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Necropsy findings in two marine bird species with paretic syndrome reveal severe damage

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Necropsy findings in two marine bird species with paretic syndrome reveal severe damage

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ABSTRACT

Climate change, as well as polluted and degraded environments have been the focus of concern for a long time. The degradation caused by human activities has been noted to cause various problems in various ecosystems. In this study, the degradation in the environment is suggested to be noted in necropsy findings performed to two marine bird species. The Algarve area in Southern Portugal has witnessed more and more cases of paretic syndrome (PS) within wild animals; debilitated, unable to move and in worst cases barely breathing. The animals have been received by the RIAS Wildlife Rehabilitation and Research Centre. The preliminary results obtained by RIAS suggest the ingestion of *Clostridium botulinum* -bacteria being behind the syndrome. Here, abnormalities found in necropsies (n=446) performed to Yellow-legged gulls (*Larus michahellis*) and Lesser black-backed gulls (*Larus fuscus*), who died due to PS, demonstrate the damage the birds have experienced. The majority of the birds were found to be emaciated, third of the gulls were suffering from renal damage and ceased excretory system that manifested as abnormalities in cloaca occurred in 10 % of the gulls. Other, less common findings (<10 %) were pancreatic damage and aspergillosis. The voluntary movement of the gulls and processes of autonomic nervous system were obstructed and the birds went through a gradual death. In those cases, where the bird had supposedly digested a large amount of the causative agent, the condition worsened rapidly, leading to suffocation due to paralysed muscular and autonomic nervous system. Various anthropogenic activities have been shown to favour the growth of *C. botulinum* bacteria, which naturally exists in soils, aquatic sediments as well as low-acid, warm and anaerobic conditions. Favourable conditions may cause high concentrations of the bacteria, resulting in deaths and altered structure of food webs. This in turn threatens the biodiversity we highly depend on.

Keywords: Paretic syndrome, Necropsy, Marine birds, Biotoxins, Environmentalism

RESUMO

As mudanças climáticas, ambientes poluídos e degradados são há muito alvo de preocupação. Vários estudos têm sido realizados para apontar os fatores por trás da degradação, como práticas agrícolas, produção de energia, destruição de zonas úmidas costeiras, urbanização em expansão, bem como práticas de processamento de resíduos urbanos. Várias melhorias foram implementadas para melhorar a situação e mais esforço tem sido feito ultimamente na proteção do meio ambiente. No entanto, o ritmo das mudanças climáticas, assim como a degradação, é rápido, e as consequências da degradação causada pelas atividades antropogénicas, na maioria das vezes representadas em números, têm sido muito amplas para que possamos assimilar e o bem-estar económico dos humanos superou o interesse pela manutenção de ambientes intocados. O valor da própria natureza é desconsiderado e o interesse global visa práticas que exploram a natureza ao invés de valorizá-la. A justificativa para o comportamento destrutivo está disfarçada em operações que melhoram o bem-estar da espécie humana e, portanto, a nossa atenção é canalizada para sentir empatia com nossa espécie, em vez de ecossistemas inteiros que estão sendo destruídos sob pressão antropogênica.

Neste estudo, são analisadas duas espécies bioindicadoras que refletem o estado do meio ambiente e o problema reflete-se em problemas de saúde que ocorrem nas espécies de aves marinhas em estudo; Gaivota-de-dorso-preto (Larus fuscus) e Gaivota-de-patas-amarelas (Larus michahellis). O sul de Portugal, uma área em rápida urbanização e uma atração turística popular, tem testemunhado cada vez mais casos de síndrome parética (SP) em animais selvagens; esta produz debilidade e incapacidade de movimento e, na pior das hipóteses, dificuldades respiratórias. Os animais foram recebidos pelo Centro de Reabilitação e Investigação da Vida Selvagem RIAS, onde os animais são reabilitados e devolvidos à natureza. Infelizmente, em alguns casos a síndrome já é muito grave, resultando em morte lenta e gradual do animal. As etiologias por trás da síndrome na literatura incluem biotoxinas produzidas por bactérias e algas, doenças infecciosas, deficiências nutricionais e poluição. Embora a ocorrência natural dessas substâncias no ambiente possa por si só causar PS, todas elas também podem estar associadas ao aumento da atividade antropológica destrutiva, como práticas que causam mudanças climáticas, eutrofização e destruição de habitats naturais. Embora não confirmados neste estudo, os resultados preliminares obtidos pela RIAS apontam o

botulismo causado pela ingestão da bactéria Clostridium botulinum como etiologia da síndrome.

Aqui, anormalidades encontradas em necropsias realizadas em L. michahellis e L. fuscus, que morreram devido a PS, permitiram observar os danos que as aves sofreram. L. fuscus é uma espécie migradora que utiliza o Sul de Portugal como zona de invernada e de paragem, enquanto L. michahellis habita a área durante todo o ano. Destas espécies, apenas L. michahellis se reproduz no Sul de Portugal, mas como outras aves migratórias, L. fuscus depende muito da alimentação que o Sul de Portugal tem para oferecer. Os achados nestas aves demonstram a ameaça que existe à sua saúde no ambiente em que estas aves vivem. No entanto, a ameaça não diz respeito apenas a estas duas espécies, mas a todo o ecossistema em que existe, embora algumas espécies apenas indirectamente. O estudo de espécies bioindicadoras, como a gaivota, dá-nos valiosas informações físicas, químicas e biológicas que nos permitem desenhar procedimentos adequados para o trabalho de conservação e, assim, evitar o agravamento do problema e, de preferência, melhorar a situação.

Os exames post-mortem desempenham um papel crucial no estudo da mortalidade. Eles datam de milhares de anos atrás e nos permitiram construir uma compreensão profunda da anatomia. Além disso, o diagnóstico patológico, composto pelo exame post-mortem e exames laboratoriais de apoio, permite-nos formar dados de monitorização das espécies em estudo e do ambiente em que vivem; reconhecer precocemente doenças infecciosas emergentes; melhorar o atendimento ao paciente; e promover conhecimento sobre anatomia e manifestação de doenças. Nas necropsias realizadas, a condição física da ave foi avaliada com base na presença de reservas de gordura, sendo registadas quaisquer anormalidades encontradas nos órgãos da ave. Além disso, foram registados parâmetros populacionais como sexo, idade e espécie, e estudada a suscetibilidade à síndrome desses diferentes grupos.

As aves neste estudo foram encontradas frequentemente emaciadas, sofrendo de desidratação, fome, danos renais, sistema excretor cessado e danos pancreáticos. A maioria das aves não conseguiu procurar comida devido à sua condição, resultando em desidratação e fome, contribuindo para os danos observados em seus corpos e enfraquecendo-os ainda mais. Seu metabolismo foi obstruído e as aves sofreram uma morte

gradual e dolorosa que durou vários dias. As aves que foram diagnosticadas com boa pontuação de condição corporal (BCS) e não demonstraram anormalidades na necropsia, terão ingerido uma grande quantidade do agente causador muito recentemente.

Isso resultou em rápida progressão da condição e terá levado à asfixia devido à paralisia muscular e do sistema nervoso autônomo. Quer a quantidade da toxina ingerida tenha sido grande ou pequena, as últimas horas ou dias da ave foram, sem dúvida, fisicamente desagradáveis. Devido ao comportamento defensivo em gaivotas ainda alertas, é mais provável que as aves tenham permanecido conscientes das mudanças ocorridas em seus corpos desde o aparecimento dos primeiros sintomas até a morte.

L. michahellis habita a área durante todo o ano, razão pela qual foi mais representada que L. fuscus. O sexo da ave demonstrou não ter significância na suscetibilidade da síndrome. As diferenças nos hábitos alimentares entre as diferentes idades e durante a reprodução, incubação e época de criação dos adultos, bem como o maior número de adultos habitando a área de estudo, resultaram em maior número de PS nos adultos em comparação aos filhotes, juvenis e subadultos. Os hábitos alimentares da ave foram apontados como a característica mais notável que determina a ocorrência da síndrome.

Neste estudo, as consequências da PS em aves são apresentadas de forma detalhada, e não apenas em números, também com registo fotográfico. Espera-se com isso mostrar que a ciência não descarta os valores imensuráveis, aos quais devemos prestar mais atenção. Pelo contrário, a informação baseada na experiência que está fortemente construída em nossa biologia e que nós, como cientistas, somos capazes de avaliar objetiva e criticamente, é um acréscimo valioso que pode apoiar os valores mensuráveis e seu papel deve ser mais enfatizado nos estudos científicos.

Embora C. botulinum ocorra naturalmente no meio ambiente e a saúde humana não esteja diretamente ameaçada por isso, o aumento das atividades antrópicas está associado ao crescimento dessa bactéria, resultando em frequentes e recorrentes mortes em massa de animais, especialmente aves. Além disso, isso tende a alterar a estrutura das teias alimentares, causando desequilíbrio na estrutura trófica. Esses eventos, por sua vez, deterioram a biodiversidade global da qual dependemos profundamente.

Palavras-chave: Síndrome parética, Necropsia, Aves marinhas, Biotoxinas, Ambientalismo

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TABLE OF CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

LIST OF ABBREVIATIONS

ABPA - Allergic bronchopulmonary aspergillosis

AI – Avian influenza

ASP – Amnesic shellfish poisoning

BCS – Body condition scoring

BONTs – Botulinum neurotoxins

CKD – Chronic kidney disease

DSP – Diarrheal shellfish poisoning

EIDs – Emerging infectious diseases

EPPPs – Environmentally persistent pharmaceutical pollutants

HABs – Harmful algal blooms

HPAI – High pathogenic avian influenza

LPAI – Low pathogenic avian influenza

NDV – Newcastle disease virus

NSP – Neurotoxic shellfish poisoning

OC – Organochlorine compounds

POPs – Persistent organic pollutants

PPCPs – Pharmaceuticals and personal care products

PS – Paretic syndrome

PSP – Paralytic shellfish poisoning

SST – Sea surface temperature

USUV – Usutu virus

VIP – Vasoactive intestinal polypeptides

WNV – West-Nile virus

STATE OF THE ART

Both marine and terrestrial ecosystems face the very noted threat of eutrophication due to increased nutrient inputs (Smith *et al.* 1999) and higher sea surface temperatures SST (Nazari-Shabarian *et al.* 2018) caused by climate change (Scafetta 2010). The pollution of coastal waters due to cultural eutrophication i.e., eutrophication of human origin, has been studied to result from population growth (Wassmann 2004), food production -especially dietary changes to more meat-based diet (Steinfeld *et al.* 2006) as well as from use of inorganic fertilizers (Howarth *et al.* 2002), energy production, especially use of fossil fuels (Nazari-Shabarian *et al*. 2008) and consumption of non-food products overall (Hamilton *et al.* 2018). In many areas, agricultural pollution threatening the inland and coastal waters has grown to exceed those of industrial and municipal pollution (Mateo-Sagasta *et al.* 2017).

Livestock production has been causing water contamination due to ammonia emissions that count 64% of total anthropogenic ammonia emissions and nitrous oxide, counting 65% of total cultural nitrous oxide emissions (Steinfeld *et al.* 2006). Both of these resulting greatly from deposited and applied manure of livestock (Steinfeld *et al.* 2006). Besides these, agricultural activities associated with water contamination include runoff from crop fields that include eutrophicating fertilizers ((Howarth *et al.* 2002) and veterinary medicines used in animal agriculture (Steinfeld *et al.* 2006). These have been shown to find their ways to aquatic food webs and therefore accumulate in the marine organisms causing possibly invisible irreversible damages (Daughton *et al.* 1999). The changes caused by pharmaceuticals; Environmentally Persistent Pharmaceutical Pollutants (EPPPs) as well as impersistent pharmaceuticals and active compounds in personal care products (PPCPs) produced by both livestock production in case of medicines (Steinfeld *et al.* 2006) and human consumption of medicines, cleaning and hygiene products (Daughton *et al.* 1999). Both are known to affect the microbial communities in aquatic environments (Labbate *et al.* 2016) and find their ways into ground water (Jones *et al.* 2001). Although being impersistent, the PPCPs are discharged to marine environments on a continuous cycle, maintaining a high concentration within the ecosystem, possibly causing multigenerational changes

(Daughton *et al*. 1999). Animal agriculture threatening the aquatic ecosystems is not limited in cattle, poultry and pig farming. It can also be associated with fish farming, and thus contamination by associated EPPPs threatens also the marine ecosystems besides damaging the terrestrial ecosystems (He *et al*. 2016 and references within). Also, coastal wetland destruction under the pressure of aquacultural demands causes great damage to the vulnerable ecosystems, such as mangroves, coral reefs and seagrass beds, as well as to the health of aquatic and terrestrial organisms, that depend on these ecosystems (Aure *et al*. 1990, Steinfeld *et al.* 2006).

The cultural changes that have altered these coastal marine ecosystems to be more productive in a human perspective rather than in a nature-friendly perspective generate imbalances that manifest as reduction of our physical and mental well-being (Kellert 2003). Rather more visible imbalances can manifest as harmful algal blooms (HABs) (Watson *et al.* 2015) or excessive growth of bacteria such as botulinum neurotoxin (BoNT) producing *Clostridium botulinum* (Woodford 2009). Although both HABs and *C. botulinum* exist naturally in our environment, the excess growth of these have been studied to cause a severe condition affecting both waterfowl and other animals, named as paretic syndrome (PS). In literature, the causative agents behind PS in marine birds, are studied to include BoNTs caused by *C. botulinum* and less occurrent strains of other *Clostridium* species, cyanotoxins associated with cyanotoxin producing prokaryotes, such as *Anabaena, Microcyctis* and *Planktothrix* (Lopez-Rodas *et al.* 2008, Eriksson *et al.* 1986, Stewart *et al.* 2008), domoic acid by diatoms of the genus *Pseudonitzchia* (Work *et al.* 1993), saxitoxin by dinoflagellates of the genus *Alexandirium, Gymnodinium, Pyrodinium* and others (Dusek *et al*. 2021). Also, various infectious diseases, environmental pollutants and nutrient deficiencies have been studied to cause PS in wild birds. Thiamine (vitamin B1) deficiency, which has also been linked to botulism in humans (Balk *et al.* 2009, Ringe *et al.* 2014), as well as vitamin D deficiency, have been studied to cause gradual paralysis in wild birds (Jordan 1990). Of these agents, besides infectious diseases, the BoNT-producing *clostridium,* have been studied to cause the most devastation within the wild bird populations as well as within domestic animals.

Besides climate change (Espelund *et al.* 2014 and references within), eutrophication and practices within animal agriculture (Notermans *et al.* 1981), the excess growth of

C. botulinum has been linked to landfill sites (Ortiz *et al.* 1994), fish farming (Cann *et al.* 2006) and improper waste water treatment (Anza et al. 2014). Anza *et al.* observed in their study in 2014 the correlation between botulism outbreaks and water treatment plants that output their chemically processed waste water into lakes or wetlands. They noted that *C. botulinum* was significantly more prevalent in wetlands that received waste water input compared to control areas. They also noted, that this practice alters the salinity of the wetland, increases eutrophication due to nutrient input, alters the natural flooding and draining of the wetland and exposes the inhabiting fauna to bacterial pathogens if the wastewater is improperly processed, all together inciting the growth of *C. botulinum* (Anza *et al.* 2014).

Observing the causes behind excessive growth of BoNT-producing bacteria, we can note that besides warming climate, the factors supporting eutrophication, such as animal agriculture, fish farming and wastewater inputs, also provide more favourable conditions to harmful algae blooms -another threat to wild animal populations as well as to human health. Also, anthropogenic erosion due to agriculture has been studied to be a great threat to aquatic ecosystems, causing water-quality degradation (Steinfeld *et al.* 2006, Mateo-Sagasta *et al*. 2017). Eutrophication -resulting in abundant algae blooms have been studied to cause oxygen depletion both in fresh waters and saline waters (Foley *et al*. 2012, Rosenberg *et al.* 1988), which in turn results in higher mortality rate and more organic matter being present in an anoxic environment, that in turn favours the growth of *C. botulinum* bacteria in ecosystems with low acidity and warm temperatures.

The control of globally concerning issue; botulism, has been forwarded to individual level. The Canadian Ministry of Agriculture, Food and Rural Affairs advices to prevent botulism by following measures: “Botulism can be prevented by providing good quality feed and ensuring proper sanitation and, in humans, by following proper food canning techniques” (Pasma, 2013). Whereas Rocke and Friend (1999) gave a more comprehensive explanation to the botulism outbreaks in wetlands, where wetland flooding and draining, pesticides and pollutants from agriculture come into picture. They theorize that the act of flooding and draining as well as contaminating the environment with chemicals that are toxic to aquatic life, may result in more organic substrate for the bacteria to grow in. Other risk factors are sewage and other nutrient inputs

that can result in bloom of invertebrate populations, followed by oxygen depletion. According to the study, the most effective way of controlling botulism outbreaks would be eliminating the organic inputs to the wetlands, as well as carcass removal, thus eliminating or minimizing the growth medium of *C. botulinum*.

Other type of anthropogenic interference in wetlands may cause botulism outbreaks well; a study conducted by Circella *et al.* in 2019, demonstrated how a procedure of trying to convert a wildlife preservation area more waterfowl friendly by constructing an artificial pond with draining canals can lead to a botulism outbreak and to a mass death of various birds. The constructed pond created a new habitat for flathead grey mullets (*Mugil cephalus*), that migrated into the area and reproduced, resulting in great numbers. The summer heats of northern Italy most likely caused unsuitable temperatures for the fish, leading to their mass death and a great amount of decaying organic material in the pond. This led to an excess amount of *C. botulinum* in the pond and laboratory examination confirmed botulism mosaic type C/D being responsible for the death of the birds. In this case, the external nutrient input was the migration of *M. cephalus* -that was enabled due to human interference. Firstly, it is debatable, whether any large-scale human-driven measures should be implemented in a wildlife reserve and whether the measures made are to profit the humans visiting the area or all of the species inhabiting the area? This is a question that should be integrated in all human-driven projects that alter the natural state of an environment. It should not be completely impossible to find solutions that benefit us all. In the long-term, the measures implemented purely for human well-being e.g., economic growth, constructed environments, water reserves wasted on excrement transportation and inorganic fertilizers to name a few only deteriorate our well-being.

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Necropsy findings in two marine bird species with paretic syndrome reveal severe damage

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ABSTRACT

Climate change, as well as polluted and degraded environments have been the focus of concern for a long time. The degradation caused by human activities has been noted to cause various problems in various ecosystems. In this study, the degradation in the environment is suggested to be noted in necropsy findings performed to two marine bird species. The Algarve area in Southern Portugal has witnessed more and more cases of paretic syndrome (PS) within wild animals; debilitated, unable to move and in worst cases barely breathing. The animals have been received by the RIAS Wildlife Rehabilitation and Research Centre. The preliminary results obtained by RIAS suggest the ingestion of *Clostridium botulinum* -bacteria being behind the syndrome. Here, abnormalities found in necropsies (n=446) performed to Yellow-legged gulls (*Larus michahellis*) and Lesser black-backed gulls (*Larus fuscus*), who died due to PS, demonstrate the damage the birds have experienced. The majority of the birds were found to be emaciated, third of the gulls were suffering from renal damage and ceased excretory system that manifested as abnormalities in cloaca occurred in 10 % of the gulls. Other, less common findings (<10 %) were pancreatic damage and aspergillosis. The voluntary movement of the gulls and processes of autonomic nervous system were obstructed and the birds went through a gradual death. In those cases, where the bird had supposedly digested a large amount of the causative agent, the condition worsened rapidly, leading to suffocation due to paralysed muscular and autonomic nervous system. Various anthropogenic activities have been shown to favour the growth of *C. botulinum* bacteria, which naturally exists in soils, aquatic sediments as well as low-acid, warm and anaerobic conditions. Favourable conditions may cause high concentrations of the bacteria, resulting in deaths and altered structure of food webs. This in turn threatens the biodiversity we highly depend on.

1 INTRODUCTION

1.1 Gulls as bioindicators

The human dependence on nature in all its appearance is in modern day perceived as mainly physical and in some degrees as mental dependence (Kellert 2003). Yet, the aspects in which nature feeds our spirituality, our capacity of empathy and creativity as well as intellectuality are most often disregarded (Kellert 2003). Bioindicator species as a term originates from environmental toxicology and is defined as an organism or biological response that reflects notably the abiotic or biotic features of an environment, which can be examined by occurrence of typical symptoms or measurable responses (Mothersill *et al.* 2016 and references within). However, the dependence the bioindicator species have on their environment can be reflected on the dependence of human well-being on that very same environment (Burger *et al.* 2015). Most commonly bioindicator species are used to determine the anthropogenic effects on an ecosystem (Gerhardt 2002). It could be stated that bioindicator species are our non-subjugated test animals in an unconfined environment. Studying bioindicator species gives information on the status of the environment or its components (Burger *et al.* 1999 and references within). The information provided can also indicate environmental integrity; whether the ecosystem is biologically capable of supporting life. Besides this, they provide physical, chemical and biological information that can be used in conservation work (Egwumah *et al.* 2017).

Being ubiquitous, and many times sharing their environment, as well as diet with us humans, gulls are effective bioindicators (Burger 2010). Family *Laridae,* consisting of around 100 species, inhabit various environments from pristine lakes, marshes, deserts and seashores to urban cities, from Arctic to tropical heights (Burger *et al.* 2019). Among seabirds, gulls are considered to be some of the least specialized, feeding in various different habitats on various types of foods; fish, molluscs (Alonso *et al.* 2015), human refuse (Pierotti *et al.* 2001), fruits (Calvino-Cancela 2011), carcasses and other birds amongst others (Burger *et al.* 2019). Depending on species, their breeding habitats might vary notably as well, making them generalists (Calvino-Cancela 2011, Pierotti *et al.* 2001). These features; wide diet, that changes regarding the availability of

feed and modesty, when it comes to nesting areas, have allowed the gulls to find their function in various ecosystems and produce fledglings nearly anywhere (Sigurdsson *et al.* 2010). Due to this fact, they play an important role in dispersion of plant seeds, especially in coastal areas, and enhance the activity of ecosystems they habit by transferring resources from surrounding environments (Calvino-Cancela 2011).

Various studies note the decline in gull populations within Europe and Northern America, populations declining up to 80% within the last 30 years (Mitchell *et al.* 2004, Hario *et al.* 2016, Laurich *et al.* 2019). Whereas the overall population numbers have been decreasing, the urban populations have been studied to grow since 1970´s, possibly due to the great availability and easy access to municipal waste (Rock 2012). The decrease in overall populations has been accompanied by an increase of egg cannibalism due to decreased availability of food (Hayward *et al.* 2014). This, besides resulting from overfished fish populations, is studied to be related to rises in SST due to climate change, mitigating primary production and causing the foraging fish to move to deeper and cooler waters in search of feed and suitable habitat, where the gulls are unable to catch them (Freitas *et al.* 2021). Besides anthropogenic indirect factors threatening gull population abundance and health, direct measurements, such as culling have been implemented to control the increasing urban gull populations. Culling has been believed to control the transmission of pathogens (Bosch *et al.* 2000) and protect the sympatric, threatened bird species from predation of the gulls (Oro *et al.* 2007). However, various studies have demonstrated that in long term, the growth-rate of the colonies of these sympatric species follows positive correlation (Oro *et al.* 2007 and references within). It is also studied that colonies, where culling programs have been implemented do decrease in size. But gulls, being intellectual animals, most likely migrate to surrounding colonies and therefore transport the problem elsewhere (Bosch *et al.* 2000).

Gulls have gained a negative reputation within urban areas, where their abundance has created disgust due to their faeces and aggressive behaviour, and where their vulnerability has not been visible (Rock 2005). However, as the wild populations keep declining, more concern and study funding have been addressed to help these antagonistically viewed animals that also effectively reflect the state of the environment they inhabit (Burger 2010). Especially in the present day world, where pristine environments

are more and more scarce and where the territories of wild animals are getting smaller and smaller, studies that examine the effect of territorial overlap between wild animals and humans are valuable indicators of how anthropogenic activities alter the balance of nature (Hart *et al*. 2018). Whereas there are visual anthropogenic effects causing threat to wild animal populations, such as powerlines, traffic, traps etc., the biotoxins such as botulinum neurotoxins (BoNTs) most often remain invisible cause of debilitation or death. Yet, the biotoxins seem to possess a major threat due to their omnipresence in a more and more chemically burdened environment and due to being more difficult to trace (Rocke *et al.* 1999). It must be also noted that these very toxins are a potent threat to human health as well (Perera *et al.* 2020), accumulating in the food webs and reaching high quantities in apex predators (Corriere *et al.* 2021). Besides this, toxins, that cause mass deaths of species, disrupt the top-down control of predators, resulting in disbalance of species (Šulčius *et al.* 2017) and thus alters the food webs (Riebesell *et al.* 2018), possibly causing extinction of species (Fey *et al.* 2015) that have an important function in ecosystems they habit. We use bioindicator species to determine the state of the environment (Burger *et al.* 2001), yet it seems we have forgotten our role as bioindicators in a world where human diseases caused by polluted water, air and soil are increasing (Pimentel *et al.* 2007) and where cancer (Pimentel *et al.* 2007 and references within), pulmonary diseases (Viegi *et al.* 2006) and mental disorders (Ventriglio *et al.* 2021) are more and more common.

1.2 Study area

RIAS Wildlife Rehabilitation and Research Centre of Ria Formosa receives debilitated and wounded wild animals from all over Southern Portugal. The southern coastline of Portugal consists of estuaries formed by Arade and Guadiana rivers and Ria de Alvor and Ria Formosa lagoons, formed by coastal barrier islands. These estuaries and lagoons encompass saltmarshes, seagrass meadows and sandy beaches, enabling rich concentrations of biodiversity. Listed in the Natura 2000 network, Ria Formosa / Castro Marim reaches from Guadiana estuary to Ria Formosa. Costa Sudoeste spreads from the estuary of the Arade river in Portimão all the way to the west coast covering Ria de Alvor in Lagos (fig. 1.1). These areas are considered to play an important role in the long-term survival of many threatened species and habitats. Besides being an

important breeding and feeding site for various species as part of Natura 2000 network, the coast of Southern Portugal is also a major tourist destination. This, together with urbanization, industrialization and agriculture have caused distress to the biodiversity in this area (de Noronha Vaz *et al.* 2013).

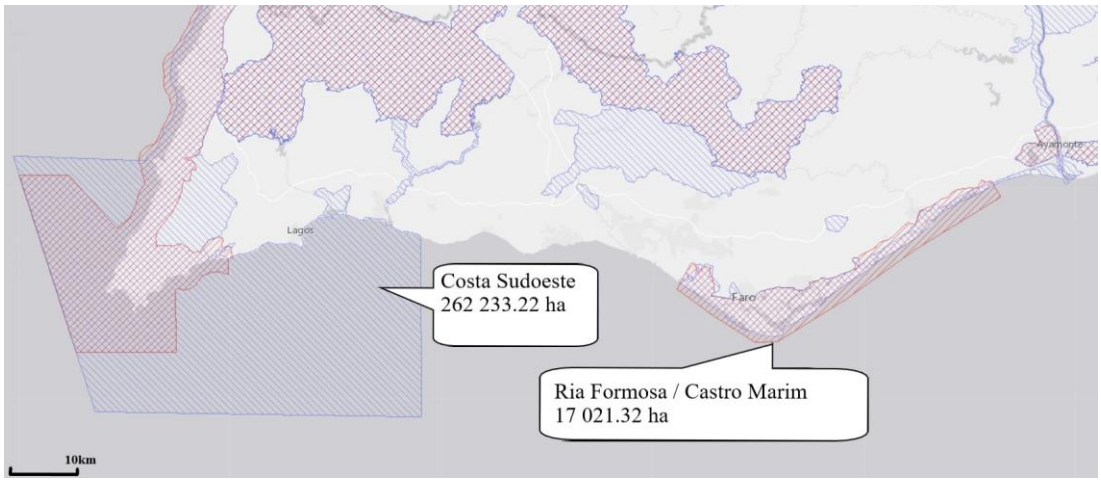


Figure 1.1 The geographical area of Southern Portugal, where represented the two Natura 2000 areas of the coastal Algarve. Retrieved from https://ec.europa.eu/environment/nature/natura2000.

Migrating birds traveling between Europe and Western Africa depend highly on Southern Portugal as a stopover to build up energy sources for the migration (Bibby *et al.* 1980). The geographical location, environmental features such as presence of wetlands and lagoons and the abundance of available feed; mosquitoes, fruits, berries and other prey offered by the wetlands, allow the birds to gather fat preserves, that enables them to continue their journey (Freitas *et al.* 2012, Thomas 1979, Vowles & Vowles *et al.* 1993). The migration periods time in spring and autumn, when the region accommodates high diversity of birds. High species density during these time periods exposes the animals to a higher risk of transmission diseases as well (Munson *et al.* 2006). These migration times coincide with the peaks of birds affected by PS being admitted to the RIAS (fig. 1.2) (Ben-Gigirey *et al.* 2012).

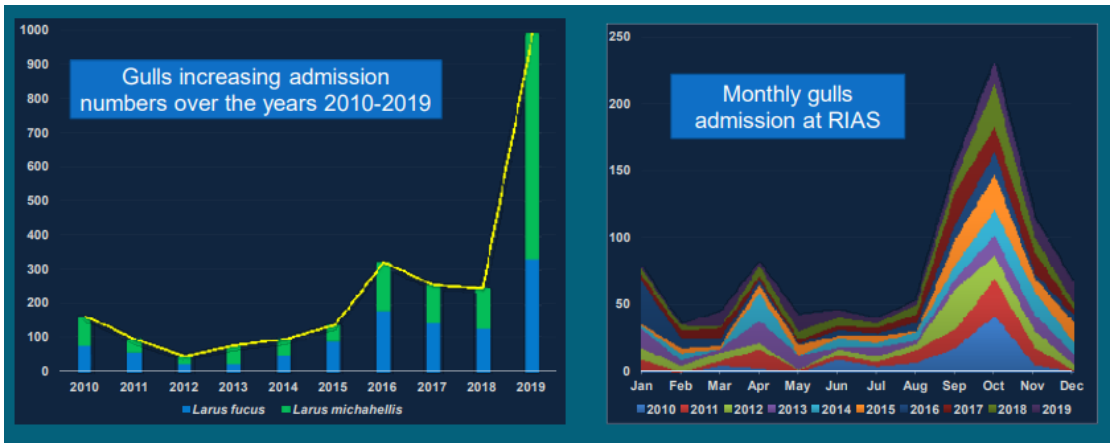


Figure 1.2 Yearly admissions of gulls *Larus fuscus* and *Larus michahellis* to RIAS (left) and monthly admissions on right demonstrating the peaks in spring and autumn. Reprinted from Paretic Syndrome in Gulls from Southern Portugal: Searching for the Causative Agent (2021) by Ben-Gigirey *et al.* First International Electronic Conference on Toxins.

1.3 Study species

L. fuscus and *L. michahellis*, both listed as least concerned by the Global IUCN 2021 Red list category, are two of the most common species admitted to RIAS due to being affected by PS. Several colonies of *L. michahellis* permanently inhabit Southern Portugal, whereas *L. fuscus* is a migratory species that breeds in summers in Northern Europe and central-northern Russia. *L. fuscus* can be found in Southern Europe, Africa and Asia during migratory periods and winters (Del Hoyo *et al.* 1996, Marques *et al.* 2009). Breeding of the species takes place only in the summer in the breeding sites listed above. Populations of *L. michahellis* are studied to be less active migrants. Many resident populations inhabit the Mediterranean Sea and coastal areas of Portugal, where breeding also occurs. Wintering grounds in migrating populations are around western coast of the Atlantic, Mediterranean coast, Red and Arabian Sea and the coast of China. Some migrating populations fly to Black Sea and Caspian Sea for breeding (Del Hoyo *et al.* 1996).

Breeding is studied to affect the dietary choices the birds make, the chicks are mainly fed with fish, whereas when not feeding chicks, the gulls depend more on terrestrial prey (Alonso *et al.* 2015). Swimming crabs (*Liocarcinus sp*.) are studied to be an important feed in the diet of both of these gulls (Alonso *et al.* 2015, Schwemmer *et al.*

2005), their high presence due to the upwelling occurring on the coast of Portugal in the summer months, high calcium-concentration and low locomotive capability is suspected to be the reason to their high consumption especially during breeding, incubation and rearing periods (Alonso *et al.* 2015).

1.4 Paretic syndrome and history of avian diseases in the area

Sudden mass deaths of birds have been recorded and studied since the beginning of the 20th century. The menace, threatening waterfowl and shorebirds, causing deaths of up to millions of wild birds from families of *Anatidae, Rallidae* and *Laridae* amongst others, was first named as Western Duck Sickness in Northern America (Giltner *et al.* 1930). It was especially noted to affect migratory waterfowl and was suspected to be caused by alkaline salts until 1930´s (Friend *et al.* 1985, Kalmbach *et al.* 1934). Since 1920´s, various studies have been conducted to define the aetiology behind the abnormal deaths of wild birds. Regularly occurring incidents of mass deaths have been studied to be caused by toxic algae, pollution, viruses, parasites, biotoxins and other anthropogenic impacts such as communication towers, powerlines, wind turbines and fireworks (Manville 2005, Stickroth 2009). Besides waterfowl, the unexplained mass deaths have been affecting humans and domestic animals, especially cattle. In the early 20th century, the condition was referred to as Midland cattle disease and as Bulbar paralysis in Australia, affecting both cattle and horses, later confirmed to be caused by BoNTs (Seddon 1922).

PS is a general name to describe the state of the animal; debilitated, unable to move, suffering from paralysis (Soares 2014 and references within). The reasons behind the syndrome can be multiple and the names given to the condition have changed within years as mentioned above. Naturally, the objective in studies regarding PS, has been to determine the aetiology behind the condition. Due to this, we have been able to recognize the risk caused to the wildlife and in some cases merge it with the cause. However, the procedures made to hinder the existing threat, have remained nearly non-existent. By date, the causes behind PS have been studied to include toxins, infectious diseases, dietary deficiencies and pollution. The condition can be as well a sum of two or more of these. The common aspects are the symptoms the syndrome causes:

diarrhoea, dropped wings, inability to fly or move, weakness, difficulties in breathing, third eyelid paralysis, possibly leading to blindness and anorexia (Ben-Gigirey *et al.* 2021).

1.4.1 Toxins

The neurotoxins produced by genus *Clostridium* are the most toxic substances known to science, due to their very small lethal dose (Bohnert *et al.* 2006). BoNT-forming anaerobic bacteria naturally exist in soil and aquatic environments and botulism outbreaks have occurred on every continent excluding Antarctica (Thomas *et al.* 2008). There are seven different known botulism types (A-G) from which C, D and a mosaic type of C/D have been associated with waterfowl, poultry, mammals and in few cases humans as well (Sidell *et al.* 1997, Oguma *et al.* 1990). The distribution of different botulinum serotypes is based on geographical location as well as environmental conditions. For example, serotype E is found in aquatic environments, both salty and fresh water (Dolman *et al.* 1963). The reason why different serotypes of BoNTs affect different species is yet unknown, as well why some species of *Clostridium* express the gene that creates the toxin and others do not, yet carry the same gene. What is interesting as well, is that the type C and D are studied to be encoded by bacteriophages (Middlebrook *et al.* 1997, Hatheway 1992).

Avian botulism (type C/D) occurs via ingestion of a potent neurotoxin either directly or via invertebrates, which are immune to the toxin and may accumulate it in their bodies (Wobeser *et al.* 1997). The time between ingestion and infection depends on the individual factors of the infected animal as well as type and quantity of toxin ingested (Lonati *et al.* 2020). The primary symptoms include incapability of flying; the bird might be standing on their tarsometatarsus, walking might be obstructed, as well as standing overall. The wings and neck are drooping, the bird suffers from diarrhoea and has difficulties with breathing. The symptoms and their severity depend on the severeness of the intoxication (Ben-Gigirey *et al*. 2021, Neimanis *et al.* 2007). The recovery of the animal depends on the severity of the condition, in other words of the entity of neuromuscular blockade caused by the toxin and of the speed of regeneration of the nervous terminals and presynaptic membranes (Lonati *et al*. 2020). Also, the most relevant factor regarding recovery, is the help provided; the conditions where it

is provided (non-stressful), the speed of it as well as the accuracy of it (Ben-Gigirey *et al.* 2021).

No confirmed outbreaks of avian botulism have occurred in Portugal, however findings from previous studies have enhanced the possibility of regularly occurring deaths of wild birds caused by *C. botulinum* (Soares 2014, Ben-Gigirey *et al.* 2021).

Besides BoNTs, toxins that are studied to cause PS are toxins produced by certain macroalgae, ingested referred to as shellfish poisoning. These toxins accumulate in immune filter feeding organisms and thus end up in food webs. The most common types of shellfish poisoning, that are caused by different organisms and toxins are:

1. Amnesic Shellfish Poisoning (ASP), resulting from ingestion of neurotoxin domoic acid (DA), produced by some strains of phytoplankton, especially strains of *Pseudo-nitzchia spp.* (Lelong *et al.* 2012 and references within).

2. Neurotoxic Shellfish Poisoning (NSP), induced by brevetoxins (BTXs) produced by dinoflagellate *Karenia brevis* and other potential strains of family *Kareniaceae* and some species of *Raphidophyceae* (Amzil *et al*. 2021 and references within).

3. Paralytic Shellfish Poisoning (PSP), induced by saxitoxins (STXs) produced by certain species of marine dinoflagellates but also freshwater cyanobacteria and some red calcareous macroalgae (Deeds *et al.* 2008 and references within).

4. Diarrheal Shellfish Poisoning (DSP), induced by okadaic acid (OA), yessotoxin (YTX) and dinophysis toxin (DTX) produced by dinoflagellates mostly from genus *Dinophysis* but also *Prorocentrum* (Aune *et al.* 1993 and references within).

From these, the two latter ones; PSP and DSP have been the most common causes of shellfish poisoning in Portugal. Due to this, recurrent seasonal bans on bivalve harvesting have been implemented in Portugal. Whereas DSP is not lethal, causing

diarrhoea, stomach ache and vomiting, PSP affects the neurological system and may be lethal by paralyzing the respiratory organs (Vale 2020).

Some species, such as Glaucous-winged gulls (*Larus glaucescens*) have learnt to avoid PSP by regurgitating feed that is poisoned by saxitoxins and to avoid the species that commonly accumulate them, and also pass the information to following generations (Kvitek 1991). Compared to other marine birds, such as European shags (*Phalacrocorax aristotelis)*, gulls seem to have a high chance of survival from PSP or a low risk of developing it (Coulson *et al.* 1968). However, various studies have noted that in the cases of bird mass deaths caused by PSP, herring gulls (*Larus argentatus*) have demonstrated to be the most vulnerable from the sympatric gull species (Coulson *et al.* 1968, Nisbet 1983).

The symptoms of PSP in marine birds vary depending on species; paralysis and convulsions seems to be common. Besides this, loss of equilibrium, inability to stand and to support the head, vomiting, and abnormal faeces have been noted in *P. aristotelis* and in *Sterna spp*. Also, inability to lay eggs and restricted functioning of pupils occur (Coulson *et al.* 1968).

1.4.2 Infectious diseases

Avian Influenza (AI) was detected in late 1800´s in poultry in Northern Italy (Lupiani *et al.* 2009) Since then, it has been repeatedly diagnosed within poultry and wild birds until 1997, when first infection within humans was diagnosed (Swayne 2009). The virus demonstrates as Low Pathogenic (LPAI) and High Pathogenic Avian Influenza (HPAI), from which the latter is responsible of various outbreaks in Europe, Russia, North America, South America, Middle East, Asia and Africa within the whole 21st century (Lupiani *et al.* 2009).

The LPAI H9N2 has been reported to become endemic in poultry in various countries, causing recurrent epidemics (Alexander 2007). It has also been confirmed to transmit to humans, causing flu-like symptoms. However, the transmission from human to human has not yet been confirmed, even though future considerations on this matter have been addressed (Lupiani *et al.* 2009). The primary transmission in poultry of LPAI

H9N2 is believed to happen via wild bird contact, waterfowl, gulls or shorebirds (Alexander 2007). The emergence of LPAI H9N2 in poultry has been controlled by vaccines, and the diagnosed infection is not considered to be a threat big enough to slay the infected population (Alexander 2007).

The HPAI is studied to arise in a mutation of LPAI that has infected the host (Alexander 2007). The clinical symptoms of HPAI vary highly amongst different bird species and regarding the virus strain (Alexander 2007). Though HPAI is rarer in wild birds than poultry, HPAI H5N1 was first diagnosed in a flock of wild geese in China in 1996 (Xu *et al.* 1999) and has since been circulating within domesticated and wild birds and in occasional mammal and human hosts (Yee *et al*. 2009, Thanawongnuwech *et al.* 2009). The infection route of HPAI H5N1 from Asia to Europe is studied to happen via migratory birds (Kilpatrick *et al.* 2006). However, the importation and exportation of poultry and exotic birds plays an important role in H5N1 transmission (Kilpatrick *et al.* 2006).

The clinical symptoms of HPAI H5N1 are studied to include cloudy eyes, straggled feathers, contraction of neck muscles, incoordination of movements and weakness in Laughing gulls (*Leucophaeus atricilla)* of family *Laridae* (Brown *et al.* 2006). The symptoms are similar with some species of family *Anatidae*, however some might not show any clinical symptoms at all. The histopathologic findings include necrotizing pancreatitis and cerebral neuronal necrosis (Brown *et al.* 2006). Ratanakorn *et al.* (2012) noted that Brown-headed gulls (*Larus brunnicephalus)* demonstrated similar symptoms to *L. atricilla*, also trembling head appeared only one day prior to death. Those gulls, who survived the H5N1 did not develop any clinical symptoms at all.

The wild bird populations in Portugal are studied to have an active circulation of AI, especially strains of H5, H1, H4, N2 and N6. The combinations of H4N6 and H1N1 were studied to be the most common (15.1 %) during a multiyear surveillance program implemented in Portugal from 2005 to 2009, although the overall prevalence of AI proved to be only 1.63 % (Henriques *et al.* 2011). Most of the circulating H5 viruses are low pathogenic, but as explained above, the virus likely mutates into a highly pathogenic form. The H1N1 combination is responsible for causing a pandemic in 1918 and 2009, resulting in 12 000 deaths in humans since 2009 and has since continued to

circulate as a seasonal virus (WHO 2005, WHO 2010). Mallard duck (*Anas platyrhynchos*) has been noted being the most susceptible to the virus, however besides *Anatidae*, families of *Laridae*, *Phasianidae*, *Ciconiidae* and *Phoenicopterus* have found to carry AI virus in Portugal (Henriques *et al.* 2011). Due to this, the Avian Influenza surveillance program commits yearly testing for birds deceased at RIAS.

Besides AI, Aspergillosis, caused by an opportunistic pathogen from various species from genus *Aspergilllus,* is a condition that causes great mortality in captive birds (Beernaert *et al.* 2010 and references within). However, amphibians (Gugnani *et al.* 1980), reptiles (Jacobson 1980), free-living birds, mammals and humans are all susceptible to this mycosis as well (Seyedmousavi *et al.* 2015). The infection happens by breathing a notable number of spores of the ubiquitous *Aspergillus* fungi. The susceptibility of the animal depends on being exposed to the spores but also factors such as migration, thermal discomfort, starvation (Young *et al.* 1998), stress due to captivity (Abrams *et al.* 2001), overcrowding and inadequate diet that leads to vitamin deficiencies (McMillan *et al.* 1989). All of these increase the probability of infection.

Aspergillus grows on organic substrates both outdoors and indoors on all continents excluding Antarctica (Beernaert *et al.* 2010 and references within). Approximately 776 humans suffer from chronic pulmonary aspergillosis (CPA) in Portugal annually. Whereas hypersensitivity to *Aspergillus*, named as allergic bronchopulmonary aspergillosis (ABPA) occurs in 12,5 thousand individuals in Portugal according to the estimates (Sabino *et al.* 2017). Animal infections are not registered and the numbers within humans are just estimates. Although the infected birds might not always show signs of disease, if not treated with antifungal medicine, though not always successful within birds, aspergillosis will cause a prolonged death (Beernaert *et al.* 2010 and references within). All the marine birds in RIAS except *L. fuscus* and *L. michahellis*, due to their high resistance against the disease are treated with antifungal medicine at arrival.

Although a smaller threat than those mentioned above, a possible future threat to bird populations in Portugal are arboviruses such as Usutu Virus (USUV) (Costa 2021), West-Nile Virus (WNV) and Newcastle Disease Virus (NDV) (Costa 2021). WNV has been confirmed in occasional human hosts in Portugal (Connel *et al.* 2004, Zé-Zé

et al. 2015) and moreover, birds and horses have studied to carry the WNV antigen in Portugal, implying for a low endemic prevalence (Barros *et al.* 2011). Migrating birds are studied to have a significant transmission role with this virus of origin Uganda (Owen *et al.* 2006). Although WNV has demonstrated low disease in avian species, cases of high mortality have been registered as well (Kramer *et al.* 2001). Most commonly, birds do not demonstrate any clinical symptoms when infected. However, if the symptoms occur, they include paresis, loss of coordination, head tilt and weakness (Jiménez-Clavero *et al.* 2008, Komar 2003, Pradier *et al.* 2012). Although rarely present, similar clinical symptoms have been reported with USUV and NDV. (Costa 2021 and references within).

The active bird migration within Portugal (Freitas *et al.* 2012), diminishing habitats (de Noronha Vaz *et al.* 2013) that both cause more dense populations, not to mention warming climate (Costa 2021), that causes species shift more north are all factors that favour the appearance of these infectious diseases.

1.4.3 Dietary deficiencies

PS is associated with thiamine deficiency, causing paralysis of the wings, followed by complete paralysis and death (Balk *et al.* 2009). Thiamine -an indispensable micronutrient used in metabolic processes, is obtained mainly from plants such as phytoplankton, but also from bacteria and fungi. Therefore, the development of the deficiency is suggested to be caused by changes and imbalances in food web structure, that may be due to pollutants and/or warming climate (Gilbert 2018).

1.4.4 Pollution

PS is studied to be caused by various environmental pollutants as well, such as naturally existing mercury and lead, that however appear in higher concentrations due to anthropogenic use (Soares 2014 and references within). Lead is ingested with ammunition and paint chips and is suggested to accumulate in the food web due to using fertilizers created as a by-product in sewage treatment, due to industrial plants, mines and waste water discharge (Fisher *et al.* 2006 and references within). Mercury is released due to various industrial practices, practices in waste treatment, mining etc.

Although not commonly in use anymore, the Persistent Organic Pollutants (POPs), such as organochlorine compounds (OC) remain in the environment and are still studied to poison wildlife (Soares 2014 and references within) causing PS.

1.5 Gout as consequence of paretic syndrome

Gout is a condition concerning humans (Garrod 1876), amphibians, reptiles (Prior *et al.* 1996, Appleby *et al.* 1960), mammals and birds (Chandler 1924). It occurs as a high amount of uric acid in blood accumulates either in the visceral or in joints causing arthritis (Guo *et al.* 2005). The reasons behind gout are various, such as renal dysfunction and ingestion of foods with high amount of nitrogen (Ragab *et al.* 2017 and references within). In birds, a calcium-rich diet, high protein and salt content and low content of phosphorus, vitamin A and D and also existing viral conditions have been studied to cause gout (Bulbule *et al.* 2014).

Although not common in wild birds, the first case of gout outside of captive, domesticated birds was reported in 1944 by Fowle. Since then, the expanding practice of agriculture has been suspected to alter the diets of wild birds, exposing them to dietary deficiencies, which together with dietary changes due to urbanization might play a crucial role in gout occurring within wild birds (Fiend *et al*. 1999). However, in the birds received by RIAS, gout is usually a consequence of a dehydration-related kidney failure. As birds do not possess a gallbladder, the nitrogenous waste and salts kidneys filter from blood is converted into uric acid in kidneys, concentrated into white paste and transported via ureters into cloaca. Also, the autonomous nervous system of birds is able to control the osmotic balance in the body by moving the urine from cloaca back to gut, where water and salts can be extracted before excreting the waste (Lovette *et al.* 2016). If the kidneys are unable to remove the excess uric acid, followed by chronic kidney failure, gout starts to appear. Therefore, gout in RIAS is considered to be a consequence of PS rather than cause of PS.

1.6 Importance of necropsies in diagnosis

Any environment consists of a centrum that determines the direct factors affecting the animals' chance to survive and reproduce, and of a web of indirectly affecting components. The indirect factors rule whether the individual can fulfil its personal purpose and purpose in an ecosystem it inhabits (Andrewartha *et al*. 1986). Each animal has its own niche, a quality that determines how the animal responds to the environment. In other words, it is the functional role of the animal, the purpose in an ecosystem (Elton 1927). Whether the animal can fulfil its niche, depends on its success against the restricting factors e.g., predation, resources, hazards (Andrewartha *et al.* 1986, Browning 1962). Hazards include infectious diseases and epidemics that naturally balance up the food webs of the ecosystems (Browning 1962, Munson *et al.* 2006). In pristine environments, the occurrence and mortality caused by infectious diseases and periodic epidemics is moderately low. However, due to human interference, the habitats are getting smaller in size, which increases the density of animals and exposes them to a higher probability of contagious diseases, exposing the habitat-sharing domestic animals and humans to some of these diseases as well (Munson *et al.* 2006). Together with this, and increasing number of environmental pollutants, the wildlife, domesticated animals and humans meet pressure both habitat-wise and health-wise.

Besides eliminating the risk factors mentioned above, one way to protect the wildlife from the threats caused by diseases is determining the cause of morbidities and mortalities by diagnostic pathology, which consists of post-mortem examination and supporting laboratory testing. Diagnostic pathology has allowed us to characterize many of the emerging diseases in wildlife and in livestock (Cooper 2002, O´Toole 2010). Emerging infectious diseases (EIDs) have caused both social and economic devastation. Their high zoonotic origin; approx. 60 %, suggests the importance of diagnostic pathology within wild animals and livestock, which helps us to diagnose these diseases early on (Jones *et al.* 2008). The appearance of zoonotic EIDs has only increased since the 1940's, encouraged by human population density and climate anomalies caused by global warming. The majority (71.8 %) of zoonotic EIDs are from wildlife origin, emerging as territories of wild animals overlap with more and more pervading human settlement (Jones *et al.* 2008, Weiss *et al.* 2004). Due to this fact, the only way to prevent the occurrence of zoonotic EIDs, besides forbearing the overtaking of pristine

environments and minimizing the quantity or environmental pollutants, is the process of wild species becoming rarer and eventually extinct, resulting in loss of biodiversity worldwide (Weiss *et al.* 2004).

Globally, the diagnostic pathology plays an important role in diagnosing and preventing the transmission of zoonotic EIDs to other wildlife, domestic animals and humans. However, the information provided by post-mortem examination does not limit in preventing the transmission of infectious diseases; nearly or completely asymptomatic diseases may only be diagnosed in post-mortem examination. As the carriers of subclinical diseases are not restricted by the disease, the dispersion potential of the disease is significant in case of an infectious disease. By early identification, the distribution of these infectious diseases, such as AI, or non-infectious diseases may be restricted and the devastation minimized (Quilliam *et al*. 2012, Cooper 2002). Post-mortem examinations also give valuable information on the overall health condition of the animal, such as size of the animal, appearance, diseases, presence of parasites and reproductive success, that can be used to monitor overall population health and the state of the environment it habits. This information allows us to construct monitoring data and an established procedure of collected samples and other conditions and lesions to look out for (Cooper 2002). Another important feature provided by post-mortem examination is the possibility to improve the patient care. The examination might give information that could not be diagnosed from clinical symptoms and thus allows us to seek for similar signals in future cases and to choose more accurate methods in the treatment (Wittekind *et al.* 2018).

As mentioned above, post-mortem examinations and supporting laboratory analyses have a significant role in recognising new arising diseases but also give valuable data in monitoring the state of the populations and the environment. Post-mortem examinations date up to 3500 years back in time, they have had a significant role in the evolution of medical science and society overall. As technology has advanced and the clinical diagnosis have become more accurate and effortless, the value given to post-mortem examinations has declined (Dada *et al.* 1996). However, various studies note 25-33 % discrepancies between clinical diagnosis and cause of death found in diagnostic pathology (Roulson *et al.* 2005, Combes *et al.* 2004). In addition, post-mortem examination can provide information that was not diagnosed before death (Roulson *et*

al. 2005). Therefore, the information given by necropsies allows us to record findings that might not have been noted in clinical diagnosis and thus make appropriate measures to improve the population health. The examinations also improve our knowledge on anatomy. Furthermore, researchers have demonstrated that necropsy findings, collected in an adept way and in high numbers, further the quality of patient care and thus improve the outcomes of patient cases (Anderson *et al.* 1989, Underwood 2010, Ioan *et al*. 2014). Noting this fact, information given by necropsies performed to animals affected by PS, allows us to determine the cause, the effects and to develop the medical care provided, so that the most problematic findings are taken in account and alternatives based on these problems can be investigated.

Studies examining post-mortem findings in birds with PS are very few. Neimanis *et al.* in 2007, examined *L. argentatus* with PS in south-eastern Sweden, capturing and decapitating affected birds from Sweden and seemingly unaffected birds from Iceland. The study noted, that the bodies of gulls suffering from botulism had tacky and dry subcutaneous tissues, the cloaca was distended, consisting of urates and faeces. The proventriculus and ventriculus were bile-stained and empty and the gall bladder was full. Various affected birds had iron storages -abundant hemosiderin in their livers, in Kupffer cells. Two affected birds from 20, had distended renal tubules with urates as well as expanded tubular epithelium. Amyloid was found both in affected birds and in the control group.

Birds received by RIAS have been formerly studied by Soares in 2014. In necropsy findings she noted feathers covered with faeces or diarrhoea, possibly resulting from malfunction of cloaca. Besides this, distended cloaca with thinned walls was a recurrent finding. Soares agreed with Coles (2007) the findings hinting towards cloacal dysfunction and enteritis. Besides cloacal findings, the gastrointestinal tract was filled with gas, faecal material and oedema-caused lesions. In a few cases gallbladder was found to be distended, the liver demonstrated hypertrophy, vascular congestion, necrosis, atrophy and pale colour. Congestions and haemorrhage were found in lungs, kidneys, heart and body cavity amongst others.

This study focuses on investigating the degradation caused by PS in the organs of affected birds, *L. fuscus* and *L. michahellis*. As mentioned before, previous studies have

processed the findings as quantitative occurrences, yet thorough explanations of the abnormalities found and their effect to the overall health of the animal have been lacking. Here, the degradation caused by PS is aimed to be explained more integrally; how does the condition manifest in the organs of the affected bird and alter the metabolism of the animal, what causes the animal to die? It is expected that the emaciated gulls demonstrate more damage in their bodies than those gulls who outwardly seem to be in good condition.

2 MATERIALS AND METHODS

2.1 Necropsy procedure

RIAS receives injured and debilitated birds, terrestrial mammals, reptiles and amphibians from Southern Portugal. The centre offers medical assistance and rehabilitation, sampling for various organizations, authorities and Universities. Performing necropsies to deceased animals is also part of the work of RIAS as well as sharing the information provided by these necropsies with the organizations in interest.

The sampling period (October 2021-January 2022) happened in the centre by performing autopsies to *L. fuscus and L. michahellis,* deceased due to PS. Based on previous data from the centre, the spring and the autumn are the periods with most gulls being admitted to the centre. Therefore, the sampling period was chosen to coalesce with the peak season. The performed gulls within this study have died prior or after being admitted to the RIAS centre, no birds were euthanized for study purpose. That is to say, the birds examined have died due to their condition, hopefully resulting in more notable abnormalities compared to those being decapitated purely for study reasons. The abnormalities demonstrated by affected gulls were analysed by their frequency. Also, the severity of the abnormalities on individual level was recorded by imaging and compared to findings in literature.

The abnormalities found in gulls during the dissection were listed and separated regarding on the organ they were noted in (table 1). Abnormalities such as lesions, parasites, hematomas etc. that could not have been assimilated with PS were not analysed.

Gulls with no notable abnormalities were marked as without apparent injuries (WAI). From those gulls, that could not have been studied properly due to autolysis, only the population parameters were analysed (body condition scoring, age, species, sex). Gout (GOT) and Aspergillosis (ASP) were also recorded.

Table 1.1 Abnormalities found in dissected birds were assimilated with the organ they were found in. The different abnormalities found within organs will be presented in results.

Abbreviation	Meaning
WAI	Without apparent injuries
CLO	Cloaca
KID	Kidney
PAN	Pancreas
LIV	Liver
GOT	Gout
ASP	Aspergillosis

After death, the gulls in RIAS were stored in the freezer to secure the preservation of the corpse for later necropsy. Prior to the dissections, the gulls were removed from the freezer and placed in room temperature until completely melted. If the dissection happened within 48 hours of the death, the animal was placed in a fridge to wait for the dissection. After the performed necropsy, due to possible presence of toxins, the dissected animals as well as all the extracted organs that were not used for sampling, were stored in the freezer until incineration.

During necropsy, following aspects were examined and recorded:

1. The Body Condition Scoring (BCS) of the bird (1-5; 1 being emaciated, 5 obese)

2. Sex of the bird

3. Macroscopic abnormalities.

Based on previous findings by Ben-Gigirey et al. 2021 and Neimanis et al. 2007 the areas to be examined in the necropsy of gulls that are suffering from PS and suspected to be suffering from botulism, include gastrointestinal tract, lungs and heart. Within the gastrointestinal tract, the organs that demonstrate the most abnormalities were suspected to include kidneys and cloaca. Macroscopic abnormalities were recorded verbally and by imaging for later analysis.

In the beginning of the post-mortem examination, the birds' identification number was written in the notes and its' physical aspects were carefully examined: presence of lesions, marks of diarrhoea in plumage e.g. The birds were dissected by removing the plumage and skin from the chest area by hands. The excess skin and plumage were cut off with scissors, maintaining the working area easily accessible. All of the extracted skin pieces etc. were disposed in a plastic bag. At this point, the body condition scoring (BCS) of the bird was assessed by evaluating the abdominal profile and the fat reserves. The profile of the sternal crest determines the richness of fat reserves, the clearer the sternal crest appears (fig. 2.1), the more malnourished the bird is. Whereas a normal-weighted bird has a round chest profile (fig. 2.2).

Figure 2.1 Severely emaciated juvenile *L. fuscus* with BCS 1.

Figure 2.2 Juvenile *L. michahellis* with BCS of 3 (normal state).

As the plumage was removed, the abdominal wall was cut open by using surgical scissors. The performed cut had a Y-shape that continued to the ribs and collarbones of the bird (fig. 2.3). If the operated bird was big in size, the use of cutting pliers was necessary in the collarbones. The sternum was removed by carefully cutting the tissues

that connect the sternum to the heart revealing the body cavity (fig. 2.4). The overall condition of the corpse was examined, as the condition of the visible organs; liver and heart, presence of aspergillosis, hematomas etc.

Figure 2.3 Red lines marking the cutting route, which ends to the collarbones of a subadult *L. michahellis.*

Figure 2.4 Body cavity revealed after the removal of sternum, where 1: trachea 2: heart 3: liver.

The heart and trachea were removed and observed for any abnormalities, such as aspergillosis, that commonly occurs around heart and lungs and in bad cases forms white tacky substance in trachea. After this, the liver was removed and the size and colour were examined. A cut was performed in the liver to see if the possible colour changes occur only on the surface of the liver (post-mortem changes) or if the possible abnormal colour occurs throughout the liver. When removing the liver, the gallbladder, which is attached to it, was removed carefully and the size of it was observed. The absence of the liver revealed the intestines (fig. 2.5), which was removed together with the oesophagus, gizzard, pancreas and cloaca. Before removal, the size of the cloaca was evaluated and if very full (fig. 2.5), the end of the cloaca was closed with hemostats to avoid the spilling of the content during removal. After removal, the content of

the cloaca was visually analysed as well as the content of the gizzard (fig. 2.6), that was cut open with surgical scissors. The colour and size of the pancreas was evaluated before the disposal of the removed organs.

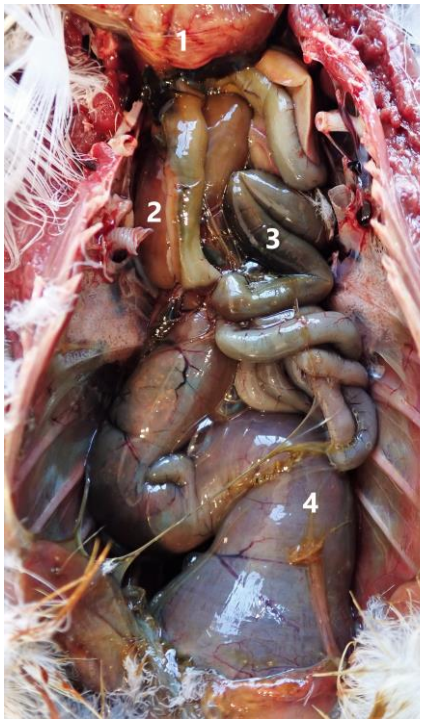


Figure 2.5 Intestines after liver removal, where 1: gizzard, 2: pancreas, 3 small and large intestine, 4: cloaca (distended).

Figure 2.6 Oesophagus and gizzard cut open revealing empty stomach content besides a piece of glass.

Cuts were performed in lungs and flanks to see for the presence of aspergillosis that can be noted as white deposits or fungi-like formations (fig. 2.7). The cavity of the corpse was cleaned from excess body liquids by using clean tissue, in order to assess the reproductive organs, that lie above the kidneys, and to determine the sex of the bird (fig. 2.8). Lastly, the condition of the kidneys (size, colour, texture) was evaluated visually. The corpse and all of the removed organs were disposed in a plastic bag and frozen to be cremated later.

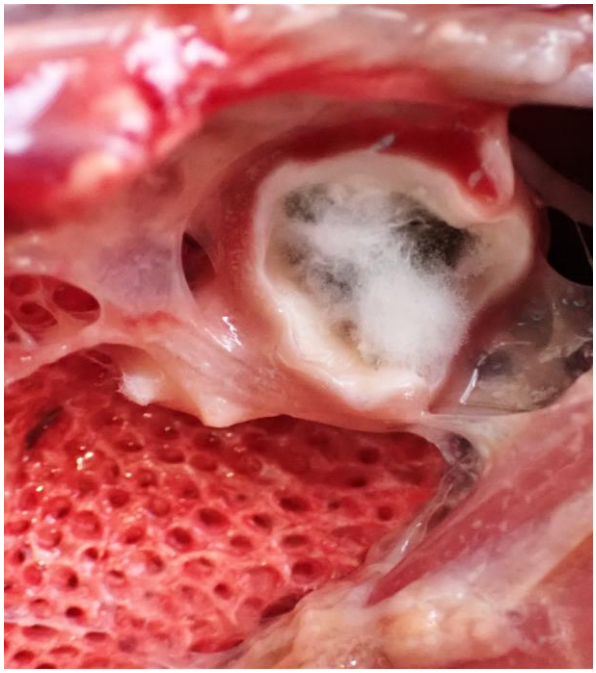


Figure 2.7 Aspergillosis in the flank of a gull.

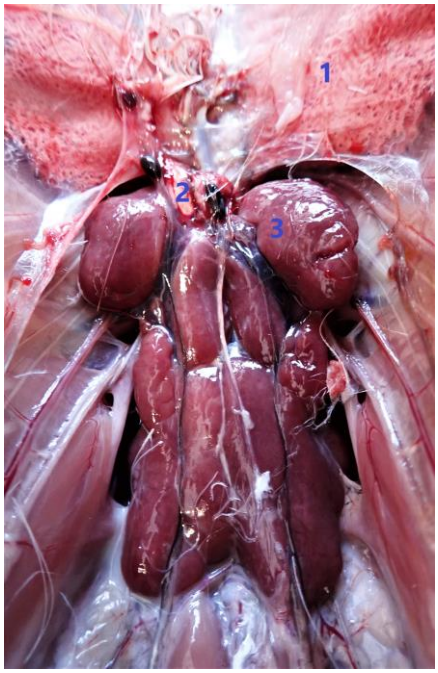


Figure 2.8 Bird cavity after the removal of intestines, where 1: Lungs 2: reproductive organs 3: kidneys.

2.2 Samples collected for histopathology and diagnosis

Botulism is difficult to diagnose by clinical observations only. Also, symptoms that refer to botulism: paralysis, diarrhoea, dyspnoea etc. (Tahseen 2019, Ben-Gigirey *et al.* 2021), can be indicators of other common avian diseases as well, such as AI, pasteurellosis, chemical toxicoses (Tahseen 2019) and PSP (Ben-Gigirey *et al.* 2021). To differentiate botulism from other avian-threatening diseases and conditions mentioned above, supporting laboratory testing should be applied. Normally, botulism, such as other biotoxins can be detected from kidney and liver, as well as faeces within live animals (Ben-Gigirey *et al.* 2021). Lung sample with aspergillosis and kidney sample were only analysed for microscopic damages.

Kidney sample and aspergillosis sample from the lungs were collected from a gull with clinically diagnosed PS. The samples were collected from a recently died individual (less than 24 h), that was kept in a refrigerator with below +3 °C. Kidneys were removed, cut in half and washed with Prontosan® wound irrigation solution to remove

the excess blood and other body substances. After this, the sample was stored in a fixation solution of buffered formaldehyde 10 % for 24 hours. After 24 hours, the sample was washed under tap water, until there was no colour released by the sample. Following this, the sample was stored for another 24 hours in tap water before storing it in distilled water for 24 hours. Lastly, it was stored in an ethanol solution of 70 % and sent for analysing. The same procedure was repeated to the aspergillosis sample. Since the aspergillosis was located in lungs, the immersion of the sample during all of the steps was secured by placing a clean piece of tissue inside the sampling bottle, keeping the sample submerged.

2.3 Review of data

The information provided by the sampling period (October 2021-January 2022) data collection as well as data from the previous necropsies performed in RIAS in 2016-2021 is the dataset of this study. The hand-written data from necropsies, limited to include *L. fuscus* and *L. michahellis* that have been clinically diagnosed with PS, as well as data from the internal database of RIAS was assessed and organized by using Microsoft Excel® 16.0.14931.20132. The information provided by the sampling period and by the review of the past necropsies were combined to be processed as one dataset. This way, the timing of the sampling period (autumn) will not have an effect on the results, rather the results concern year-round occurrence of the syndrome.

The data recorded prior to dissection and processed in this study consisted of following aspects, that were recorded in the internal database as the animal arrived to the centre:

1. State of the animal at arrival (dead, alive)

2. Species

3. Age of the bird (fledgeling, juvenile (hatch year birds), subadult (plumage no longer juvenile, but not of adult either), adult)

Latter information discussed in results, such as sex, were obtained during the dissection.

2.4 Statistical analysis

The qualitative factors of the population as well as individuals were analyzed with Microsoft Excel® 16.0.14931.20132. The population parameters that were studied, included sex, species, age, time to death from arrival and BCS. From these, the sex, age distributions were constructed to find out, whether these factors affected in the development of the syndrome. To test whether the sex or the age of the bird was a statistically significant factor in the appearance of abnormalities, a chi-squared test was performed with R studio version 1.4.1006. The species distribution was obtained to see whether the seasonal presence of *L. fuscus* and the year-round prevalence of *L. michahellis* could be seen in the affected birds. The BCS was analysed to see whether the physical condition of the bird was correlated with the occurrence of the abnormalities. The time to death from arrival was observed to evaluate, whether the birds with injuries died later than those birds who manifested no apparent injuries.

3 RESULTS

Gulls with PS, dissected between 2016 and January 2022 were in total 446, from which 245 (55 %) arrived alive to the centre and 201 (45 %) were already dead. The time from arrival to death was recorded as within the first 48 hours, after the first 48 hours and after the first 30 days (fig. 3.1). The age was registered within 98 % of the deceased gulls. (fig. 3.2).

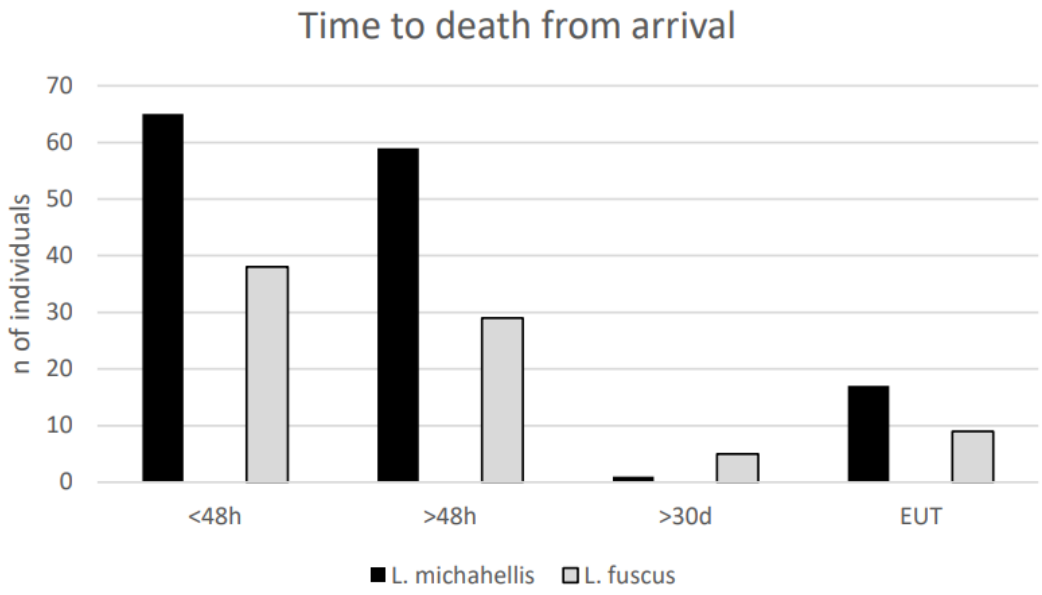


Figure 3.1 Majority of the birds died within the first 48 hours (115 gulls) *(L. fuscus, L. michahellis*), 88 after the first 48 hours, 6 after the first 30 days and 26 were euthanized due to secondary reason, such as blindness.

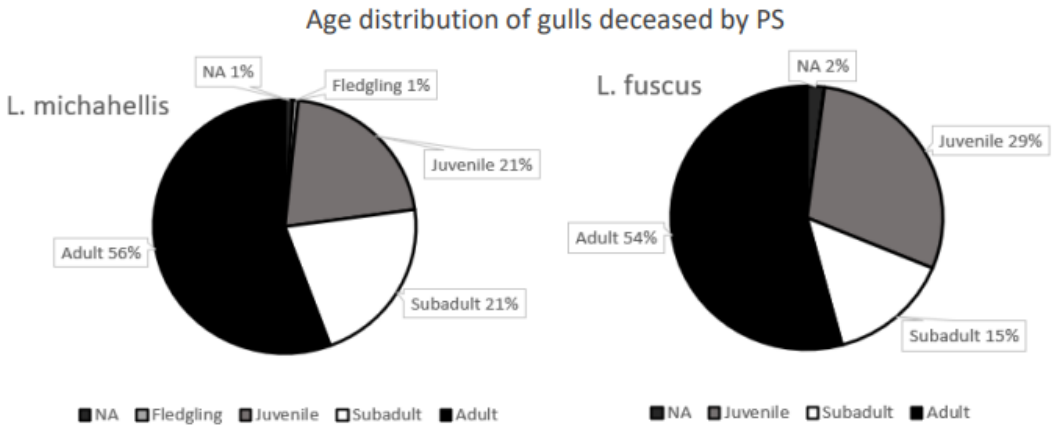


Figure 3.2 Within 6 of the gulls the age was not registered, 2 of the gulls were fledglings, 106 juveniles, 86 subadults and 246 adults.

The majority (n=201) of the birds dissected were female (fig. 3.3). Whereas the number of males was 176. Within 69 gulls, determining the sex resulted impossible due to autolysis of the corpse. Majority of the gulls dissected were *L. michahellis (68 %),* whereas *L. fuscus* represented 32 % of the gulls dissected.

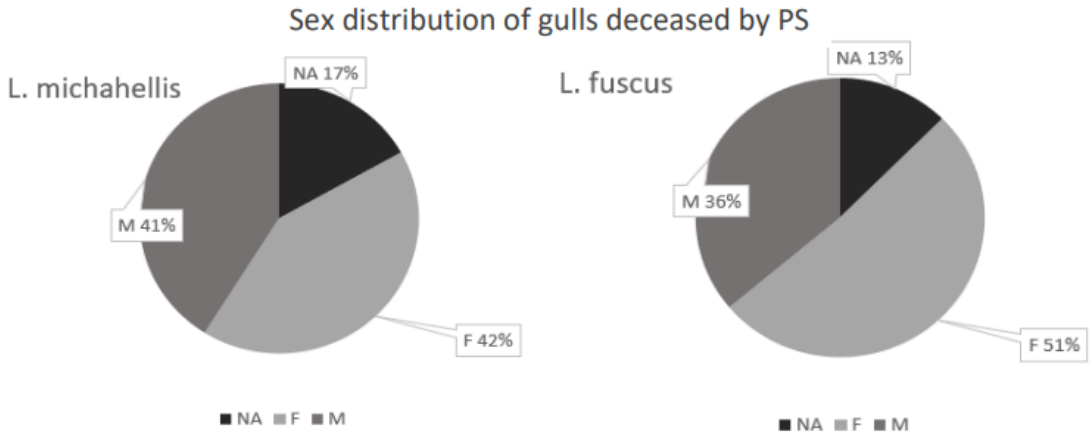


Figure 3.3 Sex distribution of the gulls dissected between January 2016 and January 2022.

The Body Condition Scoring of both alive and death gulls was evaluated within 433 gulls, the distribution being left-skewed (fig. 3.4). Majority of the gulls (n=121) were scored with BCS 1, being the most emaciated.

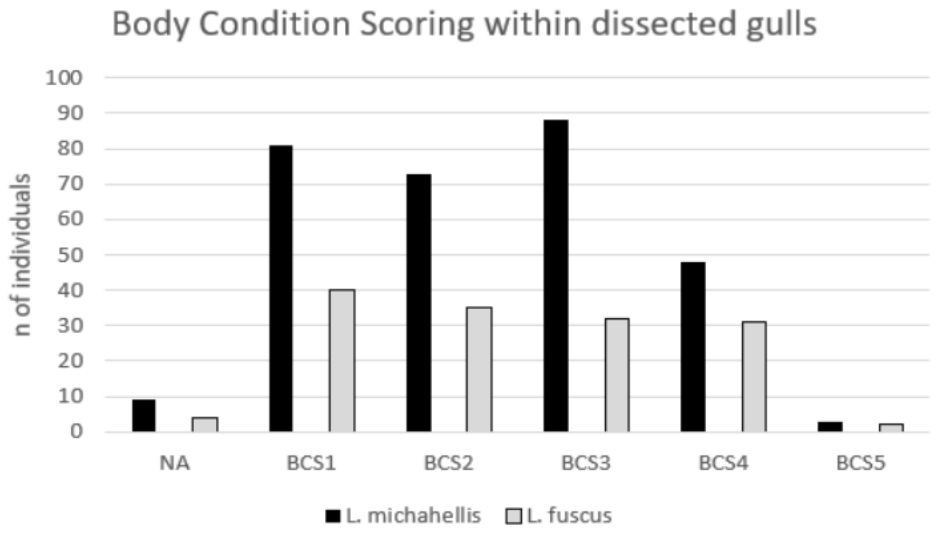


Figure 3.4 Body Condition Scoring of the gulls deceased by PS, where BCS3 is the gull with optimal body condition. 81 gulls scored more than 3, weheas 229 gulls scored less than 3.

3.1 Necropsy findings, description and significance

Of all of the 446 gulls dissected between 2016 and January 2022, nearly half (n=210) demonstrated no apparent injuries (fig. 3.5), in other words, abnormalities that could be related to PS. In birds with injuries, the most common abnormalities were associated with kidneys (n=136), also abnormalities in cloaca were common (n=46). Gout was noted in 6 of the gulls dissected and aspergillosis in 39 gulls. Other affected organs were pancreas and liver. 31 corpses were autolytic, therefore the injuries represented by these bodies could not have been assessed and their data was only used in assessing the overall information of the populations represented above.

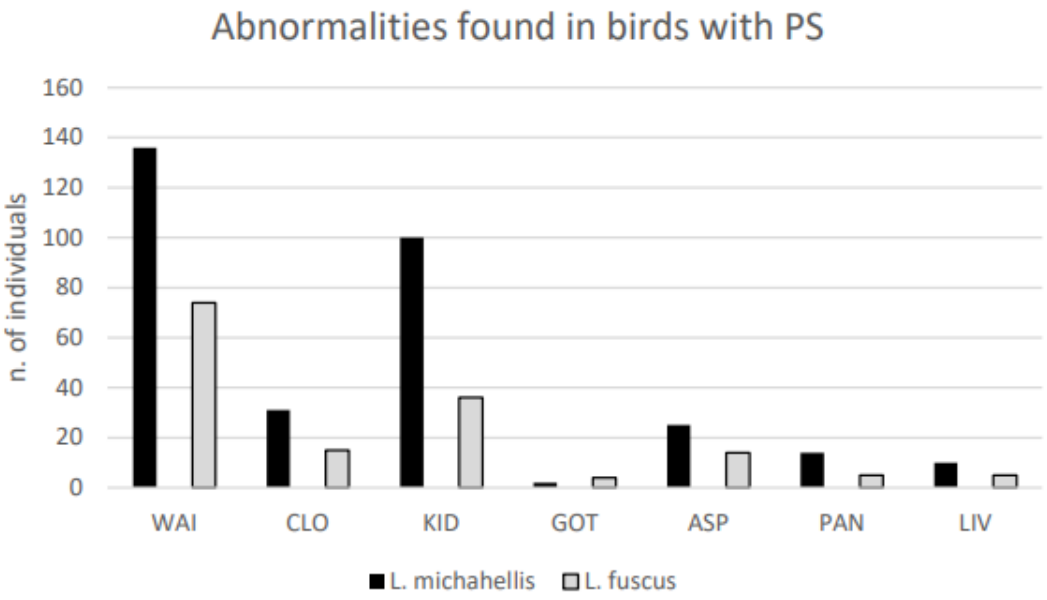


Figure 3.5 Abnormalities found in dissected gulls presented as location they were found in, excluding gout and aspergillosis, where WAI = without apparent injuries, CLO = cloaca, KID = kidney, PAN = pancreas, GOT = gout and ASP = aspergillosis

Excluding birds that had no apparent injuries and conditions that are not associated to be directly caused by PS (gout and aspergillosis), the direct conditions resulting from PS found in cloaca and indirect conditions resulting from PS in kidneys, pancreas and liver, demonstrated high emphasis on kidneys. The sex of the bird or the age of the bird had no statistical significance on the occurrence of the symptoms ($p > 0.5$).

3.1.1 Kidneys

Kidneys were the most affected organ, with 136 gulls demonstrating abnormalities (fig. 3.6). The abnormalities (nephromegaly, paleness, granular pattern) occurred either alone or together (fig. 3.7, 3.8, 3.9, 3.10). The most affected kidneys demonstrated all of these conditions together (fig. 3.10). Nephromegaly (fig. 3.7) of all the abnormalities demonstrated the highest occurence. Nephromegaly and pale colour of the kidneys are macro incjuries, whereas granular pattern (fig. 3.9) is a micro injury, where fibrosis in kidneys forms “white patches”.

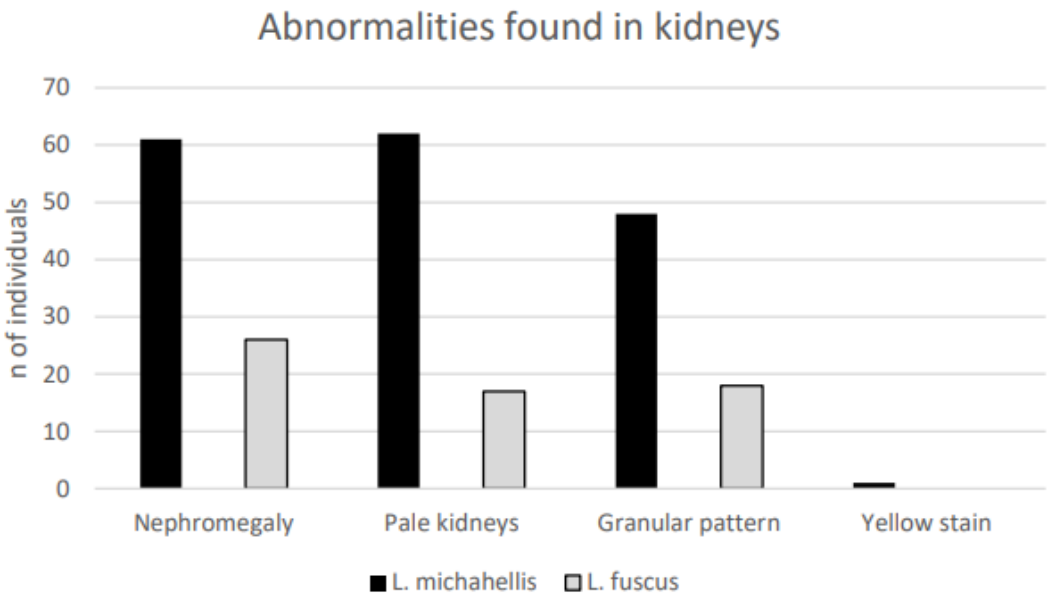


Figure 3.6 Abnormalities found in kidneys of the dissected gulls. Enlargement of the kidneys (nephromegaly, n=87 in total) was the most common symptom found where pale kidneys followed with 79 observations. 66 kidneys demonstrated a granular pattern and one had a yellow stain.

In comparison with the 30,5% of gulls demonstrating abnormalities in the kidneys between 2016 to January 2022, during the sampling period of October 2021 – January 2022, out of 53 gulls sampled, 48 (90,6%) demonstrated similar abnormalities as shown in figures 3.8, 3.9, 3.10 and 3.11.

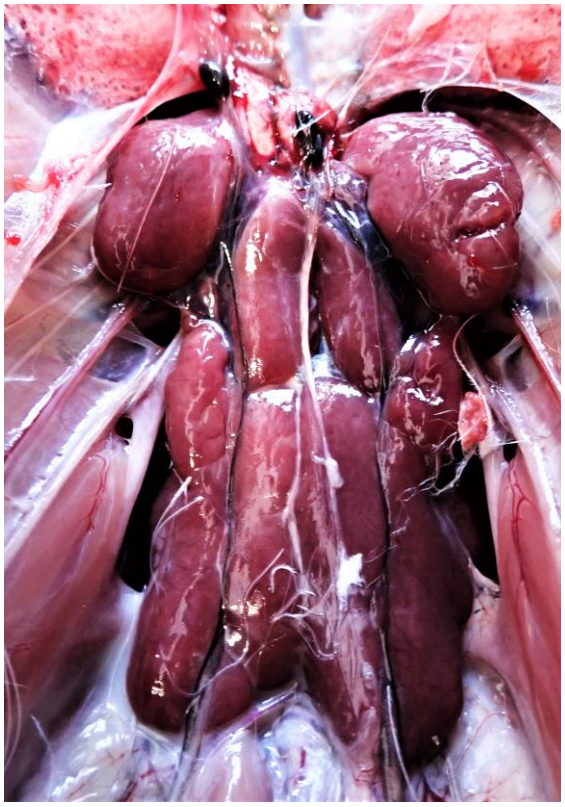


Figure 3.7 Kidneys of a gull demonstrating nephromegaly. Other than nephromegaly, the kidneys demonstrate normal color.

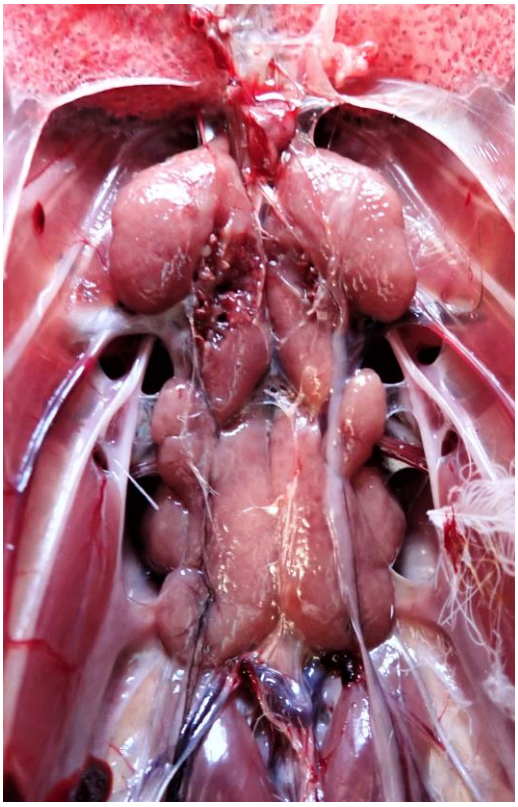


Figure 3.8 Nephromegaly accompanied by pale color.

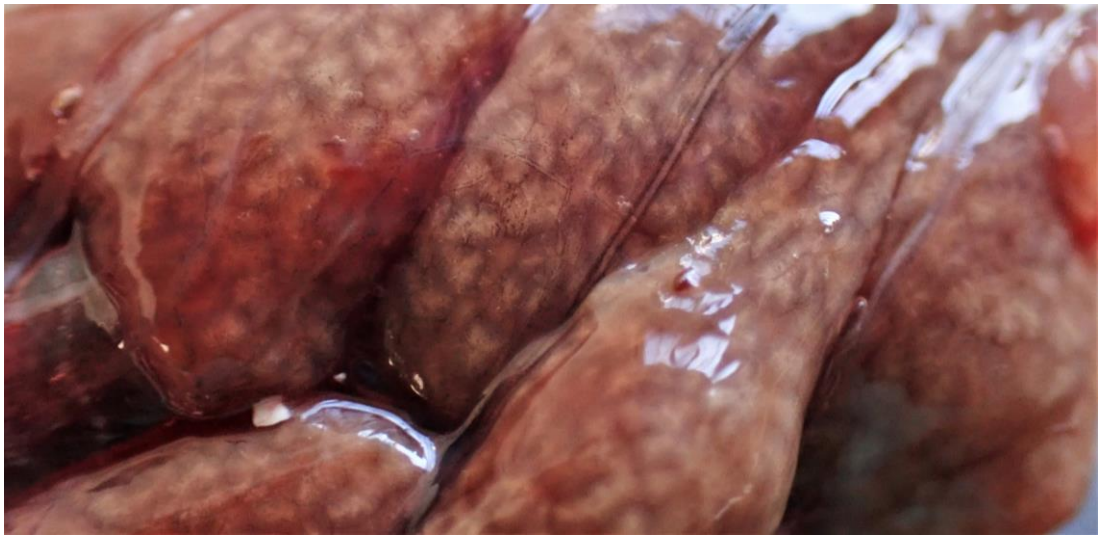


Figure 3.9 Kidneys demonstrating a granular pattern.

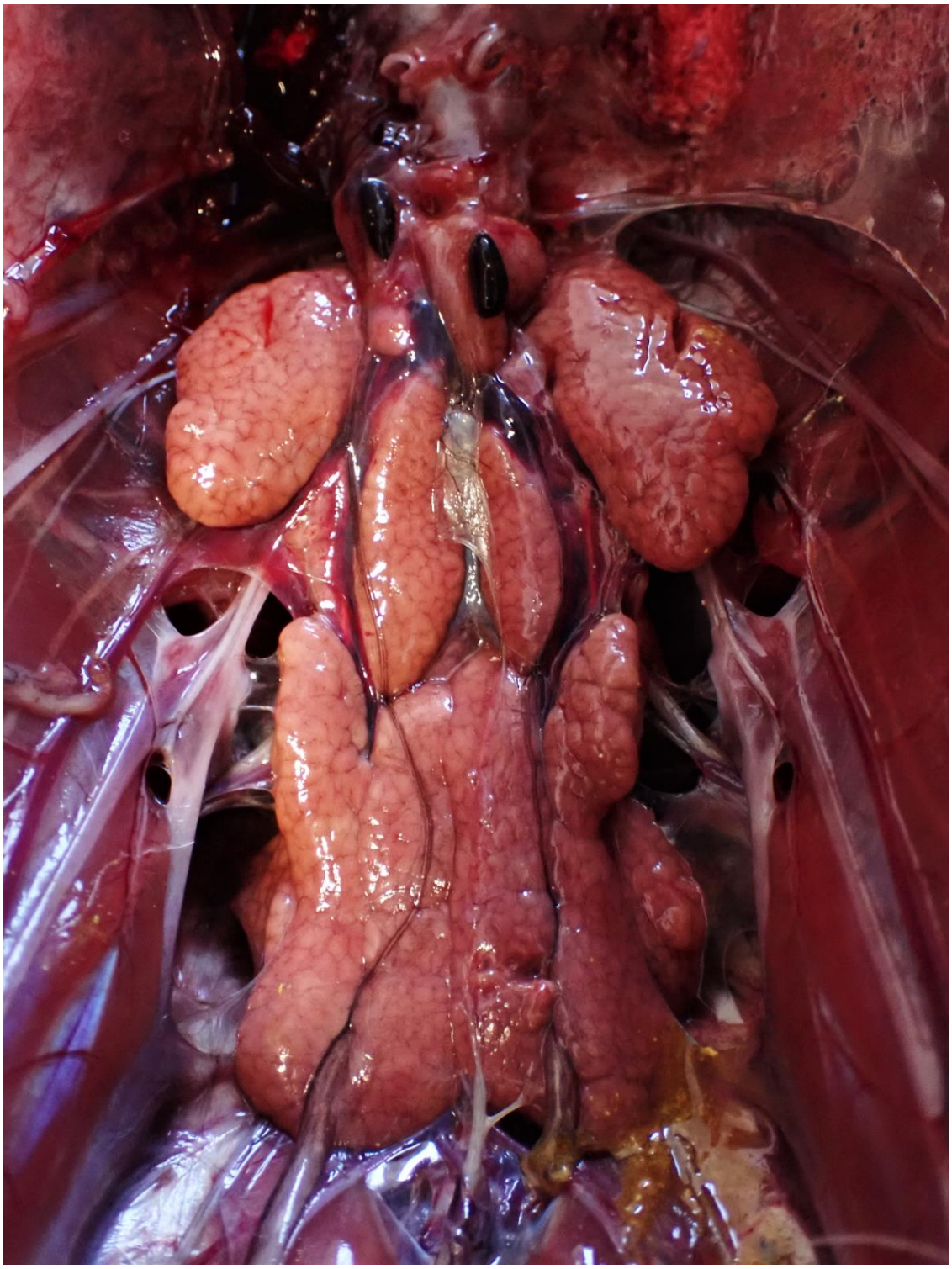


Figure 3.10 Nephromegaly, pale colour and granular pattern manifested in kidneys.

3.1.2 Cloaca

After kidneys, the most abnormalities were demonstrated by cloaca where the abnormalities were either distended form or bloody content in cloaca (fig. 3.11). Many of the birds suffered from diarrhoea (fig. 3.12). However, this finding had not been marked in necropsy notes and since the reasons behind diarrhoea can be various and gulls suffering from distended cloaca suffered also from diarrhoea, diarrhoea itself was disregarded from the abnormalities.

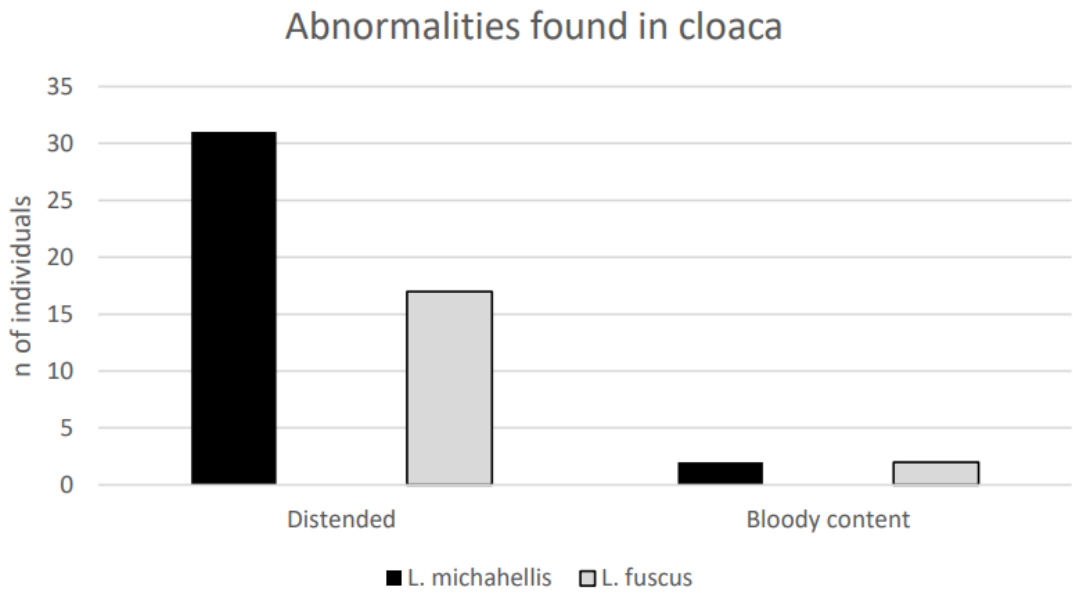


Figure 3.11 48 gulls out of 446 demonstrated distended cloaca and 4 gulls demonstrated bloody content.

The grade of the expansion of cloaca varied within the gulls, some demonstrating accumulation of up to 5 times to normal cloaca content. Often the distended cloaca was associated with full intestines as well (fig. 3.14). In worst cases, the distended cloaca appeared together with bloody content (fig. 3.15).

Again, in comparison with the sampling period, where the abnormalities demonstrated by cloaca were 27 in numbers (50,9 % of the gulls sampled), in total within 2016 to January 2022, the distended cloaca only demonstrated an occurrence rate of 10,8 %.

Figure 3.12 Juvenile *L. fuscus* suffering from diarrhoea that has stained the feathers.

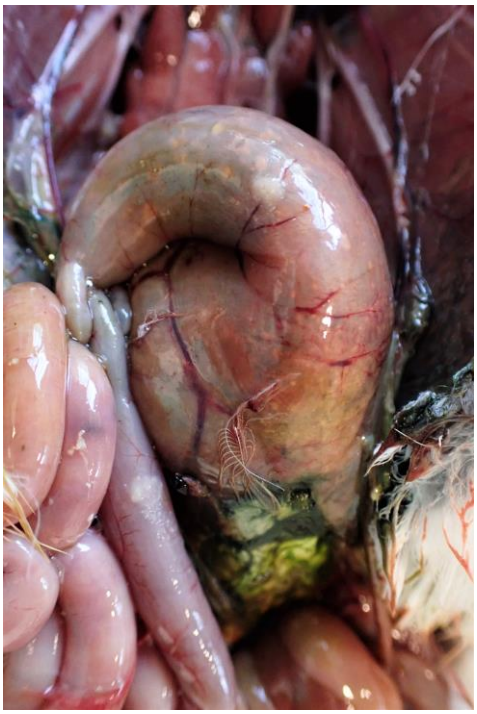


Figure 3.13 severely distended cloaca due to the body being unable to empty the accumulation of matter.

Figure 3.14 Juvenile *L. fuscus*, having her cloaca completely full of bloody liquid and clotted blood. The content exploded with pressure when opening the stomach of the bird.

3.1.3 Pancreas

In total 19 gulls out of 446 (4.3 %) demonstrated abnormalities in their pancreas (fig. 3.5). From these gulls, the most abnormalities appeared as dark patterns in pancreas (fig. 3.15), however granular pattern and enlarged pancreas were also noted in very few cases (1-2 individuals). The occurrence rate of pancreas abnormalities was again higher during the sampling period of October 2021 – January 2022 (28.3 %).

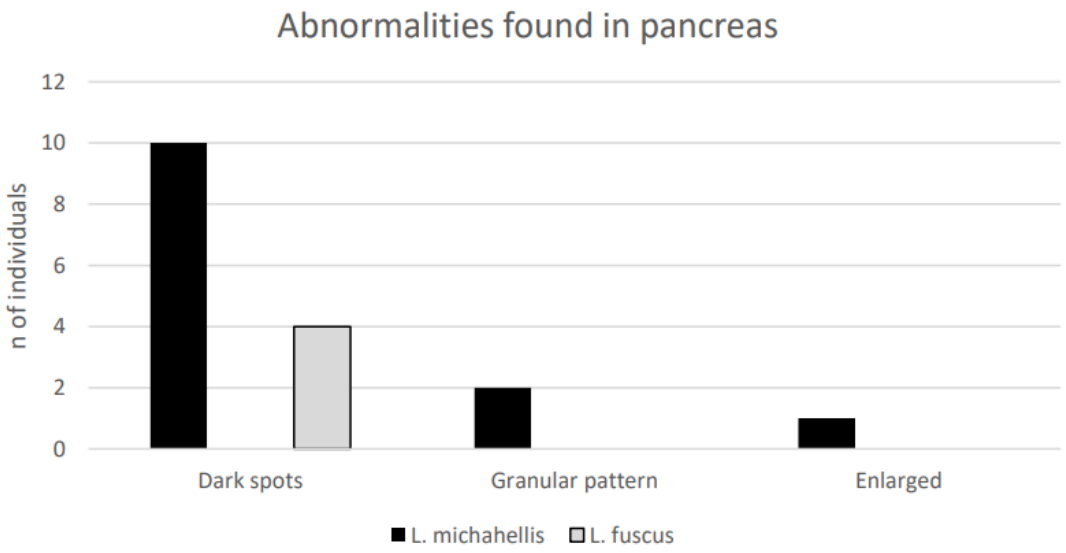


Figure 3.15 Abnormalities in numbers found in pancreas between 2015 and January 2022.

8 of the 18 gulls that demonstrated abnormalities in the pancreas were diagnosed with BCS 1, being extremely emaciated. 6 gulls had a BCS 2 and 5 were normal weight. No obese gulls with abnormalities in pancreas were diagnosed.

3.1.4 Liver

15 gulls were found to have abnormalities in the liver, from which the majority of 7 individuals had liver atrophy (fig. 3.16). Other abnormalities found were friable tissue, pale colour, hepatomegaly and white and dark spots. The overall occurrence of abnormalities found in the liver was 3.4 % whereas during the sampling period the occurrence rate was 11.3 %.

The BCSs within these 15 gulls were mainly extremely emaciated (BCS1), n=10. 3 gulls had a BCS2, one was normal weight and one gull had a BCS4. All of the gulls suffering from liver atrophy had a BCS1.

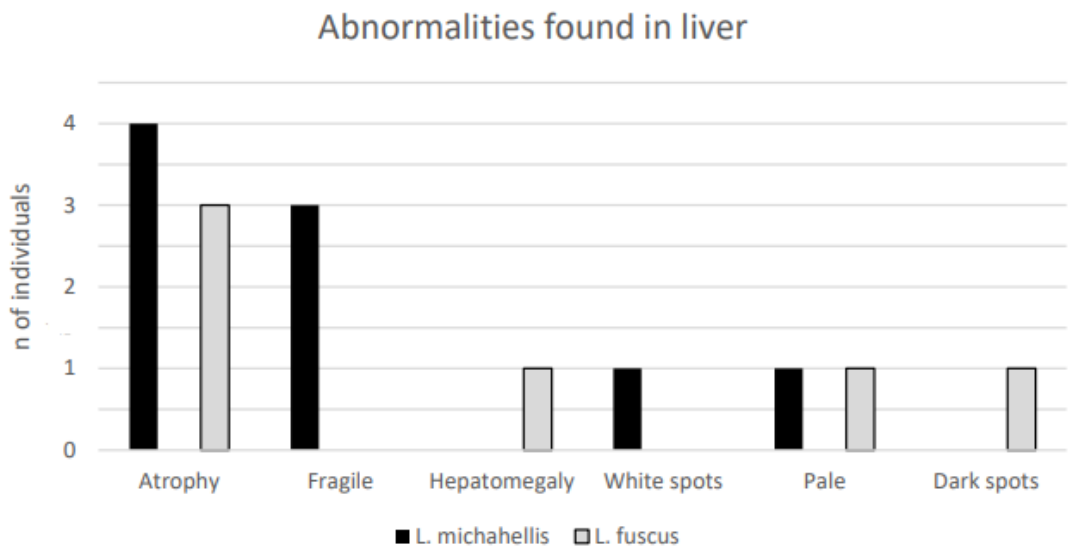


Figure 3.16 The abnormalities found in the liver between 2015 to January 2022 represented in a number of cases.

3.2 Birds without apparent injuries

The birds with no apparent injuries were gulls that manifested the clinical symptoms of PS but in necropsy demonstrated no abnormalities. These birds were 210 in total and from those, 111 birds arrived at the centre still alive. The birds who arrived already dead had either diarrhea-stained feathers or marks in their bodies demonstrating that they had been unable to move or lack of traumas that could have otherwise explained their death. From these 111 birds, 14 were euthanized due to secondary reasons. The time from arrival to death of the 97 birds that died due to paretic syndrome and had no apparent injuries was close to the arrival, 67 dying within the first 48 hours and 27 after the first 48 hours but within 30 days. The majority (60%) of the birds were adults as within birds with abnormalities. Subadults and juveniles were almost equal in quantity (fig. 3.17). One fledglig was admitted already dead.

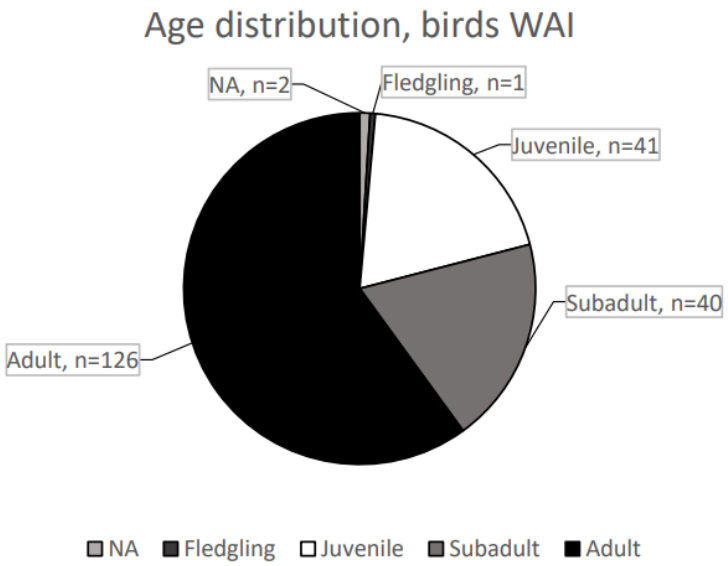


Figure 3.17 Age distribution within birds without apparent injuries.

The Body Condition Scoring (fig. 3.18) of the birds was analyzed within 205 birds of the 210 that had no apparent injuries. In this group, BCS3 and 4 were more common than within those gulls that manifested abnormalities.

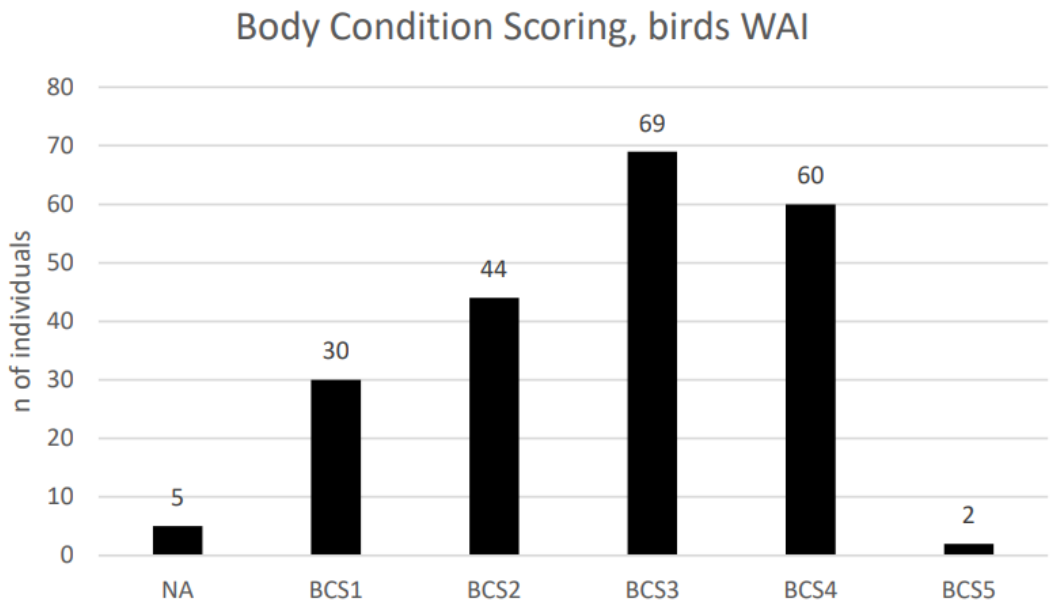


Figure 3.18 The gulls with no apparent injuries had quite evenly distributed BCS, however only 2 gulls scored 5. The majority of the gulls (n=69) were level 3 (ideal weight).

3.3 Other findings

Another common finding was parasites, especially *Reighardia sternae*; 8 gulls out of 446 were found to carry it. Besides parasites, during the sampling period, a juvenile gull suffering from PS was found to have a blockage in his digestive system caused by a condom (fig. 3.19). It had blocked the pylorus, inhibiting food to enter the intestines. Another bird dissected during the sampling period suffered from a fishing line having caused necrosis in the left leg (fig. 3.20). Since the gull had already lost the other leg as well, possibly due to same reason, euthanasia was the only option in his case.

Figure 3.19 Rumpled condom found inside the gizzard of a juvenile gull, blocking its digestive tract.

Figure 3.20 Fishing line had prevented the blood circulation and caused necrosis on the left leg of a gull.

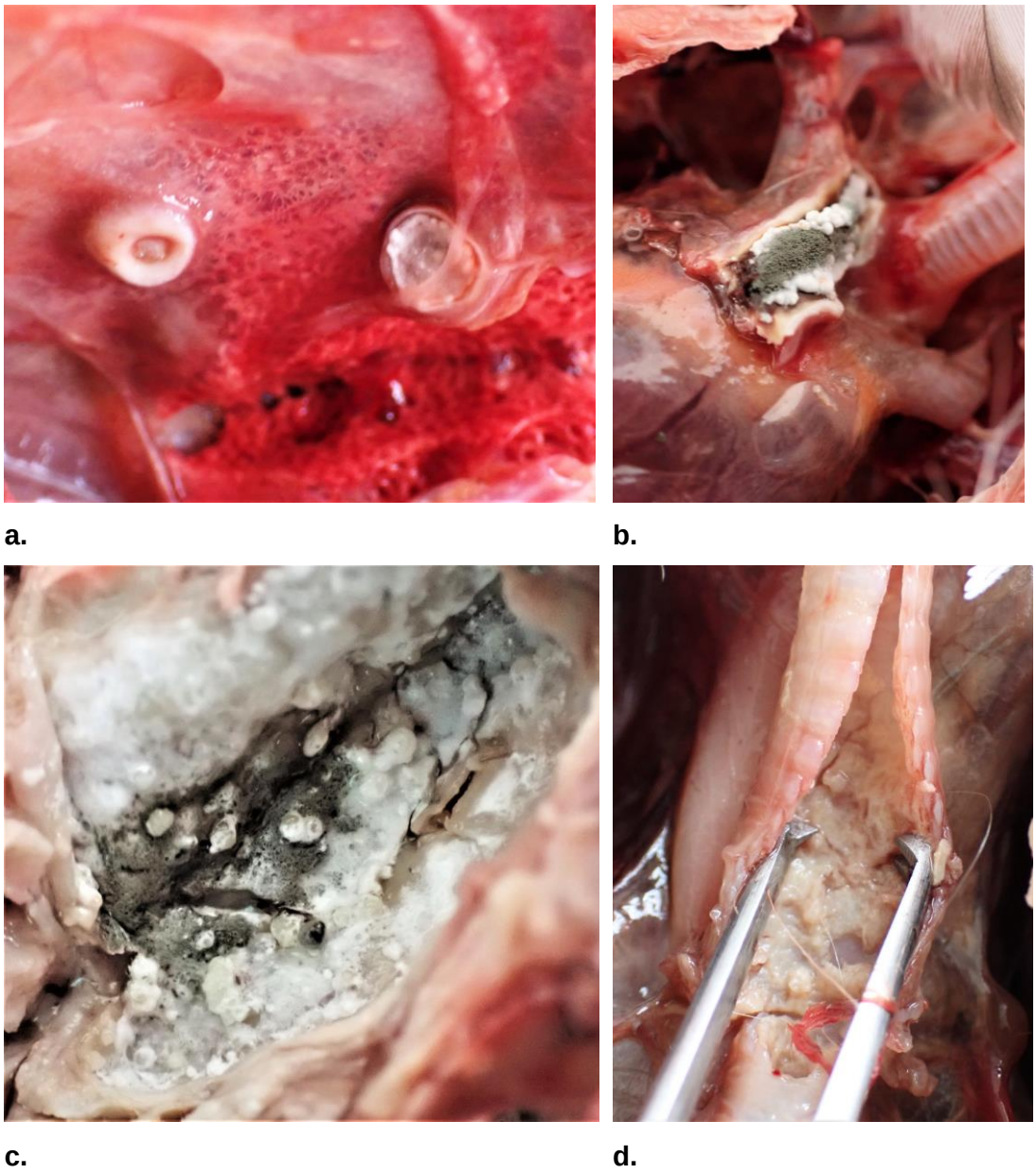
3.3.1 Kidney histopathology

The histopathological examination revealed that the gull was suffering from a chronic urolithiasis, with uric stones in the ureters. Also, the possibility of an intense bacterial infection was noted. The presence of fibrin, a protein responsible for clotting blood, accumulations of dead cells, macrophages and epithelioid cells were noted.

3.3.2 Aspergillosis

Aspergillosis was present in 39 of the 446 gulls (8,7 %) in various grades. The most severe cases covered the whole chest cavity, disrupting the ability to breath and in less severe cases small patches of aspergillosis appeared in the lungs, trachea, flanks or in the heart of the bird (fig. 3.21). In mycology identification the laboratory testing confirmed the presence of *Aspergillus spp.*

The histopathological examination of the sample of lungs collected from one gull demonstrated a dense constituent of epithelioid macrophages, fibrin and degenerated cells. Also, haemorrhagic parenchyma was present together with alveolar collapse. The bird was diagnosed with severe pneumonia and the findings were associated with an intense necrotizing fungal infection.



a. **b.**

c. **d.**

Figure 3.21 a. small deposits of *Aspergillus* in the flanks of a gull. **b.** *Aspergillus* covering the aorta. **c.** a large deposit of *Aspergillus* under the oesophagus. **d.** gull with difficult aspergillosis, having white tacky deposits inside the trachea.

3.3.3 Gout

Gout was found in 6 gulls with diagnosed PS between 2015 and January 2022, occurrence rate being as low as 1.3 %.

4 DISCUSSION

4.1 Population overall

Overall, the birds died quickly after being admitted to the centre; 46 % of all gulls died within the first 48 hours. However, it is unclear how long the bird has been suffering from PS before being admitted to RIAS. As the BCS of the birds demonstrated the majority of the birds being extremely emaciated or emaciated, this hints that the majority of the birds suffered from chronic PS and that the condition of the birds gradually weakened before being found and admitted to RIAS, hindering the capability to seek for food. Those gulls, that had no apparent injuries and were admitted alive, scored higher BCSs, than those with symptoms.

It was observed that those birds, who scored low BCS (1-2), suffered from chronic syndrome. Their state had weakened gradually and their capability to seek for food was hindered. Their condition seemed to be mostly affecting their capability to move, which resulted in gradually depleting fat storages and dehydration. Those birds with BCS 3 or higher suffered from an acute syndrome, where the bird died quite suddenly. Chronic syndrome advances slowly, debilitating the bird gradually. This can be noted in low energy preserves as the bird has been incapable of obtaining its feed for a long period of time. Whereas the acute syndrome appears fast due to ingestion of a relatively high amount of causative agent. These birds might still be in good body shape and even though they might seem alert upon arrival, their condition might worsen quickly and lead to sudden death. Overall, the gulls examined seemed to be more malnourished than in healthy body condition. The differences in feeding habits caused some gulls to obtain a small amount of toxin possibly during a longer period of time, whereas some birds might have obtained a large amount of causative agent, possibly even from the same feeding source. As hypothesized, birds with chronic syndrome and low BCSs demonstrated more abnormalities in their organs, especially in kidneys as explained later.

The differences in feeding habits due to age groups and possible sensitivity of the causative agent to different age groups was studied as the age distribution of sampled gulls, which consisted mainly of adult gulls. There is no statistical significance

between the occurring abnormalities and different abnormalities nor any indication that a specific age group would be more sensitive to PS than others. Instead, the study findings suggest that the altering feeding habits between different ages as well as the age structure of gulls in the area are thought to be responsible of the high number of adults in sampled gulls. Previously, the age structure of *L. fuscus* in same heights during the winter months has been noted to be dominated by juveniles and subadults (age > 2 years) by 59,5 % compared to adults (Marques *et al.* 2009). Whereas populations of *L. michahellis* demonstrate adult dominance by 86,2 % compared to juveniles and subadults in Western Morocco (Bellout *et al.* 2021). As the year-round prevalence of *L. michahellis* causes it to be more dominant species compared to *L. fuscus*, it is likely that there are purely more adult gulls inhabiting the area than subadults, juveniles and fledglings. Therefore, naturally more adults become ill than birds from other age groups. Also, the species sampled have a maximum recorded longevity of 19-35 years (19,2 for *L. michahellis* and 34,9 for *L. fuscus*) (Fransson 2010), highlighting the adult dominance of *L. michahellis*.

The very low number of fledglings (n=2), could be due to fledglings normally being outside of the view of humans, either in nests or bushes. Therefore, even if there were more fledglings suffering from PS, it is very unlikely that these are found alive. If not being found by predators due to being an easy catch, those fledglings that die due to PS are most likely eaten by scavengers or if found by a human, left where found. Also, the altering feeding habits affect the low number of fledglings and juveniles being studied. Where fledglings and juveniles eat mainly fish and other seafood, and adults depend more on terrestrial feed outside of the breeding, incubation and rearing season, as mentioned before, it seems that adults obtain the causative agent mainly from terrestrial sources.

Besides the age of the birds, the sensitivity to the illness due to differences in feeding habits between the two sexes were observed. Sex of the birds was not considered to be a significant factor in the occurrence of PS (p-value > 0.05). Although the majority of the gulls examined were female, the difference in numbers between female (n=201) and male (n=176) was not great. Male gulls are studied to be more vulnerable to environmental toxins due to female gulls being able to excrete toxins via fatty egg yolks, resulting in higher mortality within male gulls (Hunt & Hunt 1977). The migratory

birds could obtain environmental toxins anywhere, possibly resulting in higher presence of female gulls in the populations due to their better resilience, which could explain the higher number of deceased female gulls. Although, the difference in number could be related to differing eating habits of these two sexes as well. Studies have noted that female gulls are less competitive than male gulls when it comes to feeding sites. Females prefer to select sites with less competitive interactions or time their feeding when less gulls are present (Pons 1994, Coulson *et al.* 1985). There is a possibility that female gulls have chosen sites where the causative agent behind their condition is greater in number than on those sites, where male gulls feed. It is not viable to state that female gulls are more susceptible to PS without testing these hypotheses in the area. In here, no sex is suggested to be more immune or vulnerable than the other.

Besides female gulls being more represented in the populations, *L. michahellis* ruled in the species distribution with 302 performed necropsies compared to *L. fuscus* with 144 performed necropsies. The differences in these numbers are believed to be caused by their different migratory patterns. Southern Portugal hosts more individuals from species *L. michahellis* than *L. fuscus*, which explains the difference in their occurrence rate. It is not suggested that the migratory birds bringing the causative agent from outside of the Algarve area would be a significant cause of PS in the area. The year-round occurrence of PS in gulls of species *L. michahellis*, hints that there is a continuous source of causative agent in the area. The peaks in admission rates in autumns and springs are most likely due to a high number of gulls being present in the area, both *L. michahellis* and *L. fuscus*. The higher the bird density, the more gulls are susceptible to the condition.

4.2 Birds without apparent injuries

As mentioned in 4.1, the birds that manifested no apparent injuries, scored higher BCSs than those with visible abnormalities, meaning that they most likely recently lost their capability to move. It is likely that these gulls digested a quite large dose of causative agent, resulting in an acute syndrome that quickly proceeded to death, fortified with the fact that the time of the death was close to the arrival to the centre, as

mentioned in 4.1. The syndrome is noted to first hinder the bird from flying, followed by inability to move, leg and neck paralysis, third eyelid paralysis, dyspnoea and finally obstructed breathing caused by the paralysis. Therefore, birds without apparent injuries went through these stages rather quickly compared to those birds who suffered from chronic conditions and most likely died by suffocating slowly. This would explain why the condition could not be seen in their organs; as the bird was able to eat until recently the dehydration and starvation did not cause visible damages in the body.

Some gulls with no notable abnormalities represented a low BCS though, such as one fledgling, who was brought to the center simultaneously with another fledgling with distended cloaca and BCS1, both already dead. The fledgling with no apparent injuries had a BCS2. As fledglings rely on parental aid in feeding, it seems that the parents were unable to feed them for a while already, most likely suffering from PS as well. These two fledglings eventually died due to PS and/or starvation.

4.3 Excretory system

The most affected organ within the sampled birds was kidneys. If the kidneys are unable to excrete the metabolic waste filtered from blood, urine starts to build-up in kidneys, causing hydronephrosis and being chronic, gout. Extreme hydronephrosis is referred to as nephromegaly, found in 87 of the dissected gulls. Also, the quantity of uric acid in plasma increases due to dehydration, starvation and some bacterial and viral infections. When a bird is healthy and normally hydrated, only a small amount of the urea excreted by kidneys is reabsorbed in the renal tubules. However, if the bird suffers from dehydration, nearly all of the urea is reabsorbed, hindering the excretion of the nitrogenous waste (Coles 2007).

Another factor causing kidney nephromegaly is the failure to control the blood flow kidneys receive. Kidneys communicate with the nervous system via renal plexus that regulates the impure blood flow to the kidneys. If the communication is disrupted e.g., due to BoNTs binding with the neurons and preventing the messaging, the kidneys fail to communicate with the nervous system and the regulation of blood flow to the kidneys is hindered (Vize *et al.* 2003 and references within). Whether it is the failure of

the kidney to excrete the waste, the nerve blocking, or the cofunction of these two, that cause the nephromegaly in these birds, is unclear. Also, the pain experienced by these gulls due to the nephromegaly is difficult to estimate. The messages of pain travel from kidneys to spinal cord, and the sensory input can be sensed in the flank region (Vize *et al.* 2003 and references within). Whether the inhibited neurotransmitter release in case of botulism numbs these messages, is also unclear.

The pale colour of kidneys, observed in 78 gulls, has previously been reported with rats in cases of polycystic kidney disease together with enlarged kidneys. This hereditary renal dysfunction leads either to death or requires a renal transplantation (Kaspareit-Rittinghausen *et al.* 1989). Also, goldfish with herpesviral haematopoietic necrosis have demonstrated pale colour and enlargement of kidneys (Jung *et al.* 1995). The pale kidneys have been associated with blockage of ureters in humans, often accompanied with enlargement of the kidneys (Dash *et al.* 2019). It is the accumulation of liquids or urine in kidneys that causes it to expand, altering the liquid concentration, and most likely due to this fact the overall colour of the kidneys turns paler.

Another notable abnormality was a granular pattern, observed in 77 gulls, where kidneys enlarge wholly or partially, and subsequently possibly decrease in size. This hypertrophy, in some cases followed by atrophy, causes destruction of small blood vessels in the renal cortex, creating fibrosis, responsible for the granular pattern (Allbutt *et al.* 1877). The condition, also referred to as renal fibrosis, occurs with various chronic kidney diseases (CKD). The progression of the fibrosis is a slow process, but independent of the cause, leads to a renal failure (Liu 2006 and references within). This, in turn, requires a successful renal replacement in the form of transplant or dialysis, which currently is not a possible treatment within animals (Lees 2004).

In cloaca, the most common abnormality was the augmented size of it, with 10,8 % of the dissected gulls suffering from it. Four gulls had bloody content in their cloaca besides being augmented. Similar findings are supported by Soares 2014 and Neimanis *et al.* 2007. The distended cloaca hints that the cloacal sphincter, physically responsible for extracting the content of cloaca, is not receiving the nervous impulses that cause it to contract and/or relax. Therefore, the content of the cloaca is either emptied continuously, forming large patches of diarrhoea on the stomach feathers of a bird or it is

continuously contracted, preventing the excretion. In the first case, if the osmoregulatory function of extracting liquids and salts from the urine would still work, is hindered due to the constant free-flow of excrements. This further contributes to dehydration. In the latter case, whether the movement of urine is still functioning or not, the bird most likely experiences extremely unpleasant pressure due to the distention of the cloaca, which in worst cases causes the cloaca to tear. The movement of the cloacal sphincter as well as other autonomous processes happening in the caudal end of a bird are regulated by messages received from the thirteenth cranial nerve (Graber 1987).

Since the birds arriving to the RIAS demonstrate dehydration, renal disfunction and distention of cloaca, it is highly feasible that this process of osmoregulation is disrupted in these birds. Also, the presence of uric stones in cloaca demonstrates the high quantity of uric acid.

Based on findings represented in 4.3, three operations or rather lack of operations found in this study, that disrupt the excretory system of a bird affected by PS are:

1. If the kidneys are unable to transport the urine to the cloaca via ureters, the waste filtered from blood is trapped in kidneys, most likely leading to renal failure

2. If the autonomous nervous system of a bird is unable to move the urine between cloaca and gut, no absorption of water and salts will happen, contributing to osmotic disbalance and dehydration

3. If the cloacal sphincter is unable to receive messages from the cloacal nerve and thus fails to empty the content of the cloaca, it will gradually distend, completely blocking the excretory system of a bird.

The disrupted excretion of cloaca content supports the development of toxicosis by restraining the metabolic waste in the system. Also, as in four cases within this study, the cloacal wall weakened, causing the blood veins around cloaca to tear, further worsening the situation.

4.4 Pancreas and liver

Pancreas in 14 cases demonstrated to have abnormal dark spots and in very few cases pancreatic atrophy/lipoatrophy, often associated with chronic pancreatitis and in very few cases as well granular pattern. In pancreatitis, the pancreas becomes inflamed due to the enzymes produced by pancreas becoming active already within the pancreas instead of duodenum. The enzymes begin to break down the tissues in pancreas, a state referred as intrapancreatic proteolytic enzyme activation. The causes behind this condition are suspected to be either an obstructed pancreatic duct or a reflux event in the pancreatic duct. In first case the obstruction might lead to a rupture that activates the enzymes and in the latter case, the catalysator enterokinase, secreted in duodenum finds its route to pancreas, where it will activate the digestive enzyme trypsinogen (Steer *et al.* 1984, Arendt *et al.* 1999).

It is impossible to confirm whether the dark patches in pancreas found in this study result from pancreatitis. However, taking in account the possible development of the disease e.g., reflux in pancreatic duct, it seems very possible that the autonomous nervous system is unable to control the movement of enterokinase between duodenum and pancreas. Pancreatitis has been studied to cause colour changes in pancreas in humans and rats (Fitz 1889, Liu et al. 2009). It has also been studied to be causing pancreatic fibrosis (Masamune et al. 2009), that could be responsible for the granular pattern observed in two individuals.

Pancreas receives stimulation from the parasympathetic nervous system via the vagus nerve. It is studied that the stimuli sent by this nerve causes pancreas to secrete pancreatic juices via pancreatic ducts in dogs and pigs (Hickinson 1970). Also, the vasoactive intestinal polypeptides (VIP) hormones, produced by pancreas and appearing in especially large number in the walls of main pancreatic duct (Sundler *et al.* 1978), have been studied to activate the flow of pancreatic juices in birds (Vaillant *et al.* 1980). The production of VIP hormones and the stimuli received from vagus nerve is therefore responsible for the action of secreting the pancreatic juices, that in contact with enterokinase begin to digest the proteins in contact. In case of pancreatitis, the proteins in pancreas are in target.

As the injuries in pancreas refer to pancreatitis and based on findings in cloaca and kidneys, it is very likely that the inhibited release of neurotransmitters caused by BoNTs (Bozzi *et al.* 2006) and the disrupted production of VIP hormones leads into dilation of pancreatic duct, allowing a reflux that causes the intrapancreatic proteolytic enzyme activation. As the number of gulls suffering from abnormalities in pancreas is relatively low, it might be possible that the rich storage of VIP hormones in the walls of the main pancreatic duct keeps the juice flow active until the PS is sufficiently prolonged. As the majority of the gulls with abnormalities in pancreas had a BCS of 1 due to starvation, it hinders that the birds suffered from chronic PS. Therefore, it could be stated that the effects occurring in pancreas are low in number and that a slowly progressing PS is more likely to cause damage to pancreas than an acute one by interfering with the excretion of pancreatic juices causing an acute pancreatitis.

In the liver, the most common abnormality was liver atrophy, manifested in seven individuals, whereas other abnormalities such as fragile liver, white and dark spots, hepatomegaly and pale colour were very few in numbers (n=1-3). Although overall, the abnormalities found in the liver were scarce, it is very likely that the liver atrophy was a consequence of starvation caused by PS. Fasting accelerates the naturally occurring degradation of proteins in liver, whereas the protein synthesis referred to as hepatic regeneration (Garlick *et al.* 1975) practiced by birds and other vertebrates (Sidorova 1962), remains roughly the same. This regenerative hypertrophy is controlled by the central nervous system via autonomic nervous system (Kiba 2002 and references within). Although the relation between liver atrophy and fasting is confirmed with various studies (Samarel *et al.* 1987 and references within), it is possible that PS itself further contributed to liver atrophy by disrupting the normal regenerative process of liver. However, various conditions have been studied to cause liver atrophy, such as PCB toxicosis (Vos 1972) and fumonisin mycotoxin (Colvin *et al.* 1339). Whereas microcystins produced by some freshwater cyanobacteria species from genus *Microcystis*, have been associated with hepatomegaly and fragile liver (Stewart *et al.* 2008 and references within).

Here, the most probable cause behind the liver abnormalities, especially liver atrophy is believed to be hindered hepatic regeneration due to starvation and possibly due to inhibited neurotransmitter release. As the majority of the birds with liver abnormalities

were extremely emaciated (BCS=1, n=10), and all of the ones suffering from liver atrophy had a BCS1, it is very likely that only a chronic PS that advances gradually and very slowly, manages to affect the liver, causing liver atrophy due to starvation.

4.5 Aspergillosis

As aspergillosis was found in 8,7 % of the gulls, its role in the manifested abnormalities is considered to be very low. However, PS has most likely facilitated the spreading of aspergillosis within the individuals. As the immune system of the bird is weakened, the bird is more sensitive to *Aspergillus* (Beernaert *et al.* 2010). Also, being captivated increases the stress in birds, which leads to weakened immune system. These, as well as small premises where bird density is relatively high, compared to natural habitat, predispose the birds to a higher probability of getting infected. The infections caused by *Aspergillus* found in the birds varied greatly. Others demonstrated small patches of the fungus whereas in some birds the fungus had spread to lungs, heart, trachea and body cavity. Where *L. fuscus* and *L. michahellis* are quite resistant to the fungus, the study suggests that PS most likely contributes to the progression of the infection due to reasons mentioned above.

4.6 Gout

Gout was found to be present in 1,3 % of the birds. As gout is not common in wild birds, it is suggested that PS that leads to renal dysfunction, causes the uric acid to accumulate in the blood of the affected birds, leading to gout. However, the occurrence rate of gout was very low, whereas the damages found in kidneys notably higher. It takes weeks for a chronic kidney failure to develop, and since gout appears only with chronic kidney failure, the bird most likely dies to PS prior to developing gout.

4.7 Quality of data

There was a clear mismatch in occurrence rates between the data from 2016 to 2021 and data from the sampling period of autumn 2021 to January 2022, sampling period

demonstrating notably higher occurrence rates. The reasons behind this are various, such as the effects of storing the birds in the freezer prior to necropsy, different observers etc. Also, as in this study it was clear what was to be observed in the necropsy, it is possible that the number of findings is therefore higher than when performing the necropsies as a routine observation.

The sampling period gave information on birds that died during the migration period, whereas the dataset gave information on birds that died throughout the year. During the sampling period, the birds were mainly suffering from chronic syndrome, with a high occurrence rate of abnormalities in kidneys and pancreas, whereas between 2016-2021 the birds have demonstrated more acute syndrome; less abnormalities and higher BCSs. As the sampling period focused on a 4-month period, where bird density is high in the area, it is possible that the suspected continuous source of causative agent has been ingested in less quantities by more individuals, resulting in more frequent chronic cases. Throughout the year, as the density of birds within the area changes drastically due to migration, there are seasons where the number of gulls is notably lower, such as summers. During these seasons, it could be that the gulls manage to attain a higher amount of the causative agent due to less competition and therefore develop an acute syndrome that manifests no apparent abnormalities found in necropsies. However, the number of affected gulls is therefore lower as well.

Fampling for botulism as well as other biotoxins were excluded from the study. However, the preliminary results obtained by RIAS point towards BoNTs as the cause of the syndrome. Warm temperatures are more favourable for the *C. botulinum* to grow, possibly resulting in high concentration of BoNTs. Botulism cases have been studied to be most occurrent in the Mediterranean area during summer months, especially in July (Vidal *et al.* 2013). In areas with similar climate, such as Northern California, the presence of *C. botulinum* has been reported to be consistent in the flooded saltmarshes throughout the year. However, in seasonally flooded saltmarshes the prevalence was lower than in those that were flooded throughout the year (Sandler *et al.* 1993). The peak in numbers of gulls admitted to RIAS begins in mid-August, corresponding with findings made by Vidal *et al.* (2013). However, the numbers peak in October and keep decreasing until January. Therefore, in case of botulism, it is more likely that the

number of the birds in the area is more responsible for the occurrence of the condition rather than the seasonal variations in the occurrence of *C. botulinum*.

Although there was some data incoherence regarding the quantities, it can not be disclaimed that the effects of PS and of the causative agent behind it can be clearly seen in the gulls. The hypothesized causative agent *C. botulinum* refers to an imbalance in the environment these gulls inhabit. Though the bacteria naturally exist in the environment, the increasing number of admissions tells about the state of the environment these birds inhabit and that there exists a cause behind the increasing presence of this bacteria, that according to previous studies is likely to be caused by anthropogenic activities. Other findings in this study, such as a discarded condom that obstructed the metabolism of a gull, piece of glass that might cause cuts in the intestines and fishing line left in the nature that resulted in euthanasia of the gull speak for the anthropogenic exploitation, that besides the silent killer behind PS, does not only affect species of gulls, but other animals in the area as well.

5 CONCLUSIONS

Symptoms associated with PS and botulism have been studied for more than 100 years. The diagnostic and the progression of the illness, as well as the medical treatment are well known. The factors causing PS in birds resulting from warming climate, excessive nutrient inputs, improper waste management as well as chemical pollutants, have been studied, but the situation has not progressed. The deaths caused by the condition are processed in numbers and many times it is neglected that the animals suffer due to this condition, and that this could be prevented with adequate procedures.

This study aimed to explain the degradation caused by PS in the bodies of gulls, not only as quantitative findings but also as qualitative findings, that previously have not been addressed in studies. It was found that there were severe conditions in the bodies of the affected gulls that led to a slow gradual death of the animal. Besides the medical conditions, the affected gulls were mostly emaciated or underweight. Gulls who had most likely ingested frequently a small amount of the causative agent, demonstrated more damage and were thinner than those gulls, who had recently become ill. These

gulls had good fat reserves and an overall low number of injuries in their bodies, but their physical state weakened rapidly, leading to death via suffocation. By observing the gulls that arrived alive to the center and the body cavities during the necropsy, it could be noted that the birds were physically suffering during the whole process until death or recovery.

In here, the deteriorating effects of PS in the bodies of *L. fuscus* and *L. michahellis* are clearly demonstrated. Although animal suffering is often viewed sceptical within science due to the differentiating manners to demonstrate feeling within humans and animals, the unpleasant experience of these birds could be felt in their presence and this is suggested to support the analytical findings offered by this study. Although controversial in scientific circles, the mental experience of the animal is believed to be vital when addressing physical deterioration, and this aspect is hoped to be noted more in the science.

Although the causative agent behind the syndrome remains under study, preliminary results point out botulism as the etiology of the syndrome. In the future, a study that more profoundly examines the circulation of the causative agent is about to be published. Additionally, it would be important to study how the seasons affect the manifestation of the illness. Whether the cases are less frequent but more severe during the seasons when less birds inhabit the area compared to the migration peaks. This information, besides studying areas such as the main landfill site of Algarve, that piles unsorted waste and the water treatment plants as well as the estuaries and lagoons of Southern Portugal could lead us to the origin of the causative agent and help us implement procedures that would hinder the problem.

For appropriate legislation to be introduced, it is important that the severity of this problem is not recognized only in scientific circles but in politics and media as well. Although *C. botulinum* occurs naturally in the environment and human health is not directly under threat due to this, the increase in anthropogenic activities has been studied to encourage the growth of this bacteria, resulting in frequently recurring mass-deaths of animals, especially birds. Also, this tends to alter the structure of food webs, causing disbalance in the species structure. These events in turn deteriorate the global biodiversity that already is under threat and that we deeply depend on. Though in here,

we discuss gulls dying due to PS, this can be considered as a symbolism for all the life that is threatened due to the egoistic decisions of humans. And if one can not become more conscious in choices one makes in everyday life, how can this be expected from whole humankind where demand creates production? As long as we measure human wellbeing that focuses on economic factors rather than values that maintain us alive; such as clean water, unpolluted environments and biodiversity, we fail to give value to our actual wellbeing and that can clearly be seen in our deteriorating surroundings.

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