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SHORT COMMUNICATION

Occurrence and relevance of *Mycoplasma* spp. in free-ranging pheasants from northwestern Germany

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Abstract

Since 2008/2009, the population of free-ranging ring-necked pheasants was recorded to decrease all over Germany. Various *Mycoplasma* (*M.*) spp. are causing severe respiratory signs in captive game bird species. Furthermore, *M. gallisepticum* is responsible for massive die-offs in consequence to severe conjunctivitis in house finches in the USA. Therefore, the prevalence of mycoplasmas in free-ranging pheasants was investigated and a potential impact on the population decline of pheasants discussed. Within this study, 150 free-ranging pheasants were sampled via tracheal swabs and tissue samples of the trachea and the periorbital skin, as the latter displayed inflammatory alterations in previous studies. In total, 177 samples were investigated for the presence of mycoplasmas using cultural and molecular biological methods. In 76 birds, necropsy was performed additionally. In total, 73.7% (51/76) of the examined pheasants had periorbital skin alterations. Furthermore, 64.4% (114/177) of the samples tested positive for mycoplasmas via PCR. Overall, 102/177 samples (57.6%, 78/105 tracheal swabs, 19/51 skin tissue, 5/21 trachea tissue) tested positive for mycoplasmas via culture. *Mycoplasma gallinaceum* ($n=50$), *M. pullorum* ($n=45$), *M. glycophilum* ($n=43$), *M. iners* ($n=11$), and *M. gallinarum* ($n=5$) were frequently isolated. In 45 cases (45.9%), multiple *Mycoplasma* spp. were isolated from one sample. All examined samples tested negative for *M. gallisepticum*. Of 51 skin samples investigated for mycoplasmas, 24 (47.1%) showed inflammatory skin alterations in histology, and 58.3% (14/24) of these samples tested positive for *Mycoplasma* spp. additionally. Overall, there was a significant correlation between inflammatory altered skin samples and the detection of mycoplasmas in periorbital skin samples. Based on the present results, the isolated *Mycoplasma* spp. may play a role as facultative agents for the observed inflammatory skin alterations. However, additional investigation is needed to confirm this presumption.

Keywords Dermatitis · Periorbital inflammation · Facultative pathogens · Mycoplasma · Clinical relevance · Environmental effects

Pheasants are ground-breeding birds living mainly in the agricultural landscape of the northwestern part of Germany. As this bird species underlies the German Hunting Law, hunting bag statistics go back over several centuries showing a massive decline in the population since its plateau between 1960 and 1970 (Gehle 2011). Previous studies focused on potential infectious and non-infectious causes for this decline (Curland et al. 2018; Liebing et al. 2020) as well as on potential contributory factors (e.g., pesticides, predation, increasing traffic, changes in agricultural landscape) (Aebischer 1997; Moreby and Southway 1999). Thereby, it was shown that there is rather not a single pathogen but several factors contributing to the decline of pheasants in Germany, e.g., a reduced supply on protein-rich diet (e.g., insects) for pheasant chicks resulting in immunosuppression (Curland et al. 2018; Liebing et al. 2020).

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Upper respiratory tract infections including conjunctivitis and sinusitis are commonly noted in captive bred pheasants (Benčina et al. 2003; Welchman et al. 2002). Serological evidence and cultural findings suggested *Mycoplasma gallisepticum* (MG) as one of the possible causes of these disease outbreaks (Bradbury et al. 2001; Cookson and Shivaprasad 1994). Histologically, the affected birds exhibited lymphoplasmacytic inflammation of the conjunctiva, sinus, and trachea (Forrester et al. 2011; Ganapathy and Bradbury 1998). Via experimental infections with MG respiratory disease was reproduced in other game bird species (McMartin 1996). Besides MG, other *Mycoplasma* spp. were commonly isolated in pheasants showing respiratory disease: *M. gallinaceum* (Poveda et al. 1990; Bradbury et al. 2001; Chin and Goshgarian 2001; Welchman et al. 2002), *M. gallinarum* (Poveda et al. 1990), *M. glycophilum* (Bradbury et al. 2001; Chin and Goshgarian 2001), *M. iners* (Bradbury et al. 2001), and *M. pullorum* (Bradbury et al. 2001; Kempf et al. 1991; Shimizu et al. 1979). However, the clinical relevance of these fast-growing *Mycoplasma* spp. was unknown as some species were isolated in clinically healthy pheasants, too (Gerlach 1994; Poveda et al. 1990). Except for several studies in captive bred pheasants, investigations in free-ranging populations are rare. However, infectious agents may have been transferred to free-ranging game bird populations by releasing captive bred birds into the wild (De Marco et al. 2002).

Since 1994, massive outbreaks of MG-related conjunctivitis cause high losses in free-ranging populations of house finches (*Haemorhous mexicanus*) in the USA (Dhondt et al. 2014; Ley et al. 1996). In the following years, MG infections causing severe conjunctivitis were also seen in several other passerine species (Fischer et al. 1997; Hartup et al. 2000; Mikaelian et al. 2001). In some regions, the disease caused population declines in free-ranging house finches of up to 50% (Hochachka and Dhondt 2000). Affected birds showed conjunctivitis and encrusted eye lids. In histopathology, lymphoplasmacytic inflammation and epithelial hyperplasia around the eyes were seen just like in starlings being infected with *M. sturni* (Forsyth et al. 1996; Fischer et al. 1997). Phylogenetic studies showed that MG strains from free-ranging house finches may have been evolved from MG strains that were found in poultry by host shift (Delaney et al. 2012; Hochachka et al. 2013).

Investigations in possible causes for the population decline of pheasants in northwestern Germany showed skin alterations around the eyes in numerous birds (Curland et al. 2018). Whether MG or other *Mycoplasma* spp. are involved in the development of these lesions was investigated within this study.

Samples of respiratory tract and periorbital skin were obtained from free-ranging pheasants caught by live traps, shot during hunting, or found dead in northwestern Germany. Animals caught by live traps and being released again were sampled via tracheal swabs in the field. Dead pheasants were sampled at the hunting bag in the field or within the post-mortem examination. Therefore, tissue samples from trachea and/or skin were obtained, too (Curland et al. 2018). Findings of the post-mortem examination, especially regarding inflammatory skin lesions in the head region, were documented.

All samples were cultured using SP4 liquid and agar media as described by Bradbury (1998b). Each sample was immersed in the SP4 broth and afterwards removed and stored for further investigations. The broth was diluted (tenfold dilution up to 10^{-2}), and an aliquot of 50 µl each was transferred onto agar media. Liquid and solid media were incubated at 37 °C under microaerophilic conditions in a humidified environment for up to 10 days. Broth was examined for color change and agar plates for colony growth daily. In case of mycoplasmal growth, single colony subcultures were performed three times in order to ensure pure cultures, respectively. Each single colony subculture was stored at −80 °C until further investigation.

For DNA extraction, swabs were soaked and rubbed in 350 µl phosphate-buffered saline (PBS). Of this liquid, 100 µl was used for DNA extraction using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For DNA extraction of tissue samples and single colony subcultures, the fluid medium from culturing was centrifuged at 4000 G for 45 min. The remaining pellet was incubated with 180 µl lysis buffer (ATL Buffer, Qiagen, Hilden, Germany) and 20 µl proteinase K (Qiagen, Hilden, Germany) for 2 h at 56 °C.

All samples and single colony subcultures were screened via *Mycoplasma* genus–specific PCR (target: 16S rRNA gene sequence) as described by Van Kuppeveld et al. (1992) modified by Lierz et al. (2007). Furthermore, all samples were examined via MG-specific PCR (Hagen et al. 2004). Of all single colony subcultures, an additional PCR (target: 16S-23S rRNA intergenic transcribed spacer region (ISR) sequence) was performed (Ramírez et al. 2008). The PCR products obtained from both PCRs were sequenced by a commercial DNA sequencing service (LGC Genomics, Berlin, Germany). These sequences were aligned with sequences of *Mycoplasma* spp. in the NCBI database using BLAST (NCBI, USA) algorithm (Altschul et al. 1990).

Statistical analysis was carried out by using the program GraphPad Prism, version 5.0 for windows (GraphPad Software Inc.). Chi-square tests were used to explore the relation between presence of lesions and the following factors: detection of *Mycoplasma* spp., location of origin, month of collection, age of the sampled bird, and type of harvest (hunted, trapped, or dead found).

Within this study, 177 samples (105 tracheal swabs, 51 periorbital skin tissue, and 21 trachea tissue) were obtained from 150 pheasants. Ninety-seven birds were caught via live

trap, 34 birds were shot during the hunting season, and 19 birds were found dead. In total, 94 birds were adult and 56 juvenile. Fifty-eight pheasants were female, 22 male, and in 70 birds, gender was not determined. In total, 77/150 pheasants were examined via post-mortem examination (supplemental material Table 2).

All samples were investigated via culturing and molecular biological methods. In total, 102/177 samples (57.6%, 78/105 tracheal swabs, 19/51 skin tissue, 5/21 trachea tissue) tested positive for mycoplasmas via culturing. From these samples, several *Mycoplasma* spp. were isolated and identified: *M. gallinaceum* ($n=50$), *M. pullorum* ($n=45$), *M. glycophilum* ($n=43$), *M. iners* ($n=11$), and *M. gallinarum* ($n=5$). In 45 cases, multiple of these *Mycoplasma* spp. were isolated from the same sample. Via genus-specific PCR 114/177 samples tested positive (64.4%, 82/105 tracheal swabs, 21/51 skin tissue, 11/21 trachea tissue). There was a significant correlation between the detection of mycoplasmas (via culture and PCR) and the source of birds (trap, hunting, found dead) (rs 0.39 and 0.41 ($p<0.001$). However, there was no significant correlation between the detection of *Mycoplasma* spp. and the location, age, season of sampling, or if birds were healthy or diseased. All examined samples tested negative for MG via species-specific PCR.

Of all juvenile ($n=56$) and 21/94 adult birds, a pathological examination was performed. In 66.2% (51/77) of the examined pheasants, periorbital inflammatory alterations were observed in the dermis macroscopically. Mostly, non-purulent perivascular inflammations with different cellular compositions and gradual variable infiltrations of lymphocytes, plasma cells, and macrophages were detected histologically (Fig. 1).

In total, 41.1% (21/51) skin samples were tested positive for mycoplasmas via culture and/or PCR while 58.9% (30/51) were tested negative. Of *Mycoplasma*-positive skin samples, 14/21 (66.7%) revealed inflammatory alterations in histology while 7/21 (33.3%) did not. However, while 10/30 (33.3%) *Mycoplasma*-negative skin samples showed inflammatory alterations in histology, 20/30 (66.7%) did not (supplemental material table 1). Therefore, a significant correlation between the occurrence of inflammatory skin alterations and the presence of mycoplasmas was shown (chi-square test).

A decline of the population of free-ranging pheasants of northwestern Germany was observed since 2008/2009. In previous studies, no indication of a specific pathogen as a sole cause for this decline was identified; instead, several potential pathogens were detected (Curland et al. 2018). Within the study of Curland et al. (2018), almost half of the examined birds (49.7%) displayed inflammatory alterations of the periocular dermis. However, the number of birds examined for mycoplasmas was quite low ($n=5$), so the focus of this study was to concentrate on the investigation of the periorbital skin alterations and the association with mycoplasmas as possible cause.

The investigated birds were mainly obtained via trapping and hunting, but also dead found birds were sampled. Juvenile birds were mostly caught via live trap, while one-third of the adult pheasants were shot by hunting. Therefore, all juvenile birds ($n=56$) were investigated via post-mortem

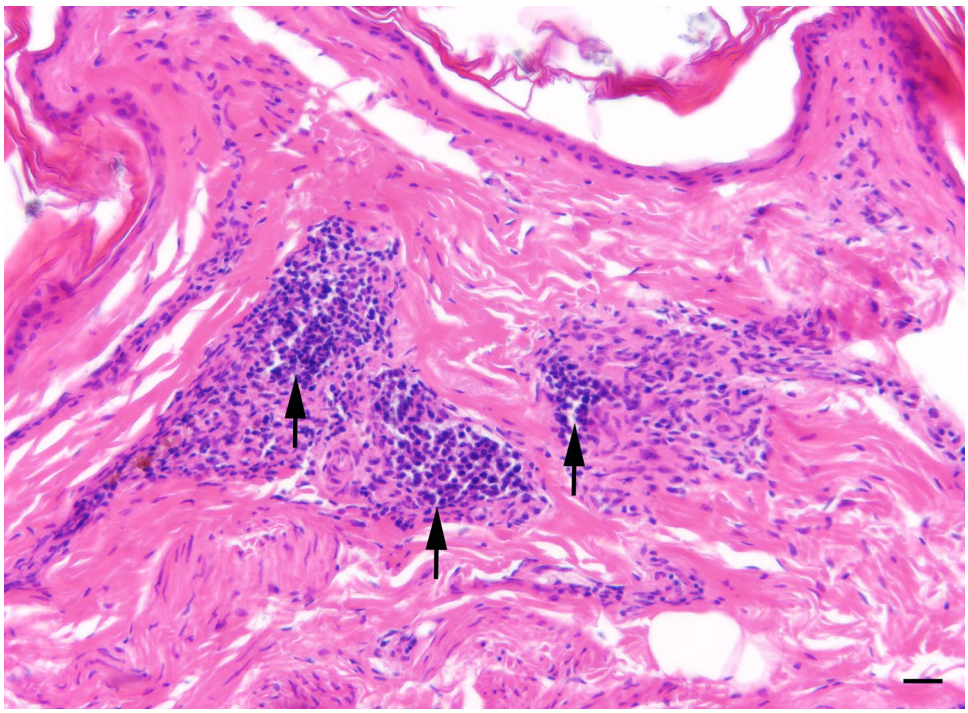

Fig. 1 Pheasant, periocular skin, perivascular predominantly lymphocytic infiltration of the dermis (arrows), HE, bar = 100 µm

examination, while this was possible in only 21/94 adult pheasants due to further use of the hunting bags. However, investigation for mycoplasmas was technically possible in all 150 pheasants.

Via culture, 57.6% of the samples tested positive for mycoplasmal growth while 64.4% tested positive via molecular biological methods. These differences in sensitivity are well known and may be related to the number of mycoplasmas in the sampled material and the viability of the organism (Bradbury 1998b). In this study, tissue samples and swabs were transported cooled down in cultural medium for 12 to 24 h so that even slowly growing *Mycoplasma* spp. are cultivatable afterwards (Zain and Bradbury 1996). However, transport of the samples may have led to a decreased viability of mycoplasmas. Furthermore, contamination with other microbial agents occurred in some cases.

In total, 66.7% of the pheasants tested positive for mycoplasmas. *Mycoplasma gallinaceum*, *M. gallinarum*, *M. glycophilum*, *M. iners*, and *M. pullorum* were regularly isolated. These species were also isolated from the respiratory tract and/or the eyes of captive, healthy, and diseased pheasants (Bradbury et al. 2001; Chin and Goshgarian 2001; Kempf et al. 1991; Poveda et al. 1990; Shimizu et al. 1979; Welchman et al. 2002). However, MG was not detected in this study. As MG induces lymphoplasmacytic inflammation of the conjunctiva in certain host species, other *Mycoplasma* spp. may represent facultative pathogens causing similar alterations when causing disease. *Mycoplasma sturni* is responsible for severe conjunctivitis in various bird species (Forsyth et al. 1996; Frasca et al. 1997; Ley et al. 1998; Wellehan et al. 2001), but was also found in healthy corvids (Ziegler et al. 2017). Furthermore, corvids suffering from stressful conditions showed a higher prevalence (31%) than healthy individuals (7%). Therefore, as *M. sturni* in corvids, the *Mycoplasma* spp. isolated in the periorbital skin of pheasants may play a role as facultative pathogens in this bird species.

In the present study, inflammatory alterations in the periorbital region were found more often (73.7%) as in prior studies on pheasants in northwestern Germany (49.7%) (Curland et al. 2018). Furthermore, there was a significant correlation between the detection of mycoplasmas in the periorbital skin and the occurrence of inflammatory skin alterations around the eyes. As the alterations displayed a lymphoplasmacytic character, isolated *Mycoplasma* spp. are possibly involved in the genesis of the observed alterations (Gerlach 1994). However, to demonstrate the role of the detected mycoplasmas in the inflamed tissue, further investigations would be necessary (e.g., immunofluorescence (Bradbury 1998a)). Differential causes for periorbital skin alterations include ectoparasites, allergic reactions, and/or irritation by toxic agents (e.g., pesticides (Pass 1989)) or immunosuppression associated with secondary bacterial infections (Huff et al. 2013; Thachil et al. 2014). Furthermore, a contribution of infectious bronchitis virus (IBV) in the formation of skin alterations in the head region was discussed in prior studies (Liebing et al. 2020). Furthermore, the time being caught in the live traps may lead to injuries especially at the bird's head due to escape reflex. However, as birds of all groups (caught in live trap, dead found, and shot) showed similar periorbital skin alterations, this possibility seems unlikely, especially as traumatic injuries would have resulted in different macroscopic and microscopic findings. In total, none of the dead found pheasants died due to the detected periorbital skin alterations as seen in house finches in the USA (Nolan et al. 1998).

As a higher percentage of the investigated chicks showed alterations associated with the detection of mycoplasmas in the periorbital skin (53.3%) compared to the investigated adult pheasants (13.8%), an age-dependency seems possible. As an immunosuppression of pheasant chicks due to a deficient protein uptake via nourishment was confirmed recently (Liebing et al. 2020), a higher percentage of *Mycoplasma*-positive chicks showing periorbital skin alteration would support the theory of an involvement of the isolated *Mycoplasma* spp. as facultative pathogens.

In conclusion, mycoplasmas may play a role as possible cause of the observed periorbital alterations, as they may represent facultative pathogens in free-ranging pheasants. However, there are no indications for the transmission of MG from captive bred pheasants to the free-ranging population. As the habitat of free-ranging pheasants is closely connected with human activities and their effects (habitat loss, decreased food availability, pesticides), they are steadily exposed to multiple stressors. The combination of these endo- and exogenous infectious and noninfectious factors may lead to general weakening with immunosuppression which may contribute to infectious diseases possibly caused by facultative pathogens like mycoplasmas (Fairbrother et al. 2004).

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Declarations

Conflict of interest The authors declare no competing interests.

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