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Microorganisms, flight, reproduction, and predation in birds

Zaid Al Rubaiee

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Par

Zaid Shaker Naji AL-RUBAIEE

Microorganisms, flight, reproduction and predation in birds

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Composition du Jury :

Dr. Tatiana Giraud	ESE, Université Paris Saclay, FRANCE	Président
Dr. Marcel Lambrechts	CEFE, Montpellier, FRANCE	Rapporteur
Pr. Marion Petrie	Université de Newcastle, ROYAUME-UNI	Rapporteur
Pr. Juan J. Soler	CSIC, Almeria, ESPAGNE	Examineur
Dr. Anders Møller	ESE, Université Paris Saclay, FRANCE	Directeur de thèse

Title

Microorganisms, flight, reproduction and predation in birds

Abstract

The fitness costs that macro- and micro-parasites impose on hosts can be explained by three main factors: (1) Hosts use immune responses against parasites to prevent or control infection. Immune responses require energy and nutrients to produce and/or activate immune cells and immunoglobulins, and that is costly, causing trade-offs against other physiological processes like growth or reproduction. (2) The host's metabolic rate can be increased because tissue damage and subsequent repair from infection caused by parasite may be costly. (3) The metabolic rate of hosts may increase and hence also increase their resource requirements. Competition between macro-parasites and hosts may deprive resources from host.

Birds are hosts for many symbionts, some of them parasitic, that could decrease the fitness of their hosts. There is huge diversity in potential parasites carried in a bird's plumage and some can cause infection.

Nest lining feathers are chosen and transported by adult birds including barn swallows *Hirundo rustica* to their nests, implying that any heterogeneity in abundance and diversity of microorganisms on feathers in nests must arise from feather preferences. We found that the effects of microorganisms on the behaviour of birds may be a combination of positive and negative effects. There may be positive effects of antimicrobial activity on birds through the process of bacterial interference, consisting of certain bacteria impeding the establishment of competing bacterial strains by producing antibiotic substances. Meanwhile, the negative effects may imply that pathogenic or/and feather-degrading microorganisms may reduce fitness components of their hosts. These effects of microorganisms and hence the microbiome can be affected by behaviour of bird hosts.

Keywords: *Accipiter gentilis*; arrival date; *Bacillus megatherium*; bacteria; barn swallow; feather lining of nests; fungi; goshawk; *Hirundo rustica*; laying date; microorganisms.

Titre

Microorganismes, vol, reproduction et prédation chez les oiseaux

Synthèse en français

Les coûts de remise en forme que les macro et micro parasites imposent aux hôtes peuvent s'expliquer par trois facteurs principaux : (1) Les hôtes utilisent des réponses immunitaires contre les parasites pour prévenir ou contrôler l'infection. Les réponses immunitaires nécessitent de l'énergie et des nutriments pour produire et / ou activer les cellules immunitaires et les immunoglobulines, ce qui est coûteux, provoquant des compromis avec d'autres processus physiologiques comme la croissance ou la reproduction. (2) Le taux métabolique de l'hôte peut être augmenté parce que les dommages aux tissus et la réparation ultérieure de l'infection causée par le parasite peuvent être coûteux. (3) Le taux métabolique des hôtes peut augmenter et donc augmenter également leurs besoins en ressources. La compétition entre macro-parasites et hôtes peut priver les ressources de l'hôte.

Les oiseaux sont hôtes de nombreux symbiotes, certains parasitaires, qui pourraient diminuer la capacité de leurs hôtes. Il existe une grande diversité de parasites potentiels transportés dans le plumage d'un oiseau et certains peuvent causer une infection.

Les plumes de la nidification sont choisies et transportées par les oiseaux adultes, y compris les hirondelles *Hirundo rustica* à leurs nids, ce qui implique que toute hétérogénéité dans l'abondance et la diversité des microorganismes sur les plumes dans les nids doit provenir des préférences des plumes. Nous avons constaté que les effets des microorganismes sur le comportement des oiseaux peuvent être une combinaison d'effets positifs et négatifs. Il peut y avoir des effets positifs de l'activité antimicrobienne sur les oiseaux par le processus d'interférence bactérienne, consistant en certaines bactéries empêchant l'établissement de souches bactériennes concurrentes en produisant des substances antibiotiques. En attendant, les effets négatifs peuvent impliquer que les microorganismes pathogènes et / ou dégradant les plumes peuvent réduire les composantes de fitness de leurs hôtes. Ces effets

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des micro-organismes et donc du microbiome peuvent être affectés par le comportement des hôtes d'oiseaux.

Mots-clés

Accipiter gentilis ; date d'arrivée ; *Bacillus megatherium* ; Bactéries ;
Hirondelle de grange ; Doublure en plumes de nids ; Champignons ; autour ;
Hirundo rustica ; Date de ponte ; Micro-organismes.

Section 1 : Date d'arrivée et microorganismes dans les hirondelles

La migration entre les sites de reproduction et les quartiers d'hiver constitue une stratégie majeure d'histoire de vie chez les oiseaux. Les avantages de ces migrations doivent au moins égaler les coûts d'un tel comportement pour évoluer et être maintenus. Nous avons testé s'il y avait une relation entre l'abondance et la diversité des microorganismes sur les plumes et le moment de l'arrivée par les hirondelles *Hirundo rustica*.

Les plumes de la douceur sont choisies et transportées par des hirondelles de grange adultes jusqu'à leurs nids avant et pendant la ponte, ce qui implique que toute hétérogénéité et diversité de microorganismes sur les plumes dans les nids doivent provenir des préférences de plumes et des microorganismes sur le corps de ces individus. Il y a eu une relation négative entre la date d'arrivée et le nombre total d'alevins montrant que l'arrivée anticipée est avantageuse.

La date d'arrivée des hirondelles adultes a été significativement corrélée positivement avec l'abondance de bactéries spécifiques (*Bacillus licheniformis*) et corrélée positivement avec le champignon *Trichoderma reesei* et corrélée négativement avec le champignon *Mucor circinelloides*. En outre, nous avons trouvé une relation significative positive entre la date d'arrivée et le nombre total moyen de colonies bactériennes dans le milieu TSA. Pendant ce temps, nous avons trouvé une relation négative significative entre la date d'arrivée et le nombre total moyen de colonies bactériennes en milieu FMA, et l'indice de diversité de Simpson de l'abondance de bactéries dans le milieu FMA.

En revanche, il n'y avait pas de relation significative entre la date d'arrivée et l'âge des hôtes individuels. Ces résultats sont cohérents avec l'hypothèse selon laquelle les hirondelles de la grange arriérée têt diffèrent en abondance et en diversité des microorganismes des constellés tardifs et qu'ils choisissent des plumes pour leurs nids qui diffèrent en termes de microorganismes de ceux choisis par les personnes arrivant tard.

Section 2 : Date de prise, microorganismes et avantages de la reproduction précoce

Les microorganismes ont été supposés affecter le moment de la reproduction de leurs hôtes soit directement parce qu'ils sont pathogènes, soit indirectement parce qu'ils éliminent de manière concurrentielle les microbes pathogènes.

Nous avons testé s'il y avait une relation entre l'abondance et la diversité des microorganismes et le moment de la pose dans les hirondelles semi-coloniales *Hirundo rustica*. Les microorganismes provenant des plumes de la couche de nid ont été extraits, cultivés et soumis à une identification moléculaire en utilisant des procédés de PCR d'ARNr 16S et 18S.

Il y a eu un mélange d'associations positives et négatives entre la date de ponte et l'abondance et la diversité des microorganismes suggérant que les effets des microorganismes sur le moment de la pose peuvent être une combinaison d'effets positifs de l'interférence microbienne et des effets pathogènes négatifs. Une méta-analyse pondérée par la taille de l'échantillon de la relation entre la date de ponte chez les oiseaux et l'abondance de microorganismes a montré une relation positive générale d'une petite taille moyenne d'effet, ce qui suggère que la ponte moyenne est retardée en présence de plus de microorganismes.

Section 3 : Microorganismes, taille d'embrayage et succès reproductif dans l'hirondelle de grange

La limitation des ressources devrait être attribuée aux exigences concurrentes afin de maximiser la réussite en matière de reproduction. L'évolution de la taille de l'embrayage a été pendant des décennies un problème central dans la théorie de l'histoire de la vie, bien que, de façon surprenante, il y ait eu peu de tentatives pour aborder le rôle potentiel des microorganismes.

Les oiseaux ont été un groupe fréquemment utilisé d'organismes pour les études de facteurs qui déterminent la taille de l'embrayage. David Lack a émis l'hypothèse que la taille de l'embrayage dans les oiseaux altruistes a évolué pour être le résultat du plus grand nombre de descendants survivants, finalement réglementés par la capacité d'alimentation des parents, ce qui entraîne un mécanisme déterminant la limite supérieure à la taille de l'embrayage étant la capacité parentale de fournir des aliments pour les nichoirs .

Nous avons testé s'il existait une relation entre l'abondance et la diversité des microorganismes et le nombre total d'alevins produits par les hirondelles *Hirundo rustica* lors de leurs événements reproductifs annuels totaux. Nous avons trouvé des relations positives entre le nombre total d'alevins (contrôlés pour les effets du nombre d'œufs) et l'abondance des microorganismes.

Étonnamment, nous n'avons pas trouvé de relation significative entre le nombre total de jeunes gens et l'âge des individus. Enfin, nous avons trouvé des relations positives entre le nombre total d'alevins et l'activité antimicrobienne des microorganismes. Ces résultats sont cohérents avec l'hypothèse selon laquelle la taille de l'embrayage et le succès de la reproduction ont évolué en réponse à l'abondance et à la diversité des microorganismes.

Section 4 : Probabilité de récupération, morphologie de vol et Microorganismes

Les microorganismes sur et au sein des organismes sont omniprésents et les interactions avec leurs hôtes varient de mutualistes sur commensal, de pathogènes. Nous avons émis l'hypothèse que les microorganismes pourraient affecter la capacité des hirondelles *Hirundo rustica* à échapper à des prédateurs potentiels, avec des associations positives entre l'abondance de microorganismes et la capacité d'échappement impliquant des effets mutualistes, alors que les associations négatives impliqueraient des effets antagonistes.

Nous avons quantifié les comportements d'évasion comme la capacité d'éviter la capture dans un réseau de brouillard et donc comme un petit nombre de recaptures. Étant donné que la probabilité de récupération peut également dépendre du moment de la reproduction et de la réussite en matière de reproduction, nous avons également testé si l'association entre la récupération et les microorganismes était médiée par une association entre la récupération et l'histoire de la vie.

Nous avons trouvé des rapports positifs intermédiaires à forts entre la probabilité de récupération et l'abondance de *Bacillus megaterium*, mais pas l'abondance d'autres bactéries ou des champignons. L'abondance de *B. megaterium* a été associée à une avance dans la date de ponte et à une augmentation de la réussite en matière de reproduction. Cependant, ces effets étaient indépendants du nombre de recaptures.

Cette interprétation est étayée par le fait qu'il n'y avait pas de corrélation directe entre la date de ponte et le succès de la reproduction d'une part et le nombre de recaptures d'autre part. Ces résultats ont des implications non seulement pour les interactions prédateur-proie, mais aussi pour l'analyse de la capture-repère-capture des taux vitaux tels que la survie et la dispersion.

Section 5 : Microorganismes, capacité de vol et risque de prédation

La prédation est une puissante force sélective ayant des effets importants sur le comportement, la morphologie, l'histoire de vie et l'évolution de la proie. Les effets des parasites sur leurs hôtes peuvent consister en des modifications de l'état corporel, de l'état de santé et de la capacité d'échapper ou de se défendre contre les prédateurs. Prey obtient des avantages immédiats de se cacher et d'échapper aux prédateurs.

Cependant, une fois qu'une personne de proie a été détectée, elle peut compter sur une diversité de moyens d'échapper à la poursuite par le prédateur. Ici, nous avons testé si la proie d'un rapace commun différait en termes de microorganismes de non-proies enregistrés sur les mêmes sites en utilisant le goshawk *Accipiter gentilis* et sa proie aviaire en tant que système modèle. En outre, la probabilité d'avoir des plumes endommagées a augmenté avec le nombre de colonies fongiques, et en particulier l'abondance de *Myceliophthora verrucos* et *Schizophyllum* sp.

Était positivement corrélé avec la probabilité d'avoir des plumes endommagées. La découverte principale de cette étude de la diversité des champignons sur les plumes de la proie des êtres humains était une association positive entre la probabilité de tomber la proie du rapace et la présence et l'abondance des champignons.

La proie Goshawk avec une composition spécifique de la communauté de champignons avait une probabilité plus élevée de tomber en proie à un goshawk que les hôtes individuels avec moins de champignons. Ces résultats impliquent que les microorganismes peuvent jouer un rôle important dans les interactions prédateur-proie.

En outre, nous avons constaté un effet significatif du taux de croissance de la plume sur les proies goshawk, plus les champignons étant plus susceptibles d'être détruits. Ces résultats concordent avec l'hypothèse selon laquelle l'efficacité de survie et de vol est liée à l'abondance et à la diversité des champignons.

plumes peuvent réduire les composantes de fitness de leurs hôtes. Ces effets des micro-organismes et donc du microbiome peuvent être affectés par le comportement des hôtes d'oiseaux.

Mots clés

Accipiter gentilis; date d'arrivée; Bacillus megatherium; Bactéries;
Hirondelle de grange; Doublure en plumes de nids; Champignons; autour;
Hirundo rustica; Date de ponte; Micro-organismes.

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General introduction

Antoni van Leeuwenhoek in 1668 improved the microscopes being manufactured in Europe to study small biological objects (Madigan *et al.* 1997). Although the microscope of von Leeuwenhoek made a revolution in biology, to support the cell theory and germs as a cause of disease, the potential of the microscope attracted the attention of most ornithologists. Thus, many researchers have used microscopes to study feathers since the 19th and early 20th centuries (Fox 1976; Prum 1999). The first isolates of microbes from feathers were made more than 50 years ago, (Gierløff *et al.* 1961; Pugh and Evans 1970), although the study of microorganisms in feathers was dormant until Burt and Ichida (1999) were able to isolate *Bacillus* spp. as feather-degrading bacteria. Feathers may harbour a diverse microflora, although the ecological role of the microflora remains a mystery. Goldstein *et al.* (2004) used a standard microbiological method to prove that feather-degrading bacteria can degrade white feathers (unmelanised) more quickly and completely than black feathers (melanised) *in vitro*. These results showed that melanin can protect feathers against feather-degrading bacteria, and that feathers with melanin are more resistant to abrasion than unmelanised feathers (Burt 1979, 1986; Bonser 1995; Butler and Johnson 2004). Melanin-based feathers can be used in social signalling (Rohwer and Rohwer 1978), thermoregulation (Walsberg 1983) and camouflage (Wallace 1889; Zink and Remsen 1986). Goldstein *et al.* (2004) found that melanin may play a role in resistance to the degrading effects of bacteria, and this observation could be important in the evolution of plumage colour. Feather-degrading bacteria may be evolving due to clonal variation in melanin-based colours. Many studies suggest that birds living in environments with high humidity are darker than conspecifics in environment with low humidity (Gloger 1833; Zink and Remsen 1986). This may be caused by bacteria benefiting from humid environments, but

also because melanin in feathers can resist feather-degrading bacteria better than light feathers that lack melanin (Burt and Ichida 2004).

Many strains of bacteria degrade feathers with *Bacillus licheniformis* being particularly important, while *B. pumilis* is less important (Williams *et al.* 1990; Burt and Ichida 1999). Feather-degrading microorganisms such as lice, mites, bacteria and fungi may lead to feather-degradation, which occurs as the edges of feathers are dented, feather barbules missing or losing function, or feather barbs becoming detached, leaving holes that can reach the size of more than one square-centimeter in a feather (Møller *et al.* 2010; Fig. 1).

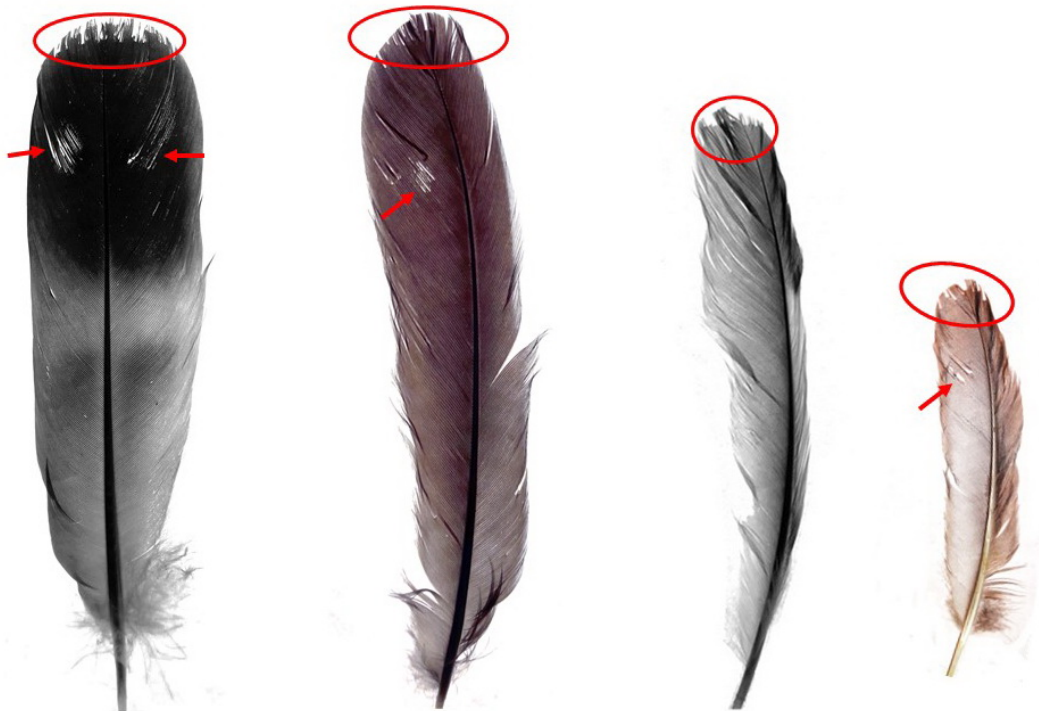


Fig. 1. Feather damage due to degradation.

Why birds should have such costly feather-degrading bacteria on their plumage is thus a puzzling observation. If these microorganisms are acquired passively as a consequence of foraging habits associated with the ground or the vegetation, there should be selection against such costly foraging habits. *Bacillus licheniformis* degrades feathers, but also produces antimicrobial substances (Haavik 1974; Simlot *et al.* 1972) that are active against bacteria (Galvez *et al.* 1994) and fungi (Lebbadi *et al.* 1994; Patel *et al.* 2004). Therefore, because of the antimicrobial properties of such substances, *B. licheniformis* may impede the establishment of other more virulent bacteria. In other words, feather-degrading bacteria are not necessarily only costly, but may entail certain benefits in terms of protection against pathogenic, but competitively inferior strains of microorganisms.

The term bacterial interference refers to antagonism and synergism that maintain the balance with the members of the normal endogenous flora (Falagas *et al.* 2008). Bacteria have developed many mechanisms that interfere with the capability of other antagonistic bacteria to colonize and infect hosts (Reid *et al.* 2001; Brook 1999). The ability to interfere in the microflora could be the most important line of defense along with the other mechanisms of defense by the host.

Bacterial interactions take place when microorganisms compete to the establish themselves and control their environment (Rotimi and Duerden 1981). Some of these interactions are antagonistic while others are synergistic, as organisms can reduce the growth of each other and thereby compete for space. Bacterial interference can play a main role for maintenance of the normal flora of the host by preventing colonization and subsequent invasion by potentially pathogenic bacteria (Brook 2005). This phenomenon is most important for preventing other bacterial infections.

Costs and consequences of parasitism for hosts

The signs of sickness and pathogenesis are typically associated with micro-parasite infections caused by multiplication inside the host by viruses, bacteria and fungi (Clayton and Moore 1997; Hudson *et al.* 2002). Since the 1990s, many experimental studies have shown that macro-parasites are costly for host (Loye and Zuk 1991; Clayton and Moore 1997; Hudson *et al.* 2002; Møller *et al.* 2009; Tompkins *et al.* 2011). Even non-lethal impacts can have effects on hosts' resource use, but can also affect many other aspects of hosts including ecology, behaviour and life-history.

Tolerance to infections is known as the ability of a host to override the harmful effect of parasites, that influence performance, host health and ultimately fitness. Tolerance of infections is one of the mechanisms that is used by the host as a means to struggle against parasites that colonized the host (Simms 2000). Biologists recognized that host defence versus parasites could be divided into two components: Resistance is a measure of the ability of the host to decrease parasite load, and tolerance is a measure of the ability of the host to "deal with" a given parasite establishment (Cobb 1894 cited in Schafer 1971; Caldwell *et al.* 1958; Clarke 1986).

Understanding the relationship between tolerance and resistance could help manipulate host defences by means of genetic manipulation. If tolerance and resistance are negatively correlated, enhancing their resistance may reduce the health of hosts (Råberg *et al.* 2009). The main difference between tolerance and resistance is that tolerance can save the host from harmful infection without any direct effects on parasites, while for tolerance resistance protects the host at the expense of parasites. Because of this fundamental difference, the ecological and evolutionary consequences of tolerance and resistance should differ.

The fitness costs that macro- and micro-parasites impose on hosts is reflected by the costly use of immune responses of host against parasites to prevent or control infection. Immune responses need energy and nutrients to produce and/or activate immune cells and immunoglobulins, and that is costly (e.g. Hasselquist and Nilsson 2012), causing a trade-off against other processes of physiology like growth or reproduction (Soler *et al.* 2003; Råberg *et al.* 2000). The host's metabolic rate can be increased because tissue damage and subsequent repair from infection caused by parasite may be costly. The metabolic rate may increase and hence their resource requirements likewise increase (Colditz 2008). Competition between macro-parasites and hosts may deprive resources from host (Atkinson *et al.* 2009).

Birds are hosts to many symbionts, some of them are parasitic and hence could decrease the fitness of their hosts. A huge diversity in potential parasites is carried in bird's plumage and some of them can cause infections (Hubálek 2004). There are two major types of parasite: Ectoparasites and endoparasites and both are costly to their hosts. Ectoparasites can play an important role in bird's lives. Among these parasites, mites offer unparalleled potential of damage to hosts because of their extraordinary ecological and evolutionary diversity (Proctor and Owens 2000).

Many studies have shown there is an immediate negative effect of parasitism on chicks, with a focus on bird/ectoparasite systems, by reducing chick survival, delaying fledging or slowing growth (e.g. Brown and Brown 1986; Richner *et al.* 1993; Bize *et al.* 2004).

Birds are colonized by various types of microorganisms, and the entire assemblage of these microorganisms is usually referred to as the "microbiome" (Morgan *et al.* 2013). The microbiome is constituted of the microorganisms on and within a host. Any human harbours approximately 1 kg of bacteria, fungi and other microorganisms (Turnbaugh *et al.* 2007). The effects of this diversity and

the abundance of microorganisms range from beneficial over neutral, to highly pathogenic. A number of diseases such as ulcer, obesity, inflammatory bowel disease, diabetes and vaginal disease among many others have been linked to the microbiome (Forsythe *et al.* 2010; Larsen *et al.* 2010; Fettweis *et al.* 2011; Greenblum *et al.* 2011; Foster and Neufeld 2013). Whether similar phenomena occur in wild animals remain to be determined.

Bacteria which are the oldest, structurally simplest and the most abundant forms of life on earth represent the vast majority of this colonization, and bacteria are often crucial to host health and their fitness, and they act as a strong selective pressure on life-history traits (Clayton and Moore 1997). Several factors have been suggested to influence the prevalence and the abundance of pathogenic microorganisms including extrinsic and intrinsic factors. Communities of microorganisms in birds constitute a dynamic ecosystem open to the influence of environmental factors (i.e. humidity or temperature) (Bisson *et al.* 2009), but also ecological features of the host (solitary vs. colonial and migratory vs. sedentary) (Waldenström *et al.* 2002 ; Bisson *et al.* 2009 ; Benskin *et al.* 2009), diet (Maul *et al.* 2005; Blanco *et al.* 2006) and antimicrobial defence mechanisms (Hawley and Altizer 2011).

An important cost of bird migration is the increased exposure to different groups of microorganisms during the annual cycle due to encounters with different environments at different times of the year. Thus, migrants encounter more different strains of parasites including microorganisms than residents (Møller 1998), and this should select for extraordinary defences against such pathogenic diversity (Møller 1998), but also for development of defences against strains of parasites encountered throughout the annual life cycle of hosts (Møller and Erritzøe 2000; Møller and Szép 2011). An unnoticed, but potentially dangerous group of microorganisms is feather-degrading fungi and bacteria that can damage the flying apparatus of birds by degrading the structure of the feathers

on which flight and hence migration relies (Burt and Ichida 1999; Møller and Erritzøe 1998).

Many bacterial taxa have the ability to degrade feather components such as *Bacillus licheniformis*, *Bacillus pumilis*, *Streptomyces fradiae*, *S. pactum* and various fungi (Williams *et al.* 1990; Burt and Ichida 1999; Pugh 1964, 1965; Hubálek 1976, 1978; Kunert 1989). Such damage to the plumage provides individual hosts that are able to control the abundance and diversity of microorganisms with a considerable advantage. In other words, feather-degrading bacteria are not necessarily only costly, but may provide benefits in terms of protection against pathogens by producing antimicrobial substances or competitive interference with inferior strains of microorganisms (Haavik 1974; Brook 1999).

The timing of reproduction is determined by availability of resources for reproducing females, but also for successful rearing of offspring. Thus, reproductive phenology is determined by availability of sufficient resources for start of reproduction on one hand, and suitable environmental conditions on the other (Perrins 1965). Many experimental studies show that temperature can affect laying date (Meijer *et al.* 1999; Nager and Van Noordwijk 1995). Crick *et al.* (1997) have shown that 31% of 67 bird species have advanced their timing of reproduction due to climate change. Dunn (2004) showed that 79% of 57 bird species have a significant negative relationship between air temperature and laying date. Timing of reproduction may be affected by the diversity and the abundance of parasites including microorganisms because such hetero-thermic organisms proliferate under warm and humid environmental conditions. Thus, pathogenic microorganisms and other parasites may prevent some host females from laying early (Møller *et al.* 2015).

Microorganisms may affect birds directly or indirectly thereby delaying timing of reproduction. Feather degradation by microorganisms can decrease the efficiency of flight (Soler *et al.* 2015). Microorganisms could damage flight

morphology or disrupt flight directly (Møller *et al.* 2009). Microorganisms may also be beneficial by improving the quality of feathers by outcompeting detrimental microorganisms that cause degradation of feathers (Martín-Vivaldi *et al.* 2009). The bacterial abundance in nests could be affected by nest material, because different nest materials may harbour different microorganism communities, and the nest material used to line the nest cup may indirectly influence the environment of microorganisms in nests with effects on thermal conditions (Mertens 1977).

A subsequent analysis revealed that the abundance of bacteria on the feathers of prey was considerably larger than that of non-prey (Møller *et al.* 2012). Microorganisms also seem to play a role in delaying laying date of their goshawk hosts (Møller *et al.* 2015). This has consequences for hatching success (Møller *et al.* 2010a) and survival prospects (Møller *et al.* 2013; Benskin *et al.*, 2015). The birds with more microorganisms suffer from the costs of microorganisms causing disease in their hosts. Alternatively, birds with more microorganisms suffer from more damage to their plumage caused by feather degrading microorganisms. Either of these mechanisms may impair flight and escape ability and hence increase the probability of capture and recapture.

The ultimate test of the fitness consequences of microorganisms for prey is whether an individual survives or falls prey to a predator. Any damage to the plumage that reduces the efficiency of flight would be costly and hence selected against, with damage to the plumage reaching such extremes as complete degradation and hence disappearance of barbules, barbs or even loss of entire segments of feathers (Møller *et al.* 2013). Previous studies of birds have shown that prey species that are disproportionately likely to be captured by predators have relatively large uropygial glands that may better control or reduce the abundance of microorganisms (Møller *et al.* 2010b).

Mechanisms of defence

One important fitness consequence of microorganisms for birds is the increased exposure to different kinds of microorganisms during their life (Møller 1998) that should lead to selection for extraordinary defences against such pathogenic diversity (Møller 1998; Soler *et al.* 2010). Birds have evolved antimicrobial defenses to avoid pathogen infections and maintain plumage integrity, and development of defenses against strains of parasites encountered throughout the life cycle of hosts (Møller and Erritzøe 2000; Ruiz-Rodríguez *et al.* 2012). Thus, we predict that birds have evolved means to regulate the microorganisms on their plumage.

The uropygial gland is an exocrine gland of birds secreting waxes with antimicrobial properties that is smeared on the feathers during preening. The uropygial gland produces a huge diversity of biochemicals (Jacob and Ziswiler 1982). Many experimental and comparative studies have shown that gland secretions have antibacterial and antifungal properties (Jacob and Ziswiler 1982; Møller *et al.* 2010; Soler *et al.* 2011).

The chemical composition of gland secretions has been well characterized for many species, and their high diversity is indicative of divergent selection between microorganisms and their hosts. The secretion of uropygial glands is a complex mixture of chemicals composed of aliphatic monoesters, waxes which are made mainly from fatty acids (with various methyl branchings) and monohydroxy wax alcohols. In addition, several diester waxes contain hydroxyfatty acids and/or alkane-diols (Jacob and Ziswiler 1982; Downing 1986; Jacob 1978). Many researchers have assumed that the main function of uropygial gland is to synthesize lipids (Kolattukudy and Rogers 1978; Urich 1994).

The secretions of uropygial glands could affect the abundance of microorganisms that inhabit feathers, as shown in an experimental study by Jacob

and Ziswiler (1982) by removal of the uropygial gland. In addition, comparative analyses have shown that the mass of the uropygial gland is associated with the production of natural antimicrobial substances (Soler *et al.* 2011). Large uropygial glands could have fitness consequences in terms of hatching success in birds by producing antimicrobial substances against abundant virulent microbes on eggs (Møller *et al.* 2010), while a small size of uropygial glands is characteristic of bird species that are more likely to fall prey to predators (Møller *et al.* 2010).

Dust or sand bath is an important animal behaviour that consists of rolling and moving around in sand, to clean and remove parasites from feathers (Baicich and Harrison 2005). Experiments on quail *Coturnix coturnix* show that birds that take frequent dust baths can help maintain an optimum amount of preen gland secretions on their feathers (Mexicano *et al.* 2014). In addition, dust can absorb excess uropygial gland secretions and keep their feathers in prime condition, while dusting may help discourage lice and other parasites in birds (Nestler 1949). Bird species that do not have uropygial glands such as bustards, kiwis, emu and ostrich depend on dust bathing to clean and dry their feathers.

Water bath is very important for feather maintenance and makes their feathers easier to preen. House sparrows *Passer domesticus* and wrens often use water bath in combination with dust bath.

Many bird species use sun bath as part of their routine feather maintenance by exposing their feathers and skin to sunlight directly. The most common birds that use sun bathing are pigeons, sparrows, cormorants, vultures and doves.

Study organisms

Here I briefly present the organisms that were used for my study.

Microorganisms (Bacteria and fungi)

Bacteria are prokaryotic microorganisms that have several shapes such as bacillus (rod-shaped), coccus (spherical), spiral (twisted) and other shapes (star shape or rectangular shape). Bacteria are one of the first lifeforms on Earth, and they are present in most habitats: soil, water, acidic hot springs, inside the Earth's crust and even in radioactive waste (Fredrickson *et al.* 2004). They are considered to constitute a large abundance of prokaryotic microorganisms, and there are about 5×10^{30} bacteria on Earth (Whitman *et al.* 1998), constituting the greatest biomass that exceeds that of all animals and plants together (Lombardo 1995). Bacteria live in parasitic and symbiotic relationships with animals and plants. Some bacteria are pathogenic, and they have the ability to infect and cause disease in hosts.

During the 19th century bacteriology only relied on light microscopy. The isolation methodology has changed over the past 60 years, from the work perspective with increases in mechanization, the technologies involved, and speed and accuracy.

The first isolates of microbes from feathers were made more than 50 years ago, (Gierløff *et al.* 1961; Pugh and Evans 1970), although the study of microorganisms in feathers was dormant until Burt and Ichida (1999) were able to isolate *Bacillus* spp. as feather-degrading bacteria. Feathers may harbour a diverse microflora, although the ecological role of the microflora remains a poorly known. The dangerous group of bacteria (in our study) is feather-degrading bacteria that can permanently damage the feathers of birds by degrading their structure. These different keratinolytic bacteria all occur naturally in soil, on the

bill of birds or in old nests (Hubálek 1976, 1978; Pugh 1964; Shih 1993; Wood 1995).

The word "fungus" usually evokes the image of mushrooms. There are approximately 300,000 species of fungi in the world with many remaining undescribed taxa. They can be found worldwide. They are classified as the kingdom Fungi that are separate from other eukaryotic kingdoms of animals and plants.

Fungi are filamentous eukaryotes, and most fungi are saprophytes that obtain their nutrients from organic materials, while the other fungi are parasitic decomposers that obtain nutrients by absorbing these through their cell walls. Some fungi are parasites of plants that cause diseases leading to monetary loss for agriculture. The others cause disease in animals and humans. The term "keratinophilic fungus" is used for fungi that degrade the keratin substances on feathers as a main source of carbon and nitrogen. Moreover, these keratinophiles can tolerate the somewhat higher temperature of birds' skin surface (40–42°C), which is usually unfavourable for the growth of most other fungi (Kaul and Sumbali 1999).

Generally, birds frequently carry keratinophiles passively on intact feathers. Some of these keratinophilic fungi are well-known pathogenic dermatophytes, and they are known to cause superficial cutaneous infections (dermatophytoses) of keratinised tissues (skin, feather, hair and nail) of humans and animals (Deshmukh 2004).

Barn swallow (*Hirundo rustica*)

This is a widespread species of passerine bird distributed across the world (Turner *et al.* 1988). Barn swallows are small ca. 18 g insectivorous passerines that breed solitarily or in small colonies that may reach more than 200 pairs. Barn swallows

are migratory birds. In winter, European populations migrate to Sub-Saharan Africa. They travel across western France, through the Pyrenees, down eastern Spain into Morocco, and through the Sahara or via Italy (Brown and Brown 1999; Turner *et al.* 1988).

Since the first humans started to build houses, barn swallows have been closely associated with human habitation, and they have adapted to build their nests either inside buildings or other large constructions, causing an enormous increase in population size (Newton 1998). However, there is recent worry about the state of barn swallow populations because many populations are declining due to intensified agriculture.

Barn swallows have a long-forked tail, with white spots, and they are larger in adults especially in males. They have a blue iridescent colour on their back (Fig. 2) (Brown and Brown 1999; Kose and Møller 1999). Barn swallows are usually monogamous, with one male and one female reproducing together (Brown and Brown 1999). The nest is a mud cup that the pair makes from mud collected in the bill and mixed with grass stems and other materials to make pellets (Fig. 3). Next, they add grass lining to the mud cup. Finally, the nest is completed by adding lining feathers, hair or other materials in total lasting 5-25 days (Brown and Brown 1999). Usually, the shape of the mud cup is gourd (Petersen and Meservey 2003). Barn swallows can re-use their nest by adding lining feathers and new layers of mud every year (Brown and Brown 1999).

Barn swallows are often colonial and tend to return to their nest year after year (Turner 2010). Most barn swallows have two clutches annually, and they lay on average five eggs in the first and four eggs in the second clutch (Petersen and Meservey 2003). The female lays one egg per day, and the eggs are incubated about 12-17 days, with only the female incubating in European populations. The eggs of barn swallows are white to pinkish white colour with brown or grey spots,

the nestlings are naked with eyes closed (Fig. 4). The nestling period lasts 15-27 days, and barn swallows have a pale juvenile coloration when they fledge. The adults feed on flies, beetles, butterflies, ants, wasps, moths, bees and other flying insects that they catch in flight (Brown and Brown 1999).



Fig. 2. The identification guide to barn swallows

Left: Female barn swallow. Right: Male barn swallow.

(© Ian Montgomery birdway.com)



Fig. 3. Mud-cup nest of barn swallow made with straw

(© Photograph by Kathleen Scott) (<http://www.hillcountrymysteries.com/>)



Fig. 4. Eggs and chick of barn swallows

(www.arkive.org)

Goshawk (*Accipiter gentilis*)

The goshawk *Accipiter gentilis* is a territorial monogamous predator (Kenward 2010), and there is a distinct division of sex roles during the breeding season (Fig. 5a, b). The smaller male supplies the food to the larger female and their chicks, while females incubate their eggs and defend their nests and the chicks. Females are about three times heavier than males (Kenward 2010).

The goshawk is adept at flying through closed forests, although it prefers to nest in tree-tops where clear level access is afforded by stream courses, rides, fire-breaks, or natural glades. It favours areas where woodland is interspersed with fields or even wetlands. Ordinarily it flies low, close to trees or bushes, but in the breeding season it soars to several hundred meters (Cramp and Simmons 1979). It favours areas where woodland is interspersed with fields or even wetlands.

Usually the goshawk is a solitary hunter, although hunting in pairs has been observed (Berndt 1970). Hunting is characterized by swift, aggressive and adroit pursuit flights; usually for only short distances, up to 500 m (Brüll 1964). It takes advantage of any cover to approach prey in an attempt to catch it by surprise. Goshawks may also watch from a hidden perch. Normally it gives up if the attack is unsuccessful, although occasionally it makes a second attempt. Prey are caught in flight, on nests, branches, and on the ground (Schnurre 1934; Pielowski 1961; Brüll 1964). It will stoop from great height in an attempt to catch prey in air (Kollinger 1974; Opdam *et al.* 1977). Prey are caught and killed with claws and then usually brought to cover and plucked and eaten on the ground or a small elevation, or (especially in the nesting area) on an exposed branch or an old nest (Opdam *et al.* 1977). Considerable size differences in the sexes are also reflected in average prey weight; 277 g in males and 505 g in female during March in a forest area near Nijmegen, Netherlands (Opdam 1975).

The diet varies according to availability of prey and can be extremely diverse (Opdam *et al.* 1977). As opportunist, it appears to take any prey of convenient size, although predator size to some extent determines prey size, and the method of hunting determines vulnerability of potential prey. In Europe, mainly grouse (Tetraonidae), partridges and pheasants (Phasianidae), pigeons (Columbidae), crows (Corvidae), thrushes (Turdidae), and, among mammals, rabbit and red squirrel *Sciurus vulgaris* are common prey (Bittera 1915; Uttendörfer 1952).



Fig. 5a, b. Northern goshawk male and female.

Objectives of study

This thesis analyses the fitness consequences of the diversity and the abundance of microorganisms on birds. The causes and consequences of microorganisms isolated from the feather nest lining of barn swallows was studied to address the following four questions, while microorganisms from feathers of prey of the goshawk and moulting live birds served as controls for the final chapter investigating the link between risk of predation and microorganisms on feathers.

1. Arrival date and microorganisms in barn swallows

The objective of this chapter was to investigate the relationship between arrival date and abundance and diversity of microorganisms in a long-distance migratory bird. Specifically, we predicted that individuals that migrate the fastest and hence arrive the earliest at the breeding grounds have fewer microorganisms including bacteria and fungi on their plumage than late arriving individuals, independent of potentially confounding effects of age and other variables. We also predicted that early arriving migrants would have more biocin-producing microorganisms than late arriving individuals. We tested these predictions in the barn swallow *Hirundo rustica* for which we have collected information on microorganisms on nest lining feathers and arrival date at the breeding grounds.

2. Laying date, microorganisms and the advantages of early reproduction

The objective of this study was to determine whether timing of reproduction is related to diversity and abundance of microorganisms. Positive effects of abundance of microorganisms on timing of reproduction may be due to competition between benign and pathogenic organisms (Perrins 1970), while negative effects on timing of reproduction may be caused by pathogenic microorganisms that cause disease or reductions in body condition (Møller *et al.* 2015).

3. Microorganisms, clutch size and reproductive success in the barn swallow

The objective of this study was to assess whether microorganisms affect clutch size and reproductive success and to quantify the role of microorganisms, in particular bacteria and fungi, in the life history of barn swallows.

4. Recapture probability, flight morphology and microorganisms

The objectives of this study were to test to which extent the probability that individual birds are recaptured once they have been captured depends on the abundance and the diversity of microorganisms. The rationale behind this objective is that birds with more microorganisms suffer from the costs of microorganisms causing disease in their hosts. Alternatively, birds with more microorganisms suffer from more damage to their plumage caused by feather degrading microorganisms. Either of these mechanisms may impair flight and escape ability and hence increase the probability of capture and recapture.

5. Microorganisms, flight ability and risk of predation

The main question of this study was whether birds with high loads of microscopic fungi were more likely to fall prey to a predator than those with few. This question was based on the assumption that more fungi and/or more diverse fungi constitute a greater cost for their hosts. Here we tested this prediction by investigating the relationship between risk of predation and the abundance of fungi on feathers from four common prey species of the goshawk, wood pigeon *Columba palumbus*, Eurasian jay *Garrulus glandarius*, Song thrush *Turdus philomelos* and blackbird *Turdus merula* that are preferred prey species of the goshawk in Denmark.

Materials and methods

Study site and field work

The samples of nest lining feathers from barn swallow nests were collected by Anders Pape Møller during May-June 2014 at Kraghede (57°12'N, 10°00'E),

Denmark, in a population of barn swallows that has been followed since 1984 (Møller 1994). Adults were captured weekly (at least twice) from the beginning of the breeding season. Arrival date was defined as the date of the first capture, although this will provide an underestimate of the true arrival date (Møller *et al.* 2004). Moreover, reproductive success was recorded as the total number of fledglings produced in the first and the second clutch by each adult (Møller *et al.* 2004). Feather samples were collected by using sterile gloves, deposited in polythene bags and stored in a refrigerator until analysis.

The samples of goshawk prey collected through collaboration between Jan Tøttrup Nielsen and Anders Pape Møller were from woodpigeon, Song thrush, Eurasian jay and blackbird. Two categories of feathers were collected at the same time of the year from the same forests: Feathers from plucking sites near 50 nests of goshawks in Northern Vendsyssel (57°10'–57°40'N, 9°50'–10°50'E), Denmark, during April–August 2009 on the mean date of 16 May (SE = 2). Plucking sites are traditional sites near nests where goshawk males bring their prey before presenting the prey for the offspring or the incubating, brooding or attending female. Furthermore, moulted feathers were collected from the same sites from birds that clearly were alive (because they were moulting). In total, we had samples from more than 500 individuals. All feathers collected were only from recent prey not more than a couple of days old as reflected by the soft structure of feathers. Feathers rapidly become stiff with rain and exposure to weather. We avoided problems of contamination of feathers by nest contents by only including feathers found on the ground, as were the samples of feathers from live individual prey.

Analyses of microorganisms

We isolated and identified the bacterial and fungal species from the samples of feather lining in barn swallow nests and quantified their abundance.

Microorganism isolation

Bacterial isolation

In the laboratory, by using sterilized tweezers, each piece of feather was introduced in a 15 ml falcon tube with sterile phosphate buffer saline (pH 7.2) with a volume proportional to the length of the feather. This was followed by three shaking periods by vortex 10 s each. Free-living bacteria were washed out from the feathers and collected in PBS solution (Saag *et al.* 2011), and then performing serial tenfold dilution to 10^{-4} . To quantify cultivable and feather-degrading bacteria, duplicates were made by spreading 100 μ l of supernatant with a sterile spreader loop on two different growth media:

(1) Tryptic soy agar (TSA) which is a rich medium in which heterotrophic bacteria can grow, thus enabling assessment of total cultivable microorganism load of the feathers.

(2) Feather meal agar (FMA) which is a medium highly selective for keratinolytic bacteria because the only source of carbon and nitrogen is keratin. Hence, only bacteria that are able to digest this medium can proliferate, and that allows quantification of feather-degrading bacterial load.

Fungal isolation

The feathers were snipped into small pieces and cultured directly onto Mycobiotic Agar (which is a selective medium for isolation of pathogenic fungi) following standard procedure (Deshmukh 2004), and moistened with 0.5 ml of sterilized PBS. The cultures were incubated at $28^{\circ}\text{C} \pm 2$ and examined daily from the third day for fungal growth over a period of four weeks. The observed developing mycotic growths, under stereoscopic binocular microscope, were individually and directly transferred onto Sabouraud dextrose agar with chloramphenicol (50 mg/l). The resulting products were further incubated at $28^{\circ}\text{C} \pm 2$ for two weeks to obtain pure isolates for identification purposes.

Feather meal agar preparation

Downy feathers were extensively washed with warm water and detergent more than three times to eliminate all dirt and dust particles, and to ensure elimination of all remnant preen wax the feathers were washed with a chloroform: methanol mixture (1:1, v/v), washed again with warm tap water to get rid of remnant chloroform and methanol, and then dried in a hot air oven at 50°C overnight). The dried feathers were ground up by using a Mixer Mill (Retsch MM-301) and a tungsten carbide vial with the aid of three balls, milling the feather for 50 secs / 30 Hz. Finally, the milled feather was sterilized by autoclaving at 121°C for 15 min (Vidal *et al.* 2000).

Plates were incubated at 28°C for three days in the case of TSA, and for 14 days in the case of FMA. After incubation, we counted the number of colony-forming units (CFU) of each morph type per plate to estimate densities of viable bacteria in the sample. We distinguished morph types on the basis of colony colour, shape, size, and presence or absence of glutinous properties. The total bacterial densities were calculated by multiplying the average number of CFU by the dilution factor and by the original sample volume (Peralta-Sánchez *et al.* 2014). Plates with each media type, but without microbial suspension were incubated in order to detect any contamination of media (negative controls).

Isolate preservation

Slants of nutrient agar and 40% glycerol stocks were prepared from identified pure culture and stored at 4 and -80°C, respectively, for medium and long-term preservation.

Microorganism identification

Bacterial identification

The bacterial identification was carried out by using molecular characterization with the aid of polymerase chain reaction (PCR) for further genetic analyses.

DNA extraction

Genomic DNA was extracted from each isolate by using two different protocols.

Freeze-thaw protocol. With a wooden toothpick transfer we squashed part of a bacterial colony in a 0.5 ml Eppendorf tube containing 50 μ l Tris (10 μ M, pH 8.0). Starting of freezing and thawing by putting the Eppendorf tube in liquid nitrogen for one to two minutes until completely frozen, then quickly placing the tube in a hot water bath until completely thawed. This process is known as a freeze-thaw cycle, and we repeated the same process for a total of three cycles. We made sure that we mixed the sample tube between each cycle.

To ensure complete cell lysis we put the tubes in a microwave at 270W for 5 to 6 seconds followed by 10 seconds of waiting, repeated three times. We centrifuged the sample tube for five minutes at 12,000 x g in a micro centrifuge. Using a micropipette, we transferred the supernatant that contains the DNA into a new clean, sterile micro centrifuge tube and discarded the pellet that contains cellular debris. We stored the tubes in -20 °C for the Polymerase Chain Reaction (PCR).

DNA Extraction kit: We used the Power Soil DNA isolation kit (MOBIO Laboratories, Inc. USA) to extract DNA according to the protocol that is supplied with the kit.

PCR amplification of bacterial 16SrRNA gene

DNA isolated from samples was used as a template for PCR to amplify the bacterial 16S rRNA gene by using the forward primer 16S rDNA-27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer 16S rDNA-1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was carried out in a total volume of 25 μ l containing 16 μ l ultrapure water, 4 μ l 5x buffer, 0.4 μ l dNTPs 10nM, 1 μ l 10 μ M 27F, 1 μ l 10 μ M 1492R, 0.2 μ l Go Taq DNA polymerase and 3-5 μ l

genomic DNA. The PCR conditions consisted of an initial denaturation at 94°C for 5 min, denaturation at 94°C for 15 sec., annealing for 30 sec. at 55°C and 1.30 min of primer extension at 72°C. The cycle of denaturation, annealing and elongation was repeated 35 times. A final elongation at 72°C for 7 min was then performed. The PCR products were sent to sequencing by Beckman Coulter Genomics.

Fungal identification

The identification of fungi was done by using two protocols: The traditional fungal identification methods such as morphological characteristics, culture on various media, presence of microconidia and chlamydospores and biochemical analysis such as pigment production have been used routinely, and still several new species are described on this basis (Zhao and Wu 2007).

For molecular identification of fungi, we took a small amount of mycelium and suspended it in 200 µL 10 mM Tris-HCl (pH 8.0) in an Eppendorf tube (1.5 mL), and stored it in a freezer (-20 °C) for further processing. The genomic DNA was isolated from the fungal strains by using the PowerSoil® DNA Isolation Kit (MO BIO). DNA was eluted in a final volume of 100 µl of 10 mM Tris-HCl, pH 8.5.

Amplification of fungal 18s rDNA gene by PCR

PCR amplification was performed in 20-30 µL reaction volume containing 25 µl assay buffer containing 1.5 mM MgCl₂, dNTP (10mM) 0.5-1 µL, 0.5-1 µL of each 0.2 mM primer FR1 (5'-'CTCTCAATCTGTCAATCCTTATT-3'), 2.5 µl forward primer UF1 (5'-CGA ATC GCA TGG CCT TG-3'), Go Taq® G2 DNA polymerase (Promega Madison, WI USA) (1.25 µl) 0.5-1 µL, DNA sample 3-5 µL. The DNA genomic was amplified with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation for 15 secs at 94°C, annealing for 30 secs

at 55°C and extension for 1.30 min at 72°C, respectively, and the final extension was carried out at 72°C for 7 min.

Agarose electrophoresis

To visualize and determine the presence or absence of PCR products and to quantify the size of amplified DNA fragments. We performed a gel electrophoresis in 1% agarose using 0.5 X TAE buffer (Tris-Acetate-EDTA) for 25 min at 100V. The gel was then stained with Gel Red (BIOTIUM) for 30 min. Images were taken under UV lamp by using the photo documentation system IP-010.SD.

DNA sequencing

PCR products were sent for sequencing to Beckman Coulter Genomics, Takeley, Essex CM22 6TA, United Kingdom for DNA Sequencing.

The sequence results were processed by using the web-based blasting program, basic local alignment search tool (BLAST), at the NCBI site (<http://www.ncbi.nlm.nih.gov/BLAST>), and the data were compared with the NCBI/Gene-bank database.

Antimicrobial activity assay

Fungal and bacterial species isolated from lining feathers of barn swallow nests were subject to antimicrobial activity assays against standard bacterial strains of *Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* JM 83, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Micrococcus luteus* and *Agrobacterium tumefacienscereus* in two different ways.

For bacterial strains, we used the streak-plating technique on Mueller Hinton agar. Each isolate was streaked on agar plates in a central single line, and the plates then incubated at 30°C for 7 days in order to give the bacteria opportunity to secrete antibiotic substances on the medium. After incubation, standard bacteria

were grown in Mueller Hinton medium for 18h and properly diluted to make cross-streaks along the line of fully grown isolates. Each streaking was started near the edge of the plates and streaked towards the central bacterial growth line. The plates were then incubated for 18 hrs. at 37°C, and the zone of inhibition was measured using a millimetre scale (Shams *et al.* 2015).

For fungal isolates, we cultured each species in Sabouraud dextrose broth medium for 1-2 weeks at 30°C. The culture medium was centrifuged at 5000 g for 10 min to remove all fungal cell, and by using a Millipore filter (0.2 µm) the supernatant was collected in a sterile falcon tube.

The antimicrobial activity was tested by well-diffusion methods. After 24-48 h of incubation, the inhibitory zones (in mm) were measured (Moshafi *et al.* 2006). Three commercial antibiotic discs Vancomycin (VA 30 mcg), Tetracycline (TE 30 mcg) and Chloramphenicol (C 30 mcg) were used as positive controls.

Statistical analyses

We used JMP for the statistical analyses (SAS 2012). We \log_{10} -transformed all bacterial and fungal counts after addition of a constant of one to normalize the data. We investigated the relationship between arrival date, laying date and reproductive success using standard least squares models and in the case of reproductive success a Generalized Linear Model with Poisson distribution and log link function to account for the count data.

We report total abundance of colonies and species richness and standard errors for fungi and bacteria. We calculated Simpson's index (1-D, a measure of diversity independent of sample size) to determine the diversity of fungi and bacteria in feathers lining the nests. This index takes the number of species present into account, as well as the abundance of each species (Simpson 1949). We subsequently investigated the relationship of (arrival date, laying date and total fledge) with the abundance of different taxa of microorganisms using standard least squares models.

We conducted a meta-analysis (Rosenthal 1991; Cooper and Hedges 1994; Rosenthal 1994) of the relationship between laying date and the abundance of microorganisms relying on all reported analyses in the literature and the findings in the present study. We converted test statistics into Pearson product-moment correlation coefficients (Rosenthal 1994), and these coefficients were subsequently z-transformed before calculating the mean estimates weighted by sample size minus two.

We report total abundance of colonies and species richness and standard errors for fungi and bacteria. We tested whether the number of recaptures followed a Poisson distribution by fitting a Poisson model to the frequency data. We tested whether the abundance of fungi and bacteria predicted the frequency of recaptures using generalized linear models. Likewise, we tested whether daily feather growth increments differed between prey and non-prey.

We estimated effect sizes by using Cohen's (1988) guidelines for the magnitude of effects being small (Pearson $r = 0.10$, explaining 1% of the variance), intermediate ($r = 0.30$, explaining 9% of the variance) or large ($r = 0.50$, explaining 25% of the variance).

General results

Communities of microorganisms

We isolated 15 different fungal species from the feather lining of barn swallow nests (according to identification by PCR technique (Table S1)).

For bacteria, we isolated 10 species from TSA and 10 species from FMA (Table S2). The different bacterial and fungal taxa were widely distributed across the phylogenetic tree of bacteria and fungi (Figs. S1a, b)

The number of fungal colonies in feather lining of nests of the barn swallow ranged from 20 to 135 with a mean of 72.09 (SE = 4.34), N = 54. The number of colonies fungal species ranged from 1 to 9, mean = 5.48 (SE = 0.23), N = 54. A

total of 15 fungal species were isolated from fungal colonies with *Aspergillus* sp. being the most frequently isolated species.

The total number of bacterial colonies in TSA ranged from 10 to 350, mean (SE) = 70.13 (8.92), N = 54. The number of bacterial species (TSA) ranged from 2 to 10, mean (SE) = 4.88 (0.25), N = 54. There were 10 bacterial species isolated from TSA. *Bacillus* spp. was the most frequently isolated species.

The total number of bacterial colonies in FMA ranged from 0 to 49, mean = 19.15 (SE = 1.79), N = 54. The number of bacterial species (FMA) ranged from 0 to 7, mean = 3.92 (SE = 0.24), N = 54. There were 10 species of bacterial colonies in FMA, with *Streptomyces* spp. being the most frequently isolated species. Species richness for fungal and bacterial species is presented in Tables S1 and S2. Means, SE and ranges of abundance and number of colonies of microorganisms are reported in Tables S1 and S2.

We isolated 27 fungal species from the feathers of goshawk prey, according to identification by PCR technique (Table S5). The number of fungal colonies from the feathers of goshawk prey ranged from 0 to 9 with a mean of 2.56 (SE = 0.20), N = 87. The number of fungal species ranged from 0 to 3, mean = 1.20 (SE = 0.09), N = 87. Species richness for fungal species, means, SE and ranges of abundance of fungal species isolated are presented in Table S5. The different fungal taxa were widely distributed across the phylogenetic tree of fungi (Fig. S2).

1. Arrival date and microorganisms in barn swallows

Arrival date, age, feather damage and reproductive success

Age was on average 1.39 years (SE = 0.11), range 1-4 years, N = 54. Arrival date was not significantly related to age ($F = 1.55$, $df = 1$, 52 , $r^2 = 0.01$, $P = 0.22$).

The total number of fledglings for the season was significantly negatively related to arrival date ($F = 11.377$, $df = 5$, 53 , $r^2 = 0.54$, $P < 0.0001$). Thus, early arriving barn swallows had larger reproductive success.

Barn swallows with damaged feathers arrived almost one week later than those with undamaged feathers (damaged feathers: 18 May (SE = 0.95), N = 509 individuals from 1984-2016; undamaged feathers: 11 May (SE = 0.28), N = 4682 individuals).

Microorganisms and arrival date

We tested the prediction that arrival date was related to the abundance of microorganisms. There was a positive relationship between arrival date and the abundance of *Bacillus licheniformis* (Fig. 6; $F = 5.637$, $df = 1, 49$, $r^2 = 0.11$, $P = 0.022$). Furthermore, we found a positive relationship between arrival date and the abundance of the fungus *Trichoderma reesei*, while there was a negative relationship with *Mucor circinelloides* (Fig. 7; $F = 4.488$, $df = 2, 49$, $r^2 = 0.16$, $P = 0.017$).

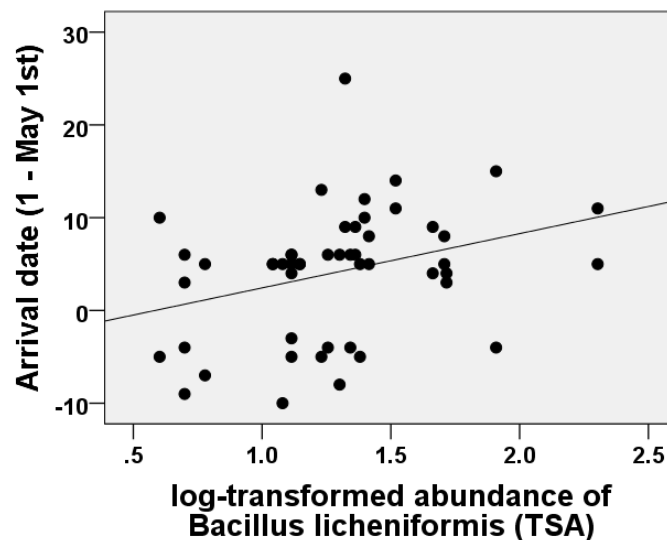


Fig. 6 : Arrival date of barn swallows (1 = May 1st) in relation to log-transformed abundance of *Bacillus licheniformis*. The line is the linear regression line.

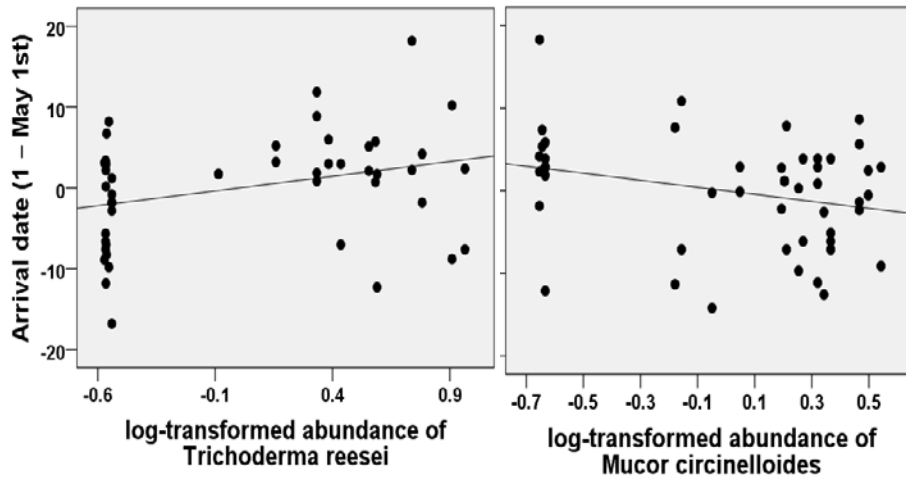


Fig. 7 : Arrival date of barn swallows in relation to log-transformed abundance of fungal species. The lines are the linear regression lines.

There was a positive relationship between arrival date and mean number of bacterial colonies in TSA medium, while there was a negative relationship between the mean number of bacterial colonies in FMA medium and Simpson’s diversity index of bacterial species in FMA medium (Fig. 8; $F = 3.981$, $df = 3$, 49 , $r^2 = 0.20$, $P = 0.013$).

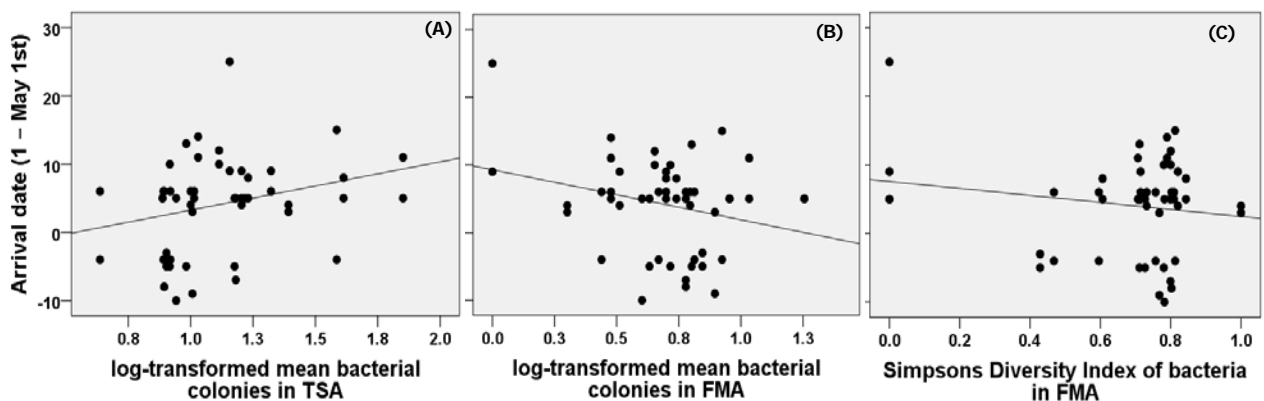


Fig. 8 : Arrival date of barn swallows in relation to (A) log-transformed mean number of bacterial colonies in TSA medium, (B) log-transformed mean number of bacterial colonies in FMA medium, and (C) Simpson diversity index of bacterial species in FMA medium. The lines are linear regression lines.

2. Laying date, microorganisms and the advantages of early arrival

Arrival date, laying date, age and reproductive success

There was a strong positive relationship between laying and arrival date (Fig. 9; $F = 63.84$, $df = 1, 49$, $r^2 = 0.57$, $P < 0.0001$). Age was on average 1.39 years (SE = 0.11), range 1-4 years, $N = 54$. Arrival date was not significantly related to age ($F = 1.55$, $df = 1, 52$, $r^2 = 0.01$, $P = 0.22$), nor was laying date ($F = 1.89$, $df = 1, 52$, $r^2 = 0.02$, $P = 0.17$). The total number of fledglings produced during the breeding season was significantly negatively related to laying date (GLM with Poisson distributed data and a log link function: $\chi^2 = 1.89$, $df = 1$, $P = 0.018$, estimate (SE) = -0.014 (0.006)), implying that there was a fitness advantage from early breeding.

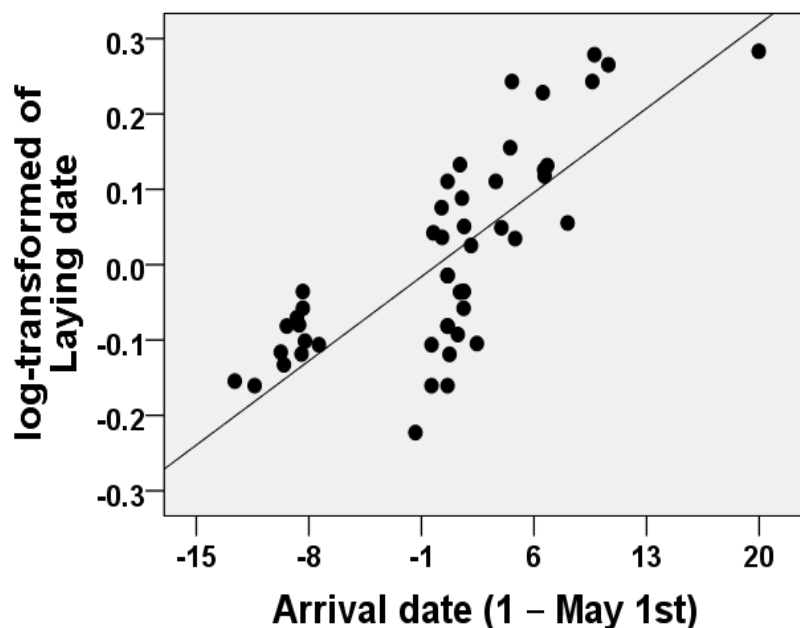


Fig. 9 : Laying date of the first clutch of barn swallows in relation to arrival date.

Dates are days since April 30th with 1 = May 1st. The line is the linear regression line

Laying date in relation to microorganism abundance and species richness

Across all microorganism taxa we tested whether there was a correlation between laying date and microorganism abundance. Next, we tested whether the mean correlation between laying date and abundance of microorganisms differed

significantly between bacteria and fungi. The distribution of correlation coefficients describing the relationship between laying date and abundance of microorganisms was approximately normal. We found a positive relationship between laying date (controlled for arrival date) and the abundance of the bacteria *Paenibacillus cookii* and *Streptomyces cacaoi*, ($t = 2.03$, $P = 0.048$, estimate = 0.06 (SE = 0.03); $t = 2.68$, $P = 0.010$, estimate (SE) = 0.14 (0.051)), respectively, while there was a negative relationship with *Methylobacterium mesophilicum* ($t = -2.08$, $P = 0.043$, estimate (SE) = -0.21 (0.1)). The whole model is (Fig. 10; Table 1; $F = 21.73$, $df = 4, 53$, $r^2 = 0.66$, $P < 0.0001$). In contrast, we did not find a significant relationship between laying date and the abundance of any of the fungal species ($F < 2.362$, $df = 1, 53$, $P > 0.130$).

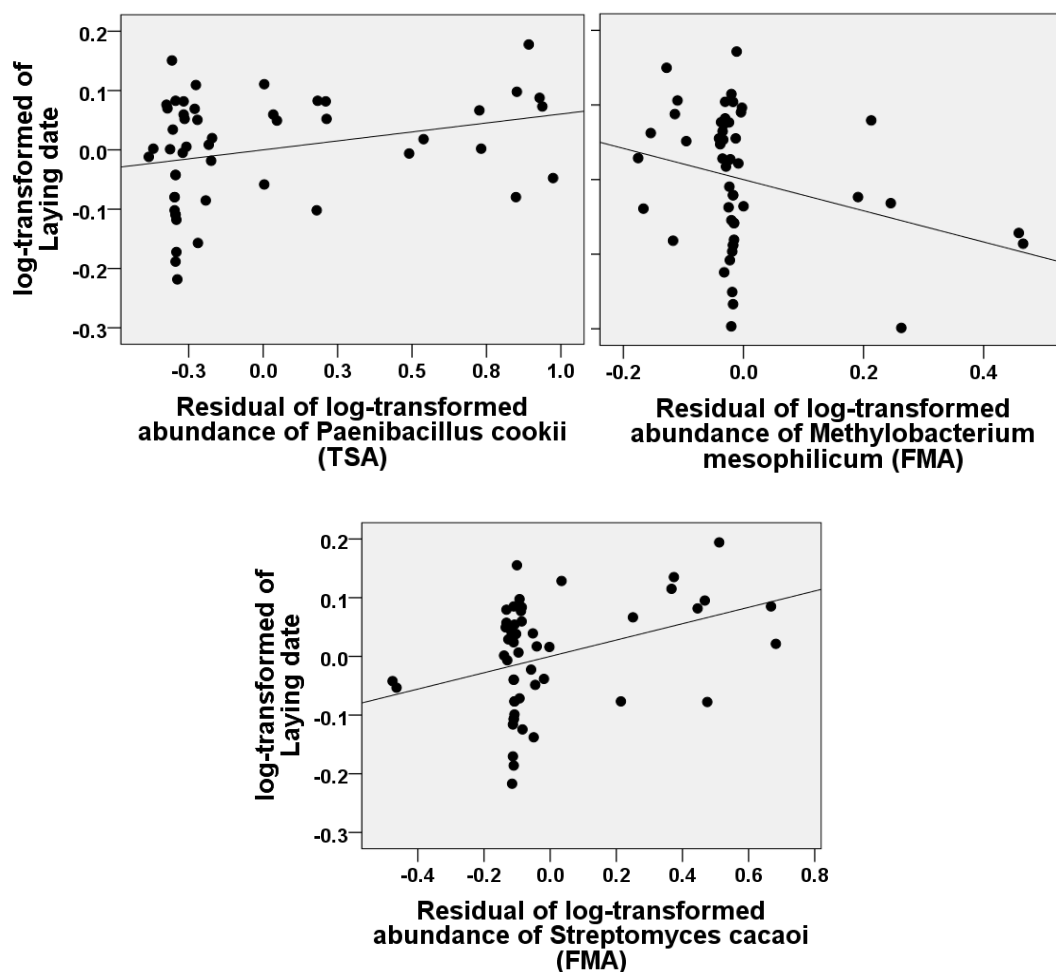


Fig. 10 : Residual laying date after controlling statistically for arrival date in relation to log-transformed abundance of bacterial species. The lines are the linear regression lines

Table 1. Relationship between laying date (response variable) and arrival date and the abundance of different bacterial taxa (predictor variables). The model had the statistics $F = 21.73$, $df = 4, 53$, $r^2 = 0.66$, $P < 0.0001$. Effect size is Pearson's product moment correlation coefficient.

Term	Estimate	SE	<i>t</i>	<i>P</i>	<i>r</i>	<i>z</i>
Intercept	1.267	0.019	67.53	< 0.0001		
Arrival date	0.016	0.002	8.55	< 0.0001	0.76	1.00
<i>Paenibacillus cookii</i> (TSA*)	0.060	0.030	2.03	0.048	0.27	0.28
<i>Methylobacterium mesophilicum</i> (FMA**)	-0.209	0.101	-2.08	0.043	-0.27	-0.28
<i>Streptomyces cacaoi</i> (FMA**)	0.139	0.052	2.68	0.010	0.35	0.36

*TSA (Tryptone Soy Agar medium)

**FMA (Feather Meal Agar medium)

Laying date, reproductive success and diversity of microorganisms

We found a positive relationship between laying date (controlled for arrival date) and Simpson's diversity index for fungal species (estimate (SE) = 0.115 (0.110)) and Simpson's diversity index for bacterial species in TSA medium (estimate (SE) = 0.206 (0.151)), while there was a negative relationship between laying date and Simpson's diversity index of bacterial species in FMA medium (estimate (SE) = -0.076 (0.070)) (Fig. 11; $F = 19.25$, $df = 4, 49$, $r^2 = 0.63$, $P < 0.0001$). This analysis shows that laying date was correlated with diversity and abundance of bacteria.

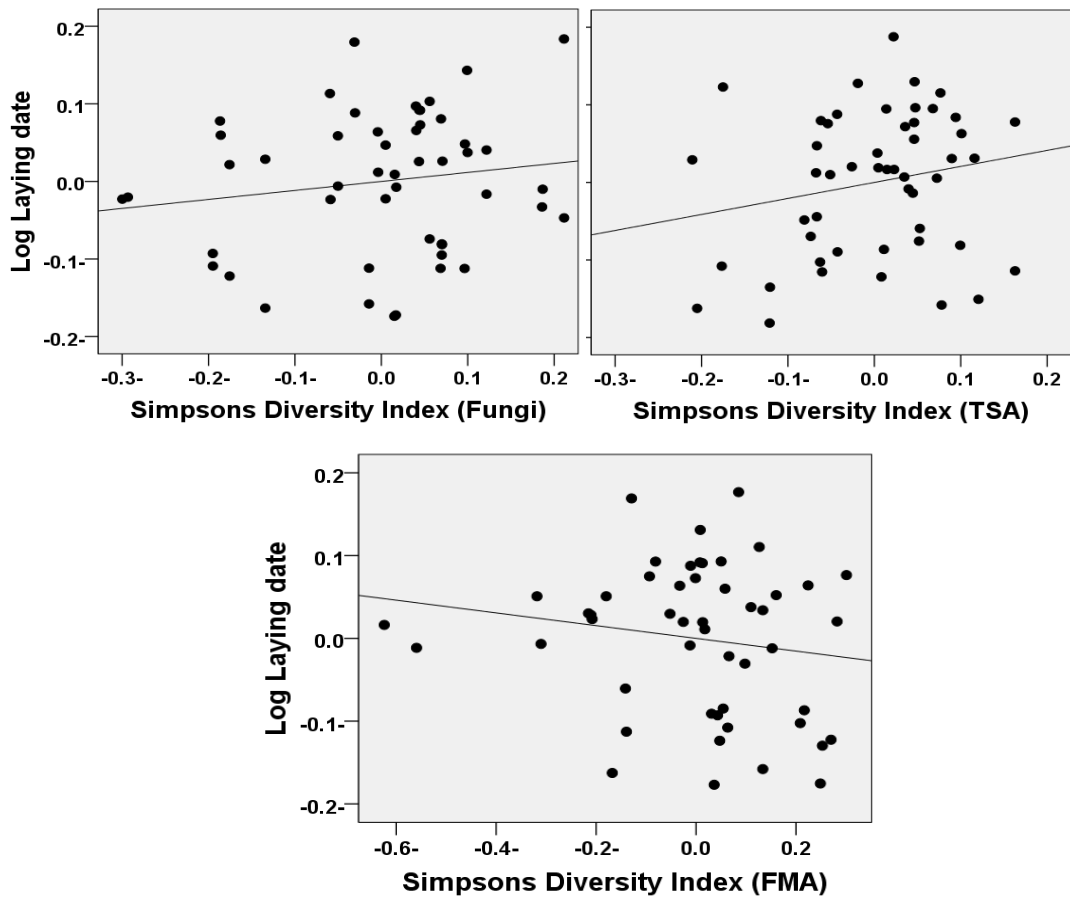


Fig. 11: Residual laying date after controlling statistically for arrival date in relation to residual Simpson's diversity index of fungi, Simpson's diversity index of bacteria in TSA medium and Simpson's diversity index of bacteria in FMA medium. The lines are the linear regression lines.

Meta-analysis of abundance of microorganisms and laying date

Two recent studies have suggested that laying date and abundance of microorganisms are positively related, implying that pairs of birds with more microorganisms suffered from delayed reproduction (Møller *et al.* 2015; Soler *et al.* 2015). Here we estimated the strength of this relationship based on 15 such estimates from these two publications and the present study. We found an overall mean effect size weighted by sample size of 0.141, SE = 0.062, 95% confidence intervals 0.009 to 0.274, Wilcoxon signed rank test = 150628.5, $P < 0.0001$ (Table 2). This suggests that the mean weighted effect size for the relationship between laying date and microorganisms is of an intermediate magnitude (Cohen 1988).

Table 2. Summary information on the relationship between abundance of microorganisms and laying date of their hosts based on a literature survey and the present study. See text for further details. Effect size is Pearson’s product-moment correlation coefficient r and the z-transformation of r .

Bird species	Microbial species	Test statistic		N	r	z	Reference
goshawk (<i>Accipiter gentilis</i>)	<i>Staphylococcus</i>	Kendall	0.60	14	0.60	0.69	Møller <i>et al.</i> (2015)
goshawk (<i>Accipiter gentilis</i>)	<i>Enterococcus</i>	Kendall	0.50	14	0.50	0.55	Møller <i>et al.</i> (2015)
goshawk (<i>Accipiter gentilis</i>)	mesophilic bacteria	Kendall	-0.14	14	-0.14	-0.14	Møller <i>et al.</i> (2015)
goshawk (<i>Accipiter gentilis</i>)	Enterobacteriaceae	Kendall	-0.08	14	-0.08	-0.08	Møller <i>et al.</i> (2015)
magpie (<i>Pica pica</i>)	mesophilic bacteria	Wald test	8.25	139	0.24	0.24	Soler <i>et al.</i> (2016)
magpie (<i>Pica pica</i>)	Enterobacteriaceae	Wald test	8.11	139	0.24	0.24	Soler <i>et al.</i> (2016)
magpie (<i>Pica pica</i>)	Staphylococci	Wald test	0.21	139	0.04	0.04	Soler <i>et al.</i> (2016)
magpie (<i>Pica pica</i>)	Enterococcic	Wald test	0.05	139	0.02	0.02	Soler <i>et al.</i> (2016)
barn swallow (<i>Hirundo rustica</i>)	<i>Lysinibacillus fusiformis</i>	t test	2.05	54	0.27	0.28	Current study
barn swallow (<i>Hirundo rustica</i>)	<i>Staphylococcus lentus</i>	t test	3.19	54	0.40	0.43	Current study
barn swallow (<i>Hirundo rustica</i>)	<i>Streptomyces cacaoi</i>	t test	2.45	54	0.32	0.33	Current study
barn swallow (<i>Hirundo rustica</i>)	<i>Streptomyces griseoaurantiacus</i>	t test	-3.39	54	-0.42	-0.45	Current study
barn swallow (<i>Hirundo rustica</i>)	<i>Paenibacillus cookii</i>	t test	2.03	54	0.27	0.28	Current study
barn swallow (<i>Hirundo rustica</i>)	<i>Streptomyces cacaoi</i>	t test	2.68	54	0.35	0.36	Current study
barn swallow (<i>Hirundo rustica</i>)	<i>Methylobacterium mesophilicum</i>	t test	-2.08	54	-0.27	-0.28	Current study

3. Microorganisms, clutch size and reproductive success in the barn swallow

Eggs, age and total number of fledglings

There was a strong positive relationship between total number of eggs and total number of fledglings produced during the breeding season (Fig. 12; $F = 88.417$, $df = 1, 53$, $r^2 = 0.62$, $P < 0.0001$, estimate (SE) = 1.278 (0.11)). Age of adult barn swallows was on average 1.39 years (SE = 0.11), range 1-4 years, $N = 54$ adults. The total number of fledglings produced during the entire breeding season was not significantly related to age (GLM with Poisson distributed data and a log link function: $\chi^2 = 1.415$, $df = 1$, $P = 0.234$, estimate = 0.077 (SE = 0.063)).

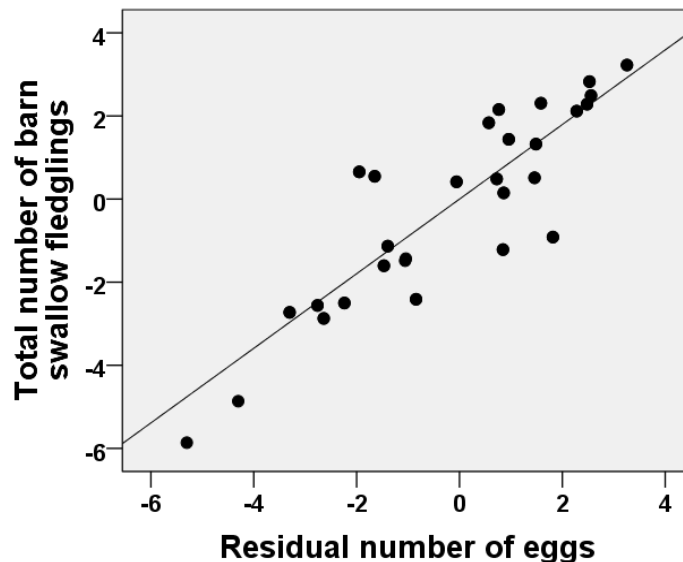


Fig. 12: Total number of barn swallow fledglings in relation to residual number of eggs after controlling statistically for laying date. The line is the linear regression line.

Microorganisms and total number of fledglings

There was a significant positive relationship between total number of fledglings (controlled for the total number of eggs laid) and abundance of *Absidia corymbifera*, *Penicillium glabrum*, *Bacillus pumilus*, *Bacillus rhizosphaerae* and *Streptomyces cacaoi*. In contrast, there was a negative relationship between total

number of fledglings (controlled for the number of eggs) and the abundance of *Trichoderma reesei* (Fig. 13; Table 3; $F = 41.819$, $df = 7, 53$, $r^2 = 0.86$, $P < 0.0001$). Implying that effects were not constant between different taxa of bacteria.

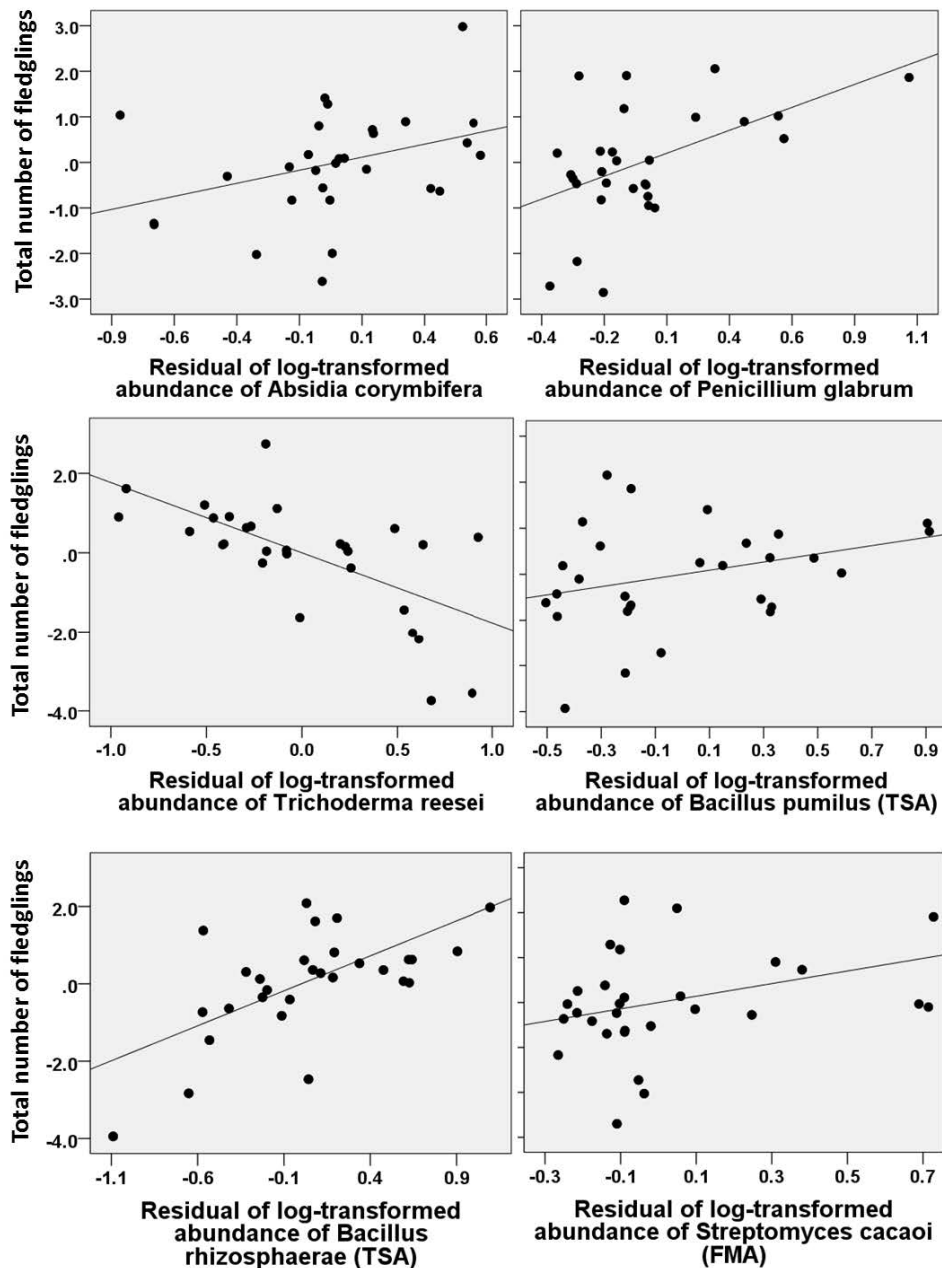


Fig. 13: Total number of barn swallow fledglings (controlled statistically for the number of eggs) in relation to log-transformed residual abundance of *Absidia corymbifera*, *Penicillium glabrum*, *Bacillus pumilus*, *Bacillus rhizosphaerae*, *Streptomyces cacaoi* and *Trichoderma reesei*. Residual abundance was controlled statistically for the number of eggs. The lines are linear regression lines.

Table 3. Relationship between total number of fledglings (response variable) and total number of eggs and the abundance of different bacterial and fungal taxa (predictor variables). The model had the statistics $F = 41.819$, $df = 7, 53$, $r^2 = 0.86$, $P < 0.0001$. Effect size is Pearson's product moment correlation coefficient r and the z -transformation of r .

Term	Estimate	SE	t	P	r	z
Intercept	-3.381	0.662	-5.11	<0.0001		
Number of eggs	0.897	0.078	11.59	<0.0001	0.85	1.24
<i>Absidia corymbifera</i>	1.146	0.470	2.44	0.0187	0.32	0.33
<i>Penicillium glabrum</i>	2.015	0.495	4.07	0.0002	0.49	0.53
<i>Bacillus pumilus</i> (TSA*)	0.891	0.393	2.27	0.0281	0.30	0.31
<i>Bacillus rhizosphaerae</i> (TSA*)	1.808	0.332	5.44	<0.0001	0.60	0.69
<i>Streptomyces cacaoi</i> (FMA**)	1.404	0.623	2.26	0.0289	0.30	0.31
<i>Trichoderma reesei</i>	-1.768	0.319	-5.55	<0.0001	-0.61	-0.70

*TSA (Tryptone Soy Agar medium)

**FMA (Feather Meal Agar medium)

Species richness of microorganisms and total number of fledglings

There was a positive relationship between total number of fledglings and the number of bacterial colonies in TSA medium and Simpson's diversity index of bacterial species in FMA medium, while there was a negative relationship between total number of fledglings and the number of fungal species (Fig. 14; $F = 12.397$, $df = 3, 53$, $r^2 = 0.63$, $P < 0.0001$). These findings show that effects were positive on diversity and abundance of bacteria and negative with number of fungal taxa.

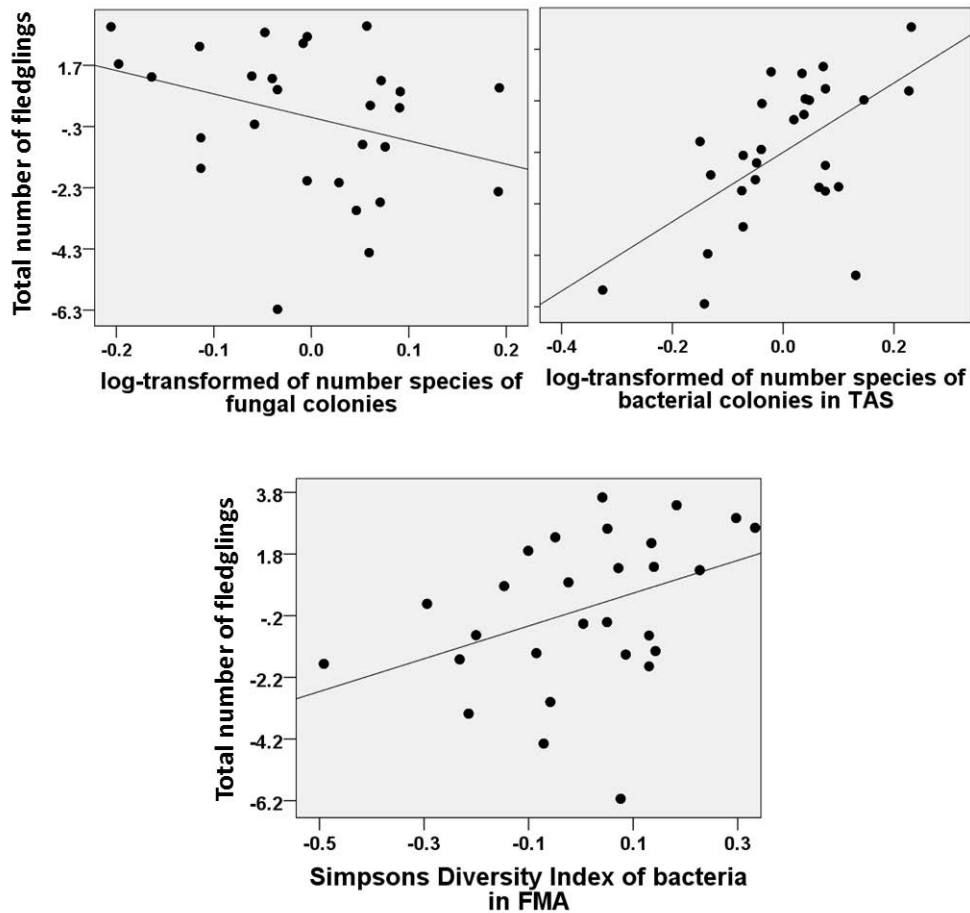


Fig. 14: Total number of barn swallow fledglings in relation to log-transformed number of fungal species, log number of bacterial colonies in TSA medium and Simpson diversity index of bacterial species in FMA medium. The lines are linear regression lines.

Antimicrobial activity and the abundance of microorganisms

We found eight species (54%) of fungal isolates that produced antibiotic substances (Table S3). In contrast, we found just three species (30%) of bacterial species isolated on TSA medium that produced antibiotic substances. We found three species (30%) of bacterial species isolated from FMA medium that produced antibiotic substance against the indicator bacterial strains (Tables S4a, b). We found a positive relationship between antimicrobial activity of *Aspergillus fumigatus* in (SDA) medium, *Streptomyces cacaoi* in (FMA) medium, *Bacillus*

pumilus in (TSA) medium, *Bacillus rhizosphaerae* in (TSA) medium. In contrast, there was a negative relationship between total number of fledglings and the antimicrobial activity of in *Bacillus licheniformis* (FMA) medium (Fig. 15; Table 4; $\chi^2 = 28.116$, $df = 5$, $P < 0.0001$). Meanwhile, we did not find a significant relationship between the antimicrobial activity of these microorganisms and each of arrival date ($F = 0.76$, $df = 15, 49$, $r^2 = 0.25$, $P = 0.703$), laying date ($F = 1.725$, $df = 15, 51$, $r^2 = 0.42$, $P = 0.089$) and recapture probability ($F = 1.59$, $df = 15, 45$, $r^2 = 0.44$, $P = 0.135$).

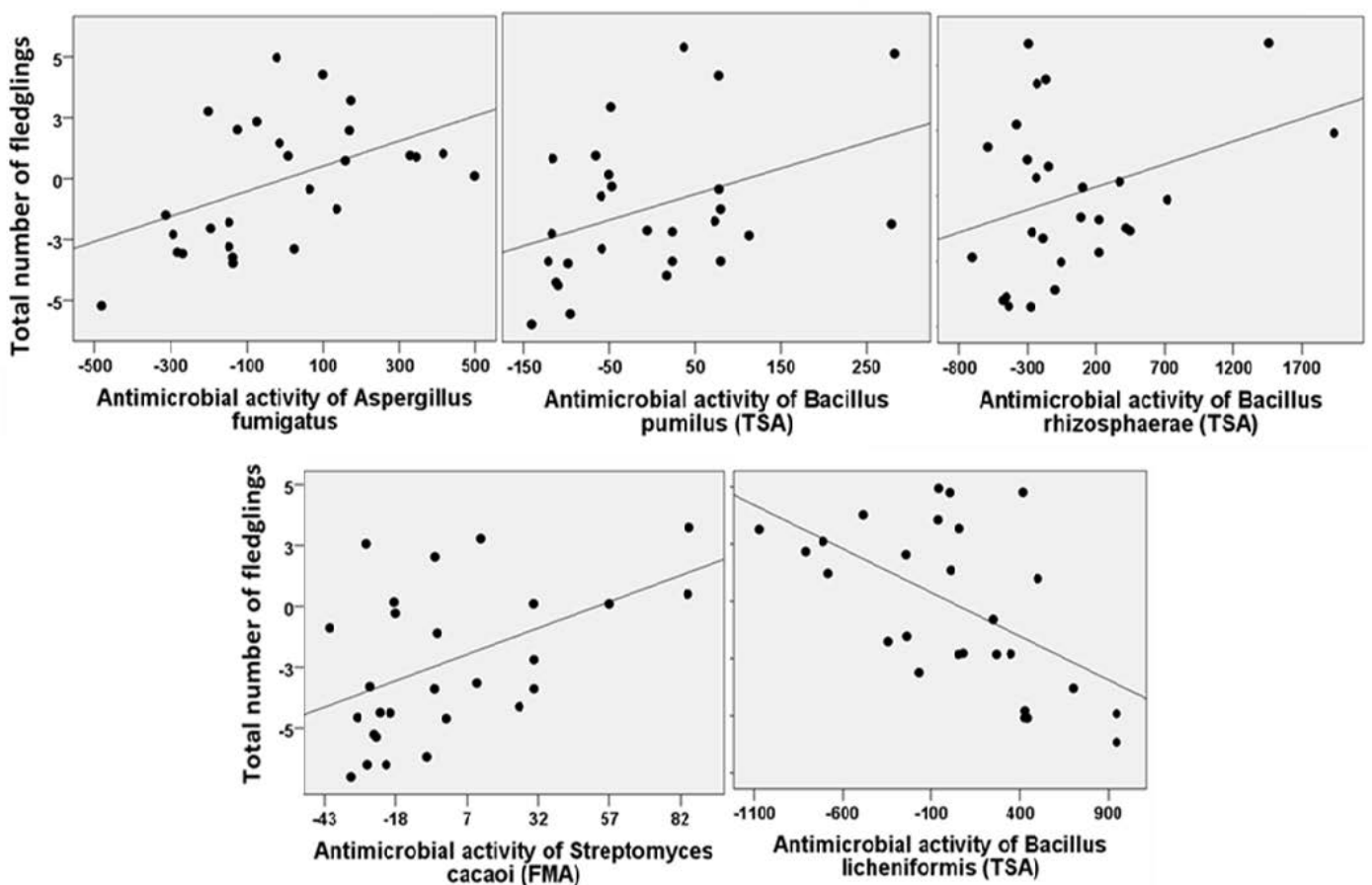


Fig. 15: Total number of barn swallow fledglings in relation to residual antimicrobial activity of *Aspergillus fumigatus*, *Streptomyces cacaoi* in TSA medium, *Bacillus pumilus* in TSA medium, *Bacillus rhizosphaerae* in TSA medium, and *Bacillus licheniformis* in FMA medium. The lines are linear regression lines.

Table 4. Antibacterial activity of fungi and bacteria in relation to total number of barn swallow fledglings in a Generalized Linear Model. The model had the statistics $\chi^2 = 28.116$, $df = 5$, $P < 0.0001$. Effect size is Pearson's product moment correlation coefficient r .

Term	Estimate	SE	t	P	r	z
Intercept	2.440	0.863	2.83	0.0069		
<i>Aspergillus fumigatus</i>	0.004	0.001	2.77	0.008	0.36	0.37
<i>Bacillus pumilus</i> (TSA*)	0.008	0.003	2.79	0.0075	0.36	0.37
<i>Bacillus rhizosphaerae</i> (TSA*)	0.002	0.0005	2.93	0.0053	0.37	0.39
<i>Streptomyces cacaoi</i> (FMA**)	0.023	0.005	4.52	<0.0001	0.53	0.59
<i>Bacillus licheniformis</i> (TSA*)	- 0.003	0.0006	- 4.72	<0.0001	-0.54	-0.61

*TSA (Tryptone Soy Agar medium)

**FMA (Feather Meal Agar medium)

4. Recapture probability, flight morphology and microorganisms

Microorganisms and recaptures

A total of 37.1% out of 1506 barn swallows was recaptured within the same breeding season. The number of recaptures ranged from 1 to 5 with a mean of 0.573 and a variance of 0.808, $N = 754$ adult barn swallows (Fig. 16). This estimate deviated from a Poisson distribution with $\lambda = 0.573$, 95% CI = 0.521, 0.629, $\chi^2 = 67.66$, $P < 0.0001$. We used the dichotomous variable recapture or not as the response variable in the subsequent analyses because data were available on recapture or not for more years.

A GLM with binomial error distribution showed significant effects of *Bacillus megaterium* with barn swallows with more bacteria being more likely to be recaptured (Fig. 17; $\chi^2 = 9.753$, $df = 1$, $P = 0.0018$). Effect size estimated as Pearson's product-moment correlation coefficient was 0.44, which can be considered a large effect (Cohen 1988). In contrast, there were only small effects

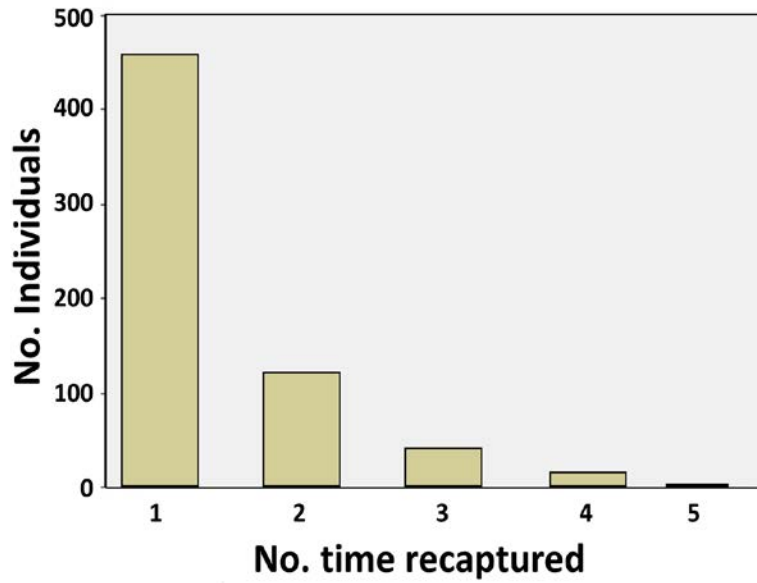


Fig. 16: Frequency distribution of number of recaptures of adult barn swallows.

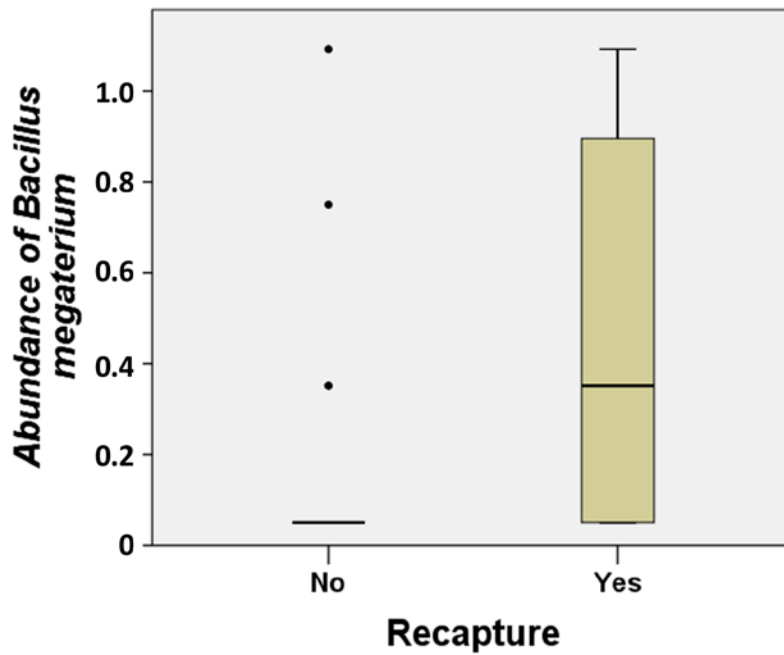


Fig. 17: Box plots of log-transformed abundance of *Bacillus megaterium* in relation to whether adult barn swallows were recaptured or not. Box plots show means, quartiles, 5- and 95-percentiles and extreme values.

for any of the other bacteria or fungi on recapture probability. *Bacillus megaterium* had a prevalence of 0.14 and a mean abundance of 1.36, SE = 0.42, range 0 to 10. Implying that barn swallow individuals with more *Bacillus megaterium* have more likely to be recaptures during the season.

Microorganisms and reproduction

Laying date was not predicted by the number of recaptures ($\chi^2 = 2.17$, df = 1, $P = 0.14$), but was predicted by the abundance of *B. megaterium* ($\chi^2 = 6.55$, df = 1, $P = 0.011$, estimate (SE) = -6.37 (2.40)). This effect increased when number of recaptures was deleted ($\chi^2 = 13.08$, df = 1, $P = 0.0003$, estimate (SE) = 23.13 (1.43)).

There was a significant relationship between the abundance of *B. megaterium* and laying date (Fig. 18; $\chi^2 = 6.59$, df = 1, $P = 0.010$, estimate (SE) = -0.20 (0.007)). The number of *B. megaterium* increased with the number of fledglings (Fig. 19; $\chi^2 = 10.19$, df = 1, $P = 0.0014$, estimate (SE) = 0.79 (0.024)). These findings may suggest that there is a trade-off between anti-bacterial defenses and investment in reproduction.

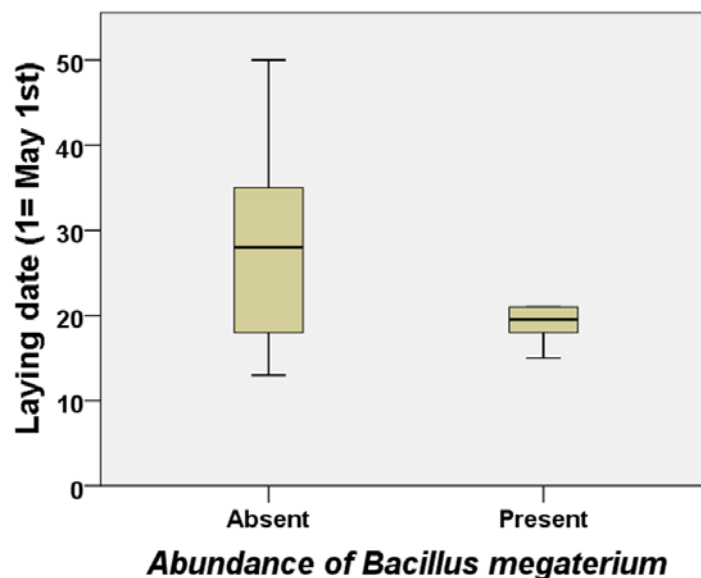


Fig. 18: Box plots of laying date (1=May 1st) in relation to whether abundance of *Bacillus megaterium* were present or absent. Box plots show means, quartiles, 5- and 95-percentiles and extreme values.

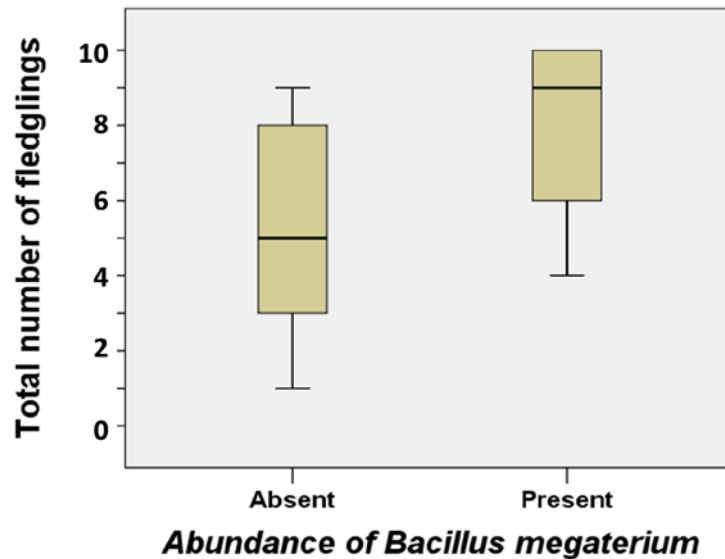


Fig. 19: Box plots of total number of fledgling in relation to whether abundance of *Bacillus megaterium* were present or absent. Box plots show means, quartiles, 5- and 95-percentiles and extreme values.

5. Microorganisms, flight ability and risk of predation

Communities of microorganisms

We isolated 27 fungal species from feathers of Goshawk prey and moulted feathers near goshawk nests according to identification by PCR technique (Fig. S8). The number of fungal colonies ranged from 0 to 9 with a mean of 2.563 (SE = 0.204), N = 87. The number of fungal species ranged from 0 to 3, mean = 1.195 (SE = 0.085), N = 87. Species richness for fungal species, means, SE and ranges of abundance of fungal species isolated are presented in Table S5.

Fungi and risk of falling prey to goshawks

Across all taxa of fungi, there was a significant positive relationship between the likelihood of birds being preyed upon and the mean number of fungi (Fig. 20; $\chi^2 = 6.18$, df = 1, p = 0.013, estimate (SE) = 2.65 (1.17)). In addition, there was an effect of prey species on the mean number of fungi ($\chi^2 = 16.58$, df = 3, p = 0.0009). Prey had almost 30% more fungal colonies on their feathers than non-prey that had molted their feathers in the same area and hence were still alive. The

interaction between species and mean number of fungi was not significant ($\chi^2 = 3.90$, $df = 3$, $p = 0.27$), and hence it was deleted from the model.

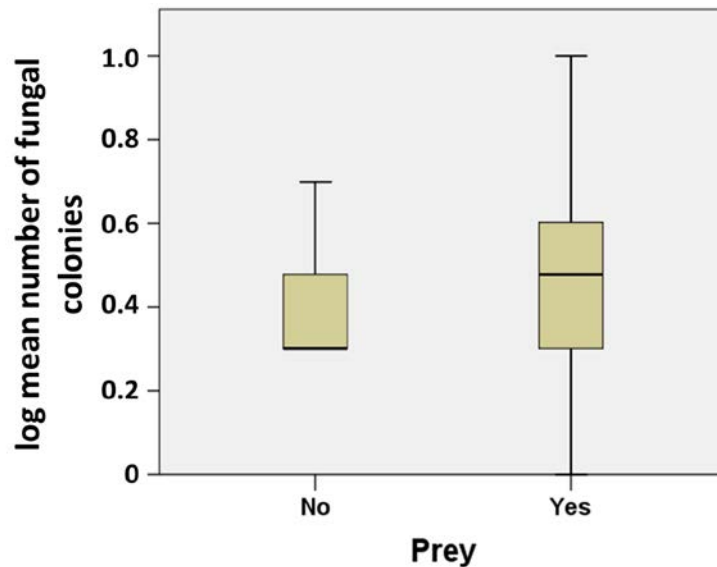


Fig. 20: Box plots of mean number of fungal colonies in relation to whether individuals were preyed upon or not. Box plots show means, quartiles, 5- and 95-percentiles and extreme values.

A GLM with binomial error distribution showed a relationship between the likelihood of birds being preyed upon and the number of colonies of *Aspergillus niger* on feathers (Fig. 21; $\chi^2 = 5.29$, $df = 1$, $p = 0.021$, estimate (SE) = 2.46 (1.20)), and there was a significant effect of prey species ($\chi^2 = 19.39$, $df = 3$, $p = 0.0002$).

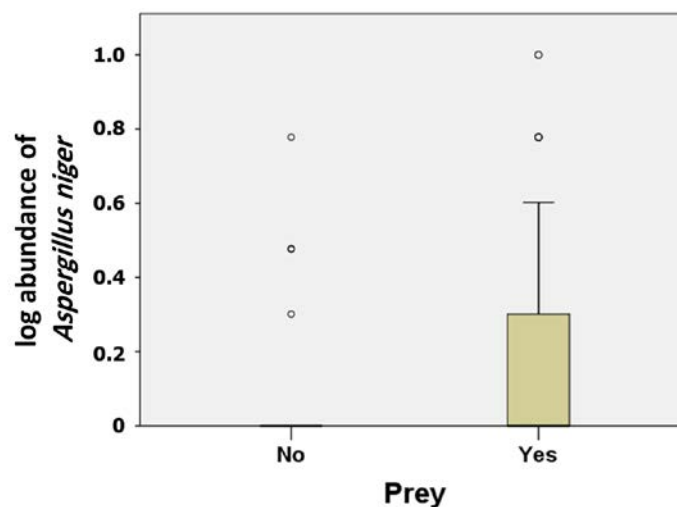


Fig. 21: Box plots of abundance of *Aspergillus niger* in relation to whether individuals were preyed upon or not. Box plots show means, quartiles, 5- and 95-percentiles and extreme values.

The interaction between species and the number of colonies of *Aspergillus niger* was not significant ($\chi^2 = 1.92$, $df = 3$, $p = 0.59$), and therefore it was deleted from the model.

Width of feather growth bars, fungi and species of goshawk prey

The mean width of daily growth bars in all individuals of different species ranged from 1.77 to 3.97 mm with a mean of 2.96 mm (SE = 0.05), N = 68. Birds with wide growth bars had a higher probability of falling prey to a goshawk. A GLM with binomial error distribution showed a significant difference in band width (daily growth increments) between prey and non-prey (Fig. 22; $\chi^2 = 5.73$, $df = 1$, $p = 0.017$, estimate (SE) = 9.43 (4.60)). Therefore, growth bars were wider in prey than in non-prey. In addition, there was a significant effect of species ($\chi^2 = 13.11$, $df = 3$, $p = 0.0044$). The interaction between species and width of growth bars was not significant ($\chi^2 = 1.91$, $df = 2$, $p = 0.59$), and it was therefore deleted from the model.

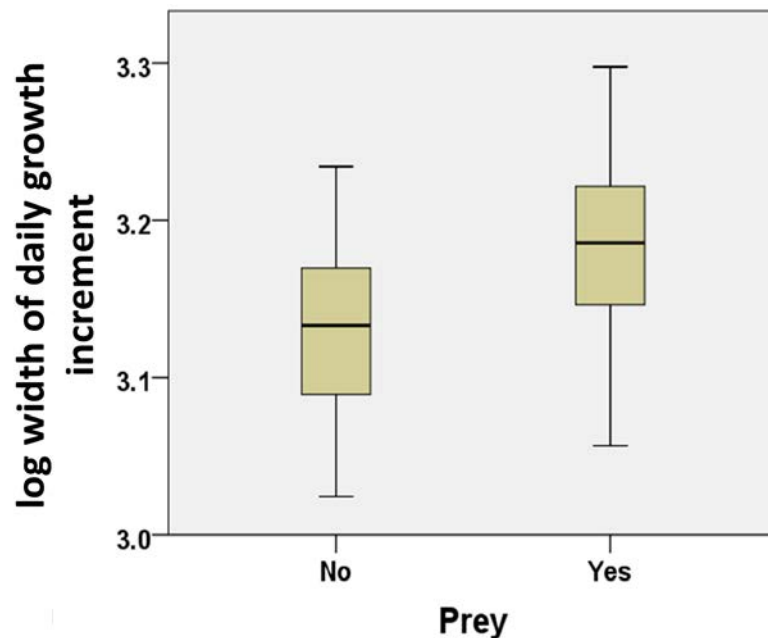


Fig. 22: Box plots of daily growth band width of feathers (mm) in relation to whether individuals were preyed upon or not. Box plots show medians, quartiles, 5- and 95-percentiles and extreme values.

A GLM with normal error distribution showed a significant difference in band width between fungal taxa (*Aspergillus fumigatus*, *Chaetomium elatum*, *Chaetomium globosum*, *Myceliophthora thermophila*, *Myceliophthora verrucos* and *Thermomyces lanuginosus*) (Table 5; $\chi^2= 30.62$, $df = 6$, $p < 0.0001$). The effect of prey species was not significant, and, therefore, it was excluded from the best model ($\chi^2 = 0.99$, $df = 3$, $p = 0.80$). We found an overall mean effect size weighted by sample size of 0.38, $SE = 0.03$, 95% confidence intervals 0.31 to 0.45, Wilcoxon signed rank test = 41718, $P < 0.0001$ (Table 5). This suggests that the mean weighted effect size for the relationship between the width of daily growth increments of feathers and the abundance of different fungal taxa is of an intermediate magnitude (Cohen 1988).

Table 5. Relationship between the width of daily growth increments of feathers (response variable) and the abundance of different fungal taxa (predictor variables). The GLM model with binomial error distribution had the statistics ($\chi^2 = 30.624$, $df = 6$, $P < 0.0001$). Effect size is Pearson's product moment correlation confession r and the z-transformation of r .

Term	Estimate	SE	χ^2	r	z	P
Intercept	2.46	0.01	509.31			<.0001
<i>Aspergillus fumigatus</i>	-0.28	0.09	9.70	0.38	0.40	0.0018
<i>Chaetomium elatum</i>	0.35	0.10	11.56	0.41	0.44	0.0007
<i>Chaetomium globosum</i>	-0.26	0.07	13.76	0.45	0.48	0.0002
<i>Myceliophthora thermophila</i>	0.10	0.04	6.52	0.31	0.32	0.0106
<i>Myceliophthora verrucos</i>	0.18	0.06	5.56	0.29	0.29	0.0184
<i>Thermomyces lanuginosus</i>	-0.46	0.16	7.94	0.34	0.36	0.0048

Damage to feathers and fungi

The probability of having damaged feathers increased with the number of fungal colonies (Fig. 4; $\chi^2 = 4.14$, $df = 1$, $p = 0.042$, estimate (SE) = 4.58 (2.72)). Two fungal taxa were positively correlated with the probability of having damaged feathers (*Myceliophthora verrucos*: $\chi^2 = 4.13$, $df = 1$, $p = 0.042$, estimate (SE) = 4.84 (3.44); *Schizophyllum* sp.: $\chi^2 = 4.13$, $df = 1$, $p = 0.042$, estimate (SE) = 5.62 (4.00)).

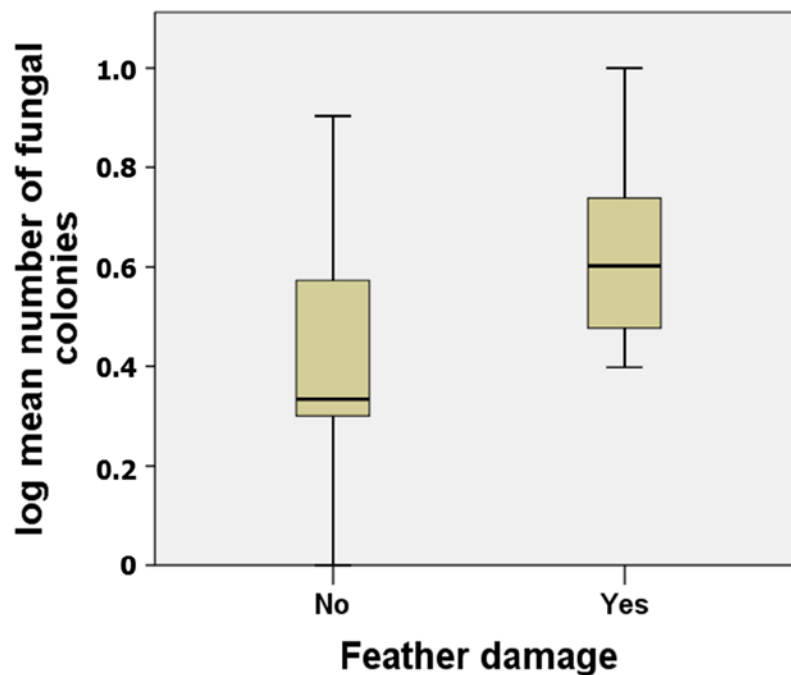


Fig. 23: Box plots of total number of fungal colonies in relation to damage of feathers.

Box plots show medians, quartiles, 5- and 95-percentiles and extreme values.

General discussion

Barn swallows are long-distance migratory birds, and as such we hypothesized that that variation in migration ability may be linked to the abundance or the diversity of microorganisms. Therefore, we predicted a negative relationship between the prevalence of feather degrading microorganisms and arrival date because these microorganisms can have severe negative effects on the quality of the plumage (Jacob and Ziswiler 1982). Colonial birds or hole nesters both facilitate direct transmission of microorganisms due to the presence of many different host individuals each with a non-negligible probability of being infected (Alexander 1974; Møller and Erritzøe 1996). Therefore, colonial and hole nesting bird species were expected to have higher levels of feather-degrading bacteria than solitary and open nesting species. This prediction was partly supported because the barn swallow is a semi-colonial bird that reuses nests for many years.

We found a positive relationship between arrival date and the mean number of bacterial colonies in TSA medium. This relationship may reflect that later laying takes place during warmer and more humid environmental conditions that favour growth and reproduction in microorganisms. Furthermore, we found a negative relationship between arrival date and both the mean number of bacterial colonies in FMA medium and the diversity of bacteria in FMA as reflected by the Simpson diversity index for bacteria. This relationship may relate to specific bacteria (*B. licheniformis*) and fungi (*Trichoderma reesei*) secreting antibiotic substances to inhibit growth of other microorganisms (see Chapter 1).

Pathogenic microorganisms are commonly identified from wild birds (Hubálek and Halouzka 1996; Hubálek 2004; Benskin 2009), although their consequences for the fitness of hosts remain poorly understood.

Obviously, feathers used in birds' nests have insulating properties that help and facilitate thermoregulation (Baicich and Harrison 2005). However, such feathers may also have negative impacts on their hosts because feathers are carriers of microorganisms that in some cases may be pathogenic. A common

reason for finding feathers in nature is that an individual bird has been killed by a predator, and such dead individuals may have fallen prey to predators because they are sick (Møller *et al.* 2015).

Arrival date and laying date were strongly positively correlated in the present study, implying that any relationship between microorganisms and laying date must be controlled for the potentially confounding effects of arrival date. We found a positive relationship between laying date and the diversity of microorganisms by using the Simpson diversity index for bacteria and fungi (Chapter 2). This relationship may reflect that later laying takes place during warmer and more humid environmental conditions that favour growth and reproduction for microorganisms. Hence it is beneficial for birds to reproduce early in spring as commonly shown for many different species (e.g. Lack 1954; Perrins 1975) in order to avoid the rapid growth and reproduction by microorganisms later during summer. Our findings that early laying date was associated with fewer microorganisms are consistent with this novel hypothesis. Several recent studies have also suggested that laying date and abundance of microorganisms are positively related (Møller *et al.* 2015; Soler *et al.* 2015). Individual birds that migrate faster and arrive earlier at the breeding grounds, which may reflect their prime condition, may harbour fewer microorganisms than individuals that arrive late (Macdonald *et al.* 2016; Langin *et al.* 2009; McKinnon *et al.* 2016). Such late arrivals may have more pathogenic microorganisms, but they may also compete more with conspecifics over access to feathers for nest lining material.

There are a number of alternative interpretations for the relationship between laying date and microorganisms. (1) Host individuals differing in abundance and diversity of microorganisms may breed at different times. It is possible that birds with few microorganisms and hence in better health breed earlier than others. (2) Later breeders may be exposed to warmer and more humid conditions that favour early start of reproduction. However, not all individuals may be able to start

reproduction early if they for example are in poor condition due to poor health. (3) There may be positive effects of antimicrobial activity on timing of reproduction by hosts. A number of studies have found that bird hosts harbour microorganisms with antimicrobial activity (Shawkey *et al.* 2008; Lee *et al.* 2014; Martín-Vivaldi *et al.* 2009). (4) The community of microorganisms on feathers will depend on the community of birds in the neighbourhood that have lost feathers and hence are potential recipients of nest lining material. This raises questions about the choice of breeding habitat to optimize the use of feathers as nest material, but also to minimize the risk of pathogenic infections.

Feathers could be a source of bacteria transferred from eggs to fledglings, and, therefore, this could increase the probability of colonization of fledglings by bacteria. Furthermore, some bacteria grow on feathers (i.e. keratinolytic bacteria) (Peralta-Sanchez *et al.* 2010). Parents can considerably affect the fitness of their offspring through the additive influences of inherited traits and parental effects (Rossiter 1996; Mousseau and Fox 1998). These latter, non-genetic factors may be adaptive phenotypic responses to environmental variation thereby increasing offspring growth and survival (Schwabl 1993, 1996; Jarvis *et al.* 1997; Mousseau and Fox 1998; Eising *et al.* 2001).

Feathers used to line nests may reduce the density of microorganisms capable of growing on egg-shells. Therefore, a larger number of feathers would contribute to a more sterile nest environment. However, occasionally pathogenic bacteria are found growing on feathers (Shawkey *et al.* 2003; Gunderson 2008). Moreover, birds prevent nest feather contamination by microorganisms from preening (Shawkey *et al.* 2003; Soler *et al.* 2008). However, nest lining feathers are unprotected, which predicts an even larger bacterial load of nest lining feathers in comparison with active feathers present on birds.

In Chapter 3, we found a positive relationship between the total number of fledglings and the abundance of bacteria and fungi. The abundance of two fungal taxa (*Absidia corymbifera* and *Penicillium glabrum*) and three bacterial taxa

(*Bacillus pumilus*, *Bacillus rhizosphaerae* and *Streptomyces cacaoi*) was positively related to the total number of fledglings produced during a single breeding season, and that may be due to these fungal taxa producing antimicrobial substances that affect other microorganisms (Peralta-Sanchez *et al.* 2010).

This relationship between the abundance of microorganisms and the total number of fledglings may reflect that eggs hatch during warmer and more humid environmental conditions that favour growth and reproduction of microorganisms. In addition, rapid growth and reproduction by microorganisms in late summer may cause accumulation of more microorganisms in the nest. While we found a negative relationship between the total number of fledglings and the abundance of *Trichoderma reesei*, the abundance of this species may be inhibited by antimicrobial substances secreted by other species in the microbial community of birds' nests. Also, the nest is considered a suitable environment for the growth of different microorganism taxa, and hence competition between microorganisms, which mainly depends on the secretion of antimicrobial substance, would impede the establishment of virulent microorganisms. We found positive relationships between the total number of fledglings and antimicrobial activity of *Aspergillus fumigatus* in SDA medium, *Streptomyces cacaoi* in FMA medium, *Bacillus pumilus* in TSA medium and *Bacillus rhizosphaerae* in TSA medium.

The frequency of recaptures during the same season was highly skewed with most barn swallows never being recaptured again. Here we have shown that microorganisms seem to play a role in whether individuals are recaptured. Because the abundance of microorganisms was associated with an increase in the probability of recapture, we can consider the effects reported here to be parasitic. Is it feasible that recaptures directly affected the abundance of microorganisms? Perhaps both recapture and microorganisms were associated with a third variable. However, we cannot see any mechanism by which such an effect could arise.

The abundance of *B. megaterium* was associated with an advance in laying date and an increase in reproductive success. These effects were independent of the number of recaptures. We hypothesize that barn swallows trade early and heavy investment in reproduction at the expense of anti-bacterial defences. This interpretation is supported by the fact that there was no direct correlation between laying date and reproductive success on one hand and the number of recaptures on the other.

Barn swallows prefer white feathers over other colours for lining their nests (Peralta-Sanchez *et al.* 2010, 2011, 2014). Feathers with such white coloration have positive effects on the bacterial community by improving hatching success in the host (Peralta-Sanchez *et al.* 2010, 2011, 2014). Most bacteria living on feathers are benign and effectively prevent pathogenic bacteria from establishment and replication (e.g. Shawkey, Pillai, and Hill 2003, 2009). The frequency of recaptures during the same season was highly skewed with most barn swallows never being recaptured again and the frequency of recaptures decreasing strongly. In Chapter 4, we have shown that the probability of recapture of adult barn swallows was significantly correlated with the abundance of specific bacteria, in particular *Bacillus megaterium*. The findings reported here have potential future prospects.

First, the findings suggest that barn swallows are more likely to be recaptured and, by inference, more likely to fall prey to a predator when having a larger abundance of specific microorganisms in their nests. We are unaware of any alternative explanation for the patterns described in the present paper. Obviously, this hypothesis requires a direct test by comparison of the abundance of these specific microorganisms in conspecific prey and non-prey. It also requires a direct experimental test of whether elimination of these microorganisms reduces the probability of recapture.

Second, studies of demography of wild organisms rely on random sampling of individuals in populations in order to obtain unbiased estimates of

demographic parameter based on capture-mark-recapture analyses (Lebreton *et al.* 1992). Several recent studies have demonstrated violations of this assumption (Garamszegi 2009; Biro and Dingemanse 2009; Møller 2010). For example, Garamszegi *et al.* (2009) showed for collared flycatchers *Ficedula albicollis* that individuals with a bold personality were over-represented, while shy individuals were less likely to be captured than expected by chance. The associations between capture probability and microorganisms that we have reported here may suggest that the microbiome plays a direct role in the estimation of demographic parameters. This interpretation is supported by human studies linking personality to the gut microbiome (Forsythe *et al.* 2010; Foster and Neufeld 2013).

Feathers of wood pigeons, jays, song thrushes and blackbirds differ in abundance of fungi between prey and live individuals that moulted feathers in the same area (Chapter 5). These findings are consistent with a previous study by Møller *et al.* (2012) showing that goshawks are differentially successful in their capture of prey when prey individuals harbour a large abundance of fungi on their plumage.

Microorganisms of feathers are generally thought not to be harmful (Gunderson 2008, Shawkey *et al.* 2009), although they can cause breakage of feather barbs. Hence, the abundance of microorganisms on feathers is probably a risk factor associated with probability of predation. In wild birds, pathogenic microorganisms are common (Hubalek and Halouzka 1996; Hubalek 2004). Thus, predators should differentially capture prey infected by pathogenic microorganisms because that will determine whether an individual survives a predatory pursuit. Here, we found that the abundance of fungi on feathers was elevated in prey of goshawks compared to non-prey.

There was a large difference in abundance of *Aspergillus niger* between prey and non-prey. *A. niger* is a filamentous fungus that is regarded as one of the most important industrial microorganisms that produces many enzymes such as amylases (Mitidieri *et al.* 2006), cellulase and xylanase (Couri *et al.* 2000, Farinas

et al. 2010), peptidases (Morya *et al.* 2012) and phytases (Bhavsar *et al.* 2011). For several decades, enzymes from *Aspergillus niger* have been used in food production, and there are a few reports of production of keratinase by *Aspergillus niger* strains (Lopes *et al.* 2008). Hence, we hypothesized that *Aspergillus niger* may affect feathers of goshawk prey by reducing their flight efficiency. Many microorganisms have a negative impact by altered feather integrity due to degradation of feather barb keratin. That is also the case for keratinolytic fungi, which may lead to reduced fitness of their bird hosts by reducing thermoregulation and flight maneuverability making such individuals more likely to be depredated (Swaddle *et al.* 1996; Clayton 1999; Shawkey *et al.* 2007; Scott and McFarland 2010).

Feather quality can affect individual fitness in terms of mate choice, late arrival from migration, delayed timing of reproduction during the breeding season and escape from predators (Hedenström 2003; Kose and Møller 1999; Pap *et al.* 2005). The rate of feather growth and feather quality can be affected by many factors like nutritional status, physiological stress, body condition and disease (DesRochers *et al.* 2009; Moreno-Rueda 2010; Vágási *et al.* 2012). Here, we found a positive relationship between the rate of feather growth and the risk of falling prey to a common raptor. These costly effects of rapid moult are condition-dependent, so that only birds in prime condition could make a fast moult without compromising their feather quality (Vágási *et al.* 2012). Here, we found a significant negative relationship between the width of daily growth increments in feathers and abundance of fungal species. In an experimental study on barn swallow offspring Romano *et al.* (2011) showed that individuals with parasite infection produced feathers of lower quality. Since many fungal species are pathogenetic, we can suggest that the fungal infection in birds during feather growth may cause variation in feather quality.

Feather damage could be used as an indicator of feather quality, since we found a positive relationship between the degree of feather damage and the

number of fungal colonies. Among 27 fungal species 14 (52%) secrete keratinase and hence have the ability to degrade feathers (Table S6a). Furthermore, there were 16 (59%) pathogenic fungal species (Table S6b) that can elicit an immune response, and this is costly in terms of energy requirements, thereby reducing feather quality.

The results reported here require experimental manipulation of the abundance of fungi in feathers for a formal verification. This could be done by use of antimicrobial substances on adult feathers. Another possibility is to treat adult feathers with antimicrobial agents to terminate the negative effect of feather-degrading fungi.

Prospects

Microbial communities on feathers have recently received attention by many scientists (Gunderson 2008; Møller *et al.* 2012) because of their effect on the efficiency of flight, and because they use nest lining material that may influence the bacterial environment in their nests (Soler *et al.* 2010; Peralta-Sánchez *et al.* 2010). Some bacteria have the ability to growing on feathers and they are known as feather-degrading bacteria (Williams *et al.* 1990; Bisson *et al.* 2009). These bacteria could affect sexually selected feather coloration, due to the relationship between the density of keratinolytic bacteria on feathers and brightness of feather colors (Shawkey *et al.* 2007, 2009). In addition, the coloration of feathers could affect the bacterial community because black and white feathers are degraded differentially by *B. licheniformis* (Gunderson *et al.* 2009; Grande *et al.* 2004).

Competition among bacteria may change the efficiency of antimicrobials (Brook 1999; Becker *et al.* 2012). Because white feathers lack melanin *B. licheniformis* grows better on white than on melanized feathers (Gunderson *et al.* 2008; Goldstein *et al.* 2004). Therefore, we expect that antimicrobials from bacteria in high-density communities on white feathers would be more efficient than antimicrobials from bacteria with lower-density communities on black

feathers (Soler *et al.* 2010). White feathers used to line nests could reduce the bacterial infection of nestlings, explaining why barn swallows favour white feathers as nest lining material (Peralta-Sánchez *et al.* 2011). Previous experimental studies (Peralta-Sánchez *et al.* 2011, 2010) showed that nests lined with dark feathers when replaced by white feathers had lower bacterial densities than those with coloured feathers. These effects could be mediated by higher bacterial densities on white feathers, and/or by antimicrobials from white feathers being more efficient than those from bacteria on coloured feathers.

The results reported here require tests based on experimental manipulation of the abundance of microorganisms in nests and on adults. This could be done by use of antimicrobial substances, or indirectly by manipulating the composition of the colour of nest lining feathers (Peralta-Sanchez *et al.* 2011, 2014). The hypothesis that antimicrobials from keratinolytic bacteria may affect the bacterial environment of nests, therefore, predicts that bacteria on white feathers from the nest lining will be more efficient against other feather-degrading bacteria than those from coloured feathers.

Finally, the hypothesis that the increase in interference and competition would lead to the production of antimicrobial substances. In turn, that would affect the bacterial community in nests, and, therefore, decrease bacterial load in feathers lining the nest.

Conclusions

In conclusion, we found that the effects of microorganisms on the behaviour of birds may be a combination of positive and negative effects. There may be positive effects of antimicrobial activity on birds through the process of bacterial interference, consisting of certain bacteria impeding the establishment of competing bacterial strains by producing antibiotic substances. Meanwhile, the negative effects may imply that pathogenic or/and feather-degrading microorganisms may reduce fitness components of their hosts. These effects of

microorganisms and hence the microbiome can be affected by behaviour of bird hosts.

1. There was a significant correlation between arrival date of adult barn swallows and the abundance of specific bacteria and fungi found on nest lining feathers even though these feathers are not encountered until after migration and arrival. If late arrival of some individual birds enhances the ability to modify the abundance of microorganisms, we can conclude that microorganisms could affect the behaviour of birds.

2. There was a significant correlation between laying date of adult barn swallows and the abundance of specific bacteria. Furthermore, we found a significant relationship between laying date and Simpson's diversity index for bacterial and fungal species. In contrast, we did not find a significant relationship between laying date and the abundance of fungal species. Furthermore, there was no significant relationship between laying date and the age of individual hosts. If the probability of late laying by barn swallows reflects the ability of an individual to modify the abundance of microorganisms in nests, we can conclude that microorganisms can be affected by laying date and hence the behaviour of birds.

3. We have shown that the total number of fledglings (controlled for the potentially confounding statistical effects of the number of eggs) of barn swallows was significantly correlated with the abundance of specific bacteria and fungi species. If the number of fledglings is affected by the abundance of microorganisms in the nest environment, we can conclude that the microorganisms may play an important role for adult birds and their offspring.

4. The probability of recapture of adult barn swallows was significantly correlated with the abundance of specific bacteria, in particular *Bacillus*

megaterium. In contrast, we did not find such a relationship in other taxa of bacteria and fungi. Capture of an individual barn swallow multiple times may reflect the probability of falling prey to a predator, implying that bacteria play an important role in predator-prey interactions.

5. We have shown that the probability of individual birds falling prey to a predator increased with the mean number of fungal colonies on feathers. In addition, we found a significant relationship between the risk of predation and the abundance of *Aspergillus niger*. Moreover, the probability of individuals falling prey to a predator was significantly positively correlated with the width of the daily growth bars of feathers from goshawk prey. Finally, we found a positive relationship between damage of feathers and the number of fungal colonies. The abundance of two fungal species (*Myceliophthora verrucos* and *Schizophyllum* sp.) was significantly correlated with damage of feathers. Hence, we conclude that the abundance of fungi on the feathers of goshawk prey and hence their microbiome is involved in predator-prey interactions.

Author contribution

APM collected the feathers samples. ZAR conducted all lab analysis in collaboration with HAM. The statistical analyses were made by ZAR and APM. ZAR wrote the text with input from APM.

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Chapter 1:

Arrival date and microorganisms in barn swallows

Zaid Al Rubaiee, Haider Al Murayati and Anders Pape Møller

*Ecologie Systématique Evolution, Université Paris-Sud, CNRS,
AgroParisTech, Université Paris-Saclay, F-91405 Orsay Cedex, France*

Word count: 6814

Correspondence to ZAR:

Tel: (+33) 1 69 15 56 96

Fax: (+33) 1 69 15 56 96

E-mail: zaid.iraq@gmail.com

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Abstract

Migration between breeding sites and winter quarters constitute a major life history strategy in birds. The benefits of such migrations must at least equal the costs for such behavior to evolve and be maintained. We tested whether there was a relationship between abundance and diversity of microorganisms on feathers and timing of arrival by barn swallows *Hirundo rustica*. Nest lining feathers are chosen and transported by adult barn swallows to their nests just before and during egg laying, implying that any heterogeneity in abundance and diversity of microorganisms on feathers in nests must arise from feather preferences and microorganisms on the body of these individuals. There was a negative relationship between arrival date and the total number of fledglings showing that early arrival is advantageous. The arrival date of adult barn swallows was significantly positively correlated with the abundance of specific bacteria (*Bacillus licheniformis*) and positively correlated with the fungus *Trichoderma reesei* and negatively correlated with the fungus *Mucor circinelloides*. Moreover, we found a significant positive relationship between arrival date and mean total number of bacterial colonies in TSA medium. Meanwhile, we found a significant negative relationship between arrival date and mean total number of bacterial colonies in FMA medium, and Simpson's diversity index of the abundance of bacteria in FMA medium. In contrast, there was no significant relationship between arrival date and the age of individual hosts. These findings are consistent with the hypothesis that early arriving barn swallows differ in abundance and diversity of microorganisms from late arriving conspecifics, and that they choose feathers for their nests that differ in terms of microorganisms from those chosen by late arrival individuals.

Keywords: arrival date, barn swallow, birds feather, microorganisms, migration

Introduction

Migration has evolved as a consequence of intraspecific competition arising when the benefits of staying in areas with more competitors exceed the costs of migration (Alerstam and Högstedt 1982). Long distance migration has evolved gradually from such resident populations eventually resulting in migrations that cover tens of thousands of kilometres. Migration has evolved independently in numerous taxa such as mammals, birds, fish, reptiles, insects, amphibians and crustaceans (Dingle and Drake 2007). Migration is by definition costly in terms of time and energy, and individuals in prime condition generally migrate faster than others with individuals in the best condition arriving first to the breeding grounds (e.g. Flood 1984; Francis and Cooke 1986; Lozano *et al.* 1996). The benefits of such early arrival are access to nest sites and territories of superior quality providing early arriving individuals of the sex competing the most intensely for mating success, usually males, with a fitness advantage (Enstrom 1992; Lundberg and Alatalo 1992; Lozano *et al.* 1996). Early arrival allows for early start of reproduction and hence larger seasonal reproductive success and an earlier termination of reproduction (e.g. Daan *et al.* 1990; Verhulst and Tinbergen 1991; Verboven and Visser 1998). Competition for early arrival is stronger in species with greater variance in mating success (e.g. Hasselquist 1998), with males of the highest phenotypic quality arriving first (Møller 1994a; Rätti *et al.* 1993; Marra *et al.* 1998; Møller and de Lope 1999). The extent of such condition-dependent migration is revealed by early arriving individuals having superior survival prospects despite the costs of early arrival, and the costs of early arrival are commonly larger for individuals in poorer condition (Møller 1994a; Kokko 1999).

Bird feathers are known to be carriers of microorganisms, which are capable of infecting animals (Camin *et al.* 1998; Anbu *et al.* 2004). Birds and their environments are regarded as large reservoirs for the occurrence and growth of keratin-degrading microorganisms (Mandeel *et al.* 2009; Deshmukh 2004).

Colonisation of keratin by saprophytes is uncommon as is competition for access to keratin (Parihar and Kushwaha 2000). Keratinophilic microorganisms can tolerate temperatures above that of bird skin (40–42°C) (Kaul and Sumbali 1999). Keratinase enzymes that degrade the keratin of the plumage of birds are produced by many microorganisms (Onifade *et al.* 1998). Generally, birds frequently carry keratinophilic microorganisms passively on intact feathers. Some of these keratinophilic microorganisms are known pathogenic dermatophytes that can lead to superficial skin infection (dermatophytoses) of keratinised tissues (Deshmukh 2004; Mbata *et al.* 2005).

Any damage to the plumage of migratory species that reduces the efficiency of flight would be costly and hence selected against, and damage to the plumage may reach such extremes as complete degradation and hence disappearance of barbules, barbs or even loss of entire segments of feathers (Møller and Erritzøe 1998). Migration between breeding sites and winter quarters constitutes a major life history strategy in birds. Long-distance migration is also costly because of risks of predation and parasitism during migration and at stopover sites (Alerstam and Lindström 1990; Møller and Szép 2011).

An important cost of migration is the increased exposure to microorganisms of different kinds during the annual cycle due to encounters with different environments at different times of the year. Thus, migrants encounter more different strains of parasites including microorganisms than residents (Møller 1998), and this should select for extraordinary defences against such pathogenic diversity (Møller 1998), but also for development of defences against strains of parasites encountered throughout the annual life cycle of hosts (Møller and Erritzøe 2000; Møller and Szép 2011). Migratory birds contain a variety of keratinophilic microorganisms that may be considered potential sources of transfer of microorganisms from one location to another (Deshmukh 2004). An unnoticed, but potentially virulent group of microorganisms is feather-degrading

fungi and bacteria that can damage the flying apparatus of birds by degrading the structure of the feathers on which flight and hence migration relies.

Many strains of microorganisms degrade feathers such as *Bacillus licheniformis*, *Bacillus pumilis*, *Streptomyces fradiae*, *S. pactum* and various fungi (Williams *et al.* 1990; Burt and Ichida 1999; Pugh 1964, 1965; Hubálek 1976, 1978; Kunert 1989). Feather-degradation occurs by the edges of feathers being dented, feather barbules missing or losing function, or feather barbs becoming detached leaving holes that can reach the size of more than one square-centimetre in a feather (Noval and Nickerson 1959; Böckle *et al.* 1995). Such damage to the plumage provides individual hosts that are able to control the abundance and diversity of microorganisms with a considerable advantage. Why some birds have such feather-degrading bacteria on their plumage while others do not is thus a puzzle given the benefits arising from their absence. If such microorganisms are acquired passively as a consequence of foraging habits, there should be selection against such costly foraging behaviour. *Bacillus licheniformis* degrades feathers, but also produces antimicrobial substances in the form of biocins (Haavik 1974; Simlot *et al.* 1972) that can affect bacteria such as *Bacillus*, *Micobacterium*, *Enterococcus* and *Corynebacterium* (Galvez *et al.* 1994) and fungi (Lebbadi *et al.* 1994; Patel *et al.* 2004). The antimicrobial properties of such substances produced by *Bacillus licheniformis* may inhibit the growth of other more virulent bacteria. Such symbionts compete through interference with pathogenic microorganisms (Brook 1999), preventing these from becoming established (Riley and Wertz 2002), or modifying their environment for symbiotic bacteria (for example Hackstein *et al.* 1996). In other words, feather-degrading bacteria are not necessarily only costly, but may provide benefits in terms of protection against pathogenic, but competitively inferior strains of microorganisms.

The objective of this study was to investigate the relationship between arrival date and abundance and diversity of microorganisms in a long-distance migratory bird. Specifically, we predicted that individuals that migrate the fastest

and hence arrive the earliest at the breeding grounds would have fewer microorganisms including bacteria and fungi in their plumage than late arriving individuals, independent of potentially confounding effects of age. We also predicted that early arriving migrants would have more biocin-producing microorganisms than late arriving individuals. We tested these predictions in the barn swallow *Hirundo rustica* for which we have collected information on microorganisms on nest lining feathers and arrival date at the breeding grounds.

Materials and methods

Study organisms

Barn swallows (*Hirundo rustica*) are socially monogamous, with pairs often remaining together for subsequent broods and in successive years if still alive. The breeding season lasts from May to August, and pairs usually raise two broods per summer (Turner 1994).

The nest is built by both sexes from mud lined with grass and feathers. The female lays 3-7 eggs and incubate these for two weeks, followed by a nestling period of three weeks (McWilliams 2000; Brown and Brown 1999; Perrins 1989; Terres 1980).

Study site and field work

The samples of nest lining feathers from barn swallow nests were collected by Anders Pape Møller during May-June 2014 at Kraghede (57°12'N, 10°00'E), Denmark, in a population that has been followed since 1971 (Møller 1994a). Feathers were collected using sterile gloves, before being placed in sterile zip-lock bags and kept in a refrigerator until analyses in the lab. Adults were captured weekly (at least twice) from the beginning of the breeding season. Arrival date was defined as the date of the first capture, although this will provide an underestimate of the true arrival date (Møller *et al.* 2004). Moreover, reproductive success was recorded as the total number of fledglings produced by each adult

(Møller *et al.* 2004). Feather samples were collected by using sterile gloves, deposited in polythene bags and stored in a refrigerator until analysis.

Analyses of microorganisms

We isolated and identified the bacterial and fungal species from the samples of feather lining in barn swallow nests and quantified their abundance.

Microorganisms isolation

Bacterial isolation

In the laboratory, by using sterilized tweezers, each piece of feather was introduced in a 15-ml falcon tube with sterile phosphate buffer saline (pH 7.2) with a volume proportional to the length of the feather. This was followed by three shaking periods by vortex 10 s each. Free-living bacteria were washed out from the feathers and collected in PBS solution (Saag *et al.* 2011), and then performing serial tenfold dilution to 10^{-4} . To quantify cultivable and feather-degrading bacteria, duplicates were made by spreading 100 μ l of supernatant with a sterile spreader loop on two different growth media:

Tryptic soy agar (TSA) is a rich medium in which heterotrophic bacteria can grow, thus enabling assessment of total cultivable microorganism load of the feathers.

Feather meal agar (FMA) is a medium highly selective for keratinolytic bacteria because the only source of carbon and nitrogen is keratin. Hence, only bacteria that are able to digest this medium can proliferate, and that allows quantification of feather-degrading bacterial load.

Preparation feather meal agar

We washed downy feathers extensively with warm water and detergent more than three times to remove all dirt and dust particle, and to ensure elimination of all remnant preen wax on the feathers were washed with a chloroform: methanol

mixture (1:1, v/v), and washed again with warm tap water to get rid of remnant chloroform and methanol, then dried in a hot air oven at 50°C overnight. The dried feathers were milled by using a Mixer Mill (Retsch MM-301) and with the aid of three iron balls for 50 s / 30 Hz. Finally, the milled feather was sterilized by autoclaving at 121°C for 15 min (Vidal *et al.* 2000).

Plates were incubated at 28°C for 14 days. After incubation, we distinguished color, shape, size, and presence or absence of glutinous aspects of colonies. The total bacterial densities were calculated by multiplying the average number of CFU by the dilution factor and by the original sample volume (Peralta-Sánchez *et al.* 2014). Plates with FMA media were incubated in order to detect any contamination of media (negative controls).

Fungal isolation

The feathers were snipped into small pieces and cultured directly onto Mycobiotic Agar (which is a selective medium for isolation pathogenic fungi) following standard procedure (Deshmukh 2004), and moistened with 0.5 ml of sterilized PBS. The cultures were incubated at 28°C ± 2 and examined daily from the third day for fungal growth over a period of four weeks. The observed developing mycotic growths, under stereoscopic binocular microscope, were individually and directly transferred onto Sabouraud dextrose agar with chloramphenicol (50 mg/l). The resulting products were further incubated at 28°C ± 2 for two weeks to obtain pure isolates for identification purposes.

Feather meal agar preparation

Downy feathers were extensively washed with warm water and detergent more than three times to eliminate all dirt and dust particles, and to ensure elimination of all remnant preen wax the feathers were washed with a chloroform: methanol mixture (1:1, v/v), washed again with warm tap water to get rid of remnant chloroform and methanol, and then dried in a hot air oven at 50°C overnight). The

dried feathers were ground up by using a Mixer Mill (Retsch MM-301) and a tungsten carbide vial with the aid of three balls, milling the feather for 50 secs / 30 Hz. Finally, the milled feather was sterilized by autoclaving at 121°C for 15 min (Vidal *et al.* 2000).

Plates were incubated at 28°C for three days in the case of TSA, and for 14 days in the case of FMA. After incubation, we counted the number of colony-forming units (CFU) of each morph type per plate to estimate densities of viable bacteria in the sample. We distinguished morph types on the basis of colony color, shape, size, and presence or absence of glutinous properties. The total bacterial densities were calculated by multiplying the average number of CFU by the dilution factor and by the original sample volume (Peralta-Sánchez *et al.* 2014). Plates with each medium type, but without microbial suspension were incubated in order to detect any contamination of media (negative controls).

Isolate preservation

Slants of nutrient agar and 40% glycerol stocks were prepared from identified pure culture and stored at 4 and -80°C, respectively, for medium and long-term preservation.

Microorganism identification

Bacterial identification

The bacterial identification was carried out by using molecular characterization with the aid of polymerase chain reaction (PCR) for further genetic analyses.

DNA extraction

Genomic DNA was extracted from each isolate by using two different protocols.

Freeze-thaw protocol: With a wooden toothpick transfer we squashed part of a bacterial colony in a 0.5 ml Eppendorf tube containing 50 µl Tris (10 µM, pH

8.0). We started freezing and thawing by putting the Eppendorf tube in liquid nitrogen for one to two minutes until completely frozen, then quickly placing the tube in a hot water bath until completely thawed. This process is known as a freeze-thaw cycle, and we repeated the same process for a total of three cycles. We made sure that we mixed the sample tube between each cycle. To ensure complete cell lysis we put the tubes in a microwave at 270W for 5 to 6 s followed by 10 s of waiting, repeated three times. We centrifuged the sample tube for five minutes at 12,000 x g in a micro-centrifuge. Using a micropipette, we transferred the supernatant that contains the DNA into a new clean, sterile micro-centrifuge tube and discarded the pellet that contains cellular debris. We stored the tubes in -20°C for the Polymerase Chain Reaction (PCR).

DNA Extraction kit: We used the Power Soil DNA isolation kit (MOBIO Laboratories, Inc. USA) to extract DNA according to the protocol that is supplied with the kit.

PCR amplification of bacterial 16SrRNA gene

DNA isolated from samples was used as a template for PCR to amplify the bacterial 16S rRNA gene by using the forward primer 16S rDNA-27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer 16S rDNA-1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was carried out in a total volume of 25 µl containing 16 µl ultrapure water, 4 µl 5x buffer, 0.4 µl dNTPs 10nM, 1 µl 10 µM 27F, 1 µl 10 µM 1492R, 0.2 µl Go Taq DNA polymerase and 3-5 µl genomic DNA. The PCR conditions consisted of an initial denaturation at 94°C for 5 min, denaturation at 94°C for 15 s., annealing for 30 s at 55°C and 1.30 min of primer extension at 72°C. The cycle of denaturation, annealing and elongation was repeated 35 times. A final elongation at 72°C for 7 min was then performed. The PCR products were sent to sequencing by Beckman Coulter Genomics.

Fungal identification

The identification of fungi was done by using two protocols: The traditional fungal identification methods such as morphological characteristics, culturing on various media, presence of microconidia and chlamydospores and biochemical analysis such as pigment production have been used routinely, and still several new species are described on this basis (Zhao and Wu 2007).

For molecular identification of fungi, we took a small amount of mycelium suspended in 200 μ L 10 mM Tris-HCl (pH 8.0) in an Eppendorf tube (1.5 mL), and stored in a freezer (-20°C) for further processing. The genomic DNA was isolated from the fungal strains by using the PowerSoil® DNA Isolation Kit (MO BIO). DNA was eluted in a final volume of 100 μ l of 10 mM Tris-HCl, pH 8.5.

Amplification of fungal 18s rDNA gene by PCR

PCR amplification was performed in 20-30 μ L reaction volume containing 25 μ l assay buffer containing 1.5 mM MgCl₂, dNTP (10mM) 0.5-1 μ L, 0.5-1 μ L of each 0.2 mM primer FR1 (5'-'CTCTCAATCTGTCAATCCTTATT-3'), 2.5 μ l forward primer UF1 (5'-CGA ATC GCA TGG CCT TG-3'), Go Taq® G2 DNA polymerase (Promega Madison, WI USA) (1.25 μ l) 0.5-1 μ L, DNA sample 3-5 μ L. The DNA genomic was amplified with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation for 15 s at 94°C, annealing for 30 s at 55°C and extension for 1.30 min at 72°C, respectively and the final extension was carried out at 72°C for 7 min.

Agarose electrophoresis

To visualize and determine the presence or absence of PCR products and to quantify the size of amplified DNA fragments. We performed a gel electrophoresis in 1% agarose using 0.5 X TAE buffer (Tris-Acetate-EDTA) for 25 min at 100V. The gel was then stained with Gel Red (BIOTIUM) for 30 min.

Images were taken under a UV lamp by using the photo documentation system IP-010.SD.

DNA sequencing

PCR products were sent for sequencing in to (Beckman Coulter Genomics, Takeley, Essex CM22 6TA, United Kingdom) for DNA Sequencing.

The sequence results were processed by using the web-based blasting program, basic local alignment search tool (BLAST), at the NCBI site (<http://www.ncbi.nlm.nih.gov/BLAST>), and the data were compared with the NCBI/Gene-bank database.

Antimicrobial activity assay

Fungal and bacterial species isolated from lining feathers of barn swallow nests were subject to antimicrobial activity assays against standard bacterial strains of *Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* JM 83, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Micrococcus luteus* and *Agrobacterium tumefaciens* in two different ways.

For bacterial strains, we used the streak-planting technique on Mueller Hinton agar. Each isolate was streaked on agar plates in a central single line, and the plates then incubated at 30°C for 7 days in order to give the bacteria opportunity to secrete antibiotic substances on the medium. After incubation, standard bacteria were grown in Mueller Hinton medium for 18 h and properly diluted to make cross-streaks along the line of fully grown isolates. Each streaking was started near the edge of the plates and streaked towards the central bacterial growth line. The plates were then incubated for 18 h at 37°C, and the zone of inhibition was measured using a millimeter scale (Shams *et al.* 2015).

For fungal isolates, we cultured each species in Sabouraud dextrose broth medium for 1-2 weeks at 30°C. The culture medium was centrifuged at 5000 g

for 10 min to remove all fungal cell, and by using a Millipore filter (0.2 μm) the supernatant was collected in a sterile falcon tube.

The antimicrobial activity was tested by well-diffusion methods. After 24-48 h of incubation, the inhibitory zones (in mm) were measured (Moshafi *et al.* 2006). Three commercial antibiotic discs Vancomycin (VA 30 mcg), Tetracycline (TE 30 mcg) and Chloramphenicol (C 30 mcg) were used as positive controls.

Statistical analyses

All statistical analyses were made with JMP (2012). We report total abundance of colonies and species richness for fungi and bacteria. We tested in a generalized linear model whether date of arrival followed a Poisson distribution by fitting a Poisson model with a log link function to the frequency data. We calculated the Simpson's index (a measure of diversity independent of sample size) to determine the diversity of microorganisms in feathers lining the nests, which takes into account the number of species present, as species richness and evenness increase, so does diversity (Magurran 2004). We tested whether the abundance of microorganisms predicted arrival date using a generalized linear model.

We estimated effect sizes by using Cohen's (1988) guidelines for the magnitude of effects being small (Pearson $r = 0.10$, explaining 1% of the variance), intermediate ($r = 0.30$, explaining 9% of the variance) or large ($r = 0.50$, explaining 25% of the variance).

Results

Arrival date, age, feather damage and reproductive success

Age was on average 1.39 years (SE = 0.11), range 1-4 years, N = 54. Arrival date was not significantly related to age ($F = 1.55$, $df = 1, 52$, $r^2 = 0.01$, $P = 0.22$). The total number of fledglings for the season was significantly negatively related to arrival date ($F = 11.377$, $df = 5, 53$, $r^2 = 0.54$, $P < 0.0001$). Thus, early arriving barn swallows had larger reproductive success.

Barn swallows with damaged feathers arrived almost one week later than those with undamaged feathers (damaged feathers: 18 May (SE = 0.95), N = 509 individuals from 1984-2016; undamaged feathers: 11 May (SE = 0.28), N = 4682 individuals).

Communities of microorganisms

We isolated 15 fungal species among which the most abundant was *Aspergillus* sp. according to identification by PCR technique (Table S1). The number of fungal species per nest ranged from 1 to 9, mean = 5.48 (SE = 0.23), N = 54. The number of fungal colonies per nest ranged from 20 to 135 with a mean = 72.09 (SE = 4.34), N = 54.

We also isolated 10 bacterial species from TSA medium, and the most frequent species was *Bacillus* spp. (Table S2). The number of bacterial species (TSA) ranged from 2 to 10, mean = 4.88 (SE = 0.25), N = 54. The total number of bacterial colonies in TSA ranged from 10 to 350, mean = 70.13 (SE = 8.92), N = 54. Moreover, we isolated 10 bacterial species from FMA medium and the most frequent species was *Streptomyces* spp. (Table S2). The number of bacterial species (FMA) ranged from 0 to 7, mean = 3.92 (SE = 0.24), N = 54. The total number of bacterial colonies in FMA ranged from 0 to 49, mean = 19.15 (SE = 1.79), N = 54. Antimicrobial activity of fungal and bacterial species is presented in Tables S1 and S2. The different bacterial and fungal taxa were widely distributed across the phylogenetic tree of bacteria and fungi (Figs. 1a, b)

Means, SE and ranges of abundance and mean number of colonies of the different microorganisms are reported in Tables S1-S2. From 54 samples, we found a range of arrival dates from 21st April to 25th May with a mean = May 4th (SE = 1).

Microorganisms and arrival date

We tested the prediction that arrival date was related to the abundance of microorganisms. There was a positive relationship between arrival date and the abundance of *Bacillus licheniformis* (Fig. 1; $F = 5.637$, $df = 1, 49$, $r^2 = 0.11$, $P = 0.022$). Furthermore, we found a positive relationship between arrival date and the abundance of the fungus *Trichoderma reesei*, while there was a negative relationship with *Mucor circinelloides* (Fig. 2; $F = 4.488$, $df = 2, 49$, $r^2 = 0.160$, $P = 0.017$).

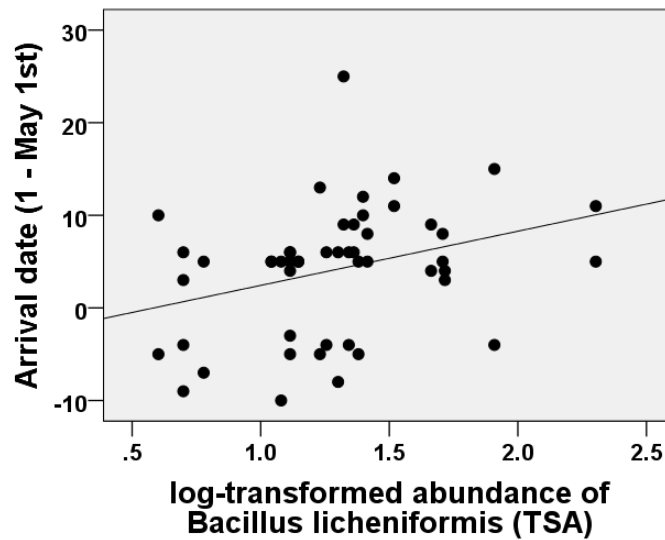


Fig. 1 : Arrival date of barn swallows (1 = May 1st) in relation to log-transformed abundance of *Bacillus licheniformis*. The line is the linear regression line.

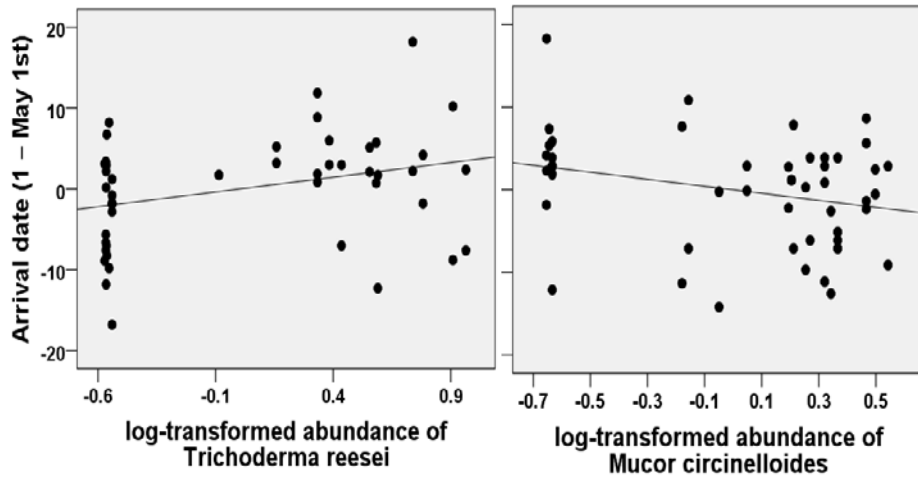


Fig. 2 : Arrival date of barn swallows in relation to log-transformed abundance of fungal species. The lines are the linear regression lines.

There was a positive relationship between arrival date and mean number of bacterial colonies in TSA medium, while there was a negative relationship between the mean number of bacterial colonies in FMA medium and Simpson’s diversity index of bacterial species in FMA medium (Fig. 3; $F = 3.981$, $df = 3, 49$, $r^2 = 0.20$, $P = 0.013$).

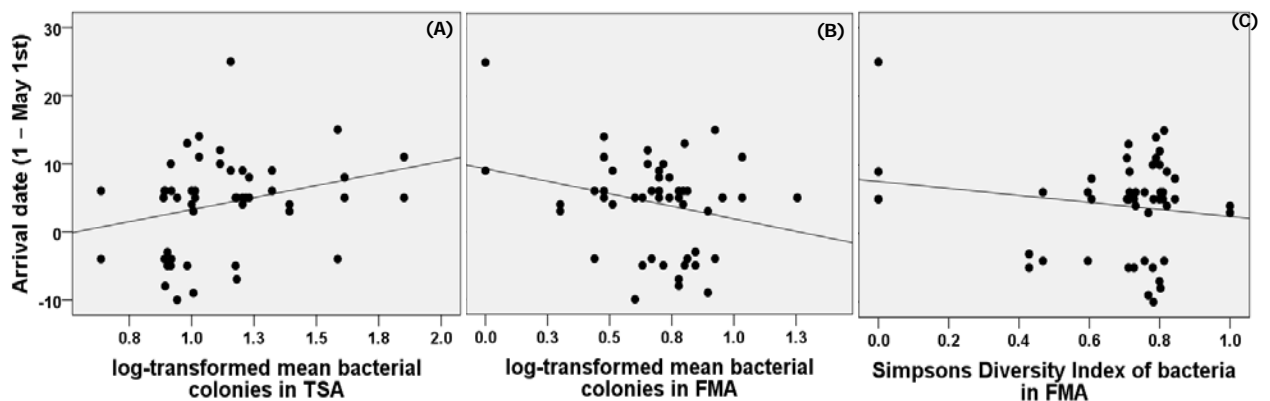


Fig. 3 : Arrival date of barn swallows in relation to (A) log-transformed mean number of bacterial colonies in TSA medium, (B) log-transformed mean number of bacterial colonies in FMA medium, and (C) Simpson diversity index of bacterial species in FMA medium. The lines are linear regression lines.

Antimicrobial activity assay

Among fifteen fungal species isolates we found nine species (60%) that produced antibiotic substance against all indicator strains (Table S3). In contrast, among the ten-bacterial species isolated on TSA medium we found just three species (30%) that produced antibiotic substance against four indicator strains, while in the FMA medium we found three species (30%) that produced antibiotic substance against five indicator strains (Tables S4a, b). There was no significant relationship between antimicrobial activity and arrival date ($F = 0.765$, $df = 15, 49$, $r^2 = 0.252$, $P = 0.703$).

Discussion

The main finding of this study of microorganisms on feathers from barn swallow nests was a positive association between arrival date and the abundance of *Bacillus licheniformis*. We found an independent positive association between arrival date and the abundance of the fungi *Trichoderma reesei* and a negative association with abundance of *Mucor circinelloides*. Finally, there was a positive association between arrival date and mean number of bacterial colonies in TSA medium, while we found a negative association between arrival date and mean number of bacterial colonies in FMA medium, and Simpson's diversity index of bacterial colonies in FMA medium.

Barn swallows arrive at the breeding grounds in early spring where they subsequently construct or refurbish an old nest with fresh mud, straw and a nest lining of feathers. We collected feathers at a standard time of the breeding cycle from the nest lining and analyzed the community of microorganisms on these feathers. We emphasize that microorganisms on nest lining feathers must at least partly derive from birds that lost these feathers near farms where barn swallows breed. These microorganisms are subsequently transferred to the nest, or new microorganisms are transferred from nest lining feathers to the nest.

Long-distance migration was predicted to be negatively related to the prevalence of feather degrading bacteria because such bacteria can have severe negative effects on the quality of the plumage (Jacob and Ziswiler 1982). Colonial birds or hole nesters both facilitate direct transmission of parasites due to the presence of many different host individuals each with a non-negligible probability of being infected (Alexander 1974; Møller and Erritzøe 1996). These species reuse nest sites year after year, and re-visit nests thereby causing transmission of feather-degrading bacteria that typically are found in old nests (Hubálek 1976; Pugh 1964). Therefore, colonial and hole nesting bird species were expected to have higher levels of feather-degrading bacteria than solitary and open nesting species. This prediction was partly fulfilled because the barn swallow is a semi-colonial bird that reuses nests for many years.

Burt and Ichida (1999) suggested that foraging mode of different bird species accounted for interspecific differences in prevalence of feather-degrading bacteria. This observation was prompted by the fact that feather degrading bacteria and fungi naturally are found in soil (Hubálek 1976, 1978; Pugh 1964; Shih 1993; Wood 1995). Hence, any bird that regularly is in contact with soil will run an elevated risk of being infected with feather-degrading bacteria. This would apply to the barn swallow that collects mud from the ground for construction of nests.

Many strains of bacteria degrade feathers with *Bacillus licheniformis* being particularly important, while *B. pumilis* is less important (Williams *et al.* 1990; Burt and Ichida 1999). *Bacillus licheniformis* degrades feathers, but also produces antimicrobial substances (Haavik 1974; Simlot *et al.* 1972) that are active against bacteria of the genera *Bacillus*, *Corynebacterium*, *Enterococcus* and *Micobacterium* (Gálvez *et al.* 1994) and fungi (Lebbadi *et al.* 1994; Patel *et al.* 2004). Here, we found a positive association between arrival date and the abundance of the bacterial species *Bacillus licheniformis* implying that individual barn swallows with more *B. licheniformis* arrive later to the breeding grounds.

Furthermore, we found a positive association between arrival date and the abundance of the fungal species *Trichoderma reesei*, which produces antimicrobial substances (Brückner and Graf 1983).

The antimicrobial properties of substances produced by *Bacillus licheniformis* may impede the establishment of more virulent bacteria. Symbionts such as *B. licheniformis* generally are superior competitors compared to pathogenic microorganisms through the process of bacterial interference (Brook 1999). This occurs when certain bacteria impede the establishment of competing bacterial strains by producing antibiotic substances (Riley and Wertz 2002), or modification of the environment for symbiotic bacteria by hosts (Hackstein *et al.* 1996). Moreover, feather-degrading bacteria are not necessarily only costly, but may entail benefits in terms of protection against pathogenic, but competitively inferior strains of microorganisms.

Here we found a positive relationship between arrival date and the mean number of bacterial colonies in TSA medium. This relationship may reflect that later laying takes place during warmer and more humid environmental conditions that favor growth and reproduction for microorganisms. Furthermore, we found a negative relationship between arrival date and both the mean number of bacterial colonies in FMA medium and the diversity of bacteria in FMA as reflected by the Simpson diversity index for bacteria. This relationship may relate to specific bacterial (*B. licheniformis*) and fungal (*Trichoderma reesei*) species that secrete antibiotic substances that inhibit growth of other microorganisms.

Migratory birds may be colonized by more microorganism species than resident species making them more susceptible to feather damage. There are at least two interpretations of the relationship between arrival date and microorganisms. (1) Individual birds that migrate faster and arrive earlier at the breeding grounds should have fewer microorganisms in their plumage than late arriving individuals. (2) Young individuals with late arrival compared to old individuals may differ in abundance and diversity of microorganisms on their

plumage, and the oldest senescent individuals may again start to accumulate microorganisms on their plumage if their anti-microbial defenses deteriorate at old age. However, here we did not find an association between arrival date and the age of barn swallows, implying that age cannot account for any of the relationships.

In conclusion, barn swallows have higher reproductive success when arriving early. Here we have shown that the arrival date of adult barn swallows was significantly correlated with the abundance of specific bacteria and fungi found on nest lining feathers. This effect was not confounded by the effects of age of individual hosts. Moreover, we found a significant relationship between arrival date and mean total number of bacterial colonies in TSA medium, mean total number of bacterial colonies in FMA medium and Simpson's diversity index of bacteria in FMA medium. If the opportunity of early arrival of some individuals enhances the ability to modify the abundance of microorganisms on their plumage and in the nest, we can conclude that microorganisms can affect the behavior of birds.

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Chapter 2

Laying date, microorganisms and the advantages of early reproduction

Zaid Al Rubaiee, Haider Al Murayati and Anders Pape Møller

*Ecologie Systématique Evolution, Université Paris-Sud, CNRS,
AgroParisTech, Université Paris-Saclay, F-91405 Orsay Cedex,
France*

Word count: 8742

Correspondence to ZAR:

Tel: (+33) 1 69 15 56 96

Fax: (+33) 1 69 15 56 96

E-mail: zaid.iraq@gmail.com

Running headline:

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Abstract

Microorganisms have been hypothesized to affect the timing of reproduction of their hosts either directly because they are pathogenic, or indirectly because they competitively eliminate pathogenic microbes. We tested whether there was a relationship between abundance and diversity of microorganisms and timing of laying in semi-colonial barn swallows *Hirundo rustica*. Microorganisms from nest lining feathers were extracted, cultivated and subjected to molecular identification using PCR methods of 16S and 18S rRNA. There was a mixture of positive and negative associations between laying date and the abundance and the diversity of microorganisms suggesting that the effects of microorganisms on the timing of laying may be a combination of positive effects of microbial interference and negative pathogenic effects. A meta-analysis weighted by sample size of the relationship between laying date in birds and abundance of microorganisms showed a general positive relationship of a small mean effect size, suggesting that on average laying is delayed in the presence of more microorganisms.

Keywords: Early reproduction. Laying date. Microorganisms. Phenology

Introduction

A major component of fitness in many organisms is the timing of reproduction as determined by suitable weather on one hand and peak availability of food on the other. Timing of reproduction is a major life history decision with important fitness consequences, because in seasonal environments there is a relatively short period of the year when conditions are optimal for successful reproduction (Lack 1968; Roff 1992).

Timing of reproduction is determined by availability of resources for reproducing females, but also for successful rearing of offspring. Thus, reproductive phenology is affected by availability of sufficient resources for start of reproduction on one hand, and suitable environmental conditions on the other. Therefore, timing of reproduction has to coincide with peak food availability for the offspring in order to maximize reproductive output (Perrins 1965). A large number of experimental studies has shown that advancement or delay in timing of reproduction has significant consequences for subsequent reproductive success (Verhulst and Nilsson 2008). The seasonal decline in individual fitness is caused by seasonal peaks in food availability, and early produced offspring enjoy an advantage in terms of being able to become fully independent before the end of the breeding season. Many studies suggest that individual and environmental quality drive this relationship between timing of reproduction and reproductive success (Parsons 1975; de Forest and Gaston 1996; Verhulst and Nilsson 2008). This rule is generally not followed by species reproducing twice or more times per year because the optimal timing of reproduction in that scenario is earlier than that resulting in a match between timing of reproduction and peak food availability (Magrath *et al.* 2007).

Additional factors may contribute to the optimal decision about timing of reproduction. These include responses to weather, but also responses to biotic factors such as competitors, predators and parasites. Each of these factors may on its own or in combination affect the timing of reproduction. First, many experimental studies show that temperature can affect laying date (Meijer *et al.* 1999; Nager and Van Noordwijk 1995). Crick *et al.* (1997) have shown that 31% of 67 bird species have advanced their timing of reproduction due to climate change. Dunn (2004) showed that 79% of 57 bird species have a significant negative relationship between air temperature and laying date. For example, many bird species that live-in holes have considerably advanced their timing of reproduction during recent decades, regardless of whether they are residents or migrants (Winkel and Hudde 1997). However, changing weather may affect timing of migration and hence timing of breeding. Indeed, many migratory bird species breed earlier in warmer springs (Gordo 2007; Lehikoinen and Sparks 2010). This implies that weather, but also changing patterns of weather can have important effects on timing of reproduction independent of the effects of food availability.

Second, competitors may preemptively extract resources that will prevent or delay reproduction by conspecifics. Such intraspecific competitive effects on timing of reproduction have been commonly reported as shown by effects of density-dependence (Lack 1966). However, such effects of competition on timing of reproduction may also occur in response to interspecific competition (Dhondt 2011). Third, predators also experience peak consumption of resources during the breeding season, and this may have consequences for the optimal timing of reproduction by their prey. Earlier reproduction by prey may allow prey to reproduce before predators reach peak food requirements for successful reproduction. Finally, timing of reproduction may be affected

by the diversity and the abundance of parasites including microorganisms because such hetero-thermic organisms proliferate under warm and humid environmental conditions. Thus, pathogenic microorganisms and other parasites may prevent some host females from laying early (Møller *et al.* 2015). The cost of late reproduction is the presence of large numbers of pathogenic microorganisms. Alternatively, offspring may suffer from the effects of microorganisms that are present in the nest if parents reproduce late during warmer and more humid conditions (Møller *et al.* 2015).

Microorganisms prefer humid and warm environmental conditions for optimal growth (Stolp 1988). Therefore, hosts should benefit from early reproduction under dry and cold environmental condition where pathogenic microorganisms experience difficulties of reproduction. Warm and humid environmental conditions make nests suitable sites for offspring of hosts, but they also provide suitable conditions for reproduction and spread of pathogenic microorganisms. Pathogenic microorganisms appear to be important selective agents that reduce fitness components of their hosts. Some studies have shown that pathogenic microorganisms are an important cause of mortality in many species of birds (Hubálek 2004; Benskin *et al.* 2009). Likewise, pathogenic microorganisms are a common cause of disease or death in humans and domestic animals (Beaver and Jung 1985; Evans and Brachman 1998; Strauss and Strauss 2002). However, the generality of these patterns under field conditions remains to be determined.

Barn swallows *Hirundo rustica* usually line their nests with feathers. Microorganisms may affect birds directly or indirectly thereby delaying timing of reproduction. Feather degradation by microorganisms can decrease the efficiency of flight in birds (Soler *et al.* 2015). Moreover, nests in general constitute optimal habitats for propagation of bacteria and other

microorganisms, mainly because of high temperatures and humidity. This selection pressure is likely to be at the origin of a number of antimicrobial defenses in birds' nests (Soler *et al.* 2015; Møller *et al.* 2015).

Birds' feathers harbor a variety of fungi (e.g. Hubálek 1978; Pugh 1972) and bacteria (e.g. Bisson *et al.* 2007; Burt and Ichida 1999; Shawkey *et al.* 2005). Feather-degrading microorganisms originate from the soil (Lucas *et al.* 2003), but may colonize the plumage of birds (Bisson *et al.* 2007; Burt and Ichida 1999). Sociality (Møller *et al.* 2009b), reproduction (Lucas *et al.* 2005), migration (Bisson *et al.* 2009) and foraging (Burt and Ichida 1999) affect the microorganism assemblage of feathers. In addition, geographic location and local environmental factors (humidity, temperature and radiation level) may drive the composition of plumage-associated microbial communities (Bisson *et al.* 2007; Burt and Ichida 2004). Pathogenic microorganisms may have negative impact on the physiology of their hosts thereby deteriorating their flight ability and hence their ability to out-manuever and ultimately escape from predators (Møller and Fiedler 2010). Microorganisms may cause disease including fever (Møller 2010). Alternatively, microorganisms could damage flight morphology or disrupt flight directly (Møller *et al.* 2009a). Microorganisms may also be beneficial by improving the quality of feathers by outcompeting detrimental microorganisms that cause degradation of feathers (Martín-Vivaldi *et al.* 2009).

The objective of this study was to determine whether timing of reproduction is related to diversity and abundance of microorganisms. Positive effects of abundance of microorganisms on timing of reproduction may be due to competition with pathogenic organisms (Perrins 1970), while negative effects may be caused by pathogens that cause disease or reductions in body condition of their hosts (Møller *et al.* 2015).

Materials and methods

Study organisms

Barn swallows are native to all biogeographic regions except Antarctica and Australia, and they winter in the tropics or the southern temperate zone (Turner 1994). They exploit open habitats of all types, including farms and are commonly found in barns and other outbuildings.

Barn swallows are socially monogamous, with pairs rearing broods together and often remaining paired for subsequent broods and in successive years. The breeding season ranges between May and August, and they usually raise two broods per summer. Male and female build a nest of mud lined with grass and feathers. The female lays 3 to 7 eggs (average 5). Only females incubate the eggs, which hatch after 13 to 15 days. Both parents feed and protect the chicks, as well as remove their fecal sacs from the nest to keep it clean. The young stay in the nest for about 20 days (Terres 1980; Brown and Brown 1999; McWilliams 2000; Perrins 2009).

Study sites and field work

APM collected feathers from barn swallow nests during May-June 2014 at Kraghede (57°12'N, 10°00'E), Denmark, in a population that has been followed since 1971 (Møller 1994). This study area consists of open farmland with scattered trees, hedges and plantations, where barn swallows breed in farms. APM checked nests at least weekly, collecting information on arrival date, laying date, clutch size and reproductive success. Feather samples were collected using sterile gloves, deposited in polythene bags and stored in a refrigerator until analysis. A breeding site was defined as a single farm or a house where barn swallows interacted on a daily basis with each other.

Neighboring farms were separated by 75–3,000 m, and barn swallows only rarely move between sites during or between breeding seasons.

Microorganism isolation

We isolated and identified the taxa of bacteria and fungi and quantified their abundance on feathers used as nest lining in barn swallow nests. We used a sterilized tweezer to cut each piece of feather and then introduced it in a 15-ml falcon tube with sterile phosphate buffer saline (pH 7.2), and we add volume proportional to the length of the feather, then shaking each tube by vortex 10 s for three periods. All bacteria were washed out from feathers and collected in PBS solution (Saag *et al.* 2011), and then we did a serial tenfold dilution to 10^{-4} . To determine the cultivable and feather-degrading bacteria, we made twice by spreading 100 μ l of supernatant by sterile spreader loop on two different growth media: Tryptic soy agar (TSA) is a rich medium for growth of heterotrophic bacteria that enable assessment of total cultivable bacterial load on feathers. Feather meal agar (FMA) is a medium highly selective for growing keratinophilic bacteria because keratin is the only source of carbon and nitrogen.

All feather samples were snipped in small fragments and cultured directly in mycobiotic agar (selective medium for fungi) by following a standard procedure (Deshmukh 2004), adding a 0.5 ml of sterilized PBS. These samples were incubated at 28°C, SE = 2 and checked daily from the third day for fungal growth until four weeks. We observed the development of mycotic growths under stereoscopic binocular microscope, and we transferred these individually into Sabouraud dextrose agar with chloramphenicol (50 mg/l), and incubated at 28°C, SE = 2 for two weeks to get a pure isolate for identification purposes.

Bacterial identification

Bacterial identification was made by using molecular characterization with polymerase chain reaction (PCR) for genetic analyses. Genomic DNA was extracted from each isolate by using two different protocols. In the freeze-thaw protocol we started freezing and thawing by putting the Eppendorf tube in liquid nitrogen for one to two minutes, then quickly placing the tube in a hot water bath. We repeated the same process for a total of three cycles. By using a micropipette, we transferred the supernatant that contains the DNA into a new clean sterile micro centrifuge tube and discarded the pellet that contains cellular debris. We stored the tubes in -20°C for the Polymerase Chain Reaction (PCR). Second, we used the Power Soil DNA isolation kit (MOBIO Laboratories, Inc. USA) to extract DNA according to the protocol supplied with the kit.

DNA isolated from samples was used as a template for PCR to amplify the bacterial 16S rRNA gene by using the forward primer 16S rDNA-27F and the reverse primer 16S rDNA-1492R according to Barghouthi (2011). The cycle of denaturation, annealing and elongation was repeated 35 times. A final elongation at 72°C for 7 min was then performed. The PCR products were sent to sequencing by Beckman Coulter Genomics.

Fungal identification

Identification of fungi by traditional fungal identification methods such as morphological characteristics, culturing on various media, presence of microconidia and chlamydospores and biochemical analysis such as pigment production have been used routinely, and still several new species are described on this basis (Zhao *et al.* 2007). A small amount of mycelium of

each fungal species was suspended in an Eppendorf tube, and stored in a freezer (-20°C) for further processing.

For molecular identification, genomic DNA was isolated from the fungal strains by using the PowerSoil® DNA Isolation Kit (MO BIO). DNA was eluted in a final volume of 100 µl of 10 mM Tris-HCl, pH 8.5.

PCR amplification was performed in 25 µL reaction volume containing 25 µl assay buffer containing 1.5 mM MgCl₂, dNTP (10mM) 0.5-1 µL, 0.5-1 µL of each 0.2 mM primer FR1, 2.5 µl forward primer UF1, Go Taq® G2 DNA polymerase (Promega Madison, WI USA) (1.25 µl) 0.5-1 µL, and DNA sample 3-5 µL. A final extension at 72°C for 7 min was then performed. The PCR products were sent to sequencing by Beckman Coulter Genomics.

We performed gel electrophoresis in 1% agarose using 0.5 X TAE buffer (Tris-Acetate-EDTA) for 25 min at 100V. The gel was then stained with Gel Red (BIOTIUM) for 30 min. Images were taken under UV lamp by using the photo documentation system IP-010.SD.

PCR products were sent to Beckman Coulter Genomics, Takeley, Essex CM22 6TA, United Kingdom for DNA Sequencing. The sequence results were processed by using the web-based blasting program, basic local alignment search tool (BLAST), at the NCBI site (<http://www.ncbi.nlm.nih.gov/BLAST>), and the data were compared with the NCBI/Genbank database.

Statistical analyses

We used JMP for the statistical analyses (SAS 2012). We investigated the relationship between arrival date, laying date and reproductive success using standard least squares models and in the case of reproductive success a

Generalized Linear Model with Poisson distribution and log link function to account for the number of fledglings being count data.

We report total abundance of colonies and species richness and standard errors for fungi and bacteria. We calculated Simpson's index (a measure of diversity independent of sample size) to determine the diversity of fungi and bacteria in feathers lining the nests. This index takes the number of species present into account, as well as the abundance of each species (Simpson 1949). We subsequently investigated the relationship between laying date and the abundance of different taxa of microorganisms using standard least squares models.

We conducted a meta-analysis (Rosenthal 1991; Cooper and Hedges 1994; Rosenthal 1994) of the relationship between laying date and the abundance of microorganisms relying on all reported analyses in the literature and the findings in the present study. We converted test statistics into Pearson product-moment correlation coefficients (Rosenthal 1994), and these coefficients were subsequently z-transformed before calculating the mean estimates weighted by sample size minus two.

We estimated effect sizes by using Cohen's (1988) guidelines for the magnitude of effects being small (Pearson $r = 0.10$, explaining 1% of the variance), intermediate ($r = 0.30$, explaining 9% of the variance) or large ($r = 0.50$, explaining 25% of the variance).

Results

Arrival date, laying date, age and reproductive success

There was a strong positive relationship between laying and arrival date (Fig. 1; $F = 63.84$, $df = 1, 49$, $r^2 = 0.57$, $P < 0.0001$). Age was on average 1.39 years (SE = 0.11), range 1-4 years, $N = 54$. Arrival date was not significantly

related to age ($F = 1.55$, $df = 1, 52$, $r^2 = 0.01$, $P = 0.22$), nor was laying date ($F = 1.89$, $df = 1, 52$, $r^2 = 0.02$, $P = 0.17$). The total number of fledglings produced during the breeding season was significantly negatively related to laying date (GLM with Poisson distributed data and a log link function: $\chi^2 = 1.89$, $df = 1$, $P = 0.018$, estimate (SE) = -0.014 (0.006)), implying that there was a fitness advantage from early breeding.

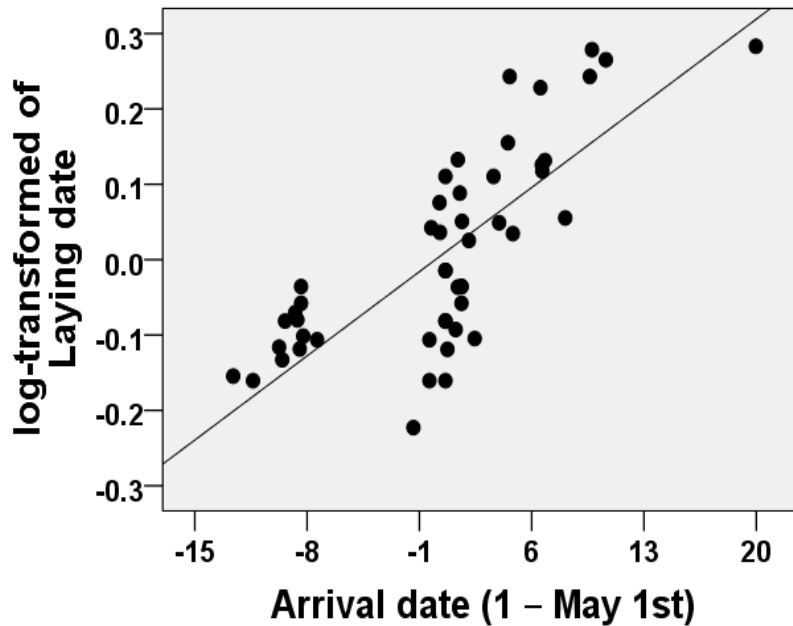


Fig. 1 : Laying date of the first clutch of barn swallows in relation to arrival date. Dates are days since April 30th with 1 = May 1st. The line is the linear regression line

Communities of microorganisms

The number of bacterial colonies ranged from 20 to 135 with a mean of 72.09 (SE = 4.34), $N = 54$. The number of fungal species ranged from 1 to 9, mean = 5.48 (SE = 0.23), $N = 54$. A total of 15 fungal species were isolated from fungal colonies with *Aspergillus* sp. being the most frequently isolated species.

The total number of bacterial colonies in TSA medium ranged from 10 to 350, mean = 70.13 (SE = 8.92), N = 54. The number of bacterial species (in TSA) ranged from 2 to 10, mean = 4.88 (SE = 0.25), N = 54. There were 10 bacterial species isolated from TSA. *Bacillus* spp. was the most frequently isolated species.

The total number of bacterial colonies in FMA medium ranged from 0 to 49, mean = 19.15 (SE = 1.79), N = 54. The number of bacterial species (in FMA) ranged from 0 to 7, mean = 3.92 (SE = 0.24), N = 54. There were 10 species of bacterial colonies in FMA, with *Streptomyces* spp. being the most frequently isolated species. Species richness for fungal and bacterial species is presented in Tables S1-S2. Means, SE and ranges of abundance and number of colonies of microorganisms are reported in Tables S1-S2. We isolated 15 fungal species from feather lining of barn swallow nests based on identification with the PCR technique (Table S1). For bacteria, we isolated 12 species from TSA medium and for FMA 10 species (Tables S2a, b). The different bacterial and fungal taxa were widely distributed across the phylogenetic tree of bacteria and fungi (Figs. S1a, b).

Laying date in relation to microorganism abundance and species richness

Across all microorganism taxa we tested whether there was a correlation between laying date and microorganism abundance. Next, we tested whether the mean correlation between laying date and abundance of microorganisms differed significantly between bacteria and fungi. The distribution of correlation coefficients describing the relationship between laying date and abundance of microorganisms was approximately normal. We found a positive relationship between laying date (controlled for arrival date) and the

abundance of the bacteria *Paenibacillus cookii* and *Streptomyces cacaoi*, ($t = 2.03$, $P = 0.048$, estimate = 0.06 (SE = 0.03); $t = 2.68$, $P = 0.010$, estimate (SE) = 0.14 (0.051)), respectively, while there was a negative relationship with *Methylobacterium mesophilicum* ($t = -2.08$, $P = 0.043$, estimate (SE) = -0.21 (0.1)). The whole model is (Fig. 2; Table 1; $F = 21.73$, $df = 4, 53$, $r^2 = 0.66$, $P < 0.0001$). In contrast, we did not find a significant relationship between laying date and the abundance of any of the fungal species ($F < 2.362$, $df = 1, 53$, $P > 0.130$).

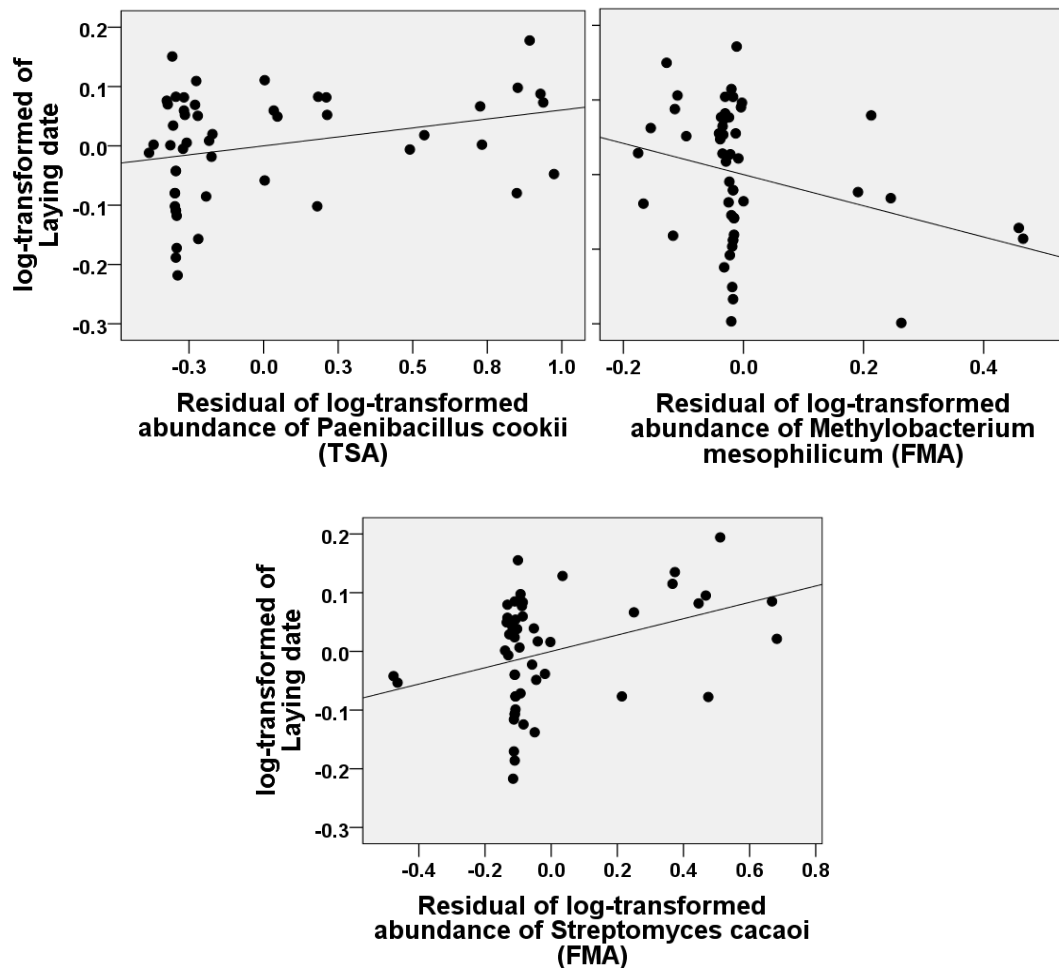


Fig. 2 : Residual laying date after controlling statistically for arrival date in relation to log-transformed abundance of bacterial species. The lines are the linear regression lines

Table 1. Relationship between laying date (response variable) and arrival date and the abundance of different bacterial taxa (predictor variables). The model had the statistics $F = 21.73$, $df = 4, 53$, $r^2 = 0.66$, $P < 0.0001$. Effect size is Pearson's product moment correlation coefficient.

Term	Estimate	SE	<i>t</i>	<i>P</i>	<i>r</i>	<i>z</i>
Intercept	1.267	0.019	67.53	< 0.0001		
Arrival date	0.016	0.002	8.55	< 0.0001	0.76	1.00
<i>Paenibacillus cookii</i> (TSA*)	0.060	0.030	2.03	0.048	0.27	0.28
<i>Methylobacterium mesophilicum</i> (FMA**)	-0.209	0.101	-2.08	0.043	-0.27	-0.28
<i>Streptomyces cacaoi</i> (FMA**)	0.139	0.052	2.68	0.010	0.35	0.36

*TSA (Tryptone Soy Agar medium)

**FMA (Feather Meal Agar medium)

Laying date, reproductive success and diversity of microorganisms

We found a positive relationship between laying date (controlled for arrival date) and Simpson's diversity index for fungal species (estimate (SE) = 0.115 (0.110)) and Simpson's diversity index for bacterial species in TSA medium (estimate (SE) = 0.206 (0.151)), while there was a negative relationship between laying date and Simpson's diversity index of bacterial species in FMA medium (estimate (SE) = -0.076 (0.070)) (Fig. 3; $F = 19.25$, $df = 4, 49$, $r^2 = 0.63$, $P < 0.0001$). This analysis shows that laying date was correlated with diversity and abundance of bacteria.

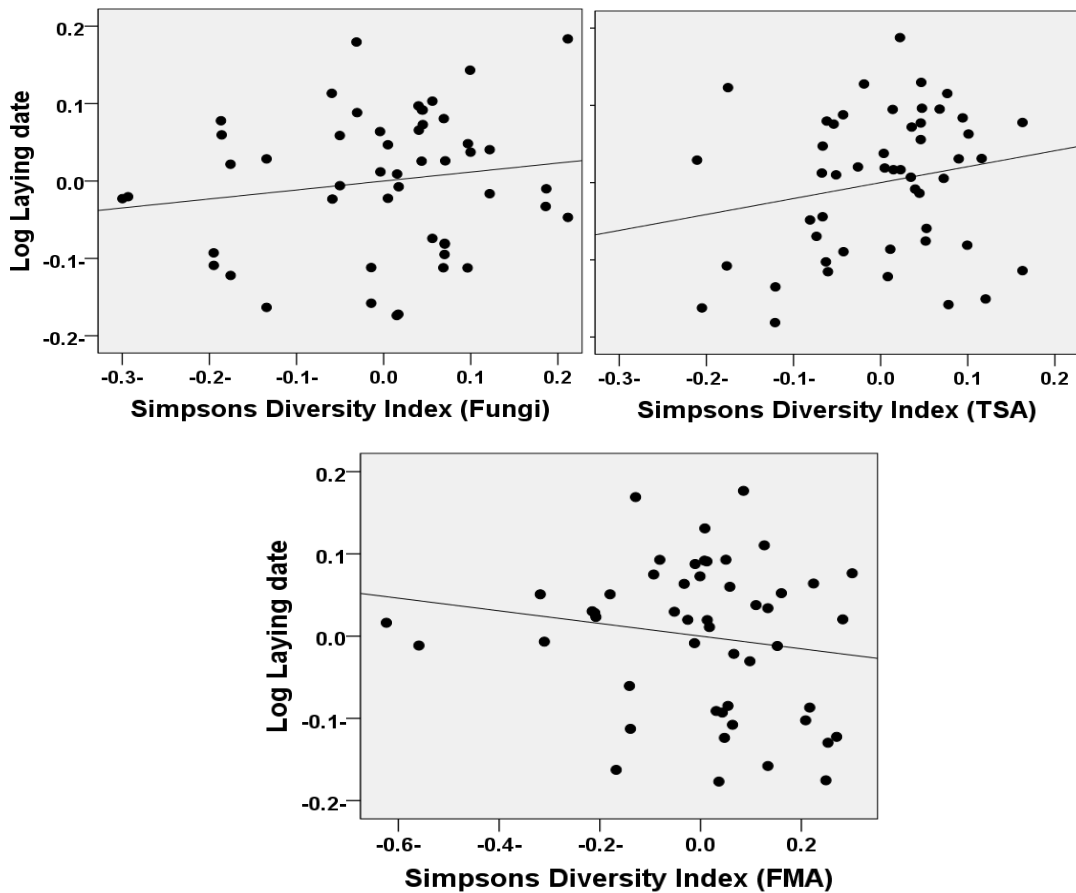


Fig. 3: Residual laying date after controlling statistically for arrival date in relation to residual Simpson's diversity index of fungi, Simpson's diversity index of bacteria in TSA medium and Simpson's diversity index of bacteria in FMA medium. The lines are the linear regression lines.

Meta-analysis of abundance of microorganisms and laying date

Two recent studies have suggested that laying date and abundance of microorganisms are positively related, implying that pairs of birds with more microorganisms suffered from delayed reproduction (Møller *et al.* 2015; Soler *et al.* 2015). Here we estimated the strength of this relationship based on 15 such estimates from these two publications and the present study. We found an overall mean effect size weighted by sample size of 0.141, SE = 0.062, 95% confidence intervals 0.009 to 0.274, Wilcoxon signed rank test =

150628.5, $P < 0.0001$ (Table 2). This suggests that the mean weighted effect size for the relationship between laying date and microorganisms is of an intermediate magnitude (Cohen 1988).

Table 2. Summary information on the relationship between abundance of microorganisms and laying date of their hosts based on a literature survey and the present study. See text for further details. Effect size is Pearson’s product-moment correlation coefficient r and the z-transformation of r .

Bird species	Microbial species	Test statistic	N	r	z	Reference
goshawk (<i>Accipiter gentilis</i>)	<i>Staphylococcus</i>	Kendall 0.60	14	0.60	0.69	(Møller <i>et al.</i> 2015)
goshawk (<i>Accipiter gentilis</i>)	<i>Enterococcus</i>	Kendall 0.50	14	0.50	0.55	(Møller <i>et al.</i> 2015)
goshawk (<i>Accipiter gentilis</i>)	mesophilic bacteria	Kendall -0.14	14	-0.14	-0.14	(Møller <i>et al.</i> 2015)
goshawk (<i>Accipiter gentilis</i>)	Enterobacteriaceae	Kendall -0.08	14	-0.08	-0.08	(Møller <i>et al.</i> 2015)
magpie (<i>Pica pica</i>)	mesophilic bacteria	Wald test 8.25	139	0.24	0.24	(Soler <i>et al.</i> 2016)
magpie (<i>Pica pica</i>)	Enterobacteriaceae	Wald test 8.11	139	0.24	0.24	(Soler <i>et al.</i> 2016)
magpie (<i>Pica pica</i>)	Staphylococci	Wald test 0.21	139	0.04	0.04	(Soler <i>et al.</i> 2016)
magpie (<i>Pica pica</i>)	Enterococcic	Wald test 0.05	139	0.02	0.02	(Soler <i>et al.</i> 2016)
barn swallow (<i>Hirundo rustica</i>)	<i>Lysinibacillus fusiformis</i>	t test 2.05	54	0.27	0.28	Current study
barn swallow (<i>Hirundo rustica</i>)	<i>Staphylococcus lentus</i>	t test 3.19	54	0.40	0.43	Current study
barn swallow (<i>Hirundo rustica</i>)	<i>Streptomyces cacaoi</i>	t test 2.45	54	0.32	0.33	Current study
barn swallow (<i>Hirundo rustica</i>)	<i>Streptomyces griseoaurantiacus</i>	t test -3.39	54	-0.42	-0.45	Current study
barn swallow (<i>Hirundo rustica</i>)	<i>Paenibacillus cookii</i>	t test 2.03	54	0.27	0.28	Current study
barn swallow (<i>Hirundo rustica</i>)	<i>Streptomyces cacaoi</i>	t test 2.68	54	0.35	0.36	Current study
barn swallow (<i>Hirundo rustica</i>)	<i>Methylobacterium mesophilicum</i>	t test -2.08	54	-0.27	-0.28	Current study

Discussion

The main findings of this study of the diversity of microorganisms on feathers from barn swallow nests was a delay in laying date (controlled for the effects of arrival date) and abundance of *Paenibacillus cookii* and *Streptomyces cacaoi*, and an advancement in laying date with the abundance of *Methylobacterium mesophilicum*. However, there was no significant relationship between laying date and the abundance of fungal species. Finally, there was a delay in laying date (controlled for the effects of arrival date) and Simpson's diversity index of fungal species and Simpson's diversity index for bacterial species in TSA medium, while laying was advanced with Simpson's diversity index of bacterial species in FMA medium.

Pathogenic microorganisms are commonly identified from wild birds (reviews in Hubálek and Halouzka 1996; Hubálek 2004; Benskin 2009), although their consequences for the fitness of hosts remain poorly understood. Here we analyzed these relationships and found a strong positive association between laying date (controlled for potentially confounding effects of arrival date) and the abundance of two bacterial species (*Paenibacillus cookii* and *Streptomyces cacaoi*). These correlations may be related to pathogenicity of these bacterial taxa for the host (Smith 1933; Deinhofer and Pernthaner 1995; Pellegrini *et al.* 2012), although some opportunistic taxa under particular circumstances turned out to be pathogenic, while others may have beneficial effects (Soler *et al.* 2010; Peralta- Sanchez *et al.* 2010; Møller *et al.* 2011).

Obviously, feathers used in birds' nests have insulating properties that facilitate thermoregulation (Baicich and Harrison 2005). However, such feathers may also have negative impacts on their hosts because feathers are

carriers of microorganisms that in some cases may be pathogenic. A common reason for finding feathers in nature is that an individual bird has been killed by a predator, and such dead individuals may have fallen prey to predators because they are sick (Møller *et al.* 2015). Here we provide a test of the extent to which feathers collected for nest material constitute a source of microorganisms in birds' nests. Nest lining feathers harbor microorganisms that in most cases are feather degrading bacteria or fungi (Pugh and Evans 1970; Shawkey *et al.* 2003; Cristol *et al.* 2005). These microorganisms associated with feathers could also affect the probability of trans-eggshell pathogenic infection of the embryo. For instance, microorganisms occupy space and/or produce antimicrobial substances against such egg pathogens. Growth of feather-degrading bacteria is mainly controlled or prevented by uropygial secretions that birds spread on feathers during preening (Shawkey *et al.* 2003). However, feathers carried to nests as lining material are not preened and consequently microorganisms would grow more quickly in such nest lining feathers.

Host individuals in prime condition may harbor fewer microorganisms than individuals in poor condition because the latter may be less able to defend themselves against microorganisms. Because early breeding individuals are in better body condition than late breeders (Perrins 1965), our findings that early laying date was associated with fewer microorganisms are consistent with this hypothesis. We conducted a meta-analysis of available data on the relationship between laying date and abundance of microorganisms, and we found a small, but highly significant mean effect weighted by sample size.

Arrival date and laying date were strongly positively correlated in the present study, implying that any relationship between microorganisms and laying date must be controlled for the potentially confounding effects of

arrival date. Here we found a positive relationship between laying date (controlled for the effects of arrival date) and the diversity of microorganisms by using Simpson's diversity index for bacteria and fungi. This relationship may reflect that delayed laying takes place during warmer and more humid environmental conditions that favor growth and reproduction for microorganisms. The reasons why it is beneficial for birds to reproduce early in spring, as commonly shown for many different species (e.g. Lack 1954; Perrins 1965), may be avoidance rapid growth and reproduction by microorganisms in the nest later during summer. Individual birds that migrate faster and arrive earlier at the breeding grounds, which may reflect their prime condition, harbor fewer microorganisms than individual that arrive late (Hubálek 2004; Møller *et al.* 2004). Such late arriving individuals may have more pathogenic microorganisms, but they may also compete more with conspecifics over access to feathers for nest lining material.

There are a number of alternative interpretations for the relationship between laying date and microorganisms described here. (1) Host individuals differing in abundance and diversity of microorganisms may breed at different times. It is possible that birds with few microorganisms and hence in better health breed earlier than others. (2) Later breeders may be exposed to warmer and more humid conditions that favor early start of reproduction. However, not all individuals may be able to start reproduction early if they for example are in poor condition due to poor health. (3) There may be positive effects of antimicrobial activity on timing of reproduction by hosts. A number of studies have found that bird hosts harbor microorganisms with antimicrobial activity (Shawkey *et al.* 2008; Lee *et al.* 2014; Martín-Vivaldi *et al.* 2009). (4) The community of microorganisms on feathers will depend on the community of birds in the neighborhood that have lost feathers and hence are potential

recipients of nest lining material. This raises questions about the choice of breeding habitat to optimize the use of feathers as nest material, but also to minimize the risk of pathogenic infections. For example, a bird that collects feathers from the neighborhood of a raptor nest may be more at risk from becoming infected by pathogens derived from prey of this predator than a bird collecting feathers elsewhere.

The results reported here require experimental manipulation of the abundance of microorganisms in nests and on adults for a formal verification. This could be done by use of antimicrobial substances, or indirectly by manipulation of the composition of the color of nest lining feathers (Peralta-Sanchez *et al.* 2011). Another possibility is to treat the white feathers of the nest lining with antimicrobial agents to terminate the negative effect of feather degrading microorganisms (Soler *et al.* 2012).

In conclusion, we have shown that the laying date of barn swallows was significantly positively correlated with the abundance of specific bacteria. In contrast, we did not find a significant relationship between laying date and the abundance of fungal species. Furthermore, there was no significant relationship between laying date and the age of individual hosts. Moreover, we found a significant positive relationship between laying date (controlled for the potentially confounding effects of arrival date) and Simpson's diversity index of bacterial and fungal species. If the probability of late laying reflects the ability of an individual barn swallow to modify the abundance of microorganisms, we can conclude that microorganisms and hence the microbiome can be affected by bird behavior.

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Chapter 3:

Microorganisms, clutch size and reproductive success in the barn swallow

Zaid Al Rubaiee, Haider Al Murayati and Anders Pape Møller

*Ecologie Systématique Evolution, Université Paris-Sud, CNRS, AgroParisTech,
Université Paris-Saclay, F-91405 Orsay Cedex, France*

Word count: 6026

Correspondence to ZAR:

Tel: (+33) 1 69 15 56 96

Fax: (+33) 1 69 15 56 96

E-mail: zaid.iraq@gmail.com

Running headline:

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Abstract

Limiting resources should be allocated to competing demands in order to maximize reproductive success. The evolution of clutch size has for decades been a central issue in life history theory, although surprisingly there have been few attempts to address the potential role of microorganisms. Birds have been a frequently used group of organisms for studies of factors that determine clutch size. David Lack hypothesized that clutch size in altricial birds has evolved to be that resulting the largest number of surviving offspring, ultimately regulated by feeding capacity of the parents, resulting in a mechanism determining the upper limit to clutch size being parental ability to provide food for nestlings. We tested whether there was a relationship between abundance and diversity of microorganisms and total number of fledglings produced by barn swallows *Hirundo rustica* during their total annual reproductive events. We found positive relationships between total number of fledglings (controlled for the effects of number of eggs) and the abundance of microorganisms. Surprisingly, we did not find a significant relationship between total number of fledglings and the age of individuals. Finally, we found positive relationships between the total number of fledglings and antimicrobial activity of microorganisms. These findings are consistent with the hypothesis that clutch size and reproductive success have evolved in response to abundance and diversity of microorganisms.

Keywords: barn swallow, clutch size, microorganisms, reproductive success.

Introduction

The evolution of clutch size has for decades been a central issue in life history theory. Birds have been a frequently used group of organisms for studies of factors that determine clutch sizes. For species with variable clutch size, the number of eggs could be influenced by availability of breeding resources, individual quality, or environmental conditions (Godfray *et al.* 1991).

Food, energy, time and other resources are limited and should be allocated optimally to account for competing demands (Ricklefs 2000). The probability of survival from various risks associated with reproduction will most likely decrease as parental investment increases because time and other resources may be allocated to adult maintenance and avoidance of predation instead of producing offspring. Natural selection favors individuals that optimize lifetime reproductive success, and optimal investment in a breeding attempt is affected by lifespan. However, individuals in species with a short life expectancy may maximize their lifetime fitness by investing more in reproduction, while individuals in species with longer lifespans enhance their fitness by investing less in reproduction.

Lack (1947, 1948) hypothesized that optimal clutch size in altricial birds is that which produces the most surviving offspring, ultimately regulated by feeding capacity of parents, resulting in mechanisms determining the upper limit to clutch size being parental ability to provide food for nestlings (Lack 1954). He further argued that individual birds can lay clutches of optimal size, matching the maximum number of offspring, which could survive from provisioning of parental care. Numerous studies have tested this hypothesis by experimentally enlarging or reducing brood size. In the majority of studies, clutches with high productivity are larger than actual modal clutches (reviews by Murphy and Haukioja 1986; Price and Liou 1989; Malacarne *et al.* 1992; VanderWerf 1992).

Individual optimization of clutch size implies that phenotypic plasticity in clutch size is adaptive (Perrins and Moss 1975; Drent and Daan 1980; Högstedt 1980; Pettifor *et al.* 1988; Lessells 1991). This means that individuals adjust

clutch size to their condition, and that any difference from the chosen clutch size reduces fitness.

From experimental studies, where the fitness consequences of variation in clutch size were measured, evidence has accumulated suggesting that clutch sizes were optimized at the individual level in a number of species. In magpies *Pica pica*, Högstedt (1980) showed that fledging success was maximized for the original clutch size. In great tits *Parus major*, Perrins and Moss (1975) showed that artificially enlarged clutches had lower recapture rates per offspring than naturally large clutches, while reduced clutches did better when compared with naturally small clutches. These data point at a positive relationship between the ability to raise offspring and original clutch size. Pettifor *et al.* (1988) did not find an effect of manipulation of brood size on parental survival. The rate of recruitment (offspring returning to breed) peaked around non-manipulated clutch sizes per brood, again pointing at individual optimization. In the blue tit *Parus caeruleus*, Pettifor (1993) showed similar results of recapture rates of offspring (after three months) per brood. In a Dutch study of great tits, Tinbergen and Daan (1990) showed that both offspring and parental fitness were affected by manipulation. Total fitness can be reduced by brood size reduction and enlargement, but fitness was positively related to natural differences in clutch size. All of these results suggest individual optimization of clutch size. In collared flycatchers *Ficedula albicollis*, Gustafsson and Sutherland (1988) showed that unmanipulated clutch sizes produced more recruits than reduced or enlarged clutches, and, also, in natural clutch sizes there was a positive relationship between the number of recruits and clutch size. In the kestrel *Falco tinnunculus*, Daan *et al.* (1990) showed that the seasonal decline in clutch size could be explained by individual optimization. Verhulst (1995) showed that brood reduction increased fitness, leading to the conclusion that clutches were larger than optimal. The availability of resources is a very important determinant of the demography and allocation in many species, and it also affects energy distribution

strategies among individuals. The energy involved in acquiring food and process it is tightly related to the physiology and behaviour of individuals, and the rate of total energy and nutrients that is assigned to reproduction (reproductive effort) causes differences in life histories among individuals (Martin 1987).

The importance of food limitation in nature continues to constitute an enigma among ecologists, as it can differ in time and space, breeding stage and among environments (Newton 1998). Food limitation is probable less important in systems where predation or critical resources other than food keeps populations below their carrying capacity (Osenberg and Mittelbach 1996). In contrast, food limitation can be inferred when a shortage of food or other critical resources result in lower reproductive performance and/or reduced survival (Martin 1987; Sinclair 1989; Newton 1998) that ultimately leads to a decline in growth rate of the population. The limitation of food can have short- (e.g., reproductive effort) and long-term (e.g., adult survival and future reproduction) life history consequences. Variation in food availability in space and time is therefore an important cue for animals to adjust their reproductive decisions: It can provide information on where and when to breed, with whom to breed, and how much to invest in offspring (Martin 1987; Asdell 1964; Lack 1966).

Birds commonly use feathers for lining their nests (Cramp 1998), and Peralta-Sánchez *et al.* (2010) showed that the abundance of feathers was negatively related to bacterial abundance on the eggshells of barn swallows *Hirundo rustica*. In addition, bacterial communities which inhabit nest-lining feathers produce antimicrobial substances (Soler *et al.* 2010), that might eliminate pathogenic microorganisms on eggshells of birds (Peralta-Sánchez *et al.* 2010; Soler *et al.* 2010) and also increase hatching success (Peralta-Sánchez *et al.* 2011).

The abundance of bacteria in nests could be affected by nest materials, because different materials may harbor different microorganism communities, and the nest material used to line the nest cup may indirectly influence the

environment of microorganisms in nests with effects on thermal conditions (Mertens 1977).

The objective of this study was to assess whether microorganisms affect clutch size and reproductive success and to quantify the role of microorganisms, in particular bacteria and fungi, in the life history of their barn swallow hosts.

Materials and methods

Study organisms

The barn swallow is the most common species of swallow in the world. It is a passerine bird with blue upper parts, a long, deeply forked tail and pointed wings. It is present in Europe, Asia, Africa and the Americas (Stevenson 1978). The barn swallow is an open country bird that normally uses man-made structures for breeding. It builds a cup-shaped nest from mud pellets in buildings and feeds on insects caught in flight. They catch most of their prey while flying and feed their young at the nest (Brown and Brown 1999; McWilliams 2000; Perrins *et al.* 1989; Terres 1980).

Barn swallows usually breed during May to August and they commonly raise two broods each summer with the same pair sometimes mating for several years. The female lays 4 - 6 eggs. Generally, second clutches are smaller than first clutches (Campbell *et al.* 1997; Brown and Brown 1999). Females incubate the eggs while both parents care for the young. The eggs take 14 days to hatch and the chicks fledge when about 21 days old. The parents will continue to feed them for up to 7 days after fledging.

Study site and field work

The nest lining feathers from barn swallow nests were collected by Anders Pape Møller in May-June 2014 at Kraghede (57°12'N, 10°00'E), Denmark, in a population which has been followed since 1971 (Møller 1994). Adults were

captured at least twice each week from the beginning of the breeding season. Reproductive success was defined as the total number of fledglings that were produced by each adult (Møller *et al.* 2004). We collected feather samples by using sterile gloves, deposited in polythene bags and stored in a refrigerator at 4°C until analysis.

Analyses of microorganisms

We isolated bacterial and fungal species that were cultured in different media from the samples of feathers lining barn swallow nests and quantified their abundance and identified them by using the PCR technique.

Microorganism isolation

Bacterial and fungal isolation

We used sterilized tweezers, and each piece of feather was introduced in a falcon tube with sterile phosphate buffer saline (pH 7.2) with a volume relative to the length of the feather. This was followed by shaking in vortex 10 s each. Free-living bacteria were washed out from the feathers and collected in PBS solution (Saag *et al.* 2011), and then making a serial tenfold dilution to 10^{-4} . To quantify cultivable and feather-degrading bacteria, duplicates were made by spreading 100 µl of supernatant with a sterile spreader loop on two different growth media: (1) Tryptic soy agar (TSA) is a rich medium in which heterotrophic bacteria can grow, thus enabling assessment of total cultivable microorganism load of the feathers. (2) Feather meal agar (FMA) is a medium highly selective for keratinolytic bacteria because the only source of carbon and nitrogen is keratin.

The feathers were clipped into small pieces and cultured directly onto Mycobiotic Agar (which is a selective medium for isolation of pathogenic fungi) following standard procedure (Deshmukh 2004). The cultures were incubated at $28^{\circ}\text{C} \pm 2$ and examined daily from the third day for fungal growth over a period of 4 weeks. The observed the mycotic growths, were individually and directly

transferred onto Sabouraud dextrose agar (SDA) with chloramphenicol (50 mg/l). The resulting products were further incubated at $28^{\circ}\text{C} \pm 2$ for two weeks to obtain pure isolates for identification purposes.

Microorganism identification

Bacterial and fungal identification

Bacterial and fungal identification was carried out by using molecular characterization with the aid of polymerase chain reaction (PCR) for further genetic analyses. Genomic DNA was extracted from each isolate by PowerSoil® DNA Isolation Kit (MO BIO). DNA was eluted in a final volume of 100 μl of 10 mM Tris-HCl, pH 8.5.

PCR amplification of bacterial (16SrRNA) and fungal (18SrRNA) gene

DNA isolated from samples was used as a template for PCR to amplify the bacterial 16S rRNA gene by using the forward primer 16S rRNA-27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer 16S rRNA-1492R (5'-GGTTACCTTGTTACGACTT-3'), and for fungi 18S rRNA gene by using the forward primer 18S rRNA-FR1 (5'-CTCTCAATCTGTCAATCCTTATT-3'), 2.5 μl forward primer UF1 (5'-CGA ATC GCA TGG CCT TG-3'), PCR was carried out in a total volume of 25 μl containing 16 μl ultrapure water, 4 μl 5x buffer, 0.4 μl dNTPs 10nM, 1 μl 10 μM 27F, 1 μl 10 μM 1492R, 0.2 μl Go Taq DNA polymerase and 3-5 μl genomic DNA. The PCR conditions consisted of an initial denaturation at 94°C for 5 min, denaturation at 94°C for 15 s, annealing for 30 s at 55°C and 1.30 min of primer extension at 72°C . The cycle of denaturation, annealing and elongation was repeated 35 times. A final elongation at 72°C for 7 min was then performed. The PCR products were sent to sequencing by Beckman Coulter Genomics.

Agarose electrophoresis

To determine the presence or absence of PCR products and to quantify the size of amplified DNA fragments, we performed gel electrophoresis in 1% agarose using 0.5 X TAE buffer (Tris-Acetate-EDTA) for 25 min at 100 V. The gel was then stained with Gel Red (BIOTIUM) for 30 min. Images were taken under UV lamp by using the photo documentation system IP-010.SD.

DNA sequencing

All PCR products were sent to Beckman Coulter Genomics, Takeley, Essex CM22 6TA, United Kingdom for DNA Sequencing. The sequence results were processed by using the web-based blasting program, basic local alignment search tool (BLAST), at the NCBI site (<http://www.ncbi.nlm.nih.gov/BLAST>), and the data were compared with the NCBI/Genbank database.

Antimicrobial activity assay

In bacterial strains, we used the streak-plating technique on Mueller Hinton agar. Each isolate was cultured on agar plates in a central single line, then incubated at 30°C for 7 days in order to allow the bacteria to secrete antibiotic substances on the media. After incubation, standard bacteria were grown in Mueller Hinton medium for 18 h and properly diluted to make cross-streaks along the line of fully grown isolates. Each culture was started near the edge of the plates and streaked towards the central bacterial growth line. Then incubated for 18 h at 37°C, and the zone of inhibition was measured using a millimeter scale (Shams *et al.* 2015).

In fungal isolates, we cultured each isolate in Sabouraud dextrose broth medium for 1-2 weeks at 30°C. The culture medium was centrifuged at 5000 g for 10 min to remove all fungal cells, and by using a Millipore filter (0.2 µm) the supernatant was collected in a sterile falcon tube. The antimicrobial activity was tested by well-diffusion methods. After 24-48 h of incubation, the inhibitory zones (in mm) were measured (Moshafi *et al.* 2006).

Statistical analyses

We used the statistical software JMP (SAS 2012) to make all statistical analyses. We \log_{10} -transformed all bacterial and fungal counts after addition of a constant of one to normalize the data. We investigated the relationship between total number of fledglings and antimicrobial activity of microorganisms by using Generalized Linear Models (GLM) with Poisson distribution and log link function to account for the count data.

We report total abundance of colonies and species richness for fungi and bacteria. We calculated Simpson's index (a measure of diversity independent of sample size) to determine the diversity of microorganisms in feather lining of the nest, which takes into account the number of species present, since as species richness and evenness increase, so does diversity (Magurran 2004).

We tested whether the abundance of fungi and bacteria predicted the frequency of recaptures using a GLM. We estimated effect sizes by using Cohen's (1988) guidelines for the magnitude of effects being small (Pearson $r = 0.10$, explaining 1% of the variance), intermediate ($r = 0.30$, explaining 9% of the variance) or large ($r = 0.50$, explaining 25% of the variance).

Results

Eggs, age and total number of fledglings

There was a strong positive relationship between total number of eggs and total number of fledglings produced during the breeding season (Fig. 1; $F = 88.417$, $df = 1, 53$, $r^2 = 0.62$, $P < 0.0001$, estimate (SE) = 1.278 (0.11)). Age of adult barn swallows was on average 1.39 years (SE = 0.11), range 1-4 years, $N = 54$ adults. The total number of fledglings produced during the entire breeding season was not significantly related to age (GLM with Poisson distributed data and a log link function: $\chi^2 = 1.415$, $df = 1$, $P = 0.234$, estimate = 0.077 (SE = 0.063)).

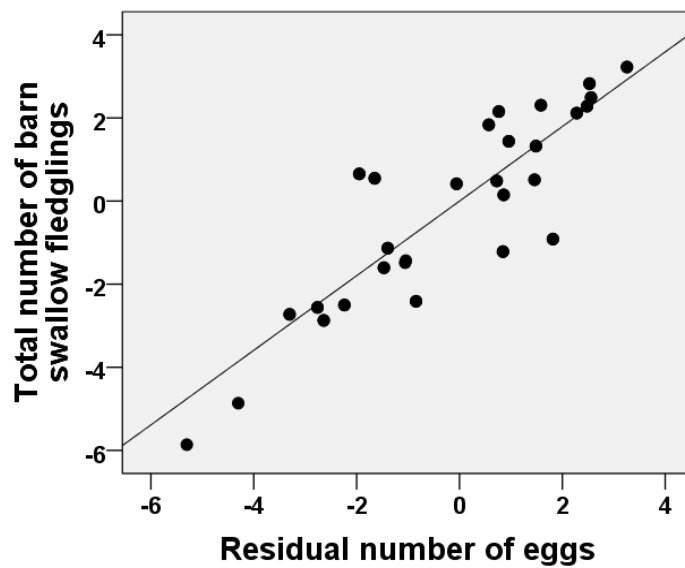


Fig. 1: Total number of barn swallow fledglings in relation to residual number of eggs after controlling statistically for laying date. The line is the linear regression line.

Communities of microorganisms

We isolated fifteen fungal species from feather lining of barn swallow nests using identification with the PCR technique (Table S1). Twelve species were isolated from TSA medium and ten species from FMA medium (Table S2). The different bacterial and fungal taxa were widely distributed across the phylogenetic tree of bacteria and fungi (Figs. S1a, b).

The most frequently isolated species of fungus was *Aspergillus* sp. The number of fungal species per sample ranged from 1 to 9, mean = 5.48 (SE = 0.23), N = 54 nests. The number of fungal colonies ranged from 20 to 135 with a mean of 72.09 (SE = 4.34), N = 54 nests.

The most frequently isolated species from TSA medium was *Bacillus* spp. The number of bacterial species (TSA) ranged from 2 to 10, mean = 4.88 (SE = 0.25), N = 54 nests. The total number of bacterial colonies in TSA medium ranged from 10 to 350, mean = 70.13 (SE = 8.92), N = 54 nests.

The most frequently isolated species from FMA medium was *Streptomyces* spp. The number of bacterial species from FMA medium ranged from 0 to 7, mean

= 3.92 (SE = 0.24), N = 54. The total number of bacterial colonies in FMA medium ranged from 0 to 49, mean = 19.15 (SE = 1.79), N = 54. Species richness for fungal and bacterial species is presented in Tables S1-S2. Means, SEs and ranges of abundance and number of colonies of microorganisms are reported in Tables S1-S2.

Microorganisms and total number of fledglings

There was a significant positive relationship between total number of fledglings (controlled for the total number of eggs laid) and abundance of *Absidia corymbifera*, *Penicillium glabrum*, *Bacillus pumilus*, *Bacillus rhizosphaerae* and *Streptomyces cacaoi*. In contrast, there was a negative relationship between total number of fledglings (controlled for the number of eggs) and the abundance of *Trichoderma reesei* (Fig. 2; Table 1; $F = 41.819$, $df = 7, 53$, $r^2 = 0.86$, $P < 0.0001$). Implying that effects were not constant between different taxa of bacteria.

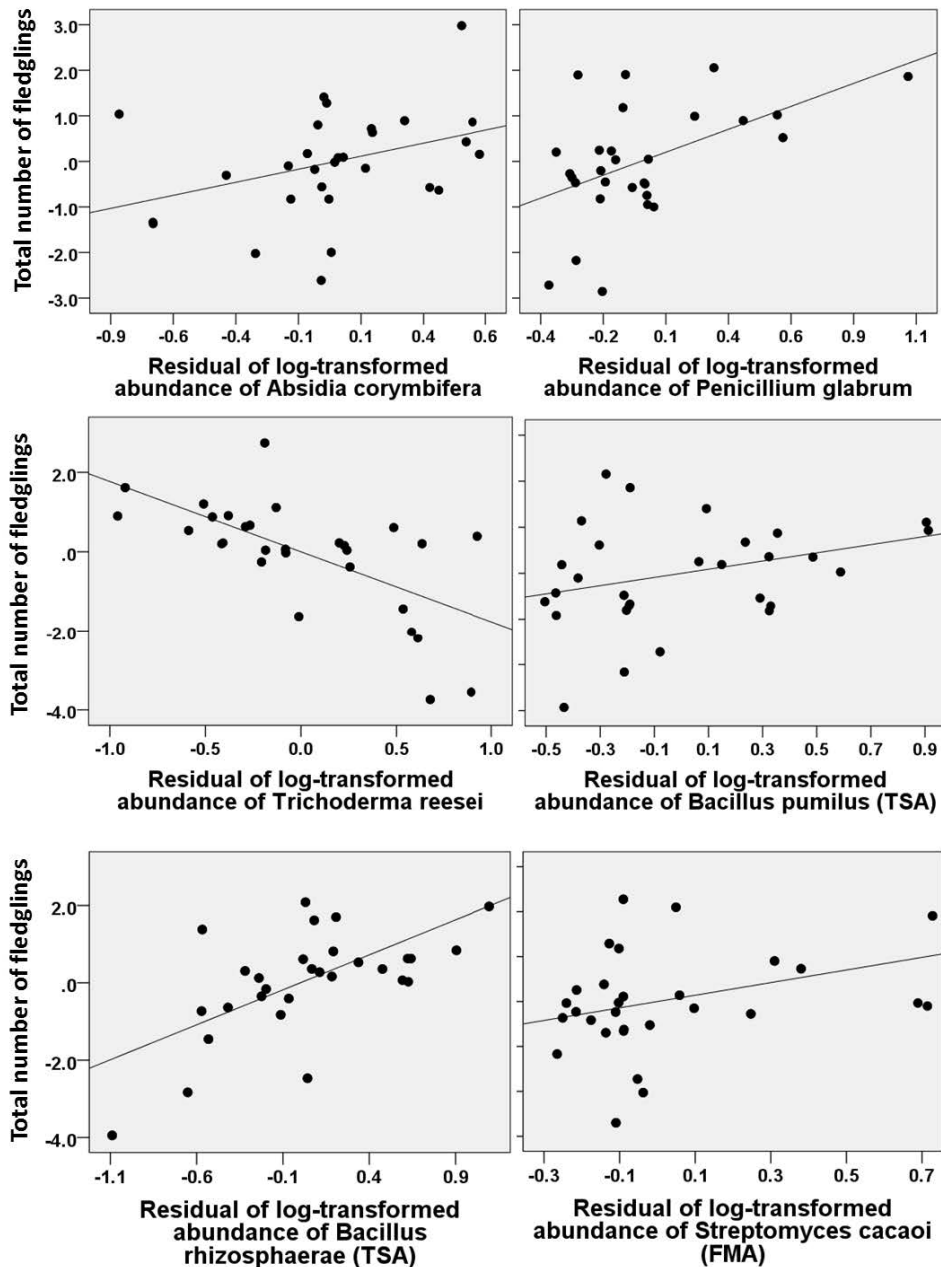


Fig. 2: Total number of barn swallow fledglings (controlled statistically for the number of eggs) in relation to log-transformed residual abundance of *Absidia corymbifera*, *Penicillium glabrum*, *Bacillus pumilus*, *Bacillus rhizosphaerae*, *Streptomyces cacaoui* and *Trichoderma reesei*. Residual abundance was controlled statistically for the number of eggs. The lines are linear regression lines.

Table 1. Relationship between total number of fledglings (response variable) and total number of eggs and the abundance of different bacterial and fungal taxa (predictor variables). The model had the statistics $F = 41.819$, $df = 7, 53$, $r^2 = 0.86$, $P < 0.0001$. Effect size is Pearson's product moment correlation coefficient r and the z-transformation of r .

Term	Estimate	SE	t	P	r	z
Intercept	-3.381	0.662	-5.11	<0.0001		
Number of eggs	0.897	0.078	11.59	<0.0001	0.85	1.24
<i>Absidia corymbifera</i>	1.146	0.470	2.44	0.0187	0.32	0.33
<i>Penicillium glabrum</i>	2.015	0.495	4.07	0.0002	0.49	0.53
<i>Bacillus pumilus</i> (TSA*)	0.891	0.393	2.27	0.0281	0.30	0.31
<i>Bacillus rhizosphaerae</i> (TSA*)	1.808	0.332	5.44	<0.0001	0.60	0.69
<i>Streptomyces cacaoi</i> (FMA**)	1.404	0.623	2.26	0.0289	0.30	0.31
<i>Trichoderma reesei</i>	-1.768	0.319	-5.55	<0.0001	-0.61	-0.70

*TSA (Tryptone Soy Agar medium)

**FMA (Feather Meal Agar medium)

Species richness of microorganisms and total number of fledglings

There was a positive relationship between total number of fledglings and the number of bacterial colonies in TSA medium and Simpson's diversity index of bacterial species in FMA medium, while there was a negative relationship between total number of fledglings and the number of fungal species (Fig. 3; $F = 12.397$, $df = 3, 53$, $r^2 = 0.63$, $P < 0.0001$). These findings show that effects were positive for diversity and abundance of bacteria and negative for number of fungal taxa.

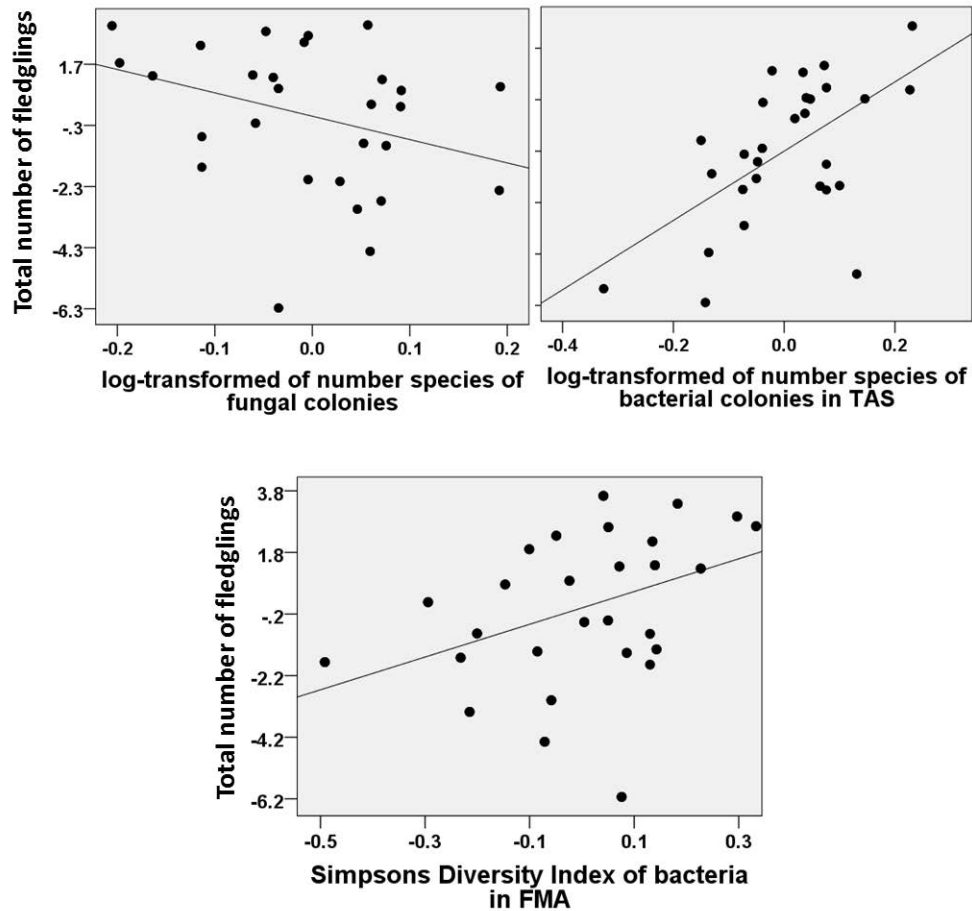


Fig. 3: Total number of barn swallow fledglings in relation to log-transformed number of fungal species, log number of bacterial colonies in TSA medium and Simpson diversity index of bacterial species in FMA medium. The lines are linear regression lines.

Antimicrobial activity and the abundance of microorganisms

We found eight species (54%) of fungal isolates that produced antibiotic substances (Table S4). In contrast, just three species (30%) of bacterial species isolated from TSA medium produced antibiotic substances. We found three species (30%) of bacterial species isolated from FMA medium that produced antibiotic substance against the indicator bacterial strains (Tables S5a, b). We found a positive relationship between the total number of fledglings and antimicrobial activity of *Aspergillus fumigatus* in SDA medium, *Streptomyces*

cacaoi in FMA medium, *Bacillus pumilus* in TSA medium and *Bacillus rhizosphaerae* in TSA medium. In contrast, there was a negative relationship between total number of fledglings and antimicrobial activity of *Bacillus licheniformis* in FMA medium (Fig. 4; Table 2; $\chi^2 = 28.116$, $df = 5$, $P < 0.0001$). Meanwhile, we did not find a significant relationship between the antimicrobial activity of these microorganisms and each of arrival date ($F = 0.76$, $df = 15, 49$, $r^2 = 0.25$, $P = 0.703$), laying date ($F = 1.725$, $df = 15, 51$, $r^2 = 0.42$, $P = 0.089$) and recapture probability ($F = 1.59$, $df = 15, 45$, $r^2 = 0.44$, $P = 0.135$).

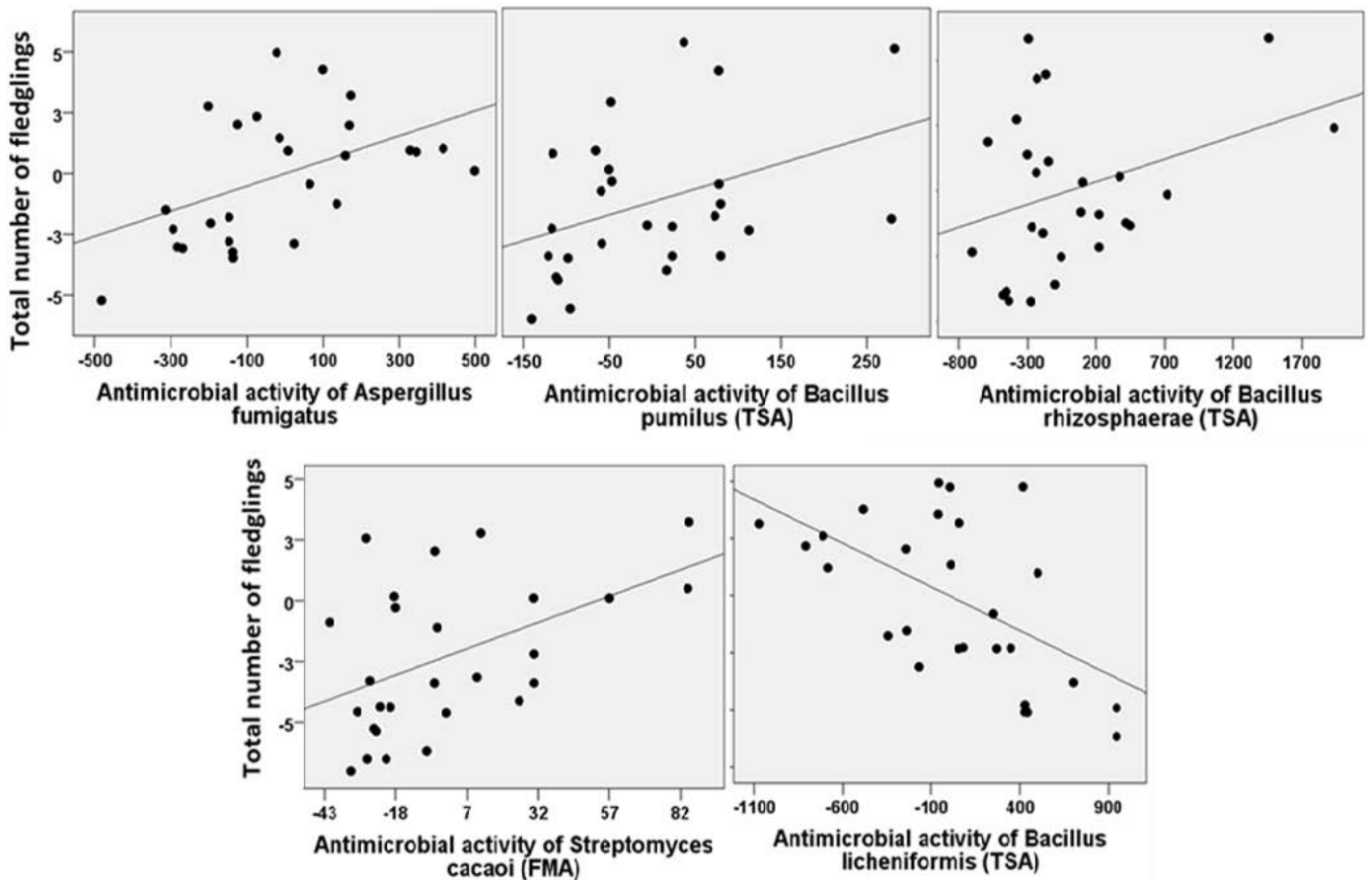


Fig. 4: Total number of barn swallow fledglings in relation to residual antimicrobial activity of *Aspergillus fumigatus*, *Streptomyces cacaoi* in TSA medium, *Bacillus pumilus* in TSA medium, *Bacillus rhizosphaerae* in TSA medium, and *Bacillus licheniformis* in FMA medium. The lines are linear regression lines.

Table 2. Antibacterial activity of fungi and bacteria in relation to total number of barn swallow fledglings in a Generalized Linear Model. The model had the statistics $\chi^2 = 28.116$, $df = 5$, $P < 0.0001$. Effect size is Pearson's product moment correlation coefficient r .

Term	Estimate	SE	t	P	r	z
Intercept	2.440	0.863	2.83	0.0069		
<i>Aspergillus fumigatus</i>	0.004	0.001	2.77	0.008	0.36	0.37
<i>Bacillus pumilus</i> (TSA*)	0.008	0.003	2.79	0.0075	0.36	0.37
<i>Bacillus rhizosphaerae</i> (TSA*)	0.002	0.0005	2.93	0.0053	0.37	0.39
<i>Streptomyces cacaoi</i> (FMA**)	0.023	0.005	4.52	<0.0001	0.53	0.59
<i>Bacillus licheniformis</i> (TSA*)	- 0.003	0.0006	- 4.72	<0.0001	-0.54	-0.61

*TSA (Tryptone Soy Agar medium)

**FMA (Feather Meal Agar medium)

Discussion

The main findings of this study of the diversity of microorganisms on feathers from barn swallow nests was a positive association between the total number of fledglings produced during the breeding season (controlled for the effects of the number of eggs) and the abundance of microorganisms. We did not find a significant relationship between the total number of fledglings and the age of adults. Meanwhile, there was a positive association between the total number of fledglings and the number of bacterial colonies in TSA medium and Simpson's diversity index of bacterial species in FMA medium. In contrast, we found a negative association between the total number of fledglings and the number of fungal species. Meanwhile, there was a positive association between the total number of fledglings and antimicrobial activity of species of bacteria and fungi. In contrast, we found a negative association between the total number of fledglings and one particular species of *Bacillus licheniformis* in FMA medium. Finally, we found a positive relationship between antimicrobial activity of

Aspergillus fumigatus in SDA medium, *Streptomyces cacaoi* in FMA medium, *Bacillus pumilus* in TSA medium and *Bacillus rhizosphaerae* in TSA medium. In contrast, there was a negative relationship between total number of fledglings and antimicrobial activity of *Bacillus licheniformis*.

This study may also have implications for the study of clutch size. Barn swallows often have two clutches, and hence they will have to optimize not only the size, but also the number of clutches (Crick *et al.* 1993). Here we have shown that seasonal production of fledglings reflecting size of both first and second clutches, but also the incidence of second clutches have implications for reproductive output. We have also shown that reproduction is partly determined by the effects of microorganisms on their avian hosts. Because microorganisms may be more abundant during warm and humid weather conditions characterized by those during the second clutch, timing of reproduction may play a crucial role in the optimization of reproduction in the barn swallows. However, this will require experimental verification.

Feathers are largely composed of keratin, a recalcitrant molecule that few organisms can degrade (Gupta *et al.* 2006; McKittrick *et al.* 2012). Bacterial strains isolated from feathers lining nests also differed in their antimicrobial properties. Thus, antimicrobials should primarily affect bacterial strains that use similar resources thereby affecting co-existing strains that are closely related (Madigan *et al.* 2004).

Feathers could be a source of bacteria transferred from eggs to fledglings, and, therefore, they could increase the probability of colonization of fledglings by bacteria. Furthermore, some bacteria grow on feathers (e.g. keratinolytic bacteria) (Peralta- Sánchez *et al.* 2009). Parents can considerably affect the fitness of their offspring through the additive influences of inherited traits and parental effects (Rossiter 1996; Mousseau and Fox 1998). These latter, non-genetic factors may be adaptive phenotypic responses to environmental variation that are invested in

the eggs at a cost thereby increasing offspring growth and survival (Schwabl 1993, 1996; Jarvis *et al.* 1997; Mousseau and Fox 1998; Eising *et al.* 2001).

Feathers used to line nests may reduce the density of microorganisms capable of growing on egg-shells. Therefore, a larger number of feathers would contribute to a more sterile nest environment. However, other sometimes pathogenic bacteria are commonly found growing on feathers (Shawkey *et al.* 2003; Gunderson 2008). Moreover, birds preen to prevent contamination of nest feather by microorganisms (Shawkey *et al.* 2003; Soler *et al.* 2008). However, nest lining feathers are unprotected, which predicts an even larger bacterial load of nest lining feathers in comparison with feathers present on birds.

We found a positive relationship between the total number of fledglings (controlled statistically for the effects of the total number of eggs) and the abundance of bacteria and fungi. The abundance of two fungal taxa (*Absidia corymbifera* and *Penicillium glabrum*) and three bacterial taxa (*Bacillus pumilus*, *Bacillus rhizosphaerae* and *Streptomyces cacaoi*) was positively related to the total number of fledglings, and that may be due to these fungal taxa producing antimicrobial substances that affect other microorganisms (Peralta- Sánchez *et al.* 2009).

This relationship between the abundance of microorganisms and the total number of fledglings may reflect that late laid eggs hatch during warmer and more humid environmental conditions that favor growth and reproduction of microorganisms. In addition, rapid growth and reproduction by microorganisms in late summer may cause accumulation of more microorganisms in the nest. While we found a negative relationship between the total number of fledglings and the abundance of *Trichoderma reesei*, the abundance of this species may be inhibited by antimicrobial substances secreted by other species in the microbial community of a specific nest.

Barn swallows prefer white feathers for lining their nests (Peralta-Sánchez *et al.* 2010), and our results show beneficial effects of this preference and suggest

a new underlying mechanism. An increased density of feather degrading bacteria from feathers lining nests may be costly because they might colonize the feathers of parents during incubation. This possibility together with our results allow us to speculate that barn swallows and other birds may attempt to collect an intermediate proportion of white and pigmented feathers relative to both thermal and bacterial environmental conditions. We found positive relationships between the total number of fledglings and antimicrobial activity of *Aspergillus fumigatus* in SDA medium, *Streptomyces cacaoi* in FMA medium, *Bacillus pumilus* in TSA medium and *Bacillus rhizosphaerae* in TSA medium. Since the nest is considered a suitable environment for growth of different microorganisms, competition between microorganisms may mainly depend on the secretion of antimicrobial substances that would impede the establishment of virulent microorganisms.

In conclusion, we have shown that the total number of fledglings (controlled for the potentially confounding statistical effects of the number of eggs) of barn swallows was significantly correlated with the abundance of specific bacteria and fungi. This effect was not caused by age affecting the total number of fledglings. If the probability of fledging reflects the ability of an individual barn swallow to modify the abundance of microorganisms in its nest environment, we can conclude that microorganisms and hence the microbiome of nests can be affected by the behavior of birds.

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Chapter 4:

Recapture probability, flight morphology and microorganisms

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Zaid Al Rubaiee, Haider Al Murayati and Anders Pape Møller

*Ecologie Systématique Evolution, Université Paris-Sud, CNRS,
AgroParisTech, Université Paris-Saclay, F-91405 Orsay Cedex,
France*

Correspondence to ZAR:

Tel: (+33) 1 69 15 56 96

Fax: (+33) 1 69 15 56 96

E-mail : zaid.iraq@gmail.com

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Article

Recapture probability, flight morphology, and microorganisms

Zaid AL RUBAIEE, Haider AL-MURAYATI, and Anders Pape MØLLER*

Ecologie Systématique Evolution, Université Paris-Sud, CNRS, AgroParisTech, Université Paris-Saclay, F-91405 Orsay Cedex, France

*Address correspondence to Anders Pape Møller. E-mail: anders.moller@u-psud.fr

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Abstract

Microorganisms on and within organisms are ubiquitous and interactions with their hosts range from mutualistic over commensal, to pathogenic. We hypothesized that microorganisms might affect the ability of barn swallows *Hirundo rustica* to escape from potential predators, with positive associations between the abundance of microorganisms and escape ability implying mutualistic effects, while negative associations would imply antagonistic effects. We quantified escape behavior as the ability to avoid capture in a mist net and hence as a small number of recaptures. Because recapture probability may also depend on timing of reproduction and reproductive success, we also tested whether the association between recapture and microorganisms was mediated by an association between recapture and life history. We found intermediate to strong positive relationships between recapture probability and abundance of *Bacillus megaterium*, but not abundance of other bacteria or fungi. The abundance of *B. megaterium* was associated with an advance in laying date and an increase in reproductive success. However, these effects were independent of the number of recaptures. This interpretation is supported by the fact that there was no direct correlation between laying date and reproductive success on one hand and the number of recaptures on the other. These findings have implications not only for predator–prey interactions, but also for capture-mark-recapture analyses of vital rates such as survival and dispersal.

Key words: bacteria, barn swallow, capture-mark-recapture analyses, fungi, *Hirundo rustica*, microbiome.

The microbiome is constituted of the microorganisms on and within a host. The effects of this diversity and abundance of microorganisms range from beneficial over neutral, to highly pathogenic. A number of diseases such as ulcer, obesity, inflammatory bowel disease, diabetes, and vaginal disease among many others have been linked to the microbiome (e.g., Forsythe et al. 2010; Larsen et al. 2010; Fettweis et al. 2011; Greenblum et al. 2012; Foster and Neufeld 2013). The extent to which organisms other than humans suffer from similar effects of perturbations of their microbiome largely remains to be determined.

Microorganisms are ubiquitous in wild and domestic animals and most are mutualistic or commensal (Krieg and Holt 1984; Hubálek 2004; Benskin et al. 2009). Microorganisms have recently been hypothesized to play an important role in predator–prey interactions. For example, uropygial glands that produce antimicrobial

substances are relatively larger in prey species that are more strongly preferred by their main predator, the goshawk *Accipiter gentilis* (Møller et al. 2010a). A subsequent analysis revealed that the abundance of bacteria on the feathers of individual prey was considerably larger than that of individual non-prey (Møller et al. 2012). Microorganisms partly derived from prey also seem to play a role in delayed laying of their goshawk hosts (Møller et al. 2015), perhaps because adults in poor condition start to reproduce later (Soler et al. 2015). This has consequences for hatching success (Møller et al. 2010b) and survival prospects (Møller et al. 2013; Benskin et al. 2015). These effects could be mediated through microorganisms causing disease including increased levels of fever (Møller 2010a), or they could be due to individual hosts in poor condition having higher abundance of pathogenic microorganisms. A 2nd possibility is that they could be acting through flight morphology, or they could

act through microorganisms disrupting flight. Microorganisms may be beneficial by improving the quality of feathers following more detrimental microorganisms that cause degradation of feathers being outcompeted (e.g. Falagas et al. 2008; Ruiz-Rodríguez et al. 2012). For example, such degradation could be controlled by uropygial secretions, or by interference competition with less pathogenic microorganisms. Alternatively, pathogenic microorganisms may have a negative impact on the physiology of their hosts thereby causing a deterioration of their flight ability and hence their ability to out-manuever and ultimately escape predators. A final possibility is that pathogenic microorganisms may be more common in hosts with poor physiological status and hence a weak immune response (Soler et al. 2011).

Behavior is known to be associated with the microbiome. For example, the gut-brain axis provides a direct link between mental health and the microbiome (Forsythe et al. 2010; Foster and Neufeld 2013). Thus, personality but also stress and depression can be linked directly to the community of microorganisms in the gut (e.g., Sudo et al. 2004; Forsythe et al. 2010; Foster and Neufeld 2013). Here we hypothesize that such a link between behavior and the microbiome also accounts for capture probability of free-living birds. Indeed, Soler et al. (2012) have previously suggested and provided evidence for an association between microbiome and personality in birds.

The objective of this study was to test to what extent the recapture probability of individual birds depends on the abundance and diversity of microorganisms. The rationale behind this objective is that birds with more microorganisms suffer from the costs of microorganisms causing disease in their hosts. Alternatively, birds with more microorganisms may suffer from more damage to their plumage caused by feather-degrading microorganisms. Feather-degrading bacteria are ubiquitous, and they are well-known for their ability to degrade feathers in wild and domesticated birds (e.g., Kent and Burt 2016). Even small amounts of damage may significantly increase the risk of birds falling prey to raptors (Møller and Nielsen 2017). Either of these mechanisms may impair flight and escape ability and hence increase the probability of capture and recapture by scientists.

The 2nd objective was to test whether the abundance of microorganisms that predicted recapture probability also predicted the timing of reproduction and reproductive success. We tested whether there was a link between number of recaptures and fitness components, and between number of microorganisms and fitness components. We did so by investigating a population of barn swallows *Hirundo rustica* that has been studied since 1971, and hence all individuals were of known age (Møller 1994). Here we analyzed capture probability and reproductive success in relation to the microbiome of feather nest lining. Barn swallows are migratory insectivorous semi-colonial passerines that mainly breed in the northern hemisphere, while wintering in the tropics or the southern hemisphere. They refurbish or construct a new nest each year, and this nest is provided with feathers collected in the neighborhood of the breeding site during 1–2 weeks before start of laying. The clutch of 3–7 eggs is incubated for 2 weeks by the female and provided with food by both parents for 3 weeks. We investigated the microbiome of the barn swallow by collecting feathers from nests during laying and incubation. Because feathers of the nest lining are derived from the neighborhood before or during laying and incubation, any association between barn swallows and the microbiome of the nest must arise from secondary infection of adult barn swallows from nest lining feathers. Previous studies of microorganisms in the feather lining

of barn swallow nests have shown significant effects of feather composition on fitness components (Peralta-Sánchez et al. 2010, 2011, 2014).

Materials and Methods

Methods

This study was conducted in a population of barn swallows at Kraghede (57°12'N, 10°00'E), Denmark (Møller 1994). Adults were captured at least once weekly by placing mist nets across doors ensuring that the birds breeding indoors were captured when exiting barns. Capture-mark-recapture analyses have shown that more than 98% of all adults are captured or sighted based on color rings (Møller and Szép 2002). All recaptures were also recorded. Adults were provided with an individually numbered aluminum ring, a color ring and an individual pattern of color markings on the white breast feathers. These markings allowed identification of nest owners with a pair of binoculars. We also recorded a number of morphological, behavioral, and parasitological variables before releasing the bird where it was initially captured.

Nesting data

We visited nests at least weekly and recorded laying date of the 1st egg of each clutch, clutch size, and brood size at hatching and fledging. If there were fewer eggs than final clutch size at the 1st visit, laying date was simply date of visit minus number of eggs because 1 egg is laid daily. If the number of eggs at 1st visit equaled subsequent clutch size, laying date was estimated from the size of nestlings at the 1st visit when nestlings were present, using a standard growth curve that links body mass to age.

Nest feather samples

We collected a sample of feathers from the nest during egg laying or early incubation using sterile gloves, while a 2nd feather sample was collected 2 weeks later during incubation in May–June 2014. The feathers from each nest were put in a numbered zip-lock bag and placed in a refrigerator until lab analyses. The two samples were subsequently analyzed separately, and the data derived from each sample were finally combined, since there was no evidence of differences between 1st and 2nd samples.

Analyses of bacteria: bacterial isolation

In the laboratory, we took a small piece of each feather sample and put it in a falcon tube and added an equal volume to the area of the feather (1 cm² feather: 1 mL PBS) with sterile phosphate buffer saline (pH 7.2). After 3 shaking periods of 5 s each in vortex, we performed serial 10-fold dilutions to 10⁻⁴. Free-living bacteria were washed out of the feathers and collected in the PBS solution (Saag et al. 2011), followed by serial 10-fold dilution to 10⁻⁴. To quantify cultivable and feather-degrading bacteria, in duplicate we spread 100 µL of supernatant with a sterile spreader loop on two different growth media. (1) Tryptic Soy Agar (TSA) which is a rich medium on which heterotrophic bacteria can grow thus enabling assessment of total cultivable microorganism load of feathers and (2) Feather Meal Agar (FMA) containing 15 g/L feather meal, 0.5 g/L NaCl, 0.30 g/L K₂HPO₄, 0.40 g/L KH₂PO₄, 15 g/L agar to quantify feather-degrading bacterial loads (Sangali and Brandelli 2000; Shawkey et al. 2003, 2009). Fungal growth was inhibited by adding cycloheximide to TSA and FMA media (Smit et al. 2001). Plates were incubated at 28 °C for 3 days in the case of TSA and for

14 days in the case of FMA. After incubation, we counted the number of colony-forming units (CFU) of each morphotype per plate by using a dissecting microscope, and we distinguished the morphotypes on the basis of colony color, shape, size, and presence or absence of glutinous aspects.

Bacterial density was calculated by multiplying the average number of CFU by the dilution factor and by the original sample volume (Peralta-Sánchez et al. 2014). Plates with each medium type, but without microbial suspension were incubated in order to detect any contamination of media (negative controls).

Bacterial identification

Bacterial identification was done by using molecular characterization with the aid of polymerase chain reaction (PCR) for further genetic analyses. The genomic DNA was extracted from each isolate by using freeze-thaw protocol: By a wooden toothpick transfer, we crush part of a bacterial colony in a 0.5-mL Eppendorf tube containing 50 μ L Tris (10 μ M, pH 8.0). We started freezing and thawing in liquid nitrogen for 1–2 min until completely frozen, then quickly placing the tube in a hot water bath until completely thawed. We repeated the same process for a total of 3 cycles. We made sure that we mixed the sample tube between each cycle. To ensure complete cell lysis we put the tubes in a microwave at 270 W for 5–6 s followed by 10 s of waiting, repeated for 3 times. Then we centrifuged the sample tube for 5 min at 12,000 \times g in a microcentrifuge. We transferred the supernatant that contains the DNA into a new clean and sterile microcentrifuge tube by using a micropipette and discarded the pellet that contains cellular debris. We stored the tubes in -20°C for the PCR.

PCR amplification of bacterial 16SrRNA gene

We used a DNA isolated from samples as a template for PCR to amplify the bacterial 16S rRNA gene by using the reverse primer 16S rDNA-1492R and the forward primer 16S rDNA-27F. PCR was done in a total volume of 25 μ L containing 16 μ L ultrapure water, 4 μ L 5X buffer, 0.4 μ L dNTPs 10 nM, 1 μ L of 10 μ M 27F, 1 μ L of 10 μ M 1492R, 0.2 μ L Go Taq DNA polymerase and 3–5 μ L genomic DNA according to Barghouthi (2011).

Microorganism isolation: fungal isolation

We snipped the feather samples accurately into small pieces and cultured them directly onto Mycobiotic Agar (which is a selective medium of fungi) following standard procedure (Deshmukh 2004), moistened with 0.5 mL of sterilized PBS. The cultures were incubated at $28^{\circ}\text{C} \pm 2$ and checked daily from the 3rd day for fungal growth until a period of 4 weeks. The observed developing mycotic growths were recorded under stereoscopic binocular microscope, and then we individually and directly transferred them onto Sabouraud dextrose agar (SDA) medium with chloramphenicol (50 mg/L). The resulting products were further incubated at $28^{\circ}\text{C} \pm 2$ for 2 weeks to obtain a pure isolate for identification purposes.

Fungal identification

Fungal identification was done by using molecular characterization with the aid of PCR for further genetic analyses. The genomic DNA was extracted from each isolate by using the PowerSoil[®] DNA Isolation Kit (MO BIO). DNA was eluted in a final volume of 100 μ L of 10 mM Tris-HCl, pH 8.5.

Amplification of fungal 18s rDNA gene by PCR

We used a DNA isolated from samples as a template for PCR to amplify the fungi 18S rRNA gene by using the forward primer 18S rRNA-UF1 and the reverse primer 16S rDNA-R1. PCR was done in a total volume of 25 μ L containing 16 μ L ultrapure water, 4 μ L 5X buffer, 0.4 μ L dNTPs 10 nM, 1 μ L of 10 μ M 27F, 1 μ L of 10 μ M 1492R, 0.2 μ L Go Taq DNA polymerase and 3–5 μ L genomic DNA.

Agarose electrophoresis

We performed gel electrophoresis in 1% agarose using 0.5 \times TAE buffer (Tris-Acetate-EDTA) for 25 min at 100 V. The gel was then stained with Gel Red (BIOTIUM) for 30 min. To determine the presence or absence of PCR products and to quantify the size of amplified DNA fragments, we take images under UV lamp by using the photo documentation system IP-010.SD.

DNA sequencing

All the PCR products were sent to Beckman Coulter Genomics, Takeley, Essex CM22 6TA, United Kingdom for DNA Sequencing. The sequence results were processed by using the web-based blasting program, basic local alignment search tool (BLAST), at the NCBI site (<http://www.ncbi.nlm.nih.gov/BLAST>), and the data were compared with the NCBI/Gene-bank database.

Statistical analyses

We used JMP (SAS 2012) for statistical analyses. We report total abundance of colonies and species richness and standard errors for fungi and bacteria. We tested whether the number of recaptures followed a Poisson distribution by fitting a Poisson model to the frequency data.

We tested whether the abundance of fungi and bacteria predicted the frequency of recaptures using a generalized linear model (GLM) with binomial error distribution when the data were classified as presence (1) or absence (0) with a logit link function, with normal error distribution when the data were normally distributed with an identity link function, or with Poisson error distribution when the data were count data with a Poisson distribution and a log link function.

We estimated effect sizes by using Cohen's (1988) guidelines for the magnitude of effects being small (Pearson $r=0.10$, explaining 1% of the variance), intermediate ($r=0.30$, explaining 9% of the variance), or large ($r=0.50$, explaining 25% of the variance).

Results

Communities of microorganisms

We isolated 15 fungal species from the feather lining of barn swallow nests according to identification by PCR technique (Supplementary Table S1). For bacteria, we isolated 10 species from TSA medium (Supplementary Table S2). The different bacterial and fungal taxa were widely distributed across the phylogenetic tree of bacteria and fungi (Supplementary Figures S1a, b).

The number of fungal species ranged from 1 to 9 per sample of feathers from each nest (mean 5.48 ± 0.23 SE), $n=54$. The number of fungal colonies ranged from 20 to 135 (mean 72.09 ± 4.34 SE), $n=54$. The most commonly isolated fungal genus was *Aspergillus* sp.

There were 10 bacterial species isolated from TSA medium. *Bacillus* spp. was the most frequently isolated species. The number of bacterial species (in TSA) ranged from 2 to 10, (mean 4.88 ± 0.25

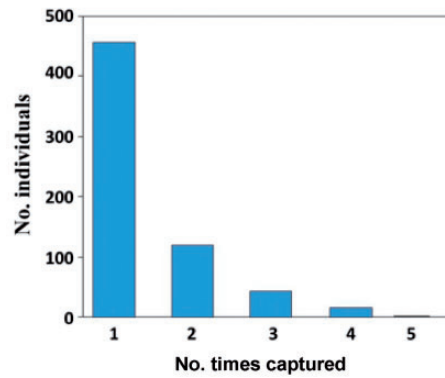


Figure 1. Frequency distribution of a number of captures of adult barn swallows.

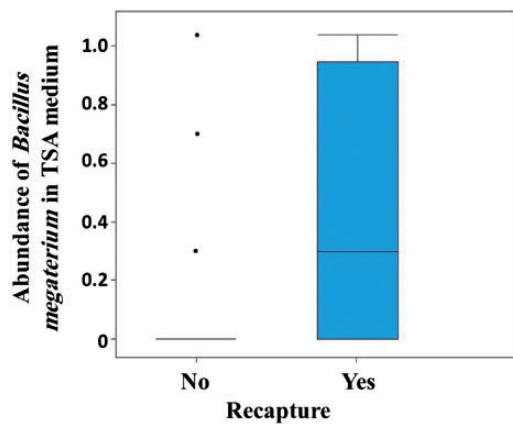


Figure 2. Box plots of the log-transformed abundance of *B. megaterium* in relation to whether adult barn swallows were recaptured or not. Box plots show means, quartiles, 5th and 95th percentiles, and extreme values.

SE), $n = 54$. The total number of bacterial colonies (in TSA) ranged from 10 to 350, (mean 70.13 ± 8.92 SE), $n = 54$.

There were 10 species of bacterial colonies in FMA medium, with *Streptomyces* spp. being the most frequently isolated species. The total number of bacterial colonies in FMA ranged from 0 to 49 (mean 19.15 ± 1.79 SE), $n = 54$. The number of bacterial species (FMA) ranged from 0 to 7 (mean 3.92 ± 0.24 SE), $n = 54$.

Microorganisms and recaptures

A total of 37.1% out of 1506 swallows were recaptured within the same breeding season. The number of recaptures ranged from 1 to 5 with a mean of 0.573 and a variance of 0.808, $n = 754$ barn swallows (Figure 1). This estimate deviated from a Poisson distribution with $\lambda = 0.573$, 95% CI = 0.521, 0.629, $\chi^2 = 67.66$, $P < 0.0001$.

A GLM with binomial error distribution and logit link function showed a significant effect of *Bacillus megaterium* with barn swallows with bacteria being more likely to be recaptured than those without ($\chi^2 = 9.753$, $P = 0.0018$; Figure 2). This model fitted the data (goodness of fit statistic $\chi^2 = 48.395$, $df = 46$, $P = 0.377$). A GLM with Poisson error distribution for count data and a log link function also showed a significant effect ($\chi^2 = 12.140$, $df = 1$, $P = 0.0005$), and this model also fitted the data (goodness of fit statistic $\chi^2 = 49.932$, $df = 46$, $P = 0.320$). Effect sizes were 0.44 and 0.50, respectively, which can be considered large effects. In contrast, there

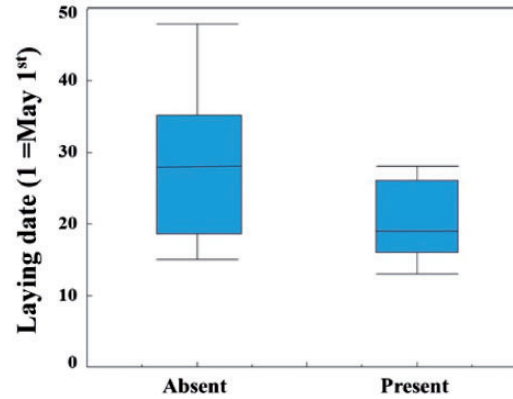


Figure 3. Box plots of laying date (1st = May 1) in relation to whether *B. megaterium* was present or absent. Box plots show means, quartiles, 5th and 95th percentiles and extreme values.

were no significant effects for any of the other bacteria or fungi on recapture probability (analyses not shown). *Bacillus megaterium* had a prevalence of 0.14 and mean 1.36 ± 0.42 SE, range 0–10.

The number of recaptures was not predicted by body mass ($\chi^2 = 0.68$, $df = 1$, $P = 0.678$) or keel length ($\chi^2 = 1.16$, $df = 1$, $P = 0.282$) in a GLM with Poisson distributed number of recaptures and a log link function.

Recaptured barn swallows may more often have attended nests or laid 2nd clutches, hence causing a link between microorganisms and recapture. Barn swallow nests with 2nd clutches had more *B. megaterium* than nests with just a single clutch ($\chi^2 = 6.51$, $df = 1$, $P = 0.011$, estimate (SE) = 0.400 (0.235)). However, there was no significant association between the probability of having a 2nd clutch and the number of times barn swallows were recaptured ($\chi^2 = 1.607$, $df = 1$, $P = 0.21$).

Microorganisms and reproduction

Laying date was not predicted by the number of recaptures ($\chi^2 = 2.17$, $df = 1$, $P = 0.14$), but was predicted by the abundance of *B. megaterium* ($\chi^2 = 6.55$, $df = 1$, $P = 0.011$, estimate (SE) = -6.37 (2.40)). This effect increased when number of recaptures was deleted ($\chi^2 = 13.08$, $df = 1$, $P = 0.0003$, estimate (SE) = 23.13 (1.43)).

There was a significant negative relationship between the abundance of *B. megaterium* and laying date ($\chi^2 = 6.59$, $df = 1$, $P = 0.010$, estimate (SE) = -0.20 (0.007); Figure 3). The number of *B. megaterium* increased with the number of fledglings ($\chi^2 = 10.19$, $df = 1$, $P = 0.0014$, estimate (SE) = 0.790 (0.024); Figure 4). These findings are consistent with a trade-off between anti-bacterial defenses and investment in reproduction, or that individuals with higher reproductive success have more nest lining feathers. However, nest lining feathers appear in nests 2 weeks before start of laying, and we found no significant association between reproductive success and the number of nest lining feathers ($\chi^2 = 0.51$, $df = 1$, $P = 0.31$). There was only a weak relationship between total number of fledglings and laying date in a GLM with Poisson distribution and a log link function ($\chi^2 = 3.86$, $df = 1$, $P = 0.050$, estimate (SE) = -0.686 (0.352)).

There were no cases of nest abandonment or mortality among adult barn swallows, excluding these factors as a cause of variation in capture probability or number of recaptures.

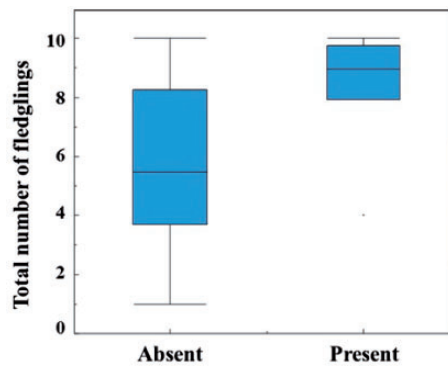


Figure 4. Box plots of the total number of fledgling in relation to whether *B. megaterium* was present or absent. Box plots show means, quartiles, 5th and 95th percentiles and extreme values.

Discussion

The main finding of this study of the diversity of microorganisms on feathers in barn swallow nests was an association between recapture probability and the abundance of the microorganism *B. megaterium*, but not any other species of microorganism. Barn swallows with more *B. megaterium* on nest lining feathers had a higher probability of being recaptured and hence falling prey to a potential predator than individual hosts with fewer microorganisms on feathers in their nests. These findings imply that the bacterial environment may play a role in predator–prey interactions (Møller et al. 2010b, 2012). We also showed that the abundance of *B. megaterium* decreased significantly with advanced laying and that the abundance of *B. megaterium* increased with increasing reproductive success measured as the number of fledglings produced during the year.

The frequency of recaptures during the same season was highly skewed with most barn swallows never being recaptured again once captured. This skew in recapture frequency is not due to lack of attempts to escape since barn swallows repeatedly fly up to the mist net and only in the very last moment turn off abruptly to avoid capture. Here we have shown that microorganisms seem to play a role in whether individuals are recaptured. Because the abundance of *B. megaterium* on feathers used as nest lining in the 1st clutch was associated with an increase in the probability of recapture, we can consider the effects reported here to be parasitic. Studies experimentally removing uropygial glands showed dramatic increases in the abundance of microorganisms on the plumage (Jacob and Ziswiler 1982). Such an increase in the abundance of microorganisms may directly impair aerodynamics since even small increases in the abundance of microorganisms on the surface of the plumage may cause turbulence. Is it feasible that recaptures directly affected the abundance of microorganisms? Perhaps both recapture and microorganisms were associated with a 3rd variable. We cannot imagine any mechanism by which such an effect could arise. However, less healthy birds may in general have higher bacterial loads and higher recapture probability. We consider this possibility unlikely given that there was no association between probability of recapture and body mass or body mass adjusted for keel length, despite this measure of body condition having been shown to repeatedly correlate with phenotypic traits in barn swallows (Møller 1994). Another possibility is that recaptured birds more often attended nests or laid 2nd clutches. Indeed, nests with 2nd clutches had more *B. megaterium* than nests with just a single clutch. However, there was no significant association between the probability of having a 2nd clutch

and the number of times recaptured. This refutes these alternative explanations.

Bacillus megaterium, which accounted for an increase in recapture probability, is one of the biggest known bacteria (cell length of up to 4 μm and a diameter of 1.5 μm , i.e., it has an up to 100-times larger volume than *Escherichia coli*). It is a common soil bacterium that during the past years has become popular in biotechnology (Bunk et al. 2010). *Bacillus megaterium* strains are known to produce antimicrobial antibiotics (Malanicheva et al. 2012). *Bacillus megaterium* is also known to control microorganisms such as those involved in plant disease (Safiyazov et al. 1995). The abundance of *B. megaterium* was associated with an advance in laying date and an increase in reproductive success. Reproductive success and laying date were only weakly correlated, implying that the 2 variables were independently associated with *B. megaterium*. These effects were independent of the number of recaptures. We hypothesize that barn swallows trade early investment in reproduction against anti-bacterial defenses. This interpretation is supported by the fact that there was no direct correlation between laying date and reproductive success on one hand, and the number of recaptures on the other. Alternatively, barn swallows may facilitate colonization and growth of potentially beneficial bacteria in their nests.

Barn swallows prefer white feathers over other colors for lining their nests (Peralta-Sanchez et al. 2010, 2011, 2014). Feathers with white coloration have positive effects on the bacterial communities of eggshells by improving hatching success in the host (Peralta-Sanchez et al. 2010, 2011, 2014). Most bacteria living on feathers are benign and effectively prevent pathogenic bacteria from establishment and replication (e.g. Shawkey et al. 2003, 2009). We still need to assess whether that also applies to *Bacillus megaterium*, or whether the relationship between recapture probability and abundance of microorganisms is simply due to direct effects of *B. megaterium* on locomotion.

The findings reported here have potential future prospects. First, the findings suggest that barn swallows are more likely to be recaptured and, by inference, more likely to fall prey to a predator when having a larger abundance of specific microorganisms in their nests. We are unaware of any alternative explanation for the patterns described in the present paper. Obviously, this hypothesis requires a direct test by comparison of the abundance of these specific microorganisms in conspecific prey and non-prey. It also requires a direct experimental test of whether the elimination of these microorganisms reduces the probability of recapture.

Second, studies of the demography of wild organisms rely on random samples of individuals in order to obtain unbiased estimates of the demographic parameter based on capture-mark-recapture analyses (Lebreton et al. 1992). Several recent studies have demonstrated violations of this assumption (Garamszegi et al. 2009; Biro and Dingemanse 2009; Møller 2010a). For example, Garamszegi et al. (2009) showed for collared flycatchers *Ficedula albicollis* that individuals with a bold personality were over-represented, while shy individuals were less likely to be captured than expected by chance. The associations between capture probability and microorganisms that we have reported here may suggest that the microbiome plays a direct role in the estimation of demographic parameters. Indeed, Soler et al. (2012) have suggested an association between microbiome and personality in birds. We have previously shown that flight initiation distance by individual barn swallows is linked to the probability of falling prey to sparrowhawks *Accipiter nisus* (Møller 2014) and that brain size predicts capture probability (Møller 2010b). These findings and those of the present study suggest that

the microbiome of nests, probability of predation, personality and cognitive skills are all related to each other. The present study assumes that the microbiome of nest lining feathers is a proxy for the microbiome of adult feathers. This assumption is likely given that the microbiome of nest lining feathers must derive from adult barn swallows since feathers are absent from nests until at most a couple of weeks before the start of egg laying. This interpretation is supported by human studies linking personality to the gut microbiome (Forsythe et al. 2010; Foster and Neufeld 2013).

In conclusion, we have shown that the probability of recapture of adult barn swallows was significantly correlated with the abundance of a specific bacterium, *B. megaterium*. If the probability of capture reflects the probability of falling prey to the capturer, we can conclude that bacteria and hence the microbiome is involved in predator-prey interactions.

Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

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Chapter 5:

Microorganisms, flight ability and risk of predation

Zaid Al Rubaiee

*Ecologie Systématique Evolution, Université Paris-Sud, CNRS,
AgroParisTech, Université Paris-Saclay, F-91405 Orsay Cedex, France*

Word count: 2902

Correspondence to ZAR:

Tel: (+33) 1 69155688

Fax: (+33) 1 69155688

E-mail : zaid.iraq@gmail.com

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Abstract

Predation is a powerful selective force with important effects on behaviour, morphology, life history, and evolution of prey. The effects of parasites on their hosts may consist of changes in body condition, health status and ability to escape from or defend themselves against predators. Prey gain immediate benefits from hiding and escaping from predators. However, once a prey individual has been detected, it can rely on a diversity of means of escape from the pursuit by the predator. Here we tested whether prey of a common raptor differed in terms of microorganisms from non-prey recorded at the same sites using the goshawk *Accipiter gentilis* and its avian prey as a model system. Furthermore, the probability of having damaged feathers increased with the number of fungal colonies, and in particular the abundance of *Myceliophthora verrucos* and *Schizophyllum* sp. was positively correlated with the probability of having damaged feathers. The main finding of this study of diversity of fungi on feathers of goshawk prey was a positive association between the probability of falling prey to the raptor and the presence and the abundance of fungi. Goshawk prey with a specific composition of the community of fungi had higher probability of falling prey to a goshawk than individual hosts with fewer fungi. These findings imply that microorganisms may play a significant role in predator-prey interactions. In addition, we found a significant effect of rate of feather growth on goshawk prey with more fungi being more likely to be depredated. These findings are consistent with the hypothesis that survival and flight efficiency are related to abundance and diversity of fungi.

Keywords: bacteria, fungi, goshawk, microorganisms, molted feathers, prey.

Introduction

Predation is known to be a powerful selective force with important effects on the behaviour, morphology, life history, and evolution of prey (Curio 1976, Endler 1986, Caro 2005). Due to the intensity of predation on many different organisms, there is an opportunity for evolutionary changes in prey with feedback on predators, which may select for further changes in prey resulting in coevolution (Vermeij 1987). However, prey often interact indirectly with predators because a large diversity of parasites may interfere with the interaction between predators and prey (Møller 2008).

The factors that determine the risk of predation for individuals, populations and prey species are poorly known because this requires knowledge of observed and expected risks of predation. Still many studies have investigated the process of predation in great detail, giving rise to a general knowledge of functional and numerical responses with consequences for ecological interactions (Holling 1965, Crawley 1992).

The effects of parasites on their hosts may consist of changes in body condition, health status and ability to escape from or defend themselves against predators. Prey gain immediate benefits from hiding and escaping from predators. However, once a prey individual has been detected, it can rely on a diversity of additional means of escape from the pursuit by the predator. These include locomotion (Irschick and Garland 2001, Møller *et al.* 2004), manoeuvrability (Venable 1996) and the ultimate fight against a predator once captured (Högstedt 1983). Prey species that have been subject to persistent predation attempts across many generations have evolved early escape behaviour that is facilitated by muscle mass, ability to accelerate and sustained running or flight.

Microorganisms can have significant effects on the quality of feathers because of feather degradation (Shawkey *et al.* 2009, Ruiz-Rodríguez *et al.* 2009, Jacob *et al.* 2014, Leclaire *et al.* 2014) and hence the flight ability of prey. The ultimate test of the fitness consequences of microorganisms for prey is whether

the probability that an individual survives or falls prey to the predator depends on the abundance or diversity of microorganisms. Any damage to the plumage that reduces the efficiency of flight would be costly and hence selected against, with damage to the plumage reaching such extremes as complete degradation and hence disappearance of barbules, barbs or even loss of entire segments of feathers (e.g. Ruiz-Rodriguez *et al.* 2009, Kim *et al.* 2001, Møller *et al.* 2013, Onifade *et al.* 1998). Because feather-degrading microorganisms naturally occur in soil, prey species that are foraging on the ground have been suggested to suffer the most from feather degradation by microorganisms (Burt and Ichida 1999).

Although predation is a common cause of mortality, there are relatively few studies investigating the factors that contribute to the risk of predator-induced mortality. As an example, woodpigeons *Columba palumbus* and other species belonging to this family are common prey believed to have been strongly selection to avoid predation (Tomialojć 1978, Kenward 1979). Previous studies of birds have shown that prey species that are disproportionately likely to be captured by predators have relatively large uropygial glands that may better control or reduce the abundance of microorganisms in the plumage and on the skin of prey (Møller *et al.* 2010). This exocrine gland produces antimicrobial substances that have been shown to have a negative effect on the abundance of microorganisms (Jacob and Ziswiler 1982, Martin-Vivaldi *et al.* 2010). Thus, a relatively larger gland for a given body size should provide superior protection against feather-degrading bacteria (Shawkey *et al.* 2003, Ruiz-Rodriguez *et al.* 2009). In addition, an intraspecific study showed that prey individuals of the goshawk *Accipiter gentilis* had feathers with more bacteria than feathers that had been moulted and hence belonged to individuals that were still alive (Møller *et al.* 2010). Microorganisms may have strong negative effects on health and fitness of their hosts. They are a common cause of disease or death in humans and domestic and wild animals (Beaver and Jung 1985, Evans and Brachman 1998, Strauss and Strauss 2002,

Benskin *et al.* 2009), and several defence mechanisms have evolved to cope with such infection.

Generally, birds frequently carry keratinophiles passively on intact feathers. Some of these keratinophilic fungi are well-known pathogenic dermatophytes, and they are known to cause superficial cutaneous infections (dermatophytoses) of keratinised tissues (skin, feather, hair and nail) of humans and animals (Deshmukh 2004).

Moult is the process of loss of old feathers and replacement with new ones (Farner *et al.* 2012). Most bird species moult their plumage annually and replace the feathers during a period that may reach half a year in the wood pigeon (Murton 1965). The term “ptilochronology” used for the width of daily growth bars of feathers as a signal of a bird’s nutritional condition (Grubb 1989). Because feathers are very important for protection, locomotion, and thermoregulation (Murphy *et al.* 1988), replacement of feathers should proceed as fast as possible, but at a cost with speed of moult being traded against quality of new feathers (Pap *et al.* 2008). If there is an optimal speed of moult, we can expect that daily growth increments of feathers will depend on microorganisms. The reason is that the presence of microorganisms may lead to a trade-off between feather growth and antimicrobial defence.

The objectives of this study were to test (1) whether birds with high loads of microscopic fungi are more likely to fall prey to predators than those with few. This question is based on the assumption that more fungi and/or a higher diversity of fungi constitute a greater cost to their hosts. Here we tested this prediction by investigating the relationship between risk of predation and the abundance of fungi on feathers from wood pigeon *Columba palumbus*, Eurasian jay *Garrulus glandarius*, Song thrush *Turdus philomelos* and blackbird *Turdus merula* that are preferred prey species of the goshawk in our study site in Denmark. In addition, we tested (2) whether the size of daily feather growth increments was related to diversity and abundance of fungi in the plumage, and (3) whether feathers that

developed faster had more damage to their plumage. While fungi are common microorganisms (Madigan *et al.* 2010), there are no studies investigating the relationship between diversity and abundance of fungi and fitness components of prey.

Material and methods

Study organisms

The goshawk *Accipiter gentilis* is a territorial predator (Cramp and Simmons 1979), and there is a distinct division of sex roles during the breeding season. The smaller male supplies food to the larger female and their chicks, while females incubate the eggs and defend the nests and chicks. Females are about three times heavier than males. The goshawk is adept at flying through closed forests, although it prefers to nest in tree-tops where clear level access is afforded by a stream course, rides, fire-breaks, or natural glades. It favours areas where woodland is interspersed with fields or even wetlands. Ordinarily it flies low, close to trees or bushes, but during the breeding season it soars to several hundred meters (Cramp and Simmons 1979).

The goshawk is a solitary hunter, with swift, aggressive and adroit pursuit flights, usually for only short distances, up to 500 m (Brüll 1964). It takes advantage of any cover to approach prey in an attempt to take it by surprise. Goshawks may also watch from a hidden perch. Normally it gives up if the attack is unsuccessful, although occasionally it makes a second attempt. Prey are caught in flight, on nests, branches, and on the ground (Schnurre 1934, Dementiev and Gladkov 1951, Pielowski 1961, Brüll 1964). It will stoop from great height in an attempt to catch prey in the air (Dementiev and Gladkov 1951, Kollinger 1974, Opdam *et al.* 1977). Prey are caught and killed with claws and then usually brought to cover and plucked and eaten on the ground or a small elevation, or especially in the nesting area on an exposed branch or an old nest (Opdam *et al.*

1977). Considerable size differences in the sexes are also reflected in average weight of prey; 277 g in males and 505 g in female during March in a forest area near Nijmegen, Netherlands (Opdam 1975).

The diet varies according to availability of prey and can be extremely diverse (Opdam *et al.* 1977). As opportunist, it appears to take any prey of convenient size, although predator size to some extent determines prey size, and the method of hunting determines vulnerability of potential prey. In Europe, mainly grouse, partridges and pheasants, pigeons, crows, thrushes are common prey (Bittera 1915, Uttendörfer 1952).

Study sites and field work

We collected feathers of woodpigeon, Song thrush, Eurasian jay and blackbird. Two categories of feathers were collected at the same time of the year from the same forests: feathers from plucking sites near 50 nests of goshawks in Northern Vendsyssel (57°10'–57°40'N, 9°50'–10°50'E), Denmark, during April–August 2009 with a mean date of May 16 (SE = 2). Traditional plucking sites were found near nests where goshawk males bring their prey before presenting it for the offspring or the incubating, brooding or attending female. Furthermore, molted feathers were collected from the same sites from birds that clearly were alive (because they were molting). All feathers collected were only from recent prey not more than a couple of days old as reflected by the soft structure of feathers. Feathers rapidly become stiff with rain and exposure to weather. We avoided problems of contamination of feathers by nest contents by only including feathers found on the ground, as were the samples of feathers from live molting birds.

Fungal isolation

We isolated the fungal species and quantified their abundance on prey feathers and molted feathers. Then we identified these by using the PCR technique.

Feathers were cultured directly onto Sabouraud dextrose agar with chloramphenicol (SDA), and moistened with 1 ml of sterilized PBS. The cultures were incubated and examined daily from the third day for fungal growth over a period of four weeks. The observed developing mycotic growths under stereoscopic binocular microscope were individually and directly transferred onto Sabouraud dextrose agar with chloramphenicol (50 mg/l). The resulting products were further incubated for two weeks to obtain pure isolates for identification purposes.

Fungal identification

All fungal strains were grown on Sabouraud's dextrose agar. A small amount of mycelium was suspended in 200 μ L 10 mM Tris-HCl, pH 8.0 in an Eppendorf tube (1.5 mL), and stored in a freezer (-20°C) for further processing.

For molecular identification, genomic DNA was isolated from the fungal strains by using the PowerSoil® DNA Isolation Kit (MO BIO). DNA was eluted in a final volume of 100 μ l of 10 mM Tris-HCl, pH 8.5.

PCR amplification was performed in 20-30 μ L reaction volume containing 25 μ l assay buffer containing 1.5 mM MgCl₂, dNTP (10mM) 0.5-1 μ L, 0.5-1 μ L of each 0.2 mM primer FR1, 2.5 μ l forward primer UF1, Go Taq® G2 DNA polymerase (Promega Madison, WI USA) (1.25 μ l) 0.5-1 μ L, and DNA sample 3-5 μ L. The DNA genomic was amplified with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation for 15 s at 94°C, annealing for 30 secs at 55°C and extension for 1.30 min at 72°C, respectively, and the final extension was carried out at 72°C for 7 min.

Slants of nutrient agar and 40% glycerol stocks were prepared from identified pure culture and stored at 4 and -80°C, respectively, for medium and long-term preservation.

To visualize and determine the presence or absence of PCR products and to quantify the size of amplified DNA fragments, we performed gel electrophoresis in 1% agarose using 0.5 X TAE buffer (Tris-Acetate-EDTA) for 25 min at 100V. The gel was then stained with Gel Red (BIOTIUM) for 30 min. Images were taken under UV lamp by using the photo documentation system IP-010.SD.

PCR products were sent to Beckman Coulter Genomics, Takeley, Essex CM22 6TA, United Kingdom for DNA sequencing. The sequence results were processed by using the web-based blasting program, basic local alignment search tool (BLAST), at the NCBI site (<http://www.ncbi.nlm.nih.gov/BLAST>), and the data were compared with the NCBI/Genbank database.

Statistical analyses

We used the statistical software JMP (SAS 2012) to make all statistical analyses. We \log_{10} -transformed all fungal counts after addition of a constant of one to normalize the data. We report total abundance of colonies and species richness for fungi.

We tested whether the abundance of fungi predicted predation using generalized linear models using a binomial response variable with a logit link function. Likewise, we tested whether daily feather growth increments and damage of feathers differed between prey and non-prey using a binomial response variable with a logit link function.

We estimated effect sizes by using Cohen's (1988) guidelines for the magnitude of effects being small (Pearson $r = 0.10$, explaining 1% of the variance), intermediate ($r = 0.30$, explaining 9% of the variance) or large ($r = 0.50$, explaining 25% of the variance).

Results

Communities of microorganisms

We isolated 27 fungal species from feathers of goshawk prey and molted feathers of the same species collected near goshawk nests according to identification by PCR technique (Fig. S1). The number of fungal colonies ranged from 0 to 9 with a mean of 2.563 (SE = 0.204), N = 87. The number of fungal species ranged from 0 to 3, mean = 1.195 (SE = 0.085), N = 87. Species richness for fungal species, means, SE and ranges of abundance of fungal species isolated are presented in Table S5.

Fungi and risk of falling prey to goshawks

Across all taxa of fungi, there was a significant positive relationship between the likelihood of birds being preyed upon and the mean number of fungi (Fig. 1; $\chi^2 = 6.18$, df = 1, p = 0.013, estimate (SE) = 2.65 (1.17)). In addition, there was an effect of prey species on the mean number of fungi ($\chi^2 = 16.58$, df = 3, p = 0.0009). Prey had almost 30% more fungal colonies on their feathers than non-prey that had molted their feathers in the same area and hence were still alive. The interaction between species and mean number of fungi was not significant ($\chi^2 = 3.90$, df = 3, p = 0.27), and hence it was deleted from the model.

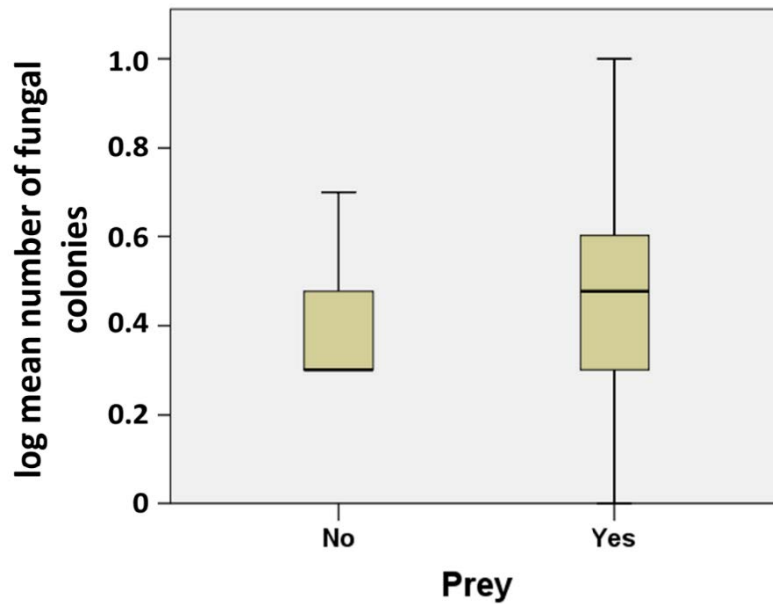


Fig. 1: Box plots of mean number of fungal colonies in relation to whether individuals were preyed upon or not. Box plots show means, quartiles, 5- and 95-percentiles and extreme values.

A GLM with binomial error distribution showed a relationship between the likelihood of birds being preyed upon and the number of colonies of *Aspergillus niger* on feathers (Fig. 2; $\chi^2 = 5.29$, $df = 1$, $p = 0.021$, estimate (SE) = 2.46 (1.20)), and there was a significant effect of prey species ($\chi^2 = 19.39$, $df = 3$, $p = 0.0002$). The interaction between species and the number of colonies of *Aspergillus niger* was not significant ($\chi^2 = 1.92$, $df = 3$, $p = 0.59$), and therefore it was deleted from the model.

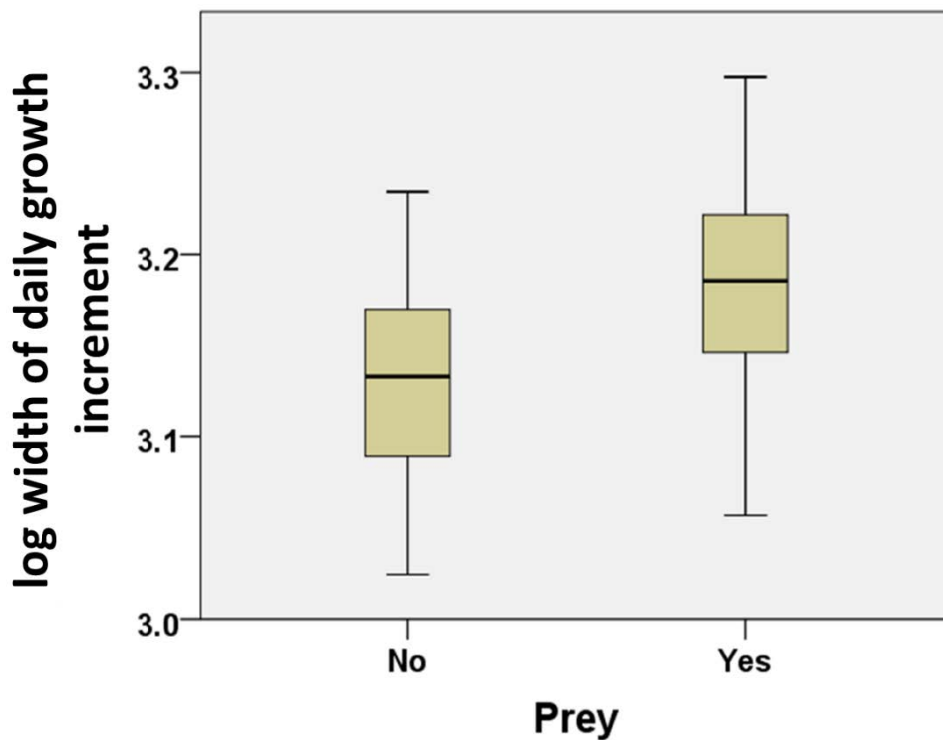


Fig. 3: Box plots of daily growth band width of feathers (mm) in relation to whether individuals were preyed upon or not. Box plots show medians, quartiles, 5- and 95-percentiles and extreme values.

A GLM with normal error distribution showed a significant difference in band width between fungal taxa (*Aspergillus fumigatus*, *Chaetomium elatum*, *Chaetomium globosum*, *Myceliophthora thermophila*, *Myceliophthora verrucos* and *Thermomyces lanuginosus*) (Table 1; $\chi^2 = 30.62$, $df = 6$, $p < 0.0001$). The effect of prey species was not significant, and, therefore, it was excluded from the best model ($\chi^2 = 0.99$, $df = 3$, $p = 0.80$). We found an overall mean effect size weighted by sample size of 0.38, $SE = 0.03$, 95% confidence intervals 0.31 to 0.45, Wilcoxon signed rank test = 41718, $P < 0.0001$ (Table 1). This suggests that the mean weighted effect size for the relationship between the width of daily growth increments of feathers and the abundance of different fungal taxa is of an intermediate magnitude (Cohen 1988).

Table 1. Relationship between the width of daily growth increments of feathers (response variable) and the abundance of different fungal taxa (predictor variables). The GLM model with binomial error distribution had the statistics ($\chi^2 = 30.624$, $df = 6$, $P < 0.0001$). Effect size is Pearson's product moment correlation coefficient r and the z-transformation of r .

Term	Estimate	SE	χ^2	r	z	P
Intercept	2.46	0.01	509.31			<.0001
<i>Aspergillus fumigatus</i>	-0.28	0.09	9.70	0.38	0.40	0.0018
<i>Chaetomium elatum</i>	0.35	0.10	11.56	0.41	0.44	0.0007
<i>Chaetomium globosum</i>	-0.26	0.07	13.76	0.45	0.48	0.0002
<i>Myceliophthora thermophila</i>	0.10	0.04	6.52	0.31	0.32	0.0106
<i>Myceliophthora verrucos</i>	0.18	0.06	5.56	0.29	0.29	0.0184
<i>Thermomyces lanuginosus</i>	-0.46	0.16	7.94	0.34	0.36	0.0048

Damage to feathers and fungi

The probability of having damaged feathers increased with the number of fungal colonies (Fig. 4; $\chi^2 = 4.14$, $df = 1$, $p = 0.042$, estimate (SE) = 4.58 (2.72)). Two fungal taxa were positively correlated with the probability of having damaged feathers (*Myceliophthora verrucos*: $\chi^2 = 4.13$, $df = 1$, $p = 0.042$, estimate (SE) = 4.84 (3.44); *Schizophyllum* sp.: $\chi^2 = 4.13$, $df = 1$, $p = 0.042$, estimate (SE) = 5.62 (4.00)).

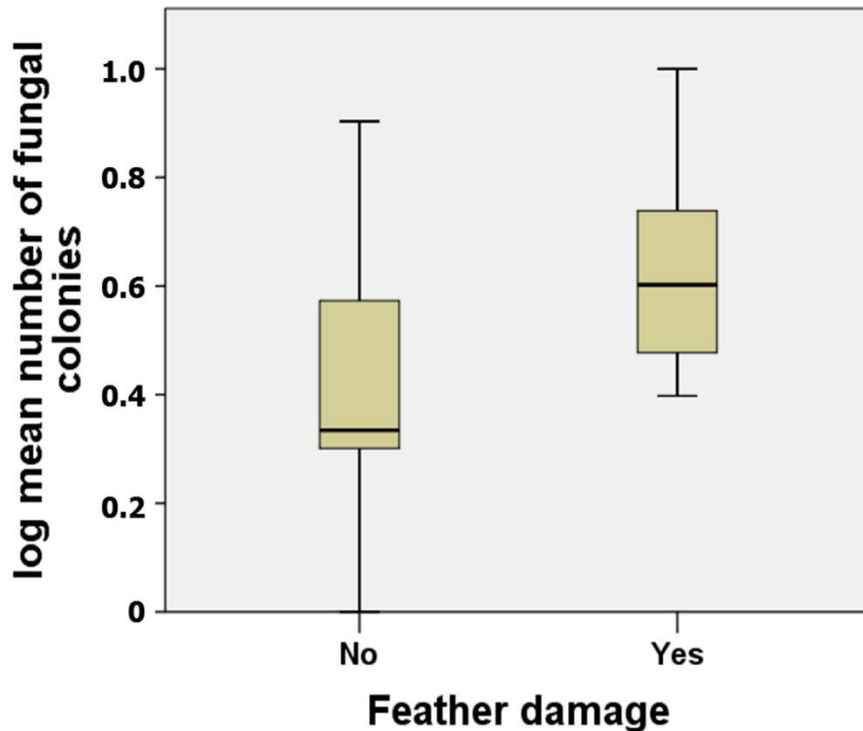


Fig. 23: Box plots of total number of fungal colonies in relation to damage of feathers.

Box plots show medians, quartiles, 5- and 95-percentiles and extreme values.

Discussion

The main findings of this study were that risk of falling prey to goshawks was related to abundance of fungi. Goshawk prey with a specific composition of the community of fungi had higher probability of being preyed upon than individual hosts with fewer microorganisms. We found a significant association between the mean number of fungal colonies and whether feathers derived from prey or non-prey. In particular, the abundance of *Aspergillus niger* was the best predictor of whether an individual was preyed upon or not. These findings imply that microorganisms may play a role in predator-prey interactions. In addition, we found a significant difference in mean daily growth increments between prey and non-prey with feathers growing faster in prey. Furthermore, feathers with more fungi differed in size of daily growth increments, and that feathers with larger growth bars were more likely to fall prey to predators. Finally, we found a

significant difference in feather damage related to the number of fungal colonies, with a positive relationship between abundance of *Myceliophthora verrucos* and *Schizophyllum* sp. and feather damage.

Feathers of wood pigeons, jays and blackbirds differed in abundance of fungi between prey and live individuals that moulted feathers in the same area. These findings are consistent with a previous study by Møller *et al.* (2012) showing that goshawks are differentially successful in their capture of prey when prey individuals harbor a large abundance of bacteria on their plumage. Here we have extended this effect to another major group of microorganisms; fungi. This is the first study to show a link between risk of predation and infection of prey with fungi.

Microorganisms of feathers are generally thought not to be harmful (Gunderson 2008, Shawkey *et al.* 2009), although they can cause breakage of feather barbs. Hence, the abundance of microorganisms on feathers is probably a risk factor associated with probability of predation. In wild birds, pathogenic microorganisms are common (Hubalek and Halouzka 1996, Hubalek 2004). Thus, predators should differentially capture prey infected by pathogenic microorganisms because that will determine whether an individual survives a predatory pursuit. Here, we found that the abundance of fungi on feathers was elevated in prey of goshawks compared to non-prey.

There was a large difference in abundance of *Aspergillus niger* between prey and non-prey. *Aspergillus niger* is a filamentous fungus that is regarded as one of the most important industrial microorganisms that produces many enzymes such as amylases (Mitidieri *et al.* 2006), cellulase and xylanase (Couri *et al.* 2000, Farinas *et al.* 2010), peptidases (Morya *et al.* 2012) and phytases (Bhavsar *et al.* 2011). For several decades, enzymes from *Aspergillus niger* have been used in food production, and there are a few reports of production of keratinase by *Aspergillus niger* strains (Lopes *et al.* 2008). Hence, we hypothesized that *Aspergillus niger* may affect feathers of goshawk prey by reducing their flight

efficiency. Many microorganisms have a negative impact through altered feather integrity due to degradation of feather barb keratin. That is also the case for keratinolytic fungi, which may lead to reduced fitness of their bird hosts by reduction in thermoregulation and flight maneuverability making them more likely to be depredated (Swaddle *et al.* 1996; Clayton 1999; Shawkey *et al.* 2007; Scott and McFarland 2010).

Feather quality can affect individual fitness in term of mate choice, late arrival from migration, delayed timing of reproduction during the breeding season and escape from predators (Hedenström 2003; Kose and Møller 1999; Pap *et al.* 2005). The rate of feather growth and feather quality can be affected by many factors like nutritional status, physiological stress, body condition and disease (DesRochers *et al.* 2009, Moreno-Rueda 2010, Vágási *et al.* 2012). Here, we found a positive relationship between the rate of feather growth and the risk of falling prey to a common raptor. These costly effects of rapid moult are condition-dependent, so that only birds in prime condition could make a fast moult without compromising their feather quality (Vágási *et al.* 2012). Here, we found a significant negative relationship between the width of daily growth increments in feathers and abundance of fungal species. In an experimental study of barn swallow offspring Romano *et al.* (2011) showed that individuals with parasite infection produced feathers of lower quality. Since many fungal species are pathogenetic, we can suggest that the fungal infection in birds during feather growth may cause variation in feather quality.

Feather damage could be used as an indicator of feather quality, since we found a positive relationship between the degree of feather damage and the number of fungal colonies. Among 27 fungal species 14 (52%) secrete keratinase and hence have the ability to degrade feathers (Table S6a). Furthermore, there were 16 (59%) pathogenic fungal species (Table S6b) due to eliciting an immune response, and this is costly in terms of energy requirements, thereby reducing feather quality.

The results reported here require experimental manipulation of the abundance of fungi in feathers for a formal verification. This could be done by use of antimicrobial substances on adult feathers. Another possibility is to treat adult feathers with antimicrobial agents to terminate the negative effect of feather-degrading fungi.

In conclusion, we have shown that the probability of individual birds falling prey to a predator increased with the mean number of fungal colonies on feathers. In addition, we found a significant relationship between the risk of predation and the abundance of *Aspergillus niger*. Moreover, the probability of individuals falling prey to a predator was significantly positively correlated with the width of the growth bars of feathers from goshawk prey. Finally, we found a positive relationship between damage of feathers and the number of fungal colonies. There were two fungal species (*Myceliophthora verrucos* and *Schizophyllum* sp.) were correlated significantly with damage of feathers. Hence, we conclude that the abundance of fungi on the feathers of goshawk prey and hence their microbiome is involved in predator-prey interactions.

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Supplementary Materials (SM)

Table S1. Identity, range in abundance, mean abundance and standard deviation in abundance of fungi from feathers obtained from nests of barn swallows.

Fungal species			
Fungal species	Range	Mean	SD
<i>Aspergillus flavus</i>	1-17	7.31	0.81
<i>Absidia corymbifera</i>	2-29	12.16	1.04
<i>Mucor circinelloides</i>	2-14	7.25	0.55
<i>Aspergillus niger</i>	2-14	6.76	0.57
<i>Aspergillus fumigatus</i>	3-85	34.22	2.73
<i>Penicillium glabrum</i>	2-24	6.83	2.37
<i>Penicillium piceum</i>	8	8	0
<i>Thermomyces lanuginosus</i>	0-4	4	0
<i>Auxarthron umbrinum</i>	3-6	4.5	0.86
<i>Trichoderma reesei</i>	2-33	13.38	1.76
<i>Aspergillus terreus</i>	2-11	6.5	2.59
<i>Arachnomyces minimus</i>	3	3	0
<i>Aspergillus candidus</i>	2	2	0
<i>Myceliophthora thermophila</i>	2	2	0
<i>Aphanoascus fulvescens</i>	2	2	0

Table S2. Identity, range in abundance, mean abundance and standard deviation in abundance of bacteria from feathers in nests of barn swallows cultivated in TSA and FMA medium.

Bacterial species (TSA)			
Bacterial species	Range	Mean	SD
<i>Paenibacillus cookii</i>	1-23	8.36	1.51
<i>Bacillus licheniformis</i>	3-200	28.51	5.38
<i>Bacillus pumilus</i>	1-14	6.33	0.81
<i>Bacillus mycoides</i>	1-25	7.33	2.08
<i>Bacillus megaterium</i>	1-23	9.41	1.49
<i>Planococcus sp.</i>	1-40	10.41	4.95
<i>Staphylococcus Saprophyticus</i>	1-38	6.72	2.29
<i>Lysinibacillus fusiformis</i>	1-30	9.88	2.24
<i>Streptomyces violascens</i>	1-30	9.57	1.60
<i>Bacillus rhizosphaerae</i>	1-100	19.82	3.76

Bacterial species (FMA)

Bacterial species	Range	Mean	SD
<i>Streptomyces cinereorectus</i>	1-23	5.27	0.78
<i>Micromonospora aurantiaca</i>	1-4	1.71	0.20
<i>Rhodococcus sp.</i>	3	3	0
<i>Kocuria sp.</i>	1-20	5.36	1.23
<i>Bacillus anyloliquefaciens</i>	1-17	5.57	0.58
<i>Methylobacterium mesophilicum</i>	1-5	2.50	0.56
<i>Streptomyces thermoviolaceus</i>	1-23	7	0.91
<i>Staphylococcus lentus</i>	1-20	5.75	3.10
<i>Streptomyces cacaoi</i>	1-8	3.14	0.65
<i>Streptomyces griseoaurantiacus</i>	1-4	1.66	0.33

Table S3. Antibacterial activity of isolated fungi against standard bacterial strains measured as the width of the inhibition zones (mm) against standard bacterial strains. – means no inhibition zone.

Isolated fungi \ Standard bacteria	<i>Bacillus cereus</i>	<i>Staphylococcus aureus</i>	<i>Enterococcus faecalis</i>	<i>Eschericia coli</i> JM 83	<i>Enterobacter aerogenes</i>	<i>Enterobacter cloacae</i>	<i>Micrococcus luteus</i>	<i>Agrobacterium tumefaciens</i>
<i>Absidia corymbifera</i>	-	-	-	-	-	-	-	-
<i>Aphanoascus fulvescens</i>	-	-	-	-	-	-	-	-
<i>Arachnomyces minimus</i>	-	-	-	-	-	-	-	-
<i>Aspergillus candidus</i>	-	-	-	-	-	-	-	-
<i>Aspergillus flavus</i>	-	-	-	-	-	-	-	-
<i>Aspergillus fumigatus</i>	-	-	-	15	-	-	-	-
<i>Aspergillus niger</i>	-	15	8	2	-	-	-	15
<i>Aspergillus terreus</i>	16	27	9	26	14	16	31	17
<i>Auxarthron umbrinum</i>	15	21	12	2	16	13	29	19
<i>Mucor circinelloides</i>	-	-	-	-	-	-	-	-
<i>Myceliophthora thermophila</i>	-	-	-	-	-	-	-	-
<i>Penicillium glabrum</i>	6	-	7	16	-	-	15	-
<i>Penicillium piceum</i>	-	-	-	15	-	-	-	-
<i>Thermomyces lanuginosus</i>	15	33	12	32	14	13	28	16
<i>Trichoderma reesei</i>	13	23	10	22	13	12	3	17

Table S4a. Antibacterial activity of bacteria isolated from TSA medium measured as the width of the inhibition zone (mm) against standard bacterial strains. – means no inhibition zone.

Isolated bacteria \ Standard bacteria	<i>Bacillus cereus</i>	<i>Staphylococcus aureus</i>	<i>Enterococcus faecalis</i>	<i>Eschericia coli JM 83</i>	<i>Enterobacter aerogenes</i>	<i>Enterobacter cloacae</i>	<i>Micrococcus luteus</i>	<i>Agrobacterium tumefaciens</i>
<i>Paenibacillus cookii</i>	-	-	-	-	-	-	-	-
<i>Bacillus licheniformis</i>	6	16	-	-	-	-	-	-
<i>Bacillus pumilus</i>	-	10	16	-	-	-	4	-
<i>Bacillus mycoides</i>	-	-	-	-	-	-	-	-
<i>Bacillus megaterium</i>	-	-	-	-	-	-	-	-
<i>Planococcus sp.</i>	-	-	-	-	-	-	-	-
<i>Staphylococcus Ssaprophyticus</i>	-	-	-	-	-	-	-	-
<i>Lysinibacillus fusiformis</i>	-	-	-	-	-	-	-	-
<i>Streptomyces violascens</i>	-	-	-	-	-	-	-	-
<i>Bacillus rhizosphaerae</i>	-	17	-	-	-	-	16	-

Table S5. Identity, range in abundance, mean abundance and standard deviation in abundance of fungi from feather samples of prey.

Fungal species			
Fungal species	Range	Mean	SD
<i>Alternaria sp.</i>	0-3	1.40	0.89
<i>Antrodia sinuosa</i>	2	2.00	0.00
<i>Ascomycota sp.</i>	0-5	3.00	2.83
<i>Aspergillus fumigatus</i>	4	4.00	0.00
<i>Aspergillus niger</i>	0-9	2.94	2.05
<i>Aspergillus ustus</i>	0-1	1.00	0.00
<i>Byssochlamys nivea</i>	0-3	1.33	0.82
<i>Chaetomium elatum</i>	0-2	1.17	0.41
<i>Chaetomium globosum</i>	0-3	1.75	0.96
<i>Chaetomium sp.</i>	0-2	1.67	0.58
<i>Coniochaeta ligniaria</i>	2	2.00	0.00
<i>Coniochaeta velutina</i>	0-2	1.67	0.58
<i>Coprinopsis atramentaria</i>	0-4	2.33	1.03
<i>Corioloopsis gallica</i>	0-4	1.78	1.20
<i>Hyphodermella rosae</i>	3	3.00	0.00
<i>Madurella mycetomatis</i>	0-1	1.00	0.00
<i>Monascus fuliginosus</i>	0-2	1.33	0.52
<i>Myceliophthora thermophile</i>	0-6	2.44	1.74
<i>Myceliophthora verrucos</i>	4	4.00	0.00
<i>Penicillium sp.</i>	2-7	3.67	2.89
<i>Pleosporaceae sp</i>	0-6	2.40	2.19
<i>Preussia sp.</i>	0-1	1.00	0.00
<i>Psathyrella candolleana</i>	2	2.00	0.00
<i>Rhizopus oryzae</i>	0-4	2.00	1.41
<i>Schizophyllum sp.</i>	3-4	3.50	0.71
<i>Stachybotrys dichroa</i>	3	3.00	0.00
<i>Thermomyces lanuginosus</i>	0-3	1.67	1.15

Table S6a. Among 27 fungal species, 14 (52%) secreted keratinase.

Fungal taxon	Reference
<i>Alternaria sp.</i>	Saber et al. 2010
<i>Ascomycota sp.</i>	Jeyaprakasam et al. 2016
<i>Aspergillus fumigatus</i>	Santos et al. 1996
<i>Aspergillus niger</i>	Lopes et al. 2011
<i>Aspergillus ustus</i>	Anbu et al. 2006
<i>Chaetomium elatum</i>	Kaul and Sumbali 1999
<i>Chaetomium globosum</i>	Kaul and Sumbali 1999
<i>Chaetomium sp.</i>	Kaul and Sumbali 1999
<i>Madurella mycetomatis</i>	de Hoog et al. 2013
<i>Myceliophthora thermophila</i>	Liang et al. 2011
<i>Myceliophthora verrucos</i>	Liang et al. 2012
<i>Penicillium sp.</i>	El-Gendy 2010
<i>Pleosporaceae sp</i>	Ghosh and Bhatt 2000
<i>Rhizopus oryzae</i>	Kumar et al. 2011

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Table S6b. Among 27 fungal species, 16 (59%) were pathogenic.

Fungal taxon	Reference
<i>Alternaria sp.</i>	Mamgain et al. 2013
<i>Ascomycota sp.</i>	Berbee 2001
<i>Aspergillus fumigatus</i>	Mirhendi et al. 2007
<i>Aspergillus niger</i>	Mirhendi et al. 2007
<i>Aspergillus ustus</i>	Mirhendi et al. 2007
<i>Chaetomium elatum</i>	Hubka 2015
<i>Chaetomium globosum</i>	Hubka 2015
<i>Chaetomium sp.</i>	Hubka 2015
<i>Madurella mycetomatis</i>	Ahmed et al. 2004
<i>Myceliophthora thermophila</i>	Farina et al. 1998
<i>Myceliophthora verrucos</i>	Revankar and Sutton 2010
<i>Penicillium sp.</i>	LoBuglio and Taylor 1995
<i>Pleosporaceae sp</i>	Manamgoda et al. 2015
<i>Rhizopus oryzae</i>	Bouchara et al. 1996
<i>Schizophyllum sp.</i>	Buzina et al. 2001
<i>Stachybotrys dichroa</i>	Andersen et al. 2003

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Figure S1a. Phylogenetic tree of bacterial species of 16s rRNA from feathers in nests of barn swallows.

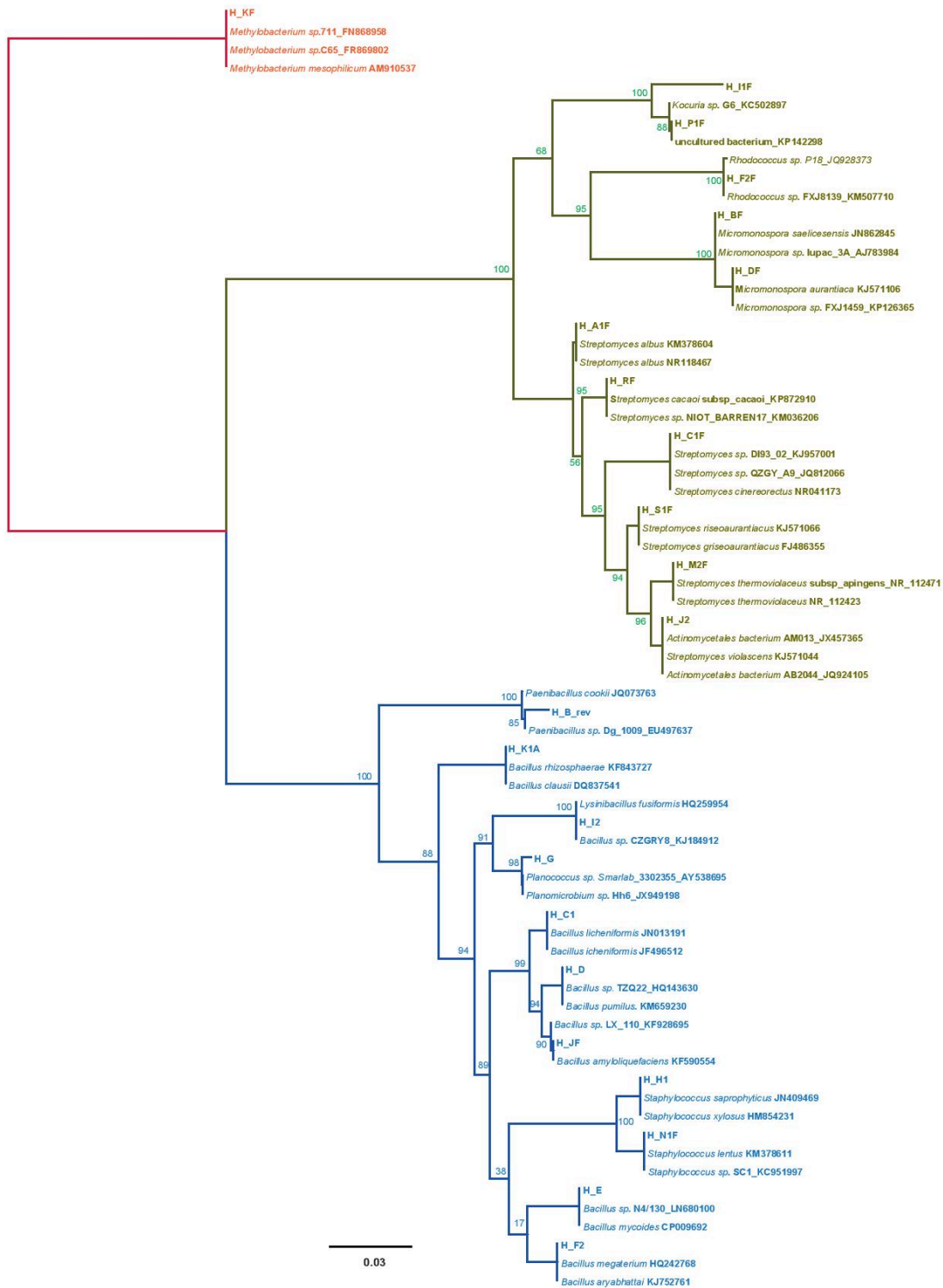


Figure S1b. Phylogenetic tree of fungal species of 18s rRNA from feathers in nests of barn swallows.

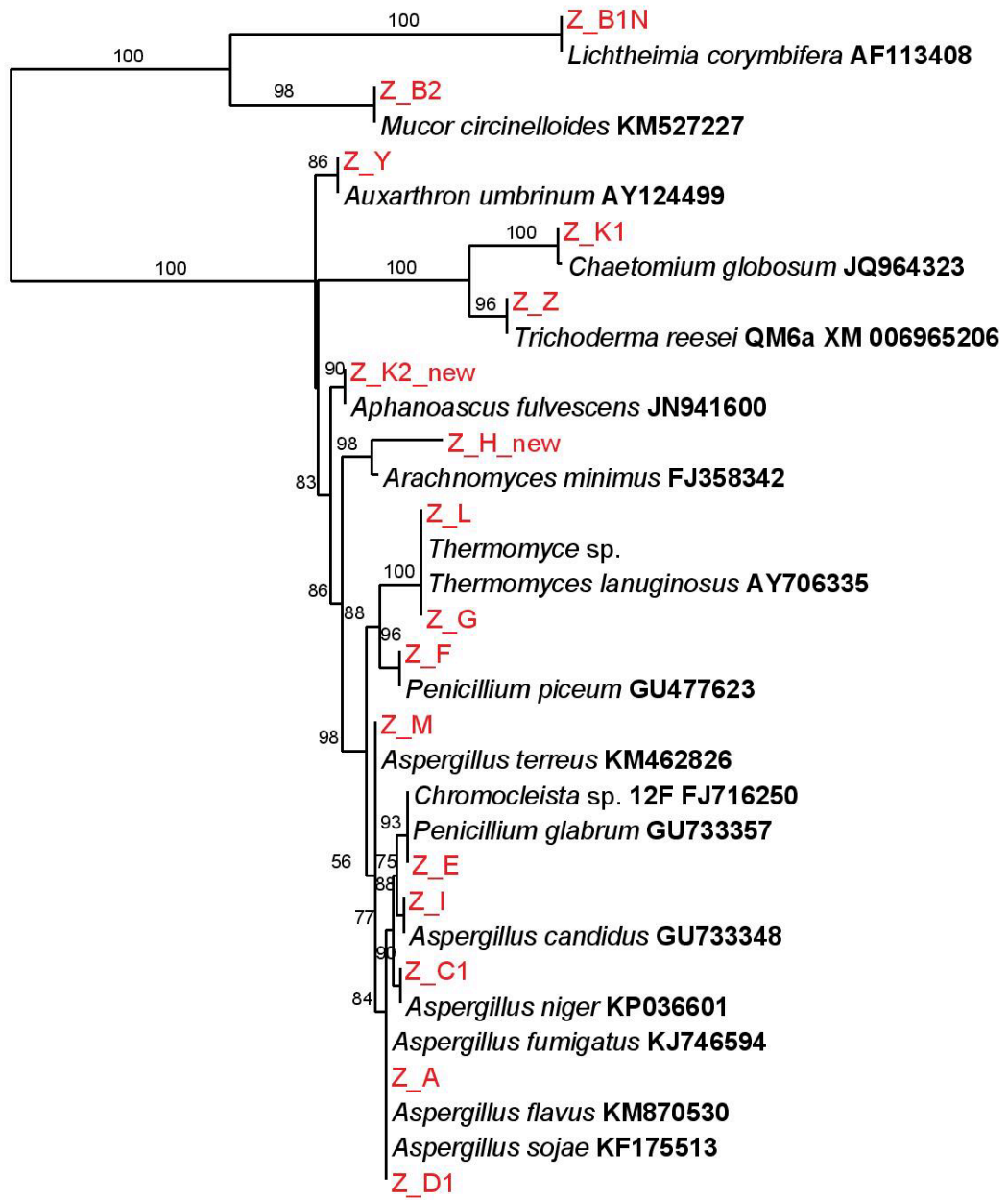
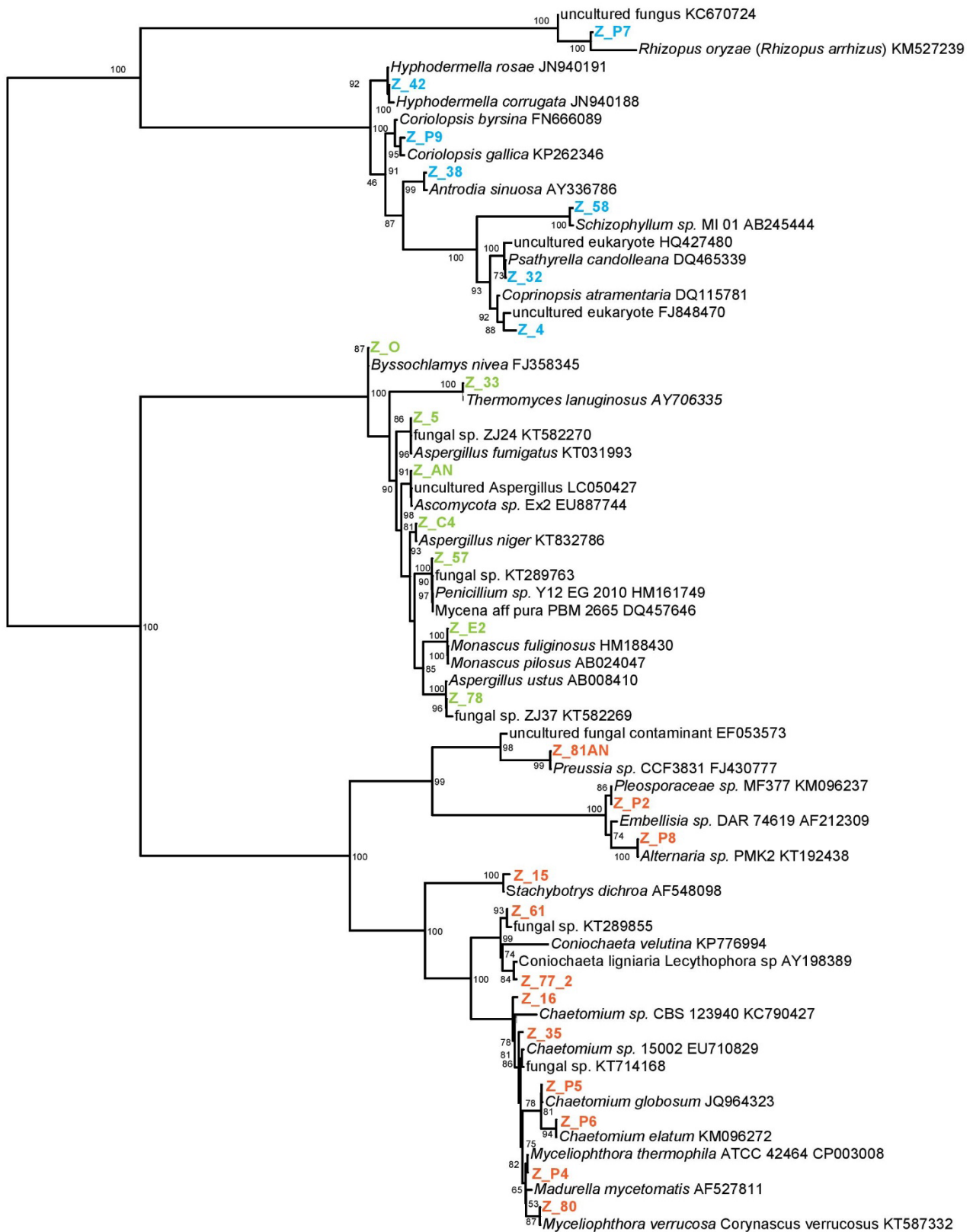


Figure S2. Phylogenetic tree of 18s rRNA from fungi isolated from feathers of goshawk prey.



Titre : Microorganismes, vol, reproduction et prédation chez les oiseaux

Mots clés : *Accipiter gentilis* ; Bactéries ; Doublure en plumes de nids ; Champignons ; *Hirundo rustica* ; Micro-organismes

Résumé : Les coûts de remise en forme que les macro et micro parasites imposent aux hôtes peuvent s'expliquer par trois facteurs principaux : (1) Les hôtes utilisent des réponses immunitaires contre les parasites pour prévenir ou contrôler l'infection. Les réponses immunitaires nécessitent de l'énergie et des nutriments pour produire et / ou activer les cellules immunitaires et les immunoglobulines, ce qui est coûteux, provoquant des compromis avec d'autres processus physiologiques comme la croissance ou la reproduction. (2) Le taux métabolique de l'hôte peut être augmenté parce que les dommages aux tissus et la réparation ultérieure de l'infection causée par le parasite peuvent être coûteux. (3) Le taux métabolique des hôtes peut augmenter et donc augmenter également leurs besoins en ressources. La compétition entre macro-parasites et hôtes peut priver les ressources de l'hôte.

Les coûts de remise en forme que les macro et micro parasites imposent aux hôtes peuvent s'expliquer par trois facteurs principaux : (1) Les hôtes utilisent des réponses immunitaires contre les parasites pour prévenir ou contrôler l'infection. Les réponses immunitaires nécessitent de l'énergie et des nutriments pour produire et / ou activer les cellules immunitaires et les immunoglobulines, ce qui est coûteux, provoquant des compromis avec d'autres processus physiologiques comme la croissance ou la reproduction. (2) Le taux métabolique de l'hôte peut être augmenté parce que les dommages aux tissus et la réparation ultérieure de l'infection causée par le parasite peuvent être coûteux. (3) Le taux métabolique des hôtes peut augmenter et donc augmenter également leurs besoins en ressources. La compétition entre macro-parasites et hôtes peut priver les ressources de l'hôte.

Title: Microorganisms, flight, reproduction and predation in birds

Keywords: *Accipiter gentilis* ; bacteria ; feather lining of nests; fungi ; *Hirundo .rustica* ; Microorganisms

Abstract: The fitness costs that macro- and micro-parasites impose on hosts can be explained by three main factors: (1) Hosts use immune responses against parasites to prevent or control infection. Immune responses require energy and nutrients to produce and/or activate immune cells and immunoglobulins, and that is costly, causing trade-offs against other physiological processes like growth or reproduction. (2) The host's metabolic rate can be increased because tissue damage and subsequent repair from infection caused by parasite may be costly. (3) The metabolic rate of hosts may increase and hence also increase their resource requirements. Competition between macro-parasites and hosts may deprive resources from host.

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