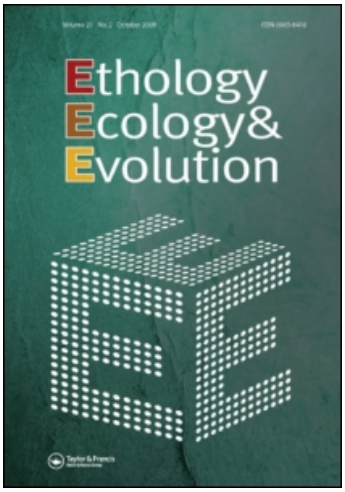

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K. Wojczulanis-Jakubas[a]; M. Dynowska[b]; D. Jakubas[a]
[a] Department of Vertebrate Ecology and Zoology, University of Gdansk, Legionow 9, Gdansk, Poland [b] Department of Mycology, University of Warmia and Mazury, Oczapowskiego 1, Olsztyn, Poland

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Fungi prevalence in breeding pairs of monogamous seabird – little auk, *Alle alle*

K. WOJCZULANIS-JAKUBAS [1,3], M. DYNOWSKA [2] and D. JAKUBAS [1]

[1] *Department of Vertebrate Ecology and Zoology, University of Gdansk, Legionow 9, 80-441 Gdansk, Poland*
[2] *Department of Mycology, University of Warmia and Mazury, Oczapowskiego 1, 10-719 Olsztyn, Poland*

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Sexually transmitted diseases have been frequently hypothesised as a cost of multiple avian mating but relatively few studies have investigated the issue in truly wild birds. The main object of this study was to determine prevalence and concordance of fungi in cloacae of pair members of a socially monogamous Arctic seabird, little auk (*Alle alle*), thereby testing whether the microbes are likely to be transmitted during copulation. Various fungi species, potentially pathogenic, occurred in the little auk cloacae. One-third of the tested individuals were found to be a host for one to three fungi species. However, half of the 19 studied pairs were found to be not concordant in fungal assemblages, suggesting that transmission of the microbes between partners is not straightforward.

KEY WORDS: *Alle alle*, fungi, little auks, monogamy, sexually transmitted microbes.

INTRODUCTION

Applying molecular tools in ecology has revolutionised the view of prevalence of monogamy in birds. A substantial proportion of the monogamous species have turned out to be sexually promiscuous, with the average frequency of extra-pair fertilisation higher than 10% of offspring and/or broods (BIRKHEAD & MØLLER 1998; PETRIE & KEMPENAERS 1998). Maximising the number of extra-pair copulation may promote fitness of both sexes. Males may sire more offspring without incurring additional rearing cost and females may benefit from increasing genetic diversity or quality of their offspring (BIRKHEAD & MØLLER 1992, 1998; PETRIE & KEMPENAERS 1998). The advantages of that strategy are so obvious that GRIFFITH et al. (2002) suggested that levels of extra-pair paternity below 5% of offspring are considered worthy of explanation.

Seabirds are particularly interesting in this respect because extra-pair paternity occurs in that group at a generally low frequency (BENNETT & OWENS 2002; GRIFFITH

[3] Corresponding author: Katarzyna Wojczulanis-Jakubas, Department of Vertebrate Ecology and Zoology, University of Gdansk, Legionow 9, 80-441 Gdansk, Poland (E-mail: biokwj@univ.gda.pl).

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et al. 2002), although with some notable exceptions (e.g. BURG & CROXALL 2006; JOUVENTIN et al. 2007). There are basically two, not necessarily mutually exclusive, hypotheses explaining the low frequency of extra-pair paternity in seabirds. The first says that high and coordinated parental investments of both sexes are required to rear the brood successfully in unpredictable, marine environments. Cuckolded males uncertain about their paternity could withhold parental care or desert the brood, which would result in reproductive failure for both mates. Hence, females, despite the mentioned benefits, might avoid the extra-pair mating/fertilisation to ensure male's parental care. The second hypothetical explanation is that birds reducing sexual contacts beyond pair-bonds may decrease the risk of infection by sexually transmitted pathogens (such as viruses, bacteria and fungi). The cloaca is the common opening to the gastrointestinal, reproductive tract and urinary system in birds. Many studies suggest that internal diseases of birds may be of cloacal origin (e.g. BONDA et al. 1998; MORRISEY 1999; SHIVAPRASAD 2002), causing mortality at levels comparable to that usually attributed to predation or starvation (ANDERSON & MAY 1979). Cloacal transmission of microbes during copulation has been demonstrated for example in domestic cockerel (*Gallus domesticus*) (PEREK et al. 1969; other examples in SHELDON 1993). In that context, sexually transmitted microbes/diseases (STM/STD) have the potential to be a potent force in the evolution of avian mating systems (SHELDON 1993; KOKKO et al. 2002).

While the first hypothesis has been considered in various studies in many bird species, providing evidence both for and against that explanation (e.g. BART & TORNES 1989; TRANSUE & BURGER 1989; GOWATY 1996; MØLLER 2000), the second one has received relatively little attention. Of those considering STM/STD, the bulk of studies related to domesticated species of economically important waterfowl (Anseriformes) and fowl (Galliformes) and only a few studies considered the issue in wild birds (reviewed in SHELDON 1993).

Several lines of evidence suggest that STM/STD may be important in wild species. The existence of various microbes in semen (e.g. LOMBARDO & THORPE 2000; WESTNEAT & RAMBO 2000) or cloaca (COOPER et al. 1980; LOMBARDO et al. 1996; STEWART & RAMBO 2000; HUPTON et al. 2003) of wild birds suggests that birds can inoculate each other with pathogens during copulation. Nevertheless, information about the prevalence of STM/STD and the effectiveness of inoculation in wild species is still far from being complete and understood.

The main object of this study was to determine the prevalence of one of the microbe types (fungi) and whether they are likely to be transmitted during copulation in the most numerous seabird species in the Arctic, the little auk (*Alle alle*, also known as dovekie). The little auk is a colonially breeding, monogamous alcid with long-term pair bonds. Both partners incubate the egg and rear one chick annually (STEMPNIEWICZ 2001). Little auks have been found to be involved in extra-pair copulations with relatively high frequency but in the great majority of the extra-pair events, cloacal contact was not achieved and that made a fairly good match with only 2% frequency of extra-pair paternity. The majority of those extra-pair copulations were unsuccessful due to lack of female interest in engaging in extra-pair events. Female reluctance to copulate outside the pair bond might be a consequence of the need for extensive male parental care according to the first-mentioned hypothesis. Alternatively it might be a way of reducing the potential risk of being infected by a sexually transmitted disease (WOJCZULANIS-JAKUBAS et al. 2009). In the present study we focused on the latter hypothesis, assuming that transmission of the microbes might be a reason for the females' reluctant behavior only if the microbes occur in the population in a noticeable number. Further, we hypothesised that if the microbes are transmitted

during copulation, the composition of the cloacal microflora will be similar in pair members.

Many microbes might circulate among seabird populations (HUBALEK 2004) although very little is known about the assemblages in Arctic birds. Polar regions are believed to be specific, less abundant in microbial organisms in comparison with other climate zones, although this is a relatively poorly studied area. In this study we focused on fungi, since they are common and widespread in the whole biosphere of all climatic zones (e.g. DEL FRATE & CARETTA 1990; LATGE 1999; BUTINAR et al. 2007). Hence, we expected to find that kind of microbe in the polar bird species. Many fungi can be pathogenic for animals, particularly in the circumstances of stress or scare food. Mycoses have been reported as an occasional disease of importance in poultry, and as a disease or an infection in numerous species of wild birds, particularly when being raised in captivity (e.g HUBBARD et al. 1985; PERELMAN & KUTTIN 1992; XAVIER et al. 2007). Fungi have also been reported among microorganisms that influence the development and mortality of nestlings (e.g. PINOWSKI et al. 1994). Thus we assumed that fungi colonisation may be a potential cost of extra-pair mating.

METHODS

The fieldwork was conducted in a large breeding colony of the little auk, situated on mountain slopes in Magdalenefjorden, NW Spitsbergen (79°30'N, 11°00'E) during the breeding season of 2009. Adult birds (with uniformly black wing coverts and flight feathers; STEMPNIEWICZ 2001) were caught in the nest chamber in second half of July (late incubation and early chick-rearing period). Cloacal swabs were collected from 56 breeding individuals (30 males and 26 females, including 19 pairs). Considering the sexual behaviour of little auks, e.g. similar frequency of occurence of within-pair and extra-pair copulations in observed individuals (WOJCZULANIS-JAKUBAS et al. 2009), we believe the sample size in the study is representative for the hypothesis tested.

A sterile cotton swab (2.5 × 120.0 mm; HAGMED, Rawa Mazowiecka, Poland) was inserted into the cloaca for 5 sec. Each swab was placed into a tube containing 6 ml of liquid agar with antibiotic (gentamycin and chloramphenicol (0.1%)) preventing bacterial reproduction. The samples were stored at 4–10 °C before being cultivated and analysed. The temperatures are sufficiently low to inhibit further microbiological reproduction but are not considered cold enough to cause fungi mortality. Each caught bird was individually marked with a metal ring (Stavanger, Norway) and blood was sampled for molecular sexing. The blood sample (ca 10 μl) was taken from the brachial vein and stored in 1 ml of 96% ethanol until the laboratory analysis. Each bird was released without any harm after a few minutes of handling.

Assuming that the taxonomic spectrum of fungi might be wide, liquid and solid Sabouraud's, Czapek-Dox's and glucose-potato media (BTL, Łódź, Poland) were used for establishing macrocultures. The initial macrocultures were run on liquid media with the addition of gentamycin and chloramphenicol (0.1%) at room temperature (23–25 °C) for 7 days (the time necessary for the adaptation of bird-originated isolates), and then they were transferred to temperatures of 37 °C and 40 °C (required for the growth of thermophilic mould fungi of the family Mucoraceae and of the genus *Aspergillus*) for 2–3 weeks (CLAYTON & MIDGLEY 1989; DYNOWSKA & KISICKA 2005; DYNOWSKA et al. 2005; KURNATOWSKA & KURNATOWSKI 2006). Yeast-like fungi and yeast were observed to appear after 48–72 hr of incubation, whereas mould fungi were appearing after 2–3 weeks (BIEDUNKIEWICZ-ZIOMEK & DYNOWSKA 2004), though there were also some isolates that were growing as fast as the yeast-like fungi. Once the fungi had proliferated, they were immediately passaged onto solid media to achieve material for biochemical tests and to

visualise macroscopic traits that serve as diagnostic criteria (morphology, colour, consistency of colonies, presence of dyes). The biochemical tests were performed to analyse the assimilation and fermentation capacity of individual isolates.

After 48–72 hr, microcultures were established onto Nickerson's medium enriched with broth bouillon with serum (1:1) (medium modified according to DYNOWSKA & EJDYS in press). The microcultures were incubated at a temperature of 37 °C for 48–72 hr and afterwards transferred to room temperature for 24 hr. Microscopic evaluation was conducted for the morphology of mycelium, size, shape and arrangement of blastospores and chlamydospores (yeast and yeast-like fungi), morphology of rhizoids, sporangiophores, sporangia and sporangiospores (family Mucoraceae), the structure of conidiophores, metules, phialides and phialoconidia (family Aspergillaceae), as well as the presence and morphology of chlamydospores, phialides and macro- and microconidia (genus *Fusarium*). The following works and keys were used for fungi identification: RAPER et al. (1949), RAPER & FENNEL (1965), GERLACH & NIRENBERG (1982), KREGER-VAN RIJ (1984), RICHARDSON & WARNOCK (1995), DE HOOG et al. (2000), KURTZMAN & FELL (2000), KURNATOWSKA & KURNATOWSKI (2006).

Molecular sexing was performed on DNA extracted with a blood mini kit (A&A Biotechnology, Gdynia, Poland) from the blood, after alcohol evaporation. Amplification of the CHD region was performed with the primer pair 2550F and 2718R (FRIDOLFSSON & ELLEGREN 1999; previously used in sexing of the little auk, e.g. WOJCZULANIS-JAKUBAS et al. 2009), according to the protocol described by GRIFFITHS et al. (1998), using 50 °C annealing temperature for the PCR reaction. The difference in the PCR product size (ca 200 bp) was clearly visible when separating on a 2% agarose gel.

RESULTS

Thirteen different fungi species were recorded (10 species of moulds: *Aspergillus candidus*, *A. fischeri*, *A. flavus*, *A. fumigatus*, *A. nidulans*, *A. proliferans*, *A. sclerotiorum*, *A. wentii*, *Pencillinum citrinum*, *P. funiculosum*; and three species of yeast: *Dipodascus albidus*, *D. armillariae* and *Cryptococcus macerans*) in 19 (34%) of 56 individuals. The females and males were infected with fungi with a frequency that did not differ significantly (chi-square test with Yates' correction, $\chi^2_1 < 0.01$, $P = 0.95$). Both partners of 10 pairs were concordant in lack of fungi in cloaca. Nine pairs (47%) were found to be not concordant, in eight cases fungi were revealed only in one mate (three males and five females), and in one pair both partners were infected with different fungi species. With the sample size of 19 pairs, the 95% confidence interval for the proportion of the non-concordant pairs increased from 27 to 68%.

The within-pair concordance in the occurence of fungi was recorded with a frequency that did not differ significantly from non-concordance (McNemary test, $P = 1.00$; the odds ratio = 0.80, with a 95% confidence interval extending from 0.159 to 3.717).

DISCUSSION

All recorded fungi species in the little auk have the potential to cause an opportunistic infection when birds are under stress or in a starvation period. Some of the fungi species, such as *Aspergillus fumigates* recorded in this study, demonstrating the highest virulence out of *Aspergillus* species, can cause very serious infections (e.g. FRIEND & FRANSON 1999). Aspergillosis, triggered by among others *A. fumigatus*, was the cause of death of ca 10% of magellanic penguins (*Speniscus magellanicus*) that were recovered (XAVIER et al. 2007). Also, yeast-like fungi, usually present in low numbers

in organisms, when increasing in number or when there is damage to the reproductive tract may cause severe diseases in birds (FRIEND & FRANSON 1999; VELASCO 2000).

One-third of the little auks studied were positive in terms of fungal occurrence in cloacae. The frequency of fungi occurrence seems to be moderate, compared with, for example, house sparrow (*Passer domesticus*), where all 16 sampled individuals were positive for fungi in cloaca (STEWART & RAMBO 2000), and tree swallow (*Tachycineta bicolor*), where fungi were recorded in 16% of the semen samples (LOMBARDO & THORPE 2000). In that context, fungi transmission might be considered as a potential risk during extra-pair copulation.

Results from studies of chickens suggest that the number of microbes transferred during copulation depends on the number of inseminations that a female receives (e.g. PEREK et al. 1969). Little auks copulate within-pair before egg-laying quite frequently (0.7–1.1 times per hr; WOJCZULANIS-JAKUBAS et al. 2009); thus the probability of transfer of microflora between mates is considerable. However, the present study revealed a lack of relationship in cloacal fungi species composition between little auk pair members. Both LOMBARDO et al. (1996) and STEWART & RAMBO (2000), testing similar ideas in two passerines species, could assign pair members correctly based on the assemblages of cloacal microbes. However, in those studies, the microbes were not determined to species level and only counts of microbial types were considered as a variable. On the other hand, HUPTON et al. (2003), comparing bacterial assemblages between pair member mates in red-wing blackbirds (*Agelaius phoeniceus*), similarly to the present study, found no correspondence between the mates.

One possibility for the non-concordance of fungi between little auk pair members is that some individuals might be strong enough to prevent fungi inoculation even when being exposed to them. Establishment and maintenance of any cloacal microbes may be under the influence of the immunocommpetence of the host (WAKELIN & APANIUS 1997). Ducks which received subcutaneous inoculations of *Aspergillus fumigatus* conidia seemed to acquire resistance to a subsequent challenge of a larger dose via the intravenous route (ASAKURA et al. 1962). Thus, there may be expected to be some possibility for active immunisation of birds against natural infection. Another possibility might be limitations of the method in detecting the fungi. Due to relatively short time of swab insertion into the cloaca (5 sec) we could not have collected fungi from individuals that were infected at a very low level. We cannot exclude the possibility that we did not detect a microfungus that was viable but non-culturable in the laboratory conditions, as is the case in some bacteria cultures (OHTOMO & SAITO 2001). Regardless of the causality, the lack of concordance between the little auk pair members in fungi prevalence indicates that the transmission is not straightforward. Clearly, more studies, especially including other microbe taxa, are needed to test the sexual transmission of microbes.

To summarise, cloacal fungi occur in the little auk population in noticeable numbers but the transmission from bird to bird seems to be insufficient to have a significant effect in shaping the sexual behaviour of birds. It is possible that the most important force driving the females' reluctance to engage in extra-pair copulations in the species is the need for the partner's care.

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