UNIVERSIDADE D
COIMBRA

Catarina Santos Lopes

THE PHYSIOLOGY AND HEALTH CONDITION OF URBAN DWELLER GULLS IN INCREASINGLY URBANIZED AREAS

Tese no âmbito do Doutoramento em Biociências, especialização em Ecologia, orientada pelo Professor Doutor Jaime Albino Ramos e co-orientada pela Doutora Ana Marta dos Santos Mendes Gonçalves e pelo Professor Doutor Carlos Manuel Marques Palmeira e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Departamento de Ciências da Vida

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Resumo

Ao longo das últimas décadas, o crescimento populacional contínuo e exponencial tem vindo a potenciar o processo de urbanização, caracterizado por um aumento do número de pessoas a viver nas áreas urbanas como consequência de uma deslocação populacional de zonas rurais para áreas urbanas. O processo de urbanização tem vindo a transformar as áreas costeiras naturais em novos ambientes urbanos, afetando os processos e a dinâmica dos ecossistemas, e tendo efeitos complexos nas populações animais. As áreas urbanas colocam novos desafios à vida selvagem, tais como perturbação humana e interações com materiais resultantes da atividade humana, entre outros fatores de stress. Ainda assim, as áreas urbanas também criam novos nichos ecológicos onde algumas espécies têm conseguido sobreviver e até prosperar, principalmente devido à disponibilidade e previsibilidade de recursos alimentares antrópicos. Espécies generalistas, como a gaivota de patas amarelas (*Larus michahellis*) e a gaivota de asa escura (*Larus fuscus*) aumentaram exponencialmente a sua presença em áreas urbanizadas ao longo das últimas décadas, dependendo dos recursos alimentares de origem humana, nutricionalmente mais pobres, procurando alimento em lixeiras e em áreas urbanas, e interagindo cada vez mais com materiais antrópicos, com consequências fisiológicas e/ou adaptações de saúde desconhecidas. O objetivo principal desta tese é caracterizar as interações das gaivotas urbanas e naturais com materiais antrópicos, tanto através da sua incorporação em ninhos com da sua ingestão, perceber as consequências fisiológicas da ingestão de tais materiais e, por fim, desvendar os impactos de uma dieta tipicamente antropogénica na fisiologia e condição corporal das gaivotas. Os principais resultados realçam: 1) a dependência das gaivotas de recursos alimentares antrópicos e como as consequentes interações com materiais relacionados com a atividade humana podem constituir uma séria ameaça à saúde das gaivotas, pois alimentos de origem humana podem atuar como uma armadilha ecológica, com benefícios imediatos para as gaivotas, mas com possíveis consequências fisiológicas a longo prazo (capítulo 1); (2) a alta diversidade e quantidade de materiais antrópicos incorporados nos ninhos das gaivotas de localizações urbanas deve resultar de uma gestão deficitária do lixo em áreas urbanas (capítulo 2); (3) os altos níveis de materiais da atividade antrópica ingeridos pelas gaivotas nas colónias urbanas de nidificação e nas lixeiras, tal como a possibilidade de ingestão acidental de detritos enquanto procuram alimento em múltiplos habitats, indicam uma necessidade de uma melhor gestão do lixo (capítulo 3); (4) a dieta de baixa qualidade das gaivotas que usam habitats urbanos para procurar alimento, caracterizada por baixas percentagens de ácidos gordos fisiologicamente importantes, pode indicar uma suscetibilidade à inflamação induzida por uma dieta de baixa qualidade (capítulo 4); (5) uma dieta baseada em recursos alimentares antrópicos influencia negativamente a composição de ácidos gordos das gaivotas e altera parâmetros hematológicos, de stress e do metabolismo mitocondrial

(Capítulo 5). A quantidade e a variedade das interações das gaivotas com materiais de origem humana, assim como a dependência das gaivotas dos recursos alimentares antropogénicos são preocupantes e podem resultar numa exposição crónica a detritos e aos efeitos fisiológicos negativos de uma dieta de origem antropogénica. Assim, a redução global de desperdício de alimentos e da poluição por detritos dentro das áreas urbanas e das lixeiras, através da implementação de medidas de gestão de lixo apropriadas, combinadas com atividades de educação ambiental e campanhas de sensibilização são cruciais para reduzir o acesso das gaivotas a grandes fontes de alimento previsível de origem antrópica.

Abstract

Over the last decades, the continuous and exponential human population growth has been enhancing the urbanization process, characterized by an increase in the number of people living in urban areas because of a population shift from rural to urban areas. The process of urbanization has been transforming natural coastal areas into novel urban environments, affecting ecosystems processes and dynamics, and exerting complex effects on animal populations. Urban areas pose new challenges to wildlife, such as human-disturbance and interaction with anthropogenic debris materials, among other stress factors. Yet, urban settlements also create new ecological niches where some species have been able to survive and even thrive, mainly due to the availability and predictability of anthropogenic food resources. Generalist species, such as the Yellow-legged (*Larus michahellis*) and Lesser black-backed (*Larus fuscus*) gulls, grew exponentially in urbanized areas over the last few decades, relying on human-derived nutritionally poorer food resources, foraging in landfills and within urban settlements, and increasingly interacting with anthropogenic debris materials, with unknown physiological consequences and/or health adaptations. The overall aim of this thesis is to characterize the interaction of urban- and natural-dwelling gulls with anthropogenic materials both through incorporation into nests and ingestion, to understand the physiological consequences of ingesting such materials and, ultimately, to unravel the impacts of a typically anthropogenic diet on the gulls' physiology and health condition. The major findings highlight: (1) the reliability of gulls on anthropogenic food resources and consequent interactions with anthropogenic debris materials may pose a serious threat to gulls' health, as such human-derived food may act as an ecological trap, with immediate benefits for gulls, but also with possible long-term physiological consequences (Chapter 1); (2) the extremely high diversity and quantity of anthropogenic materials incorporated into gull nests from urban locations may be a consequence of poor garbage management in urban locations (Chapter 2); (3) the high levels of ingested anthropogenic materials in urban breeding locations and landfills, as well as the possibility of accidental ingestion of debris while foraging at multiple habitats, indicate a need for improved waste management (Chapter 3); (4) the low-quality diet of gulls using urban habitats to forage, characterized by low percentages of physiologically important fatty acids, may indicate a diet-induced susceptibility to inflammation (Chapter 4); (5) a diet based on anthropogenic food resources impairs gulls' fatty acids composition and alters haematological, stress and mitochondrial metabolism parameters (Chapter 5). The amount and variety of gulls' interactions with anthropogenic materials, as well as the gulls' reliability on anthropogenic food resources, are concerning and could result in chronic exposure to debris and to the negative physiological effects of a human-derived diet. Thus, the overall reduction of food waste and debris pollution within urban areas and landfills, through the implementation of proper

garbage management measures, combined with environmental education and social awareness campaigns are crucial to reduce gulls' accessibility to major sources of predictable anthropogenic subsidies.

Chapter 1

GENERAL INTRODUCTION

Part of this chapter is published as part of a book chapter:

Lopes CS, Laranjeiro MI, Lavers JL, Finger A, Provencher JF (2022) Seabirds as indicators of metal and plastic pollution. In: Seabird Biodiversity and Human Activities. Ramos JA, Pereira L (eds). CRC Press, Boca Raton, Florida, USA.

The world population has experienced continuous and exponential growth, especially over the last decades. In 1950, the world population was estimated to be 2.5 billion people, in 2020 that value grew to 7.8 billion, and it is expected to grow to about 9.7 billion in 2050 and 10.9 billion in 2100 (DESA 2019). Urbanization is the process by which the amount of people living in urban areas increases through a shift of population from rural to these urban areas (Figure 1.1). Nowadays, 56.2% of the world population lives in urban areas, which is also expected to increase to 68% by 2050 (DESA 2018). In response to this population increase, natural areas were and will be continuously transformed into cities, with all anthropogenic-related facilities, including the opening of landfills. This urbanization phenomenon has complex effects on animal populations (Shochat et al. 2010), including changes on habitat, food, predators, competitors and disease patterns, and ultimately on ecosystem processes (Marzluff 2001). Urban areas pose new challenges to wildlife, such as human-disturbance, interactions with anthropogenic debris materials, among other stress factors (Partecke et al. 2006). Yet, they also create new ecological niches in which some species have been able to survive and even thrive (Marzluff 2001). Some generalist species were able to grow exponentially in urban areas, with unknown physiological consequences and/or health condition adaptations. This general introduction intends to compile some of the effects of urbanization on animal populations, as well as the consequences of interacting with anthropogenic debris materials (a consequence of the urbanization phenomenon), focusing on urban gulls.

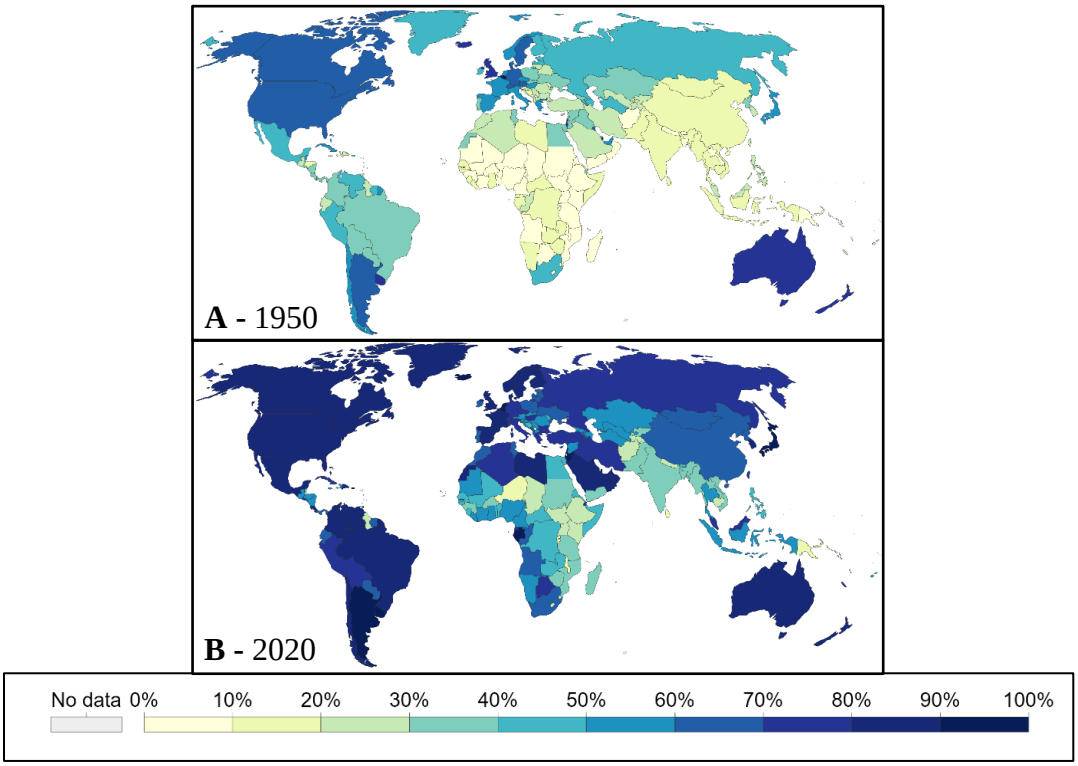

Figure 1.1. Share of the total population living in urban areas in **A)** 1950 and **B)** 2020. Source: Hannah Ritchie and Max Roser (2018) - "Urbanization". Published online at OurWorldInData.org. Retrieved from: https://ourworldindata.org/urbanization [Online Resource] in November 2021.

1.1. The novel urban habitats

Urban development and cities emergence translate into landscape transformations including the construction of residential housing, business buildings, roads for transportation, and the conversion of wildlands to agricultural fields for cultivating food (Luniak 2004), which threaten wildlife through the reduction and fragmentation of natural habitats (Marzluff 2001). Many other novel challenges are faced by wildlife due to urbanization including high levels of human disturbance, the replacement and fragmentation of natural vegetation by anthropogenic structures, the presence of non-native predators, introduced pathogens and diseases, collision with vehicles and predation by domestic animals (Bradley and Altizer 2007; Lowry et al. 2013; Loss et al. 2014). Also, urban habitats are characterized by high levels of various types of pollution: air, artificial light at night, noise, chemical (McKinney 2002; Nordt and Klenke 2013) and anthropogenic debris materials (Galgani et al. 2015; UNEP 2016; Plaza and Lambertucci 2017). Facing these challenges, animals either adapt to urban ecosystems or move away from such environments (McKinney 2002). Many bird, mammal and amphibian species are known to be negatively affected by urbanization (Marzluff 2001; Bateman and Fleming 2012). However, some species succeeded in adapting to urban live, being capable to survive and thrive, with some species even being more successful in urban areas than in their natural habitats (Luniak 2004). Urban favourable features that allow animals to favour urbanized landscapes over their traditional / natural areas include abundant and predictable food availability, lower predation pressure, warmer temperatures (known as the 'urban heat island effect', Grimm et al. 2008), vegetation complexity, novel structures to nest, and ultimately artificial illumination (Seress and Liker 2015).

Wildlife responds differently to urbanization and several authors categorized different group types along the rural-urban gradient, reflecting species' reaction to human activities (Blair 2001). The most recent categorization by Fischer et al. (2015) suggests group types based on the relative importance of natural and urban areas to population dynamics. 'Urban avoiders' includes species that rarely occur in developed/urban areas, 'urban utilisers' comprises species for occurring in developed/urban areas, but which are dependent on natural areas, and 'urban dwellers' encompass species for which persistence in urban areas is independent of natural areas (Fischer et al. 2015). Urban dwellers, often called synanthropes (Luniak 2004), are often totally reliant on anthropogenic resources, being well adapted to intensely modified urban environments.

A successful adaptation to urban environments requires a great ecological, demographic, physiological and behavioural adaptability by wildlife (Lowry et al. 2013), particularly a wide spectrum of habitat and diet requirements (Luniak 2004) including possessing high degree of feeding innovation, an opportunistic and generalist diet, large breeding ranges, fecundity and adult survival, and the capability of using a wide variety of foraging and breeding habitats (Møller 2009). Briefly, species richness (the number of species) decreases considerably with urbanization

(i.e. from non-urban areas towards the city centre), while species densities (the number of individuals of a certain species) increase following the same gradient, but usually only few superabundant species contribute to this increase (see Marzluff 2001 review for urban birds, Luniak 2008 for the urban fauna of Warsaw, Poland, and Bateman and Fleming 2012 review for urban carnivores). Towards urban centres, exotic species tend to replace the native species which are lost with urbanization (McKinney 2002), leading to a loss of diversity (Chace and Walsh 2006). Some urban-adapted species live at a much higher population densities when compared to their rural counterparts, due to spatial limitations of suitable sites, being responsible for reduced individual territories and increased levels of intra-specific aggression (Luniak 2004).

1.1.1. Effects of urbanization on animal's behaviour and life-history traits

A) Effects on behaviour, natural biorhythms and movements

Tameness toward humans is the basic barrier crossed by animals that benefit from urbanization, as coexistence with people is a condition for successfully dwelling in cities (Luniak 2004). Flushing responses, flight distances and the time it takes to a certain species to return to the nest after human disturbance are animals' behaviour parameters known to be considerably lower towards urban centres (Kenney and Knight 1992; Syrová et al. 2020), being a sign of habituation to human presence.

Animals' natural biorhythms are also susceptible to be altered in response to artificial lighting and anthropogenic noise of urban settlements. Some animals extend their foraging activity in the evening, due to artificial light, presenting nocturnal activity that was never observed in natural populations (Russ et al. 2015; Moll et al. 2018), disrupting animal behaviour, physiology and ecological interactions (Longcore and Rich 2004). Anthropogenic noise, in turn, interferes with the spread of acoustic information and is thought to be related with fitness and behavioural disruptions as some birds are known to start singing earlier in the morning when comparing to their natural conspecifics (Miller 2006; Fuller et al. 2007; Nordt and Klenke 2013). The milder microclimate and the high availability of food resources in urban habitats may affect animals' movement behaviour, allowing wintering in the city and reducing animals' migratory behaviour (Luniak 2004; Bateman and Fleming 2012).

B) Effects on reproductive success and survival

The non-migrating urban species have the ability to extend their **breeding seasons** by either anticipating their breeding onset (Schoech and Bowman 2001) or continue to breed in the winter (Luniak 2004). Earlier development of gonads (Partecke et al. 2004) and more re-nesting

situations (Jerzak 2001) of urban animals when comparing to rural conspecifics, are known to be consequences of urban breeding. Favourable foraging and nesting conditions in the cities allow increased reproductive success in urbanized areas, however this relation is not consistent as for some species it either decreased or did not change in relation to urbanization (Marzluff 2001). This may reflect the species' adaptability to urban areas and how they can benefit from the available resources. The higher the species' capability to adapt to urban environments, the higher their survival (Luniak 2004; Bateman and Fleming 2012). Urban populations may indeed have a greater longevity than rural populations (Luniak 2004), yet birds and mammals dwelling in urban areas are more prone to be victims of collisions with traffic and wires, which affects their survival (Loss et al. 2014).

C) Effects on diet and foraging behaviour

The main key factor for animal populations to survive and thrive in urban habitats is the availability and accessibility of food resources (Marzluff 2001). Urban environments provide a range of anthropogenic food sources from different origins situated within and around urban areas such as: waste food from landfills and refuse dumps, bird feeders, crop leftovers, discarded fast food, stolen food directly from people in parks and recreational areas and from trash containers, restaurants terraces and fast-food outlets (Belant 1997; Oro et al. 2013). In urban habitats, these human-derived food resources are readily available, abundant and predictable, favouring generalist and opportunistic animals which, in response to such availability of food, are known to alter their diet, distribution, activity patterns, foraging behaviour and densities (Oro et al. 2013; Parra-Torres et al. 2020). Indeed, urban animals are known to present a higher consumption of anthropogenic food, mainly provided by households, which confers a more diverse diet (Contesse et al. 2004; Murray et al. 2015), expanded foraging activities to urban locations (Murray et al. 2015) and lower foraging time but handling of more food per hour, suggesting an increased foraging efficiency of urban animals when comparing to their rural counterparts (Fleischer et al. 2003). Higher food availability in urban habitats (including from landfills) is suggested to reduce animals' starvation risk, buffering urban animals against the seasonal fluctuations in resource availability experienced in natural habitats, and to enhance reproductive success, eventually leading to higher population densities (Ross 2004; Fuller et al. 2008; Lowry et al. 2013).

Although anthropogenic food sources seem to be effectively utilized by adult birds, there is evidence that these resources are detrimental to nestlings because they are typically of poorer nutritional quality than natural food items, which may lead to a reduced growth rate and body condition (more details in section 1.1.2). Urban nestlings were reported to be smaller and nutritionally deficient (Heiss et al. 2009), with a lower assimilation of proteins and a higher probability of experiencing diseases (Murray et al. 2015) in relation to their rural conspecifics.

D) Effects on physiology and health condition

Animals that are capable to adapt to urban landscapes and to exploit anthropogenic food resources undergo physiological adaptations to survive and even to thrive in urbanized areas. Urbanization is accompanied with many stress factors and, therefore, the main physiological response investigated in the urbanization context is stress physiology (i.e. oxidative stress and corticosterone, the stress hormone, Isaksson 2018). Air pollutants typical from urbanized locations act as prooxidants, causing damage to protein, lipids and DNA, unless they are detoxified by protective antioxidants. The first response to these pollutants is the increase in levels of antioxidants that may or may not be sufficient to avoid oxidative damage, a parameter typically used as a biomarker of poor health (Salmón et al. 2018). Thus, differences in stress hormone levels and oxidative damage between urban and rural individuals are expected, but such relation is not straightforward, and it is not well understood (Shochat et al. 2010). Studies report lower levels of corticosterone (Partecke et al. 2006) and lower oxidative damage (in Blue Tits *Cyanistes caeruleus*, Isaksson et al. 2017) for urban birds when comparing to their rural conspecifics. This may be a possible effect of downregulation of birds' physiological stress response to allow them to endure the stressful urban environment. Contrarily, the same study (Isaksson et al. 2017) also detected increased levels of oxidative damage for urban House Sparrows (*Passer domesticus*) and Tree Sparrows (*Passer montanus*) when compared to their rural counterparts, indicating differences across urban-dwelling species.

Briefly, other physiological impacts that may be caused by urbanization include the shortening of telomeres (the chromosomes' protective ends that typically shorten throughout an individual's life, but this process can be faster with exposure to stress, as reported by Salmón et al. 2016), hormonal changes (some stress and reproductive hormones are likely to be more stimulated due to urban features, causing behavioural and other physiological responses on individuals, review by Bonier 2012), among others (reviews by Shochat et al. 2010 and Isaksson 2018). Some simple physiological parameters whose mechanisms of variation are well understood and known to characterize different biological functions of an organism, can be measured to address the general health condition of an individual, as well as to compare urban *vs.* natural individuals' physiology. For instance, increased number of White Blood Cells (WBC), and the ratio heterophils/lymphocytes (H/L) may be often associated with chronic stress and infection (Fokidis et al. 2008).

Urbanization appears to be responsible for negative fitness-related effects on animals, but many benefits are also noted, which are species- and context-dependent (Birnie-Gauvin et al. 2017). Urban ecology now begins to understand how the complexities of biodiversity are affected by humans' presence.

1.1.2. Opening of landfills as a consequence of urbanization

Alongside the urbanization phenomenon we witnessed the rise of the "throwaway society" during the latter half of the 20th century, which led to the opening of many landfills and refuse dumps to dispose the increasing amounts of household waste (Belant et al. 1998; Plaza and Lambertucci 2017). In 2012, Hoornweg and Bhada-Tata (2012) estimated global waste production to be 1.3 billion tonnes per year. In 2016, such value increased to 2.01 billion tonnes per year, and it is expected to grow even more to reach 3.40 billion tonnes yearly by 2050 (Kaza et al. 2018). As a result, landfills create an abundant and predictable new food source (Oro et al. 2013), with huge amounts of human-derived "junk" food which attracts multiple species of opportunistic mammals, reptilians, amphibians and birds (Belant 1997; Ramos et al. 2009, Figure 1.2). These infrastructures are distributed worldwide and are based on weekly cycles introduced by humans, triggering regular patterns and a scheduled behaviour for some species that exploit these new food resources (Oro et al. 2013). The high predictability in space and time makes anthropogenic food from landfills easier to access compared to natural sources (Bartumeus et al. 2010), reducing foraging times and energy expenditure (Fuirst et al. 2018), and improving fitness components as individuals use the available time for other life-sustaining activities (Lunn and Stirling 1985; Murray et al. 2018). In fact, there is evidence that the use of landfills by wildlife

Figure 1.2. Gulls (*Larus michahellis*, *L. fuscus* and *Chroicocephalus ridibundus*), white-storks (*Ciconia ciconia*) and cattle egrets (*Bubulcus ibis*) foraging and roosting at a landfill near Coimbra, centre of Portugal. Part of the waste available for birds to forage on and the landfill machinery are visible. © Catarina Lopes

generate impacts at both individual and population levels, with possible consequences to ecosystem functioning. For example, White Storks' (*Cicconia cicconia*) movement behaviour and home ranges were affected by the availability of landfills to forage on: they were reported to increasingly breed close to landfills (Tortosa et al. 2002), their wintering population stayed longer in breeding areas (Archaux et al. 2004) and finally they used their nests year-round instead of migrating (Gilbert et al. 2016). Positive effects of anthropogenic food subsidies on population density and size were reported by Olea and Baglione (2008) that described an increased colony growth of Rooks (*Corvus frugilegus*) strongly correlated with the availability of landfills, and by Craighead (1998) that stated increased mortalities and home range areas of Grizzly Bears (*Ursus actus*) after landfill closure.

In contrast, feeding on waste may also bring disadvantages. On one hand, it may affect predator-prey interactions as the high abundance of food sources can subsidize generalist predator populations beyond what native prey can support, increasing predation risk that may even lead to extinction of native prey, a phenomenon described as hyper-predation (DeCesare et al. 2010) and reported by Voorbergen et al. (2012), as the growth of the Kelp Gull *Larus dominicanus* population was responsible for increased predation pressure on a near-threatened Cape Cormorant *Phalacrocorax capensis* population. On the other hand, foraging on landfills may weaken individual's health through 1) the ingestion of anthropogenic materials, such as plastics, glass and metals (Lopes et al. 2022), 2) the bioaccumulation of heavy metals (de la Casa-Resino et al. 2014), 3) the quality of the food itself (usually energy denser, but nutritionally poorer than naturally available food (Pierotti and Annett 1990, 1991)) and 4) the higher risk of interacting with environmental toxins, parasites and pathogens (Murray et al. 2019). This may be responsible for altering avian body composition and nutritional physiology by increasing cholesterol (Townsend et al. 2019), modifying composition of fatty acids (Andersson et al. 2015), suppressing immune function and metabolic rate (Isaksson et al. 2017), increasing body mass (Auman et al. 2008) and increasing oxidative stress (Herrera-Dueñas et al. 2017).

1.2. Anthropogenic debris materials pollution in marine, coastal and urban ecosystems

Anthropogenic litter (hereafter anthropogenic materials or debris materials), i.e. debris items of any non-natural solid material (e.g. plastic, glass, fabric, metal, paper, rubber, among others), is a well-known anthropogenic pressure on ecosystems throughout the world (Barnes et al. 2009; Galgani et al. 2015). These anthropogenic debris materials, particularly plastics, are extremely versatile, resistant and durable, making them suitable to generate a wide range of useful products (Andrady and Neal 2009), features that also make them a serious threat to the ecosystems

throughout the world (Galgani et al. 2015). Due to the large production, intense consumption, and rapid disposal, such materials are now widespread and ubiquitous in the environment, being even suggested as a geological indicator of the Anthropocene era (UNEP 2021).

It is estimated that 9.2 billion tonnes of plastic have been manufactured since the early 1950s, of which only <10% were recycled, ~14% were incinerated, while the rest (~76%) was deposited in landfills or in natural environments (UNEP 2021). In fact, in Portugal, from the ~5 million tonnes of municipal waste produced in 2019, only just over half million (~642.000 tonnes) were recycled whereas 2.5 million tonnes were landfilled (PORDATA 2020). Waste and ultimately anthropogenic materials are increasingly concentrated in convergence points of anthropogenic activity and areas of higher population density, such as urban settlements and in proximity to waste processing sites (i.e. landfills (Hurley et al. 2020)).

Whether in a river, an ocean or on land, most debris items do not break down into their chemical components. Instead, they are exposed to mechanical and chemical weathering processes (e.g. wind, waves, UV light) that break them into smaller pieces over time (Andrady 2017), being persistent materials in the environment for very long time periods (Lambert et al. 2014). Anthropogenic debris pollution represents a critical environmental, ecological and economic problem (Wilcox et al. 2015), and a direct threat for marine fauna (Kühn et al. 2015) and for several terrestrial animals (Plaza and Lambertucci 2017). Birds, particularly seabirds, are well-known to be susceptible to the ubiquitous and increasing presence of anthropogenic litter pollution (reviews by Battisti et al. 2019a for bird species and by Kühn and van Franeker 2020 for marine megafauna, particularly seabirds).

Given the widespread occurrence and increasing concentration levels of anthropogenic debris materials in the environment, one of the fundamental challenges is how to properly assess and monitor the increasing pollution and assess its effects on organisms (Ryan et al. 2009; Lamborg et al. 2014).

1.2.1. Anthropogenic debris materials exposure in birds

Debris pollution is known to cause either direct mortality or injury, and a range of sub-lethal effects to birds which may influence species' behaviour, physiology and survival, although subtle changes in individuals' health can be difficult to detect (Rochman et al. 2016; Roman et al. 2019a). The main impacts of debris pollution on birds arise from entanglement in large items and ingestion of smaller particles (Laist 1987; Kühn et al. 2015; Gall and Thompson 2015).

Entanglement in debris, such as lost or discarded fishing gear in the ocean, nets, ropes or plastic bags (Laist 1997), is known to injure birds and to decrease their ability to obtain sufficient food or to effectively avoid predators due to an impeded mobility (Derraik 2002; Kühn et al.

2015; Ryan 2018). Battisti et al. (2019a) reported that 58.5% of the studied bird species (n = 258) were entangled in anthropogenic litter and, considering only seabirds, Kühn and van Franeker (2020) reported 27.4% of all known seabird species (n = 409) entangled in marine debris; however, these numbers are certainly higher as many entangled seabirds may die in the ocean, far from land, and are not detected (Laist 1987).

Birds are also known to directly collect anthropogenic materials which are then incorporated in their nests (reviewed by Battisti et al. 2019a), a phenomenon particularly evident in areas with increased human influence, such as urban settlements (Jagiello et al. 2019). Incorporation of plastic strings, fishing nets and other threadlike plastics, as well as other anthropogenic materials in nests (Figure 1.3A) may lead to entanglement of chicks and adults, likely to cause direct injury or death (Votier et al. 2011). The presence of debris materials, in particular plastics, in birds' nests may also lead to a dermal absorption of potentially harmful chemicals (Verlis et al. 2014), interfering with birds' physiology and causing negative effects on reproduction, behaviour and survival (Herzke et al. 2016; O'Hanlon et al. 2017).

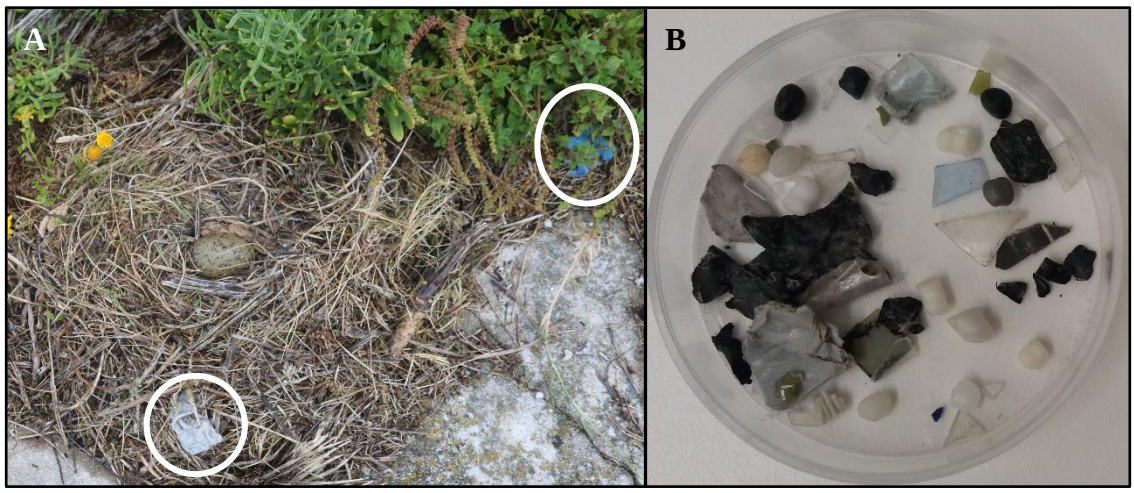

Figure 1.3. Examples of gulls' interactions with anthropogenic debris materials. **A)** Yellow-legged gull (*Larus michahellis*) nest from Peniche urban breeding colony, Portugal, with a blue plastic bag and a white fibre incorporated, pointed out with white circles. **B)** Plastic and rubber fragments found in a single Yellow-legged gull regurgitated pellet, collected within an urban breeding location, Porto, northern Portugal. © Catarina Lopes

The ingestion of indigestible anthropogenic materials (Figure 1.3B) by birds can cause stomach lesions and perforations, gastrointestinal blockage with the obstruction of food passage, reduced appetite caused by a false sensation of satiety, decreased dietary efficiency and consequently reduced growth, general debilitation often leading to death (Ryan 1988; Kühn et al. 2015; Roman et al. 2019a). Ingestion of debris is also likely to disturb the absorption and assimilation of nutrients (Gregory 2009), and birds may feed their chicks with large quantities of debris, particularly plastics (parental transfer; Cadée 2002; van Franeker et al. 2011; Verlis et al. 2013; Lavers and Bond 2016b, a). Battisti et al. (2019a) reported that 73.6% of the 258 studied

bird species ingested anthropogenic litter, while for seabirds, Kühn and van Franeker (2020) described that 44% of the 409 known seabird species ingested plastic, being Procellariiformes the best studied taxon with 41.5% of all individuals containing plastics in their digestive systems.

In addition to physical damage, debris materials, particularly plastics, may serve as vectors of plastic-associated (substances adsorbed by plastics due to their hydrophobic features (Mato et al. 2001)) and plastic-derived contaminants (chemical compounds added to plastics during their manufacture process (Hermabessiere et al. 2017)), and birds may be exposed to these chemicals either through direct ingestion (Padula et al. 2020), indirect exposure through diet (Herzke et al. 2016) or through the environment itself (Net et al. 2015). Such chemical components are likely to interfere with birds' health and physiology, including blood chemistry parameters (Lavers et al. 2019), fatty acid profiles (Puskic et al. 2019), and ultimately body condition and fitness (Lavers et al. 2014, 2019).

1.2.2. Anthropogenic debris materials pollution monitoring tools

The proportion of birds that 1) are found entangled in anthropogenic materials, 2) incorporate debris items on their nests and 3) ingest debris (directly or indirectly) are parameters that might be used to monitor debris pollution using birds. Quantifying entanglement rates and subsequent mortality can be difficult (Ryan 2018) but, despite not all bird species incorporate debris in their nests, monitoring anthropogenic materials in nests can be a rapid, non-destructive, and simple method to quantify the magnitude of debris pollution in the surrounding environment and the associated probability of entanglement (Tavares et al. 2016; Grant et al. 2018; O'Hanlon et al. 2019). Debris ingestion by birds can be assessed through both necropsies of intact birds and examination of food remains (Provencher et al. 2017). Necropsies may include beached birds, birds from human activities, such as fishery bycatch, and from rehabilitation centres. The major advantages of necropsies are the simplicity of collecting different tissues for complementary analysis (e.g. histopathological analysis) and the possibility of determining the entire debris burden, as well as birds' basal information such as age, sex, possible cause of death and body condition. However, necropsies are opportunistic, depending on the availability of deceased birds, and pre-planned sampling can be difficult (Provencher et al. 2019). Examination of food remains include regurgitations by water-offloading (lavage or flushing) or emetics that can be repeated on the same populations throughout time allowing local comparisons of the debris loads (Bond and Lavers 2013; Lavers et al. 2014). Although not all species can regurgitate, and thus a complete sample is not guaranteed, pellet collection is the simplest method allowing repeatability and regularity in sampling (Provencher et al. 2019). Also, analysing birds' guano can elucidate the

occurrence of microplastics in their system, as some items may be excreted *via* faeces (Provencher et al. 2018b; Bourdages et al. 2021).

It is extremely important to standardize sample collection, processing, debris quantification and reporting, as well as to establish long term-monitoring programs, in order to ensure data on entanglements, nest debris incorporation and ingestion of debris by birds can function as effective monitors of debris pollution.

1.2.3. Factors influencing the interactions between birds and anthropogenic debris materials

Many bird species use vegetation to build their nests and given the resemblance between some anthropogenic materials (e.g. threadlike plastics) and natural nesting materials, birds actively collect debris and incorporate them into their nests (Lavers et al. 2013; O'Hanlon et al. 2017; Battisti et al. 2019a). The prevalence of debris in bird nests is thought to be related to the availability of both debris and natural nesting materials in the surroundings of the breeding location (Bond et al. 2012; Witteveen et al. 2017).

Ingestion of debris by birds occur either accidentally while foraging on other prey items (i.e. indirectly by secondary ingestion) or directly by confounding anthropogenic materials with prey (Laist 1997; Cadée 2002). Foraging behaviour of seabirds is thought to be an important predictor of the incidence of debris ingestion, particularly plastics (Moser and Lee 1992; Avery-Gomm et al. 2013). Seabirds can be classified into three main foraging types: surface-feeders, plungers and pursuit divers (Ashmole 1971), and plastics are more concentrated at the water surface than in the subsequent layers of the water column due to buoyancy (Cózar et al. 2014; Reisser et al. 2015). Consequently, surface-feeders are more susceptible to interacting with debris than diving species that catch prey items beneath the water's surface and are less likely (but not completely immune) to ingest debris (Provencher et al. 2015; Baak et al. 2020). Species-specific regurgitation abilities (Ryan 1987) may also be responsible for variations in debris accumulation in birds' upper digestive system. Some species (e.g. skuas, albatross, gulls) are known to regurgitate indigestible prey remains, which may limit the accumulation of debris (Shealer 2002). Other species (e.g. fulmars, petrels, some auks) do not typically regurgitate indigestible prey due to a narrow passage between the proventriculus and the gizzard that prevent the returning of accumulated debris to the proventriculus, retaining ingested materials in the gizzard and acting as reservoirs (Ryan 1987; van Franeker and Law 2015).

1.3. Ecology of natural-, urban- and landfill-dwelling gulls

Animals that are extremely successful living in cities and exploiting landfills are the *Laridae* gulls (Figures 1.4 and 1.5). Gulls are generalist, highly opportunistic, and present plastic behaviour, being very flexible in exploiting novel foraging and nesting habitats (van Toor et al. 2017). Traditionally, gull species breed in islands or coastal areas and exploit many of the environments close to the sea. In the last decades, gulls have been nesting and dwelling in cities around the world and the term "urban gull" is now increasingly used by scientists, in detriment of "seagull". In fact, the first record of a roof-nesting gull was around 1894, when a European Herring Gull (*Larus argentatus*) nested on a roof in the Black Sea region, and, since then, gulls have colonized urban environments around the world, with significant populations in various cities from Europe, North America (Canada and USA) and Australia (for an overview see the book chapter Pais de Faria et al. 2022).

In Portugal, throughout the last decades, natural gull populations have been generally increasing (ICNF, unpublished data 2017), until this growth reached unsustainable levels of occupation, causing a lack of natural sites to nest, which led adult gulls, especially younger unexperienced breeders, to use non-preferred urban sites. Consequently, in the last years, gull species, mainly the Yellow-legged (*Larus michahellis*) and the Lesser black-backed (*L. fuscus*) gulls, have been increasingly dwelling in urbanized areas and landfills. These gulls seem to successfully adapt to these novel environments, which also include higher probabilities of interacting with anthropogenic debris materials. Nevertheless, the consequences of both dwelling in altered habitats and interacting with debris materials on gulls' physiology and health condition are unknown, and by assessing these impacts we are able to evaluate the environmental consequences of urbanization.

1.3.1. Gulls' urban nesting behaviour and the incorporation of anthropogenic materials in nests

Monaghan (1979) suggested that rooftop nesting provides a favourable alternative to natural breeding sites, with equal or even higher nesting quality for gulls than the traditional coastal or insular habitats. In fact, several advantages arise from the use of urban habitats to nest, such as the proximity to readily available anthropogenic sources of food that can be exploited at night due to street lighting (Rock and Vaughan 2013), warmer temperatures offered by the urban heat island effect (Pickett et al. 2011) and lower nest density, as usually each rooftop houses 1-2 nests, lowering intra-specific predation (Monaghan 1979; Vermeer et al. 1988). Urban habitats also present a higher availability of suitable nesting locations, including taller flat roofs from industrial, commercial, office or uninhabited buildings, but also bridges, pipelines, and other

suitable infrastructures (Hooper 1988; Raven and Coulson 1997). Gulls usually place their nest against some barrier, such as next to walls, chimneys stacks or any larger objects and structures (Vermeer et al. 1988; Zelenskaya 2019) which confers protection from extreme weather conditions. Roof-nesting offers protection for eggs, adults and offspring against ground predators (e.g. foxes), and provides a structural barrier that cannot be physically breached by any chicks that have not yet learned to fly (Kroc 2018).

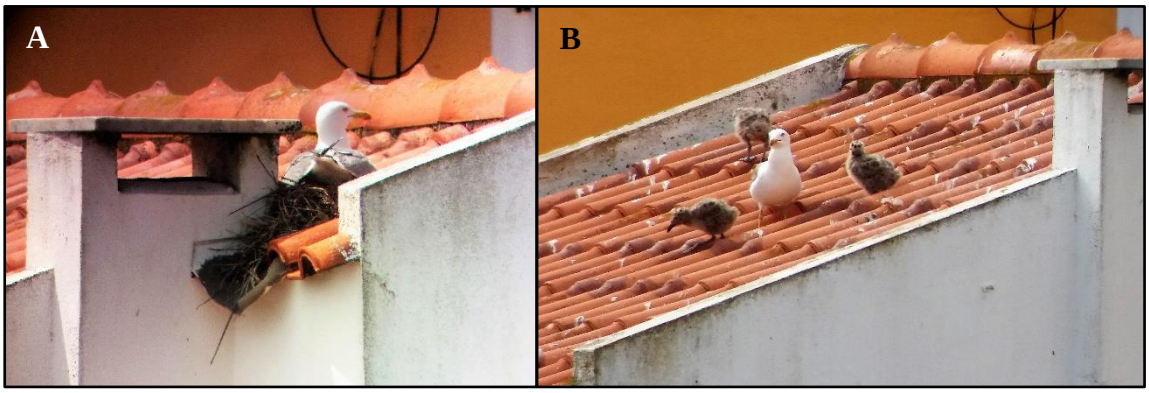

Figure 1.4. Urban Yellow-legged gull breeding pair nesting in an urban settlement, in the city of Peniche, Portugal. **A)** incubating its eggs on a rooftop nest built next to a chimney (May 25, 2018), and **B)** the same breeding pair, in the same rooftop, 11 days after photo A (June 5, 2018), with three hatched chicks. © Catarina Lopes

At the same time, urban colonies are often composed by younger and unexperienced breeders (Sydeman et al. 1991) that feed themselves and their offspring with lower quality human-derived food, which tends to decrease urban population breeding success. Also, urban nesting gulls are subject to widespread human disturbance, either through roof maintenance or active measures to control gulls and discourage nesting (e.g. removal of nests, netting, spikes, acoustic deterrents (Rock 2013)), avian predation (e.g. crows, Hooper 1988), incorporation of anthropogenic materials in nests, and exposure to several stress factors that may have physiological repercussions on their immune systems, oxidative stress, heart rate, body condition, reproductive output and behaviour among others.

Accordingly, comparisons of the reproductive success between urban and non-urban gull populations are contradictory, with reports of no differences, as well as some breeding variables being enhanced while others weakened, by nesting in urban settlements. For instance, some studies report higher fledgling success for urban gull populations compared with natural colonies (Monaghan 1979; Sellers and Shackleton 2011; Perlut et al. 2016; Kroc 2018; Zelenskaya 2019), while others described lower clutch sizes (Soldatini et al. 2008; Perlut et al. 2016; Kroc 2018) and hatching success (Pierotti and Annett 2001; Perlut et al. 2016). Hooper (1988), for instance, reported no differences on clutch initiation and size, incubation period, hatching and fledgling success between urban and non-urban gulls.

As a functional structure linked to breeding success (Mainwaring et al. 2014), a nest with anthropogenic debris materials incorporated may have a compromised functional performance, with potential alterations on its thermal properties, integrity, camouflage and/or drainage that may impact adults' and chicks' fitness-related traits (Deeming and Mainwaring 2015). Yet, the incorporation of debris materials by natural- and urban-nesting gulls, as well as its relationship with breeding success, are not known and these features may help understand the magnitude of the environmental effects of debris pollution.

1.3.2. Feeding ecology of natural-, urban- and landfill-dwelling gulls, and the ingestion of anthropogenic materials

Gulls from coastal areas or islands (non-urban populations) rely mainly on marine food resources (Tyson et al. 2015), including those derived from fishery discards (Calado et al. 2021). Non-urban individuals, however, are increasingly using terrestrial and man-made environments to forage, such as landfills, sewage outfalls and agricultural fields (Alonso et al. 2015; Gyimesi et al. 2016; Isaksson et al. 2016; Matos et al. 2018; Parra-Torres et al. 2020).

Figure 1.5. Urban Yellow-legged gull foraging on garbage bags placed outside the garbage bins, within the city of Porto, Portugal. © Catarina Lopes

Urban and landfill-dwelling gulls feed mostly on anthropogenic food remains (e.g. parts of meat, fish, chicken, fresh fruit, kitchen scraps, eggs, among others (Parfitt et al. 2010)) and refuse, including anthropogenic debris materials (Gyimesi et al. 2016; Seif et al. 2018; Battisti et al. 2019a), but they still rely on marine resources throughout the year (Pais de Faria et al. 2021a), which may help explaining the location of most urban gull colonies in cities near the coast (e.g. Huig et al. 2016; Spelt et al. 2019). In fact, the consumption of marine resources seems to be especially important during the chick rearing period, when adult gulls switch their diet from terrestrial to marine prey, to provide higher-quality food to their offspring (Alonso et al. 2015; Isaksson et al. 2016; Pais de Faria et al. 2021a). Accordingly, landfill use varies seasonally,

increasing throughout the breeding season and reaching its peak during the post-breeding season, after the chicks fledged (Ackerman et al. 2018).

The proportion of anthropogenic food items on adult and chick gulls' diets depends on the species, location and the resources available for them. Gulls relying on these anthropogenic resources typically have a more homogeneous diet (Duhem et al. 2003a; Arizaga et al. 2013), and their movements largely dictated by those food resources (Zorrozua et al. 2020b). Being highly plastic animals, gulls are able to alter their foraging strategy and timing in relation to the behaviour of people in urban settlements (Ramírez et al. 2020). For instance, gulls adjusted their foraging times with landfills' labouring hours, to match with garbage deliveries (Ackerman et al. 2018; Spelt et al. 2021), with school breaks (Spelt et al. 2021) and with meat and fishery processing plants (Yoda et al. 2012).

Some studies report a positive effect of anthropogenic food subsidies on gulls' population density and size, as well as on gulls' reproductive variables. Exponential population growth of Yellow-legged gulls positively associated with increased availability of waste in nearby landfills was registered by Duhem et al. (2008), and individuals feeding primarily on refuse from landfills were heavier and of greater body condition than individuals feeding on non-subsidized habitats (Auman et al. 2008). Closure of landfills led to decreased clutch size, fertility, last-laid egg size, body mass, body condition and overall breeding success (Pierotti and Annett 1991; Pons and Migot 1995; Kilpi and Öst 1998; Steigerwald et al. 2015).

Relying on anthropogenic food resources obtained at landfills or within urban habitats may also entail costs for gulls. Human meal leftovers are thought to have a poorer nutritional quality and to be difficult to digest by small chicks (Pierotti and Annett 1987; Hillström et al. 1994), when compared to marine resources that are generally recognized as a more profitable resource (Duhem et al. 2005) and with higher nutritional quality (Pais de Faria 2021a). Although anthropogenic food items are complex carbohydrates, rich in fat and proteins (Pierotti and Annett 1990, 1991), that allow for a greater energy intake (Patenaude-Monette et al. 2014), they might be deficient in essential nutrients which are important for adults and their offspring (Pierotti and Annett 1987). Indeed, decreased chick weight (Dosch 1997) and decreased egg quality (Hebert et al. 2020) represented some of the reported breeding costs associated with the consumption of anthropogenic food. Moreover, when foraging on waste at landfill sites and within urban environments, gulls may be increasingly exposed to contaminants (Zapata et al. 2018; Sorais et al. 2020) and pathogens, such as the human-associated microorganisms *Escherichia coli* (including antibiotic-resistant (Vredenburg et al. 2014; Varela et al. 2015)), *Enterococcus* spp., *Salmonella* (including rare types) and *Campylobacter* (Fogarty et al. 2003; Ramos et al. 2010; Converse et al. 2012), and may then act as a vector when they contact with non-contaminated areas (Alm et al. 2018).

The increased accessibility to large amounts of anthropogenic food and consequent reduction in foraging time and energy expenditure may not compensate for the lack of vital specific nutrients of human-related food (Pierotti and Annett 2001). Thus, we might question, is the anthropogenic food foraged at landfills and within urban areas suitable for gulls, or is it an ecological trap with long-term consequences for gulls' physiology and health condition?

Also, to date, there are many diet studies reporting the ingestion of anthropogenic materials, particularly plastics, by gulls (review by Battisti et al. 2019a), but studies focusing on the ingestion of such materials by urban and landfill-dwelling gulls are scarce (review by Seif et al. 2018, also see Méndez et al. 2020 and Stewart et al. 2020). The use of urban settlements and landfills to forage is thought to be associated with an increased probability of ingesting anthropogenic debris materials, and a detailed comparison of such ingested materials between gulls foraging in natural, urban and landfill sites is lacking, which may help to detect possible patterns in the intake of debris materials among habitats and seasons (Provencher et al. 2017), and, ultimately, to assess the physiological and health condition consequences of ingesting these debris materials.

1.4. Study aims and research questions

In Portugal, the number of gulls dwelling in urban environments and in landfills is increasing, especially in coastal cities such as Porto, Lisboa and Peniche (ICNF 2021, unpublished data). Because these populations have been increasingly relying on anthropogenic food resources, both foraging in landfills and within urban settlements, the frequency of occurrence and quantities of refuse and anthropogenic debris materials in their diets is thought to be increasing as well, all with unknown consequences for the physiology and health condition of gulls. The overall aim of this thesis is to qualify and quantify the interaction of urban- and natural-dwelling gulls with anthropogenic materials both through incorporation into nests and ingestion, to understand the physiological consequences of ingesting such materials, and, ultimately, to unravel the impacts of a typically anthropogenic diet on the gulls' physiology and health condition.

This thesis aims to answer the following questions:

1- How is the incorporation of anthropogenic debris materials in nests characterized in natural- and urban gulls' breeding sites? Is there any relation between the incorporation of debris materials and gulls' breeding success?

2- How is the ingestion of anthropogenic debris materials characterized for natural-, urban- and landfill-dwelling gulls? Is there any relation between the ingestion of such materials and gulls' diet?

3- Does gulls' diet quality differ between foraging habitats with different levels of urbanization? Are there any sub-lethal impacts of ingesting anthropogenic debris materials on gulls' physiology?

4- What are the effects of a typically anthropogenic diet on gulls' physiology and health condition?

1.5. Thesis outline

This thesis is organized in 6 chapters, including a general introduction and a general discussion. All chapters, except for the general discussion, are prepared as scientific articles and part of the general introduction as a section of a book chapter. Manuscripts from chapters 2, 3 and 4 are already published and chapter 5 is currently being prepared for submission.

Chapter 1 overviews the urbanization phenomenon and compiles the effects of urbanization on animal's behaviour and life-history traits, including on natural biorhythms, movements, reproductive success, survival, diet, foraging behaviour, physiology and health condition. Moreover, it describes the pollution by anthropogenic debris materials as a result of the urbanization process, how birds are exposed to such materials and the available monitoring tools, and the consequences for birds of interacting with anthropogenic debris materials.

Chapter 2 describes the incorporation of anthropogenic materials on Yellow-legged gull nests breeding in two natural and two urban breeding sites, during two consecutive years. Detailed data on the anthropogenic materials incorporated on gull nests, as well as the hatching success for the same study areas and study years, was compared between natural and urban breeding habitats. The possible physiological impacts of the incorporation of such materials in gulls' nests will also be discussed.

Chapter 3 characterizes the anthropogenic materials ingested by Yellow-legged gull in natural, urban and landfill sites, through the analysis of their pellets. More specifically, gull pellets from breeding colonies (natural and urban) were analysed to assess possible seasonal changes among three seasons (pre-breeding, breeding and post-breeding), and pellets from resting sites (urban and landfill) were analysed to detect seasonal patterns among four seasons (spring, summer, autumn and winter), in the ingestion of such materials. Also, the presence of anthropogenic materials was related with gulls' diet assessed with the analysis of the same pellets.

Chapter 4 compares the fatty acids composition of natural- and urban-dwelling Yellow-legged and Lesser black-backed gulls, in relation to the ingestion of anthropogenic materials. Gulls from three wildlife rescue centres with different levels of urbanization were necropsied and their adipose tissue fatty acids composition was compared to assess differences in birds' diet quality and birds' physiology among foraging habitats (natural *vs.* urban) and species. Also, the

possible physiological effects of ingesting anthropogenic materials (a toxicological stressor) on gulls' fatty acids composition was investigated.

Chapter 5 determines whether the consumption of a typically anthropogenic diet alters various physiological parameters of Yellow-legged and Lesser black-backed gulls. A captive feeding experiment was set up and, after an acclimatization period, gulls were subjected to either a natural or an anthropogenic diet, under controlled conditions. Then, haematological, protein, oxidative stress and mitochondrial parameters, as well as fatty acids composition were compared between gulls subjected to both diets. Also, some gull individuals were caught at a landfill and the same parameters were assessed and compared with those obtained from birds subjected to the feeding experiment.

Chapter 6 summarises the findings of the previous chapters and discuss their broader implications, limitations and future directions of study.

Chapter 2

CHARACTERIZATION OF ANTHROPOGENIC MATERIALS ON YELLOW-LEGGED GULL (*Larus michahellis*) NESTS BREEDING IN NATURAL AND URBAN SITES ALONG THE COAST OF PORTUGAL

This chapter is published as:

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Characterization of anthropogenic materials on Yellow-legged gull (*Larus michahellis*) nests breeding in natural and urban sites along the coast of Portugal

Abstract

Anthropogenic materials are a persistent pressure on ecosystems, affecting many species. Seabirds can collect these materials to construct their nests, which may modify nest characteristics and cause entanglement of chicks and adults, with possible consequences on breeding success. The incorporation of anthropogenic materials in nests of seabird species that breed in both natural and urban environments, such as gulls, is poorly known. Here, we characterize and compare anthropogenic materials incorporated in Yellow-legged gull (*Larus michahellis*) nests from two natural and two urban breeding sites across their Portuguese breeding range, and during two consecutive years. Anthropogenic materials were found in 2.6% and 15.4% of gull nests from natural locations, and in 47.6% and 95.7% of nests from urban breeding sites. No differences were found on hatching success between urban and natural breeding colonies. A significantly higher number of anthropogenic materials was found in the largest and more populated urban breeding colony, which on average included items of a greater mass but smaller size than items from the other three colonies. The higher incorporation of anthropogenic materials in urban locations could be a consequence of a lower access to natural nest construction materials and higher availability of anthropogenic debris. The quantity and diversity of anthropogenic materials incorporated in gull nests from urban locations indicates a need for improved debris management in urban settlements.

Keywords

Gulls, Laridae, *Larus michahellis*, nesting ecology, urban, plastic pollution, hatching success

2.1. Introduction

The presence of anthropogenic materials, i.e. debris items of any non-natural solid material (e.g. plastic, glass, fabric, metal, paper, among others; Seif et al. 2018), is a well identified anthropogenic pressure on ecosystems (Barnes et al. 2009; Provencher et al. 2017; Jagiello et al. 2019). These materials, especially plastics, are ubiquitous, long-lasting, and considered by the United Nations Environment Program as a critical problem for the environment (UNEP 2016). Over the last few years, plastic pollution in marine habitats has been widely studied (Law 2017) due to its rapid increase from coastlines to the open ocean, and from the sea surface to the seafloor (Barnes et al. 2009; van Sebille et al. 2015). As these anthropogenic materials spread throughout the environment, they pose a major threat to a large number of species (Provencher et al. 2017): Kühn and van Franeker (2020) reported that 914 marine species have been in contact with marine debris, including cetaceans, turtles, fish, crustaceans and seabirds. These interactions may influence species' behaviour, physiology and survival (Jagiello et al. 2019). Anthropogenic materials affect species in two major ways: ingestion and/or entanglement (Laist 1987; Gall and Thompson 2015; Kühn et al. 2015; Provencher et al. 2015, 2017; O' Hanlon et al. 2017). Ingestion can occur accidentally, while individuals forage on other prey items, or deliberately, when materials are mistaken for food (Laist 1987; Cadée 2002). Entanglement is passive in most of the cases, when individuals get stuck in lost debris materials, such as fishing nets or plastic bags, or it may be active when individuals became trapped in materials that they collect deliberately (Gregory 2009; Phillips et al. 2010).

Seabirds are especially susceptible to the increasing presence of anthropogenic materials in the environment (Battisti et al. 2019a). In addition to ingestion, debris can also be used for nest construction (O' Hanlon et al. 2017; Battisti et al. 2019a). This phenomenon is documented for birds of several groups including raptors (Sergio et al. 2011), passerines (Wang et al. 2009), crows (Townsend and Barker 2014), waterbirds, such as storks (Henry et al. 2011; Jagiello et al. 2018) and spoonbills (Lee et al. 2015), and marine birds (reviewed in Battisti et al. 2019a). Debris materials may have several purposes in the nest: 1) to strengthen the structure of the nest (e.g. plastic strings), 2) for decorative functions, 3) as a substitution of natural materials that might be unavailable (e.g. in urban settlements, natural materials may be less available) and 4) to repel parasites (reviewed by Jagiello et al. 2019; Reynolds et al. 2019). The use of debris as nesting material may also have negative consequences. Some materials, such as plastic strings, fishing nets and other threadlike materials in nests can cause direct injury or even death of chicks and adults by entanglement (Votier et al. 2011; Battisti et al. 2019b). Also, as the nest is considered a functional structure directly linked to breeding success (Mainwaring et al. 2014), any modification on its constitution that might compromise its functional performance (i.e. thermal properties, integrity, drainage, camouflage, etc), such as the incorporation of debris, may

subsequently have impacts on incubation routines and on fitness related traits for offspring (Deeming and Mainwaring 2015; Thompson et al. 2020). Hence, the incorporation of these materials in nests may alter certain breeding parameters, such as clutch size, hatching success and number of fledglings, which may reduce breeding success (Mee et al. 2007).

In terrestrial species, the incorporation of anthropogenic materials can be correlated with the level of urbanisation (Wang et al. 2009; Townsend and Barker 2014; Radhamany et al. 2016), however, studies on urban locations are scarce (but see Reynolds et al. 2019). On the other hand, seabirds are top predators and suitable as environmental indicators: their long-term monitoring may help understand the magnitude of the environmental effects of pollution (Burger and Golchfeld 2004). So far, studies on nest debris typically focused exclusively on marine birds nesting in their natural settings (e.g. Lavers et al. 2013; Grant et al. 2018; O'Hanlon et al. 2019; Tavares et al. 2019). To our knowledge, there are no studies reporting this phenomenon for seabirds nesting in both urban and natural settings, which is the case of gulls (Rock et al. 2016). The proximity of gull colonies to urban habitats may influence the amount of anthropogenic materials incorporated in nests (Witteveen et al. 2017), however studies on this subject are sparse, especially considering how well some gull species adapted to urban habitats (Duhem et al. 2003b).

Due to the increase of human population and consequent urbanization, which transformed natural coastal habitats into novel urban environments (Marzluff 2001; Aronson et al. 2014), opportunistic species have been attracted to the higher availability of human-derived food provided by these 'new' habitats (Oro et al. 2013). Gulls (*Larus* sp.) use urban environments, not only to opportunistically explore new food resources (Belant 1997; Belant et al. 1998; Winton and River 2017), but also to breed (Skorka et al. 2005; Rock 2013; Huig et al. 2016). Indeed, gulls are well established in many urban areas, using rooftops and other vantage locations to build their nests, taking advantage of lower levels of predation and disturbance (Rock 2005; Rock et al. 2016). When exploring new urban habitats, gulls have to cope with the higher presence of anthropogenic materials in their environment. As these materials are present in both natural and urban breeding colonies (Leite et al. 2014), gulls are, thus, one of the groups more likely to have contact with debris materials, whether through ingestion of by incorporating them into their nests (Hartwig et al. 2007; Nicastro et al. 2018; Seif et al. 2018; Battisti 2019, Yorio et al. 2020). The Yellow-legged gull (*Larus michahellis*) is a suitable model species to study the incorporation of anthropogenic materials in nests in both natural and urban breeding sites. It is an opportunistic and generalist species that, beyond their natural habitats, uses landfills and urban environments to search for food (Duhem et al. 2008; Ceia et al. 2014). The availability of both natural nesting materials and anthropogenic materials in breeding settlements can influence the incorporation of debris in nests (Grant et al. 2018; Brentano et al. 2020). Some litter-derived materials, especially some types of threadlike plastics, might be mistakenly collected by gulls due to their resemblance

to natural materials (Thompson et al. 2020), or they may be derived from debris picked up while foraging for food and then regurgitated at the nest (Witteveen et al. 2017).

The main goal of this study is to compare the incorporation of anthropogenic materials on Yellow-legged gull nests in two less transformed habitats, hereafter natural breeding colonies and two urban breeding sites. The chosen locations are naturally different from each other, with different access to anthropogenic and natural materials (see the characterization of study areas in materials and methods). Because this the first detailed study to assess the incorporation of debris in nests by urban gulls, it is important to characterize the materials incorporated in nests in order to detect possible patterns between both types of breeding areas, to infer about the origin of materials, and to allow a long-term monitoring of this phenomenon. Therefore, we collected, compared, and characterized debris types, sizes, weights, and colours from both breeding habitats, during two consecutive years. We analysed the effect of colony site and year on the presence/ absence, number of items, mass and size of anthropogenic materials incorporated in gull nests. We also compared the hatching success between natural and urban breeding colonies for the same studied years. With these data and considering the different accessibility to both natural and anthropogenic materials in each study site, we also infer possible origins of the materials present in nests. The abundance and diversity of incorporated materials in gull nests should increase with the increase of anthropogenic disturbance as indicated by the description of the study areas. Consequently, we expect nests from natural breeding colonies to have a lower abundance of anthropogenic materials incorporated, because gulls should have plenty of access to the natural materials typically used to construct their nests (i.e. vegetation), and lower access to anthropogenic materials. As gulls from urban colonies are likely to have lower accessibility to natural materials and are highly surrounded by debris, urban nests are expected to have higher quantity of anthropogenic materials, with a higher diversity in terms of mass, size, and colour. As the incorporation of debris in nests may have consequences on incubation routines and on fitness related traits for offspring, with possible alterations in certain breeding parameters, hatching success should be lower at urban colonies.

2.2. Materials and methods

2.2.1. Characterization of study areas

The presence of anthropogenic materials in Yellow-legged gull nests was recorded at four different locations across their Portuguese breeding range (Deserta and Berlenga islands; cities of Peniche and Porto, Figure 2.1), during the breeding seasons of 2018 and 2019.

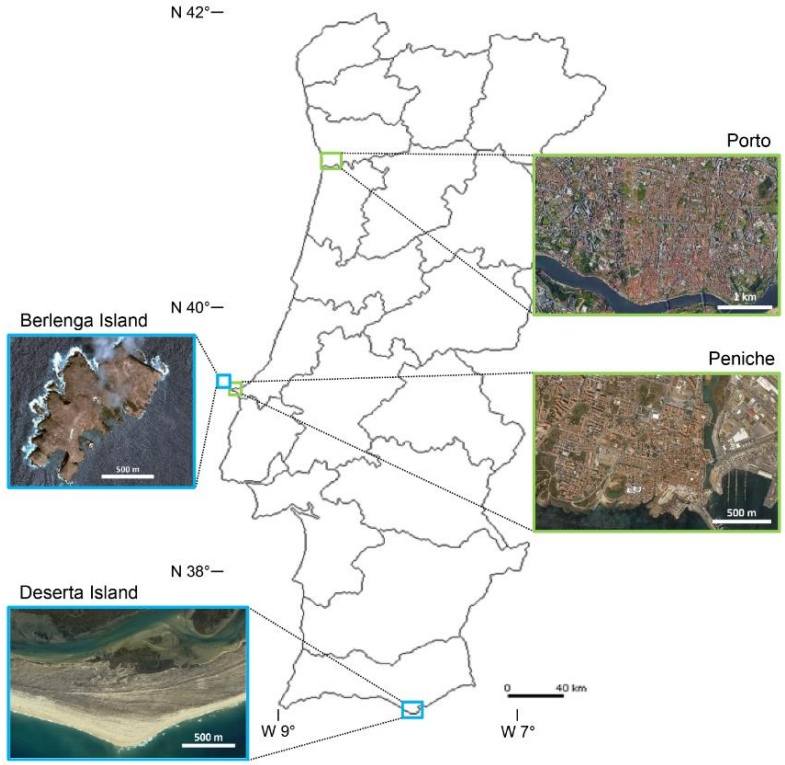


Figure 2.1. Geographical location of natural (Deserta Island and Berlenga Island, outlined in blue, on the left) and urban (Peniche and Porto, outlined in green, on the right) Yellow-legged gull (*Larus michahellis*) study colonies with aerial images of each breeding location taken from Google Earth.

Deserta Island (36° 57' 44"N, 7° 53' 23"W) is one of the five barrier sandy islands (and two peninsulas) that constitutes the Ria Formosa Natural Park, in the south of Portugal. These islands form a narrow strip of dunes which separate the lagoon from the Atlantic Ocean (Ceia et al. 2010). Deserta Island is about 7 km long, it is situated about 5.5 km from the mainland, relatively far from metropolitan and populated urban centres, and hosts an estimated population of 1200 breeding pairs of Yellow-legged gulls (Matos et al. 2018). It is uninhabited and has only a very small pressure from tourism. Berlenga Island (39° 24′ 49″N, 9° 30′ 29″W), a small rocky neritic island, is a Biosphere UNESCO Reserve located in the continental shelf, about 11 km from the Portuguese coast, in the centre of Portugal. The island is about 78.8 ha in size and is home to the largest breeding colony (about 8500 pairs, ICNF, unpublished data 2017) of Yellow-legged gulls of the Portuguese coast (Ceia et al. 2014). Berlenga Island is only inhabited by a small fishermen community. However, during late spring and summer, Berlenga is visited by a large number of tourists, leading to the increase of the tourism-related impacts. The city of Peniche is located in the centre of Portugal (39° 21' 13"N, 9° 22' 55"W): it is a small seaside city, with approximately 27750 inhabitants (PORDATA, 2011), highly dependent on fishing activities and hosting an important fishing harbour. Gull individuals are frequently seen in the city, all year round, either attracted by the fishing harbour, where they tend to forage, or due to the proximity to Berlenga Island (Morais et al. 1998). Some individuals nest in private houses' rooftops and a

small colony of about 30 breeding pairs (personal observation) nest in an abandoned part of a fortress with plenty of access to vegetation. The other urban study area is located within the Metropolitan Area of Porto. It includes the county regions of Porto, Vila Nova de Gaia and Matosinhos and it encompasses a total population of 715300 inhabitants (PORDATA 2011). Porto (41° 08' 43"N, 8° 37' 04"W) is the second largest city in Portugal, it lies on the right side of mouth of the Douro River, close to the sea, and is commonly used by gulls to construct their rooftop nests in public or private buildings. The number of breeding gulls in Porto is not known as the colonization of this area is relatively recent, but we worked in an area where about 150 breeding pairs were detected from direct observations. About 10km away from the centre of Porto is located the fishing harbour of Matosinhos, usually the second Portuguese harbour with more fish landed per year (Bueno-Pardo et al. 2020) and an essential foraging ground for gulls. Within the metropolitan area of Porto there is a high availability of anthropogenic materials that can be collected by gulls and used in nest construction.

The habitats surrounding each study colony are diverse and were characterized according to the Corine Landcover (https://land.copernicus.eu/pan-european/corine-land-cover/clc2018). Deserta Island colony is characterized by "Beaches, dunes, sands", located close to "salt marshes", "intertidal flats" and "coastal lagoons". Berlenga Island is composed by "natural grasslands". Peniche is considered a "continuous urban fabric", located close to "industrial commercial and transport units". Porto is characterized mostly as "continuous urban fabric" with "water courses", the Douro River (Figure S2.1).

Debris availability varied across study areas. During fieldwork in natural colonies (Deserta Island and Berlenga Island), we only detected the presence of a small amount of debris in the areas within and around the area of breeding. While in Deserta Island these debris were related to fishing activities (e.g. rope and fishing lines), in Berlenga Island there were some consumer waste debris (e.g. remnants of plastic bags and cling-film), possibly related to the presence of tourists. Considering the urban breeding colonies of Peniche and Porto, the urban waste collected in 2018 by the municipal services of both cities was: 407 tonnes of plastics and 609 tonnes of paper / cardboard in Peniche, and 3092 tonnes of plastics and 5431 tonnes of paper / cardboard in Porto (INE 2019). This strongly suggests that in urban areas the availability of anthropogenic materials is much higher, particularly for the larger city of Porto, than in natural breeding areas.

2.2.2. Sample collection and processing

During fieldwork, nests were chosen under the scope of other study on gulls' reproductive ecology. At all studied locations, a number of active nests were marked in 2018 and 2019 and used to capture adult nesting gulls. In Deserta Island, as part of the other study, chicks were

weighted and sampled with 5 days of age. At the same time, these nests were inspected carefully, and the few existing anthropogenic materials were removed. In this way we avoided to disturb this high-density nesting colony when chicks are older and more likely to move into nearby nesting territories. In Porto, debris were collected when chicks were about 20 days old and some had moved away from the nests. In Berlenga Island and Peniche, under the scope of a gull population control program, there are campaigns to destroy gull eggs approximately 2 weeks after the beginning of incubation, with the exception of three control areas in Berlenga Island. During such campaigns we chose nests to sample, except for Peniche, in 2019, when eggs were not destroyed, and nest debris were collected when chicks were about 20 days of age. Because anthropogenic materials in the natural colony of Deserta Island were few indeed, the slight difference in methods used at this colony site did not compromise the comparison between urban and natural sites.

Sampled nests from Deserta and Berlenga Islands were randomly chosen throughout a large area of each colony by establishing transects crossing the breeding areas, including nests from the middle and periphery of both colonies. In Peniche and Porto, all accessible nests were sampled, but these were from different buildings and covered a large area, particularly in the largest city of Porto (which also included 14 nests collected by the municipal services in 2018 following citizens complains).

All anthropogenic materials were collected from each sampled nest, stored in plastic bags, and labelled with the site, date, and nest number. Obvious regurgitated material (e.g. fish bones) was excluded from the analysis, since it was not deliberately brought as nest material. Small debris items which could originate from ingestion and then being incorporated in nests after regurgitation, could not be distinguished from non-regurgitated materials and therefore were kept in the analysis.

In the laboratory, materials were sorted and categorized into type and colour following standardized procedures established by Provencher et al. (2017). Categories of anthropogenic materials were: plastic, glass, metal (includes aluminium foil), fabric (includes different types of fibres), paper and other (uncommon items such as wood, rubber and cigarette butts). Plastics were also sub-divided in four different types: sheet plastics (e.g. plastic bags), threadlike plastics (e.g. rope, fishing lines, plastic strings and ribbons), fragment plastics (unidentifiable fragments from the break-up of larger plastic items as well as intact items) and foamed plastics (e.g. Styrofoam). Items' colours were registered using a two-step colour sorting process as recommended by Provencher et al. (2017) for plastic ingestion studies. The first colour categories were light, medium, dark and more than one colour. The second more specific categorization included: white / clear, yellow, green, blue / purple, red / pink, brown / orange, grey / silver, black and more than one colour (Verlis et al. 2014). Anthropogenic materials were weighted per category and per nest

to the nearest 0.1g using an electronic balance. The biggest axis of each debris item was measured using graph paper, with a precision of 0.5mm.

A total of 91 nests from Deserta Island (n = 62) and Porto (n = 29) were marked in 2018 and 2019 and checked every two or three days to determine the number of hatched eggs per clutch. Due to gull population control programs (i.e. egg destruction) existing in Berlenga Island and in Peniche, hatching success was not measured in these two breeding locations, nor for the nests collected by the municipal services in Porto in 2018.

2.2.3. Statistical analysis

A matrix including the number of items of each anthropogenic material category found on each nest (and therefore, per location and year) was constructed. From this, a binary matrix of presence / absence of each category of debris was also constructed. The frequency of occurrence of each category (FO, %) was calculated from the binary matrix by using the formula $FOi = n_i / n_{total} \times 100\%$, where i represents a specific category of anthropogenic debris, n_i is the number of nests in which i is present and n_{total} corresponds to the total number of analysed nests.

The presence or absence of all debris categories on gull nests was tested using a Generalized Linear Model (GLM) with binomial distribution and logit link function, which tested the influence of the following explanatory variables: (1) colony site (Deserta Island, Berlenga Island, Peniche and Porto), (2) year (2018 and 2019) and (3) their interaction on the anthropogenic materials' presence/absence on nests (response variable). The frequency of occurrence of each material category was graphically represented by Non-Metric Multidimentional Scalling (NMDS), considering the data from the two years together.

Hatching success was determined for each studied nest as the number of hatched eggs divided by the total number of eggs of the clutch and, thus, is measured in a range between 0 (no eggs hatched) and 1 (all eggs of the clutch hatched). Hatching success per breeding location (Deserta Island and Porto) and per year (2018 and 2019, total of 91 nests) is presented as mean ± standard deviation. In this study, hatching success was not measured exactly on the same nests used to study debris incorporation patterns. Thus, it should be interpreted as an assessment of the potential effects of incorporating anthropogenic materials on gull nests from colonies differing strongly in the availability of debris, on the reproduction of different gull populations. A Generalized Linear Model (GLM) with quasibinomial distribution and logit link function tested the influence of (1) colony site (Deserta Island and Porto), (2) year (2018 and 2019) and (3) their interaction on hatching success (response variable), followed by a post-hoc test using Tukey adjusted p value.

To understand how the number of items per nest, for each material category, varied between locations and years, we performed Zero Inflated Models, with negative binomial distributions to account for overdispersion. Initially, models were performed considering all breeding locations, however, as most of the categories of anthropogenic materials (Glass, Fabric, Metal, Paper and three of the four types of Plastic) do not occur on nests from both natural breeding locations (Deserta Island and Berlenga Island), models were then performed only considering urban breeding locations (Peniche and Porto). Zero inflated models tested the influence of (1) colony site (Deserta Island, Berlenga Island, Peniche and Porto or only Peniche and Porto), (2) year (2018 and 2019) and (3) their interaction on the number of items of each category incorporated on nests. Models were performed for all debris categories with and without the interaction between colony site and year, separately. The best fitting models were selected based on the lowest Akaike's Information Criterion (AIC) and Log-likelihood: models without the interaction were chosen for all debris categories, with the exception of foamed plastics, that presented a better fit with the inclusion of the interaction (Table S2.1). Number of items of each debris category on gull nests was graphically represented by Non-Metric Multidimentional Scalling (NMDS), considering the data from the two years together.

Mean mass and mean size of anthropogenic materials were calculated per nest for each colony location and year. However, as there were very few items on the nests from natural breeding locations (Deserta Island and Berlenga Island, see results), we only evaluated how mass and size of anthropogenic materials changed between the urban breeding locations (Porto and Peniche), using Generalized Linear Models (GLM). Data on masses and sizes of the most important debris categories (All Debris, All Plastic and types of plastic) were transformed to attain normality (log, $\log_{10}$, sin and square root transformations) and GLMs with Gaussian family and identity link function were performed to evaluate the effects of (1) urban colony site (Peniche and Porto), (2) year (2018, 2019) and (3) their interaction on mass and size of each mentioned category of anthropogenic materials. Mean mass and mean size of each category of anthropogenic materials incorporated in gull nests were graphically represented by Non-metric Multidimentional Scalling (NMDS), considering data only from urban colonies and from the two years separately.

The colours of the most important debris categories included per nest (All Debris, All Plastics and the four types of plastics) were represented graphically per colony site and year. To compare with our results, we searched for studies that assessed the incorporation of debris in nests of seabirds from the Laridae family. If available, we refer to data on the frequency, number of items per nest, mass, and size of anthropogenic materials.

The R statistical program (R Development Core Team 2017) was used in all analyses, with a significance level of $p < 0.05$. GLM models were performed using *lme4* R package (Bates et al. 2015), NMDS were done using *permute* and *vegan* R packages (Oksanen et al. 2019; Simpson

2019) and Zero Inflated Models were performed using *pscl* R package (Zeileis et al. 2008; Jackman 2017).

2.3. Results

2.3.1. Presence / Absence of anthropogenic materials in gull nests and association with hatching success

A total of 204 Yellow-legged gull nests were surveyed at the 4 breeding locations in 2018 and 2019 (Table 2.1). Of all surveyed nests, 32.84% (n = 67) had at least one anthropogenic material incorporated (Table 2.1). Among the studied locations, Peniche and especially Porto (urban colonies) had the highest frequency of occurrence of anthropogenic materials in both study years (50% of the nests from Peniche and 94.87% of the nests from Porto had debris incorporated), whereas only up to 16% of the nests from natural colonies had anthropogenic materials (Table 2.1). Considering each category of debris materials, plastic was the most frequently incorporated type of debris (32.63% of all incorporated materials, Figure 2.2a) and glass was the least found item (5.26% of all incorporated materials, Figure 2.2a). Regarding the different types of plastic, sheet and threadlike plastics occurred almost in the same proportions (29.14% and 28.47% of all incorporated plastics, respectively; Figure 2.2b). Fragments were the least found type of plastic (19.21% of all incorporated plastics, Figure 2.2b, Table S2.2).

GLM results testing the effect of location, year and their interaction on the presence/absence of anthropogenic materials in gull nests showed that all debris categories varied significantly among locations ($F_{3, 196} > 9.44$; $p < 0.001$, for all 11 categories), but not between years ($F_{1, 196} < 2.65$; $p > 0.10$, for all 11 categories). The interaction between location and year was also not significant for all categories of anthropogenic materials ($F_{3, 196} < 1.26$; $p > 0.29$). All debris material categories had a higher occurrence in nests from Porto when compared to other locations. NMDS considering the frequency of occurrence of each debris category in gull nests clearly showed a separation along the NMDS1 between nests of Deserta Island and the remaining colonies, mainly due to “Other” and “Plastic” categories, which were the only debris categories present on the nests of this breeding location (Figure 2.3a). NMDS2 separated Porto nests from the other breeding locations, due to the presence of “Glass” (Figure 2.3a). Considering the types of plastics, NMDS1 separated nests of Deserta Island from those of the other locations, due to the presence of threadlike plastics. Berlenga Island nests were segregated from the remaining locations by the sheet plastics along the NMDS2, and Porto nests were separated from the other locations by the relatively higher presence of fragment and foamed plastics (Figure 2.3b).

Table 2.1. Description of anthropogenic materials (debris) items present in 204 Yellow-legged gull (*Larus michahellis*) nests from four locations across the Portuguese breeding range (Deserta Island, Berlenga Island, Peniche and Porto) and during 2 years (2018 and 2019). FO = Frequency of Occurrence. SD = Standard Deviation. NA = Not Applicable.

Year	Location	Sample Size (no. of nests)	FO (%) of debris	Items per nest		Mass of debris (g)		Size of debris (cm)		Total debris items
				Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	
2018	Deserta	30	3.33	0.03 ± 0.18	0 - 1	0.2 ± NA	0.2 - 0.2	25 ± NA	25 - 25	1
	Berlenga	30	13.33	0.3 ± 0.88	0 - 4	0.275 ± 0.24	0.1 - 0.6	4.66 ± 5.72	0.4 - 18.5	9
	Peniche	19	52.63	5.63 ± 9.70	0 - 36	5.5 ± 10.89	0.1 - 35.8	10.88 ± 22.22	0.4 - 168	107
	Porto	23	95.65	108.22 ± 201.39	0 - 989	42.67 ± 98.28	0.1 - 468.5	7.85 ± 13.43	0.2 - 210	2489
2019	Deserta	39	2.56	0.05 ± 0.32	0 - 2	1.2 ± NA	1.2 - 1.2	21.3 ± 18.81	8 - 34.6	2
	Berlenga	26	15.38	0.35 ± 0.85	0 - 3	1.375 ± 1.32	0.4 - 3.2	24.66 ± 21.88	2 - 59.6	9
	Peniche	21	47.62	6.62 ± 15.64	0 - 62	2.44 ± 2.77	0.1 - 9.3	7.42 ± 15.27	0.35 - 120	139
	Porto	16	93.75	79.88 ± 78.17	0 - 316	14.03 ± 19	0.6 - 67.1	4.70 ± 7.8	0.2 - 126	1278
Overall Year	2018	102	36.27	25.55 ± 104.23	0 - 989	26.89 ± 77.73	0.1 - 468.5	8.03 ± 14.11	0.2 - 210	2606
	2019	102	29.41	14 ± 42.17	0 - 316	8.05 ± 14.63	0.1 - 67.1	5.12 ± 9.13	0.2 - 126	1428
Overall Location	Deserta	69	2.9	0.04± 0.27	0 - 2	0.7 ± 0.71	0.2 - 1.2	22.53 ± 13.47	8 - 34.6	3
	Berlenga	56	14.29	0.32 ±0.86	0 - 4	0.83 ± 1.06	0.1 - 3.2	14.66 ± 18.62	0.4 - 59.6	18
	Peniche	40	50	6.15 ± 13.01	0 - 62	3.97 ± 7.89	0.1 - 35.8	8.92 ± 18.65	0.35 - 168	246
	Porto	39	94.87	96.59 ± 161.53	0 - 989	31.06 ± 77.32	0.1 - 468.5	6.47 ± 11.42	0.2 - 210	3767
TOTAL	ALL	204	32.84	19.77 ± 79.52	0 - 989	18.46 ± 58.98	0.1 - 468.5	6.72 ± 12.21	0.2 - 210	4034

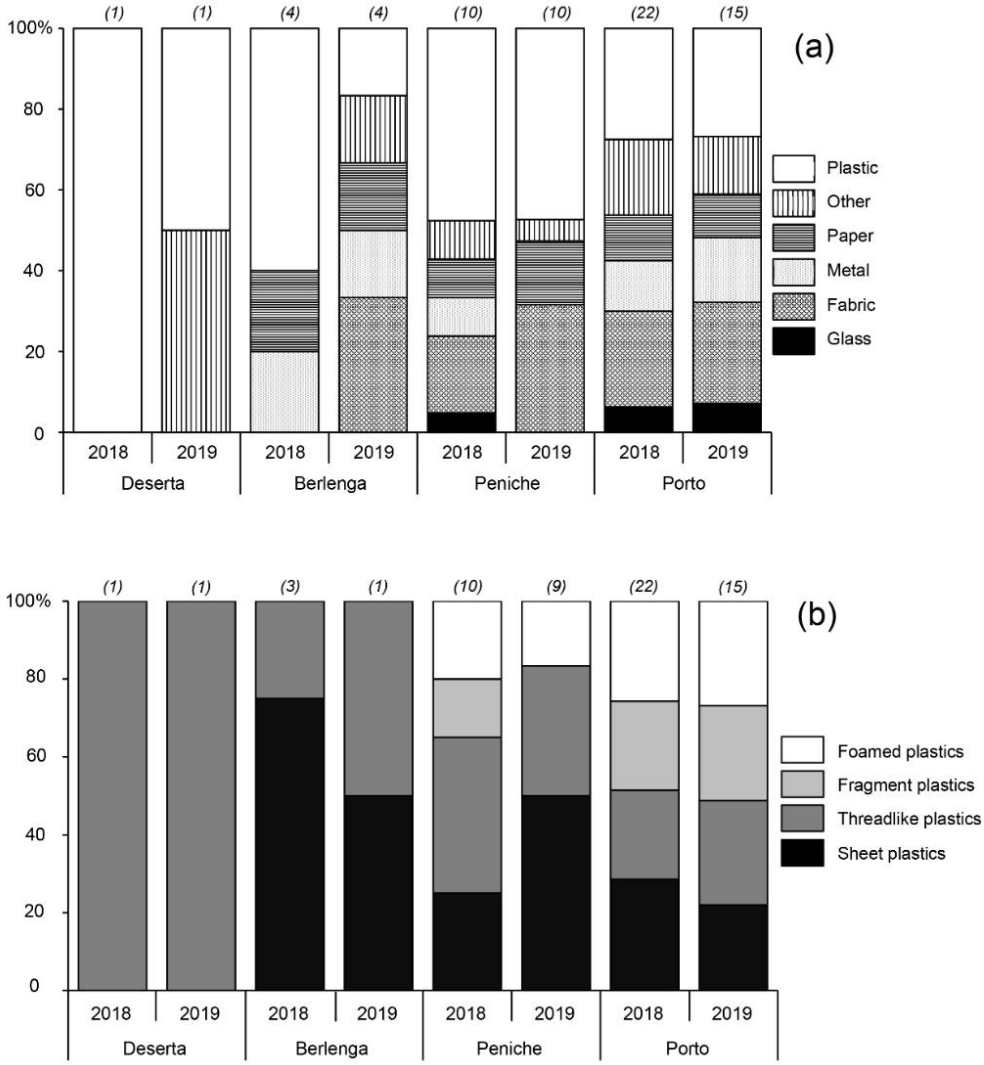


Figure 2.2. Frequency of occurrence (FO, %) of anthropogenic materials in 204 Yellow-legged gull (*Larus michahellis*) nests on 4 different locations across Portugal (Deserta Island, Berlenga Island, Peniche and Porto) during 2018 and 2019. **a)** For each category of debris (Glass, Fabric, Metal, Paper, Other and Plastic); the number of nests where debris occurred in each Location and Year is presented in the top of each bar; **b)** Plastic categories (Sheet, Threadlike, Fragment and Foamed); the number of nests where plastic items occurred in each Location and Year is presented on the top of each bar.

In Deserta Island, hatching success (mean ± standard deviation) was 0.67 ± 0.39 in 2018 (n = 22) and 0.59 ± 0.43 in 2019 (n = 40), while in Porto it was 0.57 ± 0.41 in 2018 (n = 15) and 0.88 ± 0.2 in 2019 (n = 14). There was no effect of location ($F_{1, 89}$ = 1.32, p = 0.25, GLM) and year ($F_{1, 88}$ = 0.35, p = 0.56, GLM) on hatching success. However, there was a slightly significant interaction between location and year ($F_{1, 87}$ = 5.59, p = 0.02), with a higher hatching success in Porto during 2019 (β = 2.06 ± 0.93; t = 2.23; p = 0.03), although Tukey post-hoc test showed no significant differences (all P_{adj} > 0.09).

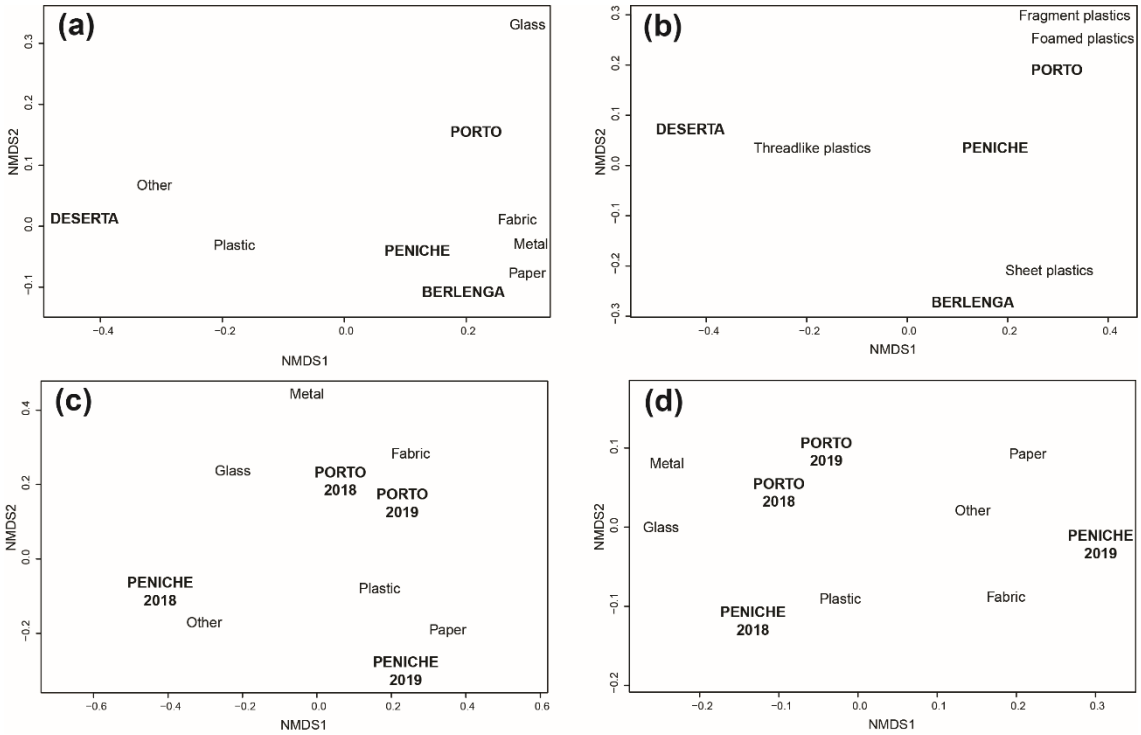


Figure 2.3. Non-metric multidimensional scaling (NMDS) using **a)** the frequency of occurrence of the main categories of debris materials (Glass, Fabric, Metal, Paper, Other and Plastic) and **b)** the frequency of occurrence for the 4 types of plastic (Sheet, Threadlike, Fragment and Foamed), in Yellow-legged gull (*Larus michahellis*) nests from breeding locations (Deserta Island, Berlenga Island, Peniche and Porto), during the years of 2018 and 2019; using **c)** the mean mass and **d)** mean size of each debris category (Glass, Fabric, Metal, Paper, Other and Plastic) in Yellow-legged gull nests from urban sites (Peniche and Porto), during the years of 2018 and 2019.

2.3.2. Number, mass, and size of anthropogenic materials in gull nests

In relation to the number of items incorporated per nest, Porto nests presented the highest values for both years (mean of 96.59 items/nest, ranging from 0 to 989, Table 2.1). Levels of incorporated debris detected in our study were also compared to those previously reported for the Laridae family (Table 2.2). A total of 4034 anthropogenic materials were included in gull nests at all locations, with a mean value of 19.77 items/nest (Table 2.1). From all anthropogenic items incorporated, 66% were plastics, mostly of foamed type (65% of the total plastic, Table S2.3 and Figure S2.2). When comparing the number of items incorporated in gull nests from natural breeding locations with those from urban sites, zero inflated models showed a clear difference between type of location (natural or urban), but not between years (Table 2.3, panel A): considering all items ("All Debris" category), the number of items incorporated varied significantly between a reference location (Berlenga Island, in this case) and Peniche (Z = 3.93; $p < 0.001$), Porto (Z = 8.09, $p < 0.001$), but not significantly with Deserta Island (Z = -0.68, p = 0.5, Table 2.3, panel A). Using 2018 as the year of reference, the number of items incorporated

in gull nests also did not vary significantly between years (Z = -0.12, p = 0.9, Table 2.3, panel A). Studying the difference in the number of included items in nests from both urban locations (Porto and Peniche), models showed a significantly higher amount of incorporated "All Debris" (Z = 6.37, $p < 0.001$), "Fabric" (Z = 5.76, $p < 0.001$), "Paper" (Z = 3.27, p = 0.001), "All Plastics" (Z = 4.79, $p < 0.001$) and, inside the plastic category, "Fragment plastics" (Z = 3.31, p = 0.001) and "Foamed plastics" (Z = 6.66, $p < 0.001$; Table 2.3, panel B) items, in nests from Porto when compared to those from Peniche. There were also significant differences between years in the number of incorporated items: 2018 had a higher amount of incorporated "Glass" (Z = 2.98, p = 0.003) and "Foamed plastics" (Z = 2.79, p = 0.005), while in 2019 "Fragment plastics" (Z = -4.09, $p < 0.001$) presented a higher amount of incorporated items (Table 2.3, panel B). The NMDS considering the number of items incorporated in gull nests showed a separation very similar to the NMDS of the frequency of occurrence, either considering each debris category or considering the types of plastics (Figure S2.3).

On average, the heaviest anthropogenic materials were found on nests from Porto in both years, ranging from 0.1 to 468.5 g (Table 2.1). The longest item measured 2,10 meters and was found on a nest from Porto (Table 2.1). More detailed information on mean masses and mean sizes of anthropogenic materials included in gull nests can be seen in Table S2.4. When testing the effects of urban location (Peniche and Porto), year of collection (2018 and 2019) and their interaction in the mean mass and size of all anthropogenic materials found on gull urban nests, materials were significantly heavier ($F_{1, 53}$ = 16.525; $p < 0.001$), but smaller ($F_{1, 53}$ = 9.16; p = 0.004) in nests from Porto, when comparing with nests from Peniche. Considering all plastics as a category, they were also significantly heavier in Porto's nests compared to those from Peniche ($F_{1,52}$ = 9.337, p = 0.0035). However, when specifying plastic subcategories, only "Foamed plastics" had a significantly larger mass ($F_{1,31}$ = 6.11, p = 0.019) and size ($F_{1,31}$ = 7.455, p = 0.01) in nests from Porto. Mass and size of "Sheet plastics" and "Threadlike plastics" were not significantly different between the two urban locations ($F_{1, 35} < 3.17$, $p > 0.08$). Year did not have a significant effect on mean mass and mean size of debris categories ($F_{1,52} < 3.55$; $p > 0.07$). A significant interaction was obtained in the mean mass of "Foamed plastics", which was significantly heavier for the nests from Porto in 2018 (Table S2.5).

Table 2.2. Compilation of studies assessing the frequency, mean (or range of means and maximum values detected) items per nest, mass, and size of debris incorporated in Laridae seabirds nests across the world. N: number of studied nests. ND: Percent of nests containing debris. Dashes indicate "no available data".

Common species names	Country	Study year(s)	N	ND (%)	Mean or range of means + Maximum value detected			Reference
					Items per nest	Mass (g)	Size (cm)	
Black-legged Kittiwake (*Rissa tridactyla*)	Denmark	1992	466	39.3	--	--	--	Clemens and Hartwig (1993)
Black-legged Kittiwake (*Rissa tridactyla*)	Denmark	2005	311	57.2	--	--	--	Hartwig et al. 2007
Kelp Gull (*Larus dominicanus*)	South Africa	2013	630	4 – 67	0.2 – 3.4 Max: 26	0.2 – 1.5 Max: 15.8	5.5 – 62.7	Witteveen et al. (2017)
Sooty Gull (*Larus hemprichii*)	United Arab Emirates	2019	258	11.2	2 Max: 11	6.1 BD[1] – 46.2	--	Yaghmour and Marashda (2020)
Yellow-legged Gull (*Larus michahellis*)	Italy	2019	307	31.3	--	--	0.5 – 18	Battisti (2019)
Yellow-legged Gull (*Larus michahellis*)	Portugal	2018 2019	204	2.6 – 95.7	0.03 – 108.22 Max: 989	0.2 – 42.67 Max: 468.5	4.66 – 25 Max: 210	This study
European Herring Gull (*Larus argentatus*)	Scotland	2018	1022	35.6	--	--	--	Thompson et al. 2020
Lesser Black-backed Gull (*Larus fuscus*)	Scotland	2018	221	~ 25	--	--	--	Thompson et al. 2020
Great Black-backed Gull (*Larus marinus*)	Scotland	2018	86	> 50	--	--	--	Thompson et al. 2020
Hartlaub's Gull (*Larus hartlaubii*)	South Africa	1996	265	0.75	--	--	--	Tavares et al. 2020
Caspian Terns (*Hydroprogne caspia*)	Senegal	2018	569	15	1	2.27	11.03	Tavares et al. 2019
Sooty Tern (*Onychoprion fuscatus*)	Brazil	2014	1800	3	1.44	11	--	Peterson et al. 2016

[1] BD = Bellow Detection

Table 2.3. Statistics from Zero Inflated Models testing the effect of year (2018 and 2019) and (A) location (Deserta Island, Berlenga Island, Peniche, Porto) and (B) urban location (Peniche, Porto) and their interaction, when significant, in the number of items of anthropogenic materials incorporated in Yellow-legged gull (*Larus michahellis*) nests. Categories (A) Berlenga Island and 2018 and (B) Peniche and 2018 were assigned references. Only results from count models are shown. Significant effects are highlighted in bold. Dashes indicate the debris categories that did not occur in gull nests when using all locations in the models.

		(count model)	All Debris	Glass	Fabric	Metal	Paper	All Plastic	Sheet plastics	Threadlike plastics	Fragment plastics	Foamed plastics	Other[1]
A	Location Deserta	β ± SE	-0.93 ± 1.37	-	-	-	-	-3.37 ± 1.08	-	-0.16 ± 1.05	-	-	-0.22 ± 1.46
		Z	-0.68	-	-	-	-	-3.12	-	-0.15	-	-	-0.15
		P	0.50	-	-	-	-	**0.002**	-	0.88	-	-	0.88
	Location Peniche	β ± SE	2.28 ± 0.58	-	-	-	-	2.18 ± 0.87	-	4.22 ± 0.88	-	-	5.00 ± 1.44
		Z	3.93	-	-	-	-	2.52	-	4.80	-	-	3.48
		P	**<0.001**	-	-	-	-	**0.01**	-	**<0.001**	-	-	**<0.001**
	Location Porto	β ± SE	4.50 ± 0.56	-	-	-	-	4.28 ± 0.82	-	5.18 ± 0.80	-	-	5.90 ± 1.08
		Z	8.09	-	-	-	-	5.22	-	6.52	-	-	5.45
		P	**<0.001**	-	-	-	-	**<0.001**	-	**<0.001**	-	-	**<0.001**
	Year 2019	β ± SE	-0.04 ± 0.31	-	-	-	-	-0.16 ± 0.39	-	-0.53 ± 0.45	-	-	0.11 ± 0.60
		Z	-0.12	-	-	-	-	-0.40	-	-1.18	-	-	0.18
		P	0.90	-	-	-	-	0.69	-	0.24	-	-	0.86
B	Location Porto	β ± SE	2.22 ± 0.35	1.75 ± 1.11	3.23 ± 0.56	-0.29 ± 1.17	2.85 ± 0.87	2.1 ± 0.44	0.89 ± 0.57	0.96 ± 0.55	2.92 ± 0.88	5.14 ± 0.77	0.97 ± 0.99
		Z	6.37	1.58	5.76	-0.25	3.27	4.79	1.56	1.75	3.31	6.66	0.99
		P	**<0.001**	0.12	**<0.001**	0.80	**0.001**	**<0.001**	0.12	0.08	**<0.001**	**<0.001**	0.33
	Location * Year	β ± SE										-4.66 ± 1.58	
		Z										-2.94	
		P										**0.003**	
	Year 2019	β ± SE	-0.09 ± 0.33	2.12 ± 0.71	0.14 ± 0.43	0.41 ± 0.6	0.72 ± 0.65	-0.14 ± 0.41	0.86 ± 0.54	-0.63 ± 0.49	-1.91 ± 0.47	3.97 ± 1.43	-0.07 ± 0.67
		Z	-0.26	2.98	0.33	0.69	1.12	-0.33	1.59	-1.29	-4.09	2.79	-0.11
		P	0.79	**0.003**	0.74	0.49	0.27	0.74	0.11	0.20	**<0.001**	**0.01**	0.91

[1] includes uncommon items such as wood, rubber, and cigarette butts

Mean mass and mean size of each category of anthropogenic materials present in gull nests from urban locations were represented graphically through NMDS. Considering mean mass, Porto and Peniche nests were clearly separated by NMDS2, mainly due to "Fabric", "Metal" and "Glass" in the case of Porto and the remaining categories for Peniche. Nests from Peniche in 2018 were segregated from the remaining nests by the NMDS1, principally attributed to the category "Other" (Figure 2.3c). As for the types of plastics, the mean mass of "Sheet plastics" separated nests from Peniche in 2018 and the mean mass of "Fragment plastics" separated those from Porto in 2018, along the NMDS2 (Figure S2.4a). Evaluating the mean size of anthropogenic materials found on gull nests, Peniche 2019 was segregated from the other locations along the NMDS1 both considering all debris categories (Figure 2.3d, mainly due to "Paper" and "Fabric"), and considering only the plastic type (Figure S2.4b, mostly due to the mean size of threadlike plastics).

The literature search revealed nine studies assessing the incorporation of anthropogenic materials in nests of seabirds from the Laridae family, encompassing ten different species (Table 2.2). Qualitative and quantitative information on the number of items per nest, mass and size of anthropogenic materials incorporated in nests are rarely included in studies (only 22% of the studies reported complete information).

2.3.3. Colour of anthropogenic materials in gull nests

The colours of the most incorporated items on gull nests are graphically represented in Figure 2.4. Considering that some locations have a small number of items, the most common colour of incorporated materials was white / clear, followed by blue / purple and green (Table S2.6). When considering plastic subcategories, white / clear and black were the most frequent colours of incorporated sheet plastics; green was a common colour for threadlike plastics and white /clear was the most frequent colour for foamed plastics (Figure 2.4, Table S2.6).

2.4. Discussion

This study presents novel knowledge about the frequency and diversity of anthropogenic materials incorporated in Yellow-legged gull nests from two natural and two urban breeding colonies in Portugal. We collected, counted, and characterized debris incorporated in gull nests at urban areas for the first time. As expected, the percentage of nests containing debris in natural sites was much lower when compared to urban sites (2.6% and 15.4% *vs.* 47.6% and 95.7%, respectively). At our natural breeding sites, this range of proportion of nests with debris incorporated is similar to previous descriptions for nests from the Laridae family (Table 2.2). The value 95.7% of nests containing debris registered on our urban site Porto was the highest recorded

so far (Table 2.2), however, our study is the first to include Laridae urban nests, as all previous nest debris studies on this family only considered nests from natural breeding colonies.

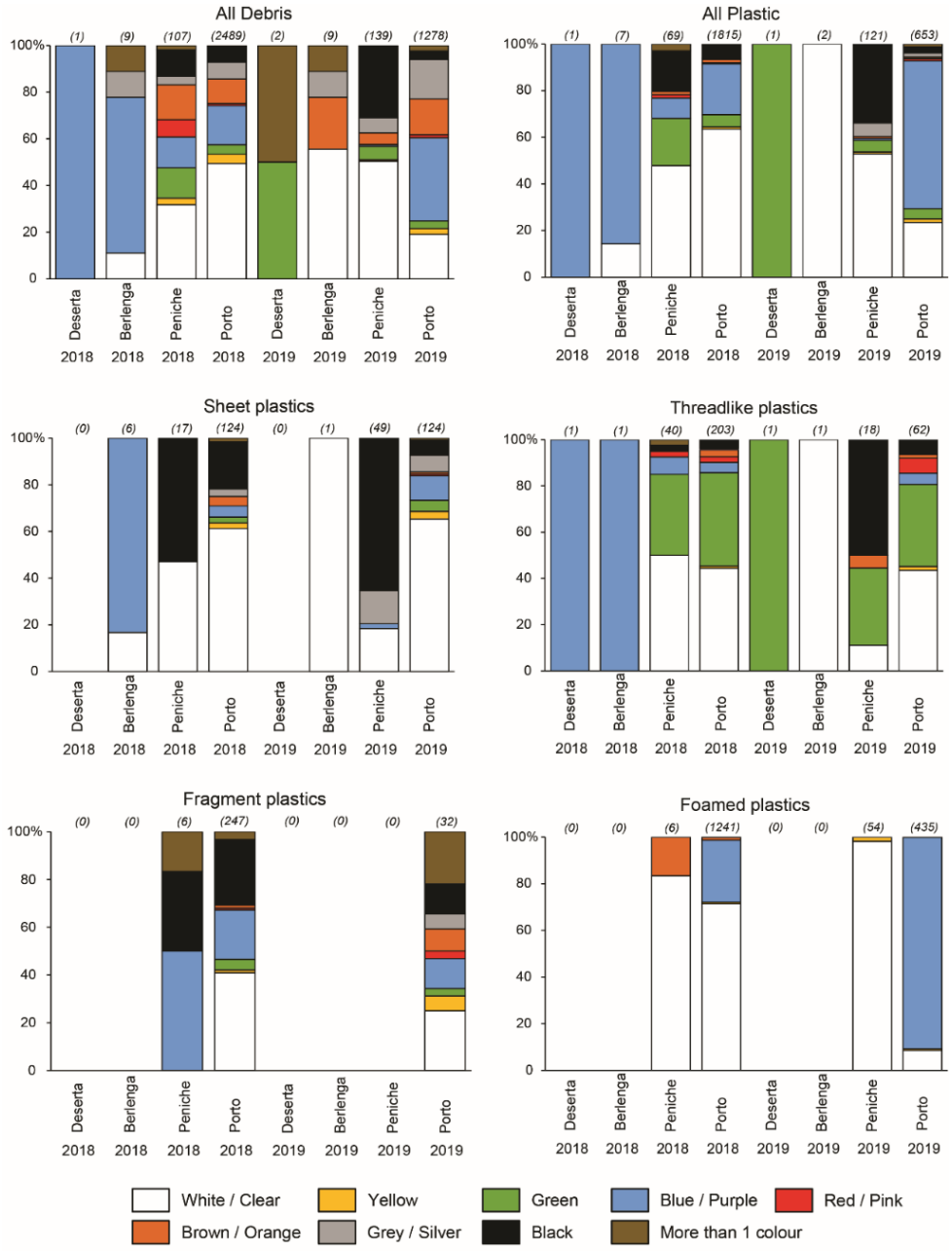


Figure 2.4. Frequency of occurrence (FO, %) of debris colours: "All Debris", "All Plastic", "Sheet plastics", "Threadlike plastics", "Fragment plastics" and "Foamed plastics" on Yellow-legged gulls (*Larus michahellis*) nests from the four breeding locations (Deserta Island, Berlenga Island, Peniche and Porto) and during two study years (2018 and 2019). Sample size (number of items) is presented on the top of each bar.

Characterization in terms of mass, sizes and colours of anthropogenic materials incorporated in gull nests is rarely reported (Table 2.2), which highlights how little we know about nest debris incorporation by birds of the Laridae family, especially for species breeding in

urban areas. As most nesting material is thought to be collected by seabirds locally (Thompson et al. 2020), long-term monitoring of debris incorporated in nests is useful to help in understanding the magnitude and the extent of anthropogenic materials present in the environment (Burger and Gochfeld 2004; Lavers et al. 2013; Grant et al. 2018; Tavares et al. 2020). A monitoring scheme of this phenomenon would allow comparisons on the same species using different breeding habitats and between different species across the globe (O'Hanlon et al. 2017). However, to allow inter-studies comparisons, it is crucial that data is collected following a standardized method. Unfortunately, there is no standardized protocol to characterize and report anthropogenic materials incorporated in seabird nests (O'Hanlon et al. 2019). Hence, we followed Provencher et al. (2017) methodology to analyse debris ingested by seabirds and adapted their guidelines to measure and characterize types of nest debris.

Although we did not perform debris analysis in the surrounding environment of the nests, we characterized in detail our study areas through the use of Corine Landcover data, as well as observations during our fieldwork and data on the quantities of garbage collected by municipal services of Porto and Peniche. Therefore, and considering the differential characterization of the study areas, we can infer about the possible origins of the debris incorporated on gull nests in our study sites in order to, in the future and after further studies on the subject, implement adequate measures in the field to reduce the amount and diversity of incorporated debris in gull nests. Gulls usually construct their nests by scrapping on the ground and collecting vegetation in the surrounding areas with which they build the walls of the nest (Cramp and Simmons 1983). Witteveen et al. (2017) found that Kelp gulls' nests had more debris incorporated in locations where there was little natural vegetation available for nest construction and where the distance to the nearest urban waste landfill was smaller. Our four studied breeding locations are different from each other in the accessibility to natural nesting materials (i.e. vegetation) and in the availability of anthropogenic debris. Both of our studied natural breeding areas have plenty of access to vegetation, however the availability of anthropogenic materials might be higher in Berlenga Island as it is visited by tourists from mid-May to mid-September, overlapping with the gull breeding season. During these periods, anthropogenic materials are improperly disposed by beachgoers, which may increase their density up to 40% (Galgani et al. 2015). Deserta Island has a small pressure from tourism and thus the availability of debris should be lower than in Berlenga Island. The high quantity and diversity of debris incorporated in nests from urban locations detected by our study may be attributed to an increase in the availability of anthropogenic nesting materials, and a reduction of natural nesting materials in urban environments, which can be supported by the characterization of the four study areas. In agreement, Lee et al. (2015) supplied Black-faced Spoonbills (*Platalea minor*) with natural nesting materials in their nest surroundings and birds reduced the use of anthropogenic materials on their nests.

Debris such as foamed plastics, fabric fibres and pieces of paper are easily disintegrable materials and may be originated from larger items brought to the nest as nesting material, which posteriorly may disintegrate into smaller items along the incubation period. This could partly explain the highest number of items per nest detected in Porto, where a single nest had, besides other items, 850 small pieces of styrofoam. Anthropogenic materials may also be accidentally ingested by gulls when foraging for food (O'Hanlon et al. 2017; Battisti 2019) and then regurgitated and become trapped in the nest bowl. In fact, previous studies showed that gulls from our four studied locations visit open disposal landfills, even during the breeding season, with debris materials appearing on their pellets (Ceia et al. 2014; Matos et al. 2018; Mendes et al. 2018). This, together with tourism pressures in natural colonies, may explain the existence of food-related bags and wrappers (sheet plastics) on gull nests at almost all locations. The high proportion of incorporated threadlike plastics in nests from Deserta Island, Berlenga Island and Peniche, suggest that debris derived mainly from commercial and recreational fishing. The loss and abandonment of fishing gear accounts approximately to 18% of the total ocean debris load worldwide (Andrady 2011). In fact, there are fishing activities close to both natural colonies as well as in Peniche. This is in agreement with other studies that report high abundance of fishing materials in nests of birds that typically collect nesting material at sea (Votier et al. 2011; Tavares et al. 2016; Grant et al. 2018). However, as gulls are opportunistic and typically collect nesting material around the breeding location (Witteveen et al. 2017), almost all, if not all, of the fishing materials found in nests probably was collected from the shoreline.

Regarding urban nests, especially those from Porto, the large amount of anthropogenic materials found in gull nests are distributed among all categories. The same happens for the proportion of plastic types (i.e. all categories of plastic were relatively common). Urban gulls tend to have opportunistic behaviour and a scavenging nature (Rock et al. 2016; Witteveen et al. 2017), which may lead gulls to collect and use materials in the surrounding environment to construct their nests, explaining the occurrence of all material categories on nests from urban locations. In fact, we registered a nest from Porto that was only constituted by "Fabric" fibres commonly used in construction, presumably originated from a nearby building that was under construction. We also found in a touristic area of Porto a nest containing 42 drinking straws probably collected by gulls on nearby coffee and restaurant terraces (personal observation, Figures S2.5 and S2.6). Overall, our results did not vary significantly between years, except for an interaction between location and year in explaining the mass of foamed plastics, that was significantly higher in 2018 for Porto nests, and in explaining the number of items of some categories when comparing only urban locations (Peniche and Porto).

We decided to characterize nest debris by their colour because some seabird species seem to demonstrate a colour preference when choosing materials to construct their nests (Lavers et al. 2013; Verlis et al. 2014). As we did not conduct an analysis of debris availability on the

surrounding environment of the nests, we were unable to form a hypothesis about colour selection. However, seabirds are known to collect debris that resemble natural nesting materials, as it is the case of Northern Gannets using fragments of fishing lines and ropes that are similar to seaweed (Votier et al. 2011). Gulls incorporated on their nests a high proportion of green threadlike plastics (Figure 2.4), which may be due to the fact that these materials may resemble vegetation in terms of colour and elongated shape (Witteveen et al. 2017). Battisti (2019) showed that some types of plastics are actively collected and brought to the nest by gulls because of their resemblance with cuttlebones of the *Sepia* cuttlefish, usually used to provide a calcium supplement to chicks (Cadée 2002). This may explain the high proportion of the white colour of debris materials incorporated in the nests of our study sites, especially of foamed plastics. Overall, our data agrees with the fact that some types and some colours of anthropogenic materials chosen by gulls for nest construction resemble natural materials that they usually collect. Colours of some materials, especially plastics, may be associated with a higher exposure to some potentially harmful chemicals (Provencher et al. 2017). Some compounds can be transferred through the skin (Zalko et al. 2011) and may interfere with an individual's physiology, causing negative impacts on reproduction, behaviour, and survival (Herzke et al. 2016; Lavers and Bond 2016b; O'Hanlon et al. 2017). Verlis et al. (2014) suggest that nesting on the top of certain items could potentially lead to the absorption of contaminants through the skin. As gulls from urban locations use a higher number and diversity of anthropogenic materials, they are possibly exposed to a wider range of harmful contaminants that may be absorbed through adults' and chicks' skin (Verlis et al. 2014; Provencher et al. 2017), nonetheless this issue needs more studies.

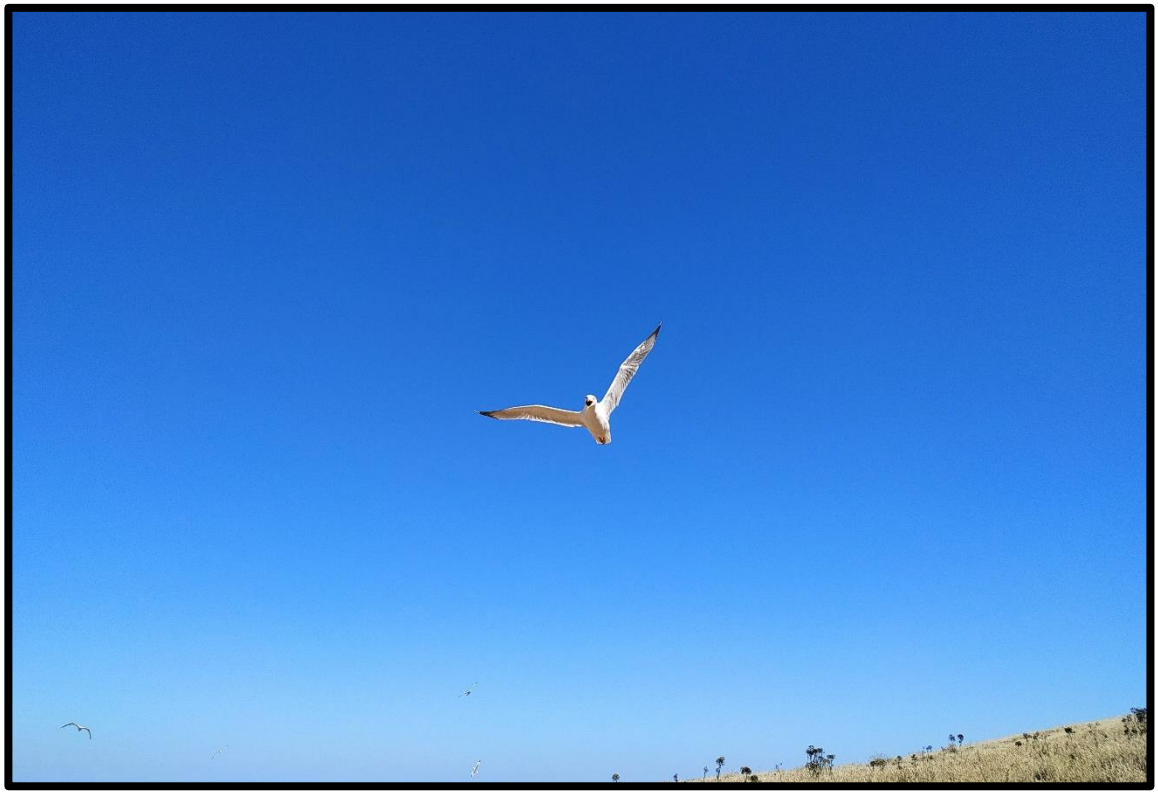
The presence of certain debris, especially plastics, in a nest may decrease its permeability and, due to lower insulation capacity in comparison with the use of vegetal material, it will provide lower buffering properties against ambient temperature variation. Such modification of nest characteristics may affect the behaviour of incubating adults, leading to nest temperature variations and possible consequences on hatching success and other fitness measures (Deeming and Mainwaring 2015). In our study, hatching success should be regarded as a coarse evaluation of the possible effects of nest debris on nest incubation, because other variables such as adult condition and quality also influence hatching success (Coulson 1968). Despite the significantly higher incorporation of anthropogenic materials in gull nests from Porto, hatching success did not vary significantly between Porto and Deserta Island. Accordingly, Jagiello et al. (2018) found no significant effect of the incorporated debris on clutch size, number of fledglings and breeding success of White Storks (*Ciconia ciconia*) breeding in Poland. As inclusion of anthropogenic materials in nests may lead to entanglement (Votier et al. 2011) and exposure to contaminants for adults and chicks (Verlis et al. 2014), it would be interesting to understand if there is a relation between the use of these materials in nest construction and gulls' fitness variables, such as

breeding, hatching and fledgling success, in order to better comprehend the costs and benefits of the incorporation of anthropogenic materials into nests (Reynolds et al. 2019).

The quantity and variety of anthropogenic materials incorporated by gulls on their nests, especially in Porto, is worrying. It may reveal a poor garbage management, giving birds access to these debris. Thus, it is crucial to identify the sources of these materials, in order to apply suitable garbage management procedures. These measures may include the improvement of the efficiency in the disposal of garbage by using closed containers, and the increase in the number of times that the garbage is collected by municipal services. Such measures may contribute to reduce the amount of available anthropogenic materials and to prevent gulls to ingest and incorporate such debris on their nests. All nest debris, both from urban areas and natural sites, seem to share a common origin: at some point, someone dealt with it wrongly or ineffectively, either unwittingly or deliberately. Therefore, wider measures that could benefit not only gulls but other animals, include education and legislation (Sheavly and Register 2007; Battisti 2018). To raise consciousness and in an attempt to change human-behaviour, awareness campaigns to promote the proper separation and the correct disposal of waste, as well as garbage collection campaigns on the beaches and cites should alert children, fishermen, tourists, waste management workers and others to this phenomenon. On the other hand, laws to prevent, reduce and control pollution should be created or intensified in order to improve environmental management.

Chapter 3

INGESTION OF ANTHROPOGENIC MATERIALS BY YELLOW-LEGGED GULLS (*Larus michahellis*) IN NATURAL, URBAN, AND LANDFILL SITES ALONG PORTUGAL IN RELATION TO DIET COMPOSITION



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Ingestion of anthropogenic materials by Yellow-legged gulls (*Larus michahellis*) in natural, urban, and landfill sites along Portugal in relation to diet composition

Abstract

Pollution is a global concern, increasing rapidly throughout marine and terrestrial ecosystems, and affecting many species. Urbanization enhances waste production, leading to the opening of landfills that constitute a spatially and temporally predictable food source for opportunistic species. Several species of gulls are known to exploit and breed in urban areas, taking advantage of accessible and diverse food resources. The exploitation of anthropogenic food subsidies at sea (e.g. fisheries discards), urban sites and landfills, lead to debris ingestion by gulls with potential negative effects. Here we characterize anthropogenic debris ingested by Yellow-legged gulls (*Larus michahellis*) along Portugal, by analysing the content of pellets collected in 1) natural and urban breeding locations, and in 2) urban and landfill resting sites, to assess seasonal patterns in the ingestion of anthropogenic debris. We also relate diet with the presence of anthropogenic debris. Debris materials were found in 28.8% of pellets from breeding locations (natural and urban) and in 89.7% of pellets from resting sites (urban and landfill). Gulls from the most urbanized breeding location exhibited higher levels of ingested materials during the entire breeding cycle, however gulls from a natural breeding site also ingested high levels of debris during the pre-breeding season. At resting sites, small seasonal differences were detected in the number and mass of debris items ingested, which were both higher during spring and summer. Gulls that typically fed on pelagic fish had significantly less sheet and fragment plastics in their pellets. The presence of certain debris categories in gull pellets was positively related to the presence of some prey items, suggesting that gulls may accidentally ingest debris while foraging at multiple habitats. The quantity of anthropogenic materials ingested by gulls from urban locations and landfills indicates a need for improved waste management.

Keywords

Laridae, gulls, diet analysis, regurgitations, pellets, plastic pollution, debris ingestion

3.1. Introduction

Anthropogenic debris pollution is one of the most acknowledged problems affecting ecosystems (Barnes et al. 2009; Kühn et al. 2015; Provencher et al. 2017). Anthropogenic materials such as glass, fabric, metal, paper and especially plastics are pervasive, long-lasting (Seif et al. 2018), and listed by the United Nations Environment Program (UNEP 2016) as a critical problem for the environment. Debris accumulation is increasing globally from coastal regions to the open ocean, and is present even in remote areas (Barnes et al. 2009; Kühn and van Franeker 2020), affecting marine and terrestrial ecosystems virtually everywhere on the planet (e.g. Thompson et al. 2009; van Sebille et al. 2015; Duis and Coors 2016; Law 2017). Given the large amounts of debris in marine ecosystems, and their unknown rate of decomposition, species including cetaceans, turtles, fish, crustaceans and seabirds are increasingly exposed to these materials, and vulnerable to the adverse impacts of their ingestion (Gall and Thompson 2015; Kühn et al. 2015; Wilcox et al. 2015; Kühn and van Franeker 2020).

Urbanization, economic development, and population growth result in increased waste production. As a result, in the last decades, a large number of refuse dumps and landfills were opened to dispose the increasing amounts of garbage (Belant et al. 1998; Plaza and Lambertucci 2017), thus creating a spatially and temporally predictable and abundant food source (Oro et al. 2013), which attracts a number of opportunistic mammals, reptilians, amphibians and birds (Belant 1997). Food subsidies from landfills can be easier to access when compared to natural sources (Bartumeus et al. 2010), allowing predators to reduce foraging time and potentially improving their fitness components, enhancing individual fecundity and survival, and favouring population growth (Oro et al. 2013). However, the use of landfills also leads to an increased probability of ingesting anthropogenic materials, higher exposure to contaminants, poisoning and pathogen infections (Seif et al. 2018; Sorais et al. 2020; Yorio et al. 2020). Despite the fact that developed countries are trying to reduce waste production and the number of functioning landfills with the European Union Landfill Directive (European Commission 2016), these anthropogenic food subsidies are still a valuable food source largely used by opportunistic species (Oro et al. 2013).

Beyond using remote islands and coastal natural areas to breed and forage, some gulls (*Larus* sp.) have become more common in urban areas, even establishing breeding populations in cities around the world, using rooftop buildings to construct their nests, which provide a more temperate and stable microclimate, and fewer natural predators than natural habitats (Auman et al. 2008; Huig et al. 2016; Spelt et al. 2019; Méndez et al. 2020). Gulls take advantage of the presence of accessible and diverse food resources, including prey of marine, terrestrial, freshwater and anthropogenic (particularly landfills) origin (Belant et al. 1998; Rock 2005; Washburn et al.

2013; Plaza and Lambertucci 2017). As opportunistic birds, gulls have adapted to the exploitation of food subsidies from landfills not only near the coast but also up to several kilometres inland, during both the breeding and non-breeding seasons (Duhem et al. 2003b, 2008; Skorka et al. 2005; Ramos et al. 2009; Weiser and Powell 2011; Arizaga et al. 2014). At sea, gulls also exploit anthropogenic subsidies derived from fishery discards (Oro et al. 2013; Calado et al. 2018).

When foraging, both at urban and landfill environments, gulls may find not only domestic waste and food scraps from human origin (remnants of cooked and raw meat, fish, fruits, vegetables, meals, eggs, among others; Parfitt et al. 2010), but also anthropogenic materials including plastic of different types, paper, rubber or pieces of metal and glass (Da Cruz et al. 2012). When exploiting resources from fishery discards, during fishing operations, gulls may also be prone to interact with anthropogenic materials floating at sea (Galgani et al. 2015). The ingestion of these non-edible materials can be deleterious for gulls, by obstructing food passage, causing stomach ulcers and perforations or a false sensation of satiety (Ryan 1987; Henry et al. 2011; Kühn et al. 2015; Kühn and van Franeker 2020). Further consequences of ingesting anthropogenic materials include disturbance in the absorption and assimilation of nutrients (Gregory 2009) or the bioaccumulation of toxins from plastic-derived chemicals (Tanaka et al. 2013; Lavers et al. 2014). Gulls, as generalist feeders, are more prone to ingest these materials either unintentionally while foraging, or deliberately as some materials look similar to natural prey being easily misinterpreted as food (Ryan 1987). However, when resting, gulls voluntarily regurgitate the remnants of food and other materials as pellets (González-Solís et al. 1997; Votier et al. 2003) and thus the impact of anthropogenic materials ingestion in these species may be lower than in other seabird groups (Acampora et al. 2016). Nevertheless, detrimental effects of temporal retention of anthropogenic debris in gulls' digestive system remain unknown (Provencher et al. 2017).

To date, most of the studies on anthropogenic debris ingestion by birds have focused on marine species as indicators of marine plastic pollution, with the ingestion of plastics as the main target (van Franeker et al. 2011; Provencher et al. 2015, 2017). Some studies have reported plastic and non-plastic ingestion by seabirds and waterbirds feeding on non-marine habitats (Henry et al. 2011; English et al. 2015; Bond 2016; Holland et al. 2016; Seif et al. 2018). Considering gulls, there is a large number of diet studies reporting the ingestion of plastics and other anthropogenic materials (review by Battisti et al. 2019a). However, studies focusing on the ingestion of anthropogenic materials by urban dwelling gulls are relatively scarce (Seif et al. 2018; Méndez et al. 2020), especially comparing foraging gulls in urban areas with those in natural areas (Weiser and Powell 2011; Bond 2016). Detailed description and characterization of anthropogenic materials ingested by gulls foraging in natural, urban and landfill environments is lacking, as well as the relationship between their diet and the intake of anthropogenic debris. Additionally, a comprehensive characterization of ingested materials may help detect possible patterns in the

intake of different debris' categories among species, habitats and seasons (Provencher et al. 2017). The collection and examination of gull pellets allow to study their diet in a non-invasive way (Karnovsky et al. 2012), using the taxonomical identification of undigested prey items. The diet assessment using this technique has some bias towards hard parts of prey, underestimating soft-bodied organisms (Shealer 2002). Pellet analysis, however, provide a reasonable representation of gulls' diet (Schmutz and Hobson 1998) and it constitutes a good contribution to study the ingestion of anthropogenic materials (Provencher et al. 2017, 2019).

The Yellow-legged gull (*Larus michahellis*) breeds in both natural and urban breeding sites (Lopes et al. 2020) and is known to use urban habitats and anthropogenic structures such as landfills to forage, roost and rest (Duhem et al. 2008; Ceia et al. 2014). The main goal of this study is to describe the anthropogenic materials (anthropogenic debris) ingested by Yellow-legged gulls in six locations across Portugal with distinct availability and accessibility to these materials, ranging from natural colonies to urban areas and landfills. More specifically, we characterize the anthropogenic debris present in gull pellets collected in 1) natural and urban breeding colonies, to assess possible seasonal changes among three seasons (pre-breeding, breeding and post-breeding), and in 2) urban and landfill resting sites, to detect seasonal patterns among four seasons (spring, summer, autumn and winter), in the ingestion of these materials. Finally, we aim to relate the presence of anthropogenic materials with the diet of gulls. The abundance of ingested materials is expected to be 1) higher in urban than in natural breeding locations, as the accessibility to these materials is higher in urban areas than in natural sites, and should be lower during the breeding season in both natural and urban breeding locations, as gulls are known to shift to a marine prey-based diet to provide good quality food to their chicks (Annett and Pierotti 1999; Alonso et al. 2015). Regarding urban and landfill resting sites, the accessibility to anthropogenic materials should be predictable and relatively constant throughout the year for gulls foraging in such sites. However, the relative amount of anthropogenic materials may vary across seasons. For instance, during the busy summer season, with more tourists and a higher consumption of take-away meals, there may be a higher concentration of anthropogenic materials in landfills and nearby urban resting sites. Also, seasonal differences in the ingestion of these materials by gulls are expected due to age-specific use of these resting sites: in the time period correspondent to the breeding season (late spring and summer seasons), these sites are primarily used by first-year immature birds, whose diet selection may differ from that of adults, which use these resting sites mostly during the non-breeding season. Finally, we expect pellets with a higher occurrence of natural prey items to have a lower occurrence of debris categories. If a positive relationship occurs between a certain natural prey item and a certain debris category, it might suggest that gulls may be mistakenly ingesting such anthropogenic material for a specific natural food item of similar appearance (Lindborg et al. 2012), or that the ingestion of debris occurs accidentally while foraging on that food item.

3.2. Materials and methods

3.2.1. Characterization of study locations

This study was conducted between 2017 and 2019 in six sites of occurrence of Yellow-legged gulls across Portugal, divided into four categories (Figure 3.1): 1) natural breeding colonies (Deserta Island and Berlenga Island), 2) urban breeding colonies (Peniche and Porto, considering only the Clérigos area in the breeding season), 3) urban resting site (St. Catarina Street area in Porto) and 4) landfill resting site (in Coimbra), with different accessibility to refuse and anthropogenic debris.

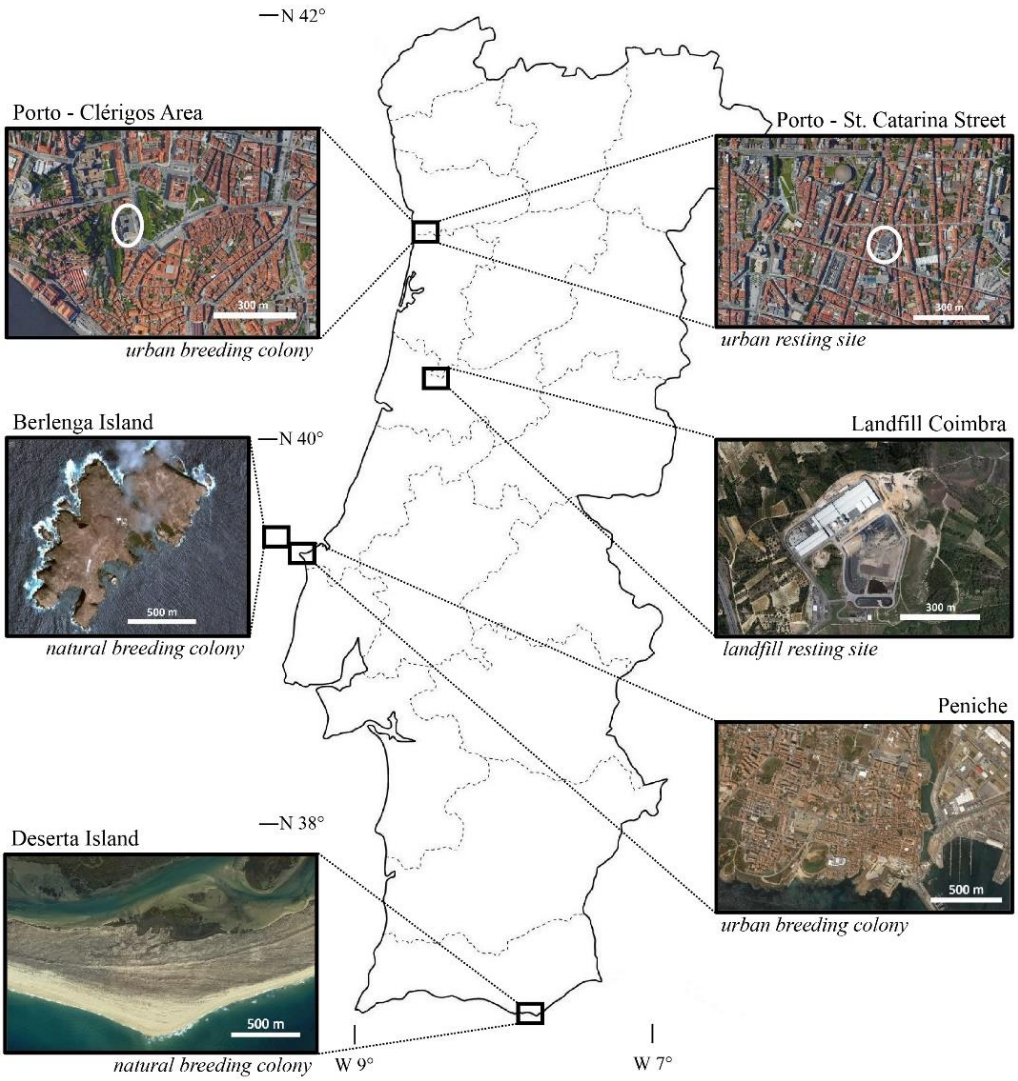

Figure 3.1. Geographical location of natural breeding colonies (Deserta Island and Berlenga Island), urban breeding colonies (Peniche and Porto Clérigos Area) and resting sites (St. Catarina Street and Landfill Coimbra) used for sampling of pellets, with aerial images of each location taken from Google Earth. For Porto aerial images, buildings where pellets were collected are marked with a white circle.

Deserta Island (36° 57' 44"N, 7° 53' 23"W), a natural breeding colony, is an uninhabited barrier sandy island that belongs to Ria Formosa Natural Park, south of Portugal. It is situated about 5.5 km from the mainland, relatively far from metropolitan and populated urban centres, and it hosts an estimated population of 1400 breeding pairs of Yellow-legged gulls (Calado et al. 2020). This island only has a very small pressure from tourism and is characterized by high fishing activity (Matos et al. 2018) that might be responsible for a higher availability of fishing related debris (e.g. rope and fishing lines).

Berlenga Island (39° 24′ 49″N, 9° 30′ 29″W), a natural breeding colony, is 11 km distant from the Portuguese coast, central Portugal, within an area of intense coastal upwelling during summer. It hosts the largest breeding colony (about 8500 pairs, ICNF, unpublished data 2017) of Yellow-legged gulls of the Portuguese coast (Ceia et al. 2014). The high number of visitors during summer leads to the increase of the tourism-related impacts, including consumer waste debris such as remnants of plastic bags and cling film.

Peniche (39° 21' 13"N, 9° 22' 55"W), an urban breeding colony, central Portugal, is a small seaside city (~26500 inhabitants, PORDATA 2011), highly dependent on fishing activities and hosting an important fishing harbour. In this urban breeding location, some gull individuals nest in rooftops of buildings and a small colony of about 30 breeding pairs nest in an abandoned part of a fortress. Gulls dwell in the city all year round, either attracted by the fishing harbour, where they usually forage, or due to the proximity to Berlenga Island (Morais et al. 1998). The municipal services of Peniche collected, in 2018, 407 t of plastics, 609 t of paper/cardboard and 615 t of glass as urban waste (INE 2019), suggesting that the availability of anthropogenic materials in Peniche is much higher than that in natural breeding colonies.

Porto (41° 08' 43"N, 8° 37' 04"W), north of Portugal, is the second largest city of the country (~215950 inhabitants, PORDATA 2011), and lies on the right side of the mouth of the Douro River, close to the sea. Gulls dwell in the city throughout the year, nest on rooftops of public and private buildings, and use certain areas of the city to rest. The colonization of this area by gulls is relatively recent and the number of breeding gulls is not known, but, from direct observations, we detected about 150 breeding individuals in the area where we worked. Pellets were collected in the Clérigos area, an urban breeding location where gulls nest in the rooftops of the Courthouse, and in St. Catarina Street area, an urban resting site, where gulls rest at LaVie Baixa Shopping throughout the year. An essential foraging ground for gulls is located about 10 km away from the centre of Porto: the fishing harbour of Matosinhos, the second Portuguese harbour with more fish landed per year (Bueno-Pardo et al. 2020). The municipal services of Porto collected, in 2018, 3092 t of plastics, 5431 t of paper/cardboard and 5406 t of glass in Porto (INE 2019), suggesting that the availability of debris materials is much higher in Porto than in Peniche.

The landfill of Coimbra (40° 17' 11"N, 8° 28' 15"W), a resting and foraging site, is part of a public company (ERSUC) that constitutes a multi cities system for treatment and valorisation of urban solid waste from the centre of Portugal. ERSUC treats approximately 300.000 t of residuals each year (ERSUC 2020). As the garbage disposal area does not have any type of coverage, and, at the time of pellets collection, no gull control occurred, refuse was fully accessible, constituting a reliable food source and attracting up to 25.000 individuals (authors' personal observation), depending on the time of the year. Beyond Yellow-legged gulls, several species occur at this landfill, especially during winter (non-breeding season), including Lesser black-backed gull (*Larus fuscus*), Black-headed gull (*Chroicocephalus ridibundus*), White Stork (*Ciconia ciconia*) and Cattle Egret (*Bubulcus ibis*). Pellets of Yellow-legged gulls and Lesser black-backed gulls are indistinguishable. Considering the relative abundance of both Yellow-legged gull and Lesser black-backed gull at this landfill, pellets from winter are expected to belong to both species roughly in the same proportion. During spring, a higher proportion of Yellow-legged gull pellets is expected, as the number of adult Lesser-black backed gulls decreased considerably. Yet, both immature Yellow-legged and Lesser-black backed gulls still occurred in the area during spring. As both gull species are opportunistic and generalist (Gyimesi et al. 2016), and both foraged exactly on the same sites inside the landfill, pellets of both species should represent similar diet items. At this site, the availability of anthropogenic materials should be the highest of all study sites.

3.2.2. Pellet analysis

Pellets were randomly collected from 2017 to 2019 across the six locations (see specific sample size for each location / season in Table 3.1). Natural (Deserta Island and Berlenga Island) and urban (Peniche and Porto) breeding colonies were visited at least once per season, and resting sites (St. Catarina Street and Landfill Coimbra) were surveyed once per month. Pellets were collected throughout the entire area of each location by establishing transects crossing each study site. Only pellets which were fresh and structurally intact were collected, to ensure that the results reflected the recent diet of the individuals and to guarantee that the maximum number of anthropogenic materials is detected as the likelihood of finding debris declines as the integrity of the pellet decreases (Provencher et al. 2019). Samples were individually stored in plastic bags until further analysis.

In the laboratory, pellets were left at room temperature until they were completely dry. Then, they were broken apart, carefully examined and debris materials were separated from natural prey items using a stereomicroscope. Anthropogenic materials were sorted and categorized into type and colour following standardized procedures established by Provencher et

al. (2017). Categories of debris were plastic, glass, metal (includes aluminium foil), fabric (includes different types of fibres), paper and other (uncommon items such as wooden toothpicks and rubber). Plastics were also sub-divided in four different types: sheet plastics (e.g. plastic bags and cling film), threadlike plastics (e.g. fishing lines, plastic strings and ribbons), fragment plastics (unidentifiable fragments from the break-up of larger plastic items as well as intact items) and foamed plastics (e.g. styrofoam). Items' colours were registered using a two-step colour sorting process as recommended by Provencher et al. (2017): light, medium, dark and more than one colour were the first colour categories. The more specific colour categorization included: white/clear, yellow, green, blue/purple, red/pink, brown/orange, grey/silver, black and more than one colour (Verlis et al. 2014). The biggest axis of each debris item was measured using graph paper, with an accuracy of 0.5 mm. Debris materials were weighted per category and per pellet to the nearest 0.0001 g using a precision balance.

Fish prey items were identified to the lowest possible taxonomic level using fish vertebrae and otoliths from a reference collection of fish from the Portuguese coast and identification guides (Assis 2004; Tuset et al. 2008). In addition to fish, other marine prey such as crabs, bivalves, cephalopods, sea-urchins, and starfishes were found in pellets. The occurrence of vegetal matter, terrestrial prey (e.g. insects) and bones of several species such as rats (*Rattus* sp.), rabbits (*Oryctolagus cuniculus*) and small birds were also detected and registered.

3.2.3. Data analysis

A matrix including the number of each debris material category found on each pellet (and therefore, per location and season) was produced. From this, a binomial matrix, of presence/absence of each category of debris was also constructed. The frequency of occurrence of each category (FO, %) was calculated from the binary matrix by using the formula $FOi = n_i / n_{total} \times 100\%$, where *i* represents a specific category of anthropogenic debris, n_i is the number of pellets in which *i* is present and n_{total} corresponds to the total number of analysed pellets.

Two study groups were considered for data analysis. Data from 1) breeding locations (Deserta, Berlenga, Peniche and Porto, considering only the Clérigos area in the breeding season) were used to analyse seasonal differences in the quantity and characteristics of anthropogenic debris ingested by Yellow-legged gulls. These data are from 2018 and encompass three seasons: pre-breeding (from January to April), breeding (from May to August) and post-breeding (from September to December). Data from 2) resting sites (St. Catarina Street area in Porto and Landfill Coimbra) were grouped to assess seasonal patterns in the quantity and characteristics of ingested anthropogenic debris by gulls. Pellets from St. Catarina Street were collected monthly from March 2018 to February 2019 and grouped into spring 2018 (March, April, May), summer 2018

(June, July, August), autumn 2018 (September, October, November) and winter 2018/19 (December 2018, January 2019 and February 2019). Pellets from Landfill Coimbra were collected monthly from December 2017 to May 2018 and grouped into winter 2017/18 (December 2017, January 2018 and February 2018) and spring 2018 (March, April, May).

The presence or absence of debris in gull pellets was tested using generalized linear models (GLMs) with binomial distribution and logit link function. We tested the effect of 1) breeding location for each season: pre-breeding (Deserta, Berlenga and Porto), breeding (Deserta, Berlenga, Peniche and Porto) and post-breeding seasons (Deserta, Berlenga, Peniche and Porto). Then we tested the effect of 2) season for each resting site (St. Catarina Street and Landfill Coimbra) to evaluate seasonal differences in the presence or absence of the total debris (all debris) and the total plastics (all plastic) in gull pellets.

To understand how the number of debris items per pellet differed between 1) breeding locations in each season and between 2) seasons in each resting site, we performed zero-inflated models, with negative binomial distributions to account for overdispersion. Zero-inflated models tested the influence of 1) breeding location (Deserta Island, Berlenga Island, Peniche and Porto) in each season of 2018 and 2) seasons in each resting site (St. Catarina Street and Landfill Coimbra). For both groups, models were performed considering the total number of items per pellet (all debris) and the total number of plastic items per pellet (all plastic). Zero inflated models use a reference category against which the remaining data is compared, thus, for 1) breeding locations, Porto was assigned as the reference location for all seasons and, for 2) resting sites, spring 2018 was assigned as the reference season for both sites.

Mean mass and mean size of anthropogenic materials were calculated per location and season, for both breeding locations and resting sites, and for each debris category. For these calculations, pellets without debris were not considered, meaning that the division was made considering only the debris-positive pellets. Data was $\log_{10}$ transformed to attain normality. Generalized linear models (GLMs) with Gaussian family and identity link function were performed to evaluate 1) for each season (pre-breeding, breeding and post-breeding) the effect of breeding locations (Deserta Island, Berlenga Island, Peniche and Porto), and 2) for each resting site (St. Catarina Street and Landfill Coimbra) the effect of season (spring, summer, autumn and winter) in the mass of ingested debris. For both groups, models were performed for the total debris ingested (all debris) and for the total plastics ingested (all plastic).

For all previous analysis (i.e. presence / absence, number of items and mass of both all debris and all plastic), further differences between 1) breeding locations for each season and 2) seasons for each resting site were investigated with post-hoc Tukey tests.

To understand the relation between the ingestion of prey and anthropogenic materials, data on the presence/absence of prey identified from the hard parts remains in pellets was added to the data on the presence or absence of debris materials categories, for exactly the same pellets. Prey

was divided in 11 groups of the most common prey found in gull pellets: pelagic fish, demersal fish, unidentified fish, total fish, Henslow's swimming crab (*Polybius henslowii*), total Crustacea (includes *Pollicipes pollicipes*, unidentified Brachyura and unidentified Crustacea), Mollusca (includes *Patella* sp., *Mytillus* sp., Gastropoda, unidentified Bivalve and unidentified Cephalopoda), Insecta (includes orders Coleoptera and Hymenoptera, and unidentified Insecta), vegetal matter, bones (includes bones of small birds, chicken, pork, *Rattus* sp., *Oryctolagus cuniculus* and unidentified bones) and others (includes Asteroidea, Echinoidea, eggshell and unidentified items). Generalized linear mixed models (GLMMs) with binomial distribution and "location" (Deserta Island, Berlenga Island, Peniche, Porto and Landfill Coimbra) as a random effect were used to test the effect of prey in the presence/absence of each debris category. Given the large number of statistical models performed and to control for the increased likelihood of type I errors (false positives), we applied the Bonferroni correction by dividing the *p* value for the number of performed tests. Each prey group was used in 11 different models, i.e. each debris category, therefore the *p* value of 0.05 was divided by 11 and the significance level was $p < 0.005$.

The R statistical program (R Core Team 2019) was used in all analyses, with a significance level of $p < 0.05$, unless stated otherwise (i.e. GLMMs). GLM models were performed using *MASS* R package (Venables and Ripley 2002), Zero-inflated models were performed using *pscl* R package (Zeileis et al. 2008; Jackman 2017), GLMM models were performed using *lme4* R package (Bates et al. 2015) and post-hoc tests were performed using *lsmeans* R package (Lenth et al. 2020).

3.3. Results

3.3.1. Seasonal changes in debris ingestion in natural and urban breeding locations

A total of 1132 Yellow-legged gull pellets were examined in natural (Deserta and Berlenga Islands, $n = 806$) and urban (Peniche and Porto, $n = 326$) breeding locations along the three seasons of 2018. Of all examined pellets, 28.8% ($n = 326$) had at least one anthropogenic material in its composition (Table 3.1, panel A). Pellets from Porto urban breeding location had the highest frequency of occurrence of anthropogenic materials for all seasons (above 81%, Table 3.1, panel A, Figure 3.2a), however pellets from Deserta in the pre-breeding season showed a similarly high frequency of occurrence of debris (almost 73%, Table 3.1, panel A, Figure 3.2a). Detailed descriptive statistics on the debris categories ingested by Yellow-legged gulls are shown in Table S3.1. Plastics were the most frequently ingested category of anthropogenic material (34.6% of all ingested debris), followed by glass (25.1% of all ingested debris), while items from the category

other were the least frequently ingested (5.5% of all ingested debris, Figure 3.2a, Table S3.2). Sheet plastics were the most frequently ingested type of plastic (33.2% of all ingested plastics, Figure 3.2b, Table S3.2).

GLM results testing the effect of breeding location on the presence/absence of anthropogenic materials in gull pellets showed that the presence of anthropogenic debris varied significantly among locations, for all seasons (pre-breeding: $F_{2, 291} = 79.7$; $p < 0.001$; breeding: $F_{3, 419} = 53.1$; $p < 0.001$; post-breeding: $F_{3, 411} = 73.6$; $p < 0.001$). The ingestion of plastics also varied significantly among locations, for all seasons (pre-breeding: $F_{2, 291} = 63.3$; $p < 0.001$; breeding: $F_{3, 419} = 50.7$; $p < 0.001$; post-breeding: $F_{3, 411} = 60.7$; $p < 0.001$). For all seasons, both anthropogenic debris and all plastics had a higher occurrence in pellets from Porto urban breeding location. Pellets from Porto always presented significant differences with each of the remaining breeding locations for all seasons (all Tukey $p < 0.01$, Table S3.3), except in the pre-breeding season when there were no significant differences in the presence/absence of debris and plastics between pellets from Porto urban location and pellets from Deserta Island (natural breeding location, Tukey $p = 0.32$, Table S3.3).

A total of 6737 anthropogenic materials were detected in gull pellets from all breeding locations with a mean number of 5.95 items per pellet (Table 3.1, panel A). Porto pellets presented the highest values for all seasons (maximum mean of 44 items/pellet in the breeding season, ranging from 0 to 664 items, Table 3.1, panel A). From all anthropogenic debris items ingested in breeding locations, 51.3% were plastics, mostly of threadlike type (42.9% of the total plastic items ingested, Table S3.4 and Figure S3.1). Zero-inflated models (Table 3.2) showed significant differences in the number of ingested items per breeding location, for all seasons. In the pre-breeding season, the number of items was significantly higher in Porto pellets (reference location) when compared to pellets from Berlenga Island ($Z = -4.92$; $p < 0.001$), and no significant differences were detected in the number of ingested items between pellets from Porto and Deserta Island (Tukey $p = 0.12$, Table S3.3). In both breeding and post-breeding seasons, Porto pellets presented the highest number of items when compared to pellets from Deserta Island, Berlenga Island and Peniche ($Z > -3.01$; $p < 0.01$, Table 3.2), always exhibiting significant differences with all the remaining breeding locations (all Tukey $p < 0.01$, Table S3.3). The same patterns were detected for the number of ingested plastic items, except that no significant differences were found between Porto and Peniche pellets in the post-breeding season (Table 3.2, Table S3.3).

Table 3.1. Description of anthropogenic debris present in **A)** 1132 Yellow-legged gull (*Larus michahellis*) pellets from 4 breeding locations (Deserta Island, Berlenga Island, Peniche and Porto), during the 3 seasons of 2018 (Pre-breeding "PBre", Breeding "Bre" and Post-breeding "PTBre"), and in **B)** 447 gull pellets from 2 resting sites (St. Catarina Street and Landfill Coimbra), from winter 2017 to winter 2018. No data for Peniche in the pre-breeding season. In Porto, only the Clérigos area was considered in the breeding season. Pellets from St. Catarina Street belong to Yellow-legged gulls only, while pellets from Landfill Coimbra belong to both Yellow-legged and Lesser black-backed gulls (*Larus fuscus*). N = Number of pellets. FO = Frequency of Occurrence. SD = Standard Deviation.

	Location	Season	N	FO (%) debris	Items per pellet		Mass of debris (g)		Size of debris (cm)		Total items
					Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	
A	Deserta	PBre	92	72.8	10.22 ± 14.25	0 - 78	0.50 ± 0.86	0.0005 - 5.12	1.74 ± 3.88	0.1 - 57.4	940
		Bre	150	12	1.62 ± 7.34	0 - 71	0.58 ± 0.69	0.0023 - 2.31	1.50 ± 1.76	0.1 - 18.5	243
		PTBre	39	7.7	0.08 ± 0.27	0 - 1	0.01 ± 0.01	0.0041 - 0.02	12.73 ± 19.33	0.3 - 35	3
	Berlenga	PBre	109	6.4	0.16 ± 0.78	0 - 6	0.17 ± 0.24	0.0017 - 0.68	1.30 ± 0.76	0.5 - 3	17
		Bre	157	1.3	0.04 ± 0.43	0 - 5	0.04 ± 0.04	0.0095 - 0.07	2.34 ± 1.95	0.4 - 5.6	7
		PTBre	259	6.6	0.59 ± 4.02	0 - 55	0.60 ± 0.99	0.0022 - 4.07	1.51 ± 1.76	0.25 - 10.7	153
	Peniche	Bre	76	23.7	1.34 ± 3.86	0 - 19	0.10 ± 0.19	0.0005 - 0.76	1.02 ± 1.61	0.15 - 14.3	102
		PTBre	30	16.7	0.87 ± 2.33	0 - 9	0.22 ± 0.31	0.0011 - 0.72	1.30 ± 1.68	0.1 - 7.8	26
	Porto	PBre	93	81.7	16.31 ± 25.08	0 - 176	0.35 ± 0.67	0.0013 - 3.87	1.52 ± 1.79	0.1 - 20	1517
		Bre	40	92.5	43.95 ± 106.47	0 - 664	0.41 ± 0.51	0.0016 - 1.87	0.95 ± 1.67	0.1 - 22.5	1758
		PTBre	87	87.4	22.66 ± 40.66	0 - 334	0.25 ± 0.38	0.0001 - 1.83	1.18 ± 1.74	0.1 - 27.3	1971
	TOTAL	ALL	1132	28.8	5.95 ± 26.45	0 - 664	0.37 ± 0.64	0.0001 - 5.12	1.35 ± 2.30	0.1 - 57.4	6737
B	St. Catarina Street	Spring18	113	83.2	15.59 ± 23.23	0 - 176	0.32 ± 0.62	0.0001 - 3.87	1.45 ± 1.74	0.1 - 20	1762
		Summer18	60	91.7	36.45 ± 89.27	0 - 664	0.32 ± 0.45	0.0016 - 1.87	1.03 ± 2.15	0.1 - 39.6	2187
		Autumn18	63	84.1	20.48 ± 24.88	0 - 125	0.25 ± 0.39	0.0001 - 1.77	1.20 ± 1.81	0.1 - 27.3	1290
		Winter18	46	95.7	22.46 ± 48.58	0 - 334	0.22 ± 0.31	0.0023 - 1.83	1.36 ± 2.21	0.1 - 29.3	1033
	Landfill Coimbra	Winter17	95	93.7	10.32 ± 7.85	0 - 33	0.21 ± 0.34	0.0012 - 1.87	1.62 ± 3.16	0.1 - 40.3	980
		Spring18	70	94.3	16.53 ± 14.54	0 - 89	0.32 ± 0.42	0.0022 – 2.01	2.26 ± 3.93	0.1 - 32	1157
	TOTAL	ALL	447	89.7	18.81 ± 40.28	0 - 664	0.28 ± 0.45	0.0001 - 3.87	1.47 ± 2.58	0.1 - 40.3	8409

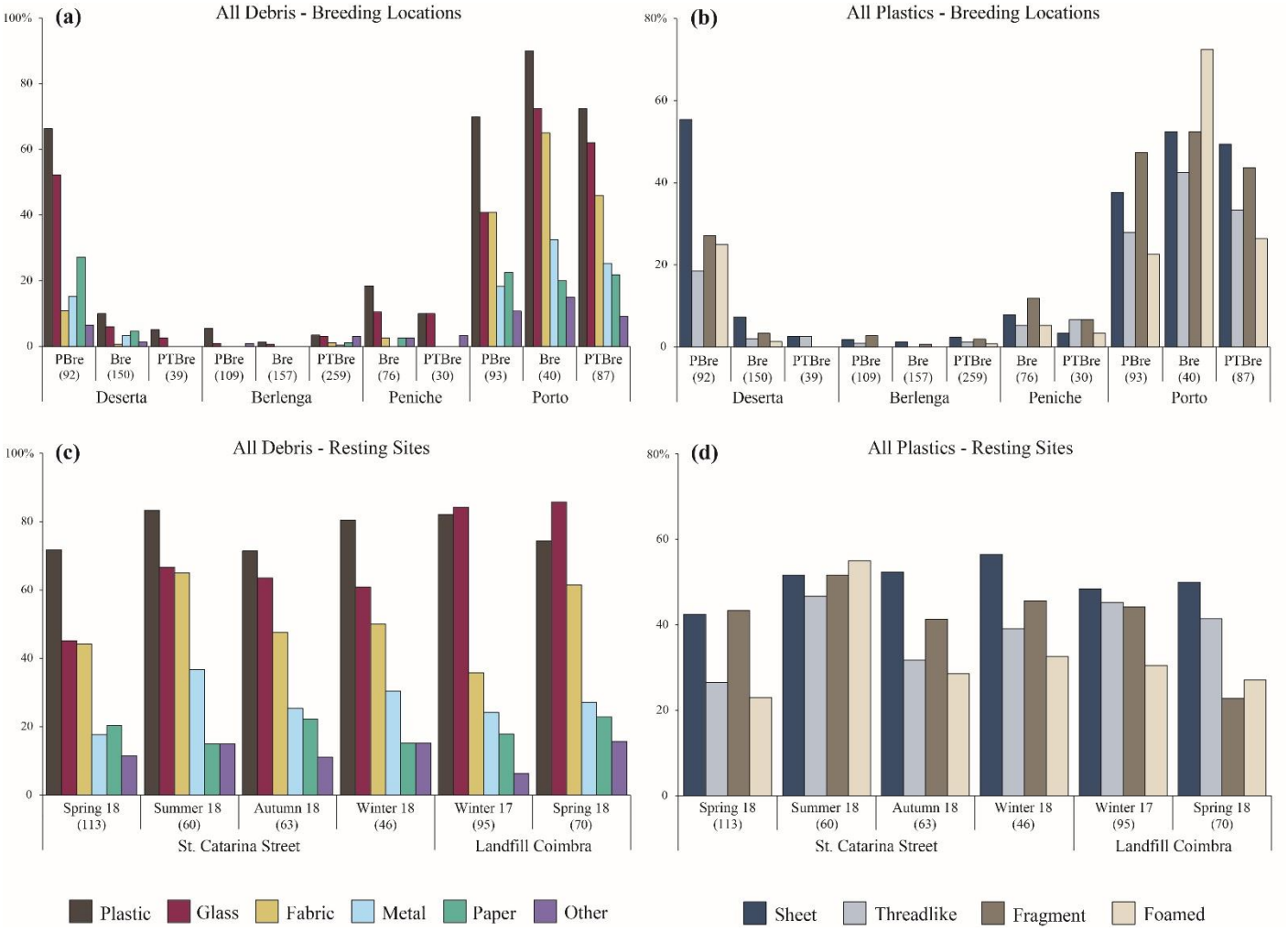

Figure 3.2. Frequency of occurrence (FO, %) of anthropogenic debris in Yellow-legged gull (*Larus michahellis*) pellets a) and b) on 4 breeding locations (Deserta Island, Berlenga Island, Peniche and Porto) during pre-breeding (PBre), breeding (Bre) and post-breeding (PTBre) seasons of 2018, and c) and d) on 2 resting sites (St. Catarina Street and Landfill Coimbra) during winter of 2017, spring, summer, autumn and winter of 2018. No data for Peniche in the pre-breeding season. In Porto, only the Clérigos area was considered in the breeding season. Pellets from St. Catarina Street belong to Yellow-legged gulls only, while pellets from Landfill Coimbra belong to both Yellow-legged and Lesser black-backed gulls (*Larus fuscus*). a) and c) For each category of anthropogenic debris (glass, fabric, metal, paper, other and plastic); the total number of pellets collected in each location / season is presented on the horizontal axis. b) and d) For plastic categories (sheet, threadlike, fragment and foamed); the total number of pellets collected in each location / season is presented on the horizontal axis.

Detailed mean mass of each debris category ingested is reported in Table S3.1. On average, the greatest mass of anthropogenic materials was found in pellets from Berlenga Island, during the post-breeding season (mean mass = 0.60 g, ranging from 0.0022 to 4.07 g, Table 3.1, panel A) especially due to metal materials (Figure 3.3a). Pellets from Deserta Island during the pre-breeding season presented the highest maximum mass value (5.12 g, Table 3.1, panel A), belonging to the category other (Figure 3.3a, Table S3.1). As for the types of plastic, on average the greatest mass of plastic was attributed to a sheet plastic found in pellets from Peniche, during the post-breeding season (mean mass = 0.33 g, Table S3.1), however fragment plastics had, on average, higher masses (Figure 3.3b). The longest item was found on a pellet from Deserta Island collected during the pre-breeding season (a sheet plastic with 57.4 cm, Table S3.1). When testing the effect of breeding location on the mean mass of all anthropogenic debris ingested, significant differences were only detected for the breeding season: pellets from Porto urban breeding location presented a greater mass of anthropogenic materials ($F_{3, 71} = 5.18$; $p < 0.01$), when compared to pellets from the remaining breeding locations and mean mass of anthropogenic items from Peniche pellets was significantly different from that of Deserta Island and Porto pellets (Tukey $p < 0.01$, Table S3.3). During the pre-breeding season pellets from Deserta Island had a significantly higher mean mass of plastics when compared to the remaining breeding locations ($F_{2, 129} = 3.54$; $p = 0.03$).

The colours of all anthropogenic materials present in gull pellets from breeding locations are graphically represented in Figure 3.4a and the colours of all ingested plastics in Figure 3.4b. The most common colour of ingested materials was white/clear, followed by black and green. The pattern is the same when considering all plastics (Figure 3.4b), sheet and foamed plastics as individual categories (Figure S3.2). Green was the most frequent colour of ingested threadlike plastics, while fragment plastics had a wider variety of colours (Figure S3.2).

Table 3.2. Statistics from zero-inflated models testing the effect of breeding location (Deserta Island, Berlenga Island, Peniche and Porto) in the number of items of debris materials ingested by Yellow-legged gull (*Larus michahellis*), for each season of the year 2018 (pre-breeding, breeding and post-breeding). No data for Peniche in the pre-breeding season. In Porto, only the Clérigos area was considered in the breeding season. Porto was assigned as reference for all models. Only results from count models are shown. Significant effects are highlighted in bold.

		(count model)	**All Debris**	**All Plastic**
Pre-breeding	Berlenga	β ± SE	-2.64 ± 0.54	-2.10 ± 0.67
		Z	-4.92	-3.15
		P	**<0.001**	**<0.01**
	Deserta	β ± SE	-0.38 ± 0.19	-0.30 ± 0.24
		Z	-1.20	-1.24
		P	0.05	0.22
Breeding	Berlenga	β ± SE	-3.13 ± 1.04	-3.56 ± 1.16
		Z	-3.02	-3.08
		P	**<0.01**	**<0.01**
	Deserta	β ± SE	-1.41 ± 0.40	-2.76 ± 0.48
		Z	-3.57	-5.75
		P	**<0.001**	**<0.001**
	Peniche	β ± SE	-2.46 ± 0.40	-2.57 ± 0.49
		Z	-6.12	-5.26
		P	**<0.001**	**<0.001**
Post-breeding	Berlenga	β ± SE	-1.19 ± 0.36	-1.83 ± 0.56
		Z	-3.35	-3.28
		P	**<0.001**	**<0.01**
	Deserta	β ± SE	-5.69 ± 0.64	-5.40 ± 0.78
		Z	-8.89	-6.92
		P	**<0.001**	**<0.001**
	Peniche	β ± SE	-1.88 ± 0.62	-1.15 ± 0.91
		Z	-3.01	-1.26
		P	**<0.01**	0.21

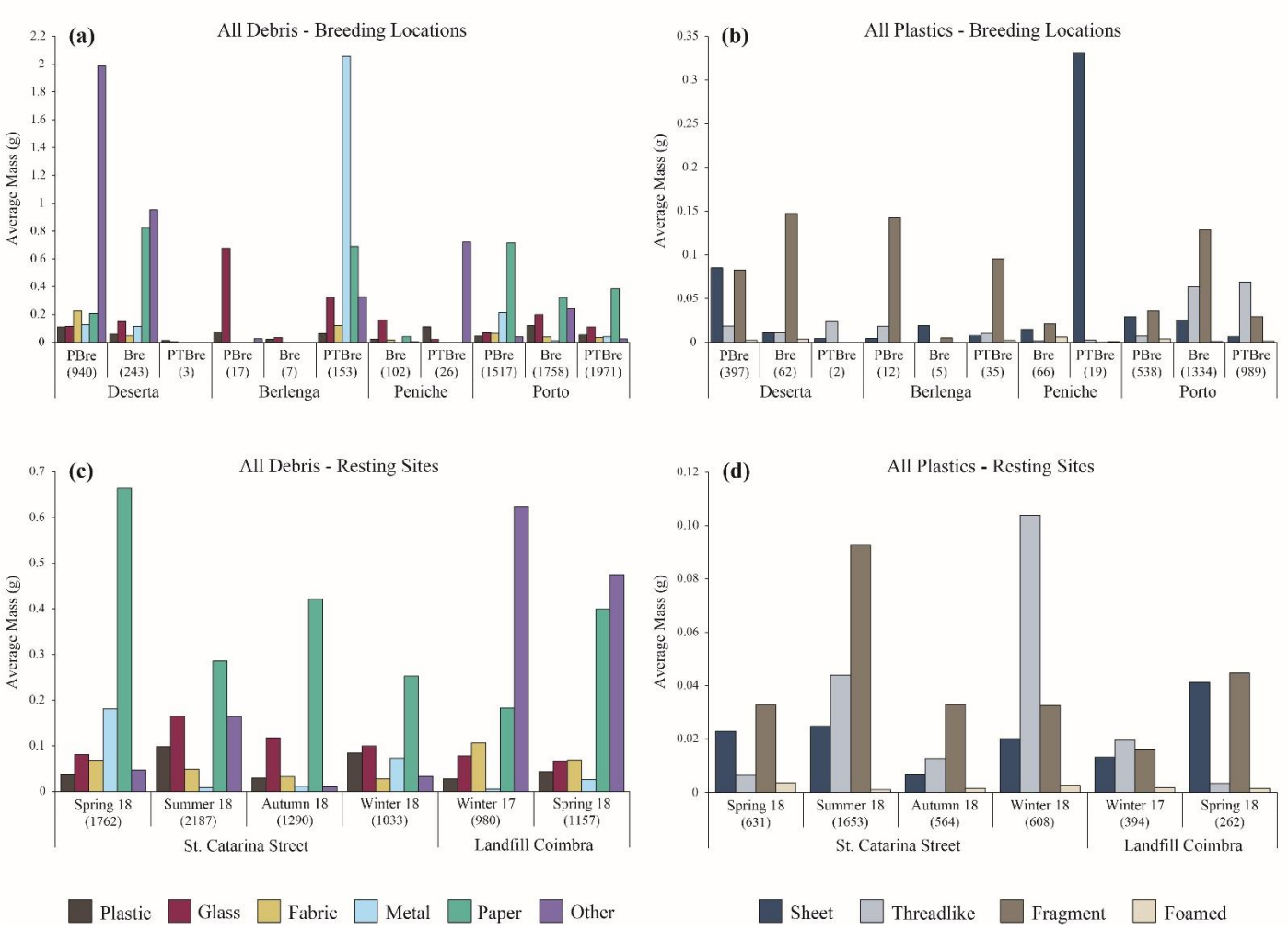

Figure 3.3. Average mass (g) of anthropogenic debris in Yellow-legged gull (*Larus michahellis*) pellets **a)** and **b)** on 4 breeding locations (Deserta Island, Berlenga Island, Peniche and Porto) during pre-breeding (PBre), breeding (Bre) and post-breeding (PTBre) seasons of 2018, and **c)** and **d)** on 2 resting sites (St. Catarina Street and Landfill Coimbra) during winter of 2017, spring, summer, autumn and winter of 2018. No data for Peniche in the pre-breeding season. In Porto, only the Clérigos area was considered in the breeding season. Pellets from St. Catarina Street belong to Yellow-legged gulls only, while pellets from Landfill Coimbra belong to both Yellow-legged and Lesser black-backed gulls (*Larus fuscus*). a) and c) For each category of anthropogenic debris (glass, fabric, metal, paper, other and plastic); the number of debris items in each location / season is presented on the horizontal axis. b) and d) For plastic categories (sheet, threadlike, fragment and foamed); the number of plastic items in each location / season is presented on the horizontal axis.

3.3.2. Seasonal patterns in debris ingestion in urban and landfill resting sites

447 gull pellets were examined in resting sites (St. Catarina Street, n = 282 and Landfill Coimbra, n = 165), from winter 2017 to winter 2018. Of all examined pellets, 89.7% (n = 401) had at least one anthropogenic material in its composition (Table 3.1, panel B). For all seasons, at both locations, the frequency of occurrence of debris was always above 83% (Table 3.1, panel B). Descriptive statistics on the debris categories ingested by gulls in both resting sites are shown

in Table S3.5. Plastics were the most frequently ingested category of anthropogenic debris (30.8% of all ingested debris), followed by glass (26.8% of all ingested debris), while items from the category other were the least frequently ingested (4.8% of all ingested debris, Figure 3.2c, Table S3.6). All types of plastics were ingested almost equally, with sheet plastics the most frequently ingested type of plastic (30.8% of all ingested plastics, Figure 3.2d, Table S3.6). When testing the effect of season on the presence/absence of anthropogenic debris in gull pellets in each resting location, no significant differences were detected, neither for the total ingested debris ($F_{3, 278}$ = 2.34; p = 0.07 for St. Catarina Street and $F_{1, 163}$ = 0.03; p = 0.87 for Landfill Coimbra), nor for the total ingested plastics ($F_{3, 278}$ = 1.40; p = 0.24 for St. Catarina Street and $F_{1, 163}$ = 1.46; p = 0.23 for Landfill Coimbra).

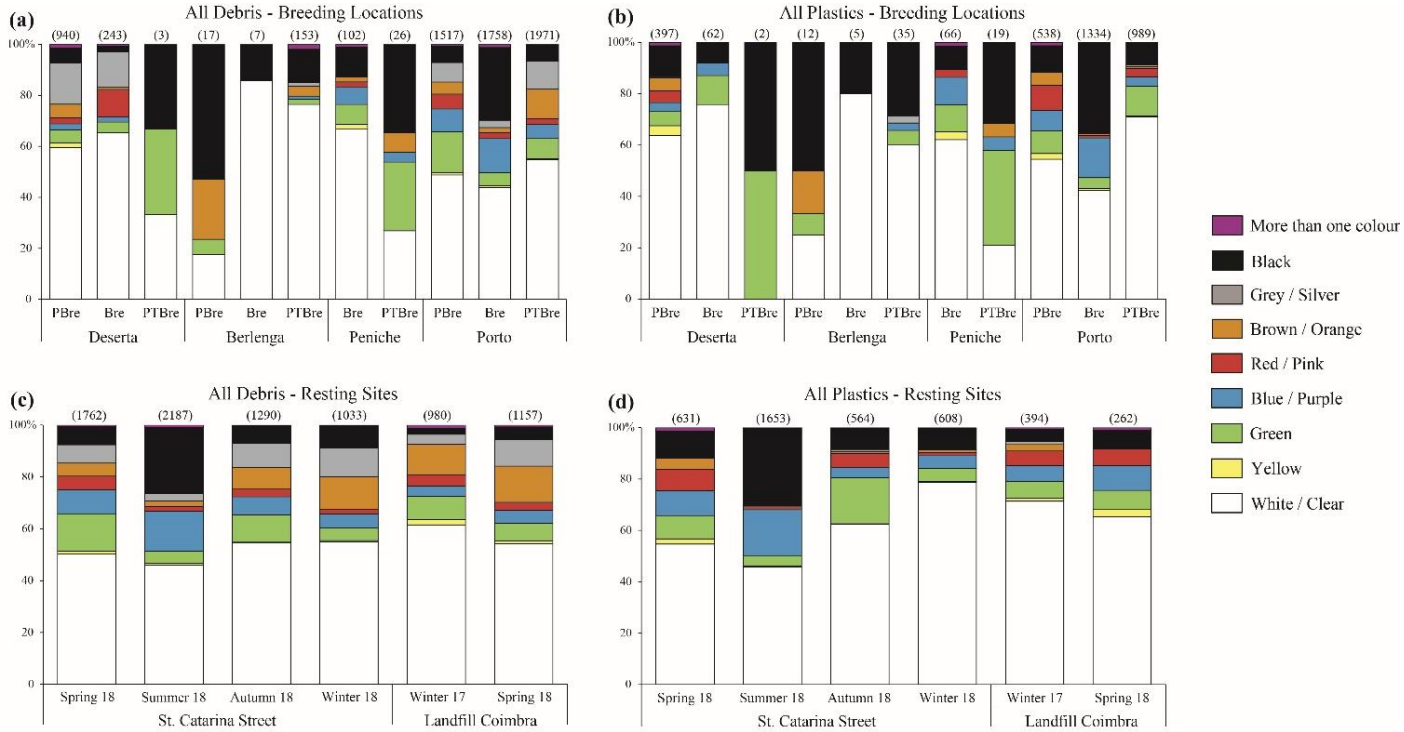

Figure 3.4. Frequency of occurrence (FO, %) of anthropogenic debris (**a** and **c**) and plastics (**b** and **d**) colours in Yellow-legged gull (*Larus michahellis*) pellets on **a)** and **b)** 4 breeding locations (Deserta Island, Berlenga Island, Peniche and Porto) during pre-breeding (PBre), breeding (Bre) and post-breeding (PTBre) seasons of 2018, and **c)** and **d)** on 2 resting sites (St. Catarina Street and Landfill Coimbra) during winter of 2017, spring, summer, autumn and winter of 2018. No data for Peniche in the PBre season. In Porto, only the Clérigos area was considered in the breeding season. Pellets from St. Catarina Street belong to Yellow-legged gulls only, while pellets from Landfill Coimbra belong to both Yellow-legged and Lesser black-backed gulls (*Larus fuscus*). Number of analysed items in each location / season presented on the top of each bar.

A total of 8409 anthropogenic debris items were detected in gull pellets from resting sites, with a mean number of 18.8 items per pellet. St. Catarina Street pellets presented the highest mean number of items per pellet (36.5 items/pellet) in summer 2018 (Table 3.1, panel B). From all anthropogenic debris items ingested in both resting sites, 48.9% were plastics, mostly of threadlike type (45.2% of the total plastic items ingested, Table S3.7 and Figure S3.3). Zero-

inflated models (Table S3.8) showed only small significant differences in the number of ingested items per season, in each resting site. In St. Catarina Street, the number of anthropogenic debris items (all debris) and plastic items (all plastic) were significantly higher during summer 2018 (Z = 3.95; $p < 0.001$ and Z = 6.28; $p < 0.001$, respectively), when compared to spring 2018 (the reference season), and the number of plastic items in gull pellets was significantly higher in winter 2018 when comparing to spring 2018 (Z = 3.08; $p < 0.01$). No significant differences in the number of all debris items were detected between the remaining seasons (all Tukey $p > 0.14$, Table S3.9), however, significant differences were found in the number of ingested plastic items between summer and autumn seasons (Tukey p = 0.01, Table S3.9). In Landfill Coimbra, the number of items (all debris) detected in gull pellets was significantly higher in spring 2018 than in winter 2017 (Z = -3.91; $p < 0.001$, Table S3.8).

On average, anthropogenic debris in pellets from resting locations weighted 0.28 g, ranging from 0.0001 to 3.87 g (Table 3.1, panel B), and the highest mean mass was detected in spring 2018 for Landfill Coimbra (0.324 g). The item with the greatest mass was registered on a pellet from St. Catarina Street in spring 2018 (a paper item with 3.87 g, Table S3.5), while the longest item was found in a pellet from Landfill Coimbra, in winter 2018 (a threadlike plastic with 40.3 cm, Table 3.1, panel B, Table S3.5). Considering the mean mass of each debris category, paper presented the highest mean mass in St. Catarina Street for spring 2018 (mean mass = 0.66 g) followed by the category other in Landfill Coimbra for winter 2017 (mean mass = 0.62 g, Figure 3.3c, Table S3.5). As for the types of plastic, threadlike plastics from St. Catarina Street in winter 2018 presented, on average, the highest mean mass (0.10 g), followed by fragment plastics from the same resting site, in summer 2018 (mean mass = 0.09 g, Figure 3.3d, Table S3.5). When testing seasonal differences in the mean mass of the total ingested anthropogenic debris (all debris), significant differences were only detected for Landfill Coimbra, with a greater mass of debris items being ingested in spring 2018 ($F_{1, 153}$ = 6.09; p = 0.01). As for the mass of the total ingested plastics, significant differences were only found for St. Catarina Street, where pellets collected in summer 2018 had plastics with the greatest mass ($F_{3, 209}$ = 3.55; p = 0.02).

The colours of all anthropogenic materials present in gull pellets from resting locations are graphically represented in Figure 3.4c and the colours of all ingested plastics are in Figure 3.4d. The most commonly found colour of all debris, all plastics and each type of plastic materials was white/clear. Black and blue/purple were the second and third most typical colours for all debris, all plastics and sheet plastics. Threadlike and fragment plastics presented a wider variety of colours (Figure S3.4).

3.3.3. Relation between the presence of anthropogenic debris and gulls' diet composition

When relating the presence of all debris categories with the presence/absence of items of gulls' diet, sheet and fragment plastics were negatively related with the presence of pelagic fish (Table 3.3): pellets with a higher occurrence of pelagic fish had a lower occurrence of these plastic categories ($Z > -3.08$; $p = 0.002$). Pellets with more paper and fragment plastics had a significantly lower presence of *Polybius henslowii* ($Z > -2.90$; $p < 0.004$). The presence of all debris, glass, all plastics, sheet, fragment and foamed plastics was positively related with the occurrence of vegetal matter ($Z > 3.09$; $p < 0.002$). Pellets with a higher occurrence of Asteroidea, Echinoidea, eggshell and unidentified items ("total others" category) had also a higher occurrence of almost all debris categories, with the exception of fabric, paper and other ($Z > 3.15$; $p < 0.002$). The presence of all debris, glass, fabric, all plastics, sheet, threadlike and foamed plastics was positively related with the occurrence of bones ($Z > 2.92$; $p < 0.004$).

3.4. Discussion

This study provides a detailed characterization of anthropogenic materials ingested by Yellow-legged gulls in Portugal, across natural, urban and landfill sites. To our best knowledge, this is the first time that the ingestion of anthropogenic materials by gulls was related with the consumption of prey items. Our results show that gulls ingested anthropogenic debris at all studied locations and at all stages of their breeding cycle, throughout the year. In agreement with previous studies, Yellow-legged gulls exhibited high levels of refuse in their pellets (Duhem et al. 2003a; Ramos et al. 2009; Alonso et al. 2015; Furtado et al. 2016; Calado et al. 2018). When studying the ingestion of anthropogenic materials through necropsies, the amount of anthropogenic debris found in individuals' stomachs are usually lower (Codina-García et al. 2013; Nicastro et al. 2018; Basto et al. 2019) as gulls have the capability to regurgitate a large part of the non-edible food remains, which includes anthropogenic materials (Barrett et al. 2007; Kühn and van Franeker 2020). Despite this ability, some items may remain in the individual's system, and the reasons behind a somehow selective regurgitation remain unknown (Provencher et al. 2017). Pellets are unlikely to represent the full debris load of an individual, and the amount of anthropogenic materials kept in the birds' stomachs cannot be quantified (Provencher et al. 2019). Even though we should be cautious when using pellets to assess anthropogenic materials ingested by gulls, this technique provides a simple, non-invasive and relatively straightforward approach that allows to compare debris ingestion across locations.

Table 3.3. Statistics from GLMM testing the effect of prey items (pelagic fish, demersal fish, unidentified fish, total fish, *Polybius henslowii*, crustacea, mollusca, insecta, vegetal matter, bones, others) in the presence/absence of debris materials ingested by Yellow-legged gull (*Larus michahellis*) with "location" (Deserta, Berlenga, Peniche, Porto and Landfill Coimbra) as a random effect. Significant effects are highlighted in bold, with significance level at $p < 0.005$ after p value correction (see methods).

		All Debris	**Glass**	**Fabric**	**Metal**	**Paper**	**Other**	**All plastics**	**Sheet plastics**	**Threadlike plastics**	**Fragment plastics**	**Foamed plastics**
Pelagic fish	β ± SE	-0.73 ± 0.35	-1.07 ± 0.43	0.39 ± 0.51	-0.79 ± 0.56	-1.51 ± 0.59	-2.57 ± 1.13	-0.71 ± 0.35	-1.21 ± 0.39	0.31 ± 0.42	-1.55 ± 0.50	-0.83 ± 0.48
	Z (*P*)	-2.10 (0.04)	-2.50 (0.01)	0.77 (0.44)	-1.41 (0.16)	-2.56 (0.01)	-2.27 (0.02)	-2.04 (0.04)	-3.11 (**0.002**)	0.75 (0.45)	-3.08 (**0.002**)	-1.72 (0.08)
Demersal fish	β ± SE	-0.27 ± 0.35	-0.73 ± 0.42	-0.41 ± 0.49	-0.63 ± 0.53	-1.07 ± 0.56	-1.37 ± 1.05	-0.32 ± 0.35	-0.57 ± 0.38	-0.35 ± 0.40	-1.32 ± 0.49	0.13 ± 0.46
	Z (*P*)	-0.77 (0.44)	-1.75 (0.08)	-0.82 (0.41)	-1.18 (0.24)	-1.89 (0.06)	-1.30 (0.19)	-0.91 (0.36)	-1.51 (0.13)	-0.88 (0.38)	-2.68 (0.007)	0.28 (0.78)
Unidentified fish	β ± SE	0.22 ± 0.36	-0.10 ± 0.43	0.06 ± 0.51	-0.42 ± 0.56	-0.84 ± 0.59	-0.97 ± 1.09	0.12 ± 0.35	-0.11 ± 0.39	0.53 ± 0.42	-0.69 ± 0.51	-0.02 ± 0.49
	Z (*P*)	0.63 (0.53)	-0.24 (0.81)	0.12 (0.90)	-0.75 (0.45)	-1.41 (0.16)	-0.89 (0.37)	0.34 (0.74)	-0.29 (0.78)	1.27 (0.20)	-1.37 (0.17)	-0.04 (0.97)
Total fish	β ± SE	-0.29 ± 0.41	0.31 ± 0.47	-0.31 ± 0.54	0.64 ± 0.59	0.60 ± 0.62	0.90 ± 1.12	-0.04 ± 0.39	0.32 ± 0.42	-0.40 ± 0.45	0.94 ± 0.53	0.13 ± 0.52
	Z (*P*)	-0.70 (0.48)	0.65 (0.51)	-0.58 (0.56)	1.08 (0.28)	0.96 (0.34)	0.80 (0.42)	-0.10 (0.92)	0.75 (0.45)	-0.89 (0.37)	1.77 (0.08)	0.25 (0.80)
Polybius henslowii	β ± SE	-1.40 ± 0.72	-1.23 ± 0.76	-0.81 ± 0.85	-0.67 ± 0.94	-2.76 ± 0.95	-2.25 ± 0.91	-0.78 ± 0.70	-0.41 ± 0.79	-1.27 ± 0.82	-2.86 ± 0.75	-0.77 ± 1.04
	Z (*P*)	-1.95 (0.05)	-1.62 (0.11)	-0.96 (0.34)	-0.71 (0.48)	-2.90 (**0.004**)	-2.49 (0.01)	-1.10 (0.27)	-0.52 (0.61)	-1.55 (0.12)	-3.81 (<**0.001**)	-0.74 (0.46)
Total Crustacea	β ± SE	-0.20 ± 0.66	-0.89 ± 0.63	-0.18 ± 0.64	-0.08 ± 0.71	0.06 ± 0.67	0.17 ± 0.78	-0.63 ± 0.61	-1.24 ± 0.69	-0.13 ± 0.63	-0.08 ± 0.56	-0.89 ± 0.81
	Z (*P*)	-0.30 (0.77)	-1.42 (0.16)	-0.28 (0.78)	-0.11 (0.91)	0.09 (0.93)	0.22 (0.82)	-1.03 (0.30)	-1.80 (0.07)	-0.20 (0.84)	-0.14 (0.88)	-1.10 (0.27)
Total Mollusca	β ± SE	0.53 ± 0.35	0.64 ± 0.33	0.80 ± 0.33	0.67 ± 0.32	0.24 ± 0.32	0.62 ± 0.38	0.42 ± 0.32	0.14 ± 0.29	0.76 ± 0.29	0.49 ± 0.28	0.75 ± 0.30
	Z (*P*)	1.53 (0.13)	1.95 (0.05)	2.46 (0.01)	2.14 (0.03)	0.75 (0.45)	1.61 (0.11)	1.30 (0.19)	0.48 (0.63)	2.63 (0.009)	1.76 (0.08)	2.48 (0.01)
Total Insecta	β ± SE	-0.05 ± 0.36	0.32 ± 0.37	0.36 ± 0.45	-0.22 ± 0.52	0.43 ± 0.41	-0.66 ± 0.62	0.33 ± 0.34	0.26 ± 0.35	0.26 ± 0.39	0.03 ± 0.36	0.58 ± 0.40
	Z (*P*)	-0.15 (0.88)	0.87 (0.39)	0.81 (0.42)	-0.43 (0.67)	1.06 (0.29)	-1.07 (0.28)	0.98 (0.33)	0.76 (0.45)	0.66 (0.51)	0.09 (0.93)	1.46 (0.15)
Total Vegetal Matter	β ± SE	1.20 ± 0.23	1.19 ± 0.19	0.42 ± 0.19	0.55 ± 0.22	0.52 ± 0.22	0.01 ± 0.27	1.08 ± 0.19	0.82 ± 0.18	0.37 ± 0.19	0.64 ± 0.18	0.63 ± 0.21
	Z (*P*)	5.14 (<**0.001**)	6.23 (<**0.001**)	2.20 (0.03)	2.54 (0.01)	2.33 (0.02)	0.05 (0.96)	5.54 (<**0.001**)	4.67 (<**0.001**)	1.94 (0.05)	3.56 (<**0.001**)	3.09 (**0.002**)
Total Bones	β ± SE	1.38 ± 0.25	0.82 ± 0.20	0.57 ± 0.19	0.26 ± 0.21	-0.19 ± 0.21	0.71 ± 0.28	1.06 ± 0.20	0.75 ± 0.18	0.55 ± 0.19	0.15 ± 0.18	0.98 ± 0.21
	Z (*P*)	5.60 (<**0.001**)	4.14 (<**0.001**)	3.01 (**0.003**)	1.25 (0.21)	-0.86 (0.39)	2.54 (0.01)	5.26 (<**0.001**)	4.22 (<**0.001**)	2.92 (**0.004**)	0.84 (0.40)	4.80 (<**0.001**)
Total Others	β ± SE	2.80 ± 0.49	1.73 ± 0.23	0.54 ± 0.20	0.82 ± 0.22	0.63 ± 0.23	0.25 ± 0.27	2.06 ± 0.27	1.29 ± 0.19	0.62 ± 0.20	0.91 ± 0.19	1.19 ± 0.21
	Z (*P*)	5.67 (<**0.001**)	7.53 (<**0.001**)	2.74 (0.006)	3.76 (<**0.001**)	2.80 (0.005)	0.91 (0.37)	7.50 (<**0.001**)	6.63 (<**0.001**)	3.15 (**0.002**)	4.79 (<**0.001**)	5.67 (<**0.001**)

Gulls from Porto urban breeding colony exhibited the highest frequency of occurrence of anthropogenic debris and plastics, a significantly higher number of items per pellet, and also ingested, on average, a higher mass of anthropogenic materials during the breeding season. Gulls opportunistically exploit the food resources which are greatly available in the colony surroundings, and the closer the colony is to urban centres, the higher the contribution of anthropogenic food subsidies for the diet of gulls (Duhem et al. 2003b; Ramos et al. 2009; Witteveen et al. 2017; Fuirst et al. 2018). In urban locations, especially in Porto, anthropogenic materials are widely available, as suggested by the amount of waste collected by the municipal services from both cites (see characterization of study locations in methods section), therefore individuals should be more exposed to the ingestion of these materials than in natural breeding colonies (Witteveen et al. 2017). Pellets from the Porto urban breeding colony always exhibited a higher frequency of occurrence and number of anthropogenic materials when compared to the other urban location, Peniche. The density of the human population in Peniche is lower than in Porto, resulting in lower anthropogenic food availability and accessibility, and a lower probability of gulls interacting with anthropogenic materials. From our data, gull pellets from Peniche had more marine items in their composition (i.e. fish, crustaceans and molluscs) than those from Porto. Peniche is surrounded by fishing grounds and has an important fishing harbour which may contribute to a higher dependency on fisheries when compared to gulls from Porto.

During the pre-breeding season, contrary to what we expected, pellets from Deserta Island natural breeding colony exhibited high levels of anthropogenic debris and had a significantly higher mass of plastics on their composition when compared to the remaining breeding locations (i.e. Berlenga Island and Porto). No statistical differences were detected in either the frequency of occurrence or in the number of items of both debris and plastics when comparing Deserta Island pellets with Porto pellets. This unusual situation may be due to weather conditions experienced in Deserta Island during the pre-breeding season, especially before sample collection (IPMA 2019). The exceptionally harsh weather, with intense rain that restricted fishing activities, forced gulls to change their foraging habits, focusing on alternative foraging habitats such as terrestrial and landfill areas; the nearest landfill used by gulls breeding in Deserta Island lays 30 km away from the colony (Matos et al. 2018). During the breeding season, especially in the chick-rearing period, gulls are known to shift their diet from anthropogenic remains towards a more energetic diet with marine prey to provide their chicks with food that better fulfil their nutritional needs (Duhem et al. 2005; Ramos et al. 2009). The ingestion of anthropogenic items during the breeding season was transversal to all breeding locations (natural and urban). Yet, Berlenga Island pellets had the lowest frequency of occurrence, mean number of items and mean mass of anthropogenic debris. This means that gulls ingest anthropogenic debris also during the breeding season when they are rearing their chicks, which may be indicative of gulls possibly delivering debris items to their chicks (Yorio et al. 2020), although most likely in low quantities. As breeding Yellow-

legged gulls also use urban and landfill environments to forage (Ramos et al. 2009), they might accidentally ingest anthropogenic debris and transfer these materials to chicks when feeding. In fact, several studies on seabirds of other taxonomic groups detected the presence of anthropogenic materials in chicks' digestive tracts and in their diets (Ryan 1988; Bond et al. 2010; Rodríguez et al. 2012; Acampora et al. 2017; Yorio et al. 2020). The presence of these materials in the chicks' system may affect their growth and body condition (Lavers et al. 2014). The occurrence of anthropogenic materials in pellets from the post-breeding season might be indicative of a higher consumption of these by juvenile individuals, with less foraging experience (Weiser and Powell 2011).

In our studied landfill resting site, collected pellets belonged to both Yellow-legged and Lesser black-backed gulls, in different proportions according to the collection date, with a larger proportion of Lesser black-backed gull pellets in winter than in spring. Both gull species are known to breed in natural and urban habitats (Spelt et al. 2019; Lopes et al. 2020), and are known to be opportunistic and generalist feeders that use marine, terrestrial, urban and landfill environments to forage (Gyimesi et al. 2016; Mendes et al. 2018). In Landfill Coimbra, adult and immature individuals of both species, were observed using the exact same sites to search for food, which may indicate that both species' pellets represent similar diet and identical ingestion of anthropogenic debris items. Even though the accessibility to anthropogenic debris is, supposedly, predictable and constant throughout the year in landfills and urban resting sites, small seasonal differences were detected in the number of debris items ingested (higher during summer in St. Catarina Street and during spring in Landfill Coimbra) and in their masses (higher during spring in Landfill Coimbra). Gulls, particularly immature birds, specialize in the consumption of refuse and exploitation of landfills and urban areas during spring, when other dietary items, such as marine prey, are less abundant (Burger and Gochfeld 1983; Belant et al. 1998; Duhem et al. 2008; Egunez et al. 2018). Although we do not have data for Landfill Coimbra other than winter 2017 and spring 2018, we may speculate that the number of debris items ingested by gulls at this resting site would be higher in summer as well due to age-specific (i.e. higher number of immature gulls) use of this site. In fact, the use of Landfill Coimbra during the beginning of the breeding season (April), was typically ruled by first-year individuals, and less by reproductive adults (authors' personal observation), a pattern also reported by Egunez et al. 2018 with first-year Yellow-legged gulls being more abundant at landfills than birds of older ages. St. Catarina Street, the urban resting location in Porto, was also used primarily by immature birds during summer season, corresponding to the period of time when adults are breeding (authors' personal observation). Immature gulls are less efficient in finding food, but their foraging success seems to improve gradually over time (Greig et al. 1983; Cristol et al. 2017), mainly due to interactions with adult gulls that may help immatures in learning and improving their foraging capabilities and effective recognition of edible items (Greig et al. 1983).

We reported mass (mean and range) of anthropogenic debris present in gull pellets in both natural and urban breeding locations, and in urban and landfill resting sites. From a biological perspective, mass of ingested debris is one of the most important metrics to be presented as it indicates information about the volume of debris in an individual (Provencher et al. 2017). As anthropogenic debris fill the stomach, the greater the mass of anthropogenic materials in an individual's stomach, the more likely it is to have negative effects caused by these materials (Kühn et al. 2015; Kühn and van Franeker 2020). In fact, gulls from Porto urban breeding location ingested the greatest mass of materials, which may indicate that gulls using large urban areas to forage and to breed may be more prone to suffer from negative consequences of ingesting these materials. Although larger and heavier items may be more likely to physically block and/or damage gulls' digestive tract, the materials described in this study were regurgitated by gulls as pellets, and therefore the items with greater masses may have less impacts on individuals' digestive system than those that remain in the digestive tracts (i.e. smaller items and microplastics that may not be regurgitated). These smaller items may interfere with birds' physiology and body condition (Puskic et al. 2019), however comprehensive studies on how these debris items affect birds' health are scarce (but see Lavers et al. 2019).

Gulls that typically fed on pelagic fish had significantly less sheet and fragment plastics in their pellets, which suggests that gulls that specialize in feeding on their natural prey in marine foraging habitats (Mendes et al. 2018) probably have less interactions with anthropogenic debris, as it is less available when comparing to highly anthropogenic environments (urban areas and landfills). The same rationale can be applied to the relation found between the ingestion of *Polybius henslowii* and certain debris categories. The Henslow's swimming crab is a well-known prey of Yellow-legged gulls, especially in Berlenga Island where gulls rely highly on this prey (Alonso et al. 2015; Calado et al. 2020). At this breeding location, as reported by Alonso et al. (2015), when this prey is highly available, the amount of refuse in gull pellets is low, meaning that gulls from Berlenga Island only choose to use a distant predictable food source (landfill) when the availability of swimming crabs (*Polybius henslowii*) in the colony surroundings is lower. Positive relations occurred between the presence of dietary items such as vegetal matter, Asteroidea, Echinoidea, eggshell and unidentified items ("others" prey category), and the presence of almost all debris categories in gull pellets. This might suggest that gulls, as a generalist species, may be using multiple foraging habitats to search for food, from both marine and terrestrial origin (Mendes et al. 2018). Asteroidea and Echinoidea are typically found in the intertidal zone where gulls were observed foraging and, given the proximity of our studied urban locations to the seashore, gulls may accidentally ingest anthropogenic debris derived from the nearby urban areas. The ingestion of bones was positively related with the majority of debris categories which suggests that the ingestion of these debris occurs accidentally while exploiting anthropogenic food remains. As an example, at Landfill Coimbra, gulls were seen exploring the

garbage, searching for food remains, parts of meat, fruit, meals and other food scraps, typically packaged in plastic or paper bags. While gulls managed to feed themselves with, for instance, meat remains (which are not detected in pellets), they also ingested bones of that meat (which are detected in pellets) and, accidentally, ingested the packages where the meat was stored (authors' personal observation).

Although we focus our research in specific locations which may not be generalized to other sites, the amount and variety of ingested anthropogenic materials at all of our studied locations, but especially in urban areas and landfills is a motive of concern and could result in chronic exposure to debris, and to the negative effects of ingesting them, including the exposure to persistent organic pollutants found especially on plastics (Hammer et al. 2016; Lavers and Bond 2016a). It reveals a poor waste management in urban areas, which allow gulls to have access to these debris, and at landfills it shows that the garbage is readily available for opportunistic species to exploit. The European Union Landfill Directive (European Commission 2016) intends to gradually reduce the biodegradable waste entering landfills by replacing open-air landfill by covered waste facilities of difficult access to birds (Gilbert et al. 2016). In Portugal, during 2018, about 40% of national plastic waste ended up in landfills (PlasticsEurope 2019), thus it is likely that, in the near future, the European Union Landfill Directive will have important consequences for birds in Portugal. To continue studying the threat of debris ingestion by Yellow-legged gulls and other birds, standardized reports of plastic and other materials ingestion are key to highlight the magnitude of pollution in the surrounding environments, to detect possible temporal, spatial and taxonomic trends and to help evaluate the population level effects of ingesting anthropogenic materials through the correlation with other demographic studies.

Chapter 4

FATTY ACIDS COMPOSITION IN YELLOW-LEGGED (*Larus michahellis*) AND LESSER BLACK-BACKED (*Larus fuscus*) GULLS FROM NATURAL AND URBAN HABITATS IN RELATION TO THE INGESTION OF ANTHROPOGENIC MATERIALS

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Fatty acids composition in Yellow-legged (*Larus michahellis*) and Lesser black-backed (*Larus fuscus*) gulls from natural and urban habitats in relation to the ingestion of anthropogenic materials

Abstract

Urban habitats offer spatially and temporally predictable anthropogenic food sources for opportunistic species, such as several species of gulls that are known to exploit urban areas and take advantage of accessible and diverse food sources, reducing foraging time and energy expenditure. However, human-derived food may have a poorer nutritional quality than the typical natural food resources and foraging in urban habitats may increase birds' susceptibility of ingesting anthropogenic debris materials, with unknown physiological consequences for urban dwellers. Here we compare the fatty acids (FA) composition of two opportunistic gull species (the Yellow-legged gull, *Larus michahellis*, and the Lesser black-backed gull, *Larus fuscus*) from areas with different levels of urbanization, to assess differences in birds' diet quality among foraging habitats, and we investigate the effects of ingesting anthropogenic materials, a toxicological stressor, on gulls' FA composition. Using GC-MS, 23 FAs were identified in the adipose tissue of both gull species. Significant differences in gulls' FA composition were detected among the three urbanization levels, mainly due to physiologically important highly unsaturated FAs that had lower percentages in gulls from the most urbanized habitats, consistent with a diet based on anthropogenic food resources. The deficiency in omega (ω)-3 FAs and the higher ω-6:ω-3 FAs ratio in gulls from the most urbanized location may indicate a diet-induced susceptibility to inflammation. No significant differences in overall FA composition were detected between gull species. While we were unable to detect any effect of ingested anthropogenic materials on gulls' FA composition, these data constitute a valuable contribution to the limited FA literature in gulls. We encourage studies to explore the long-term physiological effects of the lower nutritional quality diet for urban dwellers, and to detect the sub-lethal impacts of the ingestion of anthropogenic materials.

Keywords

Urbanization; Laridae; Diet analysis; Nutritional composition; Debris ingestion; Urban gulls

4.1. Introduction

Over the years, population growth and consequent urbanization transformed natural coastal habitats into novel urban environments (Marzluff 2001; Aronson et al. 2014) affecting ecosystems processes and dynamics (Oro et al. 2013), as well as animal physiology and behaviour (Rosenblatt and Schmitz 2016). Features from the urban environment, such as air, light and noise pollution, put urban dwelling wildlife under stress that may result in several molecular and physiological changes including altered gene expression, endocrine modifications and increased oxidative stress (Partecke et al. 2006; Salmón et al. 2016; Watson et al. 2017). The existence of suitable breeding sites and the availability of food resources are crucial for animal populations to strive and survive in urban conditions (Belant 1997; Oro et al. 2013). Human-modified environments offer high, isolated and protected buildings that allow nesting in safer sites, without human disturbance, as well as abundant, predictable and readily available anthropogenic food, which attracts a multiple number of generalist and opportunistic animals, such as gulls, rats or foxes (Belant 1997; Winton and River 2017; Parra-Torres et al. 2020).

Urban-derived food may be easier to access when compared to natural sources (Bartumeus et al. 2010), allowing opportunistic species to reduce foraging time and energy expenditure (Fuirst et al. 2018; Zorrozua et al. 2020b). However, the increase in anthropogenic food subsidies may act as an ecological trap as human-derived food has typically a poorer nutritional quality than the natural food resources (Auman et al. 2008), which may lead to a reduced growth rate and body condition (Pierotti and Annett 1991; Annett and Pierotti 1999). Animals exploiting these locations to forage may be susceptible to a higher exposure to contaminants, poisoning and pathogen infections (Seif et al. 2018; Sorais et al. 2020, Yorio et al. 2020), as well as an increased probability of interacting with anthropogenic debris materials such as glass, fabric, metal, paper and especially plastics (Lopes et al. 2020, 2021b). In fact, coastal and more generalist seabirds such as gulls are particularly exposed to such anthropogenic materials (Kühn and van Franeker 2020; Lopes et al. 2021b) and vulnerable to the direct deleterious impacts of their ingestion, which may include the obstruction to food passage, stomach ulcers and perforations of the gastrointestinal tract, disturbance in the assimilation of nutrients, damage to tissues, morbidity and starvation (Ryan 1987; Gregory 2009; Henry et al. 2011; Lavers et al. 2014; Kühn et al. 2015). In addition to these physical impacts, a range of less visible toxicological effects may be caused by the ingestion of anthropogenic materials, including a possible exposure to hazardous chemicals, especially from plastics containing known or suspected endocrine disrupting chemicals as additives (Gallo et al. 2018) which might contribute to neurological, endocrine and reproductive complications, and ultimately to death (Bouland et al. 2012; Rochman et al. 2016). In fact, examining toxicological effects of the ingestion of anthropogenic materials is difficult and evidence about the transfer of chemicals between plastics and animals is ambiguous. Most studies

report that plastic ingestion may contribute to a higher exposure of 'plastic-adhered pollutants' (Yamashita et al. 2011; Tanaka et al. 2013; Lavers and Bond 2016a; also see Herzke et al. 2016; Provencher et al. 2018a; Roman et al. 2019b). Yet, this transfer of chemicals may be bidirectional and also occur from the animal to the plastic particles, with such particles acting as "cleaning" factors and reducing the chemicals that are already present in the animal (Thaysen et al. 2020). The toxicological effects of ingested anthropogenic materials and whether they are a source or sink of chemicals to bird species are complex and dependent on the species' ecological context (e.g. exposure level and feeding ecology, Thaysen et al. 2020).

Large gulls *Laridae*, among them the Yellow-legged gull (YLG; *Larus michahellis*) and the Lesser black-backed gull (LBBG; *Larus fuscus*) have become more common in urban areas, with established breeding populations around the world, benefiting from a more temperate and stable microclimate and fewer natural predators than in natural habitats (Auman et al. 2008; Huig et al. 2016; Spelt et al. 2019; Méndez et al. 2020; Pais de Faria et al. 2021b). As opportunistic foragers, gulls use a wide variety of foraging habitats and strategies, being capable of exploiting different food types, especially anthropogenic food remains collected in landfills and within urban habitats (Ramos et al. 2009; Gyimesi et al. 2016; Matos et al. 2018; Spelt et al. 2019; Parra-Torres et al. 2020; Pais de Faria et al. 2021a). This resulted in an increase of their urban population numbers over the last few years (Vidal et al. 1998; Duhem et al. 2008; Nager and O'Hanlon 2016). Foraging gulls are known to ingest anthropogenic materials when foraging at their natural habitats (review by Battisti et al. 2019a) and at urban areas and landfills (Lopes et al. 2021b). Yet, possible invisible physiological effects that may arise from ingesting those materials are poorly known as it may not result in birds' death but in a poorer health condition, possibly only detectable at molecular and cellular organization levels (Lavers et al. 2019; Roman et al. 2019b). Many impacts from the exposure to plastics and other anthropogenic materials are perceived, but regarding subtle effects not all perceived impacts are truly demonstrated, measured and supported by evidence, and even fewer are empirically verified in realistic exposure scenarios (Rochman et al. 2016; Koelmans et al. 2017). Therefore, sub-lethal impacts of the ingestion of anthropogenic materials may be difficult to detect and may suffer from confounding bias (Roman et al. 2021), as factors other than debris ingestion might influence the observed effects at the individual level (Rochman et al. 2016). Generally, birds capable to survive and even thrive in urbanized areas are known to experience behavioural and physiological adaptations (Partecke et al. 2006; Shochat et al. 2010; Isaksson et al. 2015). Despite the known capability of gulls to exploit urban habitats and human-derived food resources, little is known about the associated physiological consequences of doing so, and if there are any consequences to their physiology from the ingestion of anthropogenic materials.

Fatty acids (hereafter FA) are the largest constituent of lipids (e.g. triglycerides, phospholipids and wax esters) which have different metabolic functions within an animal's body

from storage of energy to structural components of cell membranes (Williams and Buck 2010). FAs are obtained *via* dietary sources or by *de novo* biosynthesis, however, as birds are only capable of synthesising certain FAs, the majority of birds' FAs are acquired through their diet and, therefore, FA signatures of storage tissues largely reflect diet (Williams and Buck 2010). FA analysis has been used to assess birds' diet quality and to examine differences or changes in foraging patterns and/or diets both within and between populations of predator species (Iverson et al. 2007; Wang et al. 2009; Karnovsky et al. 2012). Recently, the potential of using FA composition as a response to toxicological factors has been explored to assess the sub-lethal impacts of plastic ingestion in seabirds (in Procellariforms, Puskic et al. 2019), after some reports of a negative correlation between ingested plastic and fat deposition in seabirds (Connors and Smith 1982; Auman et al. 1997).

FA signatures of fledgling gulls are known to differ between urban and natural habitats (Pais de Faria et al. 2021a), however, variation in FA composition has rarely been investigated in the context of urbanization, with the exception of passerines (e.g. Andersson et al. 2015; Isaksson et al. 2017). Polyunsaturated (PUFAs) and highly unsaturated fatty acids (HUFAs) are especially relevant to characterize as they are involved in regulating birds' physiological processes (Watson et al. 2017). These FAs are strictly dietary (i.e. essential fatty acids, EFAs) for all birds, mainly obtained by feeding on aquatic prey (e.g. fish; Gladyshev et al. 2009), and can affect some aspects of birds' performance (Twining et al. 2018). The ratio omega (ω)-6:ω-3 FAs is also interesting to assess in urbanization studies as it is related with inflammatory responses and oxidative stress (Romieu et al. 2008; Isaksson 2015). A high total of this ratio is associated with increased sensitivity to antigens by promoting inflammatory reactions and oxidative stress (Romieu et al. 2008). Overall, the FA composition of blood and tissues can play an important role on birds' health in urban habitats.

In this study we aim to 1) compare the FA composition of two gull species (YLG and LBBG) from three wildlife rescue centres that receive gulls from areas with different levels of urbanization, and to 2) investigate if there is any effect of ingesting anthropogenic materials on FA composition. We predict that FA composition will differ between urban and natural dwellers (i.e. between individuals from different wildlife rescue centres) mainly due to differences in diet between urban and natural habitats. As urban dwellers forage more on anthropogenic food resources than on marine prey, when compared to natural dwellers, we expect individuals from the most urbanized area to exhibit lower percentages of ω-3 PUFAs and HUFAs and a higher ω-6:ω-3 FAs ratio. As both species are known to be generalist and to forage on similar anthropogenic food subsidies, we do not anticipate major differences in overall FA composition between both gull species. We suggest that differences in diet among habitats should be the main driver for the possible differences in FA composition, however, differences in FA profiles may be also a response to toxicological stressors such as the ingestion of anthropogenic materials that may

disrupt nutritional pathways. Despite the difficulty in detecting sub-lethal impacts from the exposure to anthropogenic materials, we predict that their ingestion, if it occurs at high and toxic levels, could have physiological consequences for gulls and thus their FA composition should present differences as a response to this toxicological stress.

4.2. Materials and methods

4.2.1. Study sites and sampling processing

This study analysed 47 individuals from both Yellow-legged (YLG, *Larus michahellis*, *n* = 23) and Lesser black-backed gulls (LBBG, *Larus fuscus*, *n* = 24). All individuals used in this study were found stranded as a result of injury, illness or exhaustion, and brought by national authorities (Institute of Nature Conservation and Forests, ICNF) or by locals to one of the three

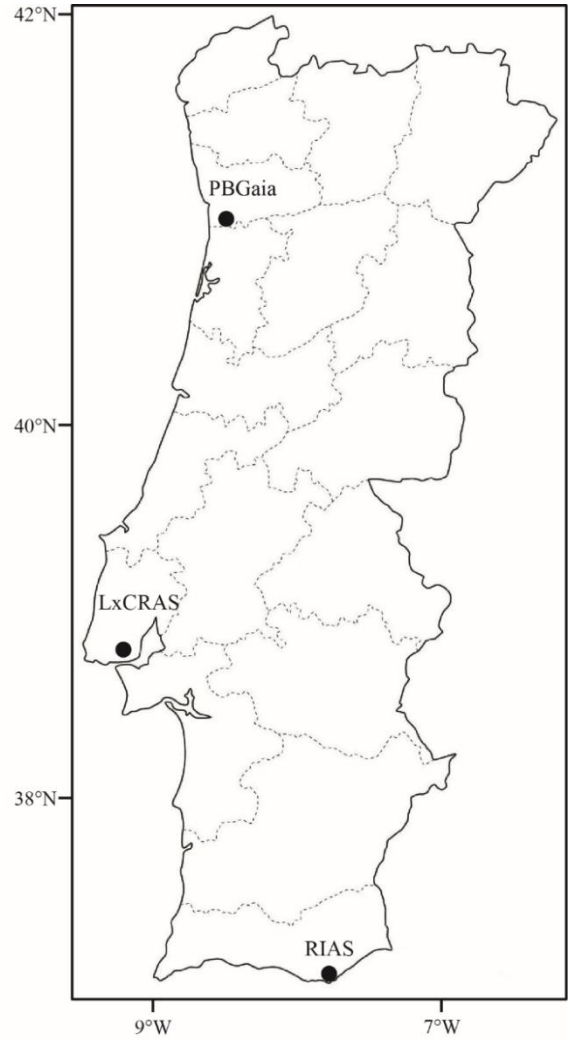

Figure 4.1. Location of the Portuguese wildlife rescue centres from where gulls were sampled: Centro de Recuperação do Parque Biológico de Gaia (PBGaia), Centro de Recuperação de Animais Silvestres de Lisboa (LxCRAS) and Centro de Recuperação e Investigação de Animais Selvagens (RIAS).

wildlife rescue centres considered for this study, located across Portugal: Centro de Recuperação do Parque Biológico de Gaia (PBGaia, 41º 05' 52'' N, 8º 33' 23'' W, *n* = 12), Centro de Recuperação de Animais Silvestres de Lisboa (LxCRAS, 38º 44' 24''N, 9º 11' 11'' W, *n* = 15) and Centro de Recuperação e Investigação de Animais Selvagens (RIAS, 37º 02' 03'' N, 7º 48' 47'' W, *n* = 20, Figure 4.1). These three rescue centres serve distinct areas of the country, with different characteristics, and animals entering these wildlife centres should be experiencing different habitats and distinct levels of urbanization prior to their admission. In addition to other areas, PBGaia mostly serves the Metropolitan Area of Porto, where Porto is the second largest city of Portugal (PORDATA 2011) that lies on the right side of the mouth of the Douro River, close to sea. A known population of urban gulls dwell in the city of Porto throughout the year, using certain areas of the city to rest (Pais de Faria et al. 2021b), and public and private buildings to construct their rooftop nests (Lopes et al. 2020). On the contrary, RIAS serves mostly the Ria Formosa Natural Park which has five barrier sandy islands and two peninsulas that form a narrow strip of dunes that separate the lagoon from the Atlantic Ocean (Ceia et al. 2010), and is located relatively far from metropolitan and populated urban centres. For this study, all studied gulls from PBGaia were collected in the urban metropolitan area of Porto, and all studied gulls from RIAS were collected in natural areas of the Ria Formosa Natural Park. LxCRAS, in turn, serves not only the metropolitan area of Lisbon but also the natural breeding and resting areas around the city. Thus, gulls entering this recovery centre should either come from the breeding population of the metropolitan area of Lisbon or from natural colonies such as Berlenga Island (39º 24' 49'' N, 9º 30' 29'' W), from which individuals are known to forage over fisheries leftovers at the seashore south of Lisbon (Ceia et al. 2014). Necropsies were performed, preferably, on recently dead animals, which either died right before or after admission (~60% of the total necropsied gulls), followed by individuals with the shortest hospitalization time possible, never longer than 2 weeks. Such selection of individuals intends to reflect the conditions of the environment in which gulls dwelled (i.e. urban *vs.* natural locations) as much as possible, rather than conditions at each rescue centre. All individuals were collected between September 2019 and January 2020, each bird was labelled and kept frozen at -20 ºC until dissection, and necropsies were performed in November 2019 at RIAS, January 2020 at PBGaia and March and May 2020 at Anatomical Pathology Laboratory, Faculty of Veterinary Medicine, University of Lisbon (FMV-UL, individuals from LxCRAS).

Necropsies were performed following the dissection techniques of van Franeker (2004) and Peleteiro (2016). Whenever possible, data on body condition, probable cause of death (clinical history), body weight, age and sex were recorded for each individual. Body condition score (BCS) was recorded based on the pectoral muscle condition, assessed by its palpation using a scale of 1 (cachexic / lean) to 5 (obese). Probable cause of death was determined considering clinical history, clinical signs and/or necropsy findings for each individual, and gulls were diagnosed with

gulls' paretic syndrome, trauma or unknown causes of death. Paretic syndrome affecting gulls in coastal Portugal has undetermined causes, outbreaks occur mainly in September and October each year, and results in gulls' inability to fly, diarrhoea, paresis, dyspnoea, stiffness of neck and dehydration (Costa et al. 2021). Individuals chosen for this study had identical degrees of the disease, with similar symptoms. Trauma category included gulls that presented fractured bones (mainly wing bone fractures), articular dislocations and open wounds most likely linked with human-related collisions (e.g. cars, boats) during their foraging activities in fishing harbours and urbanized areas. Sex was determined through direct observation of the reproductive tract at the celomic cavity and age was recorded as adult (more than 3 years old) or immature (1 - 3 years) gulls, based on their plumage evaluation. All individuals were weighted on an electronic balance to the nearest 1 g.

Birds' entire digestive system (mouth, proventriculus, gizzard, intestines and cloaca) was carefully examined for the presence of plastics and other anthropogenic materials (glass, wood, rubber, fabric, etc.). Visible anthropogenic items (>1 mm) were collected and washed in a glass petri dish with saline solution. These materials were stored in tubes with saline solution and properly labelled per bird and the respective location on the digestive system, until further analysis.

In the laboratory, anthropogenic items were left at room temperature until they were completely dry. Items were sorted, counted and categorized into several categories of materials: plastic, glass, wood, metal, fabric, rubber and paper (adapted from Provencher et al. 2017). As the last four categories were found in a small number of samples, to simplify they were grouped in a "other" category. Plastics were also sub-divided in four different types: sheetlike (e.g. plastic bags and cling film), threadlike (e.g. fishing lines, plastic strings, and ribbons), fragments (unidentifiable fragments from the break-up of larger plastic items as well as intact items), and foamed plastics (e.g. styrofoam). Items' colours were also noted following Provencher et al. (2017) and included the categories: white/clear, yellow, green, blue/purple, red/ pink, brown/orange, grey/silver, black and more than one colour. The biggest axis of each item was measured using graph paper, with an accuracy of 0.5 mm. Debris items were weighted per individual and per category to the nearest 0.0001 g using a precision balance.

4.2.2. FA quantification

From each necropsied bird, a sample of subcutaneous adipose tissue from the breast, specifically from the interior side of the pelvic limb, was collected. Fat tissues were stored in microtubes with alcohol 70% covering the sample, individually labelled and stored at -4 ºC. Fat tissues were then dried and weighted (0.03-0.6 g) and submitted to the FA extraction protocol.

The extraction of total lipids and methylation to fatty acid methyl esters (FAMEs) was performed following the methodology described by Gonçalves et al. (2012). Samples were incubated with methanol for the extraction of lipids. The nonadecanoic acid (C19:0, Fluka 74208) was added as an internal standard for further quantification. Samples were centrifuged and vacuum dried. FAMEs identification was carried out through Gas Chromatography-Mass Spectrometry (GC-MS), using a Thermo Scientific Trace 1310 Network (Waltham, MA, USA) equipment, equipped with TR-FFAP (Ton Refrigeration Free Fatty Acid Phase) column of 0.32 mm internal diameter (i.d.), 0.25 µm film thickness, and 30 m long. The sample was injected at an injector temperature of 250 ºC, lined with a split glass liner of 4.0 mm i.d. The initial oven temperature was 80 °C, followed by three ramps of linear temperature increase: 25 ºC min^{-1} until 160 ºC; 2 ºC min^{-1} until 210 ºC and finally an increase of 40 ºC min^{-1} until a final temperature of 230 ºC was reached and maintained for 10 min. Helium was used as carrier gas at a flow rate of 1.4 mL min^{-1}. A Thermo Scientific ISQ 7000 Network Mass Selective Detector at scanning m/z ranges specific for fatty acids in Selected Ion Monitoring (SIM) mode acquisition was used. The detector starts operating 3.5 min after injection, corresponding to solvent delay. The injector ion source and transfer line were maintained at 240 ºC and 230 ºC, respectively. Integration of FAME peaks were carried out using the equipment's software. Identification of each peak was performed by retention time and mass spectrum of each FAME, comparing to the Supelco®37 component FAME mix (Sigma-Aldrich, Steinheim, Germany). Finally, each peak area was extracted and then quantified as µg/g.

4.2.3. Statistical analysis

Each FA of the gulls' adipose tissue, initially in abundances (µg/g), was converted to a percentage of the total FAs, per individual.

Firstly, general linear models (GLMs) with Gaussian family and identity link were performed to evaluate the effect of the wildlife rescue centre (PBGaia, LxCRAS and RIAS) and species (YLG and LBBG) on the percentages of FA groups (SFAs, MUFAs, PUFAs, HUFAs, ω-3 and ω-6) and on the total ω-6/ω-3 ratio. When the main effect of rescue centre or species was significant in the model, we proceeded by traditional post-hoc Tukey tests.

To normalize FAs percentages data, we used the arcsine transformation. To analyse the effect of gulls' characteristics (wildlife rescue centre, species, age, sex, body condition score and clinical history) on their FA composition, we used partial least scares discriminant analysis (PLS-DA), a supervised multi-dimensional statistical model analysis that focuses on covariance while reducing dimensionality and takes into consideration both dependent and independent variables (Hadi and Ling, 1998). PLS-DA were performed using all transformed FA percentages

independently of their origin (dietary or non-dietary) and the number of double-bonds (saturated or unsaturated FA).

To understand how the number of anthropogenic materials per individual (number of items) differed between wildlife rescue centres and species, we performed zero inflated models, with negative binomial distributions to account for overdispersion. Models were performed considering the total number of items per individual (all debris) and the total number of plastic items per individual (all plastic). Zero inflated models use a reference category against which the remaining data is compared, thus, PBGaia was assigned as the reference rescue centre and LBBG was assigned as the reference species.

Mass of ingested anthropogenic materials was $\log_{10}$ transformed to attain normality. General linear models (GLMs) with Gaussian family and identity link function were performed to evaluate the effect of wildlife rescue centre and species in the mass of ingested anthropogenic materials.

The relationship between ingested anthropogenic materials' mass and number of items on YLG and LBBG body mass was investigated using linear regression. A Cook's distance of >3 identified one statistical outlier that was excluded from this analysis (Rousseeuw and Leroy 2005). To analyse possible patterns of ingested anthropogenic materials' mass effects on FA composition, we performed partial least squares regression (PLSR), also with all transformed FA percentages independently of their origin (dietary or non-dietary) and the number of double-bonds (saturated or unsaturated FA).

The R statistical program (R Core Team 2019) was used in all analyses, with a significance level of $p < 0.05$. GLM models were performed using *MASS* R package (Venables and Ripley 2002) and post hoc tests were performed using *lsmeans* R package (Lenth 2016). Zero-inflated models were performed using *pscl* R package (Zeileis et al. 2008; Jackman 2017). PLS-DA and PLSR were performed using *mixOmics* R package (Rohart et al. 2017).

4.3. Results

4.3.1. FA composition among wildlife rescue centres and species

A total of 23 FAs were found and quantified in the adipose tissue of YLG and LBBG from the three wildlife rescue centres (Table 4.1). Monounsaturated FAs (MUFAs) were the predominant FA group accounting for, on average, 48.1% of all FAs, ranging from 44.2% (for LBBG in RIAS) to 54.3% (for LBBG in PBGaia, Figure 4.2A). This was particularly due to the high percentages of the oleic acid (C18:1n-9) in all individuals (Table 4.1). The second most abundant FA group was the saturated FAs (SFAs) that accounted for, on average, 37.2% of all

FAs, ranging from 35.2% (for YLG in PBGaia) to 38.9% (for YLG in RIAS, Figure 4.2A). PUFAs (polyunsaturated FAs) presented higher percentages than HUFAs (highly unsaturated FAs) in gulls from PBGaia (9.1-10.7% *vs.* 0.38-2.1%, respectively), but this did not occur in individuals from LxCRAS (6.7-8.5% PUFAs *vs.* 6.7-9.3% HUFAs) nor from RIAS (5.4-7.3% PUFAs *vs.* 7.9-11.2% HUFAs, Table 4.1, Figure 4.2A). Individuals from PBGaia presented the lowest percentage of ω-3 FAs (0% for LBBG and 0.4% for YLG *vs.* a range of ~6% in LxCRAS to ~10% in RIAS, both for LBBG, Figure 4.2B). On the contrary, ω-6 FAs presented the highest percentage for YLGs from PBGaia (12.4%, Figure 4.2B), but the range of detected ω-6 FAs percentages was not so wide as that of ω-3 FAs (range of 5.8% in RIAS to 9.8% in LxCRAS, both for YLG, Figure 4.2B). The total ω-6/ω-3 ratio was the highest for PBGaia YLGs (38.8), and the lowest for RIAS individuals (1.8 for YLG and 4.7 for LBBG, Figure 4.2B).

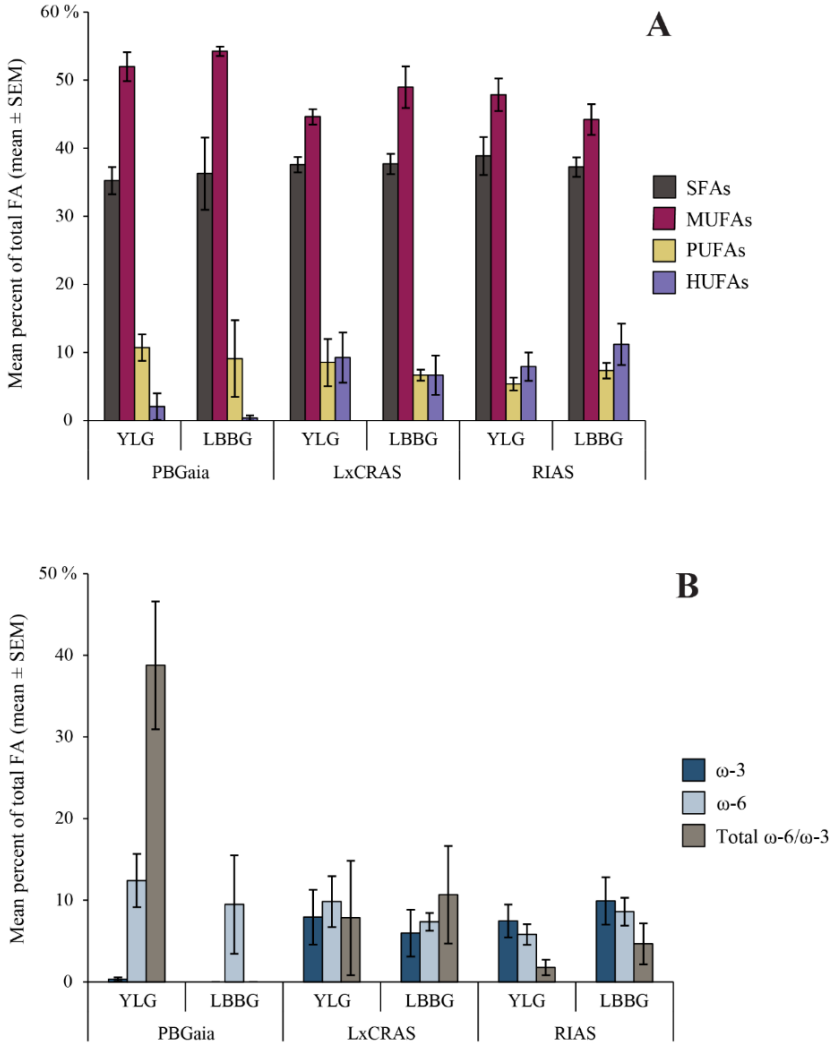

Figure 4.2. Percentages of A) saturated (SFAs), monounsaturated (MUFAs), polyunsaturated (PUFAs) and highly unsaturated (HUFAs) fatty acids and B) omega (ω)-3, ω-6 and total ω-6/ω-3 fatty acids ratio in adipose tissue of Yellow-legged (YLG, *Larus michahellis*) and Lesser black-backed gulls (LBBG, *Larus fuscus*) from three wildlife rescue centres (PBGaia, LxCRAS and RIAS, where individuals from PBGaia represent the most urbanized gull population, gulls from RIAS represent the most natural individuals and LxCRAS individuals are both natural and urban dwellers). Data are plotted as means ± standard error of the means (SEM).

Table 4.1. Relative abundance of adipose tissue fatty acids (% of the total fatty acid content) in two gull species (Yellow-legged gull, YLG, *Larus michahellis* and Lesser black-backed gull, LBBG, *Larus fuscus*) from three wildlife rescue centres (Centro de Recuperação do Parque Biológico de Gaia, PBGaia; Centro de Recuperação de Animais Silvestres de Lisboa, LxCRAS and Centro de Recuperação e Investigação de Animais Selvagens, RIAS). Individuals from PBGaia represent the most urbanized gull population, gulls from RIAS represent the most natural individuals and LxCRAS individuals are both natural and urban dwellers. Data is presented as means ± standard error of the means (SEM). C:D = number of carbon atoms:double bonds; N = number of individuals with that fatty acid detected in their adipose tissue (for Total SFA, Total MUFA, Total PUFA and Total HUFA: N = diversity of FAs per wildlife rescue centre and species, in italics); LA = linoleic acid; αLNA = α-linolenic acid; DGLA = dihomo-γ-linolenic acid; ARA = arachidonic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; SFA = saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids; HUFA = Highly unsaturated fatty acids.

		PBGaia (*n* = 12)				**LxCRAS** (*n* = 15)				**RIAS** (*n* = 20)			
		YLG (*n* = 10)		**LBBG** (*n* = 2)		**YLG** (*n* = 4)		**LBBG** (*n* = 11)		**YLG** (*n* = 9)		**LBBG** (*n* = 11)	
FA	**C:D**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**
Lauric acid	C12:0	9	0.82 ± 0.15	1	0.28 ± 0.28	2	0.43 ± 0.27	9	0.36 ± 0.09	4	0.37 ± 0.19	4	0.27 ± 0.16
Tridecylic acid	C13:0	0		0		0		2	0.02 ± 0.02	1	0.01 ± 0.01	1	0.01 ± 0.01
Myristic acid	C14:0	10	2.59 ± 0.31	2	1.37 ± 0.29	4	4.27 ± 1.38	11	4.51 ± 0.95	9	5.49 ± 0.72	11	4.67 ± 0.61
Pentadecylic acid	C15:0	10	1.13 ± 0.18	2	0.95 ± 0.26	4	0.64 ± 0.14	11	0.94 ± 0.14	9	1.01 ± 0.14	11	0.77 ± 0.09
Palmitic acid	C16:0	10	20.27 ± 2.32	2	22.15 ± 4.88	4	22.84 ± 0.74	11	22.67 ± 1.13	9	22.76 ± 1.94	11	21.75 ± 0.84
Margaric acid	C17:0	10	0.4 ± 0.03	2	0.24 ± 0.02	4	0.57 ± 0.11	11	0.61 ± 0.07	9	0.76 ± 0.09	11	0.65 ± 0.06
Stearic acid	C18:0	10	9.53 ± 0.72	2	10.79 ± 0.34	4	8.46 ± 1.2	11	8.13 ± 0.63	9	7.86 ± 0.32	11	8.46 ± 0.79
Arachidic acid	C20:0	10	0.37 ± 0.05	2	0.49 ± 0.2	4	0.37 ± 0.11	11	0.38 ± 0.06	9	0.6 ± 0.12	11	0.4 ± 0.07
Behenic acid	C22:0	1	0.03 ± 0.03	0		0		0		0		1	0.01 ± 0.01
Tricosylic acid	C23:0	1	0.09 ± 0.09	0		0		1	0.07 ± 0.07	0		1	0.24 ± 0.24
TOTAL SFA		***9***	**35.24 ± 2**	***7***	**36.27 ± 5.31**	***7***	**37.59 ± 1.14**	***9***	**37.7 ± 1.49**	***8***	**38.86 ± 2.78**	***10***	**37.24 ± 1.42**

Table 4.1. *cont.*

		PBGaia (*n* = 12)				**LxCRAS** (*n* = 15)				**RIAS** (*n* = 20)			
		YLG (*n* = 10)		**LBBG** (*n* = 2)		**YLG** (*n* = 4)		**LBBG** (*n* = 11)		**YLG** (*n* = 9)		**LBBG** (*n* = 11)	
FA	**C:D**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**
Palmitoleic acid	C16:1n-7	10	6.01 ± 1.61	2	2.3 ± 0.5	4	7.04 ± 2.44	11	6.69 ± 1.13	9	8.66 ± 1.16	11	7.8 ± 0.75
Heptadecenoic acid	C17:1n-10	8	0.33 ± 0.07	1	0.08 ± 0.08	3	0.51 ± 0.24	10	0.47 ± 0.07	8	0.7 ± 0.11	11	0.65 ± 0.08
Oleic acid	C18:1n-9	10	43.34 ± 3.02	2	50.17 ± 0.27	4	31.04 ± 4	11	34.62 ± 3.73	9	26.1 ± 2.58	11	30.04 ± 2.55
Eicosenoic acid	C20:1n-9	10	1.57 ± 0.28	2	1.7 ± 0.14	4	3.36 ± 0.8	11	3.85 ± 0.99	9	5.14 ± 0.96	11	3.28 ± 0.54
Cetoleic acid	C22:1n-11	5	0.75 ± 0.33	0		4	2.45 ± 0.72	9	3.33 ± 1.25	9	7.26 ± 3.35	9	2.34 ± 0.54
Nervonic acid	C24:1n-9	0		0		1	0.23 ± 0.23	0		0		2	0.11 ± 0.08
TOTAL MUFA		***5***	**51.99 ± 2.13**	***4***	**54.25 ± 0.71**	***6***	**44.63 ± 1.13**	***5***	**48.97 ± 3.06**	***5***	**47.85 ± 2.39**	***6***	**44.22 ± 2.24**
LA (ω-6)	C18:2n-6	10	10.5 ± 1.86	2	9.1 ± 5.64	4	7.67 ± 3.38	11	5.96 ± 0.85	9	4.68 ± 0.95	11	6.41 ± 1.21
α-LNA (ω-3)	C18:3n-3	4	0.13 ± 0.06	0		3	0.6 ± 0.21	6	0.42 ± 0.18	6	0.54 ± 0.16	10	0.64 ± 0.13
Eicosadienoic acid (ω-6)	C20:2n-6	2	0.09 ± 0.07	0		3	0.25 ± 0.09	6	0.28 ± 0.12	5	0.15 ± 0.05	8	0.26 ± 0.05
DGLA (ω-6)	C20:3n-6	0		0		0		0		0		1	0.04 ± 0.04
TOTAL PUFA		***3***	**10.72 ± 1.94**	***1***	**9.1 ± 5.64**	***3***	**8.52 ± 3.46**	***3***	**6.66 ± 0.81**	***3***	**5.37 ± 0.94**	***4***	**7.34 ± 1.15**
ARA (ω-6)	C20:4n-6	3	1.83 ± 1.76	1	0.38 ± 0.38	4	1.92 ± 1.02	7	1.13 ± 0.62	7	0.99 ± 0.5	9	1.9 ± 1.3
EPA (ω-3)	C20:5n-3	1	0.04 ± 0.04	0		3	2.14 ± 1.02	5	1.34 ± 0.76	7	1.57 ± 0.57	10	2.25 ± 0.76
DHA (ω-3)	C22:6n-3	1	0.18 ± 0.18	0		3	5.2 ± 2.42	6	4.2 ± 1.96	8	5.36 ± 1.37	10	7.04 ± 2.06
TOTAL HUFA		***3***	**2.05 ± 1.98**	***1***	**0.38 ± 0.38**	***3***	**9.26 ± 3.7**	***3***	**6.67 ± 2.89**	***3***	**7.92 ± 2.1**	***3***	**11.2 ± 3.04**
Mean number of FAs / individual		**13.5 ± 0.54**		**11.5 ± 0.5**		**16.5 ± 0.96**		**15.55 ± 0.85**		**16.11 ± 0.68**		**17 ± 0.47**	

Table 4.2. Statistics from the **A)** general linear models (GLMs) testing the effect of wildlife rescue centre (PBGaia, LxCRAS and RIAS, where individuals from PBGaia represent the most urbanized gull population, gulls from RIAS represent the most natural individuals and LxCRAS individuals are both natural and urban) and species (Yellow-legged gull, YLG, *Larus michahellis* and Lesser black-backed gull, LBBG, *Larus fuscus*) on the percentages of each fatty acids (FA) group (SFAs, MUFAs, PUFAs, HUFAs, ω-3 and ω-6) and on the total ω-6/ω-3 ratio from the adipose tissue of 47 gulls, and **B)** Tukey adjusted *p* values of pairwise post-hoc comparisons among wildlife rescue centres. Significant effects are highlighted in bold. SFAs = saturated fatty acids; MUFAs = monounsaturated fatty acids; PUFAs = polyunsaturated fatty acids; HUFAs = highly unsaturated fatty acids.

FA Group (Mean %)	A		B			Main Effect Rescue Centre
	Rescue Centre	Species	PBGaia - LxCRAS	LxCRAS - RIAS	PBGaia - RIAS	
SFAs	$F_{2, 44} = 0.8$ $p = 0.46$	$F_{1, 45} = 0.03$ $p = 0.86$	0.575	0.988	0.453	
MUFAs	$F_{2, 44} = 2.77$ $p = 0.07$	$F_{1, 45} = 0.65$ $p = 0.42$	0.279	0.734	0.06	
PUFAs	$F_{2, 44} = 3.23$ $p =$ **0.05**	$F_{1, 45} = 0.62$ $p = 0.43$	0.143	0.889	**0.045**	RIAS > PBGaia
HUFAs	$F_{2, 44} = 3.7$ $p =$ **0.03**	$F_{1, 45} = 1.12$ $p = 0.3$	0.183	0.669	**0.026**	RIAS > PBGaia
ω-3	$F_{2, 44} = 5.28$ $p =$ **0.009**	$F_{1, 45} = 1.54$ $p = 0.22$	0.08	0.617	**0.007**	RIAS > PBGaia
ω-6	$F_{2, 44} = 2.08$ $p = 0.14$	$F_{1, 45} = 0.45$ $p = 0.51$	0.263	0.949	0.132	
Total ω-6 / ω-3	$F_{2, 33} = 17.7$ $p <$ **0.001**	$F_{1, 34} = 1.58$ $p = 0.22$	**<0.001**	0.332	**<0.001**	PBGaia > Others

GLM results testing the effect of wildlife rescue centre and species on the percentages of FAs groups (SFAs, MUFAs, PUFAs, HUFAs, ω-3 and ω-6) and the total ω-6/ω-3 ratio showed that the percentage of all FA groups as well as the total ω-6/ω-3 ratio did not vary significantly among species ($F < 1.58$; $p > 0.22$, Table 4.2A). SFAs, MUFAs and ω-6 also did not vary among rescue centres ($F < 2.77$; $p > 0.07$), but PUFAs, HUFAs and ω-3 were significantly different among rescue centres ($F > 3.23$; $p < 0.05$, Table 4.2A), more specifically between PBGaia and RIAS (all Tukey $p < 0.045$, Table 4.2B). The total ω-6/ω-3 ratio was also different among rescue centres ($F_{2, 33} = 17.7$; $p < 0.001$, Table 4.2A), in particular between PBGaia and RIAS and between PBGaia and LxCRAS (all Tukey $p < 0.001$, Table 4.2B). RIAS and LxCRAS did not present significant differences for the ratio ω-6/ω-3 (Table 4.2B).

Gulls from PBGaia had a lower diversity of FAs, i.e. a lower number of FAs per individual (Table 4.1), than gulls from RIAS and LxCRAS. We identified 5 FAs that individually accounted for > 7% of the total FAs composition: C18:1n-9 (ranging from 26.1% for YLG in RIAS to 50.2% in PBGaia for LBBG), C16:0 (ranging from 20.3% in PBGaia to 22.8% in LxCRAS, both for YLG), C18:0 (ranging from 7.9% in RIAS for YLG to 10.8% in PBGaia for LBBG), C16:1n-7 (ranging from 2.3% in PBGaia to 7.8% in RIAS, both for LBBG) and C18:2n6 (ranging from 4.7% in RIAS to 10.5% in PBGaia, both for YLG, Table 4.1).

4.3.2. Influence of gulls' characteristics on FA composition

From the 47 necropsied gulls, there were more immature individuals than adults (34 immature *vs.* 13 adults) but sex was evenly distributed (23 female *vs.* 20 male gulls; the sex of 4 gulls was impossible to determine). Considering the probable cause of death, 26 gulls died from paretic syndrome complications and 18 from trauma lesions (3 gulls had unknown causes of death). Overall, 2 was the most common BCS recorded (22 gulls), followed by BCS = 3 (12 gulls) and BCS = 1 (7 gulls, Table S4.1).

A PLS-DA was run on all the percentages (arcsine transformed) of the FAs detected in the adipose tissue samples (Figure 4.3) with wildlife rescue centre as response variable. Components (comp) 1, 2 and 3 accounted for 30%, 13% and 13% of the variation in the data, respectively (Table S4.2). The three wildlife rescue centres grouped distinctly; in particular PBGaia (orange ellipse in Figure 4.3) was clearly separated from the remaining rescue centres (grey ellipse for LxCRAS and blue ellipse for RIAS in Figure 4.3).

The PLS-DA loadings (Figure 4.4) revealed that DHA (C22:6n-3), C17:0 and EPA (C20:5n-3 all higher in RIAS and LxCRAS) as well as C18:1n-9, LA (C18:2n-6) and C12:0 (all higher in PBGaia) were the FAs more important in explaining variation along comp1 and, therefore, in segregating wildlife rescue centres (Figure 4.3 and Figure 4.4A). C13:0 and C20:2n-6, negatively, as well as C20:0 and DGLA (C20:3n-6), positively, were the FAs more important in explaining variation along comp2 (Figure 4.4B). The FAs C15:0 and ARA (C20:4n-6) were the most important in explaining variation along comp3 (negatively and positively, respectively, Figure 4.4C).

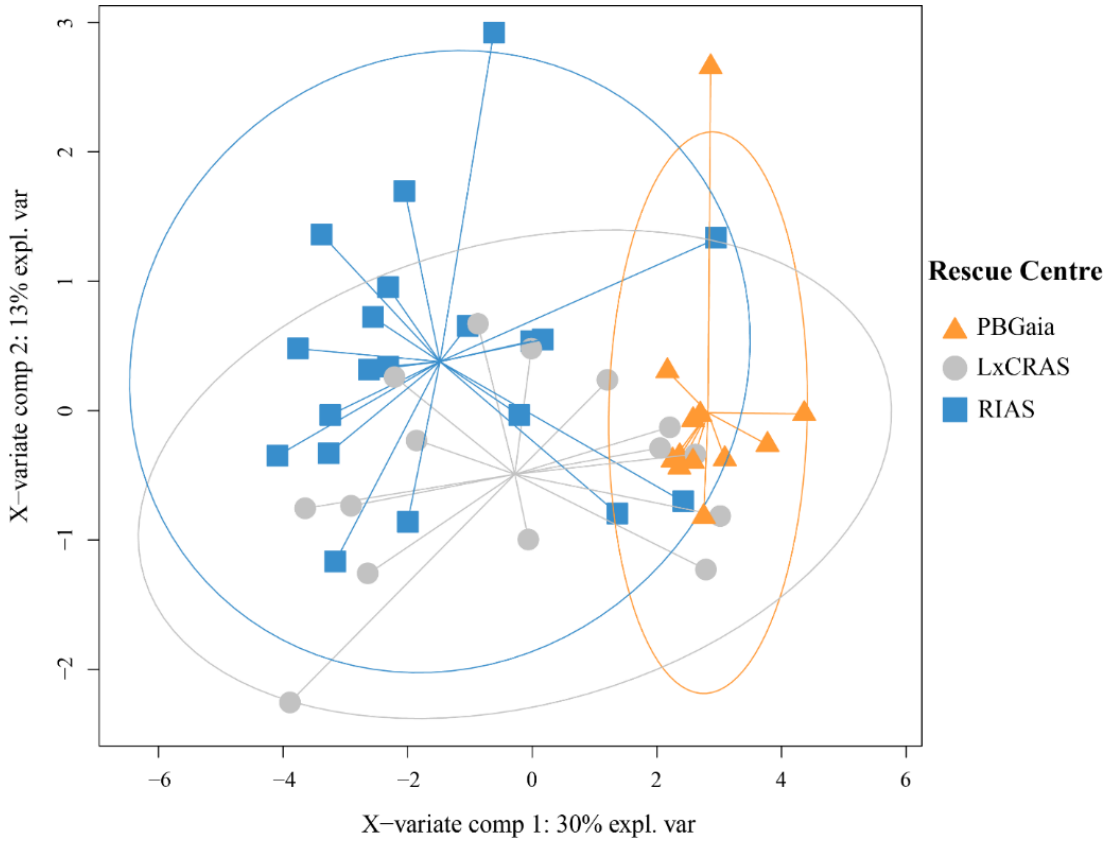

Figure 4.3. Partial Least Squares Discriminant Analysis (PLS-DA) score plot (component 1 and component 2) of 47 Yellow-legged (*Larus michahellis*) and Lesser black-backed (*Larus fuscus*) gulls' adipose tissue fatty acids mean percentages (arcsine transformed) separated according to wildlife rescue centre (PBGaia: orange triangles; LxCRAS: grey points; RIAS: blue squares, where individuals from PBGaia represent the most urbanized gull population, gulls from RIAS represent the most natural individuals and LxCRAS individuals are both natural and urban dwellers). Each triangle, point or square represents each necropsied gull. 30% and 13% of the variance in fatty acids is explained by component 1 and component 2, respectively. Coloured ellipses represent 95% confidence intervals.

Visually, YLG and LBBG did not group distinctly in the respective PLS-DA and presented a high overlap between FA percentages (for ellipses of both species, see Figure S4.1). None of the remaining gulls' characteristics (age, sex, BCS and clinical history) grouped distinctly in each corresponding PLS-DA (Figure S4.2), presenting a high overlap between age classes, sexes, body condition scores and clinical histories.

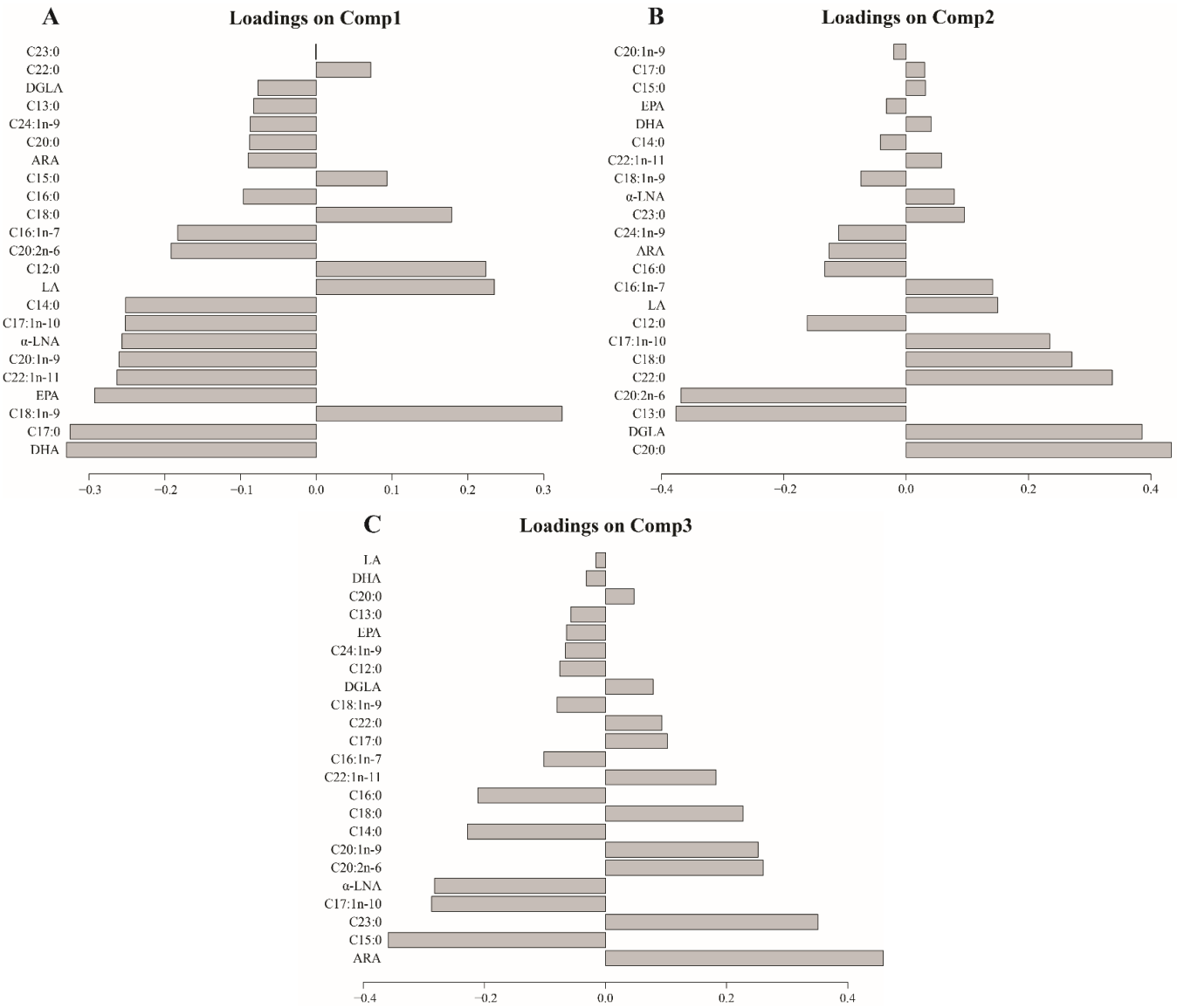

Figure 4.4. Partial Least Squares Discriminant Analysis (PLS-DA) loadings plot of each one of the first three components (**A:** component 1, **B:** component 2 and **C:** component 3) of 47 Yellow-legged (*Larus michahellis*) and Lesser black-backed (*Larus fuscus*) gulls' adipose tissue fatty acids mean percentages (arcsine transformed) separated according to wildlife rescue centre. LA = linoleic acid (C18:2n-6); αLNA = α-linolenic acid (C18:3n-3); DGLA = dihomo-γ-linolenic acid (C20:3n-6); ARA = arachidonic acid (C20:4n-6); EPA = eicosapentaenoic acid (C20:5n-3); DHA = docosahexaenoic acid (C22:6n-3).

Table 4.3. Description of anthropogenic materials (debris) items present in 47 Yellow-legged (YLG, *Larus michahellis*) and Lesser black-backed gulls (LBBG *Larus fuscus*) necropsied at three wildlife rescue centres along Portugal (PBGaia, LxCRAS and RIAS, where individuals from PBGaia represent the most urbanized gull population, gulls from RIAS represent the most natural individuals and LxCRAS individuals are both natural and urban dwellers). FO = Frequency of Occurrence. SD = Standard Deviation. NA = Not Applicable.

Rescue Centre	Species	No. individuals	FO (%) of debris	Items per individual		Mass of debris (g)		Size of debris (cm)		Total items
				Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	
PBGaia	YLG	10	80	2.5 ± 2.42	0 – 7	0.0108 ± 0.0115	0.0001 - 0.0338	5.87 ± 2.83	2.5 – 11	25
	LBBG	2	50	6 ± 8.49	0 – 12	0.0880 ± NA	0.0880 - 0.0880	16.5 ± NA	16.5 – 16.5	12
LxCRAS	YLG	4	0							0
	LBBG	11	45.5	0.91 ± 1.76	0 – 6	0.0362 ± 0.0278	0.0055 - 0.0780	6.68 ± 1.56	5 – 9	10
RIAS	YLG	9	55.6	2.22 ± 3.15	0 – 9	0.0713 ± 0.1358	0.0013 – 0.3132	4.34 ± 2.5	2.9 – 8.78	20
	LBBG	11	54.6	3 ± 4.2	0 – 14	0.1741 ± 0.3043	0.0034 – 0.7867	10.62 ± 6.45	4.75 – 23	33
PBGaia		12	75	3.08 ± 3.63	0 – 12	0.0194 ± 0.0279	0.0001 – 0.0880	7.05 ± 4.42	2.5 – 16.5	37
LxCRAS		15	33.3	0.67 ± 1.54	0 – 6	0.0362 ± 0.0278	0.0055 – 0.0780	6.68 ± 1.56	5 – 9	10
RIAS		20	55	2.65 ± 3.69	0 – 14	0.1274 ± 0.2378	0.0013 – 0.7867	7.76 ± 5.83	2.9 – 23	53
YLG		23	56.5	1.96 ± 2.62	0 – 9	0.0341 ± 0.0846	0.0001 – 0.3132	5.28 ± 2.71	2.5 – 11	45
LBBG		24	50	2.29 ± 3.8	0 – 14	0.1095 ± 0.2171	0.0034 – 0.7867	9.47 ± 5.34	4.75 – 23	55
TOTAL		47	53.2	2.13 ± 3.25	0 – 14	0.0703 ± 0.1633	0.0001 – 0.7867	7.29 ± 4.61	2.5 – 23	100

4.3.3. Influence of the ingestion of anthropogenic materials on gulls' FA composition

From the 47 individuals studied, 25 (53.2%) had anthropogenic materials in their digestive systems with a mean (±SD) number of items of 2.13 ± 3.25 per individual (range 0-14 pieces), weighting 0.0703 ± 0.1633 g (range 0.0001 – 0.7867 g, Table 4.3). Detailed description of the anthropogenic materials found in gulls' digestive tract for each species and wildlife rescue centre, and the colours of the ingested materials can be found on Table S4.3 and Figure S4.3, respectively.

No differences were detected in the number of items (all debris) found in gulls' digestive tract neither among rescue centres nor among species (Table 4.4). The number of plastic items was significantly higher for gulls from PBGaia than for gulls from LxCRAS (Z = -2.26; p = 0.02, Table 4.4). Mass of anthropogenic materials did not differ significantly among rescue centres, but LBBG had materials with greater mass in their digestive systems than YLG ($F_{1,23}$ = 6.26; p = 0.02, Table 4.4).

Table 4.4. Statistics from **A)** zero-inflated models and **B)** general linear models testing the effect of wildlife rescue centre (PBGaia, LxCRAS and RIAS, where individuals from PBGaia represent the most urbanized gull population, gulls from RIAS represent the most natural individuals and LxCRAS individuals are both natural and urban dwellers) and species (YLG, *Larus michahellis* and LBBG, *Larus fuscus*), in the number (A) and mass (B) of anthropogenic materials (all debris) and in the number (A) and mass (B) of plastic items (all plastic) detected in 47 necropsied gulls. For zero-inflated models (A), PBGaia and LBBG were assigned as reference categories for rescue centre and species, respectively, and only results from count models are shown. Significant effects are highlighted in bold.

A		**Rescue Centre**			**Species**	
		LxCRAS	**RIAS**	**Main Effect**	**LBBG**	**Main Effect**
No Items	All Debris	$\beta \pm SE$ = -1.14 ± 0.75 Z = -1.52 p = 0.13	$\beta \pm SE$ = 0.2 ± 0.5 Z = 0.40 p = 0.69	--	$\beta \pm SE$ = -0.39 ± 0.53 Z = -0.74 p = 0.46	--
	All Plastic	$\beta \pm SE$ = -2.61 ± 1.16 Z = -2.26 p = **0.02**	$\beta \pm SE$ = -0.35 ± 0.83 Z = -0.43 p = 0.67	PBGaia > Others	$\beta \pm SE$ = -0.78 ± 0.84 Z = -0.93 p = 0.35	--
B		**Rescue Centre**			**Species**	
Mass	All Debris	$F_{2,22}$ = 1.32; p = 0.29		--	$F_{1,23}$ = 6.26; p = **0.02**	LBBG > YLG
	All Plastic	$F_{2,11}$ = 0.30; p = 0.75		--	$F_{1,12}$ = 1.71; p = 0.22	--

YLG's body mass was not significantly related with the number of ingested items ($F_{1,21}$ = 0.59, p = 0.45), but the number of ingested items was positively related with LBBG's body mass (β = 9.75 ± 4.37 g number of items^{-1}, r^2 = 0.18, $F_{1,22}$ = 4.97, p = 0.04, Table S4.4). However, there was no significant relationship between the mass of ingested anthropogenic materials and gulls' body mass ($F_{1,45}$ = 1.28, p = 0.26), both in terms of wildlife rescue centre and gull species (Table S4.4). As for the number of ingested items, no significant relation was found with gulls' body mass neither considering all data ($F_{1,45}$ = 2.2, p = 0.15), nor considering each wildlife rescue centre separately (Table S4.4).

The PLSR used to address the effect of the ingestion of anthropogenic materials on gulls' FA composition showed no clear pattern between the mass of ingested materials and FA composition (Figure S4.4).

4.4. Discussion

In this study, the FA composition of two opportunistic gull species from three wildlife rescue centres representative of areas with different levels of urbanization was described and related with the ingestion of anthropogenic materials. We detected significant differences in gulls' FA composition between the three rescue centres, and therefore, among urbanization levels, but not among species. A significant positive relation between the number of ingested items and LBBG's body mass was detected, but we were unable to identify any effect of the mass of ingested anthropogenic materials on gulls' FA composition.

4.4.1. Differences in FA composition

Gulls from the rescue centre that represents the most urbanized area (PBGaia) had significantly lower percentages of physiologically important groups of FAs (HUFAs, PUFAs and ω-3 FAs) in their adipose tissue than gulls from the remaining centres (LxCRAS and RIAS). We identified the FAs responsible for the segregation between rescue centres, and therefore, important in separating highly urbanized areas from more natural habitats. The FAs DHA, C17:0 and EPA presented significantly higher percentages in RIAS and LxCRAS, which are representative of more natural habitats, whereas C18:1n-9, LA and C12:0 were higher in PBGaia, the most urbanized location.

Gulls adipose tissue revealed a clear predominance of MUFAs rather than SFAs. This is in accordance with other studies that report, for instance, higher amounts of C18:1n-9 (MUFA) than C18:0 (SFA) on seabirds' fat tissue (Dahl et al. 2003; Käkelä et al. 2006; Puskic et al. 2019), which is often related with a diet enriched on marine species (Dahl et al. 2003). However,

individuals from PBGaia (the most urbanized location) exhibited particularly higher percentages of the FA C18:1n-9, accounting for 44% of the total FAs for that rescue centre *vs.* 28% in RIAS (the least urbanized site) and 34% in LxCRAS. Urban gulls from PBGaia should have a highly diverse diet, including the presence of anthropogenic food items in their diets such as remnants of human meals, as reported for other gulls using and relying on urban habitats (Real et al. 2017; Egunez et al. 2018; Huig et al. 2016; Pais de Faria et al. 2021a). Unfavourable physiological states characterized by loss of body mass or periodic fasting associated with breeding, moult or migration, which can be enhanced by a nutritionally poorer diet in urban habitats, may result in the selective mobilization of certain FAs, and *de novo* biosynthesis of other FAs like C16:0 and C18:0, as well as their respective products C16:1n-7 and C18:1n-9 (Williams and Buck 2010). This may explain the higher proportions of C18:1n-9 in individuals from PBGaia. Still, the major factor affecting FA composition is diet and, although in smaller amounts, SFAs and MUFAs are also obtained from diet (Iverson et al. 2007). Despite the highly diverse diet reported for urban gulls from Porto, they still relied on marine resources throughout the year (Pais de Faria et al. 2021a), which also may help in understanding the higher C18:1n-9 proportions in PBGaia urban gulls.

The SFA palmitic acid (C16:0) was the second most common FA on adipose tissue of the studied individuals, in similar proportions for each species and per wildlife rescue centre. This SFA, along with the stearic acid (C18:0), are two of the most abundant FAs found in animals, and is a common released product from the *de novo* synthesis pathway of 14-carbon FAs within seabirds' liver (Dalsgaard et al. 2003; Käkelä et al. 2009). This FA may be stored in the adipose tissue or used rapidly as an energy substrate (Williams and Buck 2010). Palmitic acid was the most abundant FA found in plasma of YLG fledglings in Porto urban breeding colony and in Berlenga natural breeding colony (Pais de Faria et al. 2021a). By being biosynthesised *de novo* by birds, both SFAs and MUFAs relative levels can be controlled to a larger extent than the levels of PUFAs and HUFAs (Isaksson et al. 2017), therefore these SFAs and MUFAs results are likely a consequence of metabolic regulation combined with habitat specific diet.

Essential fatty acids (EFAs), such as the ω-3 EPA and DHA cannot be synthetised *de novo* and must be obtained through diet, being extremely important to bird physiology (Dalsgaard et al. 2003; Gladyshev et al. 2009). In fact, EPA and DHA, both ω-3 FAs, were the most important FAs in segregating wildlife rescue centres, all showing higher percentages in individuals from RIAS, the least urbanized area, and LxCRAS. The higher percentages of these ω-3 FAs in individuals from LxCRAS and RIAS is consistent with a diet based on marine resources (Dalsgaard et al. 2003; Calado et al. 2018, 2021). On the contrary, the deficiency in ω-3 FAs and the lower diversity of FAs (i.e. mean number of FAs per individual, Table 4.1) in gulls from the most urbanized location (PBGaia) are indicators of terrestrial food-webs (Taipale et al. 2014; Twining et al. 2018), suggesting a diet based on anthropogenic food resources. This FA

composition suggests that urban dwellers from PBGaia, have a poorer nutritional condition as items typically found in human meal leftovers are usually rich in fat and proteins, allowing a greater energy intake, but might be deficient in essential nutrients (Patenaude-Monette et al. 2014). The ω-3 FAs deficiency in individuals from PBGaia is in accordance with previous work performed with gull fledglings from the urban colony of Porto (Pais de Faria et al. 2021a). The higher ω-6:ω-3 FAs ratio of individuals from PBGaia may be suggestive of a higher propensity by urban gulls to an enhanced diet-induced susceptibility to inflammation when exposed to antigens, and to suffer from a higher oxidative stress status (Romieu et al. 2008; Isaksson 2015; Isaksson et al. 2017). Yet, we cannot exclude the possibility that the levels of FAs detected by this study could be within the range of healthy and normal FA variability and, therefore, may not translate into health problems. An urban diet, typically rich in anthropogenic food resources and poor in marine items, and consequently with low levels of ω-3 EFAs, may be responsible for lower egg quality and reduced chick weight in urban gulls (Dosch 1997; Hebert et al. 2020).

None of the gulls' characteristics (species, age, sex, body condition score, clinical history) seemed to be important in explaining the global variation in FA composition. Auman et al. (2008) described sex-based differences in condition of urban gulls: males were heavier and larger than the urban females of Silver Gull (*Larus novaehollandiae*). Such variation in condition could be further reflected in FA differences between males and females, as reported by Käkëla et al. (2006) for Great Skuas (*Stercorarius skua*). The Great Skuas' FA variations were attributed to sexual size dimorphism and division of labour while breeding. In YLG and LBBG species, both male and female share their nest and chick duties, and both leave the nest for feeding themselves and to provision the chicks. Still, gull males are typically larger than females (Arizaga et al. 2008) which could be responsible for sex-differences in FA composition. Both YLG and LBBG, adults and immatures, males and females, are known to benefit from reliable and predictable food sources, either by interacting with fishing boats and feeding on marine species with higher nutritional value (i.e. fishery discards; Calado et al. 2018, 2021; Mendes et al. 2018), mainly in natural habitats, or by feeding on human meal leftovers collected from trash containers or in nearby landfills, in urban habitats (Spelt et al. 2019; Lopes et al. 2021b; Pais de Faria et al. 2021b). Also, in this study, we compared FA composition between adults and immature gulls (1-3 years), and we did not consider fledglings. All gulls were captured during their non-breeding season (September to January) and adipose tissue reflects a diet integrated over a period of 1 - 2 months (Williams and Buck 2010), therefore adults and immatures of both sexes could be experiencing a similar energy-demanding status as gulls were not breeding.

We acknowledge that some individuals in each habitat may not be strict urban or natural dwellers as we stated, because the movement ecology of each individual before capture is unknown. However, our previous research indicates that gulls from Porto are largely urban

dwellers year-round (Pais de Faria et al. 2021a, b), and those from Ria Formosa forage mostly in interaction with fishing activities also year-round (Calado et al. 2021).

When comparing to pellet and bolus analysis, the use of necropsies presents several advantages including the possibility of determining age, sex, health status and cause of death of the individuals, evaluating the entire burden of anthropogenic materials, assessing potential internal pathologies (i.e. macroscopic lesions and related pathological patterns) and sampling internal tissues for subsequent histopathological or chemical analysis (Provencher et al. 2019). The use of animals from wildlife rehabilitation centres may have skewed our samples as these individuals were likely in a poorer health condition, presenting altered physiological conditions beforehand that may have been confounded with the treatment effects and introduced bias to our results. Despite being an opportunistic methodology in relation to season or species, it allows repeated sampling and constitutes a non-invasive approach, as individuals are not purposely collected or killed for scientific research, with the collection of a large amount of data on each individual (Provencher et al. 2017).

4.4.2. Debris ingestion and FA composition

Overall, our study detected that 53.2% and 29.8% of the 47 necropsied gulls had anthropogenic materials and plastics, respectively, in their digestive systems, with a mean of 2.13 debris items per individual and a mean of 0.77 plastic items per individual. These values are relatively similar to those of other gull debris studies using necropsies (review by Seif et al. 2018). In previous studies, both YLG and LBBG exhibited high levels of anthropogenic materials in their pellets (Alonso et al. 2015; Calado et al. 2018), especially in urban and landfill environments (Lopes et al. 2021b). However, the use of necropsies only allows for the detection of a smaller amount of debris in gulls' digestive system (Codina-García et al. 2013; Basto et al. 2019, this study) since gulls have the ability to regurgitate a large part of non-edible food remnants, including anthropogenic materials (Barrett et al. 2007), reducing the time that these materials are in individuals' digestive system. In fact, previous pellet analysis from breeding gulls of the same study areas indicate a large amount of regurgitated anthropogenic materials, particularly plastics by the urban gulls of Porto (Lopes et al. 2021b). This regurgitation capability allows the rejection of larger and heavier items that were more likely to physically block and/or damage gulls' digestive tract and, therefore, items with greater masses and sizes may have a lower impact on their digestive system. Still, some items are known to remain in the gulls' digestive tracts (i.e. smaller items and microplastics which may not be regurgitated, Provencher et al. 2017), with an unknown retention time, and these may be more likely to interfere with birds' physiology and

body condition (Puskic et al. 2019), but comprehensive studies on how these debris items affect birds' health are scarce, especially in an urbanization context.

Body mass of fledgling Flesh-footed Shearwaters (*Ardenna carneipes*) was inversely proportional to the mass and the number of ingested plastic items, which may indicate sub-lethal effects of plastic pollution on marine wildlife (Lavers et al. 2014). However, with a similar analysis, Puskic et al. (2019) failed to detect a relationship between ingested plastics, linear morphometrics and FA composition. Determining body condition as body mass corrected for size may not be the best metric to detect effects on animals which have ingested plastic and other anthropogenic materials, hence the reason why FA analysis are being applied to explore such problem. We were not able to detect a relation between the ingestion of anthropogenic materials and FA composition, and this may have different explanations. In fact, beyond the ingestion of debris materials, other factors may be influencing our results. First, by choosing individuals with similar symptoms and identical degrees of disease (i.e. paretic syndrome), we attempted to reduce variability regarding their health status and clinical history, still we can not exclude the possibility of bias in our samples that affected FA profile other than the ingestion of anthropogenic materials. Second, the amount of anthropogenic materials in gulls' guts turned out to be quite low comparing to what we were expecting, especially for urban gulls. Although gulls are known to ingest large amounts of anthropogenic materials, such debris may have been "excreted" *via* the production of pellets (see Lopes et al. 2021b) and, therefore, such levels of ingested anthropogenic materials may simply be below toxic levels and may not cause impairment nor sub-lethal impacts on the studied individuals. This is also a reminder of the seasonal variability in debris ingestion, the individuals' responses to ingestion, and ultimately the difficulty of identifying sub-lethal impacts of the ingestion of anthropogenic materials in seabirds (Rochman et al. 2016; Roman et al. 2019b).

In conclusion, gulls inhabiting urban habitats may have some immediate benefits when compared to gulls living in natural habitats, such as reduced foraging energetic costs due to the high availability and accessibility of anthropogenic food resources. Our study suggests that FA composition of urban gulls has lower nutritional quality than that of gulls inhabiting more natural habitats, and such nutritional costs may have long-term effects for urban dwelling populations which deserve further studies. FA analysis is thus a useful tool to elucidate how anthropogenic materials may disturb metabolic pathways and to assess the less visible impacts of their ingestion, even though our results suggest that, at least with our sample of birds from a small period of time, there was no such effect. In the long run, urban gulls may be more exposed to several contaminants, pathogens (Alm et al. 2018; Sorais et al. 2020) and anthropogenic materials (Lopes et al. 2020, 2021b) that might endanger gulls' health condition, survival and/or reproductive output.

Chapter 5

EFFECTS OF AN ANTHROPOGENIC DIET ON YELLOW-LEGGED (*Larus michahellis*) AND LESSER BLACK-BACKED (*L. fuscus*) GULLS' PHYSIOLOGY AND HEALTH CONDITION: EVIDENCE FROM A CAPTIVE FEEDING EXPERIMENT

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Effects of an anthropogenic diet on yellow-legged (*Larus michahellis*) and lesser black-backed (*L. fuscus*) gulls' physiology and health condition: evidence from a captive feeding experiment

Abstract

Several large gulls are known to exploit urban areas and routinely consume diverse and readily available anthropogenic food sources, which allows them to reduce foraging times and expenditure of energy. Such human-derived food, however, may have a poorer nutritional quality, lacking in some nutrients crucial for birds' health, with unknown physiological consequences for gulls constantly relying on such food resources. Here, we investigated the effects of a typically anthropogenic diet on gulls' physiology and health condition, by establishing a captive feeding experiment, under controlled conditions, with eight Yellow-legged (*Larus michahellis*) and Lesser black-backed (*Larus fuscus*) gulls. With a high proportion of refined carbohydrates and fat, we predicted that an anthropogenic diet would alter gulls' fatty acids composition, haematological variables (blood haemoglobin concentration, total plasma protein concentration, white blood cell count and heterophils to lymphocytes ratio), oxidative stress and mitochondrial activity parameters. To test this prediction, after an acclimatization period of 12 days, $n = 4$ gulls were submitted to an anthropogenic diet (a mix of processed food and remnants of meat), while $n = 4$ gulls were subjected to a natural diet (fish) for 14 days. Gulls were sampled after the acclimatization period and at the end of the feeding experiment of diet manipulation. Alongside, $n = 3$ gulls were captured at a landfill. No significant differences in fatty acids composition and in the evaluated health parameters were detected between groups to be established for the feeding experiment, after the acclimatization period, which suggests that the acclimatization period was successful in allowing similar conditions of individuals prior to the feeding experiment. Significant differences were detected in gulls' plasma fatty acids composition between diets, confirming the nutritionally poorer quality of anthropogenic and landfill diets. Haemoglobin concentration and oxidative stress parameters also differed significantly among diets. Mitochondrial parameters were indicative that gulls' bioenergetic function was much well preserved in gulls fed with natural diet than in gulls fed with processed food. Although the number of landfill-caught gulls is too low, we were able to detect some physiological patterns that seem to differ from the remaining dietary regimes.

Keywords

Urbanization; Laridae; Human-derived food; Nutritional composition; Urban diet

5.1. Introduction

Population growth, urbanization and consequent opening of many landfills became a global concern for the health of many wild animal populations (Marzluff 2001; Aronson et al. 2014). The presence of environmental toxins, parasites and various pathogens in urban habitats may contribute to decreased health of urban-dwelling wildlife (Murray et al. 2019). Yet, a multiple number of opportunistic and generalist animals, such as gulls, rats and foxes, are lured into urban habitats and landfills (Belant 1997; Belant et al. 1998; Parra-Torres et al. 2020) due to the high availability and easily accessible anthropogenic food sources present in these environments (Oro et al. 2013). Urban animals have access to human-derived food through recreational and intentional feeding (e.g. bread fed to birds in urban parks), and/or involuntary food waste (e.g. waste from garbage bins or landfills (Smith et al. 1993; Galbraith et al. 2015)). Consequently, urban- and landfill- dwelling animals are known to shift the types of food they consume, such as the diet shift experienced by urban Red Foxes (*Vulpes vulpes*) and urban Coyotes (*Canis latrans*) whose diet was mainly composed by human-related food when comparing to their rural counterparts (Contesse et al. 2004; Murray et al. 2015). It is unclear, however, if the consumption of these anthropogenic food resources is beneficial or detrimental to wildlife.

On the one hand, the predictability and consistent access to urban- and landfill-derived anthropogenic food has the potential to reduce animals' starvation risk, lessening the effects caused by seasonal fluctuations in resources and nutrient availability experienced in natural habitats, often considered one of the most limiting factors for wildlife (Marzluff 2001; Bartumeus et al. 2010). In fact, the continuous availability of easily accessible anthropogenic food allows opportunistic species to reduce foraging time and energy expenditure (Fuirst et al. 2018), which may enable the allocation of more energy towards life-sustaining energetically demanding processes, such as growth and reproduction, which may ultimately improve overall condition (Murray et al. 2018). For example, the supplementation of birds' diets through the consumption of human-derived food available in urban habitats was responsible for earlier egg laying and more frequent re-nesting in urban Black-billed Magpies *Pica pica* (Jerzak 2001), earlier gonadal development in urban Blackbirds *Turdus merula* (Partecke et al. 2006) and earlier breeding in urban Florida Scrub Jays *Aphelocoma coerulescens* (Schoech and Bowman 2001), when comparing to their rural conspecifics.

On the other hand, the constant consumption of human-derived food may negatively impact the physiology and general health condition of wildlife. Although anthropogenic food resources are richer in calories, fat and protein than naturally available foods, which allows a greater energy intake (Patenaude-Monette et al. 2014), they also lack in some essential nutrients, crucial for avian health, such as calcium, manganese and some amino acids (Pierotti and Annett 1991; Isaksson and Andersson 2007; Heiss et al. 2009). As a sub-optimal diet, it can be responsible for reduced

growth rate, body condition and overall health (Pierotti and Annett 1991; Annett and Pierotti 1999), as well as supressed immune function (Lawson et al. 2018) and increased oxidative stress (Partecke et al. 2006; Salmón et al. 2016; Herrera-Dueñas et al. 2017; Watson et al. 2017).

Large gulls *Laridae* are opportunistic foragers that, when inhabiting their traditional, natural habitats (i.e. coastal areas or islands) rely mainly on marine food resources, including those derived from fishery discards (Tyson et al. 2015; Calado et al. 2021). However, gulls are known to use a wide variety of habitats, food types and foraging strategies, and are increasingly using terrestrial and man-made environments to forage, such as urban settlements and landfills (Gyimesi et al. 2016; Isaksson et al. 2016; Matos et al. 2018; Parra-Torres et al. 2020). Urban- and landfill-dwelling gulls feed mostly on domestic waste and food scraps of human origin, such as remnants of cooked and raw meat, fish, fruits, vegetables, meals, eggs, among others (Parfitt et al. 2010; Lopes et al. 2021b). Moreover, within urban environments, anthropogenic food resources may also include discarded fast food, stolen by gulls directly from people in parks and recreational areas or from trash containers and restaurants terraces (Pais de Faria et al. 2021b). The regular consumption of these food resources has unclear and contradictory effects on gulls. For instance, Auman et al. (2008) reported that Silver Gulls (*Larus novaehollandiae*) that fed primarily on refuse from landfills were heavier and of greater body condition than gulls feeding on natural, non-subsidized, habitats. Also, the closure of landfills was responsible for the decrease in clutch size (Pons and Migot 1995), fertility (Pons and Migot 1995; Kilpi and Öst 1998) and last-laid egg size (Kilpi and Öst 1998) for European Herring Gulls (*Larus argentatus*), and decreased body mass, body condition and overall breeding success for Yellow-legged Gulls (*Larus michahellis*, Steigerwald et al. 2015). On the contrary, Pierotti and Annett (1991) found that herring gull adults that foraged mainly on their natural habitat laid larger and heavier clutches, hatched more eggs and had more fledging chicks in comparison to gulls that fed on refuse. Decreased chick weight on Laughing Gull (*Larus atricilla*, Dosch 1997) and decreased egg quality on Herring Gull (Hebert et al. 2020) were also reported as costs associated with the consumption of anthropogenic food. Also, when foraging on waste at landfill sites and/or within urban settlements, gulls are increasingly exposed to contaminants (Zapata et al. 2018; Sorais et al. 2020) and pathogens (Ramos et al. 2010; Converse et al. 2012; Alm et al. 2018), with potential negative consequences for their health. Despite the reduction in foraging time, the increased accessibility to large amounts of human-derived food at landfills and within urban habitats may not compensate for the lack of vital nutrients of such food (Pierotti and Annett 2001). Therefore, it is not known if these food resources are suitable for gulls or if they present an ecological trap to gulls with long-term consequences for their physiology and health condition.

Several haematological and physiological parameters that characterize different biological functions of individuals and whose mechanisms of variation are well understood, may be used to assess gulls' general health condition. The white blood cell count (WBC), particularly the ratio

heterophils/lymphocytes (H/L, Norte et al. 2022), the concentration of haemoglobin (Hb), oxidative stress measurements (Costantini 2008), fatty acids profiles (FA, Iverson et al. 2007; Andersson et al. 2015), total plasma protein concentration (Grasman 2002) and mitochondrial activity (Palmeira and Madeira 1997) are some examples of metrics that can provide crucial information in understanding the physiological effects of an anthropogenic food diet.

To date, most research on this issue has focused in the comparison of physiological parameters between birds living in urban areas and their natural counterparts (Cummings et al. 2020; Lopes et al. 2021a; Pais de Faria et al. 2021a; Basile et al. 2021) and, therefore, studies examining the physiological responses of wild birds to dietary manipulations are scarce (Basile et al. 2021). Such studies are vital to control for the potential effects of confounding variables such as temperature, pollution, water availability and pathogen exposure. The objective of this study was to determine whether the consumption of anthropogenic food resources typically present in urban and landfill settlements alters the body mass and various physiological parameters of Yellow-legged and Lesser black-backed gulls (*L. fuscus*), held in controlled environmental conditions. Also, the same physiological parameters were analysed in landfill-caught gulls and compared with the individuals from the captivity experiment. We hypothesised that gulls feeding on a human-derived diet would have altered physiological parameters, when comparing to gulls feeding on a natural diet, and the most impaired health condition should be found in landfill-caught individuals.

5.2. Materials and methods

Yellow-legged (*Larus michahellis*) and Lesser black-backed (*L. fuscus*) gulls were either part of a feeding experiment performed under controlled conditions in a wildlife rescue centre or were captured in the landfill of Coimbra. All individuals were either irrecuperable, without any possibility of being returned to nature (i.e. those included in the feeding experiment) or captured moribund (i.e. at the landfill). Three diet groups were considered for this study: the “natural” and the “anthropogenic” diet, both relative to the feeding experiment, and the “landfill” diet. This study was authorized by the Institute of Nature Conservation and Forests, ICNF (permit number: 07/2019/CAPT) and details of the procedures are given below.

5.2.1. Captive feeding experiment with irrecuperable gulls

Eight Yellow-legged (n = 4) and Lesser black-backed (n = 4) captive gulls, were subjected to a feeding experiment of diet manipulation, under controlled conditions. The eight individuals included in this study were carefully selected from the gulls admitted to Wildlife Rehabilitation

and Investigation Centre (RIAS) in the south of Portugal (37º 02' 03'' N, 7º 48' 47'' W). Usually, gulls are found stranded as a result of injury, illness or exhaustion, and brought by national authorities (ICNF) or by locals to the rescue centre. After admission, individuals are meticulously examined by the rescue centre's veterinarian, and they are either admitted for rehabilitation and subsequent release into nature, if rehabilitation is successful, or they may be euthanized when injuries are severe and without possibility of being recovered. The eight individuals chosen for this study were diagnosed with health conditions not compatible with the possibility of their return to nature. Due to trauma and other permanent lesions, these individuals were incapable to fly again, and were considered irrecoverable.

The feeding experiment (Figure 5.1) began on January 4, 2019 (day 0, T_0) with all eight individuals being housed in a large outdoor aviary (10m length x 10m width x 5m height) and provided with water *ad libitum*. For 12 days (from day 0 to day 11, January 15, 2019), all individuals were fed with Chub Mackerel *Scomber colias* and Horse/jack Mackerel *Trachurus* sp., the two fish species mostly consumed by gulls from natural colonies along the Portuguese coast (Calado et al. 2021), accounting for 150 g of food per individual each day. These 12 days of acclimatization with fish diet had the objective to allow that the gulls' initial conditions in term of diets would be the same in both groups before the change of diets (Alonso-Alvarez and Ferrer 2001). At day 12 (January 16, 2019, T_1), gulls were sampled for blood, representing the starting samples for the feeding experiment (see "gulls' sampling" section), and, after sampling, were divided into two dietary groups, the "natural diet" group (n = 4) and the "anthropogenic diet" group (n = 4), with similar species and age class ratio. Individuals from the "natural diet" group continued to be fed as in the previous 12 days, with chub and horse/jack mackerels, recreating the diet of gulls from natural, non-urbanized locations (i.e. fish). The "anthropogenic diet" group simulated a diet of anthropogenic origin, typically fed by gulls from more urbanized locations, based on previous studies and observations (Lopes et al. 2021b, a; Pais de Faria et al. 2021a), which included a mix of processed food (e.g. sausages and hamburgers) and remnants of meat (chicken and beef trimmings), in a total of 150 g of food per individual, per day. For 13 days (from day 12 to day 24, January 28, 2019), gulls were housed in two different outdoors aviaries (10 m length x 5 m width x 5 m height, each), each group of four individuals in their own cage, separated per diet, with unlimited water provided during the experiment and with food replaced daily.

At day 24 (January 28, 2019), individuals were transported individually in properly sized cardboard boxes to the laboratory and maintained in two different rooms, one per type of diet, until the next day with the respective diet and water *ad libitum*. At both rooms, birds were exposed to the normal light/dark cycle and the temperature was the same as the outside, to mimic the outside conditions and reduce stress. At day 25 (January 29, 2019, T_f) gulls were euthanized by cervical dislocation, necropsied and sampled.

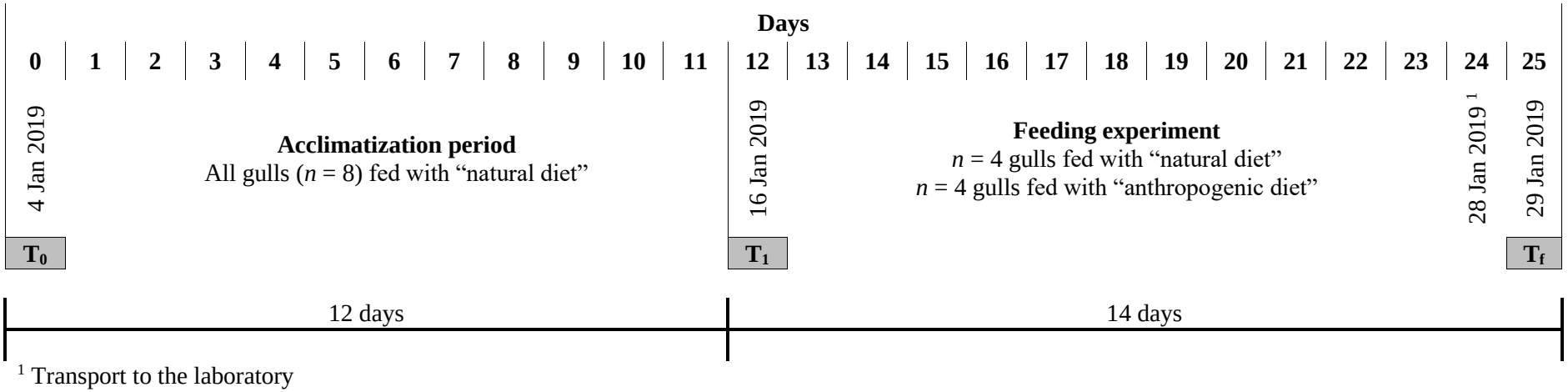


Figure 5.1. Timeline of experimental procedures and sample collection from Yellow legged (*Larus michehellis*) and Lesser black-backed (*Larus fuscus*) gulls (*n* = 8) submitted to the captivity experiment of diet manipulation, under controlled conditions in 2019. T_0 represents the beginning of the acclimatization period and no sampling occurred in this time. T_1 represents the intermediate time sampling, corresponding to the end of the 12-days acclimatization period, before changing diet regimes. After T_1 sampling, 4 gulls were submitted to a "natural diet" while 4 gulls were subjected to an "anthropogenic diet", with similar species and age class ratio. T_f represents the final time sampling, corresponding to the end of the 14-days feeding experiment.

5.2.2. Capture of moribund landfill gulls

The landfill of Coimbra (40º 17' 11'' N, 8º 28' 15''W) is part of a public company (ERSUC) responsible for the treatment and valorisation of urban solid waste from the centre of Portugal, handling approximately 300 000 t of residuals each year (ERSUC 2021). At the time of fieldwork, the garbage disposal area did not have any type of coverage and no gull control occurred, therefore, refuse was fully accessible for gulls to forage on, being a reliable food source that attracted up to 25 000 individuals especially in winter months (authors' personal observation). With such high abundances of gulls foraging and resting at this location, it was normal to find moribund gulls when performing regular visits to the landfill. During such visits, between October 2018 and November 2018, 2 moribund gulls were found, captured and transported to the laboratory in individual and properly sized cardboard boxes. Both individuals were euthanized by cervical dislocation, necropsied and sampled immediately after their arrival to the laboratory.

5.2.3. T_1 and T_f gulls' sampling

For the feeding experiment, at day 12 (i.e. after the acclimatization period and before changing diets, T_1) gulls were weighted using an electronic balance to the nearest 1 g, and a blood sample (maximum 1 mL) was taken by puncture of the tarsal vein using a syringe with a 27-Gauge needle and not-equally divided into two tubes per individual. Samples were kept in a fridge box for about 1-2h. The tube with the lower quantity of blood was used for haemoglobin concentration analysis (whole blood) and the blood from the other tube was separated into red blood cells (RBC) and plasma using a centrifuge (15 min at 2910 g); plasma was used for fatty acids, oxidative stress and protein concentrations analysis (see below). Samples were kept at -20 ºC and -80 ºC (i.e. plasma for fatty acids) until further analysis.

At day 25 (i.e. final of the experiment, T_f) all gulls were necropsied following the dissection techniques of van Franeker (2004) and Peleteiro (2016). Prior to euthanise and similarly to T_1 sampling, a blood sample was taken from the tarsal vein of all individuals and processed on the same way as in T_1 (i.e. a small portion of whole blood was used for haemoglobin concentration analysis and another portion was separated into RBC and plasma for fatty acids, oxidative stress and protein concentrations analysis). Additionally, for each individual, a drop of blood was smeared and air-dried onto a microscope slide immediately after collection, following standard procedures (Bennett 1970), to evaluate the White Blood Cell count (WBC) and the Heterophils / Lymphocytes (H/L) ratio.

During necropsies, livers of the gulls submitted to the feeding experiment were collected and submitted to the mitochondrial respiration and membrane potential protocols.

5.2.4. Fatty acids quantification

Plasma samples of each gull, including those from T_1 (i.e. feeding experiment gulls after acclimatization period) and from T_f (i.e. feeding experiment and landfill-caught gulls final sampling) were stored at -80 ºC until further analysis. The extraction of total lipids and methylation to fatty acid methyl esters (FAMEs) was performed following Gonçalves et al. (2012). Samples were incubated with methanol for the extraction of lipids and the nonadecanoic acid (C19:0, Fluka 74208) was added as an internal standard for further quantification. Samples were centrifuged and vacuum dried. FAMEs identification was carried out through Gas Chromatography-Mass Spectrometry (GC-MS), using a Thermo Scientific Trace 1310 Network (Waltham, MA, USA) equipment, equipped with TR-FFAP (Ton Refrigeration Free Fatty Acid Phase) column of 0.32 mm internal diameter (i.d.), 0.25 µm film thickness, and 30 m long. The sample was injected at an injector temperature of 250 °C, lined with a split glass liner of 4.0 mm i.d. The initial oven temperature was 80 °C, followed by three ramps of linear temperature increase: 25 °C min^{-1} until 160 °C; 2 °C min^{-1} until 210 °C and finally an increase of 40 °C min^{-1} until a final temperature of 230 °C was reached and maintained for 10 min. The carrier gas was helium at a flow rate of 1.4 mL min^{-1}. A Thermo Scientific ISQ 7000 Network Mass Selective Detector at scanning m/z ranges specific for fatty acids in Selected Ion Monitoring (SIM) mode acquisition was used. The detector starts operating 3.5 min after injection, corresponding to solvent delay. The injector ion source and transfer line were maintained at 240 °C and 230 °C, respectively, and integration of FAME peaks were carried out using the equipment's software. Identification of each peak was performed by retention time and mass spectrum of each FAME, comparing to the Supelco®37 component FAME mix (Sigma-Aldrich, Steinheim, Germany). Finally, each peak area was extracted and then quantified as µg/mL.

5.2.5. Haematological parameters and plasma protein

Haemoglobin (Hb) concentration (g/L) was measured in the whole blood samples using a Haemoglobin Assay Kit (Sigma-Aldrich) following to the manufacturer's protocol. Whole-blood samples were diluted 100x in water. Using this technique, haemoglobin is converted to a colorimetric product and the absorbance was measured at a wavelength of 405nm.

Blood smears from each bird were prepared during Tf sample collection and fixed in methanol for 2 minutes, air-dried, and latter stained using the May-Gründwalds-Giemsa procedure. Smears were scanned in a section of the smear where red blood cells were homogeneously distributed using the microscope's 1000x magnification and immersion oil (Norte et al. 2008). White Blood Cell count (WBC) was estimated by counting the total number of white blood cells per ~10.000 red blood cells (Norte et al. 2008; Cirule et al. 2012). Differential

WBC included lymphocytes (L), heterophils (H), monocytes, basophils and eosinophils (Davis et al. 2008) identified based on their morphological characteristics (Mallory et al. 2015). Thrombocytes were excluded. For each blood smear, 100 white blood cells were counted and categorized, and used to assess the H/L ratio (Mallory et al. 2015). All blood smears were examined by the same person (C.S.L.).

Total plasma protein was measured using the Bradford protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) based on the dye-binding technique described by Bradford (1976). Protein concentrations were estimated by reference to absorbances obtained for a series of standard protein dilutions that were assayed alongside the unknown samples. The manufacturer protocol was followed, absorbances were read at 595 nm and presented as mg/mL.

5.2.6. Oxidative stress

Oxidative stress was inferred from measuring both plasma Reactive Oxygen Metabolites (ROMs), using the d-ROMs assay (Diacron International, Grosseto, Italy), and the plasma non-enzymatic antioxidant capacity, using the OXY-adsorbent assay (Diacron International, Grosseto, Italy), allowing to evaluate the unbalance between oxidant and antioxidant systems in plasma (Monaghan et al. 2009).

The d-ROMs assay reflects the total oxidant capacity mostly by measuring hydroperoxides, that are intermediate oxidative damage compounds and precursors of several end-products of lipid peroxidation (Costantini 2008). The protocol provided with d-ROMs kit was followed and adapted for a 96 well microplate reader as follows: calibrator volume of 10 µL, plasma sample of 10 µL, incubation of 65 min at 37ºC. Absorbances were read at a wavelength of 540 nm and expressed as Carratelli units (CARR.U; 1 CARR.U = 0.08 mg H_2O_2/dL). To control for haemolysis in some of the samples and following the d-ROMs protocol, absorbances were also read at 450 nm and used as a co-variate, while all ROMs read at 540 nm < 11 U.CARR (n = 3 for T_1 samples and n = 5 for T_f samples) were replaced by 5.5 U.CARR.

The OXY-adsorbent assay quantifies the plasma non-enzymatic antioxidant capacity to cope with the in vitro oxidant action of hypochlorous acid (HClO, an oxidant of pathologic relevance in biological systems and endogenously produced, Costantini 2011). Manufacturer instructions were followed and adapted for a 96 well microplate reader, absorbances were read at 540 nm and presented as µMol HClO/mL.

Every spectrophotometric measurement (Hb, OXY, d-ROMs and protein) was performed at 25ºC using a 96-well microplate photometer reader (Multiskan FC, Thermo Scientific).

5.2.7. Mitochondrial activity

5.2.7.1. Mitochondrial isolation

The mitochondria were isolated in a homogenization medium comprising 250 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and 0.1% fat-free bovine serum albumin (BSA) (Palmeira et al. 1994; Varela et al. 2010). After homogenization of the minced blood-free hepatic tissue, the homogenates were centrifuged at 800 *g* for 10 min at 4°C. The supernatants were spun at 10 000 *g* for 10 min at 4°C to pellet the mitochondria that were then resuspended in a final washing medium from which EGTA and BSA were omitted, and it was adjusted to pH 7.4. The protein content was determined using the biuret method calibrated with BSA.

5.2.7.2. Mitochondrial membrane potential measurements

The mitochondrial membrane potential was estimated using an ion-selective electrode to measure the distribution of tetraphenylphosphonium (TPP+) (Rolo et al. 2000). The voltage response of the TPP+ electrode to log (TPP+) was linear with a slope of 59 ± 1, and it conformed to the Nernst equation. The mitochondria (1 mg) were suspended in standard medium (1 mL), comprising 130 mM sucrose, 50 mM potassium chloride, 5 mM magnesium chloride, 5 mM monopotassium phosphate, 50 mM EDTA, 5 mM HEPES (pH 7.4), and 2 µM rotenone, supplemented with 3 µL TPP+. A matrix volume of 1.1 µL/mg protein was assumed. The reactions were carried out at 25°C in a temperature-controlled chamber surrounded by a water jacket with magnetic stirring. The membrane potential (mV), depolarization (mV) and lag phase (s) were measured, and the readings were recorded in triplicate.

5.2.7.3. Oxygen consumption measurements

The oxygen consumption of the isolated mitochondria was determined using a Clark-type polarographic oxygen electrode (Oxygraph; Hansatech Instruments Ltd., King's Lynn, Norfolk, United Kingdom) (Rolo et al. 2000). Mitochondria (1 mg) were suspended in the standard medium (1.4 mL) with constant stirring at 25°C, as described previously. The mitochondria were energized with succinate (5 mM) and state 3 respiration was induced by adding adenosine diphosphate (ADP) (200 nmol). Oxygen consumption was also measured in the presence of 1 µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). State 3 respiration and the respiratory control ratio were calculated according to Chance and Williams (1956).

5.2.8. Data and statistical analysis

Firstly, FA composition (see below) and health parameters assessed on T_1 (Hb, Protein, OXY, ROMs and body mass) were used to evaluate whether the acclimatization period was effective in allowing similar initial conditions for both groups to be made in terms of physiology and health condition before the change of diets. Student's t-tests were used to evaluate differences among both groups for each parameter, except for ROMs where an analysis of co-variance (ANCOVA) was applied with absorbances at 450 nm used as a co-variate.

Secondly, FA composition (see below), health variables (Hb, Protein, OXY, ROMs, WBC, H/L, body mass), and mitochondrial parameters of the gulls submitted to the feeding experiment, evaluated on T_f were used to assess the effect of both diets (natural and anthropogenic) on gulls' physiology and health condition. Student's t-tests were used to assess the effect of diet (natural, anthropogenic) on the body mass, health parameters (Hb, OXY, Protein, WBC, H/L) and mitochondrial activity parameters (mitochondrial respiration and membrane potential). An ANCOVA was used to assess the effect of diet on ROMs, with absorbances at 450 nm used as a co-variate, to control for haemolysis in some samples. Due to the low number of landfill-caught gulls (n = 2), these individuals were not used for these statistical proposes but were included on several graphs as a representation.

Each FA of the gulls' plasma, initially in abundances (µg/mL), was converted to a percentage of the total FAs, per individual, and to normalize FAs percentages data, we used the arcsine transformation. To visualize the differences in FA composition 1) among the groups to be made, after the acclimatization period (T_1), and 2) among diets after the feeding experiment (T_f), we used principal component analysis (PCA). PCAs (one for each sampling time) were performed using all transformed FAs percentages independently of their origin (dietary or non-dietary) and the number of double-bonds (saturated or unsaturated FA), and FAs without variance were excluded from PCAs. Only the principal components (PCs) with eigenvalues > 1 were retained for further analysis. Student's t-tests with PC Scores as the response variable were used to examine if the segregation performed by the PCAs was statistically significant along each PC. Additionally, to visualize differences in FA composition among the three diet types (natural, anthropogenic and landfill), a PCA was performed, including the FA composition of the feeding experiment (T_f) individuals and landfill-caught gulls. Similarly, two-way ANOVAs with PC Scores as response variable were performed to assess the effect of diet (natural, anthropogenic and landfill) along each PC.

Data was tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test). Hb, ROMs and H/L were log transformed. The R statistical program (R Core Team 2019) was used in all analysis, with a significance level of $p<0.05$.

5.3. Results

5.3.1. Acclimatization period effect on gulls' physiology (T_1 sampling time)

A total of 11 FA were detected and quantified in plasma samples of captivity gulls, in the intermediate time sampling (T_1), after the acclimatization period and before changing diets (Table 5.1). Saturated FAs (SFAs) were the predominant FA group accounting for, on average 75.4% of all FAs, which was particularly due to the high percentage of palmitic (C16:0) and stearic (C18:0) acids, while polyunsaturated FAs (PUFAs) were the least predominant FA group (~0.6). In T_1 sampling time, highly unsaturated FAs (HUFAs) occurred in small percentages (~3.8%), the sum of omega (ω)-3 and ω-6 FAs also presented, on average, low percentages (0.8% and 3.6% of all FAs, respectively) and, thus, the ratio ω-6/ω-3 corresponded to 2.44 (Table 5.1).

A PCA was run on all the percentages (arcsine transformed) of the FAs detected in the captivity gulls' plasma from the T_1 sampling (Figure 5.2). From the 8 PCs that were extracted, PC1, PC2 and PC3 presented eigenvalues > 1 and accounted respectively for 44%, 33% and 14% of the variance in the data. The two groups of gulls to be established for the feeding experiment after the acclimatization period (natural and anthropogenic diets) did not differ distinctly in T_1 along PC1 (t_6 = -2.19; p = 0.07), nor along PC2 (t_6 = -0.22; p = 0.83), nor along PC3 (t_6 = -1.15; p = 0.29).

Health parameters (Hb and Protein), oxidative stress (OXY and ROMs) and body mass of gulls sampled in the intermediate time (T_1) are shown in Table 5.2. No significant differences were found between the two groups of gulls to be established for the feeding experiment (t_6 < 0.76; all p > 0.48, Table 5.2).

Table 5.1. Relative abundance of plasma fatty acids (% of the total fatty acid content) in two gull species (Yellow-legged *Larus michahellis* and Lesser black-backed *Larus fuscus* gulls, *n* = 8) submitted to a feeding experiment of diet manipulation, under controlled conditions. Intermediate time (T_1) sampling was performed in the end of the 12-days acclimatization period, before changing diet regimes and considering the groups to be formed for the feeding experiment. After T_1 sampling, 4 gulls were submitted to a "natural diet" while 4 gulls were subjected to an "anthropogenic diet", with similar species and age class ratio. Final time (T_f) sampling was performed in the end of the 14-days feeding experiment. Fatty acids composition of landfill-caught gulls (*n* = 2) is also included. Data is presented as means ± standard error of the means (SEM). C:D = number of carbon atoms:double bonds; N = number of individuals with that fatty acid detected in their plasma samples (for Total SFA, Total MUFA, Total PUFA and Total HUFA: N = diversity of FAs per sampling time and diet, in italics); LA = linoleic acid; GLA = γ-linolenic acid; DGLA = dihomo-γ-linolenic acid; ARA = arachidonic acid; EPA = eicosapentaenoic acid; AdA = Adrenic acid; DPA = Docosapentaenoic acid; DHA = docosahexaenoic acid; SFA = saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids; HUFA = Highly unsaturated fatty acids.

			Intermediate Time Sampling (T_1)				**Final Time Sampling** (T_f)					
			Natural Group (n = 4)		**Anthropogenic Group** (n = 4)		**Natural Diet** (n = 4)		**Anthropogenic Diet** (n = 4)		**Landfill Diet** (n = 2)	
FA	**FA Class**	**C:D**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**
Myristic acid	SFA	C14:0	3	1.00 ± 0.37	4	0.94 ± 0.05	4	0.77 ± 0.05	4	0.34 ± 0.06	2	0.60 ± 0.27
Pentadecylic acid	SFA	C15:0	4	0.99 ± 0.34	4	0.50 ± 0.02	4	0.26 ± 0.04	4	0.13 ± 0.02	1	0.07 ± 0.07
Palmitic acid	SFA	C16:0	4	38.48 ± 1.61	4	36.01 ± 2.05	4	25.37 ± 0.76	4	21.24 ± 0.78	2	19.63 ± 0.032
Margaric acid	SFA	C17:0	1	0.26 ± 0.26	3	0.79 ± 0.28	4	0.53 ± 0.04	4	0.34 ± 0.01	2	0.17 ± 0.00
Stearic acid	SFA	C18:0	4	31.39 ± 3.13	4	40.53 ± 3.77	4	22.61 ± 0.29	4	23.65 ± 1.42	2	19.93 ± 2.22
TOTAL SFA			***5***	**72.12 ± 3.94**	***5***	**78.76 ± 5.36**	***5***	**49.54 ± 0.64**	***5***	**45.69 ± 0.72**	***2***	**40.40 ± 1.91**
Palmitoleic acid	MUFA	C16:1n-7	4	1.68 ± 0.29	4	1.02 ± 0.11	4	1.01 ± 0.09	4	0.54 ± 0.10	2	0.68 ± 0.07
Heptadecenoic acid	MUFA	C17:1n-10	0		0		0		2	0.07 ± 0.04	1	0.21 ± 0.21
Oleic acid	MUFA	C18:1n-9	4	23.18 ± 3.58	4	14.42 ± 0.997	4	10.94 ± 0.76	4	13.63 ± 0.76	2	13.76 ± 3.75
TOTAL MUFA			***2***	**24.86 ± 3.78**	***2***	**15.44 ± 1.01**	***2***	**11.95 ± 0.73**	***3***	**14.23 ± 0.88**	**2**	**14.65 ± 4.03**
LA	ω-6 PUFA	C18:2n-6	2	0.81 ± 0.47	1	0.42 ± 0.42	4	1.10 ± 0.21	4	6.20 ± 0.88	2	11.21 ± 2.05
GLA	ω-6 PUFA	C18:3n-6	0		0		0		3	0.31 ± 0.13	0	
DGLA	ω-6 PUFA	C20:3n-6	0		0		0		1	0.11 ± 0.11	0	
Mead acid	PUFA	C20:3n-9	0		0		0		1	0.08 ± 0.08	0	
TOTAL PUFA			***1***	**0.81 ± 0.47**	***1***	**0.42 ± 0.42**	***1***	**1.10 ± 0.21**	***4***	**6.69 ± 0.92**	**2**	**11.21 ± 2.05**
ARA	ω-6 HUFA	C20:4n-6	2	2.22 ± 1.30	1	3.69 ± 3.69	4	20.86 ± 0.69	4	26.18 ± 1.75	2	22.77 ± 4.02
EPA	ω-3 HUFA	C20:5n-3	0		1	0.58 ± 0.58	4	4.41 ± 0.93	3	0.65 ± 0.24	0	
AdA	ω-6 HUFA	C22:4n-6	0		0		0		0		2	6.94 ± 0.77
DPA3	ω-3 HUFA	C22:5n-3	0		0		3	0.58 ± 0.21	3	0.45 ± 0.17	0	
DPA6	ω-6 HUFA	C22:5n-6	0		0		0		3	0.46 ± 0.16	1	0.66 ± 0.66
DHA	ω-3 HUFA	C22:6n-3	0		1	1.11 ± 1.11	4	11.56 ± 0.65	4	5.64 ± 0.39	2	3.37 ± 1.58
TOTAL HUFA			***1***	**2.22 ± 1.30**	***3***	**5.38 ± 5.38**	***4***	**37.41 ± 1.20**	***5***	**33.38 ± 1.13**	**2**	**33.74 ± 4.17**
Mean ω-6 / ω-3			0		1	2.44 ± NA	4	1.40 ± 0.23	4	5.14 ± 0.68	2	15.70 ± 7.19
Mean number of FAs / individual			7 ± 0.71		7.75 ± 1.11		11.75 ± 0.25		14 ± 1.47		11.5 ± 1.5	

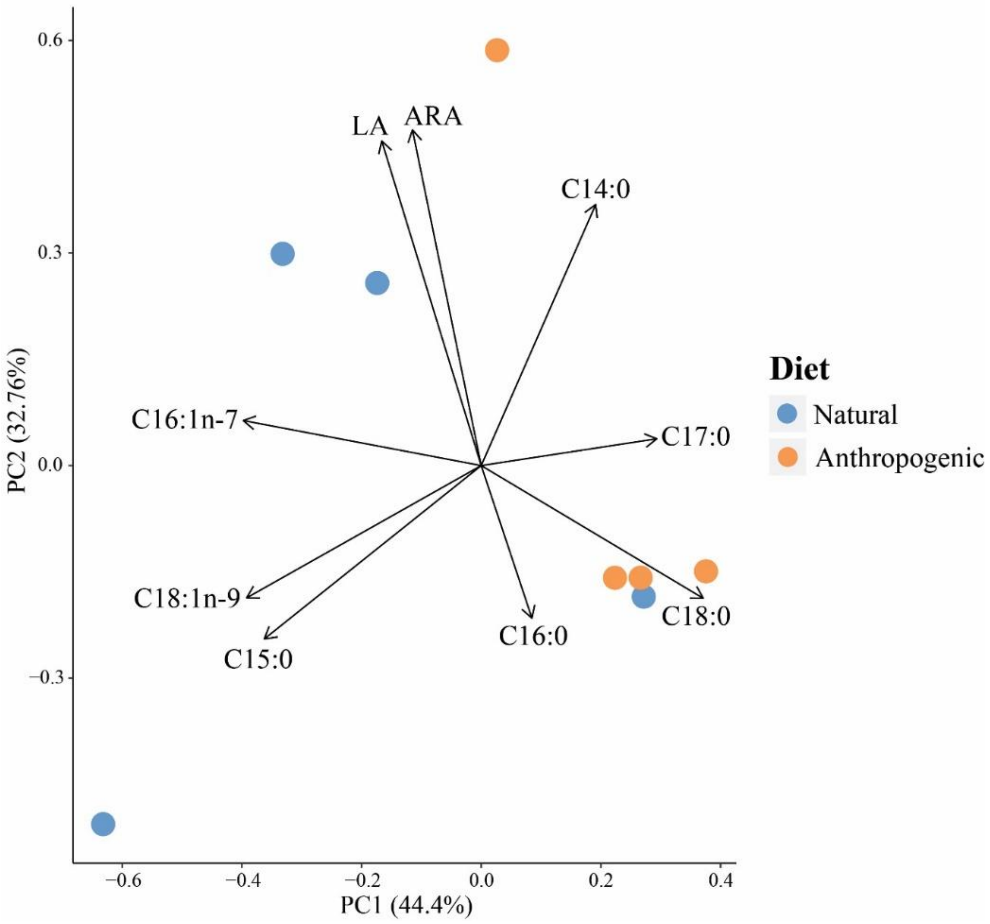


Figure 5.2. Principal component analysis (PCA) biplot (PC1 and PC2) of plasma fatty acids mean percentages (arcsine transformed) in the intermediate time sampling (T_1), after a 12-days acclimatization period and before changing diets of two gull species (Yellow-legged *Larus michahellis* and Lesser black-backed *Larus fuscus* gulls, *n* = 8) submitted to a feeding experiment of diet manipulation, under controlled conditions. The groups to be formed for the feeding experiment are defined with different colours: individuals submitted to the natural diet in blue and individuals submitted to the anthropogenic diet in orange.

5.3.2. Effect of natural and anthropogenic diets on gulls' physiology (T_f sampling time)

Certain FAs such as the ω-6 GLA, DGLA and DPA only occurred in plasma of gulls submitted to the anthropogenic diet, on the final sampling (T_f), even if in small percentages, which was also responsible for the highest diversity of FAs, i.e. number of FAs per individual, verified for this diet type (mean of 14 FAs per individual, Table 5.1). Similarly to T_1, in the final sampling time (T_f) SFAs were the predominant FA group, accounting for an average of 47.6% of all FAs, but HUFAs were the second more important group of FAs, especially for gulls submitted to the natural diet (37.4% *vs.* 33.4% for gulls submitted to the anthropogenic diet, Table 5.1). LA and

ARA, both ω-6 FAs, showed a great importance for gulls submitted to the anthropogenic diet (6.2% and 26.2% for LA and ARA, respectively *vs.* 1.1% and 20.9% registered in plasma of naturally-fed gulls), while physiologically important ω-3 FAs, such as EPA and DHA, presented higher percentages in plasma of gulls submitted to the natural diet (4.4% and 11.6% for EPA and DHA, respectively *vs.* 0.7% and 5.6% registered in plasma of gulls submitted to the anthropogenic diet, Table 5.1). The ω-6/ω-3 ratio in the final time of sampling (T_f), consequently, presented a higher value in plasma of anthropogenically-fed gulls than in gulls fed with fish.

Table 5.2. Haemoglobin concentration (Hb), total plasma protein concentration (Protein), antioxidant capacity (OXY), reactive oxygen metabolites (ROMs) and body mass in the intermediate time sampling (T_1), after a 12-days acclimatization period and before changing the diets of two gull species (Yellow-legged *Larus michahellis* and Lesser black-backed *Larus fuscus* gulls, $n = 8$) submitted to a feeding experiment of diet manipulation, under controlled conditions. Data is presented as means ± standard error of the means (SEM). Statistics assessing differences between groups to be formed for the feeding experiment (natural *vs.* anthropogenic groups) include Student's t-tests for Hb, Protein, OXY and body mass, and analysis of covariance (ANCOVA) for ROMs with absorbance at 450 nm included as co-variate. Hb and ROMs were log transformed to attain normality.

	Natural group (n = 4)		**Anthropogenic group (n = 4)**		**Statistics**	
	Mean ± SEM	**Range**	**Mean ± SEM**	**Range**	**Test value**	**p value**
Hb (g/L)	50.09 ± 8.47	38.26 – 75.09	46.05 ± 8.83	32.58 – 71.97	t = 0.414	0.694
Protein (mg/mL)	1514.45 ± 177.27	1154.86 – 2000	1524.33 ± 104.04	1247.08 – 1751.21	t = -0.048	0.963
OXY (µMol HClO/mL)	232.17 ± 13.94	195.72 – 257.00	216.47 ± 15.21	180.89 – 255.02	t = 0.761	0.476
ROMs (CARR.U)	22.45 ± 5.93	9.33 – 36.95	13.36 ± 5.24	3.39 – 26.78	F = 0.446	0.534
Body Mass (g)	734.75 ± 71.96	558 – 901	805.75 ± 61.44	690 – 951	t = -0.750	0.481

The PCA run on all the percentages (arcsine transformed) of the FAs detected in plasma of the gulls submitted to the feeding experiment and sampled at T_f allowed to separate the gulls from the two diet regimes (Figure 5.3). A total of 8 PCs were extracted, of which PC1 and PC2 accounted respectively for 64% and 24% of the variance in the data. Gulls submitted to the natural diet were significantly separated from gulls submitted to the anthropogenic diet, based on their FA composition, along PC1 (t_6 = -7.49; p = 0.0003), mainly due to DHA, C14:0, C17:0 and EPA that had higher percentages in natural-diet gulls, and due to LA, ARA and C18:1n-9 that had higher percentages in anthropogenically-fed gulls. There was no significant separation along PC2 (t_6 = 0.43; p = 0.68).

Physiological and health condition parameters from the final sampling (T_f) are shown on Table 5.3 and Figure 5.4. Gulls fed with the anthropogenic diet presented significantly higher concentrations of haemoglobin on their blood (t_6 = -4.04; p = 0.007) when compared to naturally-fed gulls. Total plasma protein was also higher in gulls submitted to the anthropogenic diet but did not differ significantly from the gulls submitted to the natural diet (t_6 = -0.89; p = 0.41). Gulls fed with anthropogenic diet had significantly lower levels of reactive oxygen metabolites (ROMs) in their plasma than gulls fed with fish ($F_{1,5}$ = 7.8; p = 0.038), and their antioxidant capacity was also lower than gulls fed with fish, but not statistically different (t_6 = 1.45; p = 0.20).

Liver mitochondria of gulls fed with natural diet (i.e. fish), upon succinate addition, develop a membrane potential of approximately -203 mV, while for those fed with anthropogenic diet the membrane potential only reached -174.4 mV, which was significantly different between diets (Table 5.4). The depolarization induced by ADP for gulls fed with processed food, 14.8 mV, was significantly lower compared to 22 mV of the natural-fed gulls. The lag phase where the phosphorylation of ADP takes place was also significantly different among diets (Table 5.4). The effects of diet on state 3 and state 4 respiration rates are shown in Figure 5.4H and Table 5.4. In gulls fed with the anthropogenic diet, state 3 mitochondrial respiration rate is decreased, and state 4 respiration rate is increased, both significantly different between diets ($p < 0.01$ for both state 3 and state 4). No significant differences were detected in mitochondrial respiration in the presence of FCCP.

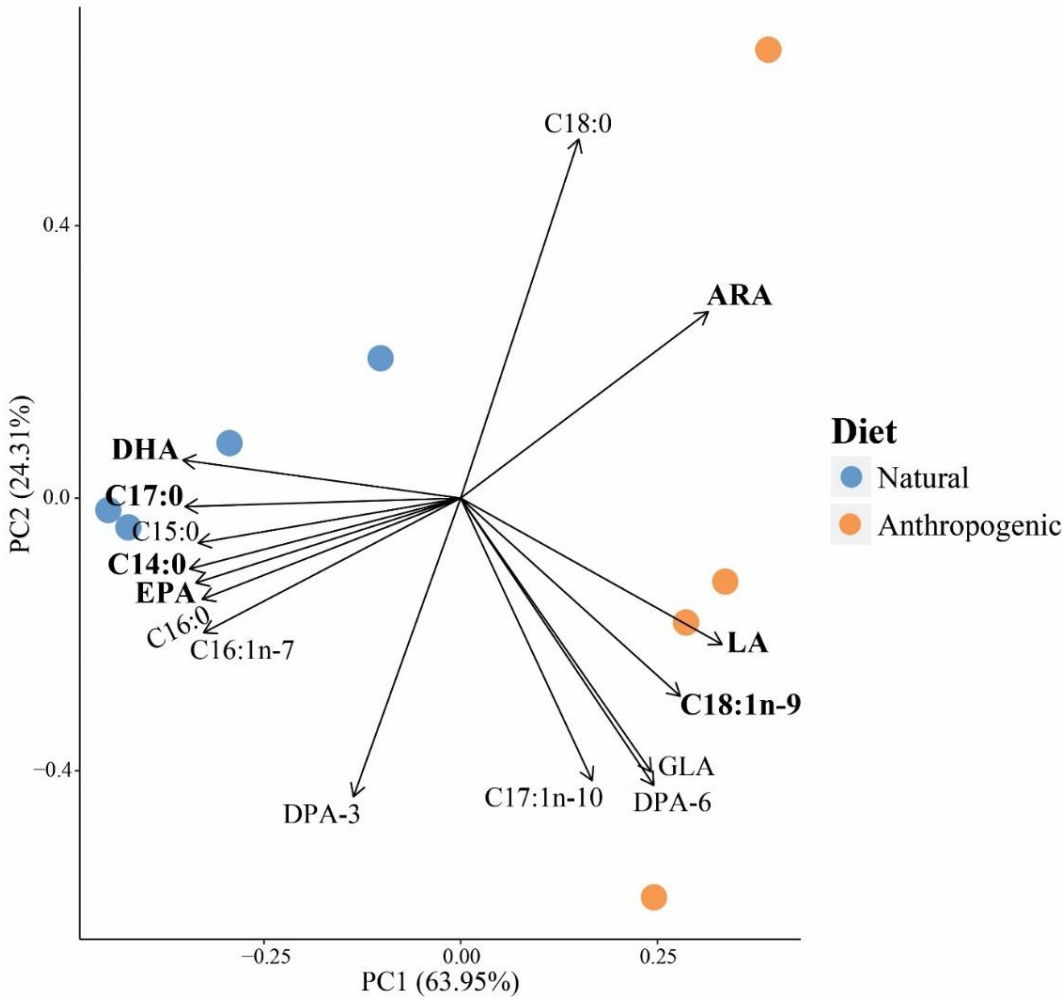
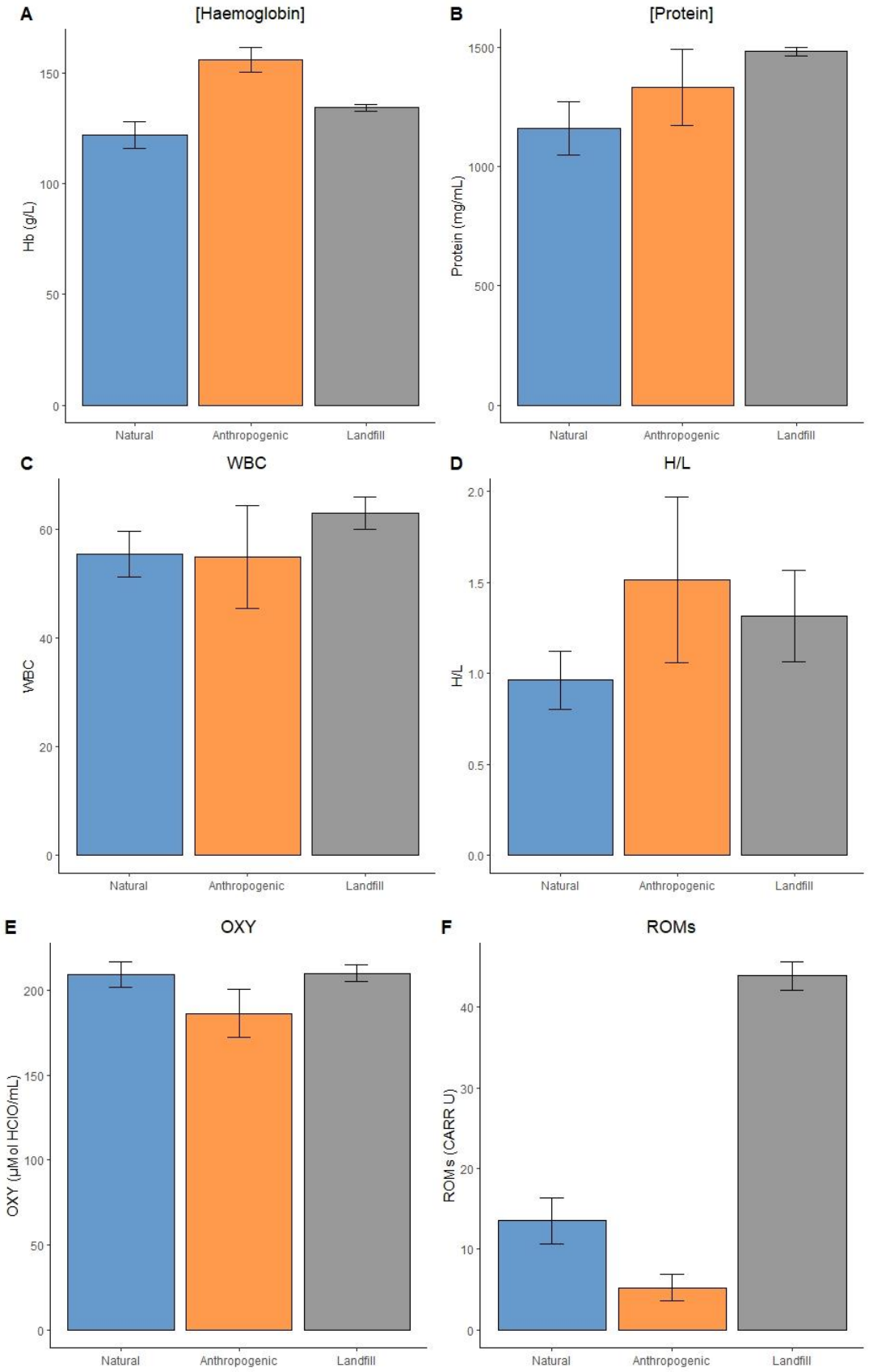


Figure 5.3. Principal component analysis (PCA) biplot (PC1 and PC2) of plasma fatty acids mean percentages (arcsine transformed) in the final time sampling (T_f), after a 14-days feeding experiment of diet manipulation of two gull species (Yellow-legged *Larus michahellis* and Lesser black-backed *Larus fuscus* gulls) being submitted to either a "natural" (n = 4) or an "anthropogenic" diet (n = 4), under controlled conditions. Individuals submitted to each diet are defined with different colours: natural diet gulls in blue and anthropogenic diet gulls in orange. The more important FAs in explaining variation along PC1 are highlighted with a larger font, in bold.

Table 5.3. Whole blood haemoglobin concentration (Hb), total plasma protein concentration (Protein), antioxidant capacity (OXY), reactive oxygen metabolites (ROMs), white blood cell count (WBC), ratio heterophils/lymphocytes (H/L) and body mass in the final time sampling (T_f), after a 14-days feeding experiment of diet manipulation of two gull species (Yellow-legged *Larus michahellis* and Lesser black-backed *Larus fuscus* gulls) being submitted to either a "natural" (*n* = 4) or an "anthropogenic" diet (*n* = 4), under controlled conditions. Parameters of landfill-caught gulls (*n* = 2) are also reported but not included in statistics. Data is presented as means ± standard error of the means (SEM). Statistics assessing differences between natural and anthropogenic diets include Student's t-test for Hb, Protein, OXY, WBC, H/L and body mass, and analysis of covariance (ANCOVA) for ROMs with absorbance at 450 nm included as co-variate. Hb, ROMs and H/L were log transformed to attain normality. Significant effects are highlighted in bold.

	Natural diet (*n* = 4)		**Anthropogenic diet (*n* = 4)**		**Statistics**		**Landfill diet (*n* = 2)**	
	Mean ± SEM	**Range**	**Mean ± SEM**	**Range**	**Test value**	**p value**	**Mean ± SEM**	**Range**
Hb (g/L)	122.04 ± 6.07	108.62 – 136.17	155.99 ± 5.60	143.94 – 171.02	*t* = -4.043	**0.007**	134.23 ± 1.37	132.86 – 135.61
Protein (mg/mL)	1160.36 ± 110.90	882.07 – 1422.09	1333 ± 159.43	1080.39 – 1760.57	*t* = -0.889	0.408	1482.74 ± 17.60	1465.15 – 1500.34
OXY (µMol HClO/mL)	209.56 ± 7.50	187.31 – 219.93	186.45 ± 14.02	169.03 – 228.34	*t* = 1.454	0.196	210.05 ± 4.94	205.11 – 214.99
ROMs (CARR U)	13.51 ± 2.87	7.75 – 21.45	5.21 ± 1.63	1.45 – 8.60	*F* = 7.798	**0.038**	43.92 ± 1.76	42.16 – 45.68
WBC	55.50 ± 4.19	43 – 61	55 ± 9.42	31 – 77	*t* = 0.049	0.963	63 ± 3	60 – 66
H/L	0.96 ± 0.16	0.58 – 1.34	1.51 ± 0.46	0.78 – 2.85	*t* = -1.177	0.284	1.32 ± 0.25	1.07 – 1.57
Body Mass (g)	772.50 ± 35.50	705 – 850	736.25 ± 56.55	640 – 870	*t* = 0.543	0.607	655 ± 35	620 – 690


A
[Haemoglobin]
Hb (g/L)
150
100
50
0
Natural
Anthropogenic
Landfill
B
[Protein]
Protein (mg/mL)
1500
1000
500
0
Natural
Anthropogenic
Landfill
C
WBC
WBC
60
40
20
0
Natural
Anthropogenic
Landfill
D
H/L
H/L
2.0
1.5
1.0
0.5
0.0
Natural
Anthropogenic
Landfill
E
OXY
OXY (µMol HClO/mL)
200
150
100
50
0
Natural
Anthropogenic
Landfill
F
ROMs
ROMs (CARR U)
40
30
20
10
0
Natural
Anthropogenic
Landfill

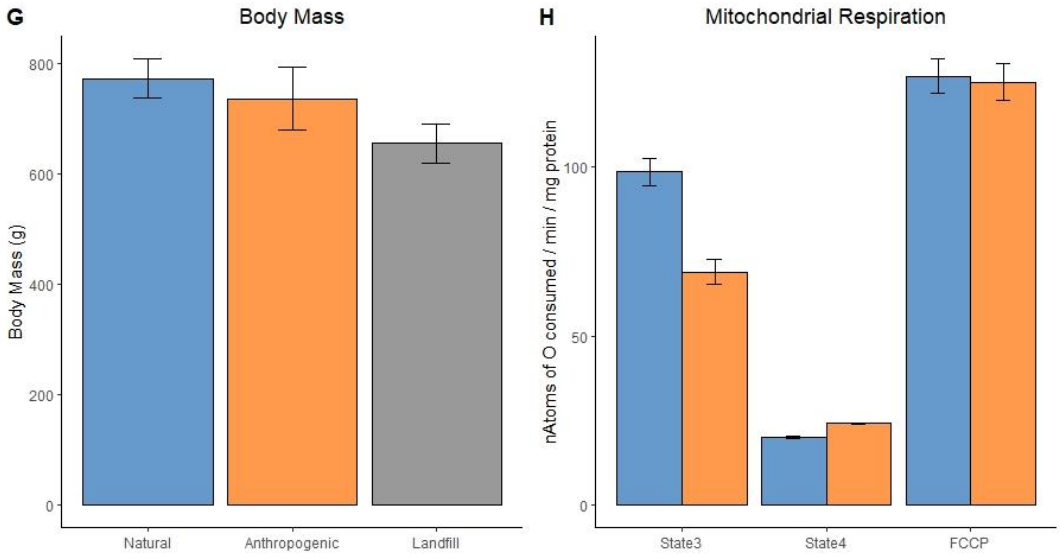


Figure 5.4. Whole blood haemoglobin concentration (Hb, **A**), total plasma protein concentration (Protein, **B**), white blood cell count (WBC, **C**), ratio heterophils/lymphocytes (H/L, **D**), antioxidant capacity (OXY, **E**), reactive oxygen metabolites (ROMs, **F**), body mass (**G**), and mitochondrial respiration (**H**) in the final time sampling (T_f), after a 14-days feeding experiment of diet manipulation of two gull species (Yellow-legged *Larus michahellis* and Lesser black-backed *Larus fuscus* gulls) being submitted to either a "natural" (n = 4, blue) or an "anthropogenic" diet (n = 4, orange), under controlled conditions. Parameters of landfill-caught gulls (n = 2, grey) presented as a comparison, when measured. Data is presented as means ± standard error of the means (SEM).

Table 5.4. Mitochondrial membrane potential and oxygen consumption rates (mitochondrial respiration) measured on gulls' liver mitochondria in the final time sampling (T_f), after a 14-days feeding experiment of diet manipulation of two gull species (Yellow-legged *Larus michahellis* and Lesser black-backed *Larus fuscus* gulls) being submitted to either a "natural" (n = 4) or an "anthropogenic" diet (n = 4), under controlled conditions. Data is presented as means ± standard error of the means (SEM). Mitochondrial respiration was stimulated by addition of succinate. FCCP: oxygen consumption in the presence of FCCP. Statistics assessing differences between natural and anthropogenic diets include Student's t-test and significant effects are highlighted in bold.

		Natural Diet (n = 4)	**Anthropogenic Diet (n = 4)**	**p value**
Mitochondrial Membrane Potential	Initial Membrane Potential (-mV)	203.4 ± 5.0	174.4 ± 2.1	< **0.01**
	Depolarization (-mV)	22.0 ± 1.3	14.8 ± 0.9	< **0.01**
	Lag phase (s)	55 ± 2.2	102.1 ± 4.8	< **0.01**
Mitochondrial Respiration	State 3 (nAtoms O/min/mg protein)	98.4 ± 4.2	68.9 ± 3.7	< **0.01**
	State 4 (nAtoms O/min/mg protein)	20.1 ± 0.4	24.1 ± 0.2	< **0.01**
	FCCP	126.7 ± 5.1	125 ± 5.5	> 0.05

5.3.3. Landfill-caught gulls' physiology

A total of 13 FAs were detected in plasma samples of landfill-caught gulls. SFAs were the predominant FA group (40.4%), followed by HUFAs (33.7%), particularly due to the high percentage of the ω-6 ARA, and MUFAs (14.7%), due to high percentage of oleic acid. The ω-6 AdA was only detected in landfill-caught individuals (Table 5.1). Similarly to gulls fed with anthropogenic diet in T_f, landfill-caught gulls had high percentages of ω-6 ARA and LA, as well as other ω-6 FAs, but the lowest percentages of ω-3 FAs, like the HUFAs DPA and DHA, such that the ω-3 EPA, a physiologically important FA, did not occur in plasma of landfill-caught gulls. Consequently, the ω-6/ω-3 FAs ratio in plasma of landfill-caught gulls was the highest of all diet regimes.

The PCA run on the FA composition of gulls caught in the landfill and those submitted to the feeding experiment and sampled at T_f allowed to separate the three diet types (Figure S5.1). A total of 8 PCs were extracted of which 3 were retained for further analysis and together accounted for 91% of the variance in the data. PC1 and PC2 accounted for 50% and 26% of the variance in the data, respectively. Gulls fed with the natural diet were significantly separated from those fed with the anthropogenic and those captured in the landfill PC1 ($F_{2,7} = 51.42$; $p < 0.001$), mainly due to DHA and EPA that had higher percentages in the plasma of gulls fed with fish, and due to LA, AdA, and oleic acid that had higher percentages in landfill gulls. The ω-6 GLA only occurred in individuals submitted to the anthropogenic diet, while AdA only occurred in plasma of landfill gulls, thus both allowed the separation between gulls of the anthropogenic and landfill diets in the PCA.

When compared to the parameters of gulls submitted to the feeding experiment, both natural and anthropogenic diet regimes, the two landfill-caught gulls presented, on average, higher levels of protein, WBC and, especially, ROMs; intermediate levels of Hb, H/L and OXY; and lower body mass (Figure 5.4 A-G).

5.4. Discussion

In this study, we aimed to understand how a typically urban anthropogenic diet and a landfill-based diet may affect the physiology and health condition of two generalist and opportunistic gull species that heavily rely on such food resources. By establishing an experiment in captivity, under controlled conditions, we aimed to monitor the potential effects of confounding variables such as temperature, pollution, water availability and pathogen exposure.

No significant differences in both FA composition and health parameters were detected between groups to be established for the feeding experiment, after the acclimatization period of 12 days. As expected, at the end of the experiment there were significant differences in FA

composition, blood haemoglobin concentration, reactive oxygen metabolites and mitochondrial parameters between gulls submitted to the natural diet and those submitted to the anthropogenic diet. Although the number of landfill-caught gulls was too low, we detected some physiological patterns that seem to differ from the remaining dietary regimes.

5.4.1. FA composition as a response to diet manipulation

In the intermediate time of the captivity experiment (T_1), after the acclimatization and before changing diets, gulls' plasma FA composition revealed no clear differences between individuals of groups to be stablished for each feeding experiment (natural *vs.* anthropogenic diets). As showed by Käkelä et al. (2005), after changing diets of Herring gulls, a clear shift in plasma FAs was found within five days, reaching equilibrium in the percentages of FAs indicators by 11-21 days. During our acclimatization period of 12 days, all individuals were fed with the same prey type (i.e. fish, simulating a natural diet), and no differences were detected in FA composition in T_1, which suggests that the acclimatization period was successful in allowing similar gulls' initial conditions of both groups, before the change of diets (Alonso-Alvarez and Ferrer 2001).

All gulls' plasma samples revealed a clear predominance of SFAs over unsaturated fatty acids (UFAs), which contrasts with previous studies that report overall higher contents of, for instance, C18:1n-9 (MUFA) than C18:0 (SFA) on birds' fat and plasma tissues (Dahl et al. 2003; Käkelä et al. 2006; Puskic et al. 2019; Lopes et al. 2021a). The pattern MUFAs > SFAs often indicates a diet enriched on marine species (Dahl et al. 2003). In our study, palmitic (C16:0) and stearic (C18:0) acids were the predominant FAs on plasma samples of gulls submitted to the natural diet, which is in accordance with Pais de Faria et al. (2021a) study on free-living yellow-legged gull chicks that reported C16:0 as the most abundant FA in chicks from Berlenga Island, a natural breeding colony. Indeed, these two FAs are the most abundant in animals, as they are a common released product from the *de novo* synthesis pathway of 14-carbon FAs within birds' liver (Dalsgaard et al. 2003; Käkelä et al. 2009), which may be stored in the adipose tissue or used rapidly as an energy substrate (Williams and Buck 2010). As biosynthesised *de novo* by birds, both SFAs and MUFAs levels can be controlled to a larger extent than PUFAs and HUFAs (Isaksson et al. 2017), thus the levels of SFAs and MUFAs measured in this study are likely a consequence of metabolic regulation.

At the final of the feeding experiment, gulls fed with processed food (i.e. anthropogenic diet) presented in their plasma increased percentages of ω-6 FAs, such as ARA and LA, and were depleted of physiologically important groups of FAs, especially HUFAs and ω-3 FAs (EPA, DHA), when comparing to gulls fed with fish (i.e. natural diet). Landfill-caught gulls presented

the highest ω-6/ω-3 FAs ratio in their plasma, with increased occurrence of ω-6 and depletion of ω-3 FAs. Essential fatty acids (EFAs), such as the ω-3 EPA and DHA, must be obtained through diet, as birds are not able to synthetize them *de novo*, and are physiologically very important (Dalsgaard et al. 2003; Gladyshev et al. 2009). In fact, Isaksson et al. (2017), working with Great Tits (*Parus major*), showed that ω-3 FAs in plasma are highly affected by its availability in the diet, with plasma amounts rapidly declining (within a few days) after restricting ω-3 FAs in diet. The results of the feeding experiment support the idea that a diet based on marine sources (the "natural diet" group) provide higher percentages of ω-3 FAs, as detected in T_f for gulls fed with fish, which is consistent with the estimates of previous studies (Lopes et al. 2021a). On vertebrates, a higher content of DHA, for instance, is often related with a higher diet quality, due to the extremely important role of this FA as a phospholipid component in cell membranes, for vision and for brain functions (Ackman and Cunnane 1992; Politi et al. 2001; Lim et al. 2005). In humans, ω-3 FAs are responsible for lower rates of heart diseases, asthma, type 1 diabetes mellitus and other beneficial health effects (Simopoulos 2010). Thus, essential FAs may be responsible to mitigate unfavourable physiological effects and energetically demanding states, such as stress or body mass loss (Williams et al. 2008).

A diet based on human-derived food (the "anthropogenic diet" group) seems to provide extremely low quantities of ω-3 FAs, as detected in T_f for gulls fed with processed food, which is in accordance with the few previous studies with urban gulls that also detected low levels of ω-3 FAs for urban individuals (Lopes et al. 2021a; Pais de Faria et al. 2021a). This is indicator of a poorer nutritional status of gulls fed with processed food, as these food items are usually rich in fat and proteins, allowing a great energy intake, but lack in essential nutrients (Patenaude-Monette et al. 2014). In support of this argument, mead acid, a suggested biomarker of malnutrition or deficiency of essential FAs (Mead and Slaton 1956; Smit et al. 2004), was detected only in gulls fed with the anthropogenic diet, although in small percentages. The higher ω-6: ω-3 FAs ratio of landfill-caught individuals may be suggestive of a higher propensity of landfill-dwelling gulls for an enhanced diet-induced susceptibility to inflammation when exposed to antigens or to suffer from a higher oxidative stress status (Romieu et al. 2008; Isaksson 2015; Isaksson et al. 2017).

In this work, although we did not quantify FAs in the diet offered to gulls, we may assume the overall differences in major FA groups between both diets from previous studies with birds from urbanized environments (Andersson et al. 2015; Isaksson et al. 2017; Lopes et al. 2021a; Pais de Faria et al. 2021a). Qualitative FA analysis does not document a detailed diet composition as other, more conventional, methods do (i.e. feeding observations, identification of prey items from collected regurgitates or pellets), as quantitative data on different food items is not obtained (Karnovsky et al. 2012). However, specific patterns of FA signatures among species, allowed the development of an ambitious technique (QFASA: Quantitative Fatty Acid Signature Analysis, Iverson et al. 2004) that allows the use of FA signatures to quantify prey proportions on a

predator's diet (Iverson et al. 2004, 2007). This technique allows a better way to estimate diet. Still, it has several assumptions to be met. It assumes that predator FA metabolism is not affected by lipid intake through diet, which include the creation of a prey library with all potential prey species or group of species FA signatures, that allow comparisons between prey and predators (Iverson et al. 2004; Budge et al. 2006; Williams and Buck 2010). Future research, including captive feeding experiments, may help in the creation of such prey library, especially for urban gulls, which may be complemented with other techniques (i.e. stable isotopes analysis) to improve the accuracy of diet estimation (Käkelä et al. 2007).

5.4.2. Physiological and health condition parameters as a response to diet manipulation

Hb, Protein, OXY, ROMs and body mass presented no statistically significant differences between both groups at the start of the feeding experiment, which, together with FAs analysis, suggest that the acclimatization period of 12 days was successful in allowing similar gulls' initial conditions of both groups, before the change of diets (Alonso-Alvarez and Ferrer 2001). Significant differences in health condition parameters between natural- and anthropogenic-fed gulls in the end of the experiment were only found for Hb and ROMs, as gulls fed with fish had lower levels of Hb and higher levels of ROMs than gulls fed with processed food. Significant differences between diets were also found for mitochondrial membrane potential and mitochondrial respiration, as gulls fed with anthropogenic diet present an impaired mitochondrial function.

Haemoglobin (Hb) concentration is considered a robust indicator of physiological condition in birds (reviews by Minias 2015 and Johnstone et al. 2017) as it reflects the blood oxygen-carrying capacity. High concentrations of haemoglobin improve aerobic capacity and are usually associated with good health and nutrition (Bańbura et al. 2007), while lower concentrations of Hb are commonly related with lower body condition, lower diet quality, less successful breeding events (e.g. egg laying) and the presence of haematophagus ectoparasites (Norte et al. 2013; Minias 2015).

Oxidative stress occurs when there is a disruption in the balance between the production of reactive oxygen metabolites (ROMs, pro-oxidant) and antioxidant defence levels, favouring the first, with antioxidants being too low to cope with ROMs production (Costantini 2008). ROMs originate naturally from normal metabolism or inflammation processes (Monaghan et al. 2009) and, as a consequence of the imbalance, they oxidize biomolecules, leading to DNA damage, lipid peroxidation and protein oxidation (Livingstone 2001; Valavanidis et al. 2006). Oxidative stress is the rate at which oxidative damage is generated to biomolecules (Costantini et al. 2014b) which

can be induced by several environmental, social and internal conditions, with potential to be severely affected by urban stressors (Partecke et al. 2006; Isaksson 2015). In fact, oxidative stress has been recently studied in association with urban environments, where the concentration of potentially oxidant pollutants tends to be higher (Salmón et al. 2018). Long-term oxidative damage can contribute to cell senescence, loss in organ and organism performance, and may influence life-history strategies (Costantini 2008; Monaghan et al. 2009).

The significantly higher levels of Hb and lower levels of ROMs detected in gulls fed with processed food were not expected, as they are indicative of a good health, nutrition and oxidative stress levels. Contrasting with our results, Pryke et al. (2011, 2012) described that haemoglobin concentration was positively affected by the quality of diet in both adults and nestlings of a captive passerine, but Wagner et al. (2008) reported no effect of diet on haemoglobin concentration, in captive zebra finches (*Taeniopygia guttata*). Divergent results suggest that haemoglobin concentration may not provide a good indication of body condition and physiological status, especially during certain circumstances like serious dehydration of an organism (Minias 2015). The oxidative stress results may be indicative of habituation and resistance to stressful conditions, features possibly intrinsic to each individual. In fact, it has been shown that urban individuals, usually subjected to different stressors within urban environments, tend to develop resistance and ability to cope with such environmental stressors (Costantini et al. 2014a).

Although without significant differences among diets, total plasma protein tended to be higher for landfill-caught gulls, except for one gull fed with anthropogenic diet in the feeding experiment. This parameter indicates short-term condition and nutritional status (Brown 1996), and plasma proteins usually increase in cases of infection / inflammation or dehydration, due to a reduction in plasma volume and consequent increase in globulins (Norte et al. 2008). Also, WBC tended to be higher for landfill-caught gulls and the H/L ratio was lower for individuals submitted to natural diet. The white blood cell count (WBC) is a broad indicator of health and immune system status, as a higher WBC may be a result of inflammation caused by infections (Norris and Evans 2000). In birds, inflammatory and stress exposure is characterized by an increase in the number of circulating heterophils (H), which play an important role during the initial stages of most infections (Bustnes et al. 2004), and a decrease in the number of lymphocytes (L) in the blood (Davis et al. 2008). Therefore, the Heterophil to Lymphocyte (H/L) ratio gives a reliable and widely used indicator of long-term exposure to multiple stressors including starvation, disease, urbanization, temperature stress, noise, injuries, among others (Davis et al. 2008; Cirule et al. 2012; Norte et al. 2021). H/L ratio is known to increase in response to stress depending on the intensity and persistence of the stressor (Averbeck 1992), and persistently elevated H/L ratios may be associated with deleterious effects for birds such as slower growth, increased risk of infection and decreased survival (Davis et al. 2008).

Among the several energy homeostatic processes, the mitochondrial oxidative phosphorylation is the major metabolic pathway, regulated by the electron transport chain (Hatefi 1985). Oxidative phosphorylation represents the metabolic activity by which cells convert enzymes to oxidize nutrients, which eventually generates molecular oxygen to release ATP, through the use of 5 functional enzymatic complexes in the inner membrane of the mitochondria (Kühlbrandt 2015). Thus, mitochondrial bioenergetics play an important role in the maintenance of equilibrium. In this study, liver mitochondria of gulls fed with anthropogenic food presented decreased mitochondrial membrane potential and a depressed mitochondrial respiration state 3. According to Chance and Williams (1956), state 3 of the mitochondrial respiration corresponds to the oxygen consumption rate in the presence of the respiratory substrate (succinate) and ADP, coinciding with the maximum flow of electrons during ATP synthesis and translated in a rapid respiration rate, whereas state 4 represents to the oxygen consumption rate after the ADP consumption, coinciding with the respiratory rate necessary to counteract the passive leak of protons. FCCP was used as a mitochondrial oxidative phosphorylation uncoupler, inducing the maximal rate of oxygen consumption, but no significant differences were detected in mitochondrial respiration in the presence of FCCP between both dietary regimes. These results suggest alterations in the mitochondrial electron transport chain complexes, comparing to gulls fed with natural diet (Palmeira and Madeira 1997; Mieiro et al. 2014), and that urban gulls relying on anthropogenic food resources may have their mitochondrial function impaired, likely to compromise energy-dependent physiological processes. Anthropogenic diet, mainly constituted by processed food, may be responsible for mitochondrial damage, by interfering with oxidative phosphorylation, leading to impairment in energy production processes (Palmeira et al. 1994; Mieiro et al. 2014).

5.4.3. Management implications

For ethical reasons, this captivity feeding experiment was constituted by the smallest possible sample size. This allowed to understand some patterns but precluded definitive statements of the effect of anthropogenic food on the physiology of gulls. Therefore, further testing of more biochemical parameters (blood chemistry: total and HDL-cholesterol, triglycerides, glucose, calcium, sodium, potassium, corticosterone and other parameters), both in captive experiments and directly on free-living birds, using larger samples and additional urban and natural sites is important. It is also unknown if such damaging effects on health condition and physiology are reflected into gulls' reproductive measures, such as clutch size, egg volume and overall breeding success, and whether mid- and long-term gulls' fitness is affected by relying on

anthropogenic food resources. Clearly there is a need to understand the long-term effects of disrupted diets on wildlife (Birnie-Gauvin et al. 2017)

Nowadays, gulls' access to anthropogenic food sources is extremely facilitated, allowing them to take advantage of such resources. The overall reduction of accessibility and availability of food waste within urban areas and landfills is essential to reduce gulls' reliability on such human-derived food. Management measures, including efficiency in garbage disposal and collection by municipal services, and covering the waste at landfills, as well as social awareness and environmental education campaigns are necessary to help in the reduction of such problem.

Chapter 6

GENERAL DISCUSSION

Part of this chapter is published as part of a book chapter:

Lopes CS, Laranjeiro MI, Lavers JL, Finger A, Provencher JF (2022) Seabirds as indicators of metal and plastic pollution. In: Seabird Biodiversity and Human Activities. Ramos JA, Pereira L (eds). CRC Press, Boca Raton, Florida, USA.

6.1. Overview of the thesis

Urbanization poses many challenges to wildlife but some opportunistic animals, such as urban gulls, are able to take advantage of urban settlements for breeding and/or foraging and are capable to survive and thrive with success in cities.

With this thesis, I aimed to provide new information about the consequences of urbanization for gulls' physiology and health condition. Several questions were framed in the general introduction, to which I have intended to answer throughout the thesis. Firstly, in general introduction (Chapter 1), I characterized the novel urban habitats and compilated the effects of urbanization on animal's behaviour and life-history traits, including on natural biorhythms, movements, reproductive success, survival, diet, foraging behaviour, physiology and health condition. I also described anthropogenic debris materials as a result of the urbanization process and focused on how birds are exposed to such materials, the available monitoring tools and the consequences for birds of interacting with anthropogenic debris materials. Secondly, through the examination of nests, pellets and digestive tracts of Yellow-legged and Lesser black-backed gulls from natural, urban and landfill settlements, I aimed to qualify and quantify the interactions of gulls with anthropogenic debris materials, both through incorporation into nests (Chapter 2) and ingested food items (Chapter 3), and to understand the physiological consequences of ingesting such materials (Chapter 4). Finally, the last section aimed to compare gulls' diet quality among urban and natural foraging habitats (Chapter 4) and, ultimately, by setting up a captive feeding experiment, I intended to unravel the impacts of a typically anthropogenic diet on the gulls' physiology and health condition (Chapter 5).

From the findings of this work, I highlight the following:

(1) The reliability of gulls on anthropogenic food resources available due to urbanization and consequent interaction with anthropogenic debris materials may pose a serious threat to gulls' health, as such human-derived food may act as an ecological trap, with immediate benefits for gulls, but also with possible long-term consequences on gulls' physiology (Chapter 1);

(2) The extremely high diversity and quantity of anthropogenic materials incorporated in gull nests from urban locations, which may be a consequence of poor garbage management in urban locations (Chapter 2);

(3) The high levels of ingested anthropogenic materials in urban breeding locations and landfills, as well as the possibility of accidental ingestion of debris while foraging at multiple habitats, indicate a need for improved waste management (Chapter 3);

(4) The low-quality diet of gulls using urban habitats to forage, characterized by low percentages of physiologically important fatty acids, may indicate a diet-induced susceptibility to inflammation (Chapter 4);

(5) A diet based on anthropogenic food resources impairs gulls' fatty acids composition and alters haematological, stress and mitochondrial physiological parameters (Chapter 5).

The scientific outputs of this thesis were important to help understand the risks to which gulls are exposed when exploiting urban settlements, either physical, through incorporation into nests and ingestion of anthropogenic materials, and physiological, through relying on a nutritionally poorer diet, based on anthropogenic food resources. This final chapter intends to summarise the findings within this thesis and discuss their wider implications. Additionally, the challenges of this work and future research directions are also debated.

6.2. Summary of the main findings

Chapter 2: How is the incorporation of anthropogenic debris materials in nests characterized in natural and urban gulls' breeding sites? Is there any relation between the incorporation of debris materials and gulls' breeding success?

In Chapter 2, I collected, counted, characterized and compared the anthropogenic debris materials incorporated on Yellow-legged gull nests from two natural (Deserta and Berlenga Islands) and two urban breeding sites (Peniche and Porto) across their Portuguese breeding range and during two consecutive years (2018 and 2019). I reported detailed data on the frequency of occurrence, number of items, mass and size of all debris materials incorporated into all studied nests, following and adapting standardized methods. As expected, the percentage of nests containing anthropogenic materials in urban breeding locations was much higher when compared to natural sites (47.6% and 95.7% *vs.* 2.6% and 15.4%, respectively), possibly as a consequence of a lower access to natural nest construction materials (i.e. vegetation) and high availability of anthropogenic debris in urban areas. A higher number of items with greater mass but smaller size was found in gulls' nests from the largest and more populated urban breeding colony. Additionally, I compared the hatching success between natural and urban breeding habitats, for the same study areas and study years, and no differences were found. Here, hatching success must be regarded as a coarse evaluation of the possible effects of nest debris on nest incubation, as other variables such as adult condition and quality may also influence hatching success. This was the first study to quantify and characterize anthropogenic debris materials incorporated in urban gulls' nests, and the value of 95.7% of the nests with incorporated debris registered for the most urbanized breeding site (Porto) was the highest recorded so far. Yet, we have to consider that previous studies on such issue only included data for gulls nesting on natural breeding colonies.

Chapter 3: How is the ingestion of anthropogenic debris materials characterized for natural-, urban- and landfill-dwelling gulls? Is there any relation between the ingestion of such materials and gulls' diet?

In Chapter 3, I characterized the anthropogenic materials ingested by Yellow-legged gulls in natural, urban and landfill sites, through the analysis of their pellets. More specifically, gull pellets from breeding colonies (natural and urban) were analysed to assess possible seasonal changes among three seasons (pre-breeding, breeding and post-breeding), and pellets from resting sites (urban and landfill) were analysed to evaluate seasonal patterns among four seasons (spring, summer, autumn and winter), in the ingestion of such materials. I presented detailed data on the frequency of occurrence, number of items, mass and size of all debris materials ingested and regurgitated by gulls in breeding colonies and resting sites, following standardized methods. Gulls from the most urbanized breeding location exhibited higher levels of ingested materials during the entire breeding cycle, as a consequence of a wider availability of anthropogenic materials in urban areas. At resting sites, only small seasonal differences were detected in the number and mass of debris items ingested, possibly related with the age of gulls (i.e. immature individuals) specialized in the use of such resting sites during spring when the availability of other dietary items is lower. Also, the presence of anthropogenic materials was related with gulls' diet assessed with the analysis of the same pellets. The presence of certain debris categories in gull pellets was positively related to the presence of some prey items, suggesting that gulls may accidentally ingest debris while foraging at multiple habitats.

Chapter 4: Does gulls' diet quality differ between foraging habitats with different levels of urbanization? Are there any sub-lethal impacts of ingesting anthropogenic debris materials on gulls' physiology?

In Chapter 4, I compared the fatty acids (FA) composition of Yellow-legged and Lesser black-backed gulls from three wildlife rescue centres representative of areas with different levels of urbanization, to assess differences in gulls' diet quality between natural and urban foraging habitats. Gulls were necropsied and the FAs of their adipose tissue were identified and quantified using GC-MS. There were significant differences in gulls' FA composition between natural and urban foraging areas, mainly due to physiologically important FAs which had lower percentages in gulls from the most urbanized habitats, consistent with a diet based on anthropogenic food resources and indicator of a diet-induced susceptibility to inflammation. No differences were detected in FA composition between gull species, as both species are known to benefit from similar food resources within each level of urbanization. Similarly to chapter 3, I collected and characterized the anthropogenic materials ingested by gulls in each location, through the examination of gulls' entire digestive system during necropsies, and I presented data on the frequency of occurrence, number of items, mass and size of all ingested debris materials. The

possible physiological impacts of ingesting such materials on gulls' FA composition were investigated, but no sub-lethal effect was detected. This was probably due to gulls' regurgitation capabilities that allows them to maintain the levels of ingested materials below toxic levels, without causing impairment nor sub-lethal impacts on studied individuals. Moreover, these data constitute a valuable contribution to the limited FA literature in gulls, especially in an urbanization context.

Chapter 5: What are the effects of a typically anthropogenic diet on gulls' physiology and health condition?

In Chapter 5, a captive feeding experiment was set up to determine whether the consumption of a typically anthropogenic diet alters various physiological parameters of Yellow-legged and Lesser black-backed gulls. During the acclimatization period of 12 days, all gulls were fed with fish and, after that gulls were subjected to either a natural or an anthropogenic diet, under controlled conditions. Gulls from the "natural diet" group were fed with fish and those from the "anthropogenic diet" group were fed with a mix of processed food and remnants of meat, to recreate a diet from urban habitats. After 14 days subjected to each diet, haematological, protein, oxidative stress and mitochondrial parameters, as well as fatty acids (FA) composition were compared between gulls subjected to each diet. Also, some gull individuals were caught at a landfill and the same health and physiological parameters were evaluated and compared with those obtained from birds fed with controlled diets, during the feeding experiment. Captive feeding experiments are essential to control for the potential effects of confounding variables such as temperature, water availability and pollution. No significant differences in FAs and evaluated health parameters were detected after the 12-day acclimatization period, which suggests that this habituation period was successful in allowing similar conditions of all individuals prior to the feeding experiment. Significant differences were detected between individuals fed with the natural diet and gulls fed with anthropogenic diet. Gulls fed with processed food had increased percentages of ω-6 FAs and were depleted of physiologically important groups of FAs, such as highly unsaturated FAs and ω-3 FAs, which is consistent with the idea that anthropogenic food resources are typically of a lower nutritional quality. Gulls fed with the anthropogenic diet presented significantly higher concentrations of haemoglobin in their blood and significantly lower levels of reactive oxygen metabolites (ROMs), which may indicate habituation and resistance to stressful conditions. Mitochondrial parameters are indicative that gulls' bioenergetic function is much well preserved in gulls fed with natural diet than in gulls fed with processed food.

6.3. Scientific advances, implications and further directions

6.3.1. Interactions with anthropogenic debris materials

To date, several studies reported gulls' interactions with anthropogenic debris materials, particularly plastics, both through ingestion and incorporation into nests (review by Battisti et al. 2019a), however rarely in an urbanization context (Seif et al. 2018). This work was the first, to my best knowledge, to focus on those interactions by urban and landfill-dwelling gulls. Cities and landfills, as highly impacted environments, provide particularly high amounts and diversity of anthropogenic materials with which gulls may interact by mistake, when those materials resemble natural nesting material or a specific natural food item, or through accidental collection, while collecting nesting material or foraging, as discussed respectively in chapters 2 and 3.

The detailed characterization of debris materials ingested by gulls or incorporated into their nests is extremely useful to detect and compare possible patterns of these two aspects among species, habitats and seasons, to infer about the origin of nest-incorporated and ingested materials, and to allow a long-term monitoring program (Provencher et al. 2017). To enable inter-studies comparisons, however, it is crucial to standardize sample collection, processing, quantification and reporting. In fact, in an attempt to do so, I followed Provencher et al. (2017) methodology to analyse debris ingested by gulls. Unfortunately, as far as I am aware, such standardized protocol is not available to characterize and report the materials incorporated into birds' nests (O'Hanlon et al. 2017). Therefore, the same guidelines as those used for ingested materials (Provencher et al. 2017) were adapted and used to characterize and measure the nest-incorporated debris. Nowadays, there are many regional, national and international strategies to prevent and mitigate debris pollution, although reviews indicate that they often do not link directly to bird populations (Linnebjerg et al. 2021). Additionally, none has a level of commitment consistent with the global magnitude and rapid growth of the problem (Borrelle et al. 2017). With the exception of the OSPAR Convention that aim to achieve less than 10% of beached Northern Fulmars (*Fulmarus glacialis*) with less than 0.1 g of ingested plastics (van Franeker et al. 2011), international policies acknowledging the globality of debris pollution, remain, most of the time, too ambitious and lack in defined and concise targets to be achieved (Borrelle et al. 2020). Only the standardisation of methods and the establishment of long-term monitoring programs will allow researchers to measure the impacts of debris in wildlife, and to reliably assess the progress and success in achieving the targets recommended by the international strategies (Provencher et al. 2020). This will provide data on the true magnitude of debris pollution, enable meta-analysis and large-scale temporal, spatial and taxonomic comparisons of debris accumulation.

In Chapter 2, on the incorporation of debris materials into nests, the availability of such anthropogenic materials in the surrounding environments was not directly assessed, but it was assumed through the Corine Landcover, observations during fieldwork in each area and the amount of urban waste collected by the municipal services in urban areas. As gulls are known to collect their nest materials in the surrounding areas of their nest (Cramp and Simmons 1983), future research should also include the examination of debris availability in the nests' surroundings (Jagiello et al. 2018), to understand whether the incorporation of debris into nests is due to a lack of natural nesting material or due to a high availability of anthropogenic materials in the surrounding environment. This should help infer about the origin of such anthropogenic materials, possibly revealing hotspots of pollution and potential pollution sources (Jambeck et al. 2015), and consequently to inform suitable waste management strategies (Weiser and Powell 2011). As the presence of debris in gulls' nests may modify their structural features and, as a result, possibly alter incubating adults' behaviour and fitness (Deeming and Mainwaring 2015), it would be interesting to evaluate if there is a relation between the use of these materials in nest construction and gulls' fitness variables, such as breeding, hatching and fledgling success, in order to better understand the costs and benefits of the incorporation of anthropogenic materials into nests (Reynolds et al. 2019). The presence of plastics in gulls' nests may also lead to a higher exposure to some potentially harmful chemicals (Lithner et al. 2011, O'Hanlon et al. 2017). As suggested by Verlis et al. (2014), nesting on top of certain debris materials, in particularly plastics, could potentially lead to the absorption of contaminants through the skin of chicks and adults. This may interfere with birds' physiology, causing negative effects on reproduction, behaviour and survival (Herzke et al. 2016, O'Hanlon et al. 2017). Despite this postulation by Verlis et al. (2014), the authors did not directly assess this issue or provide any quantitative results, hence this dermal exposure route of contaminants requires further study.

The results of Chapter 3 revealed extremely high amounts of debris in gulls' pellets, especially those from urban breeding locations and landfill resting sites, however, chapter 4 results revealed much lower levels of ingested materials, as both chapters use different techniques. In fact, because gulls have the ability to regurgitate a large part of non-edible food remnants, including debris materials (Barrett et al. 2007), necropsies only allow for the detection of a smaller amount of debris in gulls' digestive systems (Basto et al. 2019), and the levels reported by chapter 3 are similar to other gull debris studies using the same technique (review by Seif et al. 2018). Gulls' regurgitation abilities may alleviate the impacts of larger and heavier materials on their digestive system, however the smaller items are known to remain in the gulls' digestive tract, and are likely to interfere with birds' physiology and body condition (Puskic et al. 2019). Future research should focus on the retention time of these materials in gulls' digestive system, to allow a better understanding on whether and how these debris items affect birds' health and physiology, especially in an urbanization context.

Quantitative data on the complex issues of how ingested anthropogenic materials may alter birds' physiology is extremely limited, and no clear relationships have emerged from such issue, as analysis are often based on small sample sizes (Ryan 1987, Carey 2011, Lavers et al. 2014, Puskic et al. 2019). Currently, there is a lack of evidence of the sub-lethal effects and physiological harm from ingested anthropogenic materials in birds, in part due to the commonly used metrics and approaches which are not designed to detect subtle changes in health condition (Rochman et al. 2016, Roman et al. 2019b). While there are some acute effects on birds from ingested debris materials (i.e. pierced gastrointestinal tract), most are likely to be subtle, and apparently "normal" free-living birds may uncover a range of impacts associated with ingested debris that were invisible to the naked eye (Lavers et al. 2019). It is essential the application of multi-disciplinary, physio-ecotoxicological approaches and to critically review the priority issues in order to enhance our understanding of the sub-lethal impacts of debris ingestion for bird species.

6.3.2. Physiology and health condition

Generally, the "health" of wild animals is not easy to define as it may be addressed through a wide variety of methods, including physiological, chemical and biochemical analysis. In this thesis, the selected methods are complementary and chosen based on recommendations of previous studies and time frames.

Urbanization effects have wide-reaching implications and the availability and accessibility to anthropogenic food resources is the main key factor that allows animal populations to survive and thrive in urbanized environments. Gulls show remarkable flexibility in behaviours and a significant capacity to deal physiologically with anthropogenic derived food, which may be important factors for their urban-dwelling adaptation. As opportunistic feeders, gulls can easily exploit a broad variety of food resources, including novel items (Ramírez et al. 2020). Also, as eager scavengers with different foraging strategies, gulls readily take advantage of supplementary food, and easily switch between different resources when specific feeding grounds or certain food resources are not available (Tyson et al. 2015, Zorrozua et al. 2020a), allowing to increase their foraging efficiency and energy intake. As one of the fundamental mechanisms needed to exploit novel environments, behavioural flexibility as the gulls' capacity to change behaviour (diet or habitat use) in response to alterations in the external and internal environment (Wright et al. 2010, van Toor et al. 2017) is extremely useful for adapting to an urban dwelling lifestyle. From my observations during the fieldwork of this thesis in urban environments and landfills, it seems that gulls' flexibility in habitat use and their capacity of using different food sources are helping them adapt to cities. As a result, while in most cases gull populations were reported to have increased

(Duhem et al. 2008), breeding success and individual health was diminished (Pierotti and Annett 1987). Beyond poor-nutrition, consuming human-derived food may cause other detrimental effects in gulls, such as increased risk of disease and a higher exposure to bacteria and other pathogens, due to gulls' habit of foraging on refuse (Ramos et al. 2010).

Although in chapter 4 I was not able to detect a relation between the ingestion of anthropogenic materials and gulls' physiology, assessed through FA composition, it was necessary to evaluate whether the food ingested by urban-dwelling gulls was physiologically different from that consumed by gulls from natural habitats. The evaluation and comparison of gulls' FA composition confirmed significant differences in the general dietary inputs for gulls among foraging habitats. Indeed, analysis of gulls' adipose tissue FA composition suggested that non-urbanized gulls tended to feed from more marine sources, while urban gulls' FA composition was indicative of a diet based on anthropogenic food resources. The determination of dietary differences between urban and natural gulls was supported by pellet analysis from approximately the same study areas (Calado et al. 2021, Pais de Faria et al. 2021a), which also highlighted that, despite the differences in gulls' FA composition among urbanization levels, some overlap existed in their diet composition. Pais de Faria et al. (2021a) analysed natural and urban gulls' diet composition and worked exactly on the same gull pellets as those from Chapter 3. The authors concluded that gulls from natural colonies ingested a significantly higher amount of marine prey, while gulls from urban colonies ingested a significantly higher amount of refuse items, but also had considerable amounts of marine prey and terrestrial items on their diet. Gulls from natural environments seem to have access to anthropogenic foods, as they have the ability to fly long distances, if necessary, as well as urban gulls still rely on marine resources throughout the year (Pais de Faria et al. 2021a), which is in accordance with the results from Chapters 3 and 4.

Gulls using urban habitats to forage lack some physiologically important FAs and have others with increased percentages (i.e. ω-6 FAs). This opened speculation about how these dietary differences could be manifested in individual birds and what were the possible results of a human-derived diet on gulls' physiology. Indeed, Chapter 5 explored the effects on an anthropogenic diet from a biochemical perspective, as measured by common health indicators found in blood, and whether these effects had a negative impact on the birds' health and physiology. The analysis of gulls submitted to the anthropogenic diet reported negative consequences for gulls' physiology, especially at a mitochondrial level. Liver mitochondria of gulls fed with anthropogenic food presented decreased mitochondrial membrane potential and a depressed mitochondrial respiration state 3, suggesting alterations in the mitochondrial electron transport chain complexes, comparing to gulls fed with natural diet (Palmeira and Madeira 1997, Mieiro et al. 2015). We have to bear in mind, however, the small sample sizes of the captivity feeding experiment that precluded definitive statements of the effect of anthropogenic food on the physiology of gulls. Further testing of more biochemical parameters (blood chemistry: total and HDL-cholesterol,

triglycerides, glucose, calcium, sodium, potassium, corticosterone and other parameters) in larger sample sizes, both in captive experiments and directly on free-living birds, and from additional urban and natural sites is recommended before any definitive statements can be made. Also, it remains to be tested whether such detrimental effects on health condition are visible in terms of reproductive parameters and therefore in mid- to long-term fitness of urban gulls.

6.4. Management considerations

This project was focused in specific natural, urban and landfill locations, known to be used by gulls to dwell year-round. Cities and towns can differ in size and the resources available, as well as landfills may have different features to reduce gulls' access to garbage (i.e. covering the waste or using gull control programs), and natural areas may have distinct availability of natural food resources. For instance, smaller cities, such as Peniche, may have fewer waste centres surrounding them and less food waste in the streets when comparing to larger cities, such as Porto. Hence, the results found in this study might not apply to other smaller or larger urban areas in and outside Portugal.

Nevertheless, the amount and variety of ingested anthropogenic materials by gulls, especially in urban and landfill sites, as well as the gulls' reliability on anthropogenic food resources, are a motive of concern and could result in chronic exposure to debris and to the negative physiological effects of a human-derived diet. This reveals a poor waste management in urban areas and landfills which allow opportunistic gulls to have access to debris and anthropogenic food. As gulls use a wide variety of terrestrial habitats, within and outside urban areas, focusing on reducing the accessibility of only one or two locations might not suffice. Gulls are known to switch to other food resources when specific foraging locations have been closed (Zorrozua et al. 2020a), thus the overall reduction of food waste and debris pollution within urban areas (streets, gardens, schools) and landfills is needed. To accomplish this, management measures including the improvement of the efficiency in the disposal of garbage by using closed containers and the increase in the number of times that garbage is collected by municipal services are necessary to contribute to reduce the amount of available anthropogenic materials and food resources. Educating the public about separating food waste from their recyclable waste, as well as prevent feeding individual gulls in the street and properly discarding their food waste may also be helpful in reducing debris pollution in urban areas and human-derived food availability.

Waste production is an acute problem which will presumably worsen in the coming years, with significant impacts for wildlife. It is important to weight positive and negative consequences of landfills for wildlife to adequately establish conservation and waste management practices (Plaza and Lambertucci 2017). The European Union Landfill Directive (European Commission 2016) aims to progressively reduce the volume of biodegradable waste entering landfills by

replacing open-air landfill by covered waste facilities, much less accessible to birds (Gilbert et al. 2016). The successful implementation of this measure, in the future, will be responsible for a sharp reduction in food waste availability, with predicted consequences for animals which highly depend on this foraging source.

6.5. Concluding remarks

The present study provides important findings on the interactions of urban-, natural- and landfill-dwelling gulls with anthropogenic materials, both through incorporation into nests and ingestion. It allows a better understanding of the physiological consequences of ingesting such materials and expands our knowledge on the impacts of a typically human-derived diet on gulls' physiology and health condition. With the increasing urbanization and the reducing quality and availability of natural areas, the number of animals dwelling in urban settlements will probably increase as well. Through a combination of proper garbage management measures and environmental education of the public, it is possible to reduce the availability of anthropogenic debris materials and human-derived food in urban areas and landfills. These measures, however, can only be successful with a comprehensive understanding of the ecology, behaviour, demographics and physiology of animals that breed and feed in urban areas.

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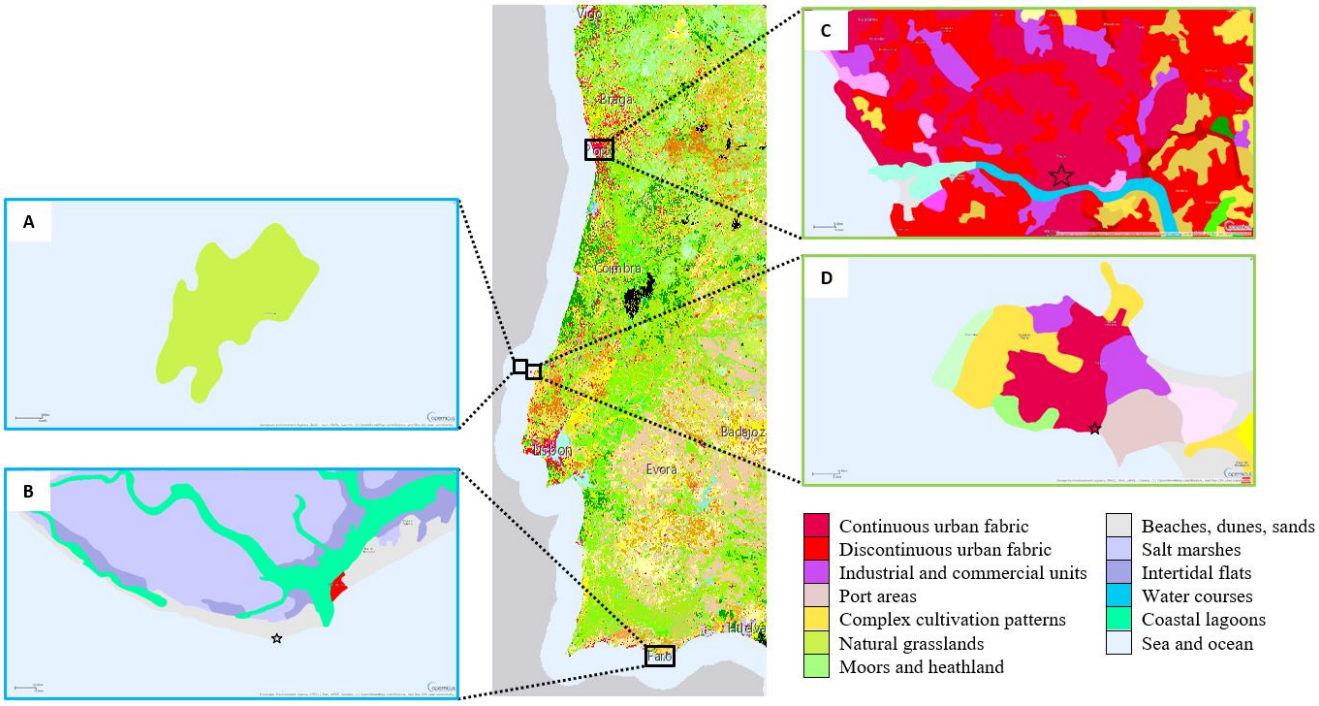


Figure S2.1. Characterization of the habitats surrounding each studied Yellow-legged gull (*Larus michahellis*) breeding colony according to Corine Landcover data. Berlenga Island (A) and Deserta Island (B) natural breeding colonies outlined in blue, on the left. Porto (C) and Peniche (D) urban breeding colonies outlined in green, on the right. Black stars indicate the exact location of the colony in each image, with exception of Berlenga Island (B) where gulls nest throughout the entire island. More detailed information about the colours can be accessed at the website: https://land.copernicus.eu/pan-european/corine-land-cover/clc2018.

Table S2.1. Comparison of negative binomial zero inflated models explaining the number of items per nest for each material category in Yellow-legged gull (*Larus michahellis*) nests, using and excluding the interaction Location * Year in the models, based on AIC and Log-likelihood. **A**) Considering all studied breeding locations (Deserta Island, Berlenga Island, Peniche and Porto) and the two studied years (2018 and 2019). **B**) Considering urban locations (Peniche and Porto) and the two studied years (2018 and 2019). Dashes indicate debris categories that did not occur in gull nests or were not enough to include the interaction in the model.

Material Category	**All Locations - A**				**Urban Locations - B**			
	With Interaction		Without Interaction		With Interaction		Without Interaction	
	AIC	Log-likelihood	AIC	Log-likelihood	AIC	Log-likelihood	AIC	Log-likelihood
All Debris	747.79	-356.9	737.75	-357.9	639.68	-310.8	636.45	-311.2
Glass	--	--	--	--	--	--	87.632	-36.82
Fabric	--	--	--	--	394.06	-188	390.80	-188.4
Metal	--	--	--	--	--	--	175.97	-80.98
Paper	--	--	--	--	209.61	-95.81	205.61	-95.81
All Plastics	650.98	-308.5	642.40	-310.2	576.08	-279	575.36	-280.7
Sheet plastics	--	--	--	--	332.15	-157.1	331.96	-159
Threadlike plastics	381.40	-173.7	370.07	-174	329.59	-155.8	325.98	-156
Fragment plastics	--	--	--	--	--	--	248.70	-117.4
Foamed plastics	--	--	--	--	369.25	-175.6	373.78	-179.9
Other	--	--	278.73	-128.4	250.68	-116.3	249.71	-117.9

Table S2.2. Percentage of occurrence (%) of each anthropogenic material category present in 204 Yellow-legged gull (*Larus michahellis*) nests on 4 different locations across the Portuguese breeding range (Deserta Island, Berlenga Island, Peniche and Porto) and during 2018 and 2019.

Year	Location	Glass (%)	Fabric (%)	Metal (%)	Paper (%)	Other (%)	Plastic (%)	Sheet plastics (%)	Threadlike plastics (%)	Fragment plastics (%)	Foamed plastics (%)
2018	Deserta	0	0	0	0	0	100	0	100	0	0
	Berlenga	0	0	20	20	0	60	75	25	0	0
	Peniche	4.76	19.06	9.52	9.52	9.52	47.62	25	40	15	20
	Porto	6.25	23.75	12.5	11.25	18.75	27.5	28.57	22.86	22.86	25.71
2019	Deserta	0	0	0	0	50	50	0	100	0	0
	Berlenga	0	33.32	16.67	16.67	16.67	16.67	50	50	0	0
	Peniche	0	31.58	0	15.79	5.26	47.37	50	33.33	0	16.67
	Porto	7.14	25	16.07	10.71	14.29	26.79	21.95	26.83	24.39	26.83
Overall Year	2018	5.61	21.5	12.15	11.21	15.89	33.64	29.47	27.37	20	23.16
	2019	4.82	26.50	12.05	12.05	13.25	31.33	28.57	30.36	17.86	23.21
Overall Location	Deserta	0	0	0	0	33.33	66.67	0	100	0	0
	Berlenga	0	18.18	18.18	18.18	9.1	36.36	66.67	33.33	0	0
	Peniche	2.5	25	5	12.5	7.5	47.5	34.38	37.49	9.38	18.75
	Porto	6.62	24.26	13.97	11.03	16.91	27.21	26.13	24.32	23.42	26.13
TOTAL	ALL	5.26	23.68	12.11	11.58	14.74	32.63	29.14	28.47	19.21	23.18

Table S2.3. Percentage of number of items (%) of each anthropogenic material category present in 204 Yellow-legged gull (*Larus michahellis*) nests on 4 different locations across the Portuguese breeding range (Deserta Island, Berlenga Island, Peniche and Porto) and during 2018 and 2019.

Year	Location	Glass (%)	Fabric (%)	Metal (%)	Paper (%)	Other (%)	Plastic (%)	Sheet plastics (%)	Threadlike plastics (%)	Fragment plastics (%)	Foamed plastics (%)
2018	Deserta	0	0	0	0	0	100	0	100	0	0
	Berlenga	0	0	11.11	11.11	0	77.78	85.71	14.29	0	0
	Peniche	0.93	9.35	8.41	2.8	14.02	64.49	24.64	57.96	8.7	8.7
	Porto	0.28	16.15	1.57	2.97	6.11	72.92	6.83	11.18	13.61	68.38
2019	Deserta	0	0	0	0	50	50	0	100	0	0
	Berlenga	0	33.34	11.11	22.22	11.11	22.22	50	50	0	0
	Peniche	0	7.91	0	4.32	0.72	87.05	40.5	14.88	0	44.62
	Porto	0.94	30.04	3.21	7.04	7.67	51.1	18.99	9.49	4.9	66.62
Overall Year	2018	0.31	15.81	1.88	2.99	6.41	72.60	7.77	12.95	13.37	65.91
	2019	0.84	27.87	2.94	6.86	7.08	54.41	22.39	10.55	4.13	62.93
Overall Location	Deserta	0	0	0	0	33.33	66.67	0	100	0	0
	Berlenga	0	16.67	11.11	16.67	5.55	50	77.78	22.22	0	0
	Peniche	0.41	8.53	3.66	3.66	6.5	77.24	34.73	30.53	3.16	31.58
	Porto	0.5	20.87	2.12	4.35	6.64	65.52	10.05	10.74	11.3	67.91
TOTAL	ALL	0.50	20.08	2.26	4.36	6.64	66.16	12.03	12.25	10.68	65.04

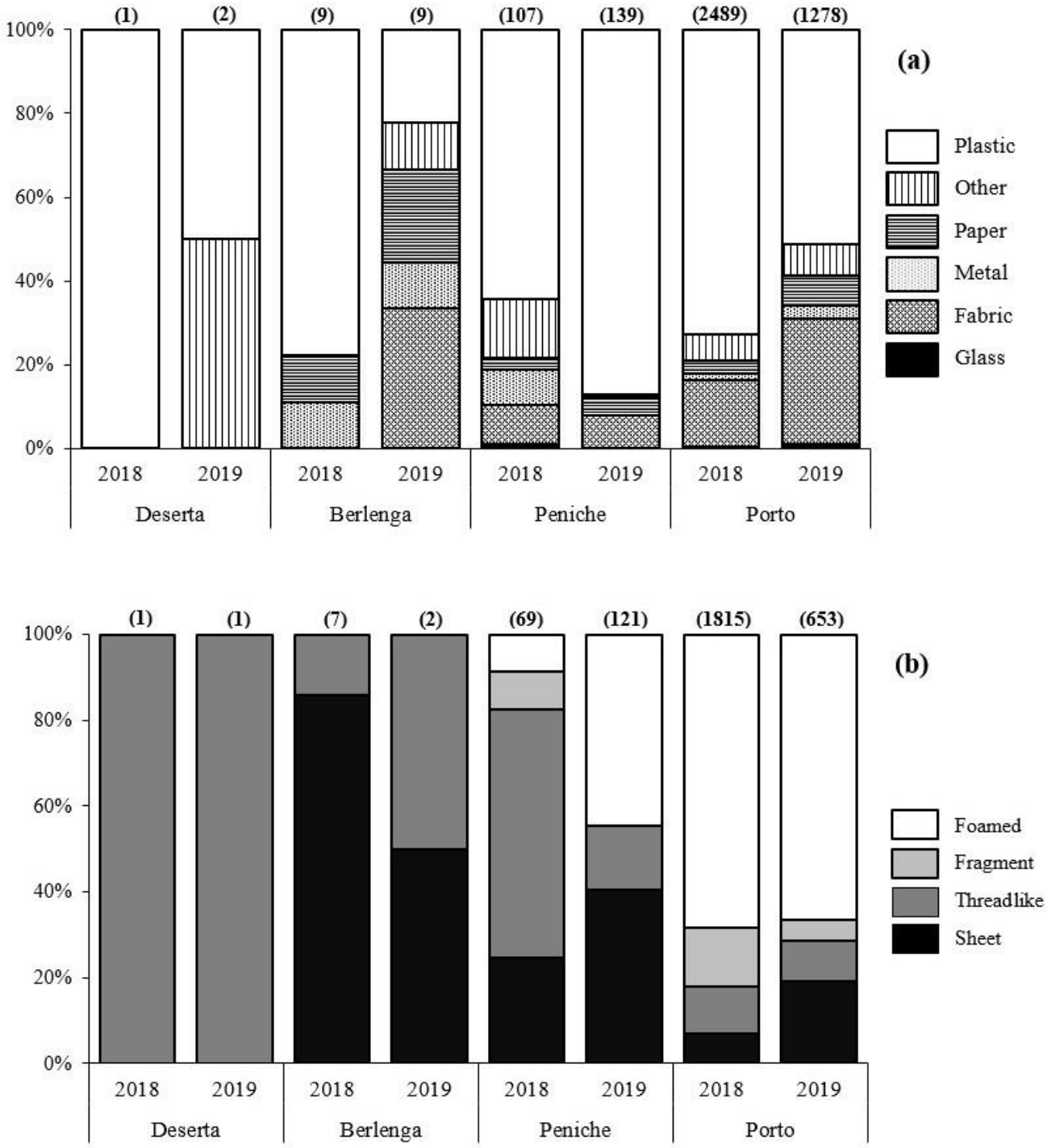


Figure S2.2. Number of anthropogenic materials (items per nest) in 204 Yellow-legged gull (*Larus michahellis*) nests on 4 different locations across the Portuguese breeding range (Deserta Island, Berlenga Island, Peniche and Porto) and during 2018 and 2019. **a)** For each category of debris (Glass, Fabric, Metal, Paper, Other and Plastic); the number of analysed debris items in each Location and Year is presented in the top of each bar; **b)** Plastic categories (Sheet, Threadlike, Fragment and Foamed); the number of analysed plastic items in each Location and Year is presented on the top of each bar.

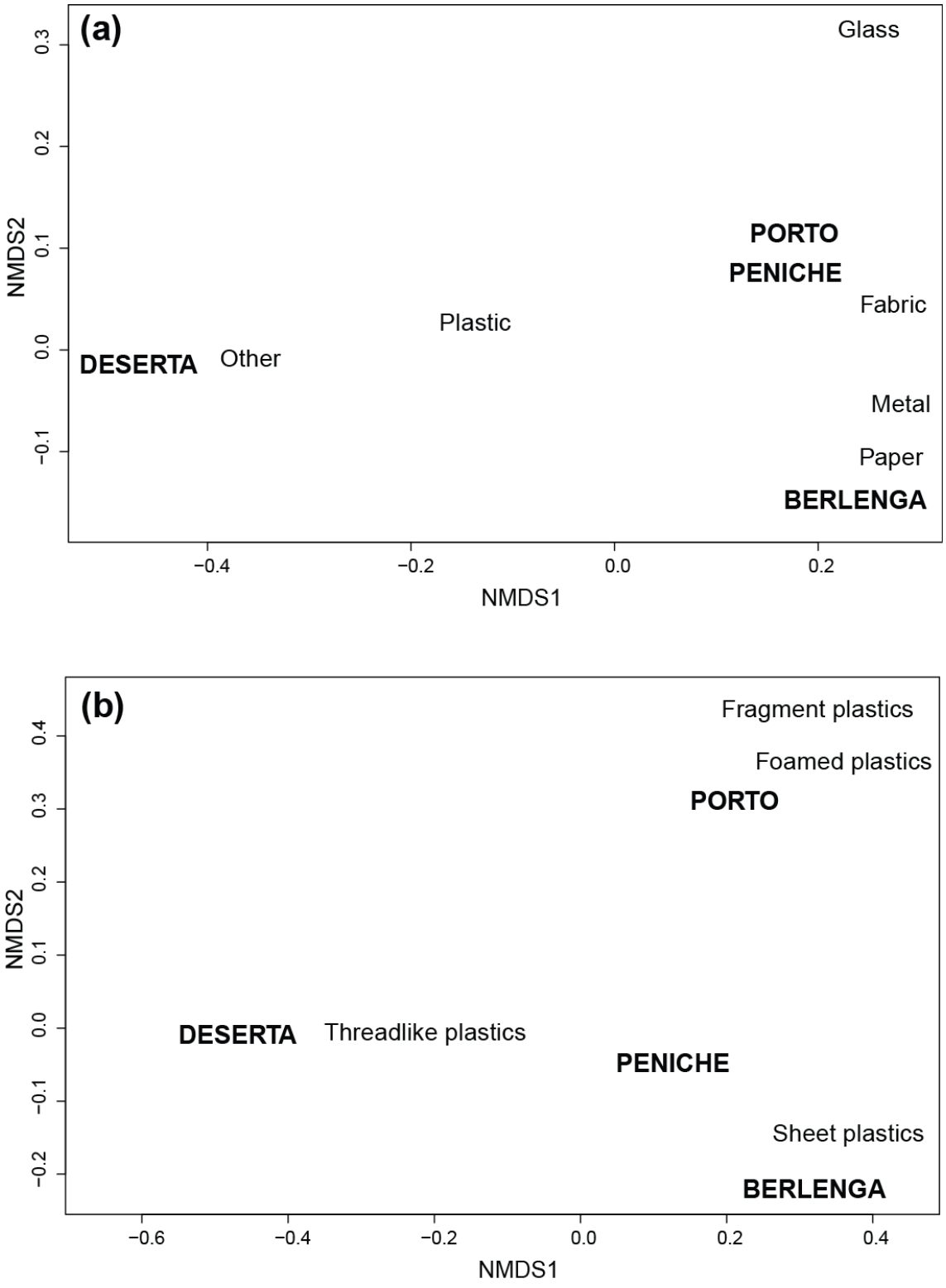


Figure S2.3. Non-metric multidimensional scaling (NMDS) using the number of items per nest of the main categories of debris materials (Glass, Fabric, Metal, Paper, Other and Plastic, **a**) and the number of items for the 4 types of plastic (Sheet, Threadlike, Fragment and Foamed, **b**) in Yellow-legged gull (*Larus michahellis*) nests from Deserta Island, Berlenga Island, Peniche and Porto, during the years of 2018 and 2019.

Table S2.4. Masses and sizes (mean per nest and range) of anthropogenic materials items present in 204 Yellow-legged gull (*Larus michahellis*) nests on 4 different locations across the Portuguese breeding range (Deserta Island, Berlenga Island, Peniche and Porto) and during 2 years (2018 and 2019). No. Nests = number of nests in which the correspondent debris category is present. N = number of measured / weighted items. SD = Standard Deviation. NA = Not Applicable.

		2018				2019				OVERALL
		Deserta	Berlenga	Peniche	Porto	Deserta	Berlenga	Peniche	Porto	
ALL DEBRIS	No. Nests	1	4	10	22	1	4	10	15	67
	N	1	9	107	2489	2	9	139	1278	4034
	Mean Size (cm) ± SD	25 ± NA	4.66 ± 5.72	10.88 ± 22.22	7.85 ± 13.43	21.3 ±18.81	24.66 ± 21.88	7.42 ± 15.27	4.70 ± 7.8	6.72 ± 12.21
	Range Size (min. – max.)	25 – 25	0.4 – 18.5	0.4 – 168	0.2 – 210	8 – 34.6	2 – 59.6	0.35 – 120	0.2 – 126	0.2 – 210
	Mean Mass (g) ± SD	0.2 ± NA	0.28 ± 0.24	5.5 ± 10.89	42.67 ± 98.28	1.2 ± NA	1.38 ± 1.32	2.44 ± 2.77	14.03 ± 19	18.46 ± 58.98
	Range Mass (min. – max.)	0.2 – 0.2	0.1 – 0.6	0.1 – 35.8	0.1 – 468.5	1.2 – 1.2	0.4 – 3.2	0.1 – 9.3	0.6 – 67.1	0.1 – 468.5
GLASS	No. Nests	0	0	1	5	0	0	0	4	10
	N	0	0	1	7	0	0	0	12	20
	Mean Size (cm) ± SD	–	–	2.2 ± NA	1.55 ± 0.90	–	–	–	1.05 ± 0.4	1.28 ± 0.67
	Range Size (min. – max.)	–	–	2.2 – 2.2	0.4 – 2.6	–	–	–	0.45 – 1.85	0.4 – 2.6
	Mean Mass (g) ± SD	–	–	0.7 ± NA	0.88 ± 1.26	–	–	–	0.53 ± 0.31	0.72 ± 0.88
	Range Mass (min. – max.)	–	–	0.7 – 0.7	0.1 – 3.1	–	–	–	0.1 – 0.8	0.1 – 3.1
FABRIC	No. Nests	0	0	4	19	0	2	6	14	45
	N	0	0	10	402	0	3	11	384	810
	Mean Size (cm) ± SD	–	–	24.75 ± 10.2	13.13 ± 11.59	–	29.5 ± 26.1	29.31 ± 32.96	7.73 ± 6.92	11 ± 11
	Range Size (min. – max.)	–	–	8 – 39	0.5 – 102	–	9.4 – 59	1.4 – 120	0.8 – 56.4	0.5 – 120
	Mean Mass (g) ± SD	–	–	1.35 ± 1.43	30.39 ± 103.41	–	1.65 ± 2.19	1.8 ± 2.4	7.8 ± 12.27	15.69 ± 67.73
	Range Mass (min. – max.)	–	–	0.1 – 3	0.1 – 454.7	–	0.1–3.2	0.1 – 6.5	0.1 – 44.2	0.1 – 454.7

Table S2.4. *cont.*

		2018				2019				OVERALL
		Deserta	Berlenga	Peniche	Porto	Deserta	Berlenga	Peniche	Porto	
METAL	No. Nests	0	1	2	10	0	1	0	9	23
	N	0	1	9	39	0	1	0	41	91
	Mean Size (cm) ± SD	–	8.8 ± NA	5.54 ± 4.38	6.16 ± 5.76	–	2 ± NA	–	3.55 ± 3	4.9 ± 4.64
	Range Size (min. – max.)	–	8.8 – 8.8	0.9 – 14.6	0.5 – 25.5	–	2 – 2	–	0.45 ± 14.35	0.45 – 25.5
	Mean Mass (g) ± SD	–	0.3 ± NA	1.2 ± 1.27	7 ± 10.84	–	0.2 ± NA	–	1.17 ± 1.2	3.63 ± 7.61
	Range Mass (min. – max.)	–	0.3 – 0.3	0.3 – 2.1	0.1 – 36.1	–	0.2 – 0.2	–	0.1 – 3.4	0.1 – 36.1
PAPER	No. Nests	0	1	2	9	0	1	3	6	22
	N	0	1	3	74	0	2	6	90	176
	Mean Size (cm) ± SD	–	2.7	2.63 ± 2.49	5.03 ± 4.49	–	28.7 ± 0.14	9.01 ± 7.26	4.29 ± 2.78	5 ± 4.61
	Range Size (min. – max.)	–	2.7 – 2.7	1.1 – 5.5	0.9 – 22.8	–	28.6 – 28.8	1.1 – 20.6	1.05 – 13.9	0.9 – 28.8
	Mean Mass (g) ± SD	–	0.1 ± NA	0.1 ± 0	2.2 ± 2.85	–	1.5 ± NA	1 ± 0.46	3.38 ± 4.49	2.04 ± 3.02
	Range Mass (min. – max.)	–	0.1 – 0.1	0.1 – 0.1	0.1 – 8.6	–	1.5 – 1.5	0.5 – 1.4	0.1 – 12.2	0.1 – 12.2
OTHER	No. Nests	0	0	2	15	1	1	1	8	28
	N	0	0	15	152	1	1	1	98	268
	Mean Size (cm) ± SD	–	–	4.77 ± 2.32	4.76 ± 10.7	8 ± NA	10.4 ± NA	7.15 ± NA	3.31 ± 2.17	4.27 ± 8.21
	Range Size (min. – max.)	–	–	1.6 – 8.7	0.3 – 93	8 – 8	10.4 – 10.4	7.15 – 7.15	1 – 14.4	0.3 – 93
	Mean Mass (g) ± SD	–	–	15.55 ± 21.28	8.24 ± 9.44	1 ± NA	0.4 ± NA	1.5 ± NA	3.25 ± 5.69	6.56 ± 9.26
	Range Mass (min. – max.)	–	–	0.5 – 30.6	0.1 – 36.3	1 – 1	0.4 – 0.4	1.5 – 1.5	0.4 – 17.2	0.1 – 36.3
ALL PLASTICS	No. Nests	1	3	10	22	1	1	9	15	62
	N	1	7	69	1815	1	2	121	653	2669
	Mean Size (cm) ± SD	25 ± NA	4.34 ± 6.33	11.38 ± 26.61	6.46 ± 14.62	34.6 ± NA	31.8 ± 39.32	5.35 ± 11.27	3.33 ± 8.99	5.49 ± 13.53
	Range Size (min. – max.)	25 – 25	0.4 – 18.5	0.4 – 168	0.2 – 210	34.6 – 34.6	4 – 59.6	0.35 – 64	0.2 – 126	0.2 – 210
	Mean Mass (g) ± SD	0.2 ± NA	0.27 ± 0.29	1.56 ± 1.85	6.55 ± 7.7	0.2 ± NA	0.2 ± NA	1.04 ± 1.55	2.86 ± 4.65	3.44 ± 5.68
	Range Mass (min. – max.)	0.2 – 0.2	0.1 – 0.6	0.1 – 5.5	0.1 – 23.8	0.2 – 0.2	0.2 – 0.2	0.1 – 4.7	0.1 – 18.3	0.1 – 23.8

Table S2.4. *cont.*

		2018				2019				OVERALL
		Deserta	Berlenga	Peniche	Porto	Deserta	Berlenga	Peniche	Porto	
SHEET PLASTICS	No. Nests	0	3	5	20	0	1	6	9	44
	N	0	6	17	124	0	1	49	124	321
	Mean Size (cm) ± SD	–	4.57 ± 6.9	8.65 ± 7.61	6.23 ± 7.2	–	4 ± NA	2.42 ± 2.74	5.03 ± 6.03	5.28 ± 6.39
	Range Size (min. – max.)	–	0.4 – 18.5	0.9 – 29	0.2 – 55	–	4 – 4	0.35 – 13.9	0.25 – 50	0.2 – 55
	Mean Mass (g) ± SD	–	0.27 ± 0.29	1.56 ± 2	0.8 ± 1.17	–	0.1 ± NA	0.37 ± 0.56	0.69 ± 1.35	0.75 ± 1.21
	Range Mass (min. – max.)	–	0.1 – 0.6	0.1 – 3.9	0.1 – 4.6	–	0.1 – 0.1	0.1 – 1.5	0.1 – 4.2	0.1 – 4.6
THREADLIKE PLASTICS	No. Nests	1	1	8	16	1	1	4	11	43
	N	1	1	40	203	1	1	18	62	327
	Mean Size (cm) ± SD	25 ± NA	3 ± NA	14.58 ± 34.3	15.5 ± 27.51	34.6 ± NA	59.6 ± NA	26.52 ± 17.61	14.28 ± 23.89	15.94 ± 27.34
	Range Size (min. – max.)	25 – 25	3 – 3	0.4 – 168	0.6 – 210	34.6 – 34.6	59.6 – 59.6	4 – 64	0.6 – 126	0.4 – 210
	Mean Mass (g) ± SD	0.2 ± NA	0.1 ± NA	0.64 ± 0.77	1.58 ± 2.89	0.2 ± NA	0.2 ± NA	0.65 ± 0.42	2.17 ± 5.32	1.34 ± 3.21
	Range Mass (min. – max.)	0.2 – 0.2	0.1 – 0.1	0.1 – 1.9	0.1 – 11.2	0.2 – 0.2	0.2 – 0.2	0.1 – 1.1	0.1 – 18.1	0.1 – 18.1
FRAGMENT PLASTICS	No. Nests	0	0	3	16	0	0	0	10	29
	N	0	0	6	247	0	0	0	32	285
	Mean Size (cm) ± SD	–	–	6 ± 5.34	7.75 ± 8.57	–	–	–	8.48 ± 8.12	7.79 ± 8.46
	Range Size (min. – max.)	–	–	0.9 – 15.1	0.4 – 39.1	–	–	–	0.5 – 24.8	0.4 – 39.1
	Mean Mass (g) ± SD	–	–	0.93 ± 1.27	6.21 ± 6.42	–	–	–	0.97 ± 1.12	3.86 ± 5.45
	Range Mass (min. – max.)	–	–	0.1 – 2.4	0.3 – 23	–	–	–	0.1 – 3.1	0.1 – 23
FOAMED PLASTICS	No. Nests	0	0	4	18	0	0	2	11	35
	N	0	0	6	1241	0	0	54	435	1736
	Mean Size (cm) ± SD	–	–	3.13 ± 3.83	1.04 ± 0.59	–	–	0.96 ± 1.32	0.9 ± 0.49	0.98 ± 0.71
	Range Size (min. – max.)	–	–	0.4 – 10.4	0.25 – 4.8	–	–	0.35 – 10.3	0.2 – 4.6	0.2 – 10.4
	Mean Mass (g) ± SD	–	–	0.18 ± 0.15	0.31 ± 0.32	–	–	2.4 ± 3.25	0.36 ± 0.44	0.43 ± 0.82
	Range Mass (min. – max.)	–	–	0.1 – 0.4	0.1 – 1.1	–	–	0.1 – 4.7	0.1 – 1.6	0.1 – 4.7

Table S2.5. Generalized Linear Models (GLM) testing the effect of the year (2018 and 2019), urban location (Peniche, Porto) and their interaction in the mass (g) and size (cm) of anthropogenic materials in Yellow-legged (*Larus michahellis*) gull nests. Data on masses of "ALL DEBRIS" and "ALL PLASTICS" were log transformed, "Sheet plastics", "Threadlike plastics" and "Foamed plastics" categories were sin transformed to attain normality. Data on sizes of "ALL DEBRIS" was square root transformed, "ALL PLASTICS" was log transformed, "Sheet plastics" and "Foamed plastics" were log transformed and "Threadlike plastics" was $\log_{10}$ transformed to attain normality. Significant effects are highlighted in bold.

		Location			***Year***		***Location * Year***		
	Debris categories	***F***	**p**	**Main Effect**	***F***	**p**	***F***	**p**	**Main Effect**
Mass (g)	ALL DEBRIS	$F_{1, 53} = 16.525$	**0.0002**	Porto > Peniche	$F_{1,53} = 1.17$	0.285	$F_{1,53} = 0.325$	0.571	
	ALL PLASTICS	$F_{1,52} = 9.337$	**0.0035**	Porto > Peniche	$F_{1,52} = 3.549$	0.065	$F_{1,52} = 0.148$	0.702	
	Sheet plastics	$F_{1,36} = 1.061$	0.31		$F_{1,36} = 0.002$	0.965	$F_{1,36} = 4.074$	0.051	
	Threadlike plastics	$F_{1,35} = 3.172$	0.084		$F_{1,35} = 1.674$	0.204	$F_{1,35} = 0.037$	0.849	
	Foamed plastics	$F_{1,31} = 6.11$	**0.019**	Porto > Peniche	$F_{1,31} = 0.716$	0.404	$F_{1,31} = 5.199$	**0.03**	Porto, 2018 > Others
Size (cm)	ALL DEBRIS	$F_{1, 53} = 9.16$	**0.004**	Peniche > Porto	$F_{1, 53} = 1.09$	0.30	$F_{1, 53} = 0.52$	0.47	
	ALL PLASTICS	$F_{1,52} = 2.959$	0.091		$F_{1,52} = 0.279$	0.6	$F_{1,52} = 0.756$	0.389	
	Sheet plastics	$F_{1,36} = 2.798$	0.103		$F_{1,36} = 1.863$	0.181	$F_{1,36} = 2.727$	0.107	
	Threadlike plastics	$F_{1,35} = 0.462$	0.501		$F_{1,35} = 1.943$	0.172	$F_{1,35} = 0.213$	0.647	
	Foamed plastics	$F_{1,31} = 7.455$	**0.01**	Porto > Peniche	$F_{1,31} = 1.679$	0.205	$F_{1,31} = 0.034$	0.854	

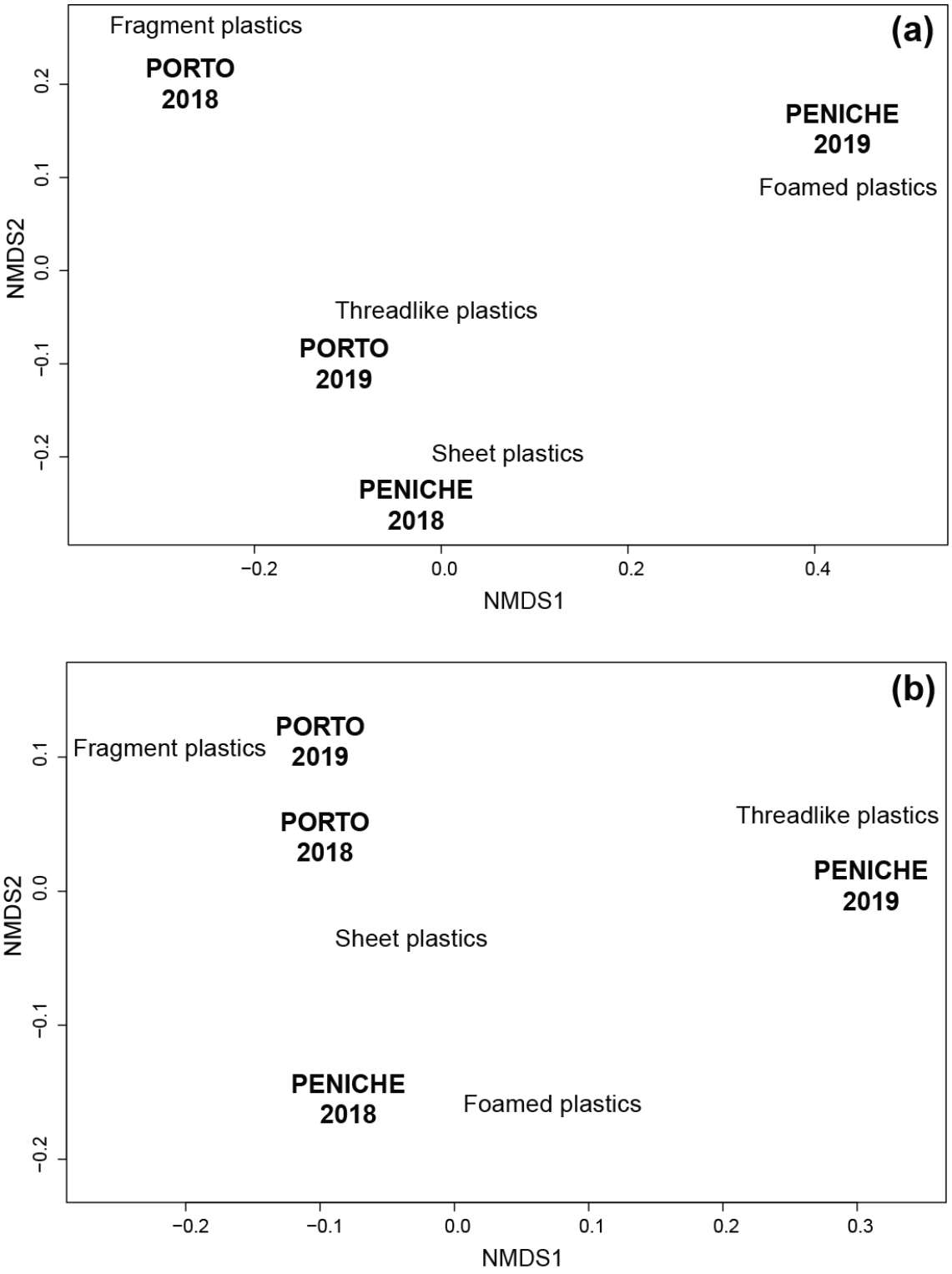


Figure S2.4. Non-metric multidimensional scaling (NMDS) using the mean mass (**a**) and mean size (**b**) of the four types of plastic (Sheet, Threadlike, Fragment and Foamed) in Yellow-legged gull (*Larus michahellis*) nests from urban sites (Peniche and Porto), during the years of 2018 and 2019.

Table S2.6. Percentage of the colours of anthropogenic materials items present in 204 Yellow-legged gull (*Larus michahellis*) nests on 4 different locations across the Portuguese breeding range (Deserta Island, Berlenga Island, Peniche and Porto) and during 2 years (2018 and 2019). Deserta Island nests: *n* = 30 in 2018 and *n* = 39 in 2019; Berlenga Island nests: *n* = 30 in 2018 and *n* = 26 in 2019; Peniche nests: *n* = 19 in 2018 and *n* = 21 in 2019; Porto nests: *n* = 23 in 2018 and *n* = 16 in 2019. NA = Not applicable. >1 = More than one colour.

	Year	Location	No. of items	White / Clear (%)	Yellow (%)	Green (%)	Blue / Purple (%)	Red / Pink (%)	Brown / Orange (%)	Grey / Silver (%)	Black (%)	>1 (%)
ALL DEBRIS	2018	Deserta	1	0	0	0	100	0	0	0	0	0
		Berlenga	9	11.11	0	0	66.67	0	0	11.11	0	11.11
		Peniche	107	31.79	2.8	13.08	13.08	7.48	14.95	3.74	11.21	1.87
		Porto	2489	49.46	3.9	4.1	16.75	0.84	10.57	7.27	6.51	0.60
	2019	Deserta	2	0	0	50	0	0	0	0	0	50
		Berlenga	9	55.56	0	0	0	0	22.22	11.11	0	11.11
		Peniche	139	50.35	0.72	5.76	0.72	0	5.04	6.47	30.94	0
		Porto	1278	19.01	2.5	3.29	35.68	1.25	15.34	16.98	3.6	2.35
GLASS	2018	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Peniche	1	100	0	0	0	0	0	0	0	0
		Porto	7	57.14	14.29	28.57	0	0	0	0	0	0
	2019	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Peniche	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Porto	12	8.33	0	8.33	0	0	75.01	8.33	0	0
FABRIC	2018	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Peniche	10	0	0	0	0	70	0	30	0	0
		Porto	402	10.44	16.92	0.75	4.48	3.23	18.66	35.57	9.95	0
	2019	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	3	33.33	0	0	0	0	66.67	0	0	0
		Peniche	11	27.27	0	18.18	0	0	27.27	9.10	18.18	0
		Porto	384	6.78	1.56	2.08	10.68	2.34	36.98	36.98	2.08	0.52

Table S2.6. *cont.*

	Year	Location	No. of items	White / Clear (%)	Yellow (%)	Green (%)	Blue / Purple (%)	Red / Pink (%)	Brown / Orange (%)	Grey / Silver (%)	Black (%)	>1 (%)
METAL	2018	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	1	0	0	0	0	0	0	100	0	0
		Peniche	9	0	0	0	88.89	0	0	11.11	0	0
		Porto	39	0	7.70	2.56	5.13	2.56	25.65	51.28	2.56	2.56
	2019	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	1	0	0	0	0	0	0	100	0	0
		Peniche	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Porto	41	0	0	0	2.44	0	7.32	46.34	0	43.90
PAPER	2018	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	1	0	0	0	0	0	0	0	0	100
		Peniche	3	0	100	0	0	0	0	0	0	0
		Porto	74	33.79	12.16	0	1.35	0	51.35	1.35	0	0
	2019	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	2	100	0	0	0	0	0	0	0	0
		Peniche	6	50	0	0	0	0	33.33	16.67	0	0
		Porto	90	70	17.78	4.44	0	1.11	1.11	3.34	0	2.22
OTHER	2018	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Peniche	15	0	0	0	0	0	100	0	0	0
		Porto	152	4.61	0	0	0	0	73.02	6.58	13.16	2.63
	2019	Deserta	1	0	0	0	0	0	0	0	0	100
		Berlenga	1	0	0	0	0	0	0	0	0	100
		Peniche	1	0	0	0	0	0	100	0	0	0
		Porto	98	0	0	0	0	0	36.73	41.84	21.43	0

Table S2.6. *cont.*

	Year	Location	No. of items	White / Clear (%)	Yellow (%)	Green (%)	Blue / Purple (%)	Red / Pink (%)	Brown / Orange (%)	Grey / Silver (%)	Black (%)	>1 (%)
ALL PLASTIC	2018	Deserta	1	0	0	0	100	0	0	0	0	0
		Berlenga	7	14.29	0	0	85.71	0	0	0	0	0
		Peniche	69	47.83	0	20.29	8.7	1.45	1.45	0	17.38	2.9
		Porto	1815	63.53	0.88	5.29	21.82	0.39	1.6	0.39	5.56	0.54
	2019	Deserta	1	0	0	100	0	0	0	0	0	0
		Berlenga	2	100	0	0	0	0	0	0	0	0
		Peniche	121	52.88	0.83	4.96	0.83	0	0.83	5.79	33.88	0
		Porto	653	23.43	1.53	4.44	63.4	0.92	0.77	1.68	2.6	1.23
SHEET PLASTICS	2018	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	6	16.67	0	0	83.33	0	0	0	0	0
		Peniche	17	47.06	0	0	0	0	0	0	52.94	0
		Porto	124	61.29	2.42	2.42	4.84	0	4.03	3.23	20.16	1.61
	2019	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	1	100	0	0	0	0	0	0	0	0
		Peniche	49	18.36	0	0	2.04	0	0	14.29	65.31	0
		Porto	124	65.32	3.23	4.84	10.47	0.81	0.81	7.26	6.45	0.81
THREADLIKE PLASTICS	2018	Deserta	1	0	0	0	100	0	0	0	0	0
		Berlenga	1	0	0	0	100	0	0	0	0	0
		Peniche	40	50	0	35	7.5	2.5	0	0	2.5	2.5
		Porto	203	44.34	0.99	40.39	4.43	2.46	2.96	0.49	3.94	0
	2019	Deserta	1	0	0	100	0	0	0	0	0	0
		Berlenga	1	100	0	0	0	0	0	0	0	0
		Peniche	18	11.11	0	33.33	0	0	5.56	0	50	0
		Porto	62	43.55	1.61	35.49	4.84	6.45	1.61	0	6.45	0

Table S2.6. *cont.*

	Year	Location	No. of items	White / Clear (%)	Yellow (%)	Green (%)	Blue / Purple (%)	Red / Pink (%)	Brown / Orange (%)	Grey / Silver (%)	Black (%)	>1 (%)
FRAGMENT PLASTICS	2018	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Peniche	6	0	0	0	50	0	0	0	33.33	16.67
		Porto	247	40.89	1.22	4.45	20.65	0.81	1.21	0	27.53	3.24
	2019	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Peniche	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Porto	32	25	6.24	3.13	12.5	3.13	9.38	6.24	12.5	21.88
FOAMED PLASTICS	2018	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Peniche	6	83.33	0	0	0	0	16.67	0	0	0
		Porto	1241	71.39	0.65	0	26.59	0	1.21	0.16	0	0
	2019	Deserta	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Berlenga	0	NA	NA	NA	NA	NA	NA	NA	NA	
		Peniche	54	98.15	1.85	0	0	0	0	0	0	0
		Porto	435	8.51	0.69	0	90.57	0	0	0	0.23	0

Figure S2.5. Drinking straws (*n* = 42) collected from a single Yellow-legged gull (*Larus michahellis*) nest from Porto, in 2018. Underneath the straws is a sheet of graph paper of 30 cm x 20 cm.

Figure S2.6. Examples of Yellow-legged (*Larus michahellis*) gull nests: **A**) from a natural breeding location (Berlenga Island), **B**) from an urban location (Peniche) and **C**) from Porto urban site (nest cup under a tile roof and not well visible). Anthropogenic materials in nests are pointed out with circles or arrows (a fabric fibre in figure B; drinking straws and plastic fragments in figure C).

Table S3.1. Means and ranges of mass, size and number of items per pellet of anthropogenic debris items present in 1132 Yellow-legged gull (*Larus michahellis*) pellets from 4 breeding locations (Deserta Island, Berlenga Island, Peniche and Porto) and during the 3 seasons of 2018 (Pre-breeding "PBre", Breeding "Bre" and Post-breeding "PTBre"). No data for Peniche in the pre-breeding season. In Porto, only the Clérigos area was considered in the breeding season. Sample sizes (total number of pellets) per location and season is presented below each season. No. Pellets = number of pellets in which the correspondent debris category is present. N = number of measured / weighted items. SD = Standard Deviation. NA = Not Applicable.

		Deserta Island			**Berlenga Island**			**Peniche**		**Porto**		
		PBre (*n* = 92)	Bre (*n* = 150)	PTBre (*n* = 39)	PBre (*n* = 109)	Bre (*n* = 157)	PTBre (*n* = 259)	Bre (*n* = 76)	PTBre (*n* = 30)	PBre (*n* = 93)	Bre (*n* = 40)	PTBre (*n* = 87)
ALL DEBRIS	No. Pellets	67	18	3	7	2	17	18	5	76	37	76
	N	940	243	3	17	7	153	102	26	1517	1758	1971
	Mean Items Per Pellet ± SD	10.22±14.25	1.62 ± 7.34	0.08 ± 0.27	0.16 ± 0.78	0.04 ± 0.43	0.59 ± 4.02	1.34 ± 3.86	0.87 ± 2.33	16.31±25.08	43.95±106.47	22.66±40.66
	Range Items Per Pellet (min. - max.)	0 - 78	0 - 71	0 - 1	0 - 6	0 - 5	0 - 55	0 - 19	0 - 9	0 - 176	0 - 664	0 - 334
	Mean Mass (g) ± SD	0.50 ± 0.86	0.58 ± 0.69	0.01 ± 0.01	0.17 ± 0.24	0.04 ± 0.04	0.60 ± 0.99	0.10 ± 0.19	0.22 ± 0.31	0.35 ± 0.67	0.41 ± 0.51	0.25 ± 0.38
	Range Mass (min. - max.)	0.0005 - 5.12	0.0023 - 2.31	0.0041 - 0.02	0.0017 - 0.68	0.0095 - 0.07	0.0022 - 4.07	0.0005 - 0.76	0.0011 - 0.72	0.0013 - 3.87	0.0016 - 1.87	0.0001 - 1.83
	Mean Size (cm) ± SD	1.74 ± 3.88	1.50 ± 1.76	12.73±19.33	1.30 ± 0.76	2.34 ± 1.95	1.51 ± 1.76	1.02 ± 1.61	1.30 ± 1.68	1.52 ± 1.79	0.95 ± 1.67	1.18 ± 1.74
	Range Size (min. - max.)	0.1 - 57.4	0.1 - 18.5	0.3 - 35	0.5 - 3	0.4 - 5.6	0.25 - 10.7	0.15 - 14.3	0.1 - 7.8	0.1 - 20	0.1 - 22.5	0.1 - 27.3
GLASS	No. Pellets	48	9	1	1	1	8	8	3	38	29	54
	N	167	29	1	2	2	24	18	4	111	149	202
	Mean Items Per Pellet ± SD	1.82 ± 2.87	0.19 ± 0.86	0.03 ± 0.16	0.02 ± 0.19	0.01 ± 0.16	0.09 ± 0.62	0.24 ± 0.88	0.13 ± 0.43	1.19 ± 2.31	3.73 ± 4.25	2.32 ± 3.77
	Range Items Per Pellet (min. - max.)	0 - 15	0 - 6	0 - 1	0 - 2	0 - 2	0 - 7	0 - 5	0 - 2	0 - 16	0 - 21	0 - 22
	Mean Mass (g) ± SD	0.11 ± 0.13	0.15 ± 0.21	0.004 ± NA	0.68 ± NA	0.03 ± NA	0.32 ± 0.63	0.16 ± 0.24	0.02 ± 0.02	0.07 ± 0.11	0.20 ± 0.33	0.11 ± 0.20
	Range Mass (min. - max.)	0.0024 - 0.53	0.0142 - 0.69	0.004-0.004	0.68 - 0.68	0.03 - 0.03	0.3217 - 0.63	0.0074 - 0.69	0.0011 - 0.04	0.001- 0.51	0.0004 - 1.28	0.0001 - 1.11
	Mean Size (cm) ± SD	0.54 ± 0.31	0.53 ± 0.31	0.3 ± NA	1.28 ± 0.04	0.65 ± 0.21	0.73 ± 0.48	0.61 ± 0.51	0.49 ± 0.20	0.49 ± 0.24	0.50 ± 0.34	0.43 ± 0.37
	Range Size (min. - max.)	0.1 - 1.9	0.2 - 1.5	0.3 - 0.3	1.25 - 1.3	0.5 - 0.8	0.3 - 2.1	0.2 - 2.4	0.2 - 0.65	0.15 - 1.25	0.1 - 2.35	0.1 - 3.9

Table S3.1 *cont.*

		Deserta Island			Berlenga Island			Peniche		Porto		
		PBre (*n* = 92)	Bre (*n* = 150)	PTBre (*n* = 39)	PBre (*n* = 109)	Bre (*n* = 157)	PTBre (*n* = 259)	Bre (*n* = 76)	PTBre (*n* = 30)	PBre (*n* = 93)	Bre (*n* = 40)	PTBre (*n* = 87)
FABRIC	No. Pellets	10	1	0	0	0	3	2	0	38	26	40
	N	22	1	0	0	0	4	4	0	374	108	272
	Mean Items Per Pellet ± SD	0.24 ± 0.87	0.007 ± 0.08	--	--	--	0.02 ± 0.15	0.05 ± 0.32	--	4.02 ± 17.3	2.7 ± 4.31	3.13 ± 7.82
	Range Items Per Pellet (min. - max.)	0 - 6	0 - 1	--	--	--	0 - 2	0 - 2	--	0 - 150	0 - 20	0 - 57
	Mean Mass (g) ± SD	0.23 ± 0.59	0.05 ± NA	--	--	--	0.12 ± 0.20	0.02 ± 0.02	--	0.07 ± 0.21	0.04 ± 0.06	0.03 ± 0.10
	Range Mass (min. - max.)	0.0001 - 1.91	0.05 - 0.05	--	--	--	0.0021 - 0.36	0.0023 - 0.03	--	0.0001 - 1.18	0.0005 - 0.20	0.0001 - 0.54
	Mean Size (cm) ± SD	6.84 ± 12.50	2.3 ± NA	--	--	--	5.86 ± 3.43	1.90 ± 1.23	--	2.06 ± 1.95	3.03 ± 3.93	2.63 ± 3.27
	Range Size (min. - max.)	0.4 - 47.4	2.3 - 2.3	--	--	--	2.3 - 8.8	0.5 - 3.5	--	0.3 - 20	0.15 ± 22.5	0.3 - 27.3
METAL	No. Pellets	14	5	0	0	0	1	0	0	17	13	22
	N	151	34	0	0	0	1	0	0	117	59	199
	Mean Items Per Pellet ± SD	1.64 ± 7.15	0.23 ± 1.64	--	--	--	0.004 ± 0.06	--	--	1.26 ± 6.31	1.48 ± 3.54	2.29 ± 10.86
	Range Items Per Pellet (min. - max.)	0 - 47	0 - 17	--	--	--	0 - 1	--	--	0 - 53	0 - 17	0 - 82
	Mean Mass (g) ± SD	0.13 ± 0.28	0.11 ± 0.23	--	--	--	2.06 ± NA	--	--	0.21 ± 0.56	0.01 ± 0.02	0.04 ± 0.11
	Range Mass (min. - max.)	0.0001 - 1.08	0.0019 - 0.53	--	--	--	2.06 - 2.06	--	--	0.0001 - 1.83	0.0001 - 0.08	0.0001 - 0.44
	Mean Size (cm) ± SD	0.54 ± 0.48	1.66 ± 2.07	--	--	--	6.5 ± NA	--	--	1.28 ± 1.81	0.60 ± 0.46	0.63 ± 0.61
	Range Size (min. - max.)	0.15 - 4.1	0.1 - 8.3	--	--	--	6.5 - 6.5	--	--	0.1 - 9	0.2 - 2.2	0.1 - 5.5
PAPER	No. Pellets	25	7	0	0	0	3	2	0	21	8	19
	N	193	115	0	0	0	76	9	0	352	91	285
	Mean Items Per Pellet ± SD	2.10 ± 7.38	0.77 ± 5.36	--	--	--	0.29 ± 3.6	0.12 ± 0.92	--	3.78 ± 13.25	2.28 ± 6.15	3.28 ± 8.16
	Range Items Per Pellet (min. - max.)	0 - 61	0 - 58	--	--	--	0 - 55	0 - 8	--	0 - 112	0 - 33	0 - 47
	Mean Mass (g) ± SD	0.21 ± 0.42	0.82 ± 0.85	--	--	--	0.69 ± 0.72	0.04 ± 0.06	--	0.71 ± 0.98	0.32 ± 0.29	0.38 ± 0.46
	Range Mass (min. - max.)	0.0008 - 1.94	0.0152 - 2.31	--	--	--	0.1614 - 1.52	0.0001 - 0.08	--	0.0118 - 3.87	0.0464 - 0.89	0.0014 - 1.77
	Mean Size (cm) ± SD	1.31 ± 0.90	1.29 ± 0.67	--	--	--	1.30 ± 0.65	0.96 ± 0.32	--	2.02 ± 1.96	1.31 ± 1.10	1.13 ± 0.84
	Range Size (min. - max.)	0.25 - 6.2	0.4 - 5.4	--	--	--	0.5 - 3.4	0.4 - 1.5	--	0.3 - 15.6	0.2 - 8.3	0.2 - 7.6

Table S3.1. *cont.*

		Deserta Island			Berlenga Island			Peniche		Porto		
		PBre (*n* = 92)	Bre (*n* = 150)	PTBre (*n* = 39)	PBre (*n* = 109)	Bre (*n* = 157)	PTBre (*n* = 259)	Bre (*n* = 76)	PTBre (*n* = 30)	PBre (*n* = 93)	Bre (*n* = 40)	PTBre (*n* = 87)
OTHER	No. Pellets	6	2	0	1	0	8	2	1	10	6	8
	N	10	2	0	3	0	13	5	3	25	17	24
	Mean Items Per Pellet ± SD	0.11 ± 0.56	0.01 ± 0.12	--	0.03 ± 0.29	--	0.05 ± 0.36	0.07 ± 0.47	0.1 ± 0.55	0.27 ± 1.49	0.43 ± 1.77	0.28 ± 1.21
	Range Items Per Pellet (min. - max.)	0 - 5	0 - 1	--	0 - 3	--	0 - 5	0 - 4	0 - 3	0 - 14	0 -11	0 - 9
	Mean Mass (g) ± SD	1.99 ± 1.93	0.95 ± 0.19	--	0.03 ± NA	--	0.33 ± 0.44	0.004±0.004	0.72 ± NA	0.04 ± 0.07	0.24 ± 0.33	0.02 ± 0.04
	Range Mass (min. - max.)	0.1924 - 5.04	0.8174 - 1.08	--	0.03 - 0.03	--	0.0011 - 0.92	0.0013-0.007	0.72 - 0.72	0.0005 - 0.18	0.0078 - 0.85	0.0006 - 0.13
	Mean Size (cm) ± SD	9.2 ± 5.90	6.75 ± 4.60	--	0.62 ± 0.20	--	2.36 ± 3.79	0.37 ± 0.03	1.45 ± 0.68	0.88 ± 1.57	1.44 ± 1.57	0.36 ± 0.25
	Range Size (min. - max.)	3.9 - 24	3.5 - 10	--	0.5 - 0.85	--	0.25 - 10.7	0.35 - 0.4	0.8 - 2.15	0.15 - 6.8	0.35 - 7	0.15 - 1.15
ALL PLASTICS	No. Pellets	61	15	2	6	2	9	14	3	65	36	63
	N	397	62	2	12	5	35	66	19	538	1334	989
	Mean Items Per Pellet ± SD	4.32 ± 5.88	0.41 ± 1.62	0.05 ± 0.22	0.11 ± 0.63	0.03 ± 0.29	0.14 ± 0.96	0.87 ± 2.83	0.63 ± 2.01	5.78 ± 8.64	33.35±106.94	11.37±36.75
	Range Items Per Pellet (min. - max.)	0 - 28	0 - 13	0 - 1	0 - 6	0 - 3	0 - 12	0 - 19	0 - 8	0 - 41	0 - 663	0 - 332
	Mean Mass (g) ± SD	0.11 ± 0.26	0.06 ± 0.18	0.01 ± 0.01	0.08 ± 0.08	0.02 ± 0.02	0.06 ± 0.14	0.02 ± 0.04	0.11 ± 0.19	0.04 ± 0.08	0.12 ± 0.27	0.05 ± 0.22
	Range Mass (min. - max.)	0.0005 - 1.36	0.0023 - 0.71	0.0043 - 0.02	0.0017 - 0.20	0.0095 - 0.03	0.0007 - 0.44	0.0005 - 0.13	0.0001 - 0.33	0.0001 - 0.28	0.0001 - 1.17	0.0001 - 1.73
	Mean Size (cm) ± SD	2.43 ± 4.65	2.07 ± 2.65	18.95±22.70	1.48 ± 0.82	3.01 ± 1.92	1.54 ± 1.71	1.14 ± 1.93	1.44 ± 1.93	1.12 ± 1.53	0.65 ± 0.83	1.04 ± 1.24
	Range Size (min. - max.)	0.2 - 57.4	0.3 - 18.5	2.9 - 35	0.6 - 3	0.4 - 5.6	0.3 - 9.2	0.15 - 14.3	0.1 - 7.8	0.1 - 14.7	0.1 - 8.6	0.1 - 11.5
SHEET PLASTICS	No. Pellets	51	11	1	2	2	6	6	1	35	21	43
	N	226	44	1	2	4	16	18	4	178	107	189
	Mean Items Per Pellet ± SD	2.46 ± 4.28	0.29 ± 1.28	0.03 ± 0.16	0.02 ± 0.13	0.03 ± 0.23	0.06 ± 0.55	0.24 ± 0.92	0.13 ± 0.73	1.91 ± 3.9	2.68 ± 4.03	2.17 ± 4.56
	Range Items Per Pellet (min. - max.)	0 - 26	0 - 9	0 - 1	0 - 1	0 - 2	0 - 8	0 - 5	0 - 4	0 - 22	0 - 17	0 - 30
	Mean Mass (g) ± SD	0.09 ± 0.26	0.01 ± 0.01	0.004 ± NA	0.005±0.004	0.02 ± 0.01	0.008 ± 0.01	0.02 ± 0.02	0.33 ± NA	0.03 ± 0.06	0.03 ± 0.05	0.007 ± 0.01
	Range Mass (min. - max.)	0.0001 - 1.36	0.0023 - 0.03	0.004 - 0.004	0.0017-0.008	0.0095 - 0.03	0.0078 - 0.01	0.0005 - 0.06	0.33 - 0.33	0.0001 - 0.27	0.001 - 0.21	0.0001 - 0.06
	Mean Size (cm) ± SD	3.39 ± 5.79	2.04 ± 1.75	2.9 ± NA	2.6 ± 0	3.66 ± 1.44	2.18 ± 2.24	2.68 ± 3.16	3.73 ± 2.79	1.89 ± 1.74	1.57 ± 1.47	1.15 ± 0.93
	Range Size (min. - max.)	0.4 - 57.4	0.4 - 10	2.9 - 2.9	2.6 - 2.6	2.45 - 5.6	0.5 - 9.2	0.3 - 14.3	1.7 - 7.8	0.15 - 14.7	0.2 - 8.6	0.15 - 7

Table S3.1. *cont.*

		Deserta Island			Berlenga Island			Peniche		Porto		
		PBre (*n* = 92)	Bre (*n* = 150)	PTBre (*n* = 39)	PBre (*n* = 109)	Bre (*n* = 157)	PTBre (*n* = 259)	Bre (*n* = 76)	PTBre (*n* = 30)	PBre (*n* = 93)	Bre (*n* = 40)	PTBre (*n* = 87)
THREADLIKE PLASTICS	No. Pellets	17	3	1	1	0	3	4	2	26	17	29
	N	48	5	1	1	0	9	11	5	72	802	531
	Mean Items Per Pellet ± SD	0.52 ± 1.54	0.03 ± 0.27	0.03 ± 0.16	0.01 ± 0.1	--	0.03 ± 0.4	0.14 ± 0.84	0.17 ± 0.75	0.77 ± 1.83	20.05±104.66	6.10 ± 35.96
	Range Items Per Pellet (min. - max.)	0 - 10	0 - 3	0 - 1	0 - 1	--	0 - 6	0 - 7	0 - 4	0 - 10	0 - 662	0 - 331
	Mean Mass (g) ± SD	0.02 ± 0.04	0.01 ± 0.02	0.02 ± NA	0.02 ± NA	--	0.01 ± 0.02	0.002±0.001	0.003±0.001	0.007 ± 0.01	0.06 ± 0.23	0.07 ± 0.31
	Range Mass (min. - max.)	0.0001 - 0.15	0.0008 - 0.03	0.02 - 0.02	0.02 - 0.02	--	0.0001 - 0.03	0.0004-0.003	0.0018-0.003	0.0001 - 0.07	0.0001 - 0.96	0.0001 - 1.70
	Mean Size (cm) ± SD	2.62 ± 2.72	4.96 ± 7.58	35 ± NA	3 ± NA	--	1.41 ± 0.95	1.13 ± 0.78	2.10 ± 1.12	1.88 ± 2.37	0.54 ± 0.49	1.75 ± 1.74
	Range Size (min. - max.)	0.2 - 11	1.4 - 18.5	35 - 35	3 - 3	--	0.4 - 2.85	0.5 - 2.9	1.6 - 4.1	0.2 - 13.5	0.1 - 3.2	0.1 - 11.5
FRAGMENT PLASTICS	No. Pellets	25	5	0	3	1	5	9	2	44	21	38
	N	76	9	0	9	1	6	14	9	247	366	232
	Mean Items Per Pellet ± SD	0.83 ± 1.94	0.06 ± 0.44	--	0.08 ± 0.61	0.01 ± 0.08	0.02 ± 0.17	0.18 ± 0.56	0.3 ± 1.32	2.66 ± 5.49	9.15 ± 29.5	2.67 ± 8.46
	Range Items Per Pellet (min. - max.)	0 - 10	0 - 5	--	0 - 6	0 - 1	0 - 2	0 - 3	0 - 7	0 - 33	0 - 174	0 - 68
	Mean Mass (g) ± SD	0.08 ± 0.18	0.15 ± 0.31	--	0.14 ± 0.06	0.005 ± NA	0.10 ± 0.19	0.02 ± 0.04	0.0003±0.0003	0.04 ± 0.06	0.13 ± 0.28	0.03 ± 0.06
	Range Mass (min. - max.)	0.0025 - 0.87	0.005 - 0.71	--	0.0812 - 0.20	0.005 - 0.005	0.0031 - 0.44	0.0005 - 0.13	0.0001-0.0005	0.0001 - 0.21	0.0001 - 1.16	0.0001 - 0.28
	Mean Size (cm) ± SD	0.72 ± 0.47	1.38 ± 1.04	--	1.06 ± 0.36	0.4 ± NA	0.83 ± 0.49	0.50 ± 0.39	0.18 ± 0.08	0.46 ± 0.38	0.43 ± 0.32	0.45 ± 0.38
	Range Size (min. - max.)	0.2 - 2.55	0.3 - 2.9	--	0.6 - 1.65	0.4 - 0.4	0.4 - 1.8	0.15 - 1.6	0.1 - 0.35	0.1 - 3.3	0.1 - 2.8	0.1 - 3.55
FOAMED PLASTICS	No. Pellets	23	2	0	0	0	2	4	1	21	29	23
	N	47	4	0	0	0	4	23	1	41	59	37
	Mean Items Per Pellet ± SD	0.51 ± 1.43	0.03 ± 0.26	--	--	--	0.02 ± 0.20	0.30 ± 2.19	0.03 ± 0.18	0.44 ± 1.18	1.48 ± 1.47	0.43 ± 0.94
	Range Items Per Pellet (min. - max.)	0 - 11	0 - 3	--	--	--	0 - 3	0 - 19	0 - 1	0 - 7	0 - 6	0 - 6
	Mean Mass (g) ± SD	0.002±0.003	0.004±0.002	--	--	--	0.002±0.0004	0.006 ± 0.01	0.0009 ± NA	0.004±0.007	0.001 ± 0.001	0.001±0.001
	Range Mass (min. - max.)	0.0003 - 0.01	0.0028-0.005	--	--	--	0.0019-0.003	0.0005 - 0.02	0.0009-0.0009	0.0001 - 0.02	0.0001-0.005	0.0001-0.006
	Mean Size (cm) ± SD	0.37 ± 0.09	0.39 ± 0.12	--	--	--	0.35 ± 0.04	0.33 ± 0.07	0.35 ± NA	0.41 ± 0.20	0.28 ± 0.07	0.31 ± 0.13
	Range Size (min. - max.)	0.25 - 0.75	0.3 - 0.55	--	--	--	0.3 - 0.4	0.2 - 0.45	0.35 - 0.35	0.15 - 1.15	0.15 - 0.45	0.1 - 0.7

Table S3.2. Percentage of occurrence (%) of each anthropogenic debris category present in 1132 Yellow-legged gull (*Larus michahellis*) pellets from 4 breeding locations (Deserta Island, Berlenga Island, Peniche and Porto) during the 3 seasons of 2018 (Pre-breeding "PBre", Breeding "Bre" and Post-breeding "PTBre"). No data for Peniche in the pre-breeding season. In Porto, only the Clérigos area was considered in the breeding season.

Location	Season	Glass (%)	Fabric (%)	Metal (%)	Paper (%)	Other (%)	Plastic (%)	Sheet plastics (%)	Threadlike plastics (%)	Fragment plastics (%)	Foamed plastics (%)
Deserta	PBre	29.3	6.1	8.5	15.2	3.7	37.2	44.0	14.6	21.6	19.8
	Bre	23.1	2.6	12.8	17.9	5.1	38.5	52.4	14.3	23.8	9.5
	PTBre	33.3	0	0	0	0	66.7	50.0	50.0	0	0
Berlenga	PBre	12.5	0	0	0	12.5	75.0	33.3	16.7	50.0	0
	Bre	33.3	0	0	0	0	66.7	66.7	0	33.3	0
	PTBre	25.0	9.4	3.1	9.4	25.0	28.1	37.5	18.7	31.3	12.5
Peniche	Bre	28.7	7.1	0	7.1	7.1	50	26.1	17.4	39.1	17.4
	PTBre	42.9	0	0	0	14.2	42.9	16.7	33.3	33.3	16.7
Porto	PBre	20.1	20.1	9.0	11.1	5.3	34.4	27.8	20.6	34.9	16.7
	Bre	24.6	22.0	11.0	6.8	5.1	30.5	23.9	19.2	23.9	33
	PTBre	26.2	19.4	10.7	9.2	3.9	30.6	32.3	21.8	28.6	17.3
Overall per Season	PBre	24.1	13.3	8.6	12.7	4.7	36.6	35.5	17.7	29.1	17.7
	Bre	25.0	15.4	9.6	9.1	5.3	35.6	29.6	17.8	26.7	25.9
	PTBre	26.6	17.3	9.3	8.9	6.9	31.0	32.5	22.2	28.7	16.6
Overall per Location	Deserta	28.2	5.3	9.2	15.5	3.9	37.9	45.3	15.1	21.6	18
	Berlenga	23.3	7.0	2.3	7.0	20.9	39.5	40.0	16.0	36.0	8.0
	Peniche	31.4	5.7	0	5.7	8.6	48.6	24.2	20.7	37.9	17.2
	Porto	23.6	20.3	10.1	9.4	4.7	31.9	28.5	20.8	29.7	21.0
TOTAL	ALL	25.1	15.1	9.0	10.7	5.5	34.6	33.2	19.1	28.3	19.4

Table S3.3. A) Tukey adjusted *p* values of pairwise post-hoc comparisons among breeding locations (Deserta Island, Berlenga Island, Peniche and Porto) for the presence/absence, number of items per pellet and mass of debris and plastic materials ingested by Yellow-legged gull (*Larus michahellis*), and for each season of the year 2018 (Pre-breeding "PBre", Breeding "Bre" and Post-breeding "PTBre"). No data for Peniche in the pre-breeding season. In Porto, only the Clérigos area was considered in the breeding season. Significant differences between breeding locations are highlighted in bold. **B)** Compact letter display from pairwise comparisons: breeding locations sharing a letter are not significantly different.

	Season	Locations	Presence / Absence		Number of items per pellet		Mass	
			All Debris	All Plastic	All Debris	All Plastic	All Debris	All Plastic
A	PBre	Porto – Berlenga	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.71	0.36
		Porto – Deserta	0.32	0.86	0.12	0.44	0.21	**0.03**
		Deserta - Berlenga	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.29	0.95
	Bre	Porto – Berlenga	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.01**	0.56	0.99
		Porto – Deserta	**< 0.001**	**< 0.001**	**< 0.01**	**< 0.01**	0.99	0.83
		Porto – Peniche	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.01**	**< 0.01**	0.29
		Peniche – Berlenga	**< 0.001**	**< 0.01**	0.86	0.7	0.99	0.92
		Peniche – Deserta	0.115	0.29	0.23	0.99	**< 0.01**	0.86
		Deserta – Berlenga	**0.01**	**0.02**	0.12	0.82	0.53	0.99
	PTBre	Porto – Berlenga	**< 0.001**	**< 0.001**	**< 0.01**	**< 0.001**	0.59	0.98
		Porto – Deserta	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.15	0.99
		Porto – Peniche	**< 0.001**	**< 0.001**	**< 0.001**	0.18	0.93	0.98
		Peniche – Berlenga	0.23	0.37	0.68	0.95	0.63	0.94
		Peniche – Deserta	0.67	0.87	0.38	0.69	0.54	0.99
		Deserta – Berlenga	0.99	0.96	0.02	0.25	0.05	0.99
B	PBre	Deserta	a	a	a	a	a	a
		Berlenga	b	b	b	b	a	ab
		Porto	a	a	a	a	a	b
	Bre	Deserta	a	a	a	a	a	a
		Berlenga	b	b	a	a	ab	a
		Peniche	a	a	a	a	b	a
		Porto	c	c	b	b	a	a
	PTBre	Deserta	a	a	a	a	a	a
		Berlenga	a	a	b	a	a	a
		Peniche	a	a	ab	ab	a	a
		Porto	b	b	c	b	a	a

Table S3.4. Percentage of number of items (%) of each anthropogenic debris category present in 1132 Yellow-legged gull (*Larus michahellis*) pellets from 4 breeding locations (Deserta Island, Berlenga Island, Peniche and Porto) during the 3 seasons of 2018 (Pre-breeding "PBre", Breeding "Bre" and Post-breeding "PTBre"). No data for Peniche in the pre-breeding season. In Porto, only the Clérigos area was considered in the breeding season.

Location	Season	Glass (%)	Fabric (%)	Metal (%)	Paper (%)	Other (%)	Plastic (%)	Sheet plastics (%)	Threadlike plastics (%)	Fragment plastics (%)	Foamed plastics (%)
Deserta	PBre	17.8	2.3	16.1	20.5	1.1	42.2	56.9	12.2	19.1	11.8
	Bre	11.9	0.4	14.1	47.3	0.8	25.5	71.0	8.1	14.5	6.5
	PTBre	33.3	0	0	0	0	66.7	50.0	50.0	0	0
Berlenga	PBre	11.8	0	0	0	17.6	70.6	16.7	8.3	75.0	0
	Bre	28.6	0	0	0	0	71.4	80.0	0	20.0	0
	PTBre	15.7	2.6	0.7	49.6	8.5	22.9	45.7	25.7	17.1	11.5
Peniche	Bre	17.6	3.9	0	8.9	4.9	64.7	27.3	16.7	21.2	34.8
	PTBre	15.4	0	0	0	11.5	73.1	21.1	26.2	47.4	5.3
Porto	PBre	7.3	24.7	7.7	23.2	1.6	35.5	33.1	13.4	45.9	7.6
	Bre	8.5	6.1	3.4	5.2	1	75.8	8.0	60.1	27.5	4.4
	PTBre	10.2	13.8	10.1	14.5	1.2	50.2	19.1	53.7	23.5	3.7
Overall per Season	PBre	11.3	16.1	10.8	22.0	1.5	38.3	42.9	12.8	35.0	9.3
	Bre	9.4	5.4	4.4	10.2	1.1	69.5	11.7	55.8	26.6	5.9
	PTBre	10.7	12.8	9.3	16.8	1.9	48.5	20.1	52.2	23.7	4.0
Overall per Location	Deserta	16.6	1.9	15.6	26	1.0	38.9	58.8	11.7	18.4	11.1
	Berlenga	15.8	2.3	0.6	42.9	9.0	29.4	42.3	19.2	30.8	7.7
	Peniche	17.2	3.1	0	7.0	6.3	66.4	25.9	18.8	27.1	28.2
	Porto	8.8	14.4	7.1	13.9	1.3	54.5	16.6	49.1	29.5	4.8
TOTAL	ALL	10.5	11.7	8.3	16.6	1.6	51.3	22.8	42.9	28.1	6.2

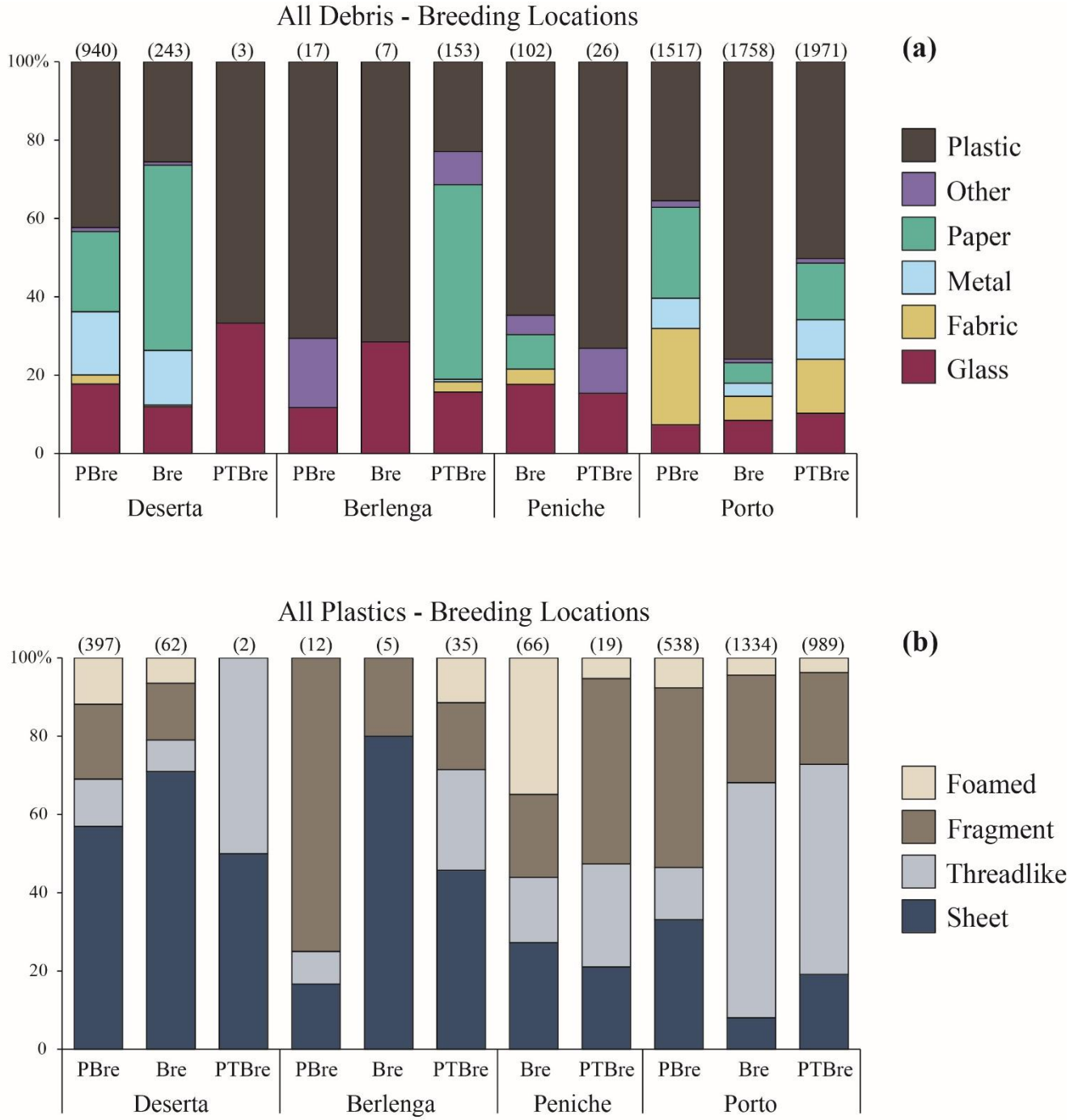


Figure S3.1. Proportion of anthropogenic debris (items per pellet) in 1132 Yellow-legged gull (*Larus michahellis*) pellets on 4 breeding locations (Deserta Island, Berlenga Island, Peniche and Porto) during the 3 seasons of 2018 (Pre-breeding "PBre", Breeding "Bre" and Post-breeding "PTBre"). No data for Peniche in the pre-breeding season. In Porto, only the Clérigos area was considered in the breeding season. **a)** For each category of anthropogenic debris (glass, fabric, metal, paper, other and plastic); the number of analysed debris items in each location / season is presented on the top of each bar. **b)** For plastic categories (sheet, threadlike, fragment and foamed); the number of analysed plastic items in each location / season is presented on the top of each bar.

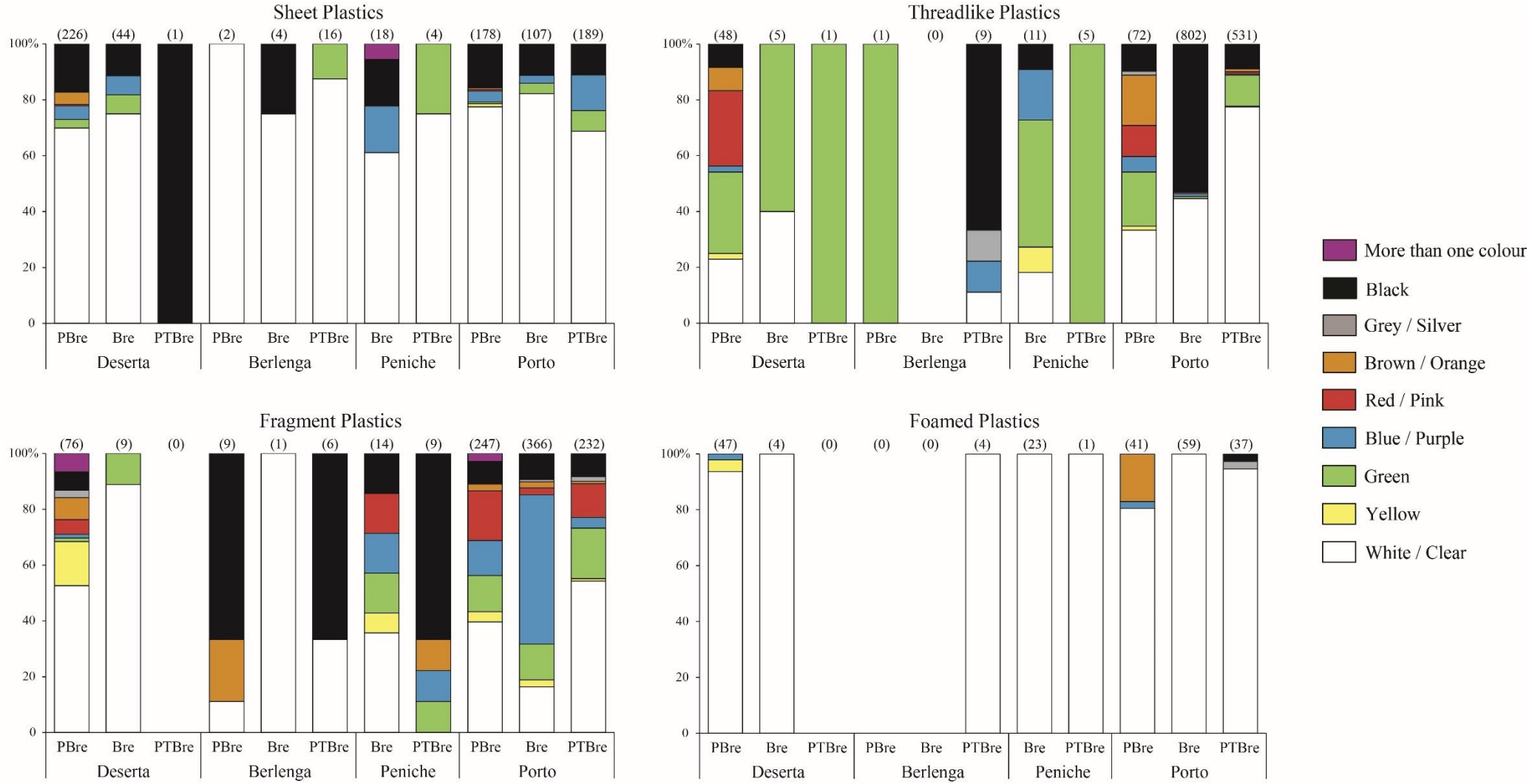


Figure S3.2. Frequency of occurrence (FO, %) of sheet, threadlike, fragment and foamed plastics colours in 1132 Yellow-legged gull (*Larus michahellis*) pellets on 4 breeding locations (Deserta Island, Berlenga Island, Peniche and Porto) during the 3 seasons of 2018 (Pre-breeding "PBre", Breeding "Bre" and Post-breeding "PTBre"). No data for Peniche in the pre-breeding season. In Porto, only the Clérigos area was considered in the breeding season. Number of items analysed in each location / season is presented on the top of each bar.

Table S3.5. Means and ranges of mass, size and number of items per pellet of anthropogenic debris items present in 447 gull pellets from 2 resting sites (St. Catarina Street and Landfill Coimbra), from winter 2017 to winter 2018. Pellets from St. Catarina Street belong to Yellow-legged gulls (*Larus michahellis*) only, while pellets from Landfill Coimbra belong to both Yellow-legged and Lesser black-backed gulls (*Larus fuscus*). Sample sizes (total number of pellets) per location and season is presented below each season. No. Pellets = number of pellets in which the correspondent debris category is present. N = number of measured / weighted items. SD = Standard Deviation. NA = Not Applicable.

		St. Catarina Street				**Landfill Coimbra**	
		Spring18 (*n* = 113)	Summer18 (*n* = 60)	Autumn18 (*n* = 63)	Winter18 (*n* = 46)	Winter17 (*n* = 95)	Spring18 (*n* = 70)
ALL DEBRIS	No. Pellets	94	55	53	44	89	66
	N	1762	2187	1290	1033	980	1157
	Mean Items Per Pellet ± SD	15.59 ± 23.23	36.45 ± 89.27	20.48 ± 24.88	22.46 ± 48.58	10.32 ± 7.85	16.53 ± 14.54
	Range Items Per Pellet (min. - max.)	0 - 176	0 - 664	0 - 125	0 - 334	0 - 33	0 - 89
	Mean Mass (g) ± SD	0.32 ± 0.62	0.32 ± 0.45	0.25 ± 0.39	0.22 ± 0.31	0.21 ± 0.34	0.32 ± 0.42
	Range Mass (min. - max.)	0.0001 - 3.87	0.0016 - 1.87	0.0001 - 1.77	0.0023 - 1.83	0.0012 - 1.87	0.0022 - 2.01
	Mean Size (cm) ± SD	1.45 ± 1.74	1.03 ± 2.15	1.20 ± 1.81	1.36 ± 2.21	1.62 ± 3.16	2.26 ± 3.93
	Range Size (min. - max.)	0.1 - 20	0.1 - 39.6	0.1 - 27.3	0.1 - 29.3	0.1 - 40.3	0.1 - 32
GLASS	No. Pellets	51	40	40	28	80	60
	N	188	194	176	66	406	350
	Mean Items Per Pellet ± SD	1.66 ± 3.16	3.23 ± 3.96	2.79 ± 4.29	1.43 ± 2.09	4.27 ± 4.46	5.00 ± 6.78
	Range Items Per Pellet (min. - max.)	0 - 20	0 - 21	0 - 22	0 - 11	0 - 18	0 - 35
	Mean Mass (g) ± SD	0.08 ± 0.12	0.17 ± 0.29	0.12 ± 0.22	0.10 ± 0.13	0.08 ± 0.12	0.07 ± 0.11
	Range Mass (min. - max.)	0.0004 - 0.60	0.0004 - 1.28	0.0001 - 1.11	0.0036 - 0.52	0.0001 - 0.82	0.0004 - 0.74
	Mean Size (cm) ± SD	0.47 ± 0.26	0.48 ± 0.32	0.40 ± 0.37	0.60 ± 0.30	0.40 ± 0.22	0.37 ± 0.21
	Range Size (min. - max.)	0.1 - 1.5	0.1 - 2.35	0.1 - 3.9	0.15 - 1.3	0.1 - 1.7	0.1 - 1.6
FABRIC	No. Pellets	50	39	30	23	34	43
	N	412	152	217	101	82	263
	Mean Items Per Pellet ± SD	3.65 ± 15.77	2.53 ± 4.24	3.44 ± 8.77	2.20 ± 3.83	0.86 ± 1.98	3.76 ± 7.31
	Range Items Per Pellet (min. - max.)	0 - 150	0 - 20	0 - 57	0 - 20	0 - 14	0 - 45
	Mean Mass (g) ± SD	0.07 ± 0.21	0.05 ± 0.08	0.03 ± 0.10	0.03 ± 0.06	0.11 ± 0.31	0.07 ± 0.14
	Range Mass (min. - max.)	0.0001 - 1.18	0.0001 - 0.35	0.0001 - 0.54	0.0006 - 0.29	0.0001 - 1.64	0.0001 - 0.79
	Mean Size (cm) ± SD	2.10 ± 1.99	3.27 ± 5.03	2.76 ± 3.31	2.68 ± 3.56	5.70 ± 5.55	5.81 ± 6.43
	Range Size (min. - max.)	0.25 - 20	0.15 - 39.6	0.4 - 27.3	0.3 - 19.8	0.65 - 28.5	0.6 - 32

Table S3.5. *cont.*

		St. Catarina Street				Landfill Coimbra	
		Spring18 (*n* = 113)	Summer18 (*n* = 60)	Autumn18 (*n* = 63)	Winter18 (*n* = 46)	Winter17 (*n* = 95)	Spring18 (*n* = 70)
METAL	No. Pellets	20	22	16	14	23	19
	N	127	74	109	110	37	111
	Mean Items Per Pellet ± SD	1.12 ± 5.75	1.23 ± 2.96	1.73 ± 10.33	2.39 ± 8.96	0.39 ± 0.82	1.59 ± 9.57
	Range Items Per Pellet (min. - max.)	0 - 53	0 - 17	0 - 82	0 - 57	0 - 4	0 - 80
	Mean Mass (g) ± SD	0.18 ± 0.52	0.01 ± 0.02	0.01 ± 0.02	0.07 ± 0.14	0.006 ± 0.008	0.03 ± 0.06
	Range Mass (min. - max.)	0.0001 - 1.83	0.0001 - 0.08	0.0001 - 0.06	0.0004 - 0.44	0.0001 - 0.04	0.0001 - 0.23
	Mean Size (cm) ± SD	1.20 ± 1.76	0.60 ± 0.46	0.40 ± 0.26	0.91 ± 0.87	1.09 ± 2.25	2.00 ± 1.43
	Range Size (min. - max.)	0.1 - 9	0.15 - 2.2	0.1 - 1.7	0.1 - 5.5	0.15 - 14	0.1 - 8
PAPER	No. Pellets	23	9	14	7	17	16
	N	376	93	201	118	54	153
	Mean Items Per Pellet ± SD	3.33 ± 12.17	1.55 ± 5.11	3.19 ± 8.22	2.57 ± 6.84	0.57 ± 1.70	2.19 ± 5.75
	Range Items Per Pellet (min. - max.)	0 - 112	0 - 33	0 - 47	0 - 28	0 - 10	0 - 26
	Mean Mass (g) ± SD	0.66 ± 0.95	0.29 ± 0.29	0.42 ± 0.52	0.25 ± 0.23	0.18 ± 0.47	0.40 ± 0.55
	Range Mass (min. - max.)	0.0118 - 3.87	0.0036 - 0.89	0.0014 - 1.77	0.0015 - 0.58	0.0011 - 1.86	0.0006 - 1.97
	Mean Size (cm) ± SD	1.97 ± 1.91	1.30 ± 1.09	1.21 ± 0.95	0.89 ± 0.48	1.58 ± 2.27	1.57 ± 1.50
	Range Size (min. - max.)	0.3 - 15.6	0.2 - 8.3	0.2 - 7.6	0.1 - 3	0.1 - 11	0.15 - 14.5
OTHER	No. Pellets	13	9	7	7	6	11
	N	28	21	23	30	7	18
	Mean Items Per Pellet ± SD	0.25 ± 1.36	0.35 ± 1.47	0.37 ± 1.41	0.65 ± 3.26	0.07 ± 0.30	0.26 ± 0.83
	Range Items Per Pellet (min. - max.)	0 - 14	0 - 11	0 - 9	0 - 22	0 - 2	0 - 6
	Mean Mass (g) ± SD	0.05 ± 0.07	0.16 ± 0.29	0.01 ± 0.009	0.03 ± 0.05	0.62 ± 0.53	0.48 ± 0.52
	Range Mass (min. - max.)	0.0005 - 0.18	0.0026 - 0.85	0.0006 - 0.03	0.0006 - 0.13	0.0006 - 1.36	0.0110 - 1.23
	Mean Size (cm) ± SD	1.01 ± 1.59	1.25 ± 1.46	0.32 ± 0.18	0.33 ± 0.17	3.54 ± 3.52	3.32 ± 5.16
	Range Size (min. - max.)	0.15 - 6.8	0.25 - 7	0.15 - 1	0.2 - 1.15	0.2 - 9.8	0.25 - 21
ALL PLASTICS	No. Pellets	81	50	45	37	78	52
	N	631	1653	564	608	394	262
	Mean Items Per Pellet ± SD	5.58 ± 8.10	27.55 ± 89.36	8.95 ± 13.52	13.22 ± 48.47	4.15 ± 4.13	3.74 ± 3.85
	Range Items Per Pellet (min. - max.)	0 - 41	0 - 663	0 - 69	0 - 332	0 - 20	0 - 15
	Mean Mass (g) ± SD	0.04 ± 0.07	0.10 ± 0.23	0.03 ± 0.06	0.08 ± 0.28	0.03 ± 0.04	0.04 ± 0.17
	Range Mass (min. - max.)	0.0001 - 0.28	0.0001 - 1.17	0.0001 - 0.29	0.0007 - 1.73	0.0001 - 0.25	0.0005 - 1.10
	Mean Size (cm) ± SD	1.09 ± 1.45	0.72 ± 1.21	1.05 ± 1.28	1.55 ± 2.51	2.05 ± 3.43	1.68 ± 2.19
	Range Size (min. - max.)	0.1 - 14.7	0.1 - 20.8	0.1 - 11.5	0.15 - 29.3	0.1 - 40.3	0.1 - 22

Table S3.5. *cont.*

		St. Catarina Street				Landfill Coimbra	
		Spring18 (*n* = 113)	Summer18 (*n* = 60)	Autumn18 (*n* = 63)	Winter18 (*n* = 46)	Winter17 (*n* = 95)	Spring18 (*n* = 70)
SHEET PLASTICS	No. Pellets	48	31	33	26	46	35
	N	219	146	144	135	111	112
	Mean Items Per Pellet ± SD	1.94 ± 3.62	2.43 ± 4.01	2.29 ± 5.06	2.93 ± 4.00	1.17 ± 1.85	1.60 ± 2.18
	Range Items Per Pellet (min. - max.)	0 - 22	0 - 18	0 - 30	0 - 16	0 - 10	0 - 8
	Mean Mass (g) ± SD	0.02 ± 0.05	0.02 ± 0.04	0.007 ± 0.01	0.02 ± 0.03	0.01 ± 0.02	0.04 ± 0.18
	Range Mass (min. - max.)	0.0001 - 0.27	0.0007 - 0.21	0.0001 - 0.06	0.0001 - 0.14	0.0001 - 0.11	0.0001 - 1.10
	Mean Size (cm) ± SD	1.81 ± 1.63	1.79 ± 1.74	1.15 ± 0.92	1.99 ± 2.28	2.58 ± 3.01	2.10 ± 2.67
	Range Size (min. - max.)	0.15 - 14.7	0.2 - 12.8	0.15 - 7	0.2 - 12.8	0.2 - 17.2	0.2 - 22
THREADLIKE PLASTICS	No. Pellets	30	28	20	18	43	29
	N	86	1011	184	373	121	85
	Mean Items Per Pellet ± SD	0.76 ± 1.94	16.85 ± 87.13	2.92 ± 8.34	8.11 ± 48.70	1.27 ± 2.36	1.21 ± 2.07
	Range Items Per Pellet (min. - max.)	0 - 11	0 - 662	0 - 50	0 - 331	0 - 13	0 - 8
	Mean Mass (g) ± SD	0.006 ± 0.01	0.04 ± 0.18	0.01 ± 0.03	0.10 ± 0.40	0.02 ± 0.04	0.003 ± 0.008
	Range Mass (min. - max.)	0.0001 - 0.07	0.0001 - 0.96	0.0001 - 0.12	0.0001 - 1.70	0.0001 - 0.24	0.0001 - 0.04
	Mean Size (cm) ± SD	1.66 ± 2.24	0.63 ± 1.44	1.77 ± 1.78	2.37 ± 4.48	3.78 ± 4.83	2.01 ± 1.89
	Range Size (min. - max.)	0.2 - 13.5	0.1 - 20.8	0.1 - 11.5	0.2 - 29.3	0.25 - 40.3	0.2 - 8.9
FRAGMENT PLASTICS	No. Pellets	49	31	26	21	42	16
	N	275	432	208	51	111	31
	Mean Items Per Pellet ± SD	2.43 ± 5.25	7.20 ± 24.61	3.30 ± 9.84	1.11 ± 1.78	1.17 ± 1.94	0.44 ± 1.00
	Range Items Per Pellet (min. - max.)	0 - 33	0 - 174	0 - 68	0 - 8	0 - 12	0 - 5
	Mean Mass (g) ± SD	0.03 ± 0.06	0.09 ± 0.24	0.03 ± 0.07	0.03 ± 0.06	0.02 ± 0.02	0.04 ± 0.12
	Range Mass (min. - max.)	0.0001 - 0.21	0.0001 - 1.16	0.0001 - 0.28	0.0013 - 0.29	0.0001 - 0.10	0.0003 - 0.50
	Mean Size (cm) ± SD	0.45 ± 0.37	0.41 ± 0.30	0.43 ± 0.40	0.76 ± 0.64	0.42 ± 0.25	0.73 ± 1.09
	Range Size (min. - max.)	0.1 - 3.3	0.1 - 2.8	0.1 - 3.55	0.25 - 3.35	0.1 - 1.65	0.1 - 5.7
FOAMED PLASTICS	No. Pellets	26	33	18	15	29	19
	N	51	64	28	49	51	34
	Mean Items Per Pellet ± SD	0.45 ± 1.18	1.07 ± 1.36	0.44 ± 0.95	1.07 ± 2.08	0.54 ± 1.11	0.49 ± 1.19
	Range Items Per Pellet (min. - max.)	0 - 7	0 - 6	0 - 6	0 - 10	0 - 7	0 - 8
	Mean Mass (g) ± SD	0.004 ± 0.006	0.001 ± 0.001	0.002 ± 0.002	0.003 ± 0.002	0.002 ± 0.002	0.001 ± 0.001
	Range Mass (min. - max.)	0.0001 - 0.02	0.0001 - 0.005	0.0001 - 0.006	0.0001 - 0.007	0.0001 - 0.007	0.0003 - 0.005
	Mean Size (cm) ± SD	0.40 ± 0.18	0.28 ± 0.07	0.32 ± 0.13	0.37 ± 0.39	0.34 ± 0.09	0.32 ± 0.09
	Range Size (min. - max.)	0.15 - 1.15	0.15 - 0.45	0.1 - 0.7	0.15 - 3	0.2 - 0.6	0.2 - 0.6

Table S3.6. Percentage of occurrence (%) of each anthropogenic debris category present in 447 gull pellets from 2 resting sites (St. Catarina Street and Landfill Coimbra), from winter 2017 to winter 2018. Pellets from St. Catarina Street belong to Yellow-legged gulls (*Larus michahellis*) only, while pellets from Landfill Coimbra belong to both Yellow-legged and Lesser black-backed gulls (*Larus fuscus*).

Location	Season	Glass (%)	Fabric (%)	Metal (%)	Paper (%)	Other (%)	Plastic (%)	Sheet plastics (%)	Threadlike plastics (%)	Fragment plastics (%)	Foamed plastics (%)
St. Catarina Street	Spring 18	21.4	21.0	8.4	9.7	5.5	34.0	31.4	19.6	32.0	17.0
	Summer 18	23.7	23.1	13.0	5.3	5.3	29.6	25.2	22.8	25.2	26.8
	Autumn 18	26.3	19.7	10.5	9.3	4.6	29.6	34.0	20.6	26.8	18.6
	Winter 18	24.1	19.9	12.1	6.0	6.0	31.9	32.5	22.5	26.2	18.8
Landfill Coimbra	Winter 17	33.6	14.3	9.7	7.1	2.5	32.8	28.7	26.9	26.3	18.1
	Spring 18	29.8	21.4	9.5	8.0	5.4	25.9	35.4	29.2	16.2	19.2
Overall per Location	St. Catarina Street	23.6	21.0	10.6	7.9	5.3	31.6	30.5	21.2	28.0	20.3
	Landfill Coimbra	31.9	17.5	9.6	7.5	3.9	29.6	31.3	27.8	22.4	18.5
TOTAL	ALL	26.8	19.7	10.2	7.7	4.8	30.8	30.8	23.6	26.0	19.6

Table S3.7. Percentage of number of items (%) of each anthropogenic debris category present in 447 gull pellets from 2 resting sites (St. Catarina Street and Landfill Coimbra), from winter 2017 to winter 2018. Pellets from St. Catarina Street belong to Yellow-legged gulls (*Larus michahellis*) only, while pellets from Landfill Coimbra belong to both Yellow-legged and Lesser black-backed gulls (*Larus fuscus*).

Location	Season	Glass (%)	Fabric (%)	Metal (%)	Paper (%)	Other (%)	Plastic (%)	Sheet plastics (%)	Threadlike plastics (%)	Fragment plastics (%)	Foamed plastics (%)
St. Catarina Street	Spring 18	10.7	23.4	7.2	21.3	1.6	35.8	34.7	13.6	43.6	8.1
	Summer 18	8.8	7	3.4	4.3	1	75.5	8.8	61.2	26.1	3.9
	Autumn 18	13.6	16.8	8.4	15.7	1.8	43.7	25.5	32.6	36.9	5
	Winter 18	6.4	9.8	10.6	11.4	2.9	58.9	22.2	61.3	8.4	8.1
Landfill Coimbra	Winter 17	41.1	8.5	3.9	5.6	0.7	40.2	28.2	30.7	28.2	12.9
	Spring 18	30.3	22.7	9.6	13.2	1.6	22.6	42.7	32.5	11.8	13
Overall per Location	St. Catarina Street	9.9	14.1	6.7	12.6	1.6	55.1	18.6	47.8	28.0	5.6
	Landfill Coimbra	35.4	16.1	6.9	9.7	1.2	30.7	34.0	31.4	21.6	13.0
TOTAL	ALL	16.4	14.6	6.8	11.8	1.5	48.9	21.1	45.2	27.0	6.7

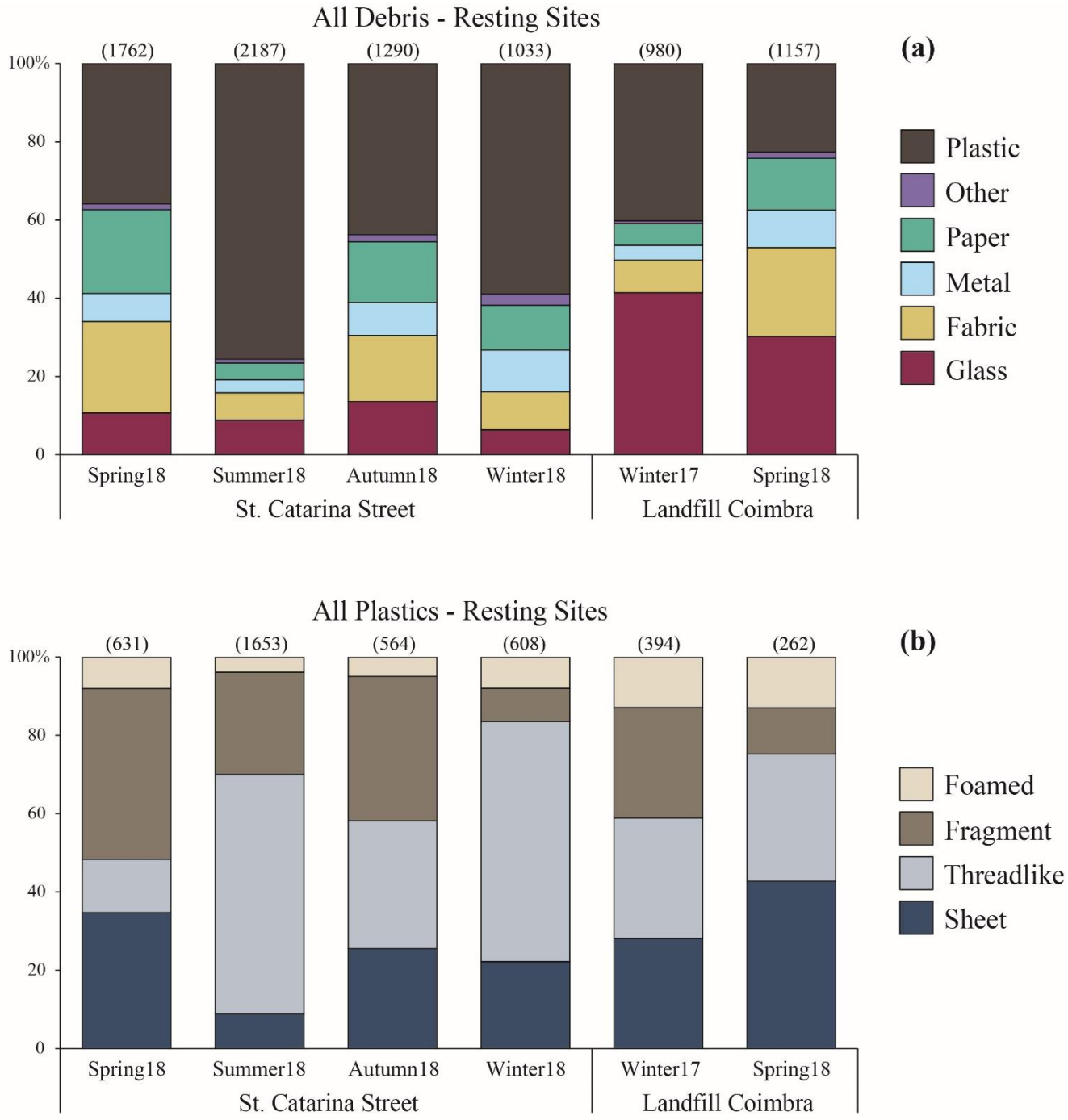


Figure S3.3. Proportion of anthropogenic debris (items per pellet) in 447 gull pellets on 2 resting sites (St. Catarina Street and Landfill Coimbra), from winter 2017 to winter 2018. Pellets from St. Catarina Street belong to Yellow-legged gulls (*Larus michahellis*) only, while pellets from Landfill Coimbra belong to both Yellow-legged and Lesser black-backed gulls (*Larus fuscus*). **a)** For each category of anthropogenic debris (glass, fabric, metal, paper, other and plastic); the number of analysed debris items in each location / season is presented on the top of each bar. **b)** For plastic categories (sheet, threadlike, fragment and foamed); the number of analysed plastic items in each location / season is presented on the top of each bar.

Table S3.8. Statistics from zero-inflated models testing the effect of season (spring18, summer18, autumn18 and winter18 for St. Catarina Street, and winter17 and spring18 for Landfill Coimbra), for each resting location (St. Catarina Street and Landfill Coimbra), in the number of anthropogenic debris items and in the number of plastic items ingested by Yellow-legged gull (*Larus michahellis*) in St. Catarina Street, and by Yellow-legged and Lesser black-backed gull (*Larus fuscus*) in Landfill Coimbra. Spring 2018 was assigned as reference category for both St. Catarina Street and Landfill Coimbra models. Only results from count models are shown. Significant effects are highlighted in bold.

		(count model)	**All Debris**	**All Plastic**
St. Catarina Street	Summer 2018	β ± SE	0.80 ± 0.20	1.60 ± 0.25
		Z	3.95	6.28
		P	< **0.001**	< **0.001**
	Autumn 2018	β ± SE	0.28 ± 0.21	0.48 ± 0.27
		Z	1.37	1.79
		P	0.17	0.07
	Winter 2018	β ± SE	0.29 ± 0.22	0.86 ± 0.28
		Z	1.33	3.08
		P	0.18	< **0.01**

		(count model)	**All Debris**	**All Plastic**
Landfill Coimbra	Winter 2017	β ± SE	-0.48 ± 0.12	0.003 ± 0.17
		Z	-3.91	0.02
		P	< **0.001**	0.99

Table S3.9. A) Tukey adjusted *p* values of pairwise post-hoc comparisons among seasons (from winter 2017 to winter 2018) for the presence/absence, number of items per pellet and mass of debris and plastic materials ingested by gulls, and for each resting site (Landfill Coimbra and St. Catarina Street). Pellets from St. Catarina Street belong to Yellow-legged gulls (*Larus michahellis*) only, while pellets from Landfill Coimbra belong to both Yellow-legged and Lesser black-backed gulls (*Larus fuscus*). Significant differences between seasons are highlighted in bold. **B)** Compact letter display from pairwise comparisons: seasons sharing a letter are not significantly different.

	Site	Seasons	Presence / Absence		Number of items per pellet		Mass	
			All Debris	All Plastic	All Debris	All Plastic	All Debris	All Plastic
A	St. Catarina Street	Spring18 – Summer18	0.43	0.33	**< 0.01**	**< 0.001**	0.3	0.051
		Spring18 – Autumn18	0.99	0.99	0.56	0.39	0.99	0.98
		Spring18 – Winter18	0.21	0.67	0.60	0.08	0.85	0.23
		Summer18 – Autumn18	0.59	0.40	0.14	**0.01**	0.51	**0.049**
		Summer18 – Winter18	0.85	0.98	0.16	0.11	0.88	0.98
		Autumn18 – Winter18	0.28	0.71	1	0.68	0.94	0.19
	Landfill Coimbra	Winter17 – Spring18	0.87	0.23	**< 0.001**	0.99	**0.01**	0.18
B	St. Catarina Street	Spring18	a	a	a	a	a	ab
		Summer18	a	a	b	b	a	b
		Autumn18	a	a	ab	a	a	a
		Winter18	a	a	ab	ab	a	ab
	Landfill Coimbra	Winter17	a	a	a	a	a	a
		Spring18	a	a	b	a	b	a

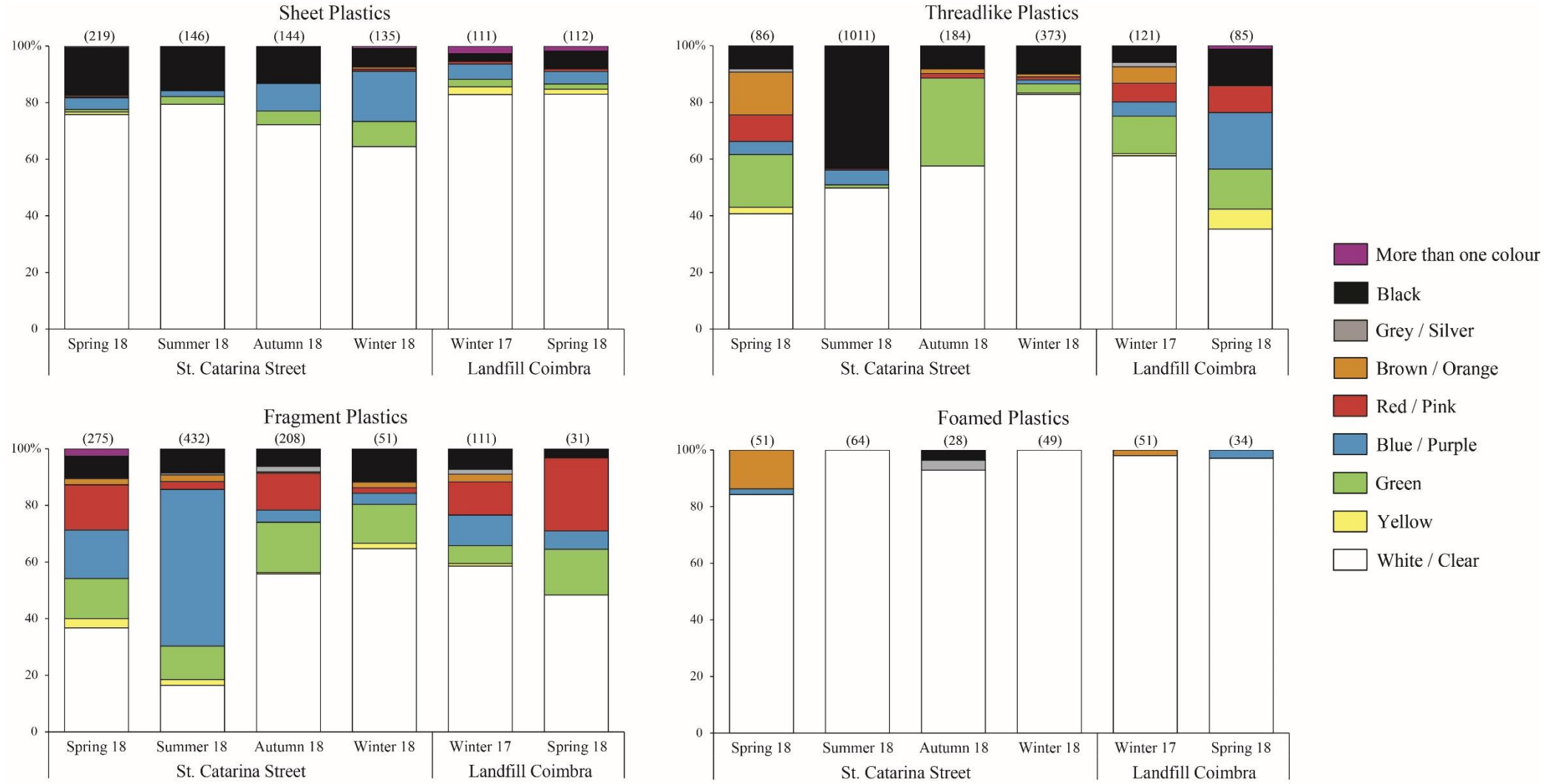


Figure S3.4. Frequency of occurrence (FO, %) of sheet, threadlike, fragment and foamed plastics colours in 447 gull pellets on 2 resting sites (St. Catarina Street and Landfill Coimbra), from winter 2017 to winter 2018. Pellets from St. Catarina Street belong to Yellow-legged gulls (*Larus michahellis*) only, while pellets from Landfill Coimbra belong to both Yellow-legged and Lesser black-backed gulls (*Larus fuscus*). Number of items analysed in each location / season is presented on the top of each bar.

Table S4.1. Individual summary of the characteristics and measurements of 47 necropsied gulls from three wildlife rescue centres (Centro de Recuperação do Parque Biológico de Gaia, PBGaia; Centro de Recuperação de Animais Silvestres de Lisboa, LxCRAS and Centro de Recuperação e Investigação de Animais Selvagens, RIAS). Individuals from PBGaia represent the most urbanized gull population, gulls from RIAS represent the most natural individuals and LxCRAS individuals are both natural and urban dwellers. Analysed characteristics were: species (YLG = yellow-legged gull *Larus michahellis*; LBBG = lesser black backed gull *Larus fuscus*); age (Im = immature, 1 to 3 years; Ad = adult, more than 3 years old); sex (M = Male; F = Female; U = Unknown / Indetermined); BCS (Body Condition Score, index from 1, lean, to 5, obese); clinical history or cause of death (PS = Paretic Syndrome; Tr = Trauma; U = Unknown / Indetermined) and body mass (at the necropsy, presented in g). The number of items of anthropogenic materials (No. Items) found on gulls' digestive system, their location in the digestive system (Location) and their mass (Materials Mass, in g) are also presented.

Rescue Centre	Gull ID	Species	Age	Sex	BCS	Clinical History	Body Mass (g)	No. Items	Location	Materials Mass (g)
PBGaia	**1**	YLG	Im	M	1	Tr	785	1	Gizzard	0.0062
	2	LBBG	Im	F	2	Tr	595	0		0
	5	YLG	Im	F	5	Tr	955	2	Gizzard	0.0025
	6	YLG	Im	U	2	Tr	695	0		0
	7	YLG	Im	M	3	PS	760	5	Gizzard	0.0229
	8	YLG	Im	U	3	Tr	795	7	Gizzard	0.0338
	10	YLG	Ad	M	3	Tr	875	1	Gizzard	0.0001
	12	YLG	Im	M	3	Tr	940	3	Gizzard	0.0080
	13	YLG	Im	F	2	PS	745	0		0
	17	YLG	Ad	F	2	Tr	680	5	Gizzard	0.0056
	19	YLG	Im	M	3	Tr	855	1	Gizzard	0.0072
	20	LBBG	Im	F	2	U	595	12	Gizzard	0.0880
LxCRAS	**1355**	LBBG	Ad	F	2	PS	550	0		0
	1359	LBBG	Im	M	2	PS	600	1	Gizzard	0.0780
	1360	LBBG	Ad	F	4	PS	550	0		0
	1367	LBBG	Ad	F	3	PS	600	0		0
	1515	YLG	Im	F	2	PS	700	0		0
	1538	LBBG	Ad	F	2	PS	650	6	Gizzard	0.0467
	1543	LBBG	Im	M	1	PS	650	1	Gizzard	0.0204
	1544	LBBG	Im	M	2	PS	650	1	Gizzard	0.0055
	1556	LBBG	Ad	M	3	Tr	600	0		0
	1560	LBBG	Im	M	1	PS	600	0		0
	1568	YLG	Im	M	3	Tr	850	0		0
	1583	LBBG	Im	F	4	Tr	750	0		0
	1589	LBBG	Im	F	2	Tr	650	1	Gizzard	0.0305
	1598	YLG	Im	M	3	Tr	750	0		0
	1599	YLG	Im	F	4	U	650	0		0

Table S4.1. *cont.*

Rescue centre	Gull ID	Species	Age	Sex	BCS	Clinical History	Body Mass (g)	No. Items	Location	Materials Mass (g)
RIAS	**2179**	LBBG	Ad	U	3	PS	795	5	Gizzard (4) Cloaca (1)	0.7867
	2269	LBBG	Ad	F	1	Tr	570	0		0
	2311	LBBG	Im	F	2	PS	655	4	Gizzard	0.0306
	2316	LBBG	Im	M	2	PS	655	0		0
	2363	YLG	Im	M	2	PS	655	1	Gizzard	0.0013
	2386	LBBG	Im	F	2	PS	570	0		0
	2434	LBBG	Im	F	2	PS	695	3	Mouth	0.0034
	2463	YLG	Im	M	1	Tr	745	5	Gizzard	0.0090
	2477	YLG	Ad	M	2	Tr	775	0		0
	2489	LBBG	Im	F	2	PS	725	5	Gizzard	0.1398
	2493	YLG	Im	U	4	PS	870	9	Gizzard	0.3132
	2497	YLG	Im	M	3	PS	980	0		0
	2504	YLG	Im	M	1	PS	545	0		0
	2528	YLG	Im	M	2	PS	705	4	Gizzard	0.0315
	2536	LBBG	Ad	F	2	Tr	755	0		0
	2538	LBBG	Im	F	1	PS	475	2	Gizzard	0.0116
	2540	YLG	Im	F	2	PS	650	0		0
	2564	LBBG	Ad	M	2	PS	850	14	Gizzard	0.0724
	2565	YLG	Im	F	4	PS	850	1	Proventriculus	0.0014
	2578	LBBG	Ad	F	3	U	705	0		0

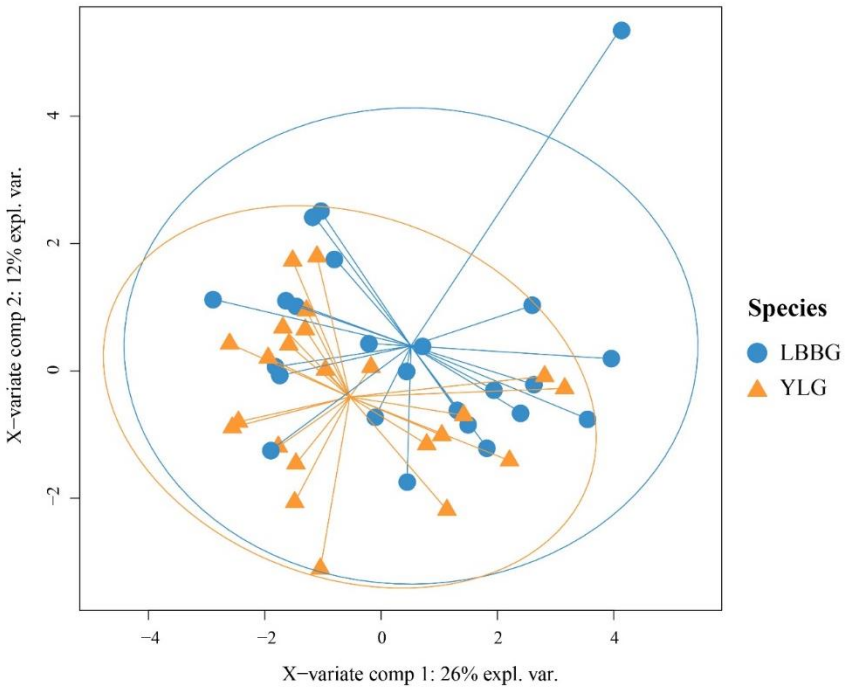
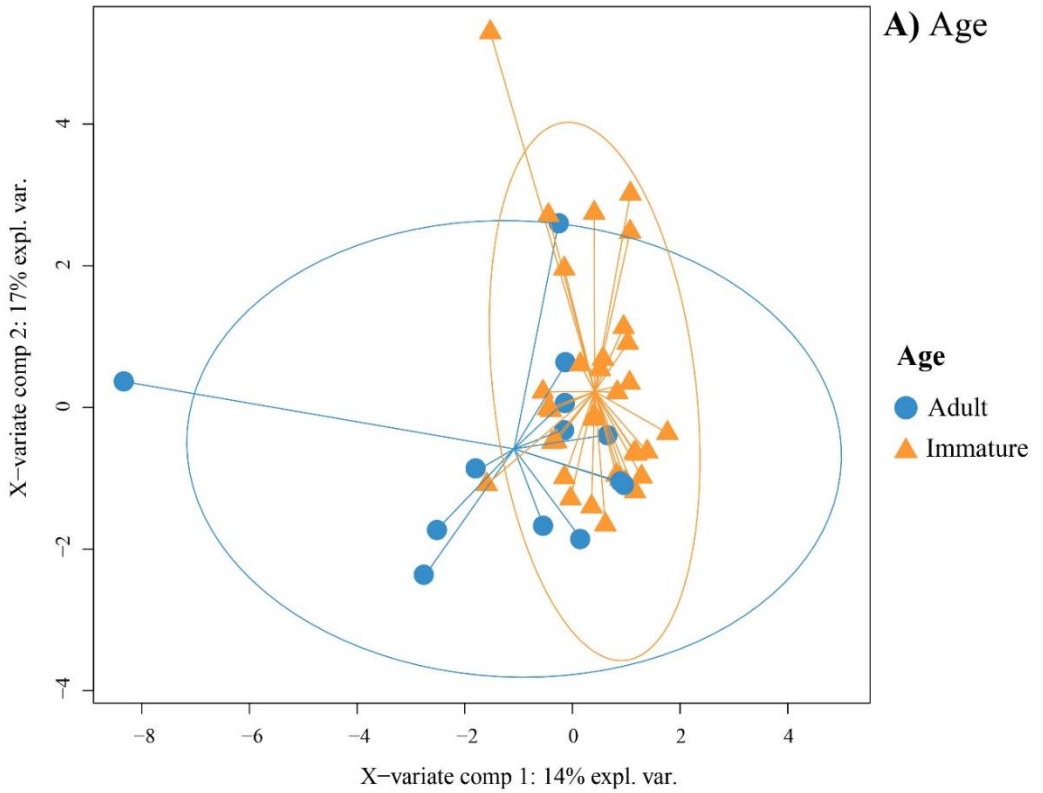
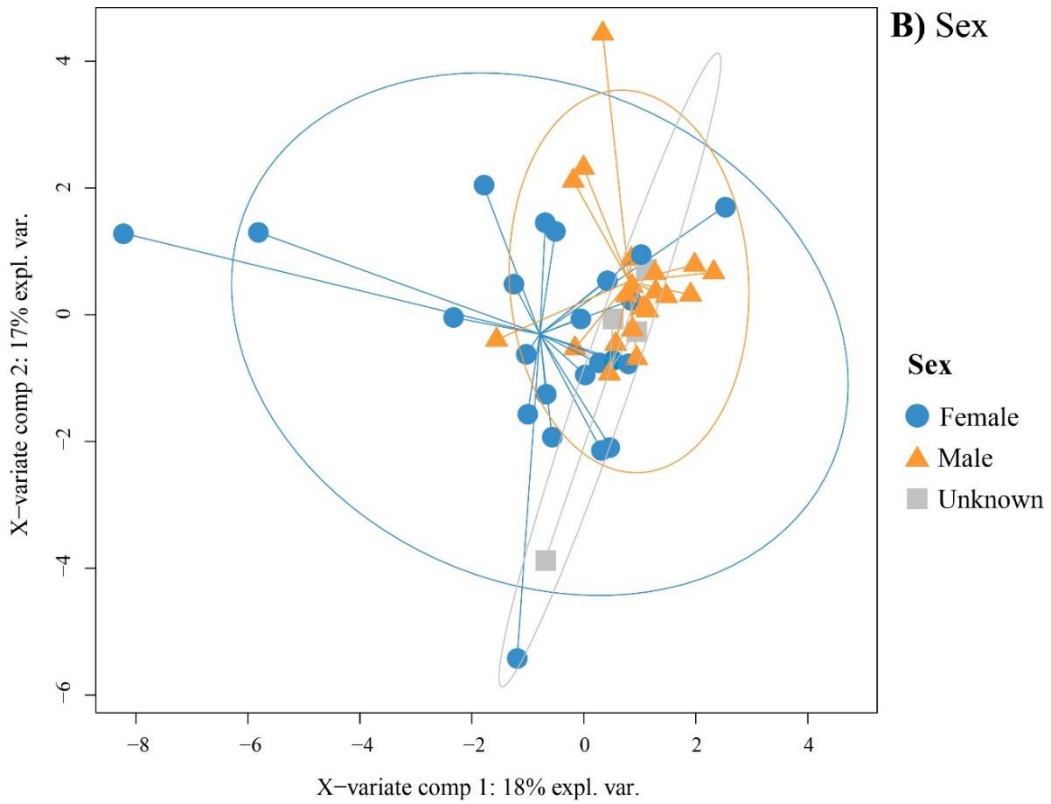


Figure S4.1. Partial Least Squares Discriminant Analysis (PLS-DA) score plot (component 1 and component 2) of 47 gulls' adipose tissue fatty acids mean percentages (arcsine transformed) separated according to species (YLG: yellow-legged gull *Larus michahellis* orange triangles and LBBG: lesser black-backed gull *Larus fuscus* blue points). Each triangle or point represents each necropsied gull. 26% and 12% of the variance in fatty acids is explained by component 1 and component 2, respectively. Coloured ellipses represent 95% confidence intervals.


A) Age
X−variate comp 2: 17% expl. var.
X−variate comp 1: 14% expl. var.
Age
Adult
Immature


B) Sex
X−variate comp 2: 17% expl. var.
X−variate comp 1: 18% expl. var.
Sex
Female
Male
Unknown

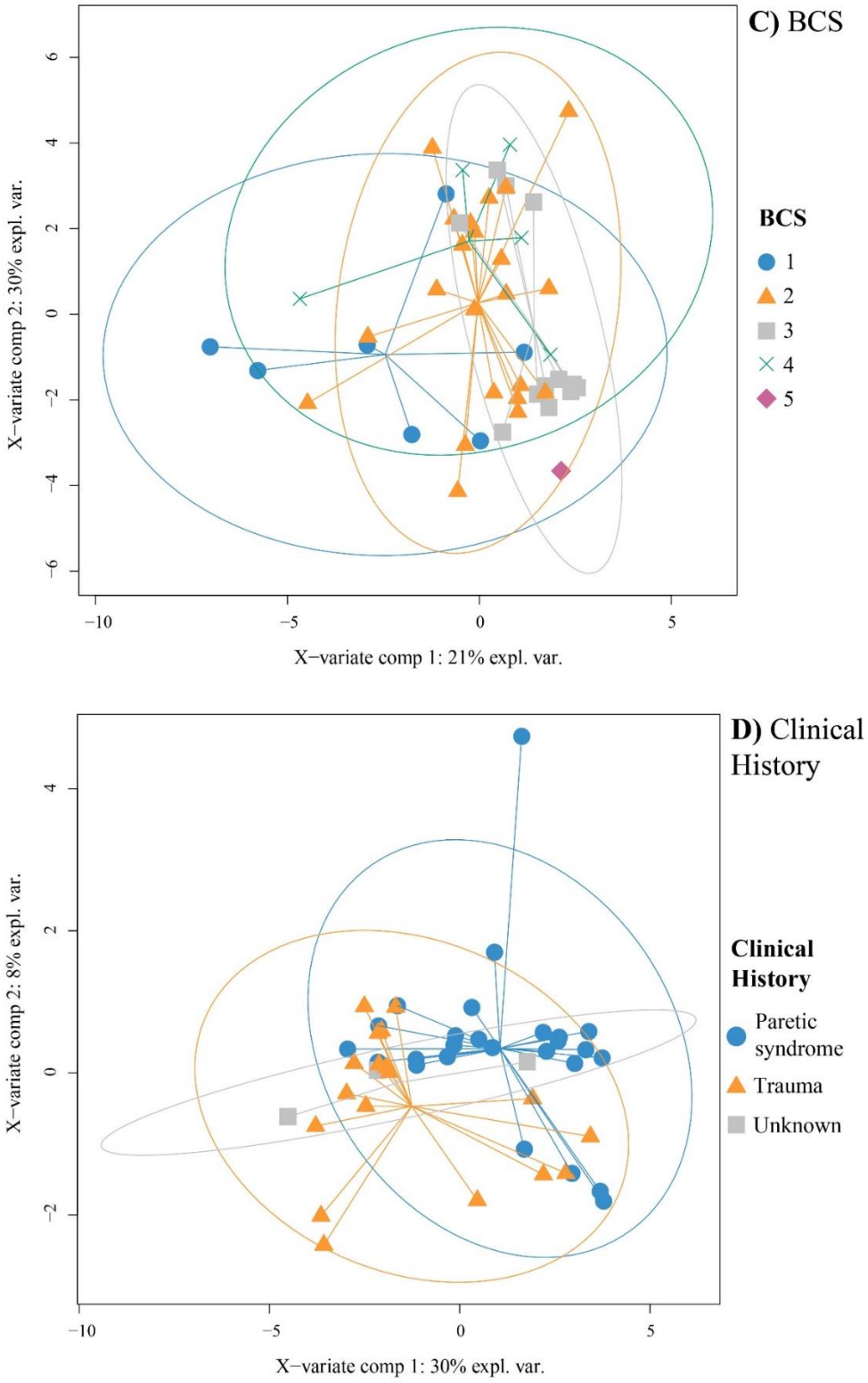


Figure S4.2. Partial Least Squares Discriminant Analysis (PLS-DA) score plots (component 1 and component 2 in each graph) of 47 gulls' adipose tissue fatty acids mean percentages (arcsine transformed) separated according to **A)** age (immature, 1 to 3 years; adult, more than 3 years old); **B)** sex (female; male; unknown / indetermined); **C)** BCS (Body Condition Score, index from 1, lean, to 5, obese) and **D)** clinical history or cause of death (paretic syndrome; trauma; unknown / indetermined). Each symbol represents each necropsied gull. The percentage of variance in fatty acids explained by component 1 and component 2 is noted below component 1 and to the left of component 2, respectively, on each graph. Coloured ellipses represent 95% confidence intervals.

Table S4.2. Variance in fatty acids (FAs) composition explained by each component in each Partial Least Squares Discriminant Analysis (PLS-DA) and Partial Least Squares Regression (PLSR) of 47 gulls' adipose tissue FAs mean percentages (arcsine transformed) separated according to wildlife rescue centre (Centro de Recuperação do Parque Biológico de Gaia, PBGaia; Centro de Recuperação de Animais Silvestres de Lisboa, LxCRAS and Centro de Recuperação e Investigação de Animais Selvagens, RIAS), species (yellow-legged gull *Larus michahellis*, lesser black-backed gull *Larus fuscus*), age (immature, 1 to 3 years, adult, more than 3 years old), sex (male, female, unknown / indetermined); BCS (Body Condition Score, index from 1, lean, to 5, obese), clinical history or cause of death (paretic syndrome, trauma, unknown / indetermined) and mass of ingested anthropogenic materials. Individuals from PBGaia represent the most urbanized gull population, gulls from RIAS represent the most natural individuals and LxCRAS individuals are both natural and urban dwellers. NA = Not Applicable (comp was not selected for further analysis).

		Comp1	**Comp2**	**Comp3**	**Comp4**	**Comp5**
PLS-DA	Wildlife Rescue Centre	0.302	0.127	0.129	0.1	NA
	Species	0.257	0.119	0.148	0.124	0.098
	Age	0.142	0.166	0.119	0.236	NA
	Sex	0.184	0.17	0.176	0.165	NA
	BCS	0.214	0.298	0.052	0.066	0.109
	Clinical History	0.298	0.081	0.174	0.093	0.096
PLSR	Refuse Mass	0.303	0.039	0.109	0.143	0.114

Table S4.3. Means and ranges of mass, size and number of items per individual of anthropogenic debris items present in 23 yellow-legged (YLG, *Larus michahellis*) and 24 lesser black-backed (LBBG, *Larus fuscus*) gulls from three wildlife rescue centres (Centro de Recuperação do Parque Biológico de Gaia, PBGaia; Centro de Recuperação de Animais Silvestres de Lisboa, LxCRAS and Centro de Recuperação e Investigação de Animais Selvagens, RIAS). Individuals from PBGaia represent the most urbanized gull population, gulls from RIAS represent the most natural individuals and LxCRAS individuals are both natural and urban dwellers. Sample sizes (total number of individuals) per rescue centre and species is presented below each species. Category "Other" includes metal, fabric, rubber and paper items. Threadlike plastics did not occur in any individual. No. Items = number of measured / weighted items. SD = Standard Deviation. NA = Not Applicable.

		PBGaia		**LxCRAS**		**RIAS**		**TOTAL**[1] (n = 47)
		YLG (n = 10)	LBBG (n = 2)	YLG (n = 4)	LBBG (n = 11)	YLG (n = 9)	LBBG (n = 11)	
ALL MATERIALS[2]	Frequency of Occurrence (FO, %)	80	50	0	45.5	55.6	54.6	53.2
	No. Items	25	12	0	10	20	33	100
	Mean Items Per Individual ± SD	2.50 ± 2.42	6.00 ± 8.49		0.91 ± 1.76	2.22 ± 3.15	3.00 ± 4.20	2.13 ± 3.25
	Range Items Per Individual (min. - max.)	0 - 7	0 - 12		0 - 6	0 - 9	0 - 14	0 - 14
	Mean Mass (g) ± SD	0.0108 ± 0.0115	0.0880 ± NA		0.0362 ± 0.0278	0.0713 ± 0.1358	0.1741 ± 0.3043	0.0703 ± 0.1633
	Range Mass (min. - max.)	0.0001 - 0.0338	0.0880 - 0.0880		0.0055 - 0.0780	0.0013 - 0.3132	0.0034 - 0.7867	0.0001 - 0.7867
	Mean Size (mm) ± SD	5.87 ± 2.83	16.5 ± NA		6.68 ± 1.56	4.34 ± 2.50	10.62 ± 6.45	7.29 ± 4.61
	Range Size (min. - max.)	2.5 - 11	16.5 - 16.5		5 - 9	2.9 - 8.87	4.75 - 23	2.5 - 23
GLASS	Frequency of Occurrence (FO, %)	10	0	0	18.2	22.2	27.3	17
	No. Items	2	0	0	2	7	5	16
	Mean Items Per Individual ± SD	0.20 ± 0.63			0.18 ± 0.40	0.78 ± 1.56	0.45 ± 0.93	0.34 ± 0.89
	Range Items Per Individual (min. - max.)	0 - 2			0 - 1	0 - 4	0 - 3	0 - 4
	Mean Mass (g) ± SD	0.0126 ± NA			0.0492 ± 0.0407	0.1664 ± 0.1918	0.1420 ± 0.1198	0.1087 ± 0.1156
	Range Mass (min. - max.)	0.0126 - 0.0126			0.0204 - 0.0780	0.0307 - 0.3020	0.0265 - 0.2657	0.0126 - 0.3020
	Mean Size (mm) ± SD	4 ± NA			7.5 ± 2.12	5.40 ± 2.45	10.56 ± 5.68	7.68 ± 4.21
	Range Size (min. - max.)	4 - 4			6 - 9	3.67 - 7.13	4.67 - 16	3.67 - 16

[1]Items were found in the gizzards of the individuals, except 1 metal item detected in the cloaca of a LBBG from RIAS, 3 paper items detected in the mouth of a LBBG from RIAS and 1 metal item detected in the proventriculus of a YLG from RIAS. These items were classified in the category "Other".

[2] Overall, most gull individuals presented also sand, small rocks and vegetation in their gizzards.

Table S4.3. *cont.*

		PBGaia		LxCRAS		RIAS		TOTAL (n = 47)
		YLG (n = 10)	LBBG (n = 2)	YLG (n = 4)	LBBG (n = 11)	YLG (n = 9)	LBBG (n = 11)	
WOOD	Frequency of Occurrence (FO, %)	50	50	0	9.1	11.1	27.3	23.4
	No. Items	18	1	0	6	1	4	30
	Mean Items Per Individual ± SD	1.80 ± 2.49	0.50 ± 0.71		0.55 ± 1.81	0.11 ± 0.33	0.36 ± 0.67	0.64 ± 1.57
	Range Items Per Individual (min. - max.)	0 - 7	0 - 1		0 - 6	0 - 1	0 - 2	0 - 7
	Mean Mass (g) ± SD	0.0110 ± 0.0129	0.0036 ± NA		0.0467 ± NA	0.0008 ± NA	0.0031 ± 0.0013	0.0105 ± 0.0151
	Range Mass (min. - max.)	0.0025 - 0.0338	0.0036 - 0.0036		0.0467 - 0.0467	0.0008 - 0.0008	0.0017 - 0.0041	0.0008 - 0.0467
	Mean Size (mm) ± SD	5.82 ± 2.04	5.5 ± NA		7.42 ± NA	3 ± NA	4.33 ± 0.76	5.27 ± 1.78
	Range Size (min. - max.)	3.71 - 8.67	5.5 - 5.5		7.42 - 7.42	3 - 3	3.5 - 5	3 - 8.67
OTHER	Frequency of Occurrence (FO, %)	0	0	0	0	22.2	36.4	12.8
	No. Items	0	0	0	0	3	15	18
	Mean Items Per Individual ± SD					0.33 ± 0.71	1.36 ± 3.01	0.38 ± 1.54
	Range Items Per Individual (min. - max.)					0 - 2	0 - 10	0 - 10
	Mean Mass (g) ± SD					0.0018 ± 0.0005	0.1416 ± 0.2320	0.0950 ± 0.1936
	Range Mass (min. - max.)					0.0014 - 0.0021	0.0034 - 0.4870	0.0014 - 0.4870
	Mean Size (mm) ± SD					2.75 ± 0.35	14.49 ± 10.59	10.58 ± 10.20
	Range Size (min. - max.)					2.5 - 3	7 - 30	2.5 - 30
PLASTICS	Frequency of Occurrence (FO, %)	40	50	0	18.2	33.3	36.4	29.8
	No. Items	5	11	0	2	9	9	36
	Mean Items Per Individual ± SD	0.50 ± 0.71	5.50 ± 7.78		0.18 ± 0.41	1.00 ± 1.80	0.82 ± 1.25	0.77 ± 1.87
	Range Items Per Individual (min. - max.)	0 - 2	0 - 11		0 - 1	0 - 5	0 - 3	0 - 11
	Mean Mass (g) ± SD	0.0047 ± 0.0032	0.0844 ± NA		0.0180 ± 0.0177	0.0065 ± 0.0045	0.0107 ± 0.0156	0.0144 ± 0.0226
	Range Mass (min. - max.)	0.0001 - 0.0072	0.0844 - 0.0844		0.0055 - 0.0305	0.0013 - 0.0091	0.0011 - 0.0340	0.0001 - 0.0844
	Mean Size (mm) ± SD	6 ± 3.58	17.5 ± NA		5.5 ± 0.71	7.19 ± 6.92	9.31 ± 5.92	7.95 ± 5.32
	Range Size (min. - max.)	2.5 - 11	17.5 - 17.5		5 - 6	2.9 - 15.17	3.5 - 16	2.5 - 17.5

Table S4.3. *cont.*

		PBGaia		LxCRAS		RIAS		TOTAL (n = 47)
		YLG (n = 10)	LBBG (n = 2)	YLG (n = 4)	LBBG (n = 11)	YLG (n = 9)	LBBG (n = 11)	
Sheet Plastics	Frequency of Occurrence (FO, %)	0	50	0	0	11.1	18.2	8.5
	No. Items	0	11	0	0	1	3	15
	Mean Items Per Individual ± SD		5.50 ± 7.78			0.11 ± 0.33	0.27 ± 0.65	0.32 ± 1.63
	Range Items Per Individual (min. - max.)		0 - 11			0 - 1	0 - 2	0 - 11
	Mean Mass (g) ± SD		0.0844 ± NA			0.0006 ± NA	0.0014 ± 0.0004	0.0219 ± 0.0417
	Range Mass (min. - max.)		0.0844 - 0.0844			0.0006 - 0.0006	0.0011 - 0.0016	0.0006 - 0.0844
	Mean Size (mm) ± SD		17.5 ± NA			40 ± NA	16.25 ± 0.35	22.5 ± 11.68
	Range Size (min. - max.)		17.5 - 17.5			40 - 40	16 - 16.5	16 - 40
Fragment Plastics	Frequency of Occurrence (FO, %)	20	0	0	9.1	22.2	9.1	12.8
	No. Items	3	0	0	1	3	3	10
	Mean Items Per Individual ± SD	0.30 ± 0.67			0.09 ± 0.30	0.33 ± 0.71	0.27 ± 0.90	0.21 ± 0.62
	Range Items Per Individual (min. - max.)	0 - 2			0 - 1	0 - 2	0 - 3	0 - 3
	Mean Mass (g) ± SD	0.0057 ± 0.0008			0.0305 ± NA	0.0049 ± 0.0051	0.0340 ± NA	0.0143 ± 0.0142
	Range Mass (min. - max.)	0.0051 - 0.0062			0.0305 - 0.0305	0.0013 - 0.0085	0.0340 - 0.0340	0.0013 – 0.0340
	Mean Size (mm) ± SD	8.25 ± 3.89			6 ± NA	3.13 ± 0.53	3.5 ± NA	5.38 ± 3.03
	Range Size (min. - max.)	5.5 - 11			6 - 6	2.75 - 3.5	3.5 - 3.5	2.75 - 11
Foamed Plastics	Frequency of Occurrence (FO, %)	20	0	0	9.1	11.1	18.2	12.8
	No. Items	2	0	0	1	5	3	11
	Mean Items Per Individual ± SD	0.20 ± 0.42			0.09 ± 0.30	0.56 ± 1.67	0.27 ± 0.65	0.23 ± 0.81
	Range Items Per Individual (min. - max.)	0 - 1			0 - 1	0 - 5	0 - 2	0 - 5
	Mean Mass (g) ± SD	0.0037 ± 0.0050			0.0055 ± NA	0.0090 ± NA	0.0031 ± 0.0033	0.0047 ± 0.0036
	Range Mass (min. - max.)	0.0001 - 0.0072			0.0055 - 0.0055	0.0090 - 0.0090	0.0007 - 0.0054	0.0001 - 0.0090
	Mean Size (mm) ± SD	3.75 ± 1.77			5 ± NA	2.90 ± NA	4.88 ± 0.53	4.19 ± 1.19
	Range Size (min. - max.)	2.5 - 5			5 - 5	2.90 - 2.90	4.5 - 5.25	2.5 - 5.25

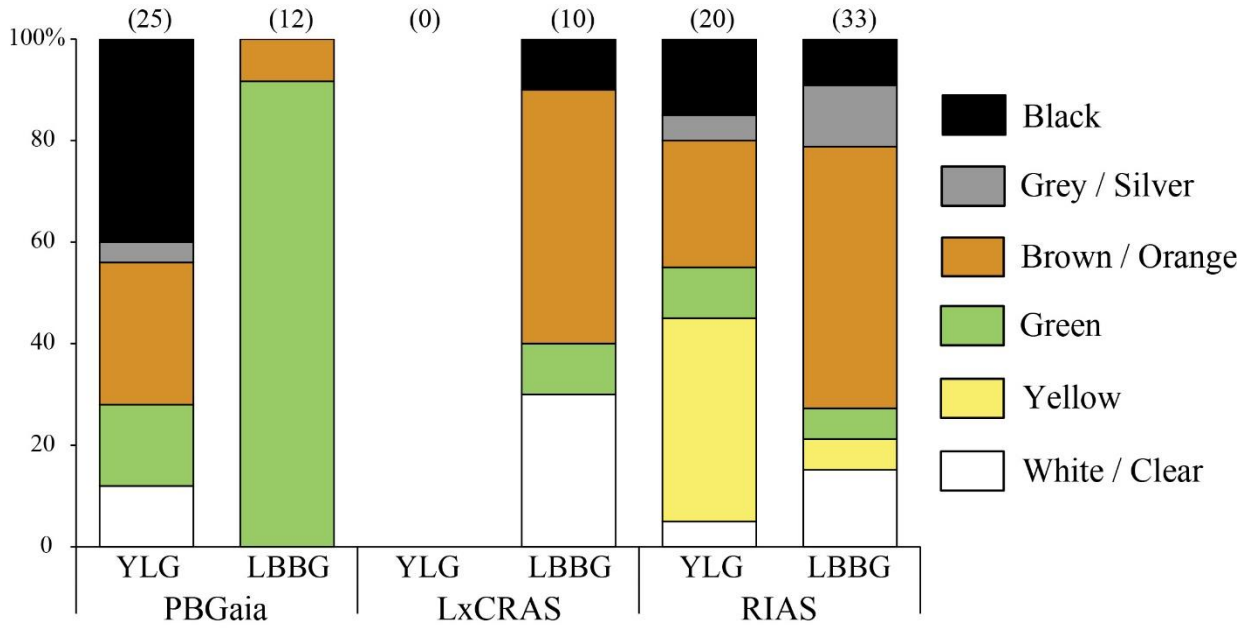


Figure S4.3. Frequency of occurrence (FO, %) of anthropogenic debris colours present in 23 yellow-legged (YLG, *Larus michahellis*) and 24 lesser black-backed (LBBG, *Larus fuscus*) gulls from three wildlife rescue centres (Centro de Recuperação do Parque Biológico de Gaia, PBGaia; Centro de Recuperação de Animais Silvestres de Lisboa, LxCRAS and Centro de Recuperação e Investigação de Animais Selvagens, RIAS). Individuals from PBGaia represent the most urbanized gull population, gulls from RIAS represent the most natural individuals and LxCRAS individuals are both natural and urban dwellers. Number of analysed items per wildlife rescue centre and species is presented on the top of each bar.

Table S4.4. Linear regression results testing the relationship between ingested anthropogenic materials' mass and number of anthropogenic items on body mass at necropsy of yellow-legged (*Larus michahellis*, YLG) and lesser black-backed (*Larus fuscus*, LBBG) gulls originating from three wildlife rescue centres (Centro de Recuperação do Parque Biológico de Gaia, PBGaia; Centro de Recuperação de Animais Silvestres de Lisboa, LxCRAS and Centro de Recuperação e Investigação de Animais Selvagens, RIAS). Individuals from PBGaia represent the most urbanized gull population, gulls from RIAS represent the most natural individuals and LxCRAS individuals are both natural and urban dwellers. 1) *per* species separately, 2) *per* wildlife rescue centre separately and 3) all data together.

	Species / Centre	Mass of Anthropogenic Materials					Number of Anthropogenic Items				
		β ± SE	r^2	p	F	df	β ± SE	r^2	p	F	df
1	YLG	321.43 ± 363.3	0.036	0.386	0.783	1. 21	6.92 ± 9.03	0.027	0.452	0.586	1. 21
	LBBG	652.4 ± 454.3	0.089	0.166	2.062	1. 21	9.75 ± 4.37	0.184	**0.036**	4.973	1. 22
2	PBGaia	-1875 ± 1371.3	0.158	0.202	1.87	1. 10	-9.99 ± 9.95	0.092	0.339	1.01	1. 10
	LxCRAS	-604.1 ± 961.6	0.030	0.541	0.395	1. 13	-2.5 ± 14.58	0.002	0.867	0.029	1. 13
	RIAS	571.4 ± 370.5	0.123	0.141	2.379	1. 17	12.91 ± 7.24	0.15	0.091	3.18	1. 18
3	ALL	158.6 ± 140	0.028	0.264	1.282	1. 45	7.8 ± 5.26	0.047	0.145	2.2	1. 45

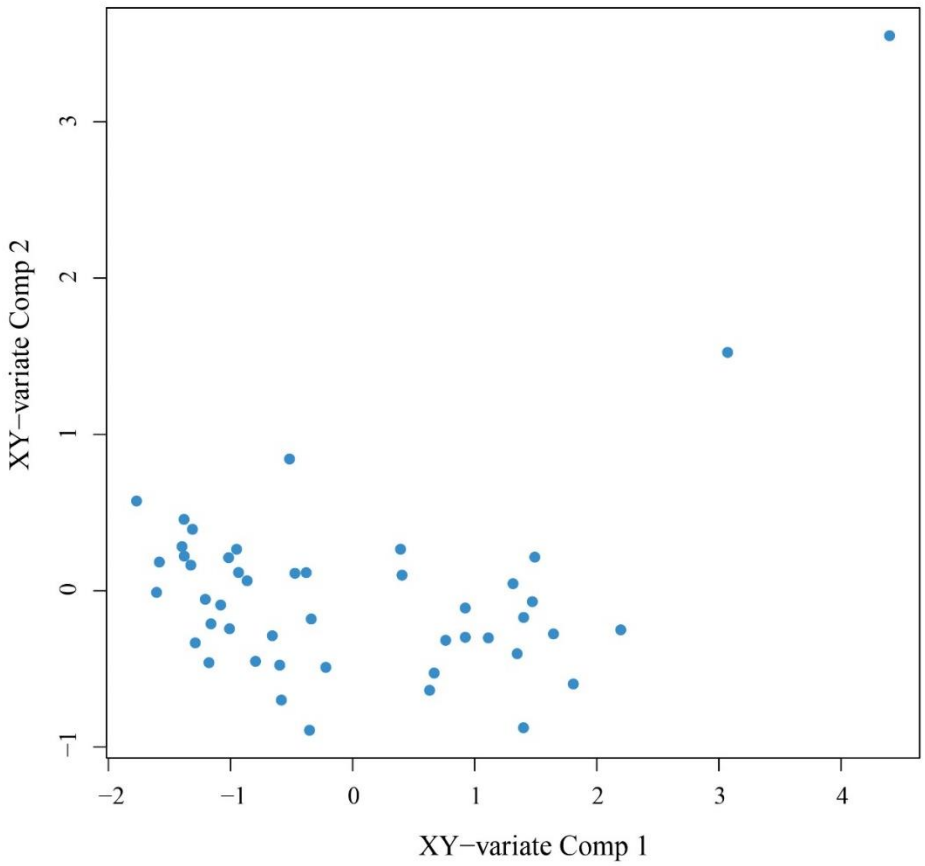


Figure S4.4. Partial Least Squares (PLS) score plot (component 1 and component 2) of 47 gulls' adipose tissue fatty acids mean percentages (arcsine transformed) plotted according to the mass of anthropogenic materials detected on each gull. Each symbol represents each necropsied gull. 30% and 4% of the variance in fatty acids is explained by component 1 and component 2, respectively.

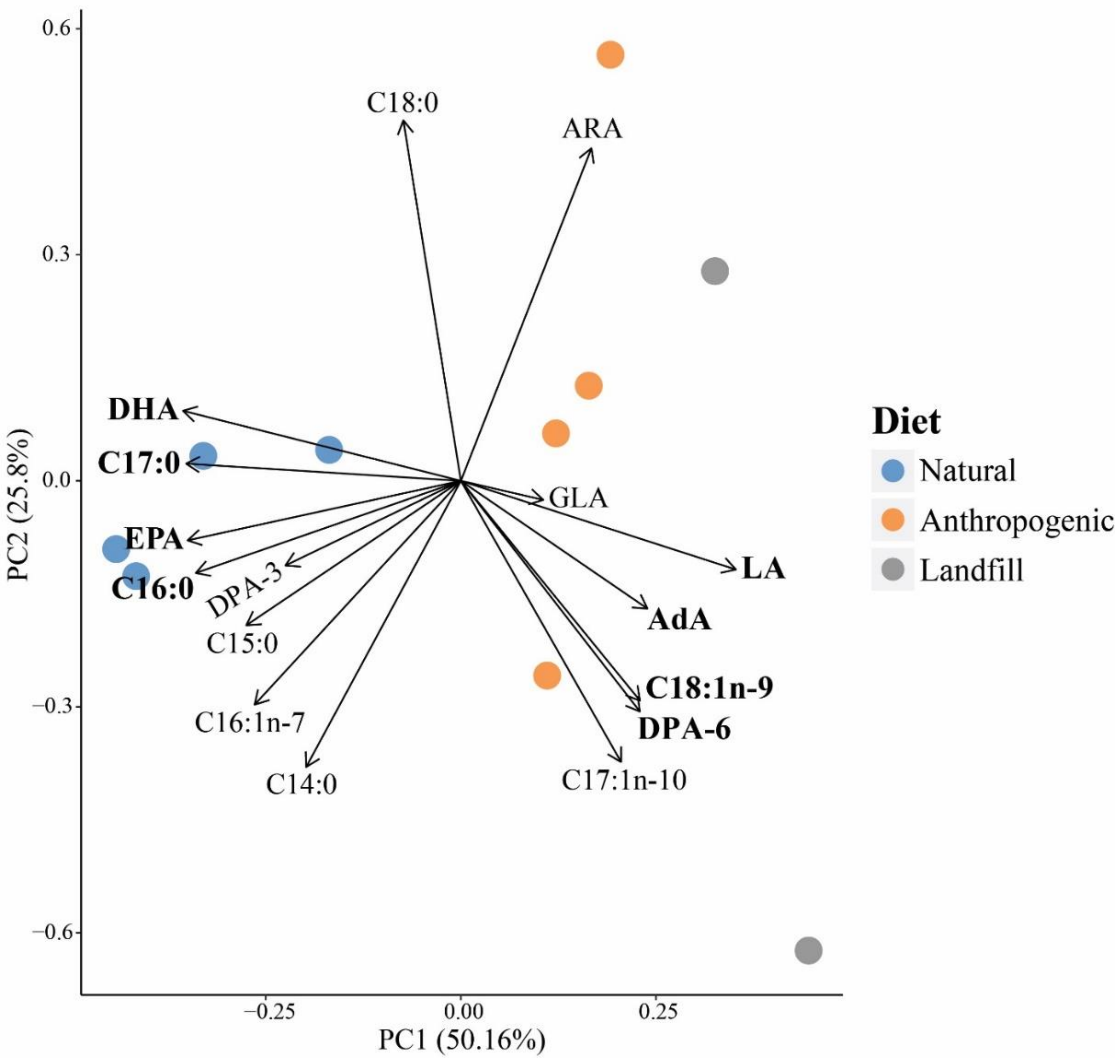


Figure S5.1. Principal component analysis (PCA) biplot (PC1 and PC2) of plasma fatty acids mean percentages (arcsine transformed) in the final time sampling (T_f), after a 14-days feeding experiment of diet manipulation of two gull species (yellow-legged gull *Larus michahellis* and lesser black-backed gull *Larus fuscus*, n = 8) being submitted to either a "natural" (n = 4) or an "anthropogenic" diet (n = 4), under controlled conditions. Landfill-caught individuals (n = 2) are also included. Individuals submitted to each diet are defined with different colours: natural diet gulls in blue, anthropogenic diet gulls in orange, landfill diet gulls in grey. The more important FAs in explaining variation along PC1 and PC2 are highlighted with a larger font, in bold.