target at large predators or prey competitors, such as *Gadus morhua* cod, influenced the stock of sandeels (family Ammodytidae), benefiting several species of seabirds, as European shag, that feed on them (Furness, 2002). Despite seeming to be a great strategy, this interaction between fishing boats and European shags may influence their feeding ecology as well as is one of the threats with the highest mortality rate for birds, due to bycatch (Oliveira et al., 2020, 2019). In South Africa, for example, the population of Cape cormorant *Phalacrocorax capensis* has already had its population of breeding pairs reduced by more than 50% (Crawford et al., 2014; Okes et al., 2009; Pichegru et al., 2009).

The recording of a material, perhaps from fishing, in a single pellet in our study, makes us question and turn on the red light on the interaction between the shags and local fishing in Portugal. Despite being just a record, continuous and detailed studies are necessary to analyse whether there is an increase in the potential effect of fishing over the shags. In a short-term perspective, changes in feeding behavior (e.g., replacements in prey preference, use of fishery to detect greater prey abundances) and reproductive success of birds are expected due to seasonal variations in prey availability. In the long term, changes in prey distribution and low recruitment of sand eels and small pelagic fish due to overfishing and environmental changes tend to drastically reduce populations around the globe (Wanless et al., 2018; Cook et al., 2014; Frederiksen et al. al., 2004).

## Conclusion

Our study suggests an opportunistic behavior arising from a possible spatial variation in prey availability for the European shag along three marine preservation areas. Our study is the first to describe the diet composition of the European shag in Arrábida Natural Park and provide preliminary information about the diet in southern Portugal, in the Southwest Alentejo and Costa Vicentina Natural Park. The higher contribution of the Ammodytidae family and *Atherina presbyter* in Arrábida and Berlengas coincides with previous records in North Atlantic and Mediterranean populations. Finally, there is a possible pattern of similarity between the diet of the populations of southern Portugal, Sagres, and the resident Mediterranean subspecies. To confirm this, it is essential that more studies are carried out continuously throughout the years-, and to verify possible interannual changes in the stock of prey and its consequences for the dynamic of shag populations. Knowing that identification through otoliths is susceptible to errors due to their erosive processes and their reduced size, a holistic approach considering barcoding seems to be a strategy to try to overcome this obstacle. Other factors to be analysed in future studies would be prey availability, environment, and effects of the species physiological state (e.g., breeding, age effects). This study provided novel information that raises several novel questions and hypotheses for future studies.

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# Appendix

## Appendix 1

Table 5: Equations for Length-weight relationship regression based on otolith's length, where TL = fish total length; OL = otolith length; TW= fish total weight.

| Family/Species | Length Equation(cm) | Biomass Equation(g) | Reference |
|---|---|---|---|
| **Ammodytidae** | | | |
| Ammodytidae | TL = (OL - 0.510)/0,141 | TW = 0,00660*(TL^2,697) | (Wilson et al., 2017; Froese & Pauly, 2018) |
| **Blenniidae** | | | |
| *Parablennius sp* | TL=(41,85*(OL^1,19))/10 | TW= 0,01223*(TL^2,7693) | (Giménez et al., 2016; Valle et al., 2002) |
| *Parablennius pilicornis* | TL=(44,31*(OL^1,02))/10 | TW= 0,01223*(TL^2,7693) | (Giménez et al., 2016; Valle et al., 2002) |
| **Atherinidae** | | | |
| *Atherina boyeri* | TL=(52,11*(OL^0,38))/10 | TW = 0,0033*(TL^3,35) | (Giménez et al., 2016; Pombo et al., 2005) |
| *Atherina presbyter* | TL=(52,11*(OL^0,38)/10 | TW(aveiro) =0,0055*(TL^3,09) / (algarve)=0,0064*(TL^3,05) | (Giménez et al., 2016); Pombo et al., 2005; Viega et al., 2009) |

| Family/Species | Length Equation(cm) | Biomass Equation(g) | Reference |
|---|---|---|---|
| **Centrocanthidae** | | | |
| *Spicara smaris* | TL=(45,43*(OL^0,73))/10 | TW= 0,046*(TL^2,538) | (Santos et al.,2002) |
| **Gobiidae** | | | |
| *Gobius cruentatus* | TL=(22,48*(OL^1,18))/10 | TW= 0,00807*(TL^3,1373) | (Giménez et al., 2016; Santos et al., 2002) |
| *Gobius sp* | TL=(29,06*(OL^0,94))/10 | TW= 0,00807*(TL^3,1373) | (Giménez et al., 2016; Santos et al., 2002) |
| **Gadidae** | | | |
| *Trisopterus luscus* | TL = (33,73*OL - 94,54)/10 | TW= 0,00000433*((TL*10)^3,19) | (Granadeiro and Silva, 2000) |
| *Trisopterus minutus* | TL=(OL-1,718)/0,362 | TW=0,00540*(TL^3,179) | (Wilson et al., 2017; Mendes et al.,2004) |
| **Labridae** | | | |
| *Coris julis* | TL=(31,63*(OL^1,56))/10 | TW=0,00001409*(TL*10)^2,946 | (Giménez et al., 2016; Gonçalves et al., 1997) |
| *Ctenolabrus rupestris* | TL=(31,63*(OL^1,56))/10 | TW=0,00001409*(TL*10)^2,946 | (Giménez et al., 2016; Gonçalves et al., 1997) |
| *Labrus bergylta* | TL=(67,97*OL-31,24)/10 | TW=0,0141*(TL^3,039) | (Härkönen, 1986; Morato et al., 2001) |
| *Labrus merula* | TL=(57,52*(OL^1,1)/10 | TW=0,0076*(TL^3,1862) | (Giménez et al., 2016; Morey et al. 2003) |
| *Labrus mixtus* | TL = (52,12*OL-4,76)/10 | TW = 0,0050*(TL^3,254) | (Härkönen, 1986; Mendes et al. 2004) |
| *Symphodus rossali* | TL=4,3583*(OL^0,9869) | TW = 0,0155*(TL^3,017) | (Altin and Ayyildiz 2017; Santos et al., 2002) |

| Family/Species | Length Equation(cm) | Biomass Equation(g) | Reference |
|---|---|---|---|
| *Symphodus cinereus* | TL=(48,27*(OL^0,94))/10 | TW = 0,0075*(TL^3,2514) | (Giménez et al., 2016; Morey et al. 2003) |
| *Symphodus mediterraneus* | TL=(31,25*(OL^1,47))/10 | TW=0,02121*(TL^2,8798) | (Giménez et al., 2016; Valle et al.,2002) |
| *Symphodus melops* | TL=(48,27*(OL^0,94))/10 | TW = 0,01120*(TL^3,17) | (Altin and Ayyildiz, 2017; Froese and Pauly, 2018) |
| *Symphodus sp* | TL=(31,25*(OL^1,47))/10 | TW= 0,0155*(TL^3,017) | (Giménez et al., 2016; Santos et al., 2002) |
| **Phycidae** | | | |
| *Phycis sp* | TL=(1,89*(OL^2,01))/10 | TW= 0,0064*(TL^3,149) | (Mendes et al., 2004) |
| **Trachinidae** | | | |
| *Trachinus draco* | TL=(15,66*(OL^1,25)/10 | TW= 0,0042*(TL^3,119) | (Giménez et al., 2016; Santos et al., 2002) |
| *Echiichtys vipera* | TL= 16,43 *(OL^1,22)/10 | TW=0,01050*(TL^3,05) | (Giménez et al., 2016; Viegas et al., 2009) |
| **Scorpaenidae** | | | |
| *Scorpaena elongata* | TL=(12,6*(OL^1,2))/10 | TW= 0,0148*(TL^3,017) | (Giménez et al., 2016; Meiners-Mandujano et al., 2018) |
| **Serranidae** | | | |
| *Serranus hepatus* | TL=(16,99*(OL^1,15))/10 | TW= 0,0110*(TL^3,190) | (Giménez et al., 2016; Santos et al., 2002) |
| **Soleidae** | | | |
| *Microchirus sp* | TL=(44,56*(OL^0,97))/10 | TW= 0,0026*(TL^3,285) | (Giménez et al., 2016; Mendes et al. 2004) |
| *Pegusa lascaris* | TL=(58,12*(OL^1,11))/10 | TW= 0,0070*(TL^3,13) | (Giménez et al., 2016; Mendes et al. 2004) |

| Family/Species | Length Equation(cm) | Biomass Equation(g) | Reference |
|---|---|---|---|
| *Solea sp* | TL=(53,18*(OL^1,17) )/10 | TW= 0,0071*(TL^3,092) | (Giménez et al., 2016; Mendes et al. 2004) |
| **Sparidae** | | | |
| *Diplodus sp* | TL=(20,04*(OL^1,27) )/10 | TW= 0,0132*(TL^3,096) | (Giménez et al., 2016; Santos et al., 2002) |
| *Diplodus annularis* | TL=(20,04*(OL^1,27) )/10 | TW= 0,0132*(TL^3,096) | (Giménez et al., 2016; Santos et al., 2002) |
| *Diplodus vulgaris* | TL=(17,18*(OL^1,33) )/10 | TW=0,00001756*(TL*10)^2,987 | (Giménez et al., 2016; Gonçalves et al., 1997) |
| *Pagrus pagrus (NorthAtlantic)* | TL=(25,83*(OL^1,01) )/10 | TW=0,00003501*((TL*10)^2,866) | (Giménez et al., 2016; Gonçalves et al., 1997) |
| *Pagrus pagrus (Mediterranean)* | TL=(18,57*(OL^1,17) )/10 | TW=0,00003501*((TL*10)^2,866) | (Giménez et al., 2016; Gonçalves et al., 1997) |
| *Spondyliosoma cantharus* | TL=(20,21*(OL^1,16) )/10 | TW= 0,0158*(TL^2,9957) | Giménez et al., 2016; Morey et al. 2003) |

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**Appendix 2:** Table of species categorised into different ecological preferences.

| Family | Species | Ecological |
|---|---|---|
| **Ammodytidae** | | Demersal |
| **Atherinidae** | | |
| | *Atherina presbyter* | Pelagic |
| | *Atherina boyeri* | Pelagic |
| **Bleniidae** | | |
| | *Parablennius sp* | Demersal |
| | *Parablennius pilicornis* | Demersal |
| **Bothidae** | | Demersal |
| **Centrocanthidae** | | |
| | *Spicara smaris* | Pelagic |
| **Clupeidae** | | |
| | *Sprattus sprattus* | Pelagic |
| **Gadidae** | | |
| | *Trisopterus luscus* | Benthopelagic |
| | *Trisopterus minutus* | Benthopelagic |
| **Gobiidae** | | |
| | *Gobius sp* | Demersal |
| | *Gobius cruentatus* | Demersal |
| **Labridae** | | |
| | *Coris julis* | Reef-associated |
| | *Symphodus sp* | Demersal |
| | *Symphodus rossali* | Reef-associated |
| | *Symphodus mediterraneus* | Demersal |
| | *Symphodus cinereus* | Demersal |
| | *Symphodus melops* | Reef-associated |
| | *Labrus bergylta* | Reef-associated |
| | *Labrus merula* | Reef-associated |
| | *Labrus mixtus* | Reef-associated |
| | *Labrus sp* | Reef-associated |
| | *Acantholabrus palloni* | Reef-associated |
| | *Ctenolabrus rupestris* | Reef-associated |
| **Lotidae** | | |
| | *Ciliata mustela* | Demersal |
| **Physicidae** | | |

|  |  |  |
|---|---|---|
|  | *Phycis sp* | Benthopelagic |
| **Scorpaenidae** |  |  |
|  | *Scorpaena elongata* | Demersal |
| **Serranidae** |  |  |
|  | *Serranus hepatus* | Demersal |
|  | *Mycteroperca sp* | Demersal |
| **Soleidae** |  |  |
|  | *Microchirus sp* | Demersal |
|  | *Solea sp* | Demersal |
|  | *Pegusa lascari* | Demersal |
| **Sparidae** |  |  |
|  | *Diplodus annularis* | Benthopelagic |
|  | *Diplodus sp* | Benthopelagic |
|  | *Diplodus vulgaris* | Benthopelagic |
|  | *Pagrus pagrus* | Benthopelagic |
|  | *Spondyliosoma cantharus* | Benthopelagic |
| **Trachinidae** |  |  |
|  | *Trachinus draco* | Demersal |
|  | *Echiichthys vipera* | Demersal |