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ORIGINAL ARTICLE

Comparative analysis reveals a possible immunity-related absence of blood parasites in Common Gulls (*Larus canus*) and Black-headed Gulls (*Chroicocephalus ridibundus*)

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Abstract Blood parasites often incur a substantial fitness cost to the infected individuals, sometimes resulting in death of the host. Some bird species, however, are apparently free of blood parasites, presumably due to the lack of exposure to blood parasite vectors. Protective immunity may be also responsible for the absence of infections by haematozoa. In this study, we tested the presence of blood parasites in Common Gulls (*Larus canus*) and Black-headed Gulls (*Chroicocephalus ridibundus*) nesting in environments with varying vector exposure. We failed to find blood parasites in Common Gulls irrespective of vector exposure, whereas infection rates of Black-headed Gulls were generally very low. We propose that the absence of haematozoa and low prevalence of blood parasites in these species of gulls is probably not a function of vector exposure and suggest alternative explanations such as enhanced immunity.

Keywords Blood parasites · Gulls · Ecological immunology · Haematozoa · Lack of parasites

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Zusammenfassung

Vergleichende Analysen zeigen eine möglicherweise mit dem Immunsystem zusammenhängende Abwesenheit von Blutparasiten bei Sturmmöwen (*Larus canus*) und Lachmöwen (*Chroicocephalus ridibundus*)

Blutparasiten bedingen oft substantielle Fitness-Aufwendungen für die betroffenen Individuen, die nicht selten zum Tod des Wirts führen. Manche Vogelarten sind jedoch offensichtlich frei von Blutparasiten, vermutlich deshalb, weil sie deren Überträgern nicht ausgesetzt sind. Diese Art von schützender Immunität ist wahrscheinlich auch dafür verantwortlich, dass es bei ihnen keine durch Hämatozoen bedingte Infektionen gibt. In unserer Studie untersuchten wir das Vorhandensein von Blutparasiten bei Sturm- und Lachmöwen, die ihre Nester in Umgebungen gebaut hatten, in denen sie in unterschiedlicher Weise einer möglichen Übertragung der Parasiten ausgesetzt waren. Unabhängig davon, wie sehr sie einer möglichen Übertragung ausgesetzt waren, konnten wir bei den Sturmmöwen gar keine Blutparasiten nachweisen, und bei den Lachmöwen war die

Infektionsrate durchweg sehr niedrig. Wir vermuten deshalb, dass bei diesen Vogelarten das Fehlen von Hämatozoen und die geringe Verbreitung von Blutparasiten nicht davon abhängen, ob und wie sehr sie den Überträgern ausgesetzt sind. Stattdessen vermuten wir hier andere Erklärungen wie z. B. ein stärkeres Immunsystem.

Introduction

Parasitism is one of the strongest forces affecting host evolution (Peirce and Prince 1980; Loye and Zuk 1991; Clayton and Moore 1997). Hosts are thought to be engaged in a never-ending arms race with parasites, in which hosts attempt to improve their defence in face of the rapidly evolving parasite attack mechanisms (Dawkins and Krebs 1979). Even more, the selective pressure on hosts to generate variability in their responses to parasites may be so strong as to explain sexual selection (Sorci 1995; Schmid-Hempel 2011). To infect a host, a blood parasite must adapt its surface antigens to resist the host's immune system. Such adaptations are not stable: vertebrate immune systems counter the adaptability of parasite antigens via rapidly evolving MHC domain (Schmid-Hempel 2011). Moreover, parasite life histories are often complex, including multiple hosts and vectors (Valkiūnas 2005), and host defences have evolved accordingly to vary from behavioural to immunological adaptations. Hence, ecological and physiological mechanisms underlying parasite and host interaction, as well as their abundance and distribution, are highly relevant to our understanding of parasite–host coexistence over evolutionary time (Sheldon and Verhulst 1996; Wakelin 1996; Zuk and Stoehr 2002).

Birds exhibit a remarkable diversity of immunological responses against parasites that have been studied in both wild and domesticated species (Isobe and Suzuki 1987; Atkinson et al. 1988; Ots and Hõrak 1998; Garvin et al. 2003; Morales et al. 2004; Tomas, Merino et al. 2005). Haemosporidians (*Plasmodium*, *Haemoproteus*, *Leucocytozoon*) are among the best-studied avian parasites. These common blood parasites use dipteran insects as definitive hosts and have been reported in most species of birds from all over the world (Valkiūnas 2005).

The existing evidence shows clear effects of haemosporidians on host physiology, as indicated by white blood cell counts (e.g. Massey et al. 1996; Ots and Hõrak 1998; Garvin and Greiner 2003) and humoral responses (e.g. Hõrak et al. 1998; Atkinson et al. 2001; Garvin and Greiner 2003; Morales et al. 2004; Tomas et al. 2005). Haemosporidians are responsible for several serious, even lethal, avian diseases (e.g. Lehmann 1993; Möller 1997; Valkiūnas 2005). In addition to direct reduction of their host's life expectancy, these parasites can decrease host's reproductive fitness (e.g. Ilmonen et al. 1999; Merino et al. 2000; Sanz et al. 2001; Marzal et al. 2005) and increase the risk of predation on their hosts (Temple 1987).

Many studies have estimated the prevalence of blood parasites in populations of wild birds (see checklists by Greiner et al. 1975; Peirce 1981a, b; Valkiūnas 2005). Although most bird species have been found to be infected with at least one species of haemosporidians, strikingly, many species appear to be apparently free from infections (Little and Earle 1994; Tella et al. 1995; Figuerola et al. 1996; Rytkönen et al. 1996; Forero et al. 1997; Gonzalez-Solis and Abella 1997; Stewart et al. 1997; Blanco et al. 1998; Merino and Minguez 1998; Engstrom et al. 2001; Jovani et al. 2001, 2002; Martinez-Abrain and Urios 2002; Quillfeldt et al. 2011). The apparent absence of blood parasites in some bird taxa is commonly explained by the absence or scarcity of parasite vectors in some habitats such as marine, saline, arid, open, or alpine/high latitude environments, or by lack of a compatible host–parasite assemblage (Bennett et al. 1992a, b; Earle and Underhill 1993; Little and Earle 1994; Bennett et al. 1995; Little and Earle 1995; Rytkönen et al. 1996; Tella et al. 1996; Gonzalez-Solis and Abella 1997; Piersma 1997; Stewart et al. 1997; Blanco et al. 1998; Figuerola 1999; Jones and Shellam 1999; Sol et al. 2000; Jovani et al. 2001; Martinez-Abrain and Urios 2002; Valera et al. 2003; Fokidis et al. 2008). Absence of blood parasites in some cases has been attributed to the inability of parasites to overcome the host immune system, and in these cases haematozoan prevalence may primarily reflect immunocompetence of the host (Ricklefs 1992; Forero et al. 1997; Merino and Minguez 1998; Tella et al. 1999). In other situations, lack of blood parasites may also be attributed to the possibility that ectoparasites might act to displace dipteran vectors required for transmission of blood parasites in bird species that suffer from a high intensity of ectoparasitism (Martinez-Abrain et al. 2004).

Failure to detect blood parasites may also be a purely methodological problem that arises because of the difficulty in detecting parasitemias. Haematozoan prevalence is traditionally recorded by microscopic inspection of blood smears (Peirce 1981a, b) where haematozoa can be observed during the patent period following infection (van Riper et al. 1994). Several studies have shown that microscopy is less sensitive than a polymerase chain reaction (PCR)-based technique and serological methods, resulting in underestimation of parasite prevalence, especially when infections are of low intensity (Jarvi et al. 2002; Ricklefs et al. 2005), whereas some other studies show that this may not be the case (Valkiūnas et al. 2006; Valkiūnas 2011).

Gulls (Charadriiformes: Laridae) are a group of medium to large opportunistic predators and scavengers of both

land and sea. Past studies have reported high prevalence and intensity of infections of *Haematozoa* in gulls (Ruiz et al. 1995; Martinez-Abrain et al. 2002; Quillfeldt et al. 2010), whereas other studies have reported a complete absence of blood parasites in several gull species (Peirce 1981a, b; Quillfeldt et al. 2010). The observed low incidence of blood parasites in some gull species is usually explained by low vector availability, which is often negatively related to distance from marine breeding sites to the coast.

In this study, we used microscopy and molecular techniques to estimate the blood parasite infection rates in the colonially breeding Common Gulls (*Larus canus*) and Black-headed Gulls (*Chroicocephalus ridibundus*). We examined adults and fledglings of the gulls at three inland colonies and one colony located on a small island in the Baltic Sea. One of the inland colonies supported both species, while the two other inland colonies consisted solely of Black-headed Gulls. The colony on the island in the Baltic Sea consisted solely of Common Gulls. The inland and sea island colonies differed in their exposure to blood parasite vectors. The abundance and activity of the vectors were high in inland colonies, while no dipteran vectors were observed on the sea island, possibly because of the low temperature and wind, which negatively affect dipteran foraging ability (Allander and Bennett 1994). We hypothesised that avian haematozoa may be absent from the blood of Common Gulls at the sea colony but present in the blood of inland birds if the absence is driven by dipteran vectors.

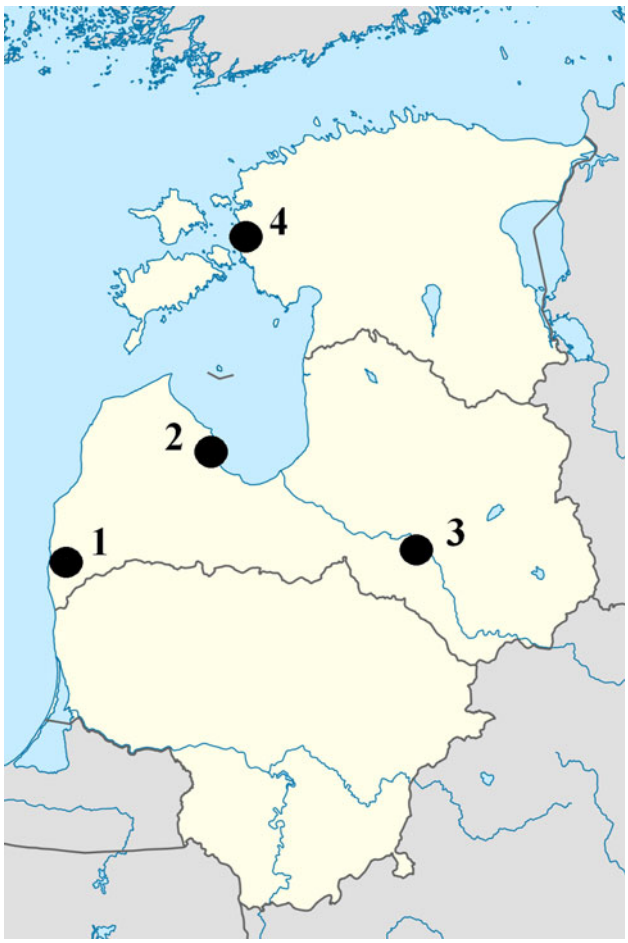

Fig. 1 Study area: *1* Lake Liepājas, *2* Lake Engure, *3* Lake Radzes, *4* Matsalu National Park

Methods

Study sites, birds and vectors

The study was conducted at three inland gull colonies in Latvia (Lakes Engure, Liepāja, and Radze) and at one colony in Estonian coastal waters in 2008 and 2009 (Fig. 1). The lake Engure (57°24′N, 23°10′E) and the lake Liepājas (56°44′N, 21°06′E) are shallow and heavily eutrophic ancient sea lagoons, divided from the Baltic Sea by a 2- to 5-km-wide bar of sand dunes covered by forests, and the distance between the colonies and the sea was about 7 km in both cases (Fig. 1). Black-headed Gulls breed at the both lakes in colonies reaching several thousand pairs. The artificial lake Radzes near the city of Jēkabpils (56°43′N, 25°86′E) is located 150 km from the Baltic Sea (Fig. 1). The gull colony consists of about 60 pairs of Common Gulls and 2,000 of Black-headed Gulls. The gull colony in the Baltic Sea is located on a small island in the Matsalu National Park in western Estonia (58°46′N, 23°26′E) and is situated 500 m from the coast (Fig. 1). This site is one of the most favourable for breeding Common Gulls in western Estonia, and it has supported a long-term study of the breeding biology of the Common Gull since 1962 (e.g. Onno 1968; Rattiste 1983; Rattiste and Lilleleht 1986, 1995). The number of breeding pairs in this site was 529 in 2008 and 550 in 2009.

Haematozoans are detectable in adult birds as well as in 14- to 20-day-old chicks. Common Gulls usually fledge 32–35 days after they hatch, while chicks of the Black-headed Gull fledge at the age of 24–26 days. We captured 19 adult Common Gulls and 67 fledglings as soon as they reached the age of 34–36 days in the mixed-species inland colony, and 250 adults and 24 chicks of Common Gulls in the colony in the Baltic Sea. We captured 12 adult Black-headed Gulls (Lake Engure only) and 29 fledglings (both at Lake Liepāja and Lake Engure) at the age of 26–30 days at the single-species colonies near the sea, and 16 adult and 40 fledgling Black-headed Gulls at the mixed-species inland colony 150 km from the sea. We obtained blood samples from each bird by puncturing the tarsal (in Latvia) and brachial (in Estonia) veins, and prepared three

individually marked microscope slides for each bird. Smears were air-dried in the field and immediately fixed with methanol and subsequently stained using Giemsa stain. We also obtained 50 µl of blood for molecular analysis. In total, we sampled 336 Common Gulls and 97 Black-headed Gulls.

According to some studies (Allander and Bennett 1994; Ruiz et al. 1995), the incidence and prevalence values of blood parasites are mainly dependent on the abundance and activity of parasite vectors. Although we did not estimate densities of vectors, we have data on the presence or absence of parasite vectors for all four colonies. Trapping with ultraviolet light suction traps at all three inland colonies revealed the presence of biting midges (Diptera: Ceratopogonidae). Blood-sucking mosquitoes (Diptera: Culicidae) and simuliid blackflies (Diptera: Simuliidae) were observed directly, and hippoboscid flies (Diptera: Hippoboscidae) were observed while handling birds. Although, especially, Lakes Engure and Liepājas are windy areas, we detected abundant numbers of blood-sucking simuliid blackflies, the vectors of *Leucocytozoon* (Valkiūnas 2005; Hellgren et al. 2008), biting midges and hippoboscid flies, the vectors of *Haemoproteus* (Valkiūnas 2005), and blood-sucking mosquitoes, the vectors of *Plasmodium* (Valkiūnas 2005), at all three colonies. In Latvia, we detected the following species of mosquitoes: *A. communis*, *A. punctor*, *A. maculatus*, *A. cataphylla*; biting midges: *Culicoides obsoletus*, *C. punctatus*, *C. pulicaris*; blackflies: *Odagmia ornate*, *Simulium verecundum*. Although blood-sucking dipterans were abundant on the mainland (Spuris 1974; Spungis 2000), no parasite vector was detected at the sea colony of Common Gulls in Estonia during the breeding seasons of 2008 and 2009.

Preparation and microscopy examination of blood smears

Blood smears were screened with a light microscope under oil immersion at ×1,000 magnification for *Haemoproteus* and *Plasmodium* and at ×500 magnification for *Leucocytozoon*, *Trypanosoma* and *Microfilaria*. Parasites were enumerated from 100 fields by moving the slide to areas where blood cells formed a monolayer for *Leucocytozoon* and from more than 200 fields for *Haemoproteus* and *Plasmodium*. Intensity of infection was estimated as a percentage by counting the number of parasites per 10,000 erythrocytes examined, as recommended by Godfrey et al. (1987). Microscopy examination of blood smears was done by T.K. and V.S.

Molecular detecting of blood parasites

The blood samples were stored in Queen's lysis buffer (0.01 M Tris, 0.01 M NaCl, 0.01 M Na-EDTA, 1 % n-lauroylsarcosine, pH 8.0). We implemented a PCR protocol designed by Bensch et al. (2000) and Hellgren et al. (2004) to screen blood samples of both gull species. DNA was extracted from the blood samples using a DNA extraction kit (Qiagen, Valencia, CA, USA). A basic assessment of the presence/quality of the extracted DNA was made by electrophoresing 2 µl of the DNA extract in 2 % agarose gel containing ethidium bromide, and visualising under UV light.

All reactions were performed in 25 µl volumes with first-round primers Haem NFI (5′-CATATATTAAGAGAAITATGGAG-3′ where I = inosine) and Haem NR3 (5′-ATAGAAAGATAAGAAATACCATTC-3′), which are general for species of *Haemoproteus*, *Plasmodium* and *Leucocytozoon*, and amplifying a fragment, including primers, 619 bp long. Each reaction contained 2 µl of genomic DNA, 0.125 mM dNTP, 0.2 µM each primer, 3 mM $MgCl_2$ and 0.25 units of Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA, USA) with the accompanying PCR buffer at 1× final concentration. The thermal profile consisted of a 2-min 94 °C enzyme activation step, followed by 20 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s, ending with an elongation step of 72 °C for 10 min. In the second round, primers Haem F (5′-ATGGTGCTTTCGATATATGCATG-3′) and Haem R2 (5′-GCATTATCTGGATGTGATAATGGT-3′) were used (Bensch et al. 2000) to amplify a fragment of 525 bp, and are specific to approximately 95 % of all *Haemoproteus* and *Plasmodium* lineages. The composition of the PCR reactions was as above, except 0.4 µM of each primer and 0.5 units of Platinum *Taq* polymerase were used, and 2 µl of the PCR product from the first round was used as template instead of genomic DNA. The thermal profile for the second-round PCR was the same as for the first round, except that the number of cycles was increased from 20 to 35. Amounts of 2–8 µl of the second-round reactions were run out on 2 % agarose stained with ethidium bromide and visualised under UV light.

Samples containing bands of the expected sizes were scored as positive. The purified PCR fragments were then sequenced directly by dye terminator cycle sequencing (Big Dye; Applied Biosystems, Foster City, CA, USA), and loaded on an ABI PRISM 3100 sequencing robot (Applied Biosystems). Sequences were edited in Sequencher v.4.2 (GeneCodes, Ann Arbor, MI, USA), and sequences were identified to the generic level of blood parasites.

Results

During microscopy examination of Black-headed Gulls, we found gametocytes of *Haemoproteus larae* (Valkiūnas 2005) in the blood of 2 adults and 3 fledglings. This was

the only haematozoan in the blood smears of Black-headed Gulls, with an overall prevalence of 5.15 % (5 infected individuals out of 97 gulls). We found 2 infected fledglings and 1 adult of the Black-headed Gull in the samples from the colonies of costal lakes, while 1 adult and 1 fledgling were infected at the inland mixed-species colony. The prevalence of infection did not differ either between adult and juvenile Black-headed Gulls (2-tailed $\chi^2 = 0.003$, $P = 0.95$, Yate's correction) or between the coastal colonies and the mixed-species colony located 150 km from the sea (2-tailed $\chi^2 = 0.13$, $P = 0.72$, Yate's correction). All of the recorded infections were light according to Valkiūnas (2005).

Molecular screening confirmed the findings of the microscopy examination, and we did not find any other infected Black-headed Gulls. By direct sequencing, we found that all were indeed attributable only to species of *Haemoproteus*.

Microscopy screening of Common Gulls did not reveal any apparent infection by bird malaria or *Leucocytozoon*, either in the inland or marine colonies. The only haematozoa found in the blood of 1 adult Common Gull of the mixed inland colony was an unidentified microfilaria. PCR techniques also failed to find any infection of *Plasmodium*, *Haemoproteus* or *Leucocytozoon*. The prevalence of infection differed significantly between Common Gulls and Black-headed Gulls (2-tailed Fisher's exact test, $P = 0.0005$), with infection rates in Black-headed Gulls higher than in Common Gulls.

Discussion

The results of this study revealed low infection prevalence of blood parasites in Black-headed Gulls and the apparent absence of blood parasites in Common Gulls, supporting previous reports of low incidence of blood parasites in gulls (Peirce 1981a, b; Randi and Spina 1987). The presence of parasites was determined by simultaneous use of microscopy and PCR techniques. Since both methods showed exactly the same estimates of *Haemoproteus* infections, we suggest that PCR diagnostics and microscopy are similarly sensitive in the detection of haemosporidian infections (Jarvi et al. 2002; Valkiūnas et al. 2006).

Whereas some bird species are heavily affected by blood parasites in the wild, others reportedly are not. The absence or scarcity of blood parasites has often been reported from avian groups such as seabirds (e.g. Peirce and Brooke 1993; Merino et al. 1997; Merino and Minguez 1998; Engstrom et al. 2000), swifts (Tella et al. 1995), waders (Figuerola et al. 1996), raptors (e.g. Tella et al. 1996), pigeons (Sol et al. 2000) and parrots (Masello et al. 2006). There is also some evidence that blood parasites are less common in certain habitats such as the arctic tundra (e.g. Bennett et al. 1992b), arid environments (e.g. Little and Earle 1995; Valera et al. 2003), island environments (e.g. Little and Earle 1994) and marine environments (e.g. Piersma 1997; Figuerola 1999; Jovani, Tella et al. 2001). In a recent review regarding the apparent absence of haematozoa in many avian species, Martinez-Abrain et al. (2004) discussed the vector-density hypothesis to explain the lack of blood parasites. In line with the vector-density hypothesis, blood parasites are generally absent from seabirds, presumably because of the absence of appropriate vectors in windy and saline environments, and distance to the coast (Bennett et al. 1992; Ruiz et al. 1995; Bosch et al. 1997; Merino et al. 1997, 2008; Figuerola 1999; Quillfeldt et al. 2010). However, the apparent lack of blood parasites in Common Gulls in our study cannot be attributed to habitat- and vector-related effects, since we did not find any parasite in blood of birds at either inland or marine colonies over the course of 2 years. Common Gulls were free of blood parasites even at the inland colony, where we found vectors of *Haemoproteus*, *Leucocytozoon* and *Plasmodium*. A study in Poland demonstrated that black fly outbreaks can affect breeding success in Common Gulls (Bukacinski and Bukacinska 2000), since black flies may consume considerable amounts of bird blood. Such detrimental effects may also explain the many dead nestlings in the inland study area. However, the attacks by vectors of blood parasites did not result in any detected infection by haemosporidians.

Some studies (Bensch et al. 2000; Ricklefs and Fallon 2001) have shown substantial host fidelity among bird malaria blood parasites, with host switching being infrequent, suggesting a strong association between hosts and parasites. This specificity suggests that, although a given blood parasite may be detected in a bird community, distantly related host species can be free of blood parasites despite the presence of appropriate vectors in the area. However, the absence of parasites in our study cannot be explained by a taxonomic barrier, as suggested by Bennett (1993) and Bennett et al. (1994), since it does not explain the presence of *Haemoproteus* in Black-headed Gulls and the lack of the parasite in closely related Common Gulls breeding at the same colony.

The absence of blood parasites in the Common Gull has been reported previously (Peirce 1981a, b), and it was explained by the scarcity of vectors. However, Common Gulls are known to use garbage dumps in cities during the breeding and non-breeding seasons. In such habitats, vectors of blood parasites can find suitable places for reproduction (Lardeux and Ottenwaelder 1997), which suggests that the lack of blood parasites must be explained by other factors than the absence of vectors of infection. It has

recently been shown that European Storm-petrels (*Hydrobates pelagicus*) have a strong response to the PHA assay, which is a measure of a T-lymphocyte-dependent component of immunocompetence (Esparza et al. 2004). This population was reported to lack blood parasites despite the presence of suitable vectors and the right host–parasite assemblages in the environment. An extremely high level of immunocompetence in European Storm-petrels may be responsible for the long-term absence of infections by haematozoa. It was also suggested that blood parasites might be lacking because these birds spend most of the year at sea where parasites are faced with problems in completing their life cycles. However, this is not the case with the lack of haematozoa in Common Gulls, since this species remains in inland habitats for most of its non-reproductive season. Studies in Estonia have shown that on average gulls breed for 5–6 years (i.e. they live for 7–8 years) (Rattiste and Lilleleht 1995), but the oldest individuals may even breed for more than 26 years. In 2011, the oldest breeder in the Estonian study area was a 30-year-old female. This suggests that most of these birds have sufficient contact with possible haematozoa vectors during their lifetime and, thus, their resistance to these parasites can probably be attributed to their immune system. However, we need to employ a broader experimental approach (Knowles et al. 2010; Palinauskas et al. 2011) to find out whether immunocompetence and the corresponding evolutionary arms-race (Schmid-Hempel 2011) are responsible for the lack of blood parasites in the Common Gull and the low prevalence in the Black-headed Gull, another long-lived species.

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