NO EVIDENCE OF AVIAN MALARIA IN TWO MEDITERRANEAN ENDEMIC SEABIRDS

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ABSTRACT

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In birds, pathogens and diseases, such as avian malaria, can have severe detrimental effects on individual fitness. Pathogen prevalence can vary across species and may differ between populations living in different localities, but screening can aid in our understanding of a disease's distribution and parasite-host interactions. Although seabirds generally exhibit low avian malaria infection patterns, blood parasites of several species and populations have never been investigated in detail. Using molecular techniques, we screened for blood parasites in two Mediterranean seabirds, the Scopoli's Shearwater *Calonectris diomedea* and the Mediterranean Storm Petrel *Hydrobates pelagicus melitensis*. In addition, we searched for and sampled potential vector insects at each seabird colony. DNA was extracted from blood samples (or whole specimens for vector insect species), and polymerase chain reaction was performed to assess the presence of *Plasmodium*, *Haemoproteus,* and *Leucocytozoon*, the most frequent infective protozoan genera. Our results showed no evidence of haemosporidians, either in the sampled species or in the vector insects. The low prevalence of parasites in these species could reflect the absence or rarity of the vector for transmission. Thus, extreme care must be taken when releasing individuals into the wild to avoid introducing infection into new seabird populations.

Key words: *Aedes mariae*, blood parasites, *Calonectris diomedea*, *Haemoproteus, Haemosporidian*, *Hydrobates pelagicus*, *Leucocytozoon*, *Plasmodium*

INTRODUCTION

In wild animals, pathogens and diseases have marked effects on individuals' fitness, modulating population processes and ultimately community structures (Johnson & Hoverman 2012). In birds, avian malaria and malaria-like diseases hold an intrinsic pathogenic potential that may negatively affect a host's fitness by diminishing flight and migration performance (Emmenegger *et al*. 2020), breeding success (Merino *et al*. 2000), survival probability, and lifespan (Asghar *et al*. 2015). Such detrimental effects could lead to the decline of entire populations or even species (Atkinson *et al*. 1995), making avian malaria parasites of important conservation concern. The agents responsible for these infections are vector-borne protozoan parasites of the haemosporidian order (*Apicomplexa: Haemosporida*). Most haemosporidian diversity is embodied into three genera, with specific dipteran families acting as their vectors. Biting midges (Ceratopogonidae) and louse flies (Hippoboscidae) transmit *Haemoproteus*, mosquitos (Culicidae) transmit *Plasmodium*, and black flies (Simuliidae) transmit *Leucocytozoon* (Valkiūnas 2005). These parasites, due to their almost cosmopolitan distribution, affect birds across a wide range of habitats and host species (Fecchio *et al*. 2021).

Seabirds are a peculiar group of long-lived birds that usually nest colonially. They spend most of their life cycle at (or in close proximity to) the sea, and they generally nest at remote and inaccessible sites (e.g., islands, cliffs; Furness 2012). Haemosporidian parasite infections in seabirds have been previously investigated, both with microscope observation of blood smears (e.g., Esparza *et al*. 2004, González-Solís & Abella 1997, Merino & Minguez 1998) and with molecular techniques (e.g., Campioni *et al*. 2018, Quillfeldt *et al*. 2010, 2014). In general, most of the screened populations have revealed a week infection pattern, and for a substantial number of species, no evidence of infection has been detected (Quillfeldt *et al*. 2011). Traditionally, haemosporidian investigation has been conducted through microscopic examination of blood smears. However, in more recent decades, polymerase chain reaction (PCR) detection protocols have been widely applied (e.g., Bensch *et al*. 2000, Drovetski *et al*. 2014, Hellgren *et al*. 2004). The advantage of PCR, compared to traditional methods, is that it can detect low parasite burdens because of its high sensitivity (Waldenström *et al*. 2004), and it also permits the genetic characterization of haemosporidian lineages and diversity (Clark *et al*. 2014). Indeed, molecular protocols have strongly accelerated research in avian malaria, leading to the development of the MalAvi open source

dedicated database (http://130.235.244.92/Malavi; Bensch *et al*. 2009) that hosts updated haemosporidian cytochrome *b* sequences and metadata related to 4 522 unique parasite lineages, 2 100 bird species, and 524 publications (Version 2.4.9, 23 April 2021).

The aim of our study was to screen for avian malaria occurrence, using PCR, in two Mediterranean seabird species previously unscreened through molecular methods: the Scopoli's Shearwater *Calonectris diomedea* and the Mediterranean Storm Petrel *Hydrobates pelagicus melitensis* (hereafter "storm petrel"). Both species are endemic to the Mediterranean basin. The shearwater's Atlantic counterpart, Cory's Shearwater *C. borealis*, has been screened through both microscopic inspection and molecular techniques (Bried *et al*. 2011, Campioni *et al*. 2018), revealing an absence of haemosporidian infection. In contrast, the storm petrel has been investigated previously, but only through inspection of blood smears (Esparza *et al*. 2004). Our final goal was to improve existing knowledge of haemosporidian-seabird relationships using up-to-date molecular techniques, ultimately providing valuable information for conservation planning.

METHODS

Blood sampling

We sampled 38 Scopoli's Shearwater (adults: n = 18; fledglings: n = 20) breeding on two small islets (< 3 km from the coast) in the Parco Nazionale Arcipelago di La Maddalena (Italy), (41°13′N, 9°14′E, 400–1 000 breeding pairs). We sampled six adult storm petrels from a colony in the Parco Naturale Regionale di Porto Conte e Area Marina Protetta di Capo Caccia-Isola Piana (40°36′N, 8°11′E, 600 breeding pairs). Storm petrels breed in deep caves that are only partially accessible to humans and are located on small islets approximately 300 m from the coast. All sampled individuals were sexed by PCR using the CHDF-CHDR primers (Fridolfsson & Ellegren 1999). Fieldwork was conducted during the 2018 and 2019 breeding seasons (July–October). Birds were caught by hand at their nest sites, then ringed with a unique metal ring to avoid double sampling and released back at their nest. Before release, 50–100 µL of whole blood was drawn from the tarsal (shearwaters) or brachial (storm petrels) vein after puncturing it with a sterile 25-gauge needle. If necessary, bleeding was stopped using sterile cotton and pressure. Blood samples were then preserved in absolute ethanol at -20 °C until further analysis. Capture, handling, and sampling procedures were carried out by the Italian Institute for Environmental Protection and Research (ISPRA), under the authorization of Law 157/1992 [Art.4(1) and Art 7(5)]. Permission was also secured from the Parco Nazionale Arcipelago di La Maddalena and the Parco Naturale Regionale di Porto Conte.

Laboratory procedures

DNA was extracted from blood samples using the E.Z.N.A. tissue extraction kit (Omega Bio-Tek, Norcross, GA, USA) following standardized protocols. PCR was used to detect haemosporidian occurrence by amplifying a fragment of the mitochondrial DNA cytochrome *b* (*cytb*) gene. We followed the molecular protocols described in Pellegrino *et al*. (2021) and utilized three primer pairs known to be successful across avian haemosporidian genetic diversity (Drovetski et al. 2014). These primers amplify all three genera of avian haemosporidia (i.e., *Haemoproteus, Leucocytozoon*, and *Plasmodium*). To verify the presence of infections, 5 µL of the PCR products were run on a 1.8% agarose gel. All reactions were performed with negative (double-distilled H_2O) and positive controls (infected individuals of other species, determined after three repeated PCRs; see Pellegrino *et al*. 2021) to evaluate the validity of the PCR and detect possible contamination.

Vector survey

In both colonies, an opportunistic vector survey was performed to verify the occurrence of any insect family that was potentially suitable for avian malaria transmission. During the ringing procedures, all sampled birds were systematically inspected for ectoparasites (i.e., louse flies; Tella *et al*. 1998) for approximately 2 min. Free-living biting insects (i.e., Culicidae mosquitoes) were collected during field operations and stored in absolute ethanol. Ceratopogonidae biting midges and Simuliidae black flies were never observed by field workers. Mosquito DNA was extracted using a E.Z.N.A. kit from each whole specimen (n = 10). Haemosporidian presence was checked with the same primers and procedure utilized for birds. The morphologic identification of insect species was confirmed by a barcode procedure, using LEPF1-LEPR1 primers for the cytochrome *c* oxidase gene (COI; Hernandez-Triana *et al*. 2019), following the PCR reaction suggested by Pellegrino *et al*. (2017) and the thermal cycling protocol described in Ilahiane *et al*. (2021).

RESULTS

All of the sampled shearwater and storm petrel individuals were negative for haemosporidian infections (Table 1). No vector species were detected during ringing operations in the storm petrel colony. In the shearwater colony, we found only one insect species potentially suitable as a haemosporidian vector: the mosquito (Culicidae) *Aedes mariae*, found both at the larval stage in nearby small ponds and as flying adults. The *Aedes mariae* mosquitoes screened through PCR for haemosporidian occurrence resulted in a negative reading for haemosporidian presence.

TABLE 1
Observed prevalence of haemosporidian infection in Scopoli's Shearwaters Calonectris diomedea and Mediterranean Storm Petrels Hydrobates pelagicus melitensis breeding at colonies in the Mediterranean

Species	Colony	Females	Males	Age	Total	Prevalence
Scopoli's Shearwater	La Maddalena	10	8	Adults	18	0%
	La Maddalena	11	9	Chicks	20	0%
Mediterranean Storm Petrel	Porto Conte	5	1	Adults	6	0%

DISCUSSION

To the best of our knowledge, this study is the first molecular screening of avian haemosporidian in either Scopoli's Shearwater or Mediterranean Storm Petrels from the Mediterranean Sea. Although the combination of microscopy and PCR is currently considered the most accurate approach to detect this group of blood parasites (see Valkiūnas *et al*. 2016, Jia *et al*. 2018), we have analysed the samples only through the PCR screening. We opted for such a protocol because both target seabird species had previously tested negative for haemosporidians through microscopy (González-Solís & Abella 1997, Merino & Minguez 1998). Indeed, it has been shown that molecular assessment of haemosporidian prevalence is suitable for studies of avian malaria because it can detect parasite occurrence at very low infection rates; in contrast, microscopic examination of blood smears is less effective when parasites are not abundant (Waldenström *et al*. 2004). Inspection of blood smears can also be time-consuming, even with the development of new automated, more rapid methods for analysis (Gering & Atkinson 2004). Moreover, molecular methods are best suited to concurrently identify haemosporidians at the genus level and to discover the occurrence of new lineages (Clark *et al*. 2014).

Although the screened sample size is modest and may affect the prevalence assessment, particularly for the storm petrel, our study supports the absence of haemosporidians in Scopoli's Shearwaters and Mediterranean Storm Petrels, and our results are consistent with former studies on procellariiforms. Indeed, previous findings of infection rates in most seabird hosts have been very low (Valkiūnas 2005, Soares *et al*. 2016, Mariano & Dantas 2021). This pattern is particularly intriguing compared to the high haemosporidian prevalence observed in terrestrial birds (Ricklefs *et al*. 2011) and could partially be explained by their peculiar life-history traits. The highly pelagic nature and/or close association with the marine environment (De Pascalis *et al*. 2020, 2021) could minimize the time available for infection (Soares *et al*. 2016). In addition, during the time they spend on land for reproduction, the probability of infection could be reduced due to the lack of suitable insect vectors at respective breeding grounds. This could be the result of local adverse conditions for insects at the larval stage. For example, freshwater habitats could be available only for short timespans that are inadequate for developing larvae, or water salinity could be too high for development (Soares *et al*. 2016). Our findings support this hypothesis because during ringing operations, we did not find ectoparasitic louse flies on the skin of any handled bird, nor did we detect free-flying biting insects of the families Ceratopogonidae (biting midges) and Simuliidae (black flies) (but see Gomez-Diaz *et al*. 2008, Stefan *et al*. 2015). The only abundant insects that were potential vectors were Culicidae mosquitoes of the species *Aedes mariae*, one of the few mosquito species adapted to develop in highly saline rock pools (Mastrantonio *et al*. 2015). However, of the insects tested, all were negative for haemosporidian presence, again suggesting that the probability of infection is very low.

Oceans and seas could also act as ecological barriers—the large and hostile environment imparted by saline waters on vector insects may hamper the flow of haemosporidians from mainland continental sources (Martínez-Abraín 2004, Ricklefs *et al*. 2011). Indeed, the four cases of avian malaria reported in procellariiforms were primarily related to infections contracted in recovery centres and zoos (Vanstreels *et al*. 2020, Inumaru *et al*. 2017, Quillfeldt *et al*. 2010, MalAvi database, Bench *et al*. 2009), involving weak and captive individuals. Nonetheless, at our study site, the ecological barrier effect was probably of minor importance. The study colonies were located relatively close to the coast of Sardinia, the second-largest Mediterranean island, which is known to host several haemosporidian taxa (Pellegrino *et al*. 2021); this territory could act as a source of haemoparasites, making our study colonies incomparable to other remote small oceanic islands located hundreds to thousands of kilometres from the mainland (Le Goff *et al*. 2014, Martinez-Abrain *et al*. 2004). Alternatively, seabirds could show a low prevalence of haemoparasites because they are equipped with a strong immune response that protects them from infection (Valkiūnas 2005). This characteristic could be an adaptation to water environments that is shared with several other waterbird species (Mariano & Dantas 2021, Larcombe & Gauthier-Clerc 2015).

Considering the lack of haemoparasitic infection in shearwaters and storm petrels (and the rarity of infection in seabirds in general), we recommend a careful check of haemosporidian occurrence in individuals harboured in recovery centres (possibly with both molecular and microscopy techniques) in order to avoid spillover of parasites and infestation of free-living populations in cases where birds are released from captivity. Moreover, future studies should assess if ongoing climate change fosters the introduction and/or spread of new vector insects (Kovats *et al*. 2001), and whether this alters the relationship between seabirds and vector-borne pathogens, increasing the occurrence of infection in this threatened avian group (Perez-Rodriguez *et al*. 2014).

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