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# Feeding ecology of an aerial Mediterranean bird: the Pallid Swift

**Sandra Eduarda Rocha Fernandes**

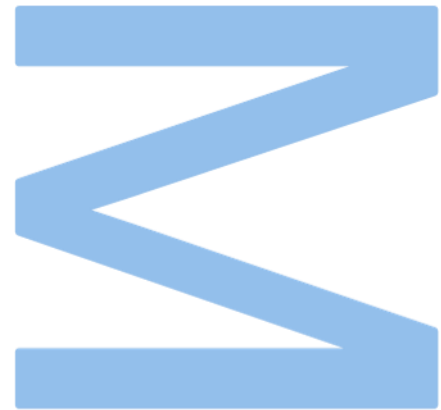
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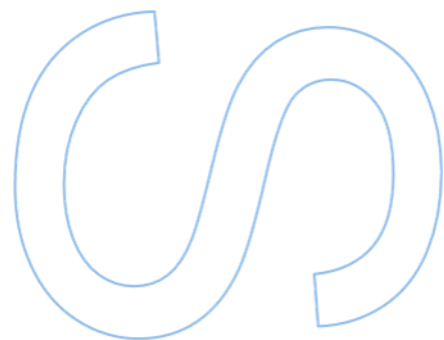
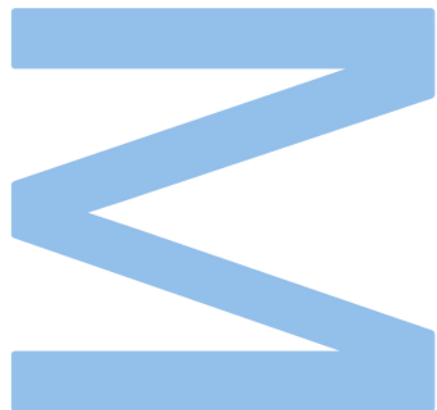
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# Sworn Statement

I, Sandra Eduarda Rocha Fernandes, enrolled in the Master Degree Biodiversity, Genetics and Evolution at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this dissertation reflects perspectives, research work and my own interpretations at the time of its submission.

By submitting this dissertation, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

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Sandra Eduarda Rocha Fernandes

Vila Nova de Famalicão, 2022

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The conclusion of such a challenging journey full of new experiences and realities was not only due to a year of hard work, but also to a set of circumstances, some happier than others, that shaped my personal, academic and professional path, making me reach the point where I am today.

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## Resumo

Os andorinhões-pálidos, tal como outras espécies de andorinhões, são aves extremamente bem-adaptadas a um estilo de vida aéreo, exibindo adaptações únicas que lhes permitem voar quase continuamente. Estes voos contínuos, por vezes a elevadas altitudes, e a sua restrita área de distribuição dificultam o estudo detalhado da ecologia alimentar desta espécie. A dieta destas aves era até recentemente estudada a partir da identificação visual das presas presentes em fezes, bolos alimentares entregues às crias e conteúdo estomacal. Estas técnicas, que não permitem identificações taxonómicas de elevada resolução, têm sido substituídas por técnicas moleculares inovadoras, como o DNA metabarcoding. Esta abordagem molecular permite identificar espécies com alto rendimento, reduz o enviesamento que as identificações morfológicas geram, apesar dos seus próprios artefactos, e evita que seja necessário conhecer previamente a presa a ser identificada.

Esta tese teve como objetivo estudar a dieta do andorinhão-pálido durante a sua época de reprodução, avaliando e caracterizando as variações temporais ao longo deste período e analisando as potenciais diferenças entre a dieta de machos e fêmeas, relativamente à riqueza e composição da dieta e ao tamanho das presas ingeridas. Para tal, foi usado DNA metabarcoding para analisar amostras fecais recolhidas mensalmente ao longo da época de reprodução de 2021 numa colónia de andorinhão-pálido em Vila Nova de Famalicão. Utilizou-se o conjunto de primers fwh2 da região COI para identificar os artrópodes presentes nos excrementos e o conjunto de primers P2P8 da região CHD para sexar as aves.

Das presas analisadas, foram identificadas 74 famílias pertencentes a 16 ordens de artrópodes, sendo as ordens Hymenoptera, Hemiptera, Coleoptera, Diptera e Psocodea as mais consumidas. Foram também encontradas variações temporais na riqueza e composição da dieta e no tamanho dos artrópodes predados. Em junho, a dieta foi composta principalmente por formigas (Hymenoptera: Formicidae) e foi neste mês em que se registou um dos valores mais baixos de riqueza específica e o segundo valor mais elevado no tamanho das presas capturadas. Em julho, pelo contrário, registou-se os valores de riqueza específica mais elevados, com os andorinhões-pálidos a alimentarem-se das presas mais pequenas. Em agosto e setembro, as dietas amostradas apresentaram uma maior incidência de Hemiptera e Coleoptera, sendo que setembro apresentou um dos valores mais baixos de riqueza específica, a par de junho, e os valores mais elevados no tamanho das presas consumidas. Por fim, a dieta consumida em outubro foi predominantemente composta por Diptera. Relativamente à

dieta dos machos e das fêmeas, embora não se tenham encontrado diferenças entre a dieta dos dois sexos na sua riqueza e composição, foram observadas diferenças no tamanho dos artrópodes consumidos pelos diferentes sexos, com os machos a alimentarem-se de presas maiores.

A variação da dieta do andorinhão-pálido durante a época de reprodução resultou provavelmente de acumulações de artrópodes, que foram oportunisticamente predados pelos andorinhões. Embora não se tenham encontrado diferenças significativas na riqueza e composição da dieta entre os sexos, o facto de os machos consumirem presas maiores pode sugerir a existência de segregação alimentar sexual neste grupo de aves. Este estudo encontrou ainda várias espécies de pragas na dieta dos andorinhões, sugerindo assim o seu importante papel ecológico na regulação de artrópodes nocivos. Finalmente, estes resultados mostram como as espécies parecem moldar o seu comportamento alimentar, provavelmente em resposta a mudanças na disponibilidade alimentar. Quando as potenciais presas ótimas estavam disponíveis, os andorinhões-pálidos aparentemente exploraram esses invertebrados, reduzindo o seu nicho alimentar. Contudo, quando as condições ambientais se deterioraram e as presas ótimas deixaram de estar disponíveis, estes começaram a alimentar-se de presas subótimas, alargando assim o seu nicho alimentar, o que resultou numa dieta mais variada.

**Palavras-chave:** Alimentação aérea, *Apus pallidus*, DNA metabarcoding, Ecologia alimentar, Variação temporal da dieta.



# Abstract

Pallid Swifts, as other swifts, are birds extremely adapted to an aerial lifestyle, showing unique adaptations that allow them to fly almost continuously. This non-stop high-altitude flight along with their restricted distribution breeding area hampers the detailed study of this species' feeding ecology. The diet of these highly aerial birds was until recently studied from the visual identification of prey items present in faeces, food boluses delivered to nestlings or stomach contents. These techniques, that fail to produce high taxonomic resolution identifications, have been replaced by avant-garde molecular techniques, such as DNA metabarcoding. This molecular approach allows high-throughput species identification, reduces the bias generated by morphological identifications, despite its own artefacts, and avoids the requirement of prior knowledge of the identified prey.

This thesis aimed to study the diet of the Pallid Swift during its breeding season, evaluating and characterising temporal variations throughout this period and assessing potential differences between the diets of males and females, with regard to diet richness, composition and prey size. For that, DNA metabarcoding was used to analyse faecal samples collected monthly during the 2021 breeding season, from a Pallid Swift colony in Vila Nova de Famalicão. The primer set fw2 COI was used for the identification of arthropods present in the faecal droppings and P2P8 CHD primer set for birds' sexing.

From the prey items retrieved, 74 families were identified belonging to 16 arthropod orders, with Hymenoptera, Hemiptera, Coleoptera, Diptera and Psocodea being the most consumed prey orders. There were temporal variations in diet richness, composition and prey size. The diet in June was mainly composed of ants (Hymenoptera: Formicidae), and this month registered one of the lowest values of prey richness and the second highest of prey size. In July, by contrast, prey richness values were the highest, with Pallid Swifts feeding on the smallest prey items. In August and September, the sampled diets showed a higher incidence of Hemiptera and Coleoptera, with September showing one of the lowest values of prey richness, and the highest values of prey size. Finally, the diet consumed by these swifts in October was predominantly composed of Diptera. Regarding the diet of males and females, although no differences were found between the diet of males and females in composition and richness, there were differences in the size of arthropods preyed by the different sexes, with males feeding, on average, upon larger arthropods.

The temporal variation in Pallid Swifts' diet during the breeding season likely resulted from spatiotemporally accumulations of aerial prey, which were opportunistically

predated by swifts. Although no significant differences were found in diet richness and composition between sexes, the fact that males consume larger prey may suggest the existence of sexual dietary segregation in this group of birds. In addition, this study has recorded the consumption of multiple pest species by swifts, thus suggesting their important ecological role in regulating harmful arthropods. At last, these results show how species appear to shape their feeding behaviour, likely in response to changes in food availability. When potential optimal prey were available, the Pallid Swifts apparently exploited these invertebrates, reducing their food niche. However, when environmental conditions deteriorated and the optimal prey were no longer available, Pallid Swifts started to feed on sub-optimal prey, thus widening their food niche, which resulted in a more diverse diet.

**Keywords:** Aerial foraging, *Apus pallidus*, Dietary temporal variation, DNA metabarcoding, Feeding ecology.

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## List of Abbreviations

BOLD	BARCODE OF LIFE DATA SYSTEMS
CEMPA	CENTRO DE ESTUDOS DE MIGRAÇÕES E PROTEÇÃO DE AVES
CHD	CHROMODOMAIN HELICASE DNA-BINDING
DNA	DEOXYRIBONUCLEIC ACID
EURING	EUROPEAN UNION FOR BIRD RINGING
GLM	GENERALIZED LINEAR MODEL
GLMM	GENERALIZED LINEAR MIXED MODEL
IUCN	INTERNATIONAL UNION FOR CONSERVATION OF NATURE
MJOLNIR	METABARCODING JOINING OBITOOLS & LINKAGE NETWORKS IN R
OTU	OPERATIONAL TAXONOMIC UNIT
PCR	POLYMERASE CHAIN REACTION
PERMANOVA	PERMUTATIONAL MULTIVARIATE ANALYSIS OF VARIANCE



# 1. Introduction

## 1.1 Swifts (Apodiformes: Apodidae)

Swifts (Apodiformes: Apodidae) are insectivorous birds extremely well-adapted to an aerial lifestyle, displaying unique morphological, behavioral, and physiological adaptations to this distinct way of living. High aspect ratio wings, provided by short arm-wings and very long hand wings, allow a leading-edge vortex which significantly increases lift on the wings, resulting in more agile and aerodynamic performances (Ben-Gida et al., 2020; Henningsson & Hedenström, 2011; Lentink et al., 2007; Videler et al., 2004). Stream-lined compact body suitable for both high-speed and energy-efficient flights (Henningsson et al., 2008; Lentink et al., 2007; Videler et al., 2004). Energy-saving flap-gliding flight, an intermittent flight mode of flapping movements intermingled with gliding phases that save them much-needed energy, allowing them to roost during flight (Muijres et al., 2012; Sachs, 2017). High hematocrit and hemoglobin concentration fulfill the oxygen requirements, in these birds with high metabolic rates (Palomeque et al., 1980). Thermoregulation during flight, through leg trailing and panting, promotes effective heat dissipation that is crucial for birds that spend most of their time in flight and are therefore particularly susceptible to solar radiation (Neumann & Neumann, 2016), are some of these adaptations.

The above mentioned traits allow these highly mobile and fast birds, that can ascend several hundred meters high (Gustafson et al., 1977; Meier et al., 2018), to fly almost continuously (up to 10 months) during the nonbreeding period (Hedenström et al., 2019; Hedenström et al., 2016; Liechti et al., 2013; Lockley, 1969). During the breeding period, collection of material to build nests, sleeping, mating, drinking and invertebrates' foraging occurs during flight (Brunton, 2018; Hedenström et al., 2016; Henningsson et al., 2009; Orłowski & Karg, 2013; Rattenborg, 2006). Invertebrates are one of the most abundant and diverse food resource, being particularly exploited by avian predators, among which insectivorous aerial birds, such as swifts, stand out as they are one of the few that can hunt flying invertebrates on the wing (Nebel et al., 2010; Nyffeler et al., 2018). Studying the ecology of swifts and in particular their diet is highly difficult, not only due to their aerial lifestyle but also because most nesting places are hard to access and some species are very difficult to distinguish since they have very similar body silhouette and plumages (Marin-Aspillaga, 1998).

### 1.1.1 Pallid Swift (*Apus pallidus*)

In the Western Palaearctic region (Cramp, 1985), there are six breeding species of swifts (del Hoyo, 2020) (Figure 1), of which Pallid Swift (*Apus pallidus*) is one of the most abundant. Pallid Swifts are medium-sized swifts, with a body length ranging from 16 to 18 cm, wingspan between 39 to 44 cm (Svensson et al., 2010), and 30 to 46 g in body weight (Cramp, 1985). The breeding range of this species is restricted to the Mediterranean region, Asia Minor, and adjoining areas (Chantler & Driessens, 2000; Keller et al., 2020), with some resident populations in Niger, Chad, and Egypt (BirdLife International, 2022b) (Figure 2). The Pallid Swift's breeding season is particularly long, often including a second clutch laid in summer, leading to a reproductive period that extends into autumn, lasting up to 8 months (Antonov & Atanasova, 2001; Boano & Cucco, 1989; Cramp, 1985). Usually, this species nests in either natural landscapes, using caves and cliffs, or in urban areas, using preferentially cavities under the eaves or ceilings of old and tall buildings (Antonov & Atanasova, 2002; Cucco & Malacarne, 1987; Thibault et al., 1987). Although the migration of this long-distance migrant is not well studied, the species is known to cover vast areas during its nonbreeding period and to winter in Western Africa (Chantler & Driessens, 2000; Finlayson et al., 2021; Hedenström et al., 2019; Kearsley et al., 2022).

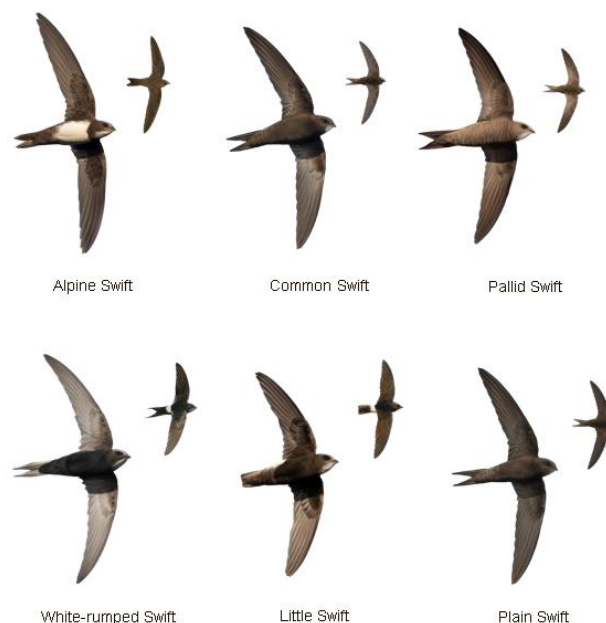


Figure 1 – Western Palaearctic region's swifts breeding species: Alpine Swift (*Thachymarpis melba*), Common Swift (*Apus apus*), Pallid Swift (*Apus pallidus*), White-rumped Swift (*Apus caffer*), Little Swift (*Apus affinis*) and Plain Swift (*Apus unicolor*). Adapted from andorin (2022).

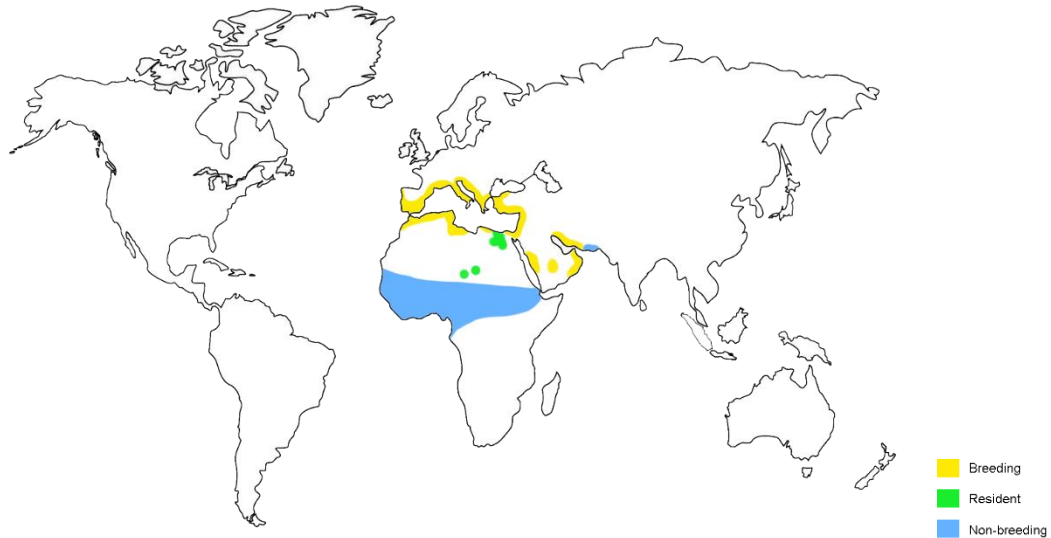


Figure 2 – Pallid Swift's geographical distribution, based on the data available at Keller et al. (2020) and at BirdLife International (2019).

The Pallid Swift is phenotypically very similar to the Common Swift (*Apus apus*) (Pellegrino et al., 2017), with evidence of gene flow between the two species (Cibois et al., 2022). Despite the need of a prolonged observation under good lighting to obtain a proper identification in the field (Malacarne et al., 1989), these species display very distinct biological traits (Pellegrino et al., 2017). Common Swifts, unlike most Pallid Swifts, only lay one clutch (Sicurella et al., 2015), thus leaving breeding grounds earlier and having longer non-breeding seasons (Boano & Cucco, 1989; Cramp, 1985; Hedenström et al., 2016). Their moulting, i.e., feather replacement, period is also dissimilar: Pallid Swifts initiate moulting at breeding sites and suspend it for the autumn migration (Boano et al., 2015; Chantler & Driessens, 2000), while the majority of Common Swifts' populations moult at wintering sites (Jukema et al., 2015). Regarding distribution, Common Swifts' breeding range is far more extensive, covering Europe, north of Morocco, Algeria and Tunisia, the northern Middle East, and temperate regions of Asia (BirdLife International, 2022a), being the most abundant and widespread swift in the Western Palearctic region. Due to these differences in distribution, studies on Common Swifts are much more abundant than those on Pallid Swifts, with many aspects of the latter's ecology being poorly understood. Besides, the misidentification of flying birds, with many Pallid Swifts being mistakenly identified as Common Swifts, undermines the reliability of distribution data (Costa & Elias, 1998).

Although both species are listed as Least Concern by the International Union for Conservation of Nature (IUCN) Red List (BirdLife International, 2016, 2019), in Portugal

(Cabral et al., 2005), European Common Swift's populations are in decline, and the species is now considered Near Threatened by the European Red List of Birds (BirdLife International, 2021). On a local scale, the situation is even more alarming, with Spain's and United Kingdom's Red Lists considering this species as Vulnerable and Endangered, respectively (SEO/BirdLife, 2021; Stanbury et al., 2021). There are several causes for the swifts' decline, namely: decline in invertebrate populations as a result of land use changes and pesticides application; destruction of nests or breeding colonies, through the restoration and demolition of old buildings; development of new building techniques and materials that do not allow birds to settle and build nests; and climate change that affects broods' growth and survival (Molina, 2021). Unlike the Common Swift, the European Pallid Swift's populations are listed as Least Concern (BirdLife International, 2021; SEO/BirdLife, 2021; Stanbury et al., 2021). Despite the positivity of these assessments, these data may be biased by the smaller distribution of the Pallid Swift and frequent confusion with the Common Swift. In addition, the known geographical distribution of Pallid Swift appears to be expanding northwards, but this expansion can be a reflection of more thorough and accurate identifications in recent times rather than a properly natural expansion of this species, as suggested by Elias (2022). If these scenarios are confirmed we may be witnessing the silent decline of the Pallid Swift's breeding populations, as the threats mentioned above affect both Common and Pallid Swifts. This lack of knowledge about the Pallid Swift is particularly notorious in the Iberian Peninsula, where the species is very abundant. Thus, studying the ecology of this highly mobile and fast predator, and in particular its diet, is a challenge, but a necessary one to provide valuable insights into this species' ecology.

## 1.2 Dietary studies

Dietary studies can not only unravel species' ecology, by profiling the diversity and composition of diets (Mata et al., 2019), but can also assess temporal changes in ecosystems (Boström et al., 2012; Cristiano et al., 2018), establish management strategies for conservation purposes (Agosta, 2002; Meena et al., 2011), and inform about provided ecosystem services (Chan et al., 2021; Cristiano et al., 2018; Kamenova et al., 2018), thus offering valuable insights into the structure and function of entire ecosystems (Estes et al., 2011). Among the wide range of ecosystem services provided by birds (Whelan et al., 2015; Whelan et al., 2008), regulation services are those that stand out (Michel et al., 2020), with insectivorous birds feeding on different types of arthropod pests, disease vectors and invasive species, hence functioning as biological

control agents. Birds, by feeding on seed predators, sap feeders, and leaf herbivores reduce herbivory, thus increasing plant growth rates and enhancing crop yields (García et al., 2021; Marquis & Whelan, 1994; Sanz, 2001). Sarwar (2015) pointed out birds and other natural enemies of dengue vector mosquitoes as a potential solution to decrease the transmission of this disease, by replacing the traditional use of insecticides, which are harmful to human health and the ecosystem, with biological control methods. Insectivorous birds, namely swifts, can also feed on invasive species, as Cristiano et al. (2018) found, and thus help to restore the ecosystem's balance. The detailed and continuous study of insectivorous birds' diets can be a powerful tool in ecosystem monitoring, including early detection; and birds such as swifts can be used as airborne invertebrate bio-surveyors in disrupted ecosystems (Cristiano et al., 2018; Orłowski & Karg, 2013).

### 1.2.1 Swifts' diet

Generally, swifts feed mainly on airborne insects and aerial plankton (Lack & Owen, 1955; Liechti et al., 2013), i.e., ensembles of small organisms that are passively dispersed by air currents (Cotoras & Zumbado, 2020). Their diets have been studied using conventional analyses based on the visual examination of ingested prey, by identifying under a dissecting microscope the prey items present in faeces (Cucco et al., 1993; Gory, 2008; Waugh, 1978); stomach contents (Beebe, 1949; Brito et al., 2015; Kopij, 2000; Marín & Stiles, 1993; Rose, 1997); or in food pellets delivered to nestlings (Bigot et al., 1984; Cristiano et al., 2018; Cucco et al., 1993; Garcia-del-Rey et al., 2010; Gory, 2008; Malacarne & Cucco, 1992). These analyses have many limitations: either they are very invasive methods, such as the analysis of stomach contents that requires swifts' euthanasia; or depend on samples that are already very digested, making visual identification of the prey quite difficult and sometimes even impossible (Chung et al., 2021), particularly for soft bodied prey (Nielsen et al., 2018).

#### 1.2.1.1 Pallid Swift's diet

Some previous studies have analysed the diet of the Pallid Swift through the visual inspection of faeces and food boluses delivered to nestlings (Bigot et al., 1984; Cristiano et al., 2018; Cucco et al., 1993; Finlayson, 1979; Malacarne & Cucco, 1992; Pulcher, 1985). Malacarne and Cucco (1992) conducted a study on Pallid Swift's diet in Northwest Italy by analysing 36 food pellets delivered to nestlings. The authors found that food

boluses were predominantly composed of arthropods belonging to the orders Hemiptera, Hymenoptera and Diptera, with Coleoptera also being commonly recorded. In addition to describing the diet composition, they also studied its temporal variation, of which the highest frequency of Hymenoptera in June and Hemiptera in August and September stood out. Cristiano et al. (2018) also conducted a study on the Pallid Swift's diet in a colony in Northwest Italy, examining the birds' diet during the 2012 and 2013 breeding seasons. During the fieldwork, 26 food boluses were obtained, resulting in 5980 prey items. From the prey items, 37 families or superfamilies were identified, belonging to the Araneae, Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Odonata, and Hemiptera orders. The Hemiptera and Coleoptera were the most consumed orders, representing more than 70% of these Pallid Swifts' diet.

### 1.2.2 DNA metabarcoding

The traditional approaches for dietary studies mentioned above (visual inspection of faecal, stomach contents and food pellets), have recently been replaced by more avant-garde procedures, such as high-throughput sequencing (Chan et al., 2019) and DNA (deoxyribonucleic acid) metabarcoding (Chan et al., 2020; Chung et al., 2021). The use of DNA metabarcoding techniques to study diets relies on the presence of DNA of taxonomically unknown prey in the faeces. A small and standard region of this DNA, i.e., DNA barcode, is then mass-amplified and species' identification is obtained through comparison against a library of known and taxonomically identified DNA barcodes (Hebert et al., 2003). This technique uses high-throughput sequencing methods to simultaneously sequence PCR (Polymerase Chain Reaction) amplified DNA of all samples in a single mixture, allowing a multi-species identification approach. Although this technique presents some limitations, such as errors during amplification; difficulty in obtaining suitable barcodes for certain taxonomic groups; need to analyse different taxonomic groups separately (especially problematic for generalist species) and requiring a good taxonomic reference database (Taberlet et al., 2012), the potential power for high-throughput species identification makes this technique a good tool for diet assessment. The major benefits of this method is the ability to provide high taxonomic resolution of the consumed species (Gibson et al., 2015; Jackson et al., 2014) and to enable more reliable discrimination of closely related taxa, reducing the bias that morphological identifications generate and avoiding the requirement for prior knowledge of the identified prey (de Sousa et al., 2019).

This technique has already been used over the last decade to assess a wide range of feeding regimes, namely carnivorous (de Jesus, 2021; Hacker et al., 2021; Nota et al., 2019; Shi et al., 2021), molluscivorous (Fraser, 2020; Huang et al., 2021), insectivorous (Aizpurua et al., 2018; Esnaola et al., 2018; McClenaghan et al., 2019; Rytönen et al., 2019), frugivorous (Volpe et al., 2022), nectarivorous (Moran et al., 2019; Spence et al., 2022) and omnivorous species (Anderson et al., 2018; da Silva et al., 2019; Stenhouse et al., 2021; Tercel et al., 2022). The high applicability of this technique, which allows the study of such a wide range of diets, enables the study of the feeding ecology of virtually any species. To date and concerning the class Aves, DNA metabarcoding has been used to study the diet of species belonging to a wide variety of bird orders, such as Galliformes (Fujii et al., 2022; Sullins et al., 2018), Caprimulgiformes (Mitchell et al., 2022), Apodiformes (Chan et al., 2019; Chung et al., 2021), Otidiformes (Cabodevilla et al., 2021; Liu et al., 2018), Pteroclitiformes (Cabodevilla et al., 2021), Gruiformes (Kataoka et al., 2022), Charadriiformes (Fraser, 2020; Gerik, 2018), Gaviiformes (Kleinschmidt et al., 2019), Sphenisciformes (Tabassum et al., 2022; Young et al., 2020), Procellariiformes (Carreiro et al., 2020; Fayet et al., 2021; Komura et al., 2018; McInnes et al., 2017; Nimz et al., 2022; Querejeta et al., 2022), Accipitriformes (Hacker et al., 2021; Nota et al., 2019), Piciformes (Stillman et al., 2022), Falconiformes (Bourbour et al., 2021), and Passeriformes (da Silva et al., 2020; McClenaghan et al., 2019; Stenhouse et al., 2021; Trevelline et al., 2018). Besides allowing the study and characterisation of the feeding ecology of a diverse range of organisms, DNA metabarcoding can also be a useful tool in the biosurveillance of invasive species (Brown et al., 2016; Montauban et al., 2021; Tercel et al., 2022). This technique enables an early and quick invasive species' detection, which allow the prompt implementation of controlling or eradication programmes, thus mitigating some of the devastating effects that these species cause in the ecosystems where they were introduced (Borrell et al., 2017; Westfall et al., 2020).

Despite its broad application and its potential to unravel swifts' ecology, only in 2019 this technique was first used to analyse the diet of an Apodidae species (Chan et al., 2019), and to date has not been applied to assess the diet of any of the Palearctic region's swift species, whose populations have been the subject of other biological and ecological studies (Cibois et al., 2022; Finlayson et al., 2021; Hedenström et al., 2019; Kearsley et al., 2022).

### 1.3 Sexual dietary differentiation

Although the diet of some swift species is already known, through the elementary methods or the most modern ones described above, dietary differences between males and females have never been considered. Sexual dietary segregation has been described in several animal groups (Borrell et al., 2011; Mata et al., 2016; Mramba et al., 2017), namely in birds (Catry et al., 2016; da Silva et al., 2020; Lewis et al., 2005; Mariano-Jelicich et al., 2007; Massaro et al., 2020; Wearmouth & Sims, 2008). These differences are usually the result of either marked morphological differences between males and females, with smaller and more agile individuals feeding on smaller prey and larger prey being exploited by larger individuals; or differences in parental care during the breeding season, males and females may have different energy requirements as a result of different roles in incubating eggs or caring for offspring (Catry et al., 2016; Lewis et al., 2005). Overall Pallid Swifts' parental care is shared by females and males, as found by Finlayson (1979) and confirmed later by Malacarne et al. (1992). Carmagnola colony's males and females shared incubation duties and feeding roles almost equally, and although Malacarne et al. (1992) noted that in 36% of pairs one partner had incubated more than the other, this was observed for both females and males. This biparental care combined with the fact that Pallid Swifts do not have sexual dimorphism suggest no significant differences between the diets of males and females. But as already mentioned, sexual dietary segregation has never been studied for swifts, and there are other birds without morphological sexual dimorphism with differences in their diet composition (da Silva et al., 2020).

Although these dietary differences can be assessed through a wide range of methods, e.g., direct observation, morphological identification of ingested food items, fatty acids and alcohols analysis, or stable isotope analysis (Hoenig et al., 2021; Nielsen et al., 2018), DNA metabarcoding can be a more powerful and informative tool, as it can detect subtle differences that would not be detected by the poor taxonomic resolution of the more conventional methods (Mata et al., 2016).

#### 1.3.1 Bird sex identification

In sexually dimorphic birds, such as the Eurasian Bullfinch (*Pyrrhula pyrrhula*), Pin-tailed Sandgrouse (*Pterocles alchata*), and Common Rock Thrush (*Monticola saxatilis*), it is very easy to distinguish between males and females. However, in birds without



morphological sexual dimorphism, such as Pallid Swifts, it is very difficult to accurately separate the sexes by visual examination of the individuals. In these non-sexual dimorphic species, individuals are often sexed by videotape inspections noting which of the parents laid the eggs. This method implies marking the individuals, installing cameras in nests and examining several hours of recordings (Boano et al., 2015; Malacarne et al., 1994). Therefore, more practical and reliable techniques, such as DNA-based sexing, have been adopted.

In DNA-based sexing in birds, the chromodomain helicase DNA-binding (CHD) gene, highly conserved in most avian species, is amplified using specific primers pairs. One gene is located on the Z chromosome (CHD-Z), whereas its homolog is on the W chromosome (CHD-W) (Cerit & Avanus, 2007). Since in birds, unlike in humans, males are the homogametic sex (ZZ), while females are the heterogametic sex (ZW) (Irwin, 2018), males exhibit two identical sized copies (both from CHD-Z gene fragment) on the agarose gel, while females generally display two different sized bands (one from CHD-Z and one from the CHD-W gene fragment) (Çakmak et al., 2017). However, depending on the primers used and the target species, the banding patterns obtained on the agarose gel may vary and in some species the analysis should focus on the size of the bands rather than the number of bands (Dubiec & Zagalska-Neubauer, 2006).

## 1.4 Objectives

The main purpose of this thesis was to evaluate the diet composition of the Pallid Swift during its breeding season in the North of Portugal using DNA metabarcoding on their faecal samples. A technique that, as previously mentioned, allows to accurately assess the diet composition in a non-invasive manner.

By using this method, I aimed to assess and characterize the temporal variations in the diet during the breeding season and evaluate whether differences exist between sexes, regarding (1) diet composition; (2) prey richness; (3) niche width; (4) niche overlap; and (5) prey size.

Finally, I aimed to compare the results with those obtained by other studies that have also studied the diet of swifts (Chung et al., 2021; Collins & Hespeneide, 2016; Hespeneide, 1975; Quang et al., 2006), namely Pallid Swifts (Cristiano et al., 2018; Finlayson, 1979; Malacarne & Cucco, 1992).

## 2. Methods

### 2.1 Study area

The studied Pallid Swift colony is installed in the arcades of the Vila Nova de Famalicão City Hall (41.4100°, -8.5203°), Braga, Portugal. The building designed by the architect Januário Godinho was inaugurated in 1961 and replaced the previous building destroyed by a fire nine years earlier. Januário Godinho's modern lines brought an opportunity for a close and unexpected coexistence between Pallid Swifts and Famalicão citizens.

The birds use the main arcade with openings to the east and west as a nesting place, building their nests in a small recess that follows the entire interior of the arcades right next to the ceiling (Figure 3).

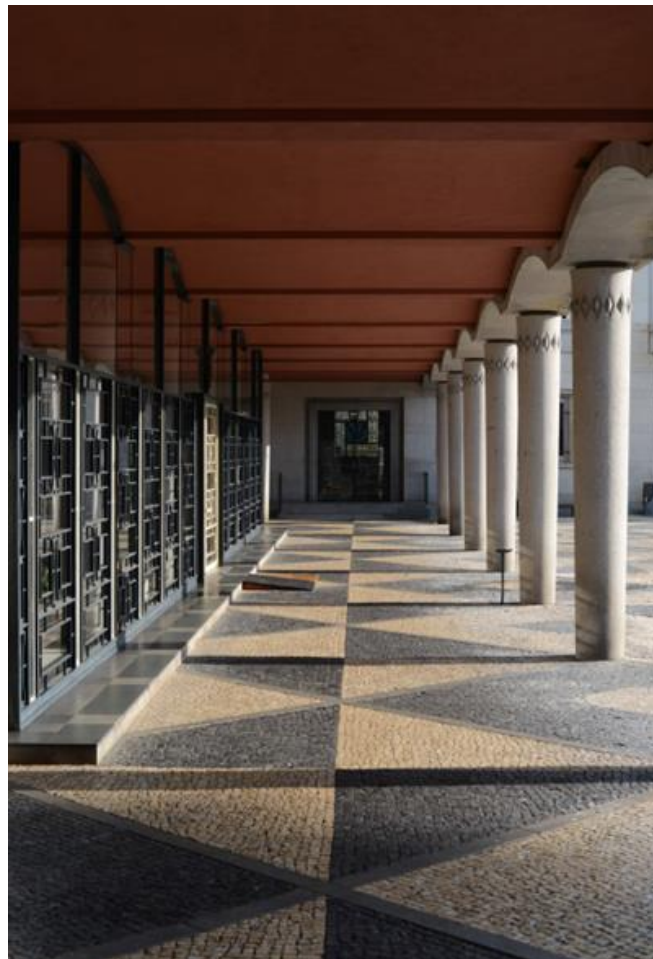


Figure 3 – East open of Vila Nova de Famalicão City Hall's arcades, where Pallid Swifts build their nests in the small recess next to the ceiling.

It is unknown for how long the swifts have been breeding there. The first reference to the existence of this colony dates back to 1997, when the Mayor Agostinho Fernandes, requested the Vento Norte Association and the Quercus Braga Regional Nucleus to improve the colony conditions, which resulted in the placement of artificial nests that are still in place today. This action, although incompatible with the birds' breeding requirements, since the artificial nests placed are not suitable for swifts, allowed researchers to know that Pallid Swifts have been using this colony for at least 25 years.

## 2.2 Field sampling

The field sampling was conducted in the above-described colony during the 2021 breeding season, from June to October. Pallid Swifts' fledglings were taken from the nests while flying individuals were captured with vertical mist nets, i.e., a finely woven mesh (16mm x 16mm) net erected to entangle and capture birds in flight, placed at the entrance of the colony before bird activity. Birds were removed from the nets immediately after being captured and placed in a clean cotton bag for 15 to 30 minutes while waiting to be processed. Captured individuals were ringed with official metal rings provided by the national ringing center (CEMPA - Centro de Estudos de Migrações e Proteção de Aves), following European Union for Bird Ringing (EURING) guidelines. Wing length, the distance from the distal portion of the carpus to the tip of the longest primary feather, was measured with a stopped wing ruler (blocked off at the 0 mm mark and with an accuracy of 0.5 mm) with the wing chord flattened and straightened, following the classic maximum chord method described by Svensson (1992). The weight was measured with a 0.1g precision on a digital mini scale. Birds' age was assigned based on the EURING codes (Du Feu et al., 2020). Regarding age, birds were assessed as adults (second year or older birds) or pulli (fledglings taken from the nests). The body condition of the birds (muscle and fat mass) was assigned according to Bairlein (1995) and Kaiser (1993), respectively. Cotton bags, from which the droppings were collected, were sterilized with 10% bleach for 1 hour and washed after every use to minimize contamination. Droppings were passed directly from the bags to 2 ml tubes with 98% ethanol without direct contact, and stored at 4°C until laboratory processing (da Silva et al., 2019).

## 2.3 Laboratory procedures

DNA was extracted from bird droppings using the Norgen Stool DNA Isolation Kit, following the manufacturer's protocol. DNA extraction was carried out in batches of 23

samples plus one negative control in which no faecal sample was added. The extracted DNA and the negative controls were distributed in 96-well plates where the last well was left empty for PCR negative control.

### 2.3.1 Prey identification

Invertebrate prey items were amplified using the COI primers fwhF2 (5'-GGDACWGGWTGAACWGTWTAYCCHCC-3') and fwhR2n (5'-GTRATWGCHCCDGCTARWACWGG-3') (Vamos et al., 2017), modified to contain Illumina adaptors. This primer set was originally designed to amplify the DNA of freshwater invertebrates (Vamos et al., 2017), but also performs well in the amplification of terrestrial arthropods' DNA and the amplification of degraded DNA samples, such as faecal droppings (Elbrecht et al., 2019; Mata et al., 2021). The PCR was carried out in volumes of 10 µl, comprised of 5 µl of Multiplex PCR Master Mix (Qiagen), 0.3 µl of each 10 pM primer, 2.4 µl of H<sub>2</sub>O, and 2 µl of DNA extract. Cycling conditions consisted of a 15 min period at 95°C, 45 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 50°C, and 30 sec extension at 72°C, and a final extension period of 10 min at 60°C. Amplification success was checked by visually inspecting 2 µl of each PCR product on a 2% agarose gel stained with GelRed. The resulting extracted DNA was cleaned using a 1:0.8 ratio of AMPure XP beads (Beckman Coulter, High Wycombe, UK) according to the manufacturer's instructions with the exception that 80% ethanol was used instead of 70%, and eluted in 25 µl of 10 mM Tris, pH 8.5. Clean PCR products went through a second PCR reaction to incorporate 7bp long indexes and P5+P7 Illumina adaptors. This second PCR was carried out in a total volume of 14 µl, comprising 7 µl of KAPA HiFi HotStart ReadyMix, 2.8 µl of cleaned PCR, 2.8 µl of H<sub>2</sub>O, and 0.7 µl of each adaptor. Cycling conditions were 3 min period at 95°C, 10 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 55°C, and 30 sec extension at 72°C, and a final extension period of 5 min at 72°C. Indexed samples were again cleaned and then pooled at equimolar concentrations and sequenced in a MiSeq run (500 cycles) together with samples from other projects.

### 2.3.2 Sex identification

The Pallid Swifts' sex identification was performed by amplifying a small amplicon of the Z and W chromosomes using P2 (5'-TCTGCATCGCTAAATCCTTT-3') and P8 (5'-CTCCAAGGATGAGRAAYTG-3') primers (Griffiths et al., 1998). This primer set was designed to amplify fragments of the (CHD) gene, providing distinct banding patterns on an agarose gel as a result of intronic regions within this gene (Çakmak et al., 2017; Griffiths et al., 1998; Quinn et al., 1990). The PCR was carried out in volumes of 11 µl, comprised of 5 µl of Multiplex PCR Master Mix (Qiagen), 0.4 µl of each primer, 2.2 µl of H<sub>2</sub>O, and 3 µl of DNA extract. P2 primers were labelled with the fluorescent dye FAM. Cycling conditions consisted of a 15 min period at 95°C, 20 cycles of 35 sec denaturation at 95°C, 45 sec annealing at 45°C, and 45 sec extension at 72°C, followed by more 25 cycles of 35 sec denaturation at 95°C, 45 sec annealing at 47°C, and 45 sec extension at 72°C and a final extension period of 10 min at 60°C. PCR products were checked on an agarose gel, and, although amplification was successful, it was not possible to visually separate the different amplicons. Therefore, PCR products were separated by capillary electrophoresis using the automatic sequencer ABI 3130xl Genetic Analyzer. Fragments were scored against Genescan-500LIZ size Standard, using GeneMapper version 4.1 (Applied Biosystems). Male individuals showed a single fragment with about 370bp length, while females showed an extra fragment of about 380bp (Figure 4). Each sample was sequenced three times, and sex was only assigned to samples that provided congruent results for at least two sequencing results. This should avoid false assignments resulting from allelic drop-out of the CHD-W, which makes females indistinguishable from males (van der Velde et al., 2017), and that can be particularly common in degraded DNA samples like bird droppings (Mitchell et al., 2012).

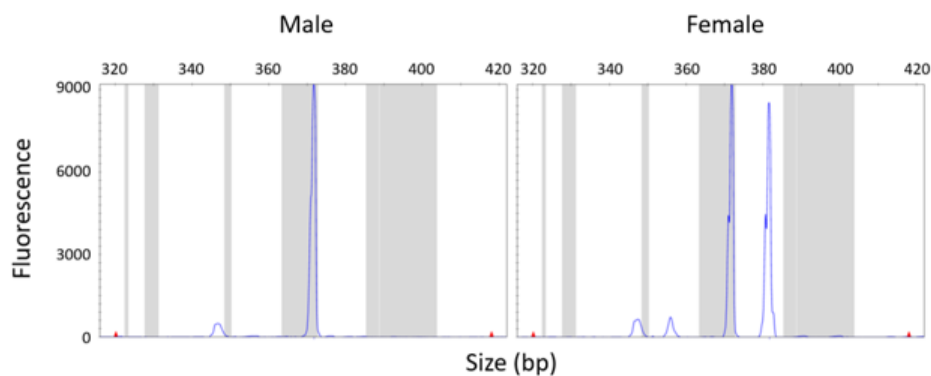


Figure 4 – Example amplicon length profile of a male and female Pallid Swift.

## 2.4 Bioinformatic analysis

Bioinformatic processing of generated Illumina reads was done using the R package Metabarcoding Joining Obitools & Linkage Networks In R (MJOLNIR: <https://github.com/uit-metabarcoding/MJOLNIR>), a tool designed to analyse and organise raw metabarcoding data into taxonomically assigned operational taxonomic units (OTUs). The two paired-end Illumina reads' alignment was done using 'illuminapairedend' from OBITools (Boyer et al., 2016). After paired-end sequences were merged, each sequence was assigned to the corresponding sample. This demultiplexing step was carried using 'ngsfilter', which was also used for primer-removal. In addition, the data was filtered to select fragments of 190-220 bp by applying the command 'obigrep'. All these steps were carried out simultaneously using the 'mjolnir2\_FREYJA()' function. Chimeric sequences from the individual sample files provided by FREYJA were removed using the 'mjolnir2\_HELIA()' function. This function uses the uchime\_denovo algorithm implemented in VSEARCH (Rognes et al., 2016) and after chimera removal joins all the samples. After this merge, reads were clustered using the 'mjolnir4\_ODIN()' function, which uses SWARM (Mahé et al., 2015) to delimit OTUs, based on linkage-networks created by step-by-step aggregation. This algorithm, which is not based on a constant, arbitrary and absolute identity threshold, can have different effective values for within-OTU identity threshold, depending on the complexity of the natural variability of the sequences present in the sample. Finally, to reduce the number of erroneous OTUs (e.g., retained PCR artefacts, sequencing errors, pseudogenes, etc.) and thus achieve more realistic biodiversity metrics, the 'mjolnir2\_LOKI()' function was used. This function uses the LULU (Frøslev et al., 2017) algorithm, which merges similar and highly occurring OTUs (identity higher than 84% and co-occurrence levels higher than 95%).

The taxonomic assignment of the haplotypes was reached using BOLDigger v1.2.5 (Buchner & Leese, 2020). This Python package compares the OTUs to the Barcode of Life Data Systems (BOLD) Identification System database, a library of known and taxonomically identified DNA barcodes, and assigns a match. The retrieved taxonomic assignment matches were then manually curated. When different OTUs matched a single taxon, these were condensed into a single taxonomic unit. In case an OTU matched different species, genera, or families at a similar identity level, this was assigned to the most inclusive taxonomic rank. OTUs assigned to higher taxonomic levels than species and with more than 98% of similarity, were clustered with a neighbour-joining tree (Mata et al., 2018) into distinct OTUs (e.g., Nabidae 1, Nabidae 2, etc). OTUs assigned to items that are not part of the insectivorous diet of swifts (e.g., fungi, protists, platyhelminths, birds, mammals, etc.) were categorized as "Not diet" and discarded from

the analysis. The number of reads per OTU in the extraction and PCR blanks was subtracted to the samples associated to each extraction batch and PCR plate, to rule out possible sources of laboratory contaminations. Finally, only samples with more than 100 reads of dietary items were considered and OTUs comprising less than 1% of the total dietary reads per sample were discarded.

## 2.5 Data analysis

All statistical analyses were performed on R v4.1.1 (R Core Team, 2020). Statistical significance was considered at  $\alpha = 0.05$ . Dietary analysis was based on the presence or absence of taxa per sample, considering two different taxonomic levels: family and OTU (identified to the most resolved taxonomic level possible).

The effect of sex, month, and their interaction on the number of prey taxa detected in each dropping was tested using a generalized linear model (GLM) with a Poisson error distribution, applying the function 'glm'. The significance of explanatory variables was tested using the 'Anova' function from package car (Fox & Weisberg, 2019). Pairwise comparisons were then used to identify in which pairs the observed differences occurred, using the function 'emmeans' of the emmeans package (Lenth, 2022).

The overall prey richness consumed by both sexes, i.e., niche width, during the five months was estimated using rarefaction curves based on Hill numbers with the function 'iNEXT' of the iNEXT package (Hsieh et al., 2016), with the triple of the lower reference sample size to avoid extrapolation bias (Chao et al., 2014). Significant differences were considered if the 95% confidence intervals between groups did not overlap.

To compare the prey composition among samples of different sexes and months, a pairwise distance matrix using the Jaccard dissimilarity indices was calculated using 'vegdist' available in the package vegan (Oksanen et al., 2020). This matrix, which quantifies the differences between samples based on prey occurrence, was then tested using a Permutational Multivariate Analysis of Variance (PERMANOVA) with the Binomial method and 99999 permutations using 'adonis' function from the same R package. To verify which pair of months differ from each other, a pairwise PERMANOVA was performed. Similarity percentages were also calculated to determine the contribution of different prey groups to the observed differences in variables, using the 'simper' function of the package vegan.

To test for differences in prey size consumption between sexes and months, a literature search was conducted to characterize the size of each prey item detected. Only prey

items identified to genus, species complex and species were considered, as it is not feasible to correctly assign a size to an arthropod family or order. Prey items used in this analysis included 85% of the OTUs and 85% of the predation events, thus covering the majority of the diet. The prey sizes obtained can be found in Annex I - Supplementary material (Table S1). The average prey size of each sample was further calculated and used as a response variable in a GLM with a Gamma error distribution to test the effect of bird's sex, month of capture, and their interaction, applying the function 'glm'. The significance of explanatory variables was tested using the 'Anova' function from the package car (Fox & Weisberg, 2019). To identify in which pair of months the observed differences occurred, pairwise comparisons were done applying the function 'emmeans' of the emmeans package (Lenth, 2022). Finally, to look for a correlation between the ingested diversity and the prey size, a generalized linear mixed model (GLMM) was conducted, applying the function 'glmer' of the lme4 package (Bates et al., 2014). In this analysis, month was used as a random variable, since the diversity consumed throughout the breeding season is expected to be affected by the prey's availability across the different months.



### 3. Results

#### 3.1 Sample collection and sequencing

During the Pallid Swift monitoring a total of 226 capture events were recorded, corresponding to a total of 103 individuals. The capture of these 103 individuals resulted in the collection of 82 faecal samples. After sequencing and bioinformatic processing, only 65 samples successfully produced dietary data (Table 1). Of these 65 faecal samples, 29 were identified as belonging to female birds, 33 to male, and in 3 samples it was not possible to accurately determine the sex of the bird.

Table 1 – Number of successful and failed samples for diet analysis per sex and month of capture.

	Success	Fail	Total
<b>June</b>	<b>19</b>	<b>3</b>	<b>22</b>
Female	11	0	11
Male	8	3	11
Unknown	0	0	0
<b>July</b>	<b>13</b>	<b>4</b>	<b>17</b>
Female	6	2	8
Male	7	2	9
Unknown	0	0	0
<b>August</b>	<b>20</b>	<b>3</b>	<b>23</b>
Female	4	0	4
Male	13	2	15
Unknown	3	1	4
<b>September</b>	<b>6</b>	<b>5</b>	<b>11</b>
Female	3	4	7
Male	3	0	3
Unknown	0	1	1
<b>October</b>	<b>7</b>	<b>2</b>	<b>9</b>
Female	5	1	6
Male	2	0	2
Unknown	0	1	1
<b>Total</b>	<b>65</b>	<b>17</b>	<b>82</b>

### 3.2 Diet composition

A total of 139 OTUs were identified in the diet of the Pallid Swift, belonging to 74 different families and 16 orders. The most commonly observed OTU was *Tetramorium forte* (32% of the samples), followed by *Lasius* 1 (25%), Elateridae 1 (17%), *Tetramorium caespitum/impurum* (17%), *Nabis* 1 (15%) and *Nezara viridula* (15%; Figure 5A). Regarding families, Formicidae was the most common (62%), followed by Pentatomidae (20%), Elateridae (17%), Nabidae (17%) and Caeciliusidae (14%; Figure 5B). Finally, the most common orders were Hymenoptera (66%), Hemiptera (58%), Coleoptera (57%), Diptera (33%) and Psocodea (17%; Figure 5C).

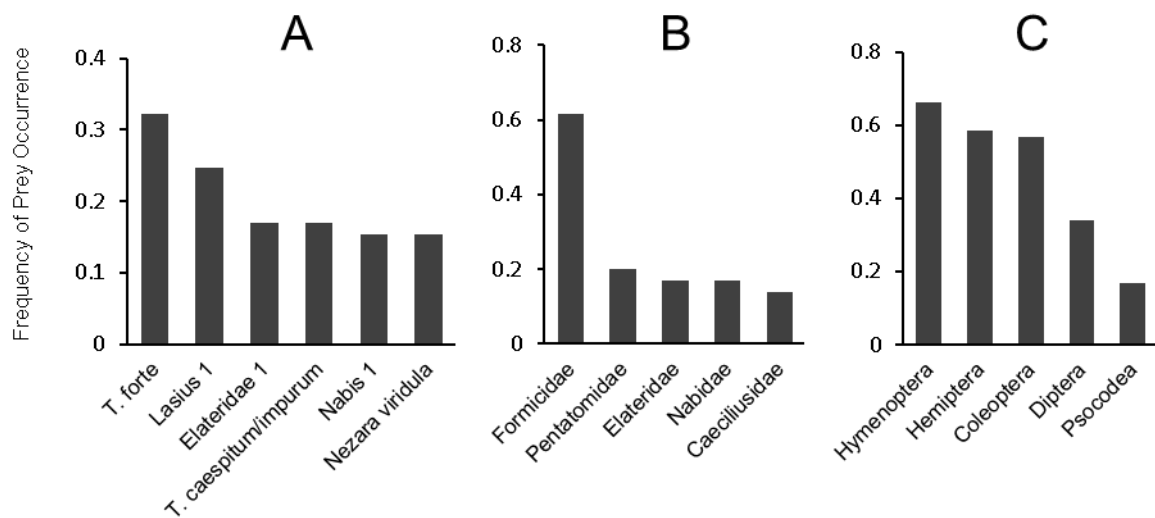


Figure 5 – Frequency of prey occurrence of mostly consumed OTUs (A), families (B), and orders (C) by Pallid Swifts throughout the breeding season.

The five most common orders were present throughout all the months, except for the order Psocodea which was not found in the samples collected in October. The order composition of the Pallid Swifts' diet over the breeding period seemed to undergo some variation, with some months presenting a greater diversity of prey and with some orders playing a more important role in the diet of these swifts in particular months (Figure 6).

In June, a substantial part of the prey (46%) belonged to the order Hymenoptera, with almost all of these OTUs (96%) belonging to the family Formicidae. In July, the five most common orders were almost equitably represented in the samples collected, unlike in the other months, each representing on average about 18% of the predation events. The remaining 10% was comprised of the other five orders that occurred in the swifts' diet in July (Lepidoptera, Araneae, Orthoptera, Odonata and Thysanoptera). In August, although birds consumed the highest number of orders (n = 11), 65% of the arthropods

consumed belonged to the orders Hymenoptera and Hemiptera. Finally, the faeces collected in September and October, despite having the least number of prey orders (6 and 5 orders detected, respectively), presented a very distinct composition. Whereas in September the orders Coleoptera and Hemiptera comprised more than 70% of the diet frequency; in October these orders only represented 18%, with the vast majority of the diet (>70%) being composed of insects belonging to the order Diptera.

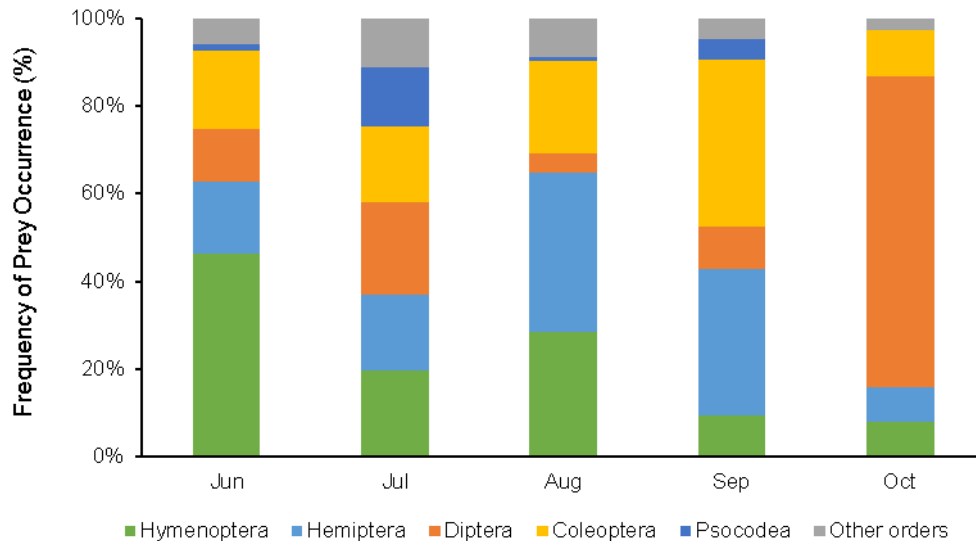


Figure 6 – Frequency of predation events of mostly consumed orders by Pallid Swifts in each sampled month. "Other orders" represents unfrequently consumed prey orders: Lepidoptera, Araneae, Orthoptera, Neuroptera, Trichoptera, Entomobryomorpha, Isopoda, Julida, Odonata, Symphypleona and Thysanoptera.

A total of 11 pest species were detected, belonging to 9 families of 4 different orders. Of these arthropods, ten were agricultural pests (*Bradysia trivittata*, *Chaetocnema hortensis*, *Drosophila suzukii*, *Geomyza tripunctata*, *Lacanobia oleracea*, *Psylliodes chrysocephalus*, *Sipha maydis*, *Sitotroga cerealella*, *Tetraneura nigriabdominalis* and *Tipula oleracea*) and one was a forest pest (*Ctenarytaina spatulata*). The most common pest was *G. tripunctata*, which was found in 6% of the samples; *D. suzukii*, *T. oleracea* and *C. spatulata* were detected in two samples and each of the remaining arthropods was only found in one sample each. Of the 17 pest predation events, more than half were with Diptera pests.

### 3.3 Prey richness

There were no differences between sexes in the number of prey items detected per sample (Figure 7), regardless of the taxonomic level considered: OTU (LR Chisq = 0.199, df = 1, p-value = 0.656) and family (LR Chisq = 0.036, df = 1, p-value = 0.850). With regard to temporal variation, differences were found in the average number of prey items detected per sample (Figure 7), both for OTU (LR Chisq = 14.634, df = 4, p-value = 0.006) and family levels (LR Chisq = 18.767, df = 4, p-value = 0.001). The significant differences were observed between June and July (OTU: z.ratio = -3.295, p-value = 0.008; family: z.ratio = -3.920, p-value = 0.001), the months with the lowest and highest average number of prey items per sample, respectively.

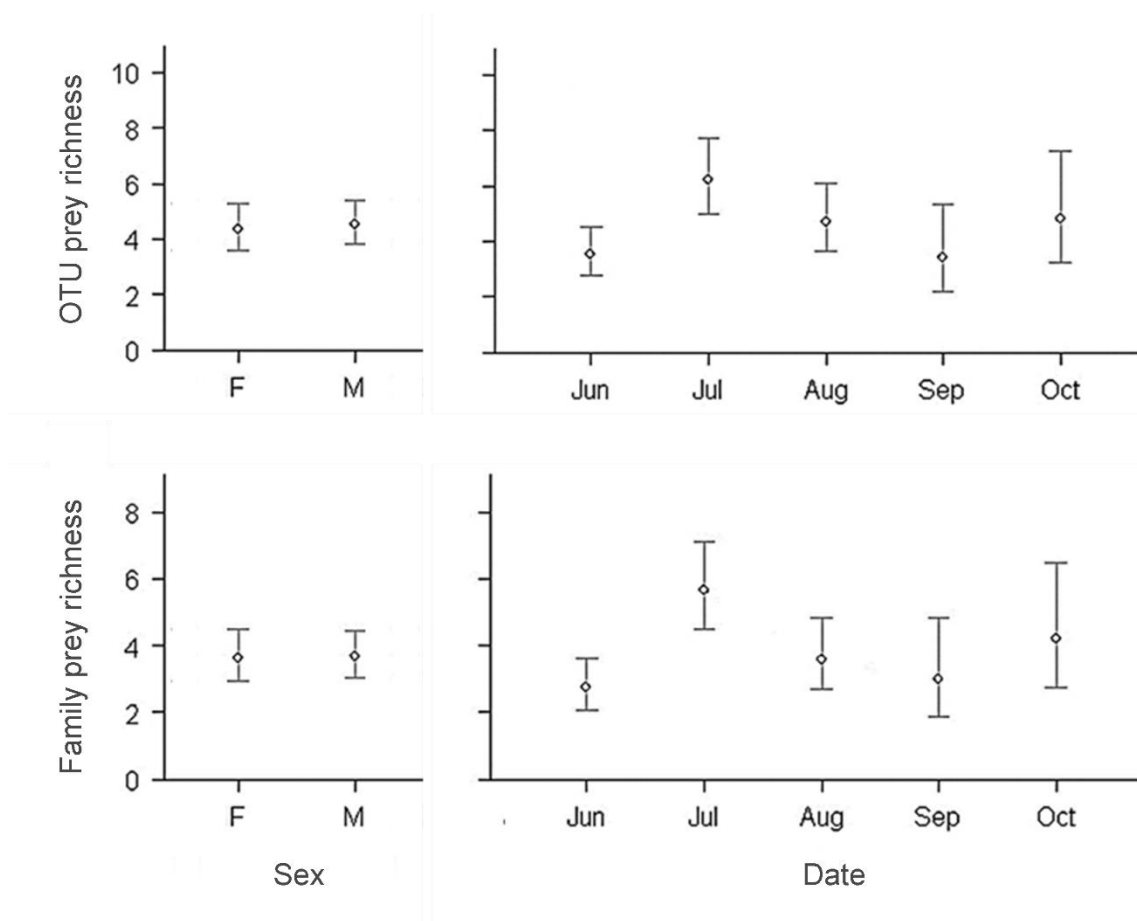


Figure 7 – Estimated prey richness of OTUs and families per sample for different sex and month classes. Whiskers represent 95% confidence intervals based on 1,000 bootstraps.

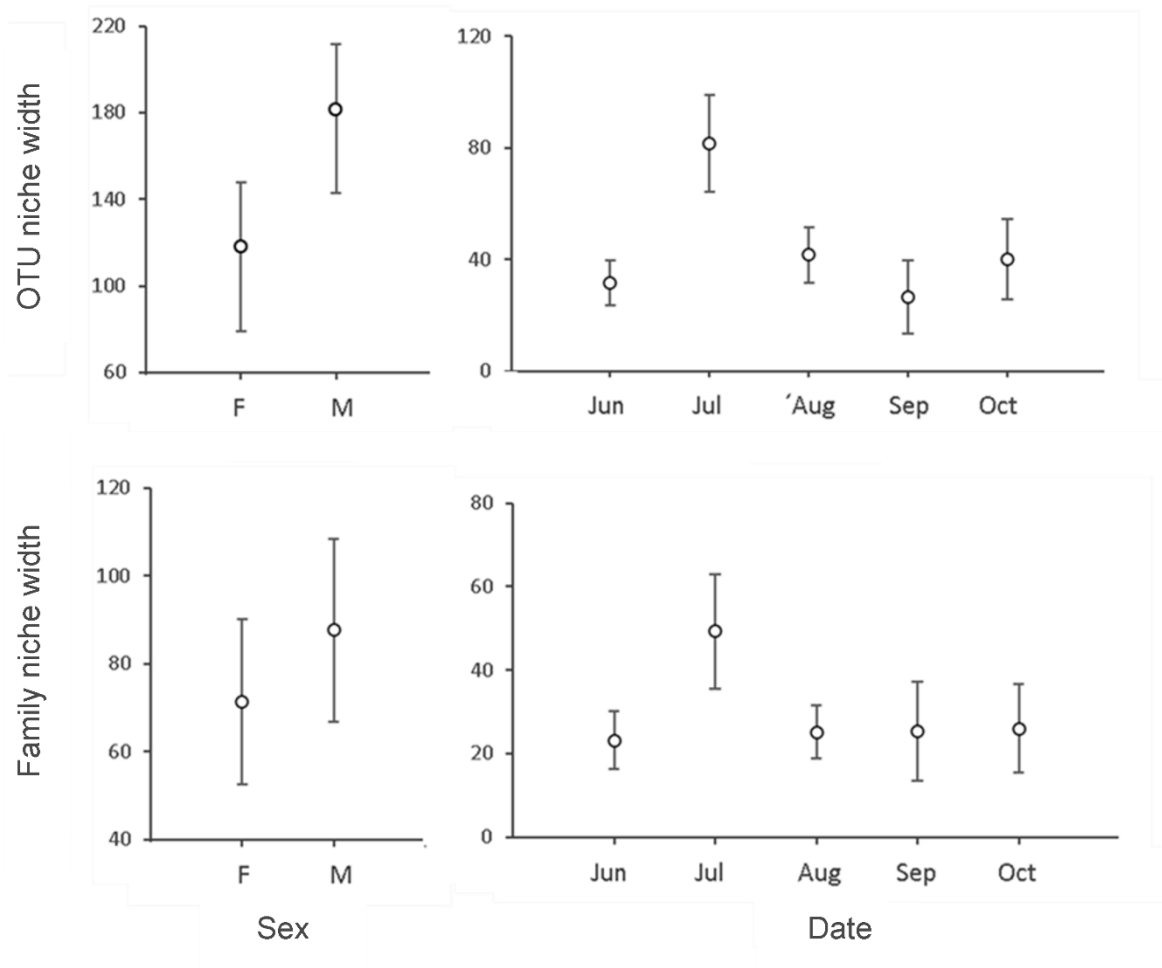


Figure 8 – Estimated niche width of OTUs and families for different sexes and months classes. Niche width of different sexes and months was estimated with 87 and 18 sampling units, respectively. Whiskers represent 95% confidence intervals based on 1,000 bootstraps.

### 3.4 Niche width

Overall diet richness was significantly different between months, but not between sexes (Figure 8). Regarding the temporal variation, for OTUs, September was the month with the lowest diversity values, followed by June, while October and August showed moderate niche width values. July was by far the month with the widest niche breadth, exhibiting more than double the average diversity values estimated for the remaining breeding season. Regarding the number of prey families consumed, July was also the month with the highest taxa richness values, standing out among the studied months, and also showing almost two times the diversity observed in the other months. The remaining four months showed similar diversity values, with October, August and September showing slightly higher values than June, which contrary to the values

obtained by the OTU analysis, was the month with the lowest prey richness values. For sexes, although the differences between the niche width of males and females was not significant, males tended to ingest a higher number of prey taxa. These differences were more pronounced when diets were analysed at the OTU level, than at the family level.

### 3.5 Niche overlap

The PERMANOVA revealed no differences in niche overlap between sexes both for OTU (df = 1, pseudo-F= 1.215, R<sup>2</sup> = 0.018, p-value = 0.151) and family level analysis (df = 1, pseudo-F = 1.195, R<sup>2</sup> = 0.017, p-value = 0.216). Regarding the sampling month, significant differences were found in niche overlap over the months, both for OTU (df = 4, pseudo-F = 2.952, R<sup>2</sup> = 0.171, p-value < 0.001) and family level analysis (df = 4, pseudo-F = 3.593, R<sup>2</sup> = 0.200, p-value < 0.001). Differences were found between all pairs of months except September and October, for both OTU and family analysis (Table 2).

Table 2 – Pairwise PERMANOVA testing for the effects of sampling month on niche overlap for OTU and family level analysis. Significant values are at bold.

Month Comparison	OTU			Family		
	Pseudo-F	R <sup>2</sup>	P value	Pseudo-F	R <sup>2</sup>	P value
Jun vs Jul	4.178	0.122	<b>&lt;0.001</b>	6.081	0.169	<b>&lt;0.001</b>
Jun vs Aug	5.535	0.140	<b>&lt;0.001</b>	4.424	0.115	<b>0.005</b>
Jun vs Sep	5.467	0.192	<b>0.001</b>	7.369	0.243	<b>0.001</b>
Jun vs Oct	4.870	0.169	<b>&lt;0.001</b>	6.206	0.205	<b>0.001</b>
Jul vs Aug	3.732	0.118	<b>&lt;0.001</b>	4.807	0.147	<b>&lt;0.001</b>
Jul vs Sep	3.042	0.152	<b>0.003</b>	3.978	0.190	<b>0.003</b>
Jul vs Oct	2.855	0.137	<b>0.003</b>	3.045	0.145	<b>0.004</b>
Aug vs Sep	3.123	0.129	<b>0.004</b>	4.160	0.165	<b>0.012</b>
Aug vs Oct	3.732	0.145	<b>&lt;0.001</b>	4.837	0.180	<b>0.001</b>
Sep vs Oct	2.871	0.207	0.070	2.921	0.210	0.076

The compositional differences at OTU level found among the diets collected over the different five months were explained by the temporal variation of the most frequently consumed OTUs (present in more than 10% of samples; Figure 9). The presence of *Tretamorium forte* and *Lasius 1* in 68% and 58% of the samples collected in June, respectively, and the negligible presence or even absence of these OTUs in the other months, explained the significant differences between the diet composition of June and the rest of the breeding season. The differences between the composition of the diet consumed in July and in the other months were due to *Valenzuela flavidus*, since it was

present in 54% of the samples collected in July, and barely present or absent in the remaining months. The compositional differences found between August and the other four months resulted from the presence of Elateridae 1 in 50% of the samples collected in this month and the absence of this OTU in the other sampled months, with the exception of September, in which its presence was residual. In addition, *Tretamorium caespitum/impurum*, *Nabis* 1 and *Macroscytus brunneus* also contributed to the differences found between August and June. Finally, the marginal presence of *Nezara viridula* in June and August and the total absence in the July and October samples, but a dominant presence in September samples (present in 67% of the faecal samples analysed), made this OTU the main responsible for the differences detected between the diets collected in September and the other months. Regarding October, the differences in diet composition between this month and the others was explained by *Lonchoptera lutea* and *Scaptomyza pallida*, both present in 57% of the samples of this month.

Concerning the temporal variation at family level analysis (Figure 9), the Formicidae family was responsible for the significant differences found between diets collected in June and those collected in the last two months of sampling, since Formicidae was in 89% of the samples collected in June and only appeared to a minor extent in September and October. Nabidae and Carabidae families also explained the compositional differences between the prey captured in June and the ones captured in August. The differences found between diets collected in July and in the other months were due to the Caeciliusidae family, since it was in 54% of the samples taken in June and was barely present or even absent in the remaining months. Additionally, Chironomidae also explained the differences observed between July and the months of June, August and October. In turn, Elateridae explained the differences between diets sampled in August and those sampled in other months, whereas Cydnidae also contributed to the compositional differences between diets collected in August and those collected in the first two sampled months. Pentatonidae and Cicadellidae were the families responsible for the significant differences between September and the other four months. The differences in diet composition between October and the others were explained by Lonchopteridae and Drosophilidae, both present in 57% of the samples of this month's diet.

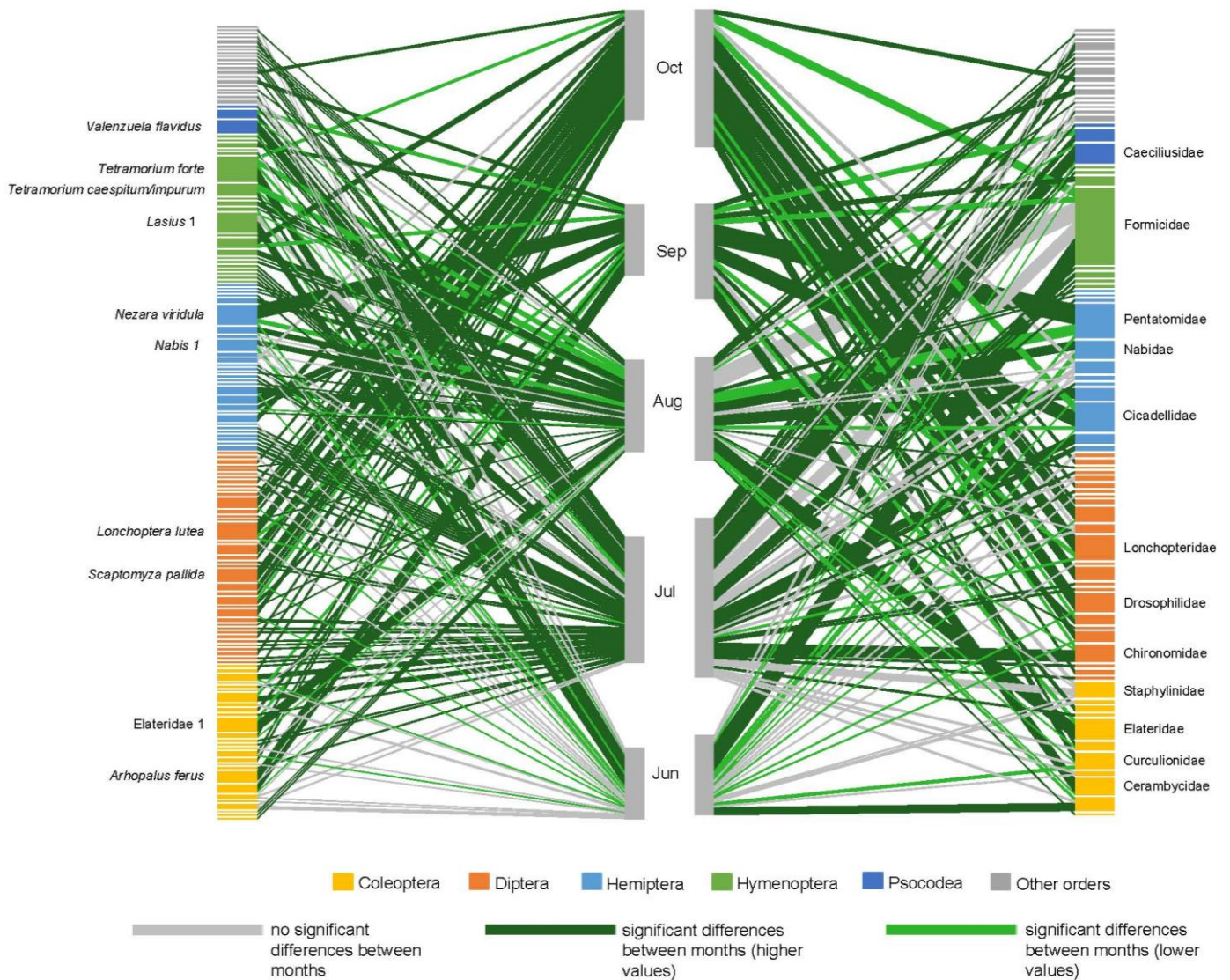


Figure 9 – Bigraph displaying the OTUs and families ingested by Pallid Swifts per sampling month. Green links indicate OTUs and families consumed in significantly different proportions over the five months, obtained from the SIMPER analysis. The width of links is proportional to their frequency of occurrence within months. Month and prey boxes are proportional to the sum of the frequency of occurrence of all interactions in that month and across months, respectively. Only the most frequent OTUs and families (more than 10% of the average monthly frequency of occurrence) have their name displayed.

### 3.6 Prey size

Arthropods between 2-4 mm in size were the prey on which Pallid Swifts fed the most, with size classes 4-6 and 6-8 mm being the second and third most preyed classes, respectively (Figure 10). Only a minor part of Pallid Swifts' diet consisted of prey smaller than 2 mm and larger than 16 mm.



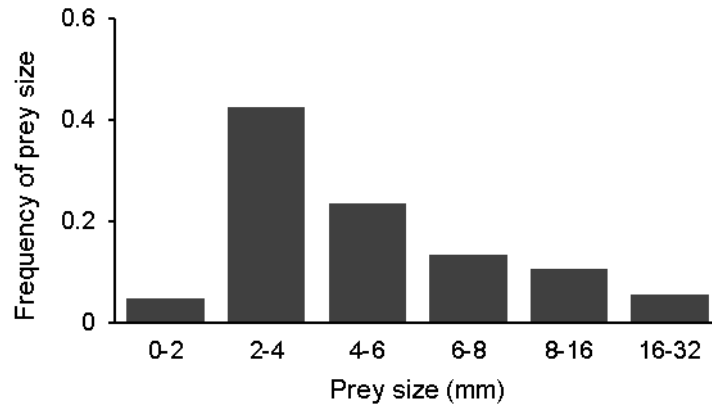


Figure 10 – Frequency of interaction (%) with each prey size class in Pallid Swifts' diet.

There were significant differences in the average size of prey consumed per sample, between sexes (LR Chisq = 4.871, df = 1, p-value = 0.027) and months (LR Chisq = 10.856, df = 4, p-value = 0.028; Figure 11). Overall, males fed on larger prey, while females tended to prey on smaller arthropods. Regarding the temporal variation in prey size, Pallid Swifts in July preyed on smaller sized arthropods when compared to the remaining breeding season. In turn, September was the month in which larger prey were consumed, with significant differences only detected between these two months (t.ratio = 2.882, p-value = 0.044). An overall significant inverse relationship between the ingested diversity and the prey size was also found (Chisq = 4.7, df = 1, p-value = 0.030), with prey diversity increasing as preys becomes smaller.

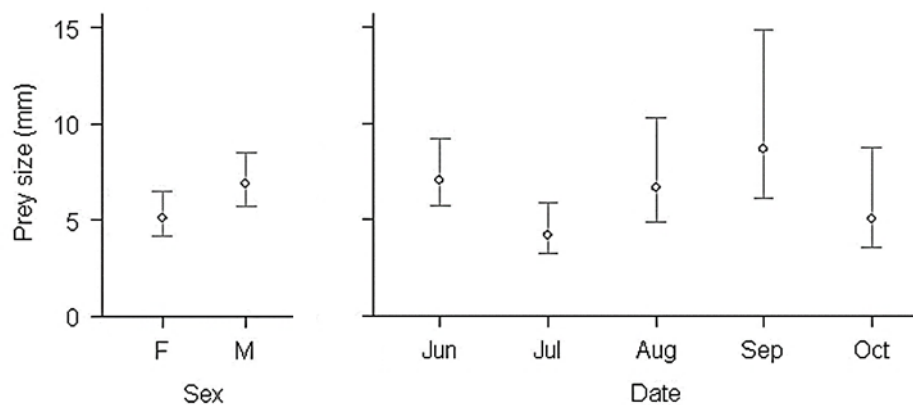


Figure 11 – Estimated average prey size (mm) of OTUs for different sex and month classes. Whiskers represent 95% confidence intervals.

## 4. Discussion

In this study, the feeding ecology of Pallid Swift was analysed using DNA metabarcoding to identify prey items in faecal samples. Through this technique, which allows high taxonomic resolution identification of prey items, the temporal variation in Pallid Swifts' feeding ecology and the diet of males and females during the breeding season was characterized.

The results suggested a marked temporal variation in prey richness, niche width, niche overlap, and prey size throughout the breeding season, likely as a consequence of the seasonality of aerial arthropods, since aerial foragers rely on spatiotemporally unpredictable accumulations of aerial prey (Arbeiter et al., 2016). As swifts display an opportunistic feeding behaviour, massive arthropod assemblages lead to high intraspecific variability in these birds' diets throughout time, as evidenced by Cucco et al. (1993). Although no significant differences in diet richness and composition were noted between males and females, there were differences between the size of arthropods preyed by the different sexes.

The diet was composed by 74 families, belonging to 16 orders. Of these orders, Hymenoptera, Hemiptera, Coleoptera, Diptera and Psocoptera were the most consumed, with particular emphasis on the first three. These results are congruent with those found in other studies of Pallid Swifts. The study by Cristiano et al. (2018) concluded that Hemiptera and Coleoptera were the most consumed orders by Pallid Swifts; and Malacarne and Cucco (1992) identified the orders Hemiptera, Hymenoptera and Diptera as the most important prey for Pallid Swifts' diet in Piedmont, northwest (NW) Italy. Regarding Hymenoptera, although Cristiano et al. (2018) did not find a large predominance of this order, the study conducted by Malacarne and Cucco (1992) and the one conducted by Chung et al. (2021), on the diet of House Swifts (*Apus nipalensis*), found the same pattern, with a predominance of hymenopterans consumed during the breeding season, being Formicidae the most prevalent family ingested. The temporal variation of these orders was also in line to that found in these previous works. Malacarne and Cucco (1992) found that the vast majority of the preyed arthropods in June belonged to the order Hymenoptera, while August and September were dominated by Hemiptera. The majority of Diptera was recorded in October and this marked presence of dipterans at the end of the breeding season is in line with the results found by Chung et al. (2021). Unlike the other orders, Coleoptera did not show a clear temporal variation and this pattern was also noted in the results obtained by Malacarne and Cucco (1992).

Most of these previous studies relied on traditional methods to study the diet of Pallid Swifts, however, the study conducted by Chung et al. (2021), was a pioneer in using DNA metabarcoding, to study the diet of Apodidae species. This modern technique offers a much higher taxonomic resolution, allowing a much more detailed study of the diet of these species, as evidenced by this study's results and the results found by Chung et al. (2021). Overall, this study found Pallid Swifts feeding on 139 prey items, while Chung et al. (2021) found 80 prey items, belonging to 44 families of 10 orders. The diversity consumed by the *Apus nipalensis* was much lower than that seen in Pallid Swifts' diet, even though the sample size of the study conducted in Hong Kong was considerably higher, more than the double that used in this study. Overall, for the same number of samples, the species richness, and the family richness, of Pallid Swifts' diet was about twice that observed in House Swifts, with order richness also being considerably higher in the *Apus pallidus*' diet. The differences in diet richness between these two *Apus* species must be interpreted carefully, since they are likely highly influenced by the use of different primer sets, which may amplify different taxa and have different taxonomic power.

Along with the composition of the diet, prey richness and niche width also underwent a sharp temporal variation. In June, both prey richness and niche width showed the lowest values, with a large portion of the prey belonging to the Formicidae family. Ants are relatively weak fliers and can be found in dense aggregations during nuptial flights (Hespenheide, 1975; Levin et al., 2008), which reduces the required capture effort and energy expenditure for their predation. Thus, swifts seem to choose to prey on these arthropods rather than on others also present in the air column (Chung et al., 2021), since according to optimal foraging theory, natural selection promotes the most energy-efficient foraging pattern in order to maximise energy intake and increase fitness (Pyke, 1984). For swifts, who have such an energy-demanding aerial lifestyle, which have even led them to develop morphological, behavioural and physiological adaptations to save the much-needed energy, this energy-efficient foraging is even more critical. In addition to the prey's ease of capture and its local density, the prey's selection also depends on its size (Hespenheide, 1975). Thus, larger, and therefore more energetically nutritious prey, should be preferred in order to maximise the energetic intake. The consumption of the largest prey was recorded in June and September, and, therefore, these were likely the months when optimal prey items were more abundant, and, as a result, swifts appeared to concentrate on them, reducing prey richness and niche width. On the other hand, when ideal prey diminishes and environmental restrictions constrain consumers to lower quality diets, predators need to obtain sufficient nourishment, thus increasing

consumption rates (Simpson & Simpson, 1990). This compensatory feeding leads to more varied diets under suboptimal conditions. As July presented the highest values of prey richness and niche width over the breeding season, and, at the same time the lowest values of prey size, it is plausible that Pallid Swifts were under unfavourable environmental conditions and the low abundance of their optimal food compelled them to explore a broader food niche.

In this thesis, not only it was found the same temporal variation pattern as in Malacarne and Cucco (1992) with respect to prey order composition, the same prey size preference was also observed, even though the overall pattern differed slightly. As in Malacarne and Cucco (1992), Pallid Swifts in this study fed mainly on prey with 2-4 mm, however in this study the second most consumed class was 4-6 mm while in their study was the 1-2 mm one. Nevertheless, the results from this study seem to fall between those obtained by Malacarne and Cucco (1992) in Piedmont and those obtained by Finlayson (1979) in Gibraltar, with the latter pointing arthropods between 4-6 mm in size as the most consumed by Pallid Swifts.

Regarding diet composition of males and females, sexual dietary segregation has been described in several animal groups (Borrell et al., 2011; Mata et al., 2016; Mramba et al., 2017), and particularly in birds (Catry et al., 2016; da Silva et al., 2020; Massaro et al., 2020). Marked morphological differences between sexes or differences in parental care during the breeding season are generally the drivers of this segregation (Catry et al., 2016; Lewis et al., 2005). Although dietary differences between sexes have never been studied in Apodidae species, the Pallid Swifts' biparental care combined with the fact that these birds do not have sexual dimorphism led to hypothesise that no significant differences would be found between the diet of males and females. The results partly confirmed my initial hypothesis, since no differences in diet richness, niche width, nor prey composition between males and females were found. However, there were significant differences in the size of consumed prey, with males preying on larger arthropods. The results obtained by compositional and prey size analysis were not congruent, since although no differences were observed in diet composition between sexes, there were differences in the size of the arthropods consumed. This non-compliance is likely related to differences in statistical power of both analyses, with PERMANOVA likely requiring a higher number of samples to detect a significant effect.

In addition to characterising Pallid Swift's diet, providing better insights into the ecology of this species, and finding evidence suggesting that these birds can turn to suboptimal prey under suboptimal conditions, this thesis also uncovered the presence of some

arthropod pests in the diet of these swifts. Among the agricultural pest species observed were *Bradysia trivittata*, a greenhouse production systems' pest that feeds on horticultural crops (Cloyd, 2015); *Chaetocnema hortensis*, a flea beetle that damages barley, flax and wheat (Coral Şahin et al., 2018); *Drosophila suzukii*, a fly that causes and spreads sour rot (Rombaut et al., 2017); *Geomyza tripunctata*, a fly pest of maize crops (Thibord, 2017); *Lacanobia oleracea*, a moth that attacks tomato, apple and many other agricultural species (Fitches et al., 2004); *Psylliodes chrysocephalus*, a beetle pest of most brassica seed crops (Winfield, 1992); *Sipha maydis*, a grass and cereal feeding aphid (Aripov, 2003); *Sitotroga cerealella*, a moth that feeds in storage grains and seeds (Bushra & Aslam, 2014); *Tetraneura nigriabdominalis*, an important aphid pest of rice and maize (Walczak et al., 2017); and *Tipula oleracea*, a pest of agricultural grasslands (Benefer et al., 2017). In addition to agricultural pests, I also found an invasive forest pest, *Ctenarytaina spatulata*, a psyllid responsible for economic damage to commercially grown *Eucalyptus* spp. (Hodkinson, 2007; Zina et al., 2015), and that can have a medium economic impact in *E. globulus* plantations in Portugal (Valente, 2017). The ability of swifts, and other aerial feeders, to provide an important regulatory service by preying on invasive and pest arthropods has already been reported in previous research (Cristiano et al., 2018; Orłowski & Karg, 2013). These studies point to aerial feeders as potential agents in biological pest management strategies, a sustainable alternative to the use of traditional pesticides, which directly and indirectly affect the populations of swifts and other aerial foragers, either by food poisoning or by food availability reduction. In addition to performing an important ecosystem service, swifts can also act as bioindicators. This biosurveillance has been enhanced by the application of DNA metabarcoding, which allows an early and quick detection (Montauban et al., 2021; Westfall et al., 2020), thereby mitigating the damaging effects that these species have on ecosystems. Thus, monitoring swifts' diet can be a useful tool in ecosystem monitoring (Cristiano et al., 2018).

## 5. Final Remarks

Overall, this thesis' results suggest a sharp temporal variation in Pallid Swifts' diet during the breeding season, with almost the whole diet being comprised of arthropods belonging to the orders Hymenoptera, Hemiptera, Coleoptera, Diptera and Psocodea. The temporal variation found in richness, niche width, composition and size of the Pallid Swifts' prey consumption likely resulted from the spatiotemporally unpredictable accumulations of aerial arthropods. Although no significant differences were found in diet richness, niche width and overlap between males and females, the finding that male birds feed on larger prey items than females, raises the question of whether birds such as swifts, with no sexual dimorphism and with shared parental caretaking, may exhibit sexual dietary segregation. This study also suggests that swifts may play an important role in pest predation. Finally, this thesis suggest how species may modify their food niche in response to changing environmental conditions and its influence on prey availability. When environmental conditions were likely good and potentially optimal prey were available, the Pallid Swifts focused on this prey, and, as a consequence, the diversity of their diet reduced. But as potential optimal prey became less available, Pallid Swifts became more opportunistic and began to incorporate sub-optimal prey items into their diet, thus broadening their food niche, which resulted in a more diverse diet.

To strengthen these results and disentangle the possible differences between the diets of males and females, future studies should extend the sampling period, to assess whether the same pattern is observed overtime; integrate more bird colonies, to avoid possible local and regional effects; and sample prey availability to study in detail prey selection in this species.

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## Annex I – Supplementary material

Table S1 – Size (mm) and frequency of occurrence (%FOO) of all genera, species complexes and species used in the prey size analysis.

Order	Family	OTU	Size (mm)	%FOO
Araneae	Cheiracanthidae	<i>Cheiracanthium elegans</i>	7.5	1.5
Araneae	Linyphiidae	<i>Agyneta rurestris</i>	2.3	3.1
Araneae	Linyphiidae	<i>Bathypantes gracilis</i>	2	1.5
Coleoptera	Anthicidae	<i>Notoxus</i> 1	4.6	1.5
Coleoptera	Anthicidae	<i>Omonadus floralis</i>	3.1	1.5
Coleoptera	Carabidae	<i>Acupalpus brunnipes</i>	3.25	1.5
Coleoptera	Carabidae	<i>Clivina collaris</i>	5	1.5
Coleoptera	Carabidae	<i>Ophonus</i> 1	11.25	4.6
Coleoptera	Cerambycidae	<i>Arhopalus</i> 1	17.5	4.6
Coleoptera	Cerambycidae	<i>Arhopalus ferus</i>	18	6.2
Coleoptera	Chrysomelidae	<i>Chaetocnema hortensis</i>	1.9	1.5
Coleoptera	Chrysomelidae	<i>Psylliodes chrysocephalus</i>	3.8	1.5
Coleoptera	Curculionidae	<i>Charagmus griseus</i>	7.75	1.5
Coleoptera	Curculionidae	<i>Hylurgus ligniperda</i>	7.5	4.6
Coleoptera	Curculionidae	<i>Sitona</i> 1	4.3	1.5
Coleoptera	Curculionidae	<i>Sitona obsoletus</i>	5	1.5
Coleoptera	Dermestidae	<i>Attagenus unicolor</i>	3.9	1.5
Coleoptera	Dermestidae	<i>Dermestes mustelinus</i>	7	3.1
Coleoptera	Nitidulidae	<i>Pria dulcamarae</i>	1.75	1.5
Coleoptera	Phalacridae	<i>Olibrus affinis</i>	2.1	1.5
Coleoptera	Scarabaeidae	<i>Calamosternus granarius</i>	4.5	3.1
Coleoptera	Staphylinidae	<i>Anotylus nitidulus</i>	2.45	6.2
Coleoptera	Staphylinidae	<i>Carpelimus bilineatus</i>	2.15	1.5
Coleoptera	Staphylinidae	<i>Nehemitropia lividipennis</i>	3.15	1.5
Coleoptera	Staphylinidae	<i>Stenus ossium</i>	4	1.5
Diptera	Anthomyiidae	<i>Adia cinerella</i>	4.5	1.5
Diptera	Anthomyiidae	<i>Delia</i> 1	3.8	1.5
Diptera	Asteiidae	<i>Asteia amoena</i>	2.75	1.5
Diptera	Chironomidae	<i>Chironomus luridus</i>	6.1	1.5
Diptera	Chironomidae	<i>Macropelopia adauca</i>	4	1.5
Diptera	Chironomidae	<i>Micropsectra atrofasciata</i>	3	1.5
Diptera	Chironomidae	<i>Paracricotopus</i> 1	1.75	1.5
Diptera	Chironomidae	<i>Tanytarsus volgensis</i>	2.8	1.5
Diptera	Chironomidae	<i>Virgatanytarsus triangularis</i>	3.05	3.1
Diptera	Chloropidae	<i>Thaumatomyia</i> 1	3.05	4.6
Diptera	Dolichopodidae	<i>Dolichopus griseipennis</i>	5.38	3.1
Diptera	Dolichopodidae	<i>Medetera saxatilis</i>	2.63	1.5
Diptera	Drosophilidae	<i>Drosophila suzukii</i>	3	3.1

Order	Family	OTU	Size (mm)	%FOO
Diptera	Drosophilidae	<i>Scaptomyza pallida</i>	2.38	7.7
Diptera	Ephydriidae	<i>Psilopa polita</i>	1.08	1.5
Diptera	Lauxaniidae	<i>Calliopus aeneum</i>	4	1.5
Diptera	Lauxaniidae	<i>Minettia lupulina/fasciata</i>	3.25	4.6
Diptera	Limoniidae	<i>Dicranomyia modesta</i>	8.8	1.5
Diptera	Lonchopteridae	<i>Lonchoptera lutea</i>	2.5	9.2
Diptera	Mycetophilidae	<i>Exechia seriata</i>	4.5	1.5
Diptera	Mycetophilidae	<i>Tarnania dziedzickii</i>	7.75	1.5
Diptera	Opomyzidae	<i>Geomyza tripunctata</i>	3.13	6.2
Diptera	Phoridae	<i>Megaselia scutellaris/tenebricola</i>	2.15	1.5
Diptera	Psychodidae	<i>Psychoda</i> 1	3.5	1.5
Diptera	Sciaridae	<i>Bradysia trivittata</i>	2.65	1.5
Diptera	Simuliidae	<i>Simulium rubzovianum</i>	2.38	1.5
Diptera	Sphaeroceridae	<i>Coproica hirticula</i>	1.7	1.5
Diptera	Syrphidae	<i>Eupeodes bucculatus/corollae/luniger</i>	9	1.5
Diptera	Tachinidae	<i>Trichopoda pennipes</i>	9	1.5
Diptera	Tephritidae	<i>Tephritis formosa</i>	5	1.5
Diptera	Tipulidae	<i>Tipula oleracea</i>	20.5	3.1
Hemiptera	Aphalaridae	<i>Ctenarytaina spatulata</i>	2	3.1
Hemiptera	Aphididae	<i>Anoecia</i> 1	2.45	1.5
Hemiptera	Aphididae	<i>Sipha maydis</i>	1.95	1.5
Hemiptera	Aphididae	<i>Tetraneura nigriabdominalis</i>	2	1.5
Hemiptera	Aphididae	<i>Tuberculatus remaudierei</i>	1.9	1.5
Hemiptera	Cicadellidae	<i>Agallia consobrina</i>	3.75	1.5
Hemiptera	Cicadellidae	<i>Aphrodes aestuarinus</i>	6.5	3.1
Hemiptera	Cicadellidae	<i>Empoasca decipiens/pteridis/vitis</i>	3.5	3.1
Hemiptera	Cicadellidae	<i>Psammotettix</i> 1	4.05	3.1
Hemiptera	Cydnidae	<i>Macroscytus brunneus</i>	7.5	10.8
Hemiptera	Cydnidae	<i>Sehirus morio</i>	10	1.5
Hemiptera	Delphacidae	<i>Muellerianella extrusa</i>	2.5	1.5
Hemiptera	Lygaeidae	<i>Kleidocerys ericae</i>	4.25	1.5
Hemiptera	Lygaeidae	<i>Nysius ericae</i>	4	1.5
Hemiptera	Miridae	<i>Macrolophus costalis</i>	3.5	1.5
Hemiptera	Miridae	<i>Orthotylus adenocarpis</i>	4.1	1.5
Hemiptera	Miridae	<i>Pinalitus cervinus</i>	4.1	3.1
Hemiptera	Miridae	<i>Trigonotylus</i> 1	5.5	3.1
Hemiptera	Nabidae	<i>Nabis</i> 1	6	15.4
Hemiptera	Nabidae	<i>Nabis ferus</i>	8	3.1
Hemiptera	Nabidae	<i>Nabis punctatus</i>	7.55	9.2
Hemiptera	Pentatomidae	<i>Nezara viridula</i>	12.5	15.4
Hemiptera	Pentatomidae	<i>Piezodorus lituratus</i>	11.5	6.2
Hemiptera	Psyllidae	<i>Acizzia uncatoides</i>	2.05	1.5
Hemiptera	Rhopalidae	<i>Liorhysus hyalinus</i>	7	1.5
Hemiptera	Rhyparochromidae	<i>Tropistethus holosericus</i>	2.65	1.5
Hymenoptera	Andrenidae	<i>Panurgus</i> 1	9.5	1.5

Order	Family	OTU	Size (mm)	%FOO
Hymenoptera	Apidae	<i>Apis mellifera</i>	12.5	1.5
Hymenoptera	Braconidae	<i>Chorebus leptogaster</i>	2.55	1.5
Hymenoptera	Braconidae	<i>Meteorus rubens</i>	3.5	1.5
Hymenoptera	Formicidae	<i>Aphaenogaster gibbosa</i>	6.5	3.1
Hymenoptera	Formicidae	<i>Camponotus cruentatus</i>	10	1.5
Hymenoptera	Formicidae	<i>Camponotus truncatus</i>	7	7.7
Hymenoptera	Formicidae	<i>Crematogaster scutellaris</i>	4.25	4.6
Hymenoptera	Formicidae	<i>Lasius</i> 1	3.85	24.6
Hymenoptera	Formicidae	<i>Linepithema humile</i>	2.4	4.6
Hymenoptera	Formicidae	<i>Myrmica rubra</i>	4.5	6.2
Hymenoptera	Formicidae	<i>Myrmica ruginodis</i>	5	1.5
Hymenoptera	Formicidae	<i>Tetramorium caespitum/impurum</i>	2.98	16.9
Hymenoptera	Formicidae	<i>Tetramorium forte</i>	4.2	32.3
Hymenoptera	Ichneumonidae	<i>Aclastus solutus</i>	3.15	1.5
Hymenoptera	Ichneumonidae	<i>Dicaelotus resplendeus</i>	3.1	1.5
Hymenoptera	Ichneumonidae	<i>Dichrogaster</i> 1	6	1.5
Hymenoptera	Megastigmidae	<i>Megastigmus amicornum</i>	3.1	1.5
Hymenoptera	Proctotrupidae	<i>Exallonyx longicornis</i>	4	1.5
Isopoda	Armadillidiidae	<i>Armadillidium vulgare</i>	13.25	1.5
Lepidoptera	Gelechiidae	<i>Megacraspedus quadristictus</i>	3	1.5
Lepidoptera	Gelechiidae	<i>Sitotroga cerealella</i>	6	1.5
Lepidoptera	Gelechiidae	<i>Sophronia semicostella</i>	18	1.5
Lepidoptera	Noctuidae	<i>Lacanobia oleracea</i>	16.5	1.5
Lepidoptera	Pyalidae	<i>Pempelia palumbella</i>	26	1.5
Lepidoptera	Zygaenidae	<i>Zygaena fausta</i>	24.5	1.5
Neuroptera	Chrysopidae	<i>Chrysoperla lucasina</i>	2.8	1.5
Neuroptera	Hemerobiidae	<i>Micromus angulatus</i>	10	1.5
Odonata	Coenagrionidae	<i>Erythromma lindenii</i>	33	1.5
Psocodea	Caeciliusidae	<i>Valenzuela flavidus</i>	2.45	13.8
Psocodea	Ectopsocidae	<i>Ectopsocus briggsi</i>	2.5	6.2
Psocodea	Peripsocidae	<i>Peripsocus phaeopterus</i>	2.15	1.5
Thysanoptera	Aeolothripidae	<i>Aeolothrips</i> 1	1.25	1.5
Trichoptera	Hydropsychidae	<i>Hydropsyche bulbifera</i>	8.5	1.5
Trichoptera	Hydropsychidae	<i>Hydropsyche lobata</i>	8.5	1.5

## Annex II – Manuscript

This manuscript has been prepared and submitted to the journal Avian Research and therefore follows its structure guidelines. The version presented here is the one submitted for revision, however, it has since been peer-reviewed and accepted for publication in the same journal. Please find the published version in <https://doi.org/10.1016/j.avrs.2022.100073>.

### Feeding ecology of a highly aerial bird during its long breeding season

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### Abstract

Pallid Swifts, as other swifts, are birds extremely adapted to an aerial lifestyle, showing unique adaptations that allow them to fly almost continuously. The diet of these non-stop high-altitude aerial birds has been mostly studied through techniques that fail to produce high taxonomic resolution identifications, and for that have been replaced by avant-garde molecular techniques, as DNA metabarcoding. Faecal samples were monthly collected during the breeding season, from a Pallid Swift colony in the north of Portugal. DNA from the faecal samples was used to sex the birds and to identify the arthropods present in the faecal samples through metabarcoding. From the prey items detected, 74 families were identified belonging to 16 orders, with Hymenoptera and Hemiptera being the most consumed prey orders. There were seasonal variations in diet richness, composition and prey size. Regarding the diet of males and females, although no differences were found

between the diet of males and females in composition and richness, there were differences in the size of arthropods preyed by the different sexes, with males feeding on larger arthropods. The large seasonal variation in Pallid Swifts' diet during the breeding season is likely a result from spatiotemporally variation of aerial prey, which must have been opportunistically predated by swifts. Although no significant differences were detected in diet richness and composition between sexes, the fact that males consume larger prey may suggest the existence of sexual dietary segregation in this group of birds. Our results show how species appear to shape their feeding composition, likely in response to changes in the environment and the food availability, according to optimal feeding theory. At last, several pest species were found in these swifts' diet, which, if studied through metabarcoding, can be used to monitor small arthropods, including airborne pests.

**Keywords:** Aerial foraging, *Apus pallidus*, Dietary seasonal variation, Feeding ecology, Metabarcoding, Optimal foraging theory.

## 1. Introduction

Swifts (Apodiformes: Apodidae) are insectivorous birds extremely well-adapted to an aerial lifestyle, displaying unique morphological, behavioral, and physiological adaptations to this distinct way of living (Henningsson & Hedenström, 2011; Neumann & Neumann, 2016; Sachs, 2017). These traits allow these highly mobile and fast birds to fly continuously during the nonbreeding period (Hedenström et al., 2019; Hedenström et al., 2016; Liechti et al., 2013). During the breeding period, collection of material to build nests, sleeping, mating, drinking and invertebrates' foraging occurs during flight (Hedenström et al., 2016; Henningsson et al., 2009; Orłowski & Karg, 2013; Rattenborg, 2006). The Pallid Swift (*Apus pallidus*) is one of the most abundant breeding species of swifts through its breeding range, restricted to the Mediterranean region, Asia Minor, and adjoining areas (Chantler & Driessens, 2000; Keller et al., 2020), with some resident populations in Niger, Chad, and Egypt (BirdLife International, 2022). Usually, this species nests in either natural landscapes, using caves and cliffs, or in urban areas, using preferentially cavities under the eaves or ceilings of tall buildings (Antonov & Atanasova, 2002; Cucco & Malacarne, 1987; Thibault et al., 1987). The Pallid Swift often lays a second clutch in summer, leading to a long reproductive period that extends into autumn (Antonov & Atanasova, 2001; Boano & Cucco, 1989; Cramp, 1985).

Some previous studies have analysed the diet of the Pallid Swift through the visual inspection of faeces and food boluses delivered to nestlings (Cristiano et al., 2018;

Cucco et al., 1993; Finlayson, 1979; Malacarne & Cucco, 1992) and concluded that the diet of this species was mainly composed of arthropods belonging to the orders Coleoptera, Diptera, Hemiptera and Hymenoptera. Nonetheless, there is a particularly notorious lack of knowledge of this species' diet in its expanding Northern distribution range, namely through the Iberian Peninsula, where it is very abundant (Keller et al., 2020). Although the diet of some swift species has already been studied, dietary differences between sexes have never been considered. Sexual dietary segregation has been described in several animal groups (Borrell et al., 2011; Mata et al., 2016; Mramba et al., 2017), namely in birds (Catry et al., 2016; da Silva et al., 2020; Massaro et al., 2020). These differences are usually the result of either sexually marked morphological traits, or differences in parental care (Catry et al., 2016; Lewis et al., 2005). Since Pallid Swift's parental care is shared by females and males (Finlayson, 1979; Malacarne et al., 1992) and this species has no sexual dimorphism, no significant differences between the diets of males and females should be expected. However, there are records of birds without major morphological sexual dimorphism, but with differences in their diet composition (da Silva et al., 2020). Moreover, the morphological dietary analyses performed have many limitations: either they are very invasive methods, or depend on samples that are already very digested, making visual identification of the prey quite difficult and sometimes even impossible (Chung et al., 2021). Therefore, these traditional approaches have been recently replaced by more avant-garde procedures, such as DNA metabarcoding (Chan et al., 2020; Chung et al., 2021). Although presenting some limitations (Taberlet et al., 2012), the ability to provide high taxonomic resolution of the consumed species (Gibson et al., 2015; Jackson et al., 2014) and avoid the requirement for prior knowledge of the identified prey (de Sousa et al., 2019) makes this technique a good tool for diet assessment. Despite its broad application and its potential to unravel swifts' ecology, to date has not been applied to assess the diet of any of the Palearctic region's swift species, whose populations, however, have been the subject of other biological and ecological studies (Cibois et al., 2022; Hedenström et al., 2019; Kearsley et al., 2022).

In this study the feeding ecology of Pallid Swift was analysed during its breeding season in the North of Portugal using DNA metabarcoding on faecal samples. We aimed to assess and characterize the temporal variations in the diet during the breeding season and evaluate whether differences exist between sexes, also molecularly determined, regarding prey richness, niche width, prey size, and diet composition. Finally, we compared our results with those obtained by other swift dietary studies (Chung et al.,

2021; Hespeneide, 1975), namely Pallid Swifts (Cristiano et al., 2018; Finlayson, 1979; Malacarne & Cucco, 1992).

## 2. Materials and Methods

### 2.1 Study area and Field Sampling

The studied Pallid Swift colony is located in the arcades of the Vila Nova de Famalicão City Hall (41.4100°, -8.5203°), Braga, Portugal. The field sampling was conducted during the 2021 breeding season, from June to October. Birds were monthly captured with mist nets or by hand. Cotton bags, from which the droppings were collected, were sterilized with 10% bleach for 1 hour and washed after every use to minimize contamination. Droppings were transferred directly from the bags to 2 ml tubes with 98% ethanol, and stored at 4°C until laboratory processing (da Silva et al., 2019).

### 2.2 Laboratory procedures

DNA was extracted from bird droppings using the Norgen Stool DNA Isolation Kit, following the manufacturer's protocol. DNA extraction was carried out in batches of 23 samples plus one negative control in which no faecal sample was added. The extracted DNA and the negative controls were distributed in 96-well plates where the last well was left empty for PCR negative control.

Invertebrate prey items were amplified using the COI primers fwhF2 (5'-GGDACWGGWTGAACWGTWTAYCCHCC-3') and fwhR2n (5'-GTRATWGCHCCDGCTARWACWGG-3') (Vamos et al., 2017), modified to contain Illumina adaptors. This primer set was originally designed to amplify the DNA of freshwater invertebrates (Vamos et al., 2017), but also performs well in the amplification of terrestrial arthropods' DNA and the amplification of degraded DNA samples, such as faecal droppings (Elbrecht et al., 2019; Mata et al., 2021). The PCR was carried out in volumes of 10 µl, comprised of 5 µl of Multiplex PCR Master Mix (Qiagen), 0.3 µl of each 10 pM primer, 2.4 µl of H<sub>2</sub>O, and 2 µl of DNA extract. Cycling conditions consisted of a 15 min period at 95°C, 45 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 50°C, and 30 sec extension at 72°C, and a final extension period of 10 min at 60°C. Amplification success was checked by visually inspecting 2 µl of each PCR product on a 2% agarose gel stained with GelRed. The resulting extracted DNA was cleaned using a 1:0.8 ratio of AMPure XP beads (Beckman Coulter, High Wycombe, UK) according to the manufacturer's instructions with the exception that 80% ethanol was used instead of

70%, and eluted in 25  $\mu$ l of 10 mM Tris, pH 8.5. Clean PCR products went through a second PCR reaction to incorporate 7bp long indexes and P5+P7 Illumina adaptors. This second PCR was carried out in a total volume of 14  $\mu$ l, comprising 7  $\mu$ l of KAPA HiFi HotStart ReadyMix, 2.8  $\mu$ l of cleaned PCR, 2.8  $\mu$ l of H<sub>2</sub>O, and 0.7  $\mu$ l of each adaptor. Cycling conditions were 3 min period at 95°C, 10 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 55°C, and 30 sec extension at 72°C, and a final extension period of 5 min at 72°C. Indexed samples were again cleaned and then pooled at equimolar concentrations and sequenced in a MiSeq run (500 cycles) together with samples from other projects.

The Pallid Swifts' sex identification was performed by amplifying a small amplicon of the Z and W chromosomes using P2 (5'-TCTGCATCGCTAAATCCTTT-3') and P8 (5'-CTCCAAGGATGAGRAAYTG-3') primers (Griffiths et al., 1998). This primer set was designed to amplify fragments of the (CHD) gene, providing distinct banding patterns on an agarose gel as a result of intronic regions within this gene (Çakmak et al., 2017; Griffiths et al., 1998; Quinn et al., 1990). The PCR was carried out in volumes of 11  $\mu$ l, comprised of 5  $\mu$ l of Multiplex PCR Master Mix (Qiagen), 0.4  $\mu$ l of each primer, 2.2  $\mu$ l of H<sub>2</sub>O, and 3  $\mu$ l of DNA extract. P2 primers were labelled with the fluorescent dye FAM. Cycling conditions consisted of a 15 min period at 95°C, 20 cycles of 35 sec denaturation at 95°C, 45 sec annealing at 45°C, and 45 sec extension at 72°C, followed by more 25 cycles of 35 sec denaturation at 95°C, 45 sec annealing at 47°C, and 45 sec extension at 72°C and a final extension period of 10 min at 60°C. PCR products were checked on an agarose gel, and, although amplification was successful, it was not possible to visually separate the different amplicons. Therefore, PCR products were separated by capillary electrophoresis using the automatic sequencer ABI 3130xl Genetic Analyzer. Fragments were scored against Genescan-500LIZ size Standard, using GeneMapper version 4.1 (Applied Biosystems). Male individuals showed a single fragment with about 370bp length, while females showed an extra fragment of about 380bp. Each sample was sequenced three times, and sex was only assigned to samples that provided congruent results for at least two sequencing results. This should avoid false assignments resulting from allelic drop-out of the CHD-W (van der Velde et al., 2017), that can be particularly common in degraded DNA samples like bird droppings (Mitchell et al., 2012).

## 2.3 Bioinformatic analysis

Bioinformatic processing of generated Illumina reads was done using the R package Metabarcoding Joining Obitools & Linkage Networks In R (MJOLNIR: <https://github.com/uit-metabarcoding/MJOLNIR>). The two paired-end Illumina reads'



alignment was done using 'illumina-paired-end' from OBITools (Boyer et al., 2016). After paired-end sequences were merged, each sequence was annotated with its corresponding sample. This step was carried using 'ngsfilter', which was also used for primer-removal. In addition, the data was filtered to select fragments of 190-220 bp by applying the command 'obigrep'. All these steps were carried out simultaneously using the 'mjoinir2\_FREYJA()' function. Chimeric sequences from the individual sample files provided by FREYJA were removed using the 'mjoinir2\_HELIA()' function. This function uses the uchime\_denovo algorithm implemented in VSEARCH (Rognes et al., 2016) and after chimera removal joins all the samples. After this merge, reads were clustered using the 'mjoinir4\_ODIN()' function, which uses SWARM (Mahé et al., 2015) to delimit OTUs, based on linkage-networks created by step-by-step aggregation. Finally, to reduce the number of erroneous OTUs (e.g., retained PCR artifacts, sequencing errors, pseudogenes, etc.) and thus achieve more realistic biodiversity metrics, the 'mjoinir2\_LOKI()' function was used. This function uses the LULU (Frøslev et al., 2017) algorithm, which merges similar and highly occurring OTUs (identity higher than 84% and co-occurrence levels higher than 95%).

The taxonomic assignment of the haplotypes was reached using BOLDigger v1.2.5 (Buchner & Leese, 2020). This compares the OTUs to the BOLD Identification System database. The retrieved taxonomic assignment matches were then manually curated. When different OTUs matched a single taxon, these were condensed into a single taxonomic unit. In case an OTU matched different species, genera, or families at a similar identity level, this was assigned to the most inclusive taxonomic rank. OTUs assigned to higher taxonomic levels than species and with more than 98% of similarity, were clustered with a neighbour-joining tree (Mata et al., 2018) into distinct OTUs (e.g., Nabidae 1, Nabidae 2, etc). OTUs assigned to items that are not part of the insectivorous diet of swifts (e.g., fungi, protists, platyhelminths, birds, mammals, etc.) were categorized as "Not diet" and discarded from the analysis. The number of reads per OTU in the extraction and PCR blanks was subtracted to the associated samples to rule out possible sources of laboratory contaminations. Only samples with more than 100 reads of dietary items were considered and OTUs comprising less than 1% of the total dietary reads per sample were discarded.

## 2.4 Data analysis

All statistical analyses were performed on R v4.1.1 (R Core Team, 2020). Statistical significance was considered at  $\alpha = 0.05$ . Dietary analysis was based on the OTU presence or absence per sample.

The effect of sex, month, and their interaction on the number of prey taxa detected in each dropping was tested using a generalized linear model (GLM) with a Poisson distribution and a log link-function, with the base function 'glm'. To test for differences in prey size consumption between sexes and months, we conducted a literature search to characterize the size of each prey item detected. Only prey items identified to genus, species complex and species were considered, as it is not feasible to correctly assign a size to an arthropod family or order. Prey items used in this analysis included 85% of the OTUs and 85% of the predation events, thus covering the majority of the diet. The prey sizes obtained can be found in Appendix Table S1. The average prey size of each sample was further calculated and used as a response variable in a GLM with a Gamma distribution and an inverse link-function to test the effect of bird's sex, month of capture, and their interaction, applying the base function 'glm'. Explanatory variables significance was tested in these models using the 'Anova' function from package car (Fox & Weisberg, 2019). Pairwise comparisons were performed to identify in which pairs the observed differences occurred, using the function 'emmeans' of the emmeans package (Lenth, 2022). To evaluate the effect of the ingested diversity on the size of the ingested prey, a generalized linear mixed model (GLMM) with a Poisson distribution and a log link-function was done with the function 'glmer' of the lme4 package (Bates et al., 2014), using month as random variable. This was done since the average prey richness is expected to vary across months.

The overall prey richness consumed i.e., niche width, was estimated by sexes, and month using rarefaction curves based on Hill numbers with the function 'iNEXT' of the iNEXT package (Hsieh et al., 2016), with the triple of the lower reference sample size to minimize extrapolation bias (Chao et al., 2014). Significant differences were considered if the 95% confidence intervals between groups did not overlap.

The package vegan (Oksanen et al., 2020) was used to evaluate differences in prey composition among sexes and months. First, a pairwise distance matrix using the Jaccard dissimilarity index was calculated with the function 'vegdist'. This matrix was then tested using a Permutational Multivariate Analysis of Variance (PERMANOVA) with the Binomial method and 99999 permutations using the 'adonis' function. To identify the months that differed from each other, a pairwise PERMANOVA was performed. Similarity percentages were also calculated to determine the contribution of different prey groups to the observed differences in variables, using the 'simper' function.

### 3. Results

### 3.1 Diet description

During the Pallid Swift monitoring a total of 226 bird captures occurred, and 82 faecal samples were collected. After sequencing and bioinformatic processing, only 65 samples successfully produced dietary data. Of these 65 faecal samples, 29 were identified as females, 33 as males, and in 3 samples it was not possible to reliably assign the sex of the bird.

We identified 139 OTUs in the diet of the Pallid Swift, belonging to 74 different families and 16 orders. The most commonly observed OTU was *Tetramorium forte* (32% of the samples), followed by *Lasius 1* (25%). Formicidae and Pentatomidae were the most frequent families, 62% and 20% respectively, and also represented the most detected orders Hymenoptera (66%) and Hemiptera (58%) (Appendix Table S1). The five most common orders were present throughout all the months, except for the order Psocodea which was not found in the samples collected in October (Fig. 1). In June, a substantial part of the prey interactions (46%) belonged to the order Hymenoptera, with almost all of these (96%) belonging to the family Formicidae. In July, the five most common orders were almost equitably represented in the samples collected, unlike in the other months. In August, 65% of the arthropods consumed belonged to the orders Hymenoptera and Hemiptera. Finally, the faeces collected in September and October, presented a very distinct composition. Whereas in September the orders Coleoptera and Hemiptera comprised more than 70% of the diet; in October these orders only represented 18%, with the vast majority of the diet (>70%) being composed by Diptera.

Additionally, 17 predation events (5.7% of the total) of 11 pest species were detected, mostly agricultural pests (Appendix Table S1). The most common pest was *Geomyza tripunctata*, which was found in 6% of the samples.

### 3.2 Prey richness and niche width

We found no differences between sexes in the number of prey items detected per sample (LR Chisq = 0.199, df = 1, p-value = 0.656). With regard to temporal variation, differences were found in the average number of prey items detected per sample (LR Chisq = 14.634, df = 4, p-value = 0.006; Fig. 2a). The significant differences were observed between June and July (z-ratio = -3.295, p-value = 0.008), the months with the lowest and highest average number of prey items per sample, respectively.

Overall diet richness, i.e., the niche width, was significantly different between months, but not between sexes (Fig. 2b). Regarding the temporal variation, there were no differences between months except for July that was the month with the widest niche

breadth, exhibiting more than double the average diversity values observed for the remaining breeding season. For sexes, although the differences between the niche width of males and females was not significant, males tended to ingest a higher number of prey taxa.

### 3.3 Prey size

Arthropods between 2-4 mm in size were the prey on which Pallid Swifts fed the most, with size classes 4-6 and 6-8 mm being the second and third most preyed classes, respectively. We found significant differences in the average size of prey consumed per sample, between sexes (LR Chisq = 4.871, df = 1, p = 0.027) and months (LR Chisq = 10.856, df = 4, p = 0.028; Fig. 3). Overall, males fed on larger prey than females. Regarding the temporal variation in prey size, Pallid Swifts in July preyed on smaller sized arthropods when compared to the remaining breeding season. In turn, September was the month in which larger prey were consumed, with significant differences only detected between these two months (t-ratio = 2.882, p-value = 0.044). A significant inverse relationship between the ingested diversity and the prey size was also found (Chisq = 4.7, df = 1, p-value = 0.030), with prey diversity increasing as preys becomes smaller.

### 3.4 Diet composition

The PERMANOVA revealed no differences in niche overlap between sexes (df = 1, pseudo-F = 1.215,  $R^2 = 0.018$ , p-value = 0.151), however, with respect to sampling month, significant differences were found in niche overlap (df = 4, pseudo-F = 2.952,  $R^2 = 0.171$ , p-value < 0.001). These significant differences were observed between all pairs of months except September and October (Appendix Table S2).

The compositional differences at OTU level found among the diets collected over the different five months were explained by the seasonal variation of the most frequently consumed OTUs (present in more than 10% of samples; Fig. 4; Appendix Table S3). The presence of *Tretamorium forte* and *Lasius* 1 in 68% and 58% of the samples collected in June, respectively, and the negligible presence or even absence of these OTUs in the other months, explained the significant differences between the diet composition of June and the rest of the breeding season. The differences between the composition of the diet consumed in July and in the other months were due to *Valenzuela flavidus*, since it was present in 54% of the samples collected in July, and barely present or absent in the remaining months. The compositional differences found between August and the other four months resulted from the presence of Elateridae 1 in 50% of the samples collected in this month and the absence of this OTU in the other sampled

months, with the exception of September, in which its presence was residual. In addition, *Tretamorium caespitum/impurum*, *Nabis* 1 and *Macroscytus brunneus* also contributed to the differences found between August and June. Finally, the marginal presence of *Nezara viridula* in June and August and the total absence in the July and October samples, but a dominant presence in the September samples (present in 67% of the faecal samples analysed), made this OTU the main responsible for the differences detected between the diets collected in September and the other months. Regarding October, the differences in diet composition between this month and the others but was explained by *Lonchoptera lutea* and *Scaptomyza pallida*, both present in 57% of the samples of this month (Appendix Table S3).

## 4. Discussion

Our results showed a marked seasonal variation in prey richness, size and composition throughout the breeding season, likely as a consequence of the seasonality of available arthropods, since aerial foragers rely on spatiotemporally unpredictable accumulations of aerial prey (Arbeiter et al., 2016). As swifts display an opportunistic feeding behaviour, massive arthropod assemblages lead to high intraspecific variability in these birds' diets throughout time (Cucco et al., 1993). Although no significant differences in diet richness and composition were noted between males and females, there were differences between the size of arthropods preyed by the different sexes.

Our results are similar with those found in other studies of Pallid Swifts. The study by Cristiano et al. (2018) concluded that Hemiptera and Coleoptera were the most consumed orders, while Malacarne and Cucco (1992) identified the orders Hemiptera, Hymenoptera and Diptera as the most important prey. Regarding Hymenoptera, the study conducted by Malacarne and Cucco (1992) and the one conducted by Chung et al. (2021), on the diet of House Swifts (*Apus nipalensis*), found the same pattern as we, with a predominance of hymenopterans consumed during the breeding season, being Formicidae (ants) the most prevalent family ingested. The seasonal variation was also in line to that found by Malacarne and Cucco (1992) where the vast majority of the predated arthropods in June belonged to the order Hymenoptera, while August and September were dominated by Hemiptera. The majority of Diptera was recorded in October and this marked presence of dipterans at the end of the breeding season as found by Chung et al. (2021). Unlike the other orders, Coleoptera did not show a clear seasonal variation and this pattern was also noted by Malacarne and Cucco (1992). The prey diversity detected cannot be compared with studies using visual identification due

to the much higher taxonomic resolution of metabarcoding (da Silva et al., 2019). Until now only Chung et al. (2021) used DNA metabarcoding to study the diet of Apodidae species, and for a similar sample size, the OTU and family richness of Pallid Swifts' diet was about twice that observed in House Swifts (Chung et al., 2021). Nonetheless, the differences in the richness between these two species must be looked carefully, because they are likely highly influenced by the use of different primer sets, which may amplify different taxa and have different arthropod identification resolution (da Silva et al., 2019).

The optimal foraging theory suggests that the natural selection promotes the most energy-efficient foraging pattern to maximise energy intake and increase fitness (Pyke, 1984). Ants are relatively weak fliers and can be found in dense aggregations during nuptial flights (Hespenheide, 1975; Levin et al., 2008), which reduces the required capture effort and energy expenditure for their predation justifying why swifts seem to choose ants to others arthropods present in the air column (Chung et al., 2021). In addition to the prey's ease of capture and its local density, the prey's selection also depends on its size (Hespenheide, 1975). Thus, larger, and therefore more energetically nutritious prey, should be preferred in order to maximise the energetic intake. The consumption of the largest prey was recorded in June and September, and, therefore, these were likely the months when optimal size prey items were more abundant, and, as a result, swifts appeared to concentrate on them, reducing prey richness and niche width. On the other hand, when ideal prey diminishes and environmental restrictions constrain consumers to suboptimal diets, predators often need to increase consumption rates, leading to more diversified diets (Simpson & Simpson, 1990). As in Malacarne and Cucco (1992), Pallid Swifts in this study fed mainly on prey with 2-4 mm, however in our study the second most consumed class was 4-6 mm while in their study was the 1-2 mm one. Nevertheless, the results from this study seem to fall between those obtained by Malacarne and Cucco (1992) in Piedmont and those obtained by Finlayson (1979) in Gibraltar, with the latter pointing arthropods between 4-6 mm in size as the most consumed by Pallid Swifts.

Sexual dietary segregation has been described in several animal groups (Borrell et al., 2011; Mata et al., 2016; Mramba et al., 2017), and particularly in birds (Catry et al., 2016; da Silva et al., 2020; Massaro et al., 2020). Marked morphological differences between sexes or differences in parental care during the breeding season are generally the drivers of this segregation. Although, to the best of our knowledge, dietary differences between sexes have never been studied in Apodidae species, the Pallid Swifts' biparental and the lack of sexual dimorphism led us to hypothesise that no relevant differences would be found between sexes. The results partly confirmed our initial hypothesis, since no

differences in diet richness or prey composition were found. However, there were significant differences in the size of consumed prey, with males preying on larger arthropods. The results obtained by compositional and prey size analysis were not congruent, since although no differences were observed in diet composition between sexes, there were differences in the size of the arthropods consumed. This non-compliance is likely related to differences in statistical power of both analyses, but a higher sample size would be desired to disentangle this incongruence. For a better understanding of swifts' diet, future studies should try to extend the sampling period across several years, assessing if the same pattern is observed overtime and integrate more bird colonies, to avoid possible local and regional effects. Ideally it would be also accessed swifts foraging areas and evaluate prey availability in these areas, to study in detail prey selection by these birds.

Our work also uncovered the presence of some arthropod pests in the diet of these swifts. In addition to agricultural pests, mostly responsible for crop damage, we also found an invasive forest pest, *Ctenarytaina spatulata*, a psyllid responsible for economic damage to *Eucalyptus* spp. plantations (da Silva et al., 2022). The ability of swifts, and other aerial feeders, to provide an important regulatory service by preying on invasive and pest arthropods has already been reported in previous research (Cristiano et al., 2018; Orłowski & Karg, 2013). Besides performing an important ecosystem service, swifts can also act as bioindicators. Biosurveillance has been enhanced by the application of DNA metabarcoding, which allows an early and quick detection (Montauban et al., 2021; Westfall et al., 2020), thereby enabling the mitigation of the damaging effects that invasive and pest species may have on ecosystems. Thus, monitoring swifts' diet can be a useful tool in ecosystem monitoring (Cristiano et al., 2018).

## 5. Conclusion

Overall, our results suggest a sharp seasonal variation in Pallid Swifts' diet during the breeding season, with almost the whole diet being comprised of arthropods belonging to the orders Hymenoptera, Hemiptera, Coleoptera, Diptera and Psocodea. The seasonal variation found in richness, niche width, size and composition of the Pallid Swifts' prey consumption likely resulted from the spatiotemporally changes of arthropods in the air currents. Although no significant differences were found in diet richness and composition between males and females, the finding that male birds feed on larger prey items than females, raises the question of whether birds such as swifts, with no sexual dimorphism

and with shared parental caretaking, may exhibit sexual dietary segregation. Our study also suggests that swifts may have a role in pest predation. Finally, this work highlights how species modify their food niche in response to changing environmental conditions. When environmental conditions were optimal and ideal prey were available, the Pallid Swifts focused on this prey, and, as a consequence, the diversity of their diet reduced. But as environmental conditions deteriorated and optimal prey were no longer available, they became more opportunistic and began to incorporate sub-optimal prey items into their diet, thus broadening their food niche, which resulted in a more diverse diet.

## **Authors' contribution**

LPS and VAM conceived the study. All authors carried the field work. SF performed the lab work and VAM the bioinformatics. Data analysis and interpretation was performed by SF and LPS with inputs from VAM. SF wrote the first draft with improvements and approval from all authors.

## **Ethics statement**

All work was carried under all the required legal requirements, namely ICNF (Portuguese Institute for the Conservation of Nature and Forests) ringing credential N° 134/2021.

## **Declaration of competing interest**

The authors declare that they have no competing interest.

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## Appendix A. Supplementary data

This is an excel document that was sent together with the manuscript when it is submitted to the Avian Research Journal.

**Table S1.** Size (mm) and frequency of occurrence (%FOO) of all genera, species complexes and species used in the prey size analysis. OTUs marked with  $\alpha$  consist in agricultural pests, while OTUs marked with  $\dagger$  are considered forest pests

**Table S2.** Pairwise PERMANOVA testing for the effects of sampling month on niche overlap. Significant values are at bold.

**Table S3.** Similarity percentage analysis of OTUs between pairs of months. Significant values are at bold.

## Figure captions

Fig. 1 – Frequency of predation events of mostly consumed orders by Pallid Swifts in each sampled month. "Other orders" represents unfrequently consumed prey orders: Lepidoptera, Araneae, Orthoptera, Neuroptera, Trichoptera, Entomobryomorpha, Isopoda, Julida, Odonata, Symphypleona and Thysanoptera.

Fig. 2 – Estimated prey richness (**A**) and niche width (**B**) of OTUs per sample for different sex and month classes. Niche width of different sexes and months was estimated with 87 and 18 sampling units, respectively. Whiskers represent 95% confidence intervals based on 1,000 bootstraps.

Fig. 3 – Estimated average prey size (mm) of OTUs for different sex and month classes. Whiskers represent 95% confidence intervals.

Fig. 4 – Bigraph displaying the OTUs ingested by Pallid Swifts per sampling month. Green links indicate OTUs consumed in significantly different proportions over the five months, obtained from the SIMPER analysis. The width of links is proportional to their frequency of occurrence within months. Month and prey boxes are proportional to the sum of the frequency of occurrence of all interactions in that month and across months, respectively. Only the most frequent OTUs (more than 10% of the average monthly frequency of occurrence) have their name displayed.



## Figures

Figure 1

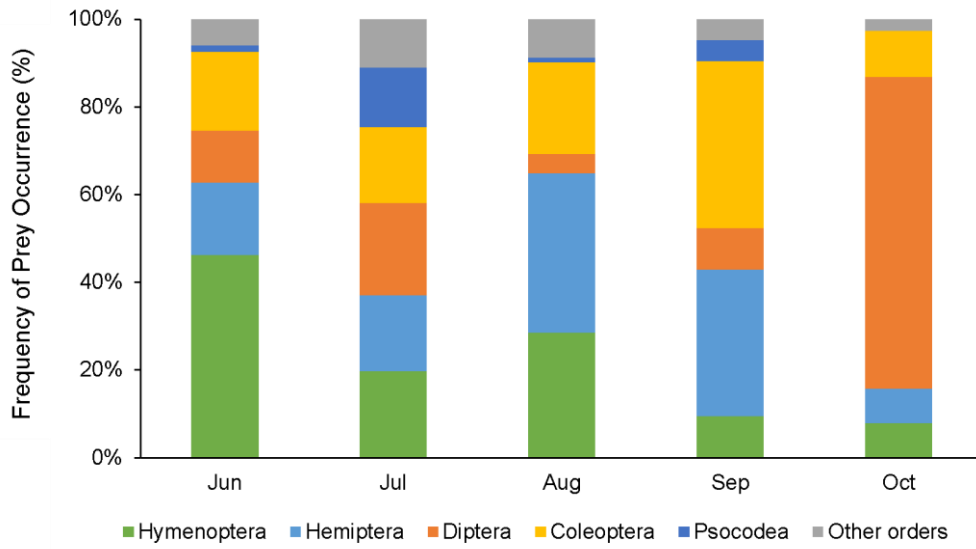


Figure 2

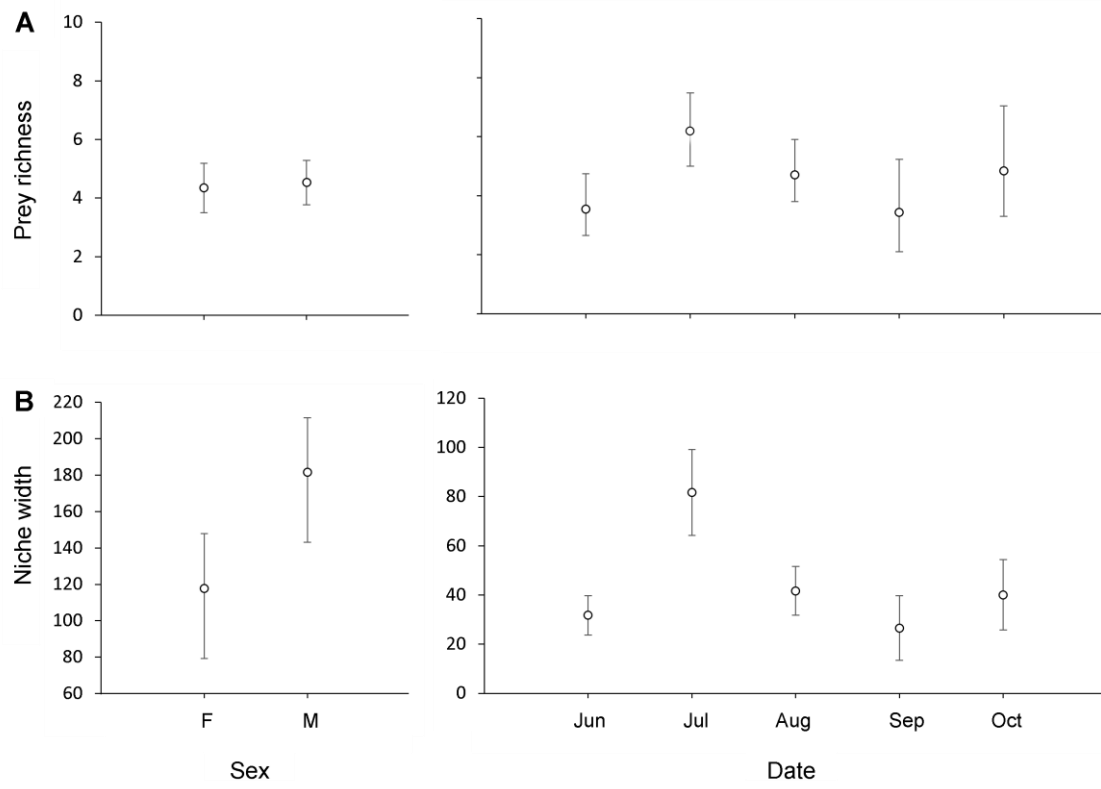


Figure 3

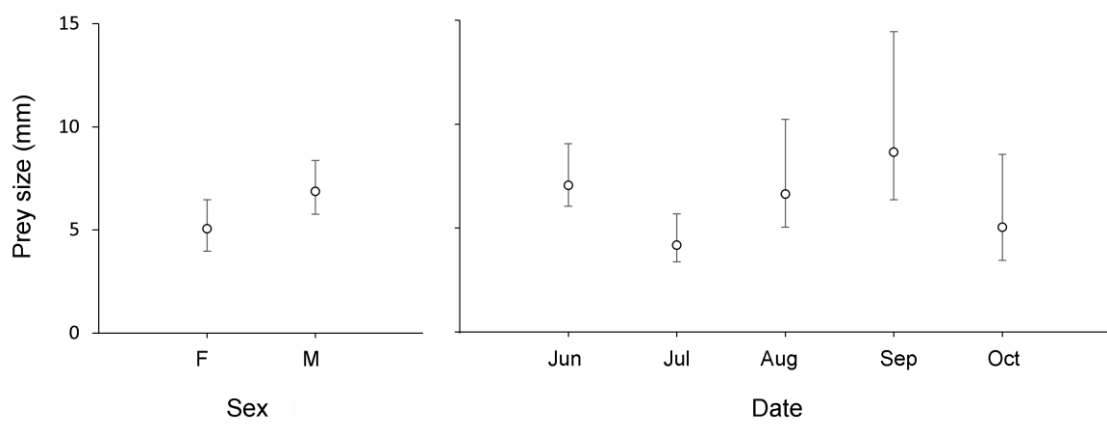


Figure 4

